Molecular Mechanisms of Cancer Pathogenesis

Beata Mladosievičová
Soňa Čierniková
Michal Mego

UNIVERZITA KOMENSKÉHO V BRATISLAVE

Molecular Mechanisms of Cancer Pathogenesis

Beata Mladosievičová
Soňa Čierniková
Michal Mego

2024
Comenius University Bratislava

The publication was partially funded by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and Slovak Academy of Sciences (VEGA), contract No. 1/0738/21 and 2/0069/22.

Prof. Beata Mladosievičová, MD, PhD [3,21 AH] Comenius University Bratislava, Faculty of Medicine, Institute of Pathological Physiology, Department of Clinical Pathophysiology, Slovak Republic

Assoc. Prof. Dr.rer.nat. Soňa Čierniková, PhD. [3,58 AH] Cancer Research Institute, Biomedical Research Center of Slovak Academy of Sciences, Bratislava , Slovak Republic

Prof. Michal Mego, MD, DSc. [1,24.AH] 2nd Department of Oncology, Faculty of Medicine, Comenius University Bratislava and National Cancer Institute, Bratislava, Slovak Republic

Reviewers

Prof. Ingrid Tonhajzerová, MD, PhD.

Assoc. Prof. Ľubica Harvanová, MD, PhD.

Cover illustration from Freepik.com

Figures were created by MSc. Aneta Ševčíková, Dr. Rer. Nat. Soňa Čierniková, PhD. with Biorender.com

https://stella.uniba.sk/texty/LF_kol_molecular_mechanisms-cancer_pathogenesis.pdf

Published by Comenius University Bratislava

ISBN 978-80-223-5881-1 (online)

Content

Introduction

Cancer is a problematic condition due to its heterogeneity in terms of genes, cell and tissue biology, pathology and varying response to treatment.

Biomedical technologies today provide an enormous amount of data. Therefore, it is necessary to update a great deal of information but also "distil" it such that it is comprehensible to future doctors.

In the avalanche of new knowledge on the pathophysiological mechanisms of the origin and development of malignant diseases, a major shift can be observed in the past decade towards new aspects of oncogenesis.

Attention is shifting from tumour cells to the tumour microenvironment; science is moving on from carcinogen-induced mutations to random *bad luck* controlling mutations during DNA replication, and the interest of genetics is expanding from the gene to the so-called the dark matter of the genome and its role in oncogenesis. New types of cells, whose functions are still being clarified, are being added to the list of important cell types in the tumour microenvironment. Microorganisms can contribute to the origin and development of oncologic diseases not only at the local but also at the systemic level. The importance of the gut microbiome in tumour pathogenesis is also underlined by the fact that polymorphic microbiomes, like non-mutational epigenetic reprogramming, phenotypic plasticity and senescent cells, were assigned in the updated concept of Cancer Hallmarks from 2022, which summarises the basic characteristics of tumours.

We believe that the new scientific knowledge presented in the third updated edition of this university textbook intended for pregraduate and postgraduate students of medical and other faculties will contribute to a better understanding of the pathogenetic mechanisms of cancer and help to identify reliable biomarkers and therapeutic targets of oncogenesis.

Authors

1 Determinants of malignant transformation

1.1 Genetic alterations of proto-oncogenes and tumour-suppressor genes

Even though a tumour is considered to be a "genetic disease", it needs to be emphasised that only 1% of the DNA sequence consists of genes that code for proteins.

In the genome (as in the universe) a large proportion of "dark matter" genetic sequences occur that were originally referred to as waste, or so-called *junk DNA*.

And although *junk DNA* was originally considered to be useless, the development of molecular and genetic methods made it possible to uncover the important role of many non-coding regions, for example, in the regulation of gene expression and chromosome stability. However, the dark matter of the genome still remains largely unknown (Fig. 1).

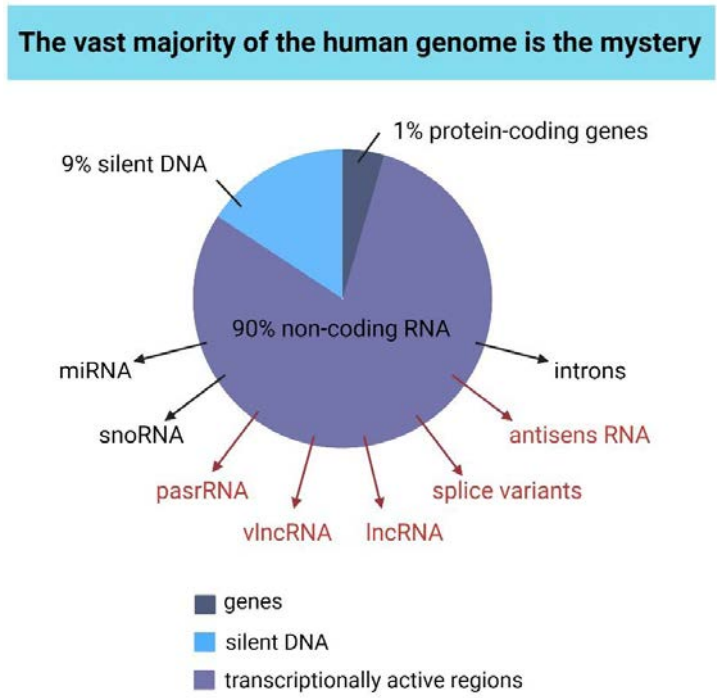

Figure 1
Components of the human genome.
Created with Biorender.com

Not all genetic aberrations present in a tumour are related to tumour transformation. Several dozen abnormally activated genes may be present in a single tumour; however, only about 10% of them actually contribute to the tumour's growth and survival. Each tumour cell has a certain set of genetic changes, some of which are controlling (tumour initiating, a so-called *driver*) mutations; others are only so-called passengers or passenger mutations unrelated to the tumour process.

The results of Tomasetti C. and Vogelstien B. suggest that approximately a third of the variation in cancer risk among tissues is attributable to environmental factors or inherited predispositions. The majority is due to "bad luck," that is, random mutations arising during DNA replication in normal, noncancerous stem cells. In their study including 32 types of tumours, most so-called "driver" mutations (66%) were found to arise randomly during DNA replication, and environmental factors induced 29% of such mutations. About 5% of these mutations were of hereditary origin. This study created vigorous scientific debate. What is today labelled as a random event will not necessarily be considered a random event in the future. Moreover, not only "bad luck" mutations are responsible for the variability of the risk of tumours in individual tissues; the function of stem cells and mutations in them play an important part, too. Clearly, some types of cancer, such as lung cancer, are heavily influenced by environmental factors, on the other hand majority of critical mutations e.g. in pancreatic cancer are due to random DNA copying errors.

Only a small number of types of malignancies have known a so-called Achilles's heel – controlling (tumour-initiating) genetic changes, such as, for example, the crucial BCR-ABL translocation in patients with chronic myeloid leukaemia.

In the majority of tumours abnormalities of many genes (both proto-oncogenes and tumour-suppressor genes) are present in several signalling pathways. The mutational complexity, redundancy and deregulation of these pathways in tumour cells are extensively studied.

A single genetic change is not sufficient to transform a normal cell into a cancerous one. Oncogenesis arises from the gradual accumulation of genetic changes in specific genes responsible for controlling growth, differentiation, migration, cell lifespan and genome stability.

For the malignant transformation of cells, an accumulation of 5 – 20 genetic changes is necessary in most tumours, namely in:

- proto-oncogenes,
- tumour suppressor genes,
- genes encoding repair systems,
- genes encoding the correct number of chromosomes.

The order of these changes is also important for the emerging of tumours. In the course of oncogenesis, selection of a cell clone capable of survival occurs. Most solid tumours arise from the division of a single malignant cell, though leukaemia's and lymphomas probably arise from multiple cell clones. Products coded by proto-oncogenes are of particular importance in proliferation and apoptosis. At present, about 350 proto-oncogenes are known, and among the most well-known are, for example, *HRAS, KRAS, NRAS, ERBB2(HER2), MYB, C-MYC, PML, RAF1,* and *SRC.*

With increased activation of proto-oncogenes by

- mutations,
- chromosomal aberrations,
- amplifications

their products are formed in excessive amounts or in changed quality.

The products of proto-oncogenes have the function of

- growth factors,
- receptors for growth factors and hormones,
- signal molecules,
- transcription factors,
- apoptosis regulators,
- chromatin modulators (Fig. 2).

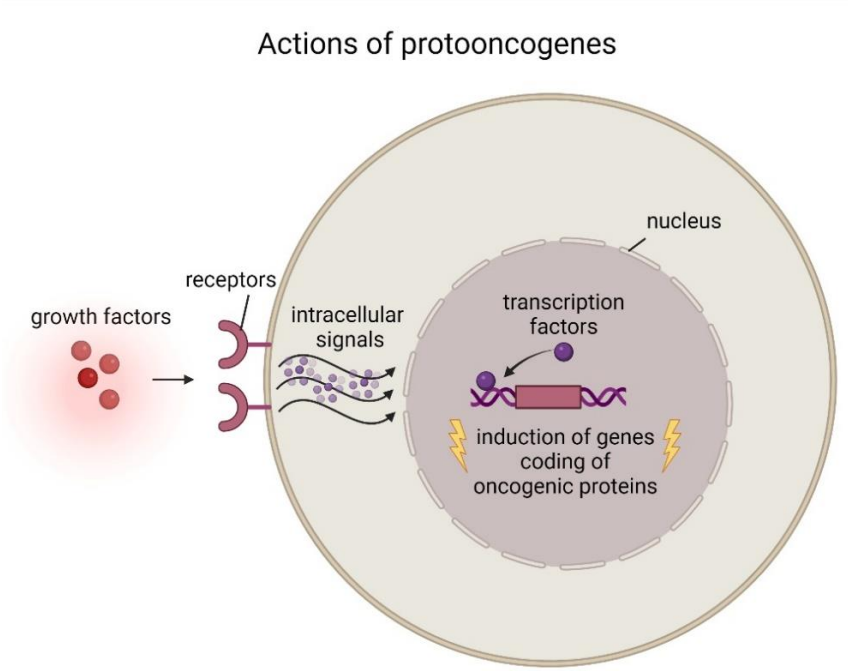

Figure 2
Working of proto-oncogenes.
Oncogenes, after conversion from proto-oncogenes, encode growth factors, receptors for growth factors, signal transduction molecules from the cell membrane to the nucleus, and transcription factors in excess or of abnormal quality. Created with Biorender.com

Recent findings indicate that activation of proto-oncogenes can also occur as a result of genetic alterations that disrupt the 3D structure of chromosomes.

Disturbances to circadian rhythms can also lead to the deregulation of oncogenes, e.g. *C-MYC*.

Another group of genes that can acquire transformation potential are tumour-suppressor genes. Approximately 150 of them are currently known, including, for example, *BRCA1*,

BRCA2, CDKN2B, CDKN3, E2F1, IGF2R, MEN1, NF1, NF2, SMAD4, TP53, VHL, WT1, and *XRCC1*.

Several genes have common properties of proto-oncogenes and tumour-suppressor genes, e.g. *EGF, ERBB2, HRAS, KRAS, MDM2, MYC, RB1, RET, TGFB1, TNF,* and *TP53*.

Under normal circumstances, the role of tumour-suppressor genes, the so-called anti--oncogenes, is

- to ensure the repair of damaged DNA,
- to limit excessive cell growth,
- to reduce invasive and metastatic potential,
- to trigger apoptosis.

Tumour-suppressor genes can acquire oncogenic potential by inactivation, namely by

- mutation,
- deletion.

For a simplified illustration, a tumour cell can be imagined as a car moving at an exceedingly high speed. Activation of proto-oncogenes is then like using the gas pedal, and deactivation of tumour-suppressor genes is akin to releasing the brakes.

Proteins that are encoded by tumour-suppressor genes take part in processes important in the repair of damaged DNA and also in signalling the initiation of programmed cell death – apoptosis. The presence of mutations in proto-oncogenes leads to the emergence of oncogenes and proteins that cause uncontrolled cell division. In the case of mutations in tumour-suppressor genes, their function is lost, and subsequent suppression of the protective and antiproliferative effect of the corresponding proteins triggers tumourigenesis.

Genetic alterations relate to somatic cells (sporadic forms of malignancies) or to germinal cells (a genetic predisposition to the development of malignancies then occurs, and the entire process of carcinogenesis can be accelerated). With the origin of hereditary forms of malignancies (which represent approximately 5 – 10% of malignancies in adults and 15 – 20% in children), alterations in tumour-suppressor genes occur. Hereditary forms of tumours are known in the context of colorectal cancer, breast cancer, retinoblastoma and other malignant diseases. In colorectal cancer with a familial occurrence, the mutation of one allele of the APC is germinal. Germinal mutations of one allele of this gene are found in all cells. After the mutation of the first allele, the next step is the so-called second hit – a mutation of the second allele, which leads to a loss of heterozygosity (LOH). In a person who does not have a germline mutation in the *APC* gene, the probability that both alleles of the same gene will be damaged in one cell is relatively low. In a person who has one allele of the *APC* gene altered in all of his cells, however, the probability is relatively high (80% or more) that the second allele will also be damaged in one of the cells in the colon mucosa. Persons with a germline mutation of the *APC* gene have an elevated risk of developing familial adenomatous polyposis (FAP), which is an autosomal dominant disease. In FAP, hundreds to thousands of polyps develop in the large intestine (possibly also more proximally and more distantly) with an almost 100% probability of their progression into colorectal cancer. A hereditarily conditioned mutation of an allele of

the MSH2 and MLH1 genes is associated with an elevated risk of developing nonpolyposis colon cancer.

Patients with a hereditary mutation of the *BRCA1* and *BRCA2* genes have an increased risk of developing breast cancer and ovarian cancer and a lower risk of other cancers, e.g. pancreatic cancer. Male carriers of the mutation have an increased risk of breast and prostate cancer (Tab. 1).

Data from a recently published study by authors Li S. et al. of more than 7,600 families with at least one family member with a *BRCA1/2* mutation showed that aside from breast and ovarian cancer in women, pathological variants of *BRCA1/2* are also linked with the risk of gastric cancer, the relative risk of which is higher in women than in men. Potential associations with colon and gallbladder cancer are also indicated.

Table 1

Cancer risk in carriers of pathological variants of *BRCA1/2* (According to Li S. et al., 2022 and Petrucelli et al., 2022)

Type of cancer	**General population**	**Cancer risk BRCA1 mutation**	**Cancer risk *BRCA2* mutation**
Breast cancer	12 %	55 – 72 % by age 70	45 – 69 %
Contralateral breast cancer	2 %	20 – 30 % in 10 yrs	40 – 50 % in 20 yrs
Ovarian cancer	1 – 2 %	39 – 44 %	11 – 17 %
Breast cancer in men	0,4 % by age 80	0,4 – 1.2 %	6 – 8 %
Prostate cancer	6 % by age 69	29 % by age 85	60 % by age 85
Pancreatic cancer	0.5 %	1 – 2.5 %	3 – 27 % by age 80

Mutations of proto-oncogenes in germinal cells are observed only very rarely, because the products they encode are of such importance, that the lack of them or a change in their quality can be lethal even during embryogenesis.

Malignancies are usually caused by somatic mutations that accumulate during the development of cancer. In typical tumours, germline mutations are also inherited from parents. Mutations in *BRCA1* and *BRCA2* (and other tumour-suppressor genes) are transmitted to offspring with a 50% risk.

The advantage of cellular selection of tumour cells for preferential growth or survival also enables them to rapidly accumulate mutations in a short time. Mutations can also occur in some normal tissues of the organism. The older a person is, the more cell divisions he or she has undergone, especially in proliferating tissues. There is a correlation between organ tissues and the number of cell divisions that occur in humans exposed to high risk of developing malignant disease. Spontaneous mutations are, of course, also present in cells that do not reproduce.

However, these mutations are caused by a failure of DNA repair, not during DNA replication. Generally speaking, mutations arise from replication errors or damage to DNA that is either incorrectly repaired or not repaired at all.

1.2 Disorders of DNA repair systems

In human beings 10^{18} repairs of damaged DNA need to be made every day. If this damage cannot be repaired, apoptosis is induced. The products of five repair pathways play a major role in DNA repair:

- the MMR system (methyl-directed mismatch repair) – the repair of incorrectly paired bases – is associated with the genes *MSH 2, 3, 6, MLH1, PMS1,* and *PMS2*;
- the NER system (nucleotide-excision-repair) – is associated with the *XPA* and *ZPG* genes;
- the BER system – base excision repair;
- the DSB system (double-strand break repair) – repair of double-strand breaks;
- the DR system (direct reversion) – direct restoration of damage.

Several anticancer drugs induce fatal DNA damage (breaks). Tumour cells, however, are capable of proliferating even if they are damaged with treatment, by the "upregulation" of DNA repair, which leads to an unfavourable treatment response and even resistance to treatment.

1.3 Disorders of the mechanisms responsible for the correct division of chromosomes into daughter cells

Chromosomal instability is associated with aneuploidy – having an abnormal number of chromosomes. Errors in the connection of the microtubules of the dividing spindle apparatus to the kinetochore region and errors in the coordination of individual phases may lead to chromosomal aberrations – chromosomes or parts of them can be lost or unevenly distributed into two daughter cells – leading to aneuploidy. Abnormalities of specific genes (e.g. *BUB1, BUBR1,* and *MAD2*) that control the proteins responsible for correct attachment of the dividing spindle apparatus to the kinetochore region in the mitotic phase of the cell cycle are responsible for the incorrect distribution of chromosomes. Also important for the correct division of chromosomes into daughter cells are aurora kinase enzymes. Aurora kinases A and B are novel targets for treatment with small molecule tyrosine kinase inhibitors, as overexpression of these kinases has been found in several malignancies.

Some authors characterise a tumour as a "chromosomal" disease.

Aneuploidy caused by losses, fragmentation or rearrangement of chromosomes can be found in almost all types of malignancies. The number of chromosomes in tumour cells is often in the range of 50–90, and tumours with aneuploidy have a worse prognosis than diploid tumours. The higher the number of chromosomes, the more severe the malignancy. Hypoploidy, for example, is also a problem with leukaemia. Changes in the structure and number of centrosomes also correlate positively with aneuploidy; for example, in patients with breast cancer, such changes

were present in 80% of cases. Aneuploidy can be induced by the action of carcinogens or it can occur spontaneously. In cells with aneuploidy several thousand genes are over- or under-expressed. According to mutation theory, a genetic change in a few specific genes suffices to induce carcinogenesis. The action of these genes, however, is unlikely with a total number of genes (approximately 19,000 protein-coding genes) with a large number of non-coding regions. The loss, fragmentation and rearrangement of chromosomes are more likely to occur. These chromosomal changes then destabilise thousands of genes. It was recently found that certain sequences of the genome, which until recently were considered *junk DNA*, are responsible for the stabilisation and repair of chromosomes.

1.4 The cell cycle

The cell cycle is a series of events that leads to the duplication of genetic material, the separation of the chromosomes and the allocation of DNA copies into daughter cells. The individual phases of the cell cycle are strictly coordinated and must have adequate speed and the ability to respond to the needs of the external environment. The processes of synthesis and mitosis are divided into phases G1 and G2 (G from the word *gap*). Cells, however, can also be found in a long period of rest (in the G0 phase), and after stimulation, they can re-enter the cell cycle. The cell passes through the G1-S transition as a response to the presence of mitogenic factors (hormones, growth factors). Mitogens bind to cell receptors, and a signal is transmitted to the nucleus via intracellular pathways, where transcription factors activate the expression of the proteins needed for the G1-S transition (e.g. specific cyclins and cyclin-dependent kinases). Other phases of the cell cycle are now independent of the presence of mitogens. In healthy cells, upon the entry from G1 to S and G2 to M – at the so-called checkpoints – the cycle slows down or stops, giving the cell time for quality control or to undergoes apoptosis. The protein encoded by the *TP53* gene and inhibitors of cyclin-dependent kinases (CDK) play an important role in this process. DNA replication takes place during the S phase, and errors in DNA replication are corrected by repair systems. With the mutation of genes encoding repair proteins in tumour cells, DNA repair is insufficient, and this results in the accumulation of genetic defects. In healthy cells, a cell with damaged DNA or incomplete DNA replication does not enter mitosis. In the mitotic phase (M), chromosomes separate, and the cell divides into two daughter cells. There is also a third checkpoint in mitosis, at the transition from metaphase to anaphase. Among the inhibitors of cell cycle regulatory proteins are CDK inhibitors, and also an inhibitor of the ubiquitin-proteasome complex, which is important in the degradation of regulatory proteins, by which the G1/S and G2/M transition is stopped. If the damage to the DNA can be repaired, the cell cycle slows down or stops for the time needed for the repair; then continues after DNA repair is completed. If the damage to the DNA is such that it is irreparable, the cell cycle does not continue, and apoptosis is initiated with subsequent DNA degradation.

Products encoded by proto-oncogenes, cyclins and CDK regulate the cell cycle positively, and products encoded by tumour-suppressor genes and CDK inhibitors slow down the cell cycle.

Passage through the restriction point is secured by the Rb protein, and passage through this point is a question of phosphorylation and dephosphorylation. The dephosphorylation of Rb leads to binding to the E2F transcription factors, thus stopping proliferation. Phosphorylation of these proteins releases E2F, initiating the cell growth phase (G1). Phosphorylation occurs via the action of CDK, the activity of which is conditioned by the formation of a complex with cyclins. Overexpression of cyclins occurs with inappropriate proliferation, which is typical for tumour cells. When the cell reaches a certain volume, the phase of DNA synthesis (S) begins. The events that take place after the S phase then continue without stimulation by growth factors. Tumour cells lose the ability to inhibit the cell cycle. DNA damage in various forms accumulate in their genome. A new and attractive area of research is the elucidation of cell cycle regulation in relation to circadian rhythms, e.g. in patients with colorectal cancer.

References

Dasari K, Somarelli JA, Kumar S, Townsend JP. The somatic molecular evolution of cancer: Mutation, selection, and epistasis. Prog Biophys Mol Biol 2021, 165:56-65.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-674.

Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov 2022; 12(1), 31-46.

Hasakova K, Vician M, Reis R, et al. The expression of clock genes cry1 and cry2 in human colorectal cancer and tumor adjacent tissues correlates differently dependent on tumor location. Neoplasma 2018; 65(6):986-992.

Hnisz D, Weintraub AS, Day DS, et al. Activation of proto-oncogenes by disruption of chromosome neighborhoods. Science 2016; 351(6280):1454-1458.

Li S, Silvestri V, Leslie G, et al. Cancer risks associate with BRCA1 and BRCA2 pathogenic variants. J Clin Oncol 2022; 40(14):1529-1541.

Martincorena I, Raine KM, Gerstung M, et al. Universal Patterns of Selection in Cancer and Somatic Tissues. Cell. 2017 Nov 16;171(5):1029-1041.e21.

Nowak MA, Waclaw B. Genes, environment, and „bad luck“. Science 2017; 355(6331):1266-1267.

Petrucelli N, Daly MB, Pal T. BRCA1- and BRCA2-Associated Hereditary Breast and Ovarian Cancer. 1998 Sep 4 [Updated 2022 May 26]. In: Adam MP, Mirzaa GM, Pagon RA, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2023. Dostupné na: https://www.ncbi.nlm.nih.gov/books/NBK1247.

Sulli G, Lam MTY, Panda S. Interplay between Circadian Clock and Cancer: New Frontiers for Cancer Treatment. Trends Cancer 2019; 5(8):475-494.

Tomasetti C, Vogelstein B. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. Science 2015; 347(6217):78-81.

Tomasetti C, Li L, Vogelstein B. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. Science 2017; 355(6331):1330-1334.

Walsh T, Casadei S, Coats KH, et al. Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. JAMA 2006 Mar 22; 295(12):1379-88.

Weinberg RA. Coming Full Circle – From Endless Complexity to Simplicity and Back Again. Cell 2014; 157:267-271.

Zhu L, Finkelstein D, Gao C, et al. Multi-organ Mapping of Cancer Risk. Cell 2016; 166:1132-1146.e7.

Zhu W, Wu S, Hannun YA. Contributions of the Intrinsic Mutation Process to Cancer Mutation and Risk Burdens. EBioMedicine 2017; 24:5-6.

2 Epigenetic changes

Epigenetic changes are changes that occur in gene expression without changing the primary genetic information (the order of the nucleotides). Epigenetic mechanisms were first thought to be predominately associated with signalling pathways in embryonic development, differentiation and organogenesis. Increasing evidence, however, has revealed their essential role in the aetiology and treatment of malignant diseases. According to the current concept, aberrant epigenetic reprogramming is one of the characteristic signs of malignant progression. Chromatin inheritance is associated with structural and chemical modifications of chromatin and plays a role in controlling gene expression, replication and repair and chromosome segregation.

The basic unit of chromatin is the nucleosome, which is made up of histone molecules. The following are responsible for chromatin remodelling:

- ATP-dependent enzymes (which change the position of nucleosomes),
- enzymes that modify the N-terminal ends of histones.

The target of modification is DNA and/or histones.

2.1 Histone modification

Events that take place in a specific sequence (in the epigenetic code) and whose result is active chromatin with transcriptional capacity are responsible for the modification of histones:

- histone acetylation (enzymes for histone acetyltransferase (HAT) and histone deacetylase (HDAC)),
- histone phosphorylation (kinases are used for this),
- methylation of histones (on lysine residues),
- ubiquitination of histones.

Post-translational modification of histone proteins regulates chromatin condensation, the positioning of nucleosomes and access of the transcription apparatus to the DNA molecule. At least nine different types of histone modifications have thus far been discovered, with acetylation, methylation, phosphorylation and ubiquitination being the most studied. Histone modifications collectively regulate the transcriptional state of a genomic region. Acetylation and deacetylation of histones are the key epigenetic signals through which gene expression is

regulated. This occurs with the help of HAT enzymes, such as CREB-binding protein (CBP), p300 and pCAF, which transfer an acetyl group to lysine residues in histones and non-histone proteins. This process leads to relaxation of the chromatin, which facilitates the accessibility of DNA for transcription factors and the whole transcription apparatus. On the other hand, the reverse reaction, called histone deacetylation, is mediated by HDAC, and this is linked with gene repression. The balance between the acetylation and deacetylation of histones is very dynamic, and both groups of enzymes can bind to gene promoters, regardless of their transcriptional activity.

Deacetylation of histones and DNA methylation lead to the repression of transcription – to transcriptionally inactive chromatin. Acetylation and deacetylation should be in balance. Inhibitors of histone deacetylation enable the expression of the so-called gene silencing, which is necessary, e.g. for the cell cycle.

Modulators of histone deacetylases are gaining ever more attention not only in therapy but also in the chemoprevention of tumours. Their importance has been confirmed in clinical studies, particularly in the treatment of haematological malignancies and cutaneous T-cell lymphomas.

2.2 Dysregulation of DNA methylation

The pathogenesis of malignancies is also influenced by processes leading to hypermethylation (i.e. repression) of tumour suppressor genes and demethylation (derepression) of proto-oncogenes.

DNA methylation is the most often studied type of epigenetic mechanism in both normal and tumour cells and means the transfer of a methyl group to a pyrimidine cytosine before a guanosine. This part of the DNA is subsequently not available to RNA polymerase, and the information encoded in this section cannot be expressed. The transfer of the methyl group itself is ensured by DNA methyltransferases (DNMTs). The method of DNA methylation is both inherited and acquired.

The silencing of gene expression by DNA methylation is catalysed by DNMTs, which add a methyl group to the 5' carbon of the cytosine pyrimidine ring within CpG islands. The addition of a methyl group occurs preferentially in the regulatory regions of genes. In the human genome, five *DNMT* genes are known; these are *DNMT1, DNMT2, DNMT3A, DNMT3B,* and *DNMT3L*. Only the proteins DNMT3A, DNMT3B and DNMT1, however, have a catalytic domain with methylation activity. DNMT1 is considered the key enzyme for maintaining methylation patterns after replication of maternal DNA, while *de novo* DNA methylation is catalysed by the enzymes DNMT3A and DNMT3B.

Similar to mutations, changes in the methylation status of the genes that regulate the cell cycle can cause a change in gene expression or function. Among the typical signs of malignancies are the so-called *silencing* of genes through hypermethylation of a promoter in CpG islands as well as the global hypomethylation of the entire genome. With some types of tumours, it has been shown that patients without mutations in those genes which in most cases are associated with the relevant type of tumour have these genes epigenetically affected. This means that their

tumour tissue contains hypermethylated promoter sequences of genes encoding the tumour suppressor proteins VHL, p16, Rb or BRCA1. Similar hypermethylation of genes encoding DNA-repair enzymes leads to increased mutation frequency and carcinogenesis. The set of genes that are hypermethylated and thereby inactivated in a given tumour is called a hypermethylome. Genomic DNA in tumours when compared to healthy tissue is hypomethylated. This fact is associated with the accelerated cell cycle, when after the duplication of nuclear DNA, there is evidently not sufficient time left for the methylation of the relevant sections for the next mitosis. The hypomethylation of the DNA of tumour cells correlates with their malignancy and facilitates mitotic recombination, leading to aneuploidies and chromosome mutations. Hypomethylation can lead to increased activation of proto-oncogenes.

Originally active genes are deactivated due to hypermethylation of cytosines in the regulatory regions of the genes. Methylation of cytosines in CpG dinucleotides, which usually accumulate in CpG islands, is crucial for the inhibition of gene transcription. In the human genome, CpG islands are localised in the promoters of most genes. A higher frequency of CpG islands elevates the probability of the methylation of DNA sequences, and the presence of repetitive sequences also increases the probability of DNA methylation. Hypermethylation tends to eliminate, e.g. tumour-suppressor genes and the repair genes responsible for DNA repair, as well as other sequences.

If for various reasons methylation does not work, the cell's genome is unstable, which is a fatal condition for the cell. The modification of histone proteins and DNA methylation interact. What's more, methylated CpG islands also interact with other proteins containing a methyl-CpG binding domain. This issue is at present also taking on a new dimension in connection with new knowledge about adenine methylation.

DNA methylation can have both diagnostic and prognostic significance and is also determined within the so-called liquid biopsy.

2.3 MicroRNA dysregulation in tumours

RNA plays a major role in the development of oncological and numerous other diseases. RNA which does not code for proteins is called non-coding RNA. Based on the number of nucleotides, we distinguish:

- long non-coding RNA *(lncRNA)*,
- small non-coding RNA *(sncRNA)*.

Small non-coding RNA includes several groups – microRNA, small nuclear RNA (*snRNA*), small nucleolar RNA (*snoRNA*) and RNA interacting with the so-called by PIWI proteins *(PIWI-interacting RNA, piRNA)*.

MicroRNA (miRNA)

MicroRNA (*miRNA*) is a group of short non-coding RNA molecules that consist of sequences of approximately 22 nucleotides long. They take part in virtually every physiological process

and play a key role in the post-transcriptional regulation of gene expression. MicroRNAs are partially complementary to several mRNAs, and their binding to mRNA results in the degradation or downregulation of gene expression through several mechanisms, including translational repression, cleavage of mRNA and deadenylation. Their post-transcriptional repression of relevant target mRNA plays a key role in the regulation of different biological processes, such as the cell cycle, differentiation, proliferation and cell death. Most miRNA sequences are located in the introns or exons of non-coding RNA molecules, and their biogenesis occurs either through a canonical or a non-canonical pathway. In the majority of cases, miRNA genes are transcribed in the nucleus by RNA polymerase II into primary miRNA (pri-miRNA), after which cleavage into precursor miRNA (pre-miRNA) structures follows. Further export to the cytoplasm and cleavage by the Dicer complex into small dsRNAs enables their binding to Argonaute (AGO) proteins and the RISC complex, which results in the formation of mature single-stranded miRNA molecules. The canonical pathway of miRNA biogenesis is dependent on Drosha and DGCR8 proteins, while some non-canonical pathways are independent of these proteins.

Roughly 2,500 miRNAs have been identified in the human genome. Genes for miRNAs are found not only in regions between protein-coding genes, but also inside already known genes. MiRNAs normally serve for the "fine-tuning" of gene expression in various tissues. They serve an important role in cell communication. They are secreted into the extracellular space by one type of cell. Another type of cell, in which they can influence gene expression, then takes them up. We find some miRNAs in body fluids; others are found in extracellular vesicles – in exosomes or apoptotic bodies, for example. Studies have shown that extracellular miRNA have a much higher stability in comparison with cellular miRNA. An important, more recent discovery was the presence of circulating miRNA in body fluids (blood, urine or stool). These molecules represent potential diagnostic and prognostic biomarkers in the case of many cancers. Even exosomes secreted from cells can contain mature or pre-miRNA forms and thereby represent "vehicles" for the transfer of miRNA to the surrounding microenvironment. Exosomes can be taken up by different cells, and the subsequent release of miRNAs could mediate changes in the gene expression of the target genes. Many experimental, but especially clinical studies have revealed the important role miRNA plays in tumourigenesis and the spread of metastases, and their dysregulation has been described in a broad spectrum of malignant diseases.

Their abnormal expression – decreased or increased – has been detected in tumour cells. Some miRNAs cause tumour initiation and progression, while others suppress oncogenesis. Several miRNAs have been reported in the processes of abnormal proliferation, invasiveness and metastasis.

It is believed that miRNA controls the expression of about 30% of genes required, e.g. for the cell cycle and cell survival. MiRNA regulates the function of protein genes in several ways:

- it can "intertwine" with a certain section of the mRNA, thereby disabling the function of the gene (the mRNA is thus "cut into pieces"),
- it temporarily binds to sections of mRNA and blocks the synthesis of proteins according to this mRNA.

If the target of the miRNA is an oncogene, it can have, e.g. the role of the tumour-suppressor gene, and vice versa. Upregulation of miRNA can be caused by amplification, deregulation of transcription and demethylation in the promoter region.

References

Baylin SB, Jones PA. Epigenetic Determinants of Cancer. Cold Spring Harb Perspect Biol 2016 Sep 1;8(9):a019505.

Fridrichova I, Smolkova B, Kajabova V, et al. CXCL12 and ADAM23 hypermethylation are associated with advanced breast cancers. Transl Res 2015; 165(6):717-30.

He B, Zhao Z, Cai Q, et al. miRNA-based biomarkers, therapies, and resistance in cancer. Int J Biol Sci 2020; 16(14):2628-2647.

Hill M and Tran N. miRNA interplay: mechanisms and consequences in cancer. Dis Model Mech 2021 Apr 1; 14(4), dmm047662.

Jurkovicova D, Smolkova B, Magyerkova M, et al. Down-regulation of traditional oncomiRs in plasma of breast cancer patients. Oncotarget 2017; 8(44):77369-77384.

Rodenhiser D, Mann M. Epigenetics and human disease: translating basic biology into clinical applications. CMAJ 2006; 174(3):341-8.

Rohlf T, Steiner L, Przybilla J, et al. Modeling the dynamic epigenome: from histone modifications toward self-organizing chromatin. Epigenomics 2012; 4(2):205-19.

Ryan BM: MicroRNAs in Cancer Susceptibility. Adv Cancer Res 2017; 135: 151-171.

Sandoval J, Esteller M. Cancer epigenomics: beyond genomics. Curr Opin Genet Dev 2012; 22(1):50.

Sebova K, Zmetakova I, Bella V, et al. RASSF1A and CDH1 hypermethylation as potential epimarkers in breast cancer. Cancer Biomark 2011; 10(1):13-26.

Sevcikova A, Fridrichova I, Nikolaieva N, et al. Clinical Significance of microRNAs in Hematologic Malignancies and Hematopoietic Stem Cell Transplantation. Cancers 2023; 15(9):2658.

Van Roosbroeck K, Calin GA. Cancer Hallmarks and MicroRNAs: The Therapeutic Connection. Adv Cancer Res 2017; 135:119-149.

Visvanathan A, Somasundaram K. mRNA Traffic Control Reviewed: N6-Methyladenosine (m6 A) Takes the Driver‘s Seat. Bioessays 2018 Jan; 40(1).

3 Selected biological characteristics of malignant cells

Tumours arise when cells break the so-called "social rules". A single "renegade" cell may not represent a problem. A problem can arise, however, if this cell, "favoured in competition" by the accumulation not only of genetic but also epigenetic changes, is not eliminated, but survives and transforms into a malignant one – it begins to divide uncontrollably; it ceases to respond correctly to growth-stimulating and inhibiting signals and increases its replicative potential; it induces the formation of new blood vessels, becomes resistant to programmed cell death, reprograms its energy metabolism and is be able to avoid destruction by the immune system; it acquires additional properties and establishes a clone of other "asocial" cells capable of colonizing other territories with a tendency to survive even at the cost of subverting the entire organism.

In the course of the individual stages, tumour cells acquire the eight main so-called *emerging* characteristics that are necessary for tumour growth and progression:

1. independence from signals stimulating growth,
2. resistance to signals inhibiting growth,
3. replication potential – reactivation of telomerase,
4. resistance to programmed cell death,
5. inducing or accessing to vasculature,
6. invasive and metastatic potential,
7. reprogramming of energy metabolism,
8. avoiding immune destruction.

The last two mentioned characteristics were included among the main characteristics of tumour cells in 2011, when these characteristics were reported to be the result of genomic instability and inflammation. Since 2022, the American Association for Cancer Research (AACR) has also included among the characteristics of tumours:

- phenotypic plasticity
- non-mutational epigenetic reprogramming.
- polymorphic microbiomes (described in detail in chapter 5)
- senescent cells in the tumour microenvironment.

3.1 Independence from growth-stimulating signals

3.1.1 Growth factors

On the surface of cells are receptors that are activated by growth factors and hormones. As soon as they are activated, a whole series of reactions are triggered in the cell. A malfunction in cell growth control by growth factors or hormones may result in increased proliferation and malignant transformation. Growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and interleukins – colony stimulating factors (CSF), play a significant role in the proliferation of normal cells. Growth factors become active after binding to receptors, further inducing a cascade of mitogenic signal transduction, culminating in the transcription of specific genes. Tumour cells have mechanisms capable of the constant activation of signals.

3.1.2 Growth-factor receptors

3.1.2.1 EGFR

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein which is expressed in normal epithelial tissues. The EGFR belongs to the ErbB family of tyrosine kinase receptors, which has four members:

- EGFR (ErbB1, HER1),
- ErbB2 (HER2),
- ErbB3 (HER3),
- ErbB4 (HER4) (Fig.3).

Inadequate activation of the EGF pathway is typically caused by increased production of ligands, overexpression of EGFR, mutations of EGFR, a loss of negative regulatory mechanisms influencing this receptor, heterodimerization of EGFR with other ErbB receptors and transactivation of heterologous signalling pathways. Inappropriate activation of EGFR (ErbB1) occurs with several solid tumours – lung, colorectal, gastrointestinal stromal tumours, among others. This is most often caused by overproduction or a change in the structure of the EGFR receptor.

Overproduction of the EGFR receptor leads to inadequate, excessive “reception” of the extracellular signal. Cells with a concentrated receptor are stimulated to enter the cell cycle. EGFR is thus activated after the binding of various ligands (EGF, TGRβ, betacellulin, amphiregulin, HB-EGF, epiregulin, and TNFα). The structurally changed receptor is independent of the ligand, and when the structure is changed (e.g. when it is shortened), the receptor is permanently active even without ligand binding. After the binding of a ligand to EGFR, a conformational

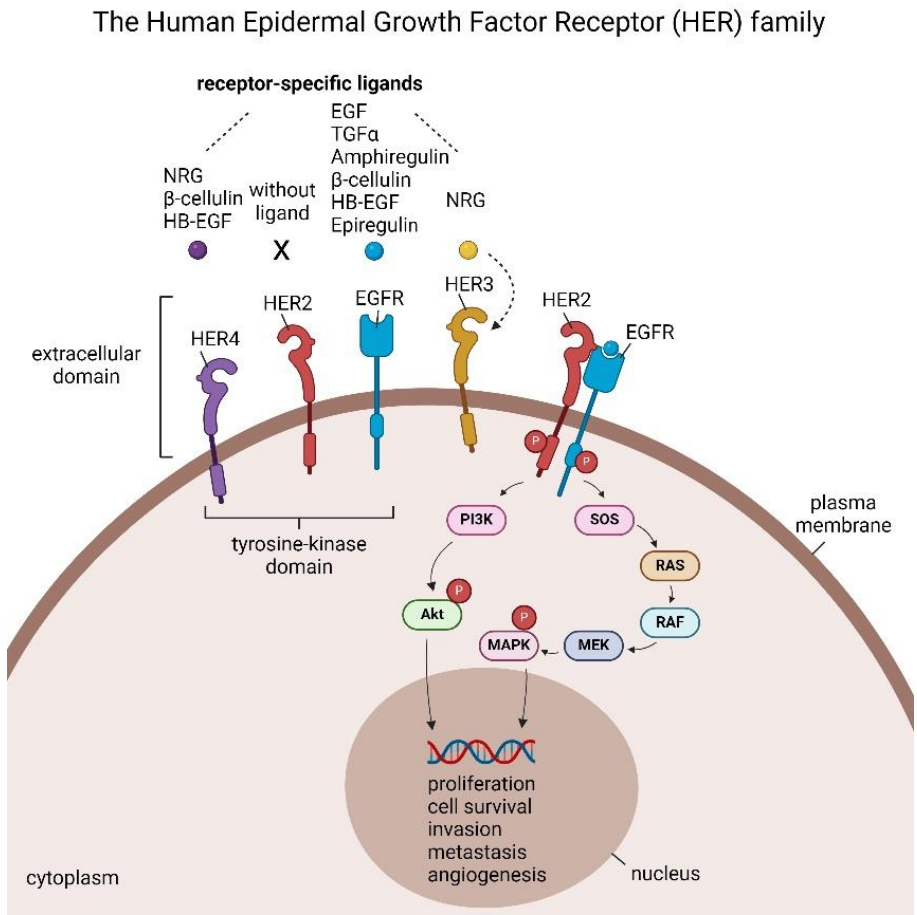

Figure 3
Tyrosine kinase receptors of the HER family.
Created with Biorender.com

change is induced which causes homo- and heterodimerization of the receptors with subsequent phosphorylation and activation of the signalling pathways responsible for cell proliferation, angiogenesis, survival and metastasis. The signalling pathway activated in patients with the same disease may be activated at different levels, which explains the unequal effectiveness of administering targeted treatments blocking a certain section of the signalling pathway.

The main pathways activated by the EGF receptor are:

- RAS-RAF-MEK-MAPK – controls transcription (c-Fos, AP-1 and ELK-1), the cell cycle (transition from G1 to S) and cell proliferation (activation of STAT-1 and STAT-3 through JAK – Janus kinase);
- PI3K-Akt-mTOR – controls translation and activates anti-apoptotic and other cascades.

The binding of monoclonal antibodies to the extracellular part of the EGFR receptor blocks the binding of ligands. Another possibility for blocking the EGFR receptors is the administration of tyrosine kinase inhibitors (TKI), which block the phosphorylation of this receptor inside the cell or block intracellular signalling pathways.

3.1.2.2 ErbB2 (HER2) receptor

The transformation of a normal cell into a malignant one cannot occur if the treatment targets either a growth factor or a receptor or affects some other link of the chain inside the cell. The overexpression of ErbB2 (HER2) serves as a prognostic and predictive marker. It is detected in 15 – 30% of patients with breast cancer and in 10 – 30% of patients with gastric cancer. Approximately 20,000 ErbB2 (HER2) receptors are present on healthy breast cells, and two million ErbB2 (HER2) receptors are present on tumour cells. The administering of human monoclonal antibodies blocking the ErbB2 receptor (anti-HER2 antibodies) shows favourable results in the treatment of malignancies with ErbB2 overexpression. At present, for example, dual EGFR and ErbB2 receptor inhibitors are being administered in the treatment of breast cancer. The monoclonal antibody trastuzumab acts against ligand-independent HER2 signalling. The tyrosine kinase inhibitor lapatinib targets both ligand-independent and ligand-dependent HER2 signalling, and the antibody pertuzumab acts against ligand-dependent HER2 signalling.

The problem with these drugs, however, is their possible toxicity, associated with the fact that, for example, ErbB2 is also found on the surface of normal tissues – e.g. epithelial cells, neuronal cells and others. ErbB2 (HER2) signalling is also necessary for the growth and survival of cardiomyocytes and maintenance of left ventricular function. Signalling activated by the binding of neuregulin ligand to the ErbB2-ErbB4 heterodimer plays a role in protecting the heart from oxidative stress damage. The administration of antibodies against the ErbB2 (HER2) receptor may therefore lead to cardiac dysfunction; therefore, administering antibodies and inhibitors against HER2 receptors to cancer patients may have adverse cardiological effects.

3.1.2.3 VEGFR

Currently, 3 tyrosine kinase receptors of the vascular endothelial factor VEGFR are known:

- VEGFR1(FLT1),
- VEGFR2 (FLK1/KDR),
- VEGFR3 (FLT4).

Knowledge about the ligands of these receptors, VEGF-A, VEGF-B, VEGF-C, VEGF-D and PIGF, is also growing. VEGFR1 has a role in the migration and proliferation of both tumour and endothelial cells; however, its activation does not lead to additional signalling to the nucleus. The VEGFR2 receptor plays a more significant role than VEGFR1. After the binding of ligands to this receptor, signalling pathways responsible for the mobilisation of endothelial progenitor cells, the proliferation, migration and survival of endothelial cells and also vascular permeability are activated. VEGF-A, which binds to VEGFR1 and VEGFR2 receptors with tyrosine kinase activity, is considered a key mediator of angiogenesis.

Under physiological conditions, after binding of the relevant ligand to the VEGFR2 receptor, the pathways responsible for, e.g. the proliferation and survival of endothelial cells, vascular permeability, as well as for the mobilisation of endothelial progenitor cells are activated.

Therefore, cardiovascular complications related to the anti-VEGF antibody or anti-VEGFR inhibitors include hypertension, cardiac dysfunction/heart failure and deep vein thrombosis.

3.1.2.4 Hormonal receptors

The inappropriate expression of hormone receptors is typical for the cells of some tumours. Oestrogen receptors (ER) are known to be associated with breast tumours, and ER expression is not observed in normal mammary gland epithelium. These receptors may be expressed in the early premalignant stages (in the hyperplasia stage). ER positivity is observed in approximately 60% of breast cancer patients. ER modulators bind to these receptors in the cytosol of mammary gland tissue cells, by which the interaction of the oestradiol/receptor is disrupted and the transduction of signals necessary for mammary cell proliferation is inhibited. The oestrogen/receptor complex stimulates proliferation through growth factors (e.g. IGF1). Not only the expression of oestrogen receptors is important, but also the identification of their subtypes. For example, in the presence of a certain subtype of receptors, even small amounts of oestrogens can have a growth-stimulating effect. Selective oestrogen receptor modulators, such as tamoxifen, toremifene and substances which help in the destruction of oestrogen receptors, e.g. fluvastatin, have anti-oestrogen effects. The enzyme aromatase is necessary for oestrogen production; thus, blocking this enzyme leads to a decrease in oestrogen levels and inhibition of the growth of oestrogen-dependent tumours. Aromatase inhibitors are administered to postmenopausal women. Anastrozole, exemestane and letrozole are used in clinical practice. Chronobiological monitoring point to the fact that the expression of hormonal receptors and sensitivity towards growth factors has a periodically fluctuating character with regard to individual biorhythms.

3.1.3 Signal transduction in cells

Pathways for signal transduction inside cells are divided into:
- non-receptor protein kinases,
- GTP-binding proteins (e.g. Ras).

Intracellular non-receptor tyrosine kinases

Approximately 540 kinases, which have a significant role in catalysing protein phosphorylation, have been thus far elucidated in the human genome. Kinases also play a role in the pathogenesis of cancer, neurodegenerative, cardiovascular, inflammatory and other diseases.

Approximately 90 of the total number of kinases are tyrosine kinases (TK), which are divided into receptor (localized in the intracellular domain of the receptor) and non-receptor (located in the cytosol, in the nucleus and on the inner side of the plasma membrane). Among the non-receptor TKs are, for example, the group c-Abl, c-Src. The reciprocal translocation t(9;22) – Philadelphia (Ph) chromosome is characteristic for chronic myeloid leukaemia (CML)

and acute lymphoblastic leukaemia (ALL), occurring in almost 100% of patients with CML and in 25% of patients with ALL. It is rare in patients with acute myeloblastic leukaemia (AML). This chromosomal translocation occurs within a gene called BCR on chromosome 22 and the region containing the *C-ABL* gene on chromosome 9. A rearrangement of broken sections of the BCR-ABL genes arises, leading to fused genes. C-Abl (*Abelson tyrosine kinase*) is a protein with tyrosine kinase activity, while BCR has serine kinase activity. Rearrangement of the *BCR-ABL* genes leads to the formation of the *BCR-ABL* gene encoding the oncoprotein p210. This oncoprotein has as a result an increase of TK activity and inappropriate growth of leukemic cells. Phosphorylation of p210 activates the RAS and STAT cascade, which also contributes to the excessive proliferation of leukaemia cells with the *BCR-ABL* gene. The P210 oncoprotein ultimately increases proliferation, leading to an escape from apoptosis and the accumulation of mutations in the malignant clone.

Abl protein with TK activity is also necessary for the viability of endothelial cells. The loss of its function leads to insufficient creation of new blood vessels and damage to existing vessels. Abl inhibition can lead to vasospasm, progression of the atherosclerotic process and arterial stenosis (e.g. the renal artery).

Imatinib is a low molecular weight TK inhibitor with an inhibitory effect against the TK domain of the *BCR-ABL* fusion oncogene. The mechanism of its action is associated with the Ph chromosome. Imatinib, a tyrosine kinase inhibitor of BCR-ABL, is used for the treatment of chronic myelocytic leukaemia, Philadelphia-positive acute lymphoblastic leukaemia, in patients with chronic eosinophilic leukaemia and in patients with c-KIT-positive stromal tumours of the GIT – GISTs. Increased activity of the non-receptor TK Src (*cellular Rous sarcoma viral oncogene homolog*) is found in several malignancies (e.g. colon, breast, pancreatic, ovarian, melanoma and other cancers). Src-kinases play a key role in mediating the signal from integrins and cadherins, thereby interrupting the communication of cells with each other and with the extracellular matrix. They also affect the organisation of the cytoskeleton and play a role in cell growth, apoptosis, invasiveness and the ability to metastasize; Src-dependent bone resorption is also observed. Among their inhibitors are dasatinib and bosutinib. In addition to TK, a group of serine and threonine kinases is also known. The regulatory protein kinase mTOR (mammalian target of rapamycin) is located predominately in the cytoplasm, and it plays an important role in cell proliferation, angiogenesis and cell metabolism. The protein kinase mTOR is a key point on which multiple signalling pathways converge; it is therefore an attractive therapeutic target. The PI3K-AKT-mTOR signalling pathway is so complex that it offers the possibility of multi-step inhibition. It affects cell proliferation through regulation of the production of cyclin D1 (which is responsible for passing through the G1-S critical point in the cell cycle). Overexpression of cyclin D1 is often present in patients with lymphoma, breast cancer, colorectal cancer, prostate cancer and melanoma. The protein kinase mTOR also controls the production of HIF1-α (and thus also expression of the regulatory genes responsible for the response to hypoxia). Under normal conditions, if a cell has an adequate supply of glucose and other nutrients, mTOR is activated and enables the synthesis of cellular proteins (cyclin D, HIF1-α, survivin, the glycolytic enzyme GLUT1 and others). In the absence of nutrients, mTOR is inactive and protein synthesis is inhibited. In times of nutritional deficiency, so-called “self-eating” (autophagy) is activated.

In tumour cells, mTOR is inappropriately activated by the effect of some signalling pathways, and as a result protein synthesis in tumour cells is not inhibited. Cells with an inappropriately activated mTOR molecules shown an increased consumption of glucose and a higher capacity for glycolysis, which correlates with a worse prognosis. The kinase mTOR can be abnormally activated due to mutations in the genes responsible for "upstream" proteins (e.g. EGFR, PDGFR, HER2, PI3K and cKIT). Inhibitors of mTOR include, e.g. rapamycin, temsirolimus and everolimus.

Kinases become oncogenic as a result of the activation of mutations (e.g. PI3K in cooperation with AKT). TKs are an attractive target for modern treatment with TK inhibitors, which block phosphorylation of the intracellular domain of the TK receptor or block intracellular signalling pathways. One of the problems of TKI treatment is the fact that their action does not have sufficient selectivity; many of them inhibit several targets at once, and they are less specific than monoclonal antibodies, which is associated with their toxicity, e.g. the multikinase TKI sunitinib (used in the treatment of renal carcinomas, GISTs and other malignancies) influences more than 50 kinases, while sorafenib (in hepatocellular carcinoma, renal carcinoma and other malignancies) inhibits more than 15 kinases. The toxicity of these pharmaceuticals is also caused by the fact that they are often combined with classical cytostatic and monoclonal antibodies.

The RAS cascade

A typical mutation used in intracellular signal transduction is mutation of the RAS proto--oncogene. The RAS product plays a key role in mitogenic stimulation within the RAS-RAF--MEK-MAPK pathway (mitogen-activated protein kinase) (Fig 4). The Ras (*Rous adenosarcoma*) cascade is activated by EGF receptors. The family of Ras proteins includes three primary members – HRas, KRas and NRas. Other small GTPase proteins, e.g. from the group Rho, Rab and others, belong to the so-called the Ras superfamily. Abnormalities of these proteins – e.g. RhoA, RhoC play a role in the motility (amoeboid movement) of individual cells, in the regulation of the cytoskeleton and in cell metastasis (as will be discussed below). After the binding of the growth factor to the TK receptor, autophosphorylation of this receptor occurs as well as the binding of the Grb2 protein. SOS binds to this complex. (Several hybrid proteins that arise as a result of gene fusion during chromosomal translocations, e.g. the bcr-abl protein, can cause inappropriate activation of Grb2 and SOS). Ras proteins have the ability to bind GTP, and its active form is created by binding the p21 protein with GTP. In a healthy cell, there is a balance between the active form of Ras GTP and the inactive form of Ras GDP. Disruption of the balance between GTP and GDP by Ras is an element of uncontrolled cell proliferation. If the cell is in stationary phase, Ras has a bound GDP molecule. If the cell is stimulated by a growth factor to proliferate, the active form – Ras GTP – is produced.

Activated p21 carries the signal through its effectors by activation of the MAPK and PI3K pathways, which lead to the phosphorylation of transcription factors in the nucleus, thereby affecting cell proliferation, differentiation and survival. Patients with a mutated form of RAS cannot benefit from anti-EGFR antibody treatment. The Ras p21 protein may take part in various processes. Its effects are determined not only by the type of cells, but also by the nature of

extracellular signals. Inappropriate activation of the RAS proto-oncogene has been observed in various types of malignancies. The most sensitive places for the arising of mutations in codons 12, 13 and 61 are specifically in the areas that interact with GTP. Mutations in RAS genes are responsible for the production of abnormal p21 protein, which is characterised by continuous activity as a consequence of the loss of the ability to inactivate. Genetic changes to RAS lead to a loss of sensitivity to GAP. If the sensitivity to GAP (GTPase-activating protein) is reduced, signalling pathways for proliferation, cytoskeletal changes and cell survival are inappropriately stimulated.

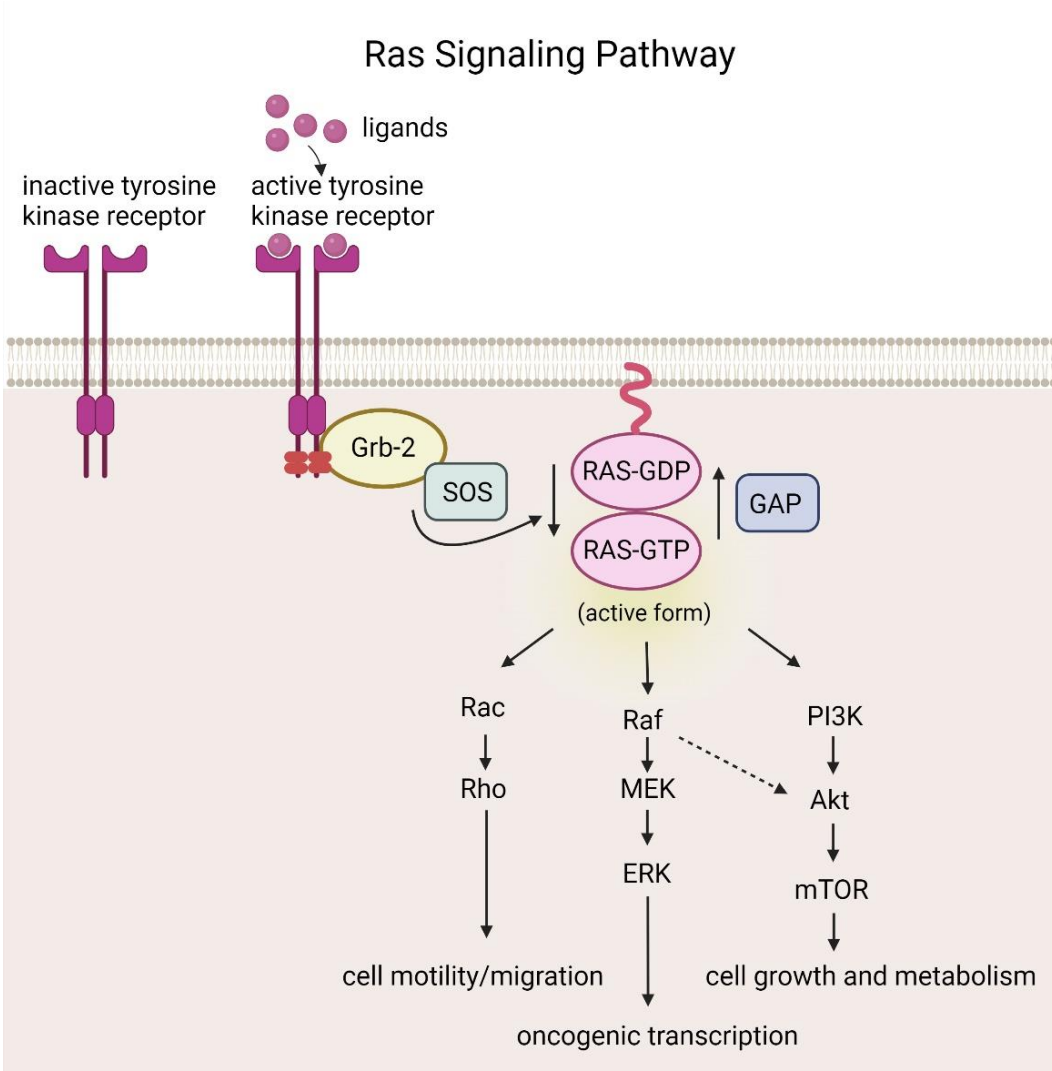

Figure 4
Role of RAS protein in signal trasnduction pathways.
After the binding of the growth factor to the tyrosine kinase receptor, the autophosphorylation of this receptor and the binding of the Grb2 protein occur. SOS is bound to this complex. Ras proteins have the ability to bind GTP. By binding the p21 protein with GTP, its active form is created. In a healthy cell, a balance exists between the active form of Ras GTP and the inactive form of Ras GDP. Created with Biorender.com

Of the total number of tumours, approximately 15 – 20% show the presence of RAS gene mutations. For example, in colon tumours, K-RAS mutations occur in 50% of cases; in lung tumours in 30%, and in pancreatic cancer in up to 90% of cases. N-RAS mutations occur in up to 25% of patients in AML and myelodysplastic syndrome. H-RAS (and K-RAS, N-RAS) Mutations tend to be non-selectively distributed in cancer of the thyroid. Mutations of RAS genes are also found in the early stages of the tumour process. Ras oncogenic stimulation of proliferation may not occur solely through the MAP kinase pathway. The Ras protein can also directly stimulate transcription factors in the nucleus.

3.1.4 Transcription factors

β-catenin

The Wnt-1/β-catenin pathway, which has a role in the pathogenesis of colon and rectal cancer, is a known signalling pathway. Beta-catenin is a proto-oncogene, bound mainly to E-cadherin. The result of the activation of this pathway after the binding of Wnt-1 to the extracellular receptor is the entry of β-catenin transcription factor into the nucleus, where the transcription of the genes responsible for cell division is started. Under normal circumstances, free β-catenin in the cytoplasm is dismantled with the participation of a negative regulator – the protein APC. The APC protein, together with other kinases, forms a complex that helps phosphorylate β-catenin, leading to its degradation. In cases of inactivation of the APC gene (by mutation or deletion), deregulation by the APC protein does not occur, and the relevant pathway is inappropriately activated, resulting in abnormal cell division. With colorectal cancers, inappropriate transcription of specific genes can also be triggered by an activating mutation of the β-catenin proto-oncogene, which as a result becomes insensitive to binding to the APC protein.

C-myc

The MYC proto-oncogene participates in several cell functions, specifically replication, growth, metabolism and differentiation. Deregulation of myc proteins takes place in more than half of malignancies and is associated with a poor prognosis. Overexpression of the transcription factor N-myc is observed in neuroblastomas, and the transcription factor L-myc is overexpressed in small cell lung tumours. With lymphoma, the most common translocation is t(8;14) (q24; q32), in which the proto-oncogene C-MYC moves to the vicinity of the IGH locus of the gene for heavy-chain immunoglobulins and thus falls under the influence of another promoter, which leads to its deregulation and excessive expression. This translocation occurs in approximately 80% of patients, and in Burkitt's lymphoma, there is a significant association with Epstein-Barr virus (EBV) infection. The product of the EBV gene increases the translocation frequency of the C-MYC gene, and the viral infection itself can increase its transcription. Increased transcription of the C-MYC gene is also observed in connection with oestrogens

in ER-positive breast tumours. Several transcription factors operate in interactions with other proteins (e.g. Fos and Jun form AP1). This complex subsequently influences the function of other genes in the process of cell division. Transcription factors are usually activated during chromosomal translocations (in lymphomas, prostate cancer and Ewing's sarcoma). More than 50 chromosomal translocations have been described in patients with leukaemia in recent years, and most of them affect transcription factors important in haematopoiesis. Transcription factors in haematopoietic cells bind to specific DNA sequences. One important transcription factor is *core binding factor* (CBF), which is comprised of subunits α (also known as AML1) and β. Translocations related to the transcription factor CBF negatively affect haematopoiesis. The translocation t(15;17)(q22;q11.2-12) occurs in nearly all patients with acute promyelocytic leukaemia (AML-M3). The gene for retinoic acid receptor on chromosome 17 rearranges with the PML gene on chromosome 15. The hybrid protein PML/RAR at t(15;17) negatively affects differentiation at the promyelocyte stage through the corepressor (N-CoR) complex. After the addition of *all-trans retinoic acid* (ATRA), which binds to the RAR receptor, the corepressor complex is released, thus allowing cell maturation and differentiation, leading to clinical remission. TEL/AML1 hybrid protein is one of the most important and frequent genetic changes in children with ALL. Another hybrid protein related to CBF is TEL/AML1, the cytogenetic basis of which is the translocation t(12;21) (p13;q22). It occurs in approximately 20% of these patients. It probably works as an inhibitor of the second allele of the AML1 gene, thus blocking the transcription of CBF-dependent genes. The monitoring of a group of paediatric patients with ALL confirmed an association between TEL/AML1 fusion of genes and a favourable prognosis. TEL/AML1 occurred more often in the group with very low risk. Aside from risk stratification, the presence of specific translocations interfering in the transcription of genes during haematopoiesis may also be an early marker of the presence of leukemic cells that have escaped antitumour treatment.

3.2 Replication potential of tumour cells – telomerase reactivation

Normal human cells in culture stop dividing after 50 to 100 generations and enter a senescence phase. It is possible to delay this aging for a certain time by inactivating the TP53 and RB genes. After a few more generations, however, chromosomal abnormalities appear in these cells, leading to death. Reactivation of the telomerase enzyme has been demonstrated in tumour cells. They replicate without limitation under *in vitro* conditions. The gradual erosion of chromosome ends limits the survival of normal cells. The erosion of the telomere is an inevitable consequence of chromosome ends not replicating. Therefore, with each replication, a gradual shortening of the DNA occurs. In germ cells and some stem cells (especially haematopoietic cells), the length of telomeres is preserved – it does not shorten – as a result of the expression of the telomerase enzyme. In the majority of somatic cells telomerase is not expressed, but reactivation of the telomerase enzyme has been demonstrated in tumour cells. The measure of this activation in some malignancies correlates with the aggressiveness of the malignancy – which has been seen mainly in lung, breast, colon and melanoma tumours. Higher telomerase activity was also found

in intestinal carcinomas in comparison to normal bowel tissue. Currently, several possibilities for influencing telomerase are being tested. In most tumours, this takes place through aberrant regulation of the enzyme telomerase, and in perhaps 10% of cases the cause is alternative lengthening of the telomere. The activity of telomerase has been shown (with the exception of germ cells) only in tumour cells, and its reactivated expression in these cells represents one of the presumed mechanisms that causes their immortality.

3.3 Dysregulation of apoptosis in tumour cells

Each cell in the body is ready to die by apoptosis if doing so is in the interest of the organism. Apoptosis – the suicide program of the cell – also ensures the elimination of cells progressing to the malignant stage; thus, dysregulation of apoptosis contributes to the tumour process.

Damage to the DNA, the activation of oncogenes, hypoxia, the absence of survival signals or the presence of death signals (via TNF, TRAIL and FASL ligands) play an important role in the inducting of apoptosis.

The key effectors of apoptosis are caspases. The signals that trigger apoptosis come:

- from intracellular sensors that detect the internal state of the cell,
- through an external route.

Apoptosis-inducing ligand 2/tumour necrosis factor-related apoptosis inducing ligand (Apo2L/TRAIL) belongs to a family of cytokines that selectively induce apoptosis in tumour cells. Apo2L/TRAIL binds specifically to 4 different surface receptors, of which only DR4 and DR5 are able to transmit the apoptotic signal. The activation of these receptors leads through the FADD (*Fas-associated death domain*) adapter protein to the activation of caspases 8 and 10, which then leads via an extrinsic pathway to apoptosis independent of p53. Activation of caspases 8 and 10 can also lead to apoptosis via a proapoptotic protein through the intrinsic mitochondrial pathway. The signal for initiating apoptosis is the release of cytochrome C from mitochondria. Activation of this pathway can increase the antitumour efficacy of chemotherapy and targeted treatment. Affecting apoptosis is possible through its inducers, which include, e.g. glucocorticoids, antioestrogens and antiandrogens.

Eluding apoptosis is associated with repression of FAS and FAS-L expression at the transcriptional level and also with overexpression of focal adhesion kinases (FAK). Apoptosis can be induced by apoptosis inducers, activation of anti-apoptotic genes and suppression of its inhibitors (products of *BCL2, MDM-2* and others).

Tumour cells are capable of avoiding apoptosis in several ways:

- by increased production of proteins blocking cell death (BCL2, IGF, Myc and AKT),
- by reduced production or inactivation of proteins supporting cell death (p53, Bax and Fas).

Even an expanding clone of tumour cells does not have to be problematic for the organism if these cells respond adequately to apoptotic signals. BCL2 proteins are divided into proapoptotic and antiapoptotic forms.

Negative regulators of apoptosis contribute to oncogenesis; for example, translocation of the *BCL2* gene has been linked with the development of follicular lymphomas. Overexpression of the anti-apoptotic gene *BCL2* also plays a role in apoptosis. The most common finding (in 70 – 95% of cases) in follicular lymphoma, for example, is the t(14; 18) (q32; q21) translocation, in which the anti-apoptotic gene *BCL2* is placed adjacent to the JH region of the locus for immunoglobulin heavy chain. The result is an overproduction of the BCL2 protein. The *BCL2* gene prevents apoptosis by stabilising the cell membrane. Overexpression of this gene leads to prolonged survival of the B-cell line, while its inhibition results in the inducing of cell apoptosis. Most B-cell malignancies express the differentiation antigen CD20 on their surface; therefore, they are suitable for treatment with rituximab, which is an anti-CD20 chimeric monoclonal antibody that selectively binds to CD20-positive (both healthy and pathological) B-cells. This causes the downregulation of the BCL2 and BCL-X proteins and activates caspases 9 and 3, thus conditioning a higher sensitivity of cells to therapy.

A whole range of stressors can affect tumour cells, with the most important cell stressors being a lack of oxygen or nutrients and acidosis. Abnormal growth of tumour tissue and imperfect vascular supply to tumours lead to hypoxia, and though tumour cells tolerate hypoxia, at the same time they attempt to escape it. Treatment resistance is associated with hypoxia. Mutations to stress response genes (e.g. *ATM* and *BRCA*) have been discovered in tumour cells. At present, additional apoptosis pathways, e.g. associated with damage to the endoplasmic reticulum, are also known. This causes the induction of the pro-apoptotic BAX protein (from the BCL2 family). Overexpression of the proapoptotic *BAX* gene in tumour cells leads to a higher sensitivity to chemotherapy. The translocation of the p53 protein from the nucleus to the mitochondria with the help of mitochondrial importing peptides also plays an important role in p53-mediated apoptosis. The loss of RB function may also contribute to p53-mediated apoptosis. Inactivation of the *TP53* can lead to a resistance to apoptosis. P53 signalling is also likely to be responsible for several toxic effects of chemotherapy. Activation of the *TP53* gene leads to modification of the state of chromatin and better accessibility of repair proteins to damaged DNA. The role of p53 is induction of cell cycle arrest in the G1 phase or inducing apoptosis (if the damage is irreparable). The *TP53* gene stimulates apoptosis by means of the transcription of specific genes or direct signalling. The *TP53* gene monitors the length of the telomeres – and when they are critically shortened, it triggers apoptosis. This also affects other effector genes in the case of cell cycle arrest, and others in the case of apoptosis. The cells contain control mechanisms that decide whether to stop the cell cycle or to repair damaged DNA. The role of *TP53* is induction of cell cycle arrest or programmed cell death. Inactivation of the *TP53* gene, which is observed in 70% of malignancies in human oncogenesis, leads to insufficient repair, and damaged DNA thus enters replication. The altered *TP53* gene is an attractive target for gene therapy. The *TP53* and *RB* genes can also be inactivated by human papillomavirus (HPV). Inactivation of these cellular regulators leads to increased proliferation activity of epithelial cells and their resistance to apoptosis. Heat shock proteins (HSP) also have an antiapoptotic effect. Their production is induced by UV radiation, hyperthermia, inflammation and reactive oxygen metabolites. These proteins operate as "chaperones" – accompanying proteins capable of binding other proteins (e.g. of the cell cycle) and maintaining their configuration. HSPs

stabilize stress-damaged pathological proteins, thereby increasing tumour aggressiveness. The administration of HSP inhibitors is promising. Apoptosis eliminates epithelial cells that lack the ability of focal anchorage to the basement membrane (*anoikis*, loss of home). Contact with the basement membrane is mediated by integrin receptors. FAK (*focal adhesion kinases*) together with TK Src play a significant role in signal transduction from integrin receptors. Disruption of FAK-signalling induces programmed cell death, and conversely, overexpression of these kinases enables apoptosis to be avoided. Many tumours are characterised by the overproduction of these kinases, thanks to which the tumour epithelial cells are able to escape the so-called *anoikis*-mediated apoptosis. The induction of apoptosis is an important goal of cancer therapy.

3.4 Evading destruction by the immune system

The human immune system is capable of recognising and destroying tumour cells and inhibiting tumour growth through innate and adaptive immunity.

Tumour cells use several mechanisms to avoid from destruction by the immune system, to which we assign, e.g. the following:

- loss of specific surface antigens,
- production of immunosuppressive cytokines (such as Il-4, TGF-β),
- creation of inhibitory proteins, e.g. programmed cell death protein-1 (PD-1) and its ligand programmed cell death protein 1 (PD-L1).

Tumour cells can escape the control of the immune system by suppressing it through immune checkpoints. Immune checkpoints are receptors that the immune system uses to sustaining tolerance for self-antigens as well as potentially damaging chronic inflammation. The immune tolerance of the organism is provided, for example, by T-regulatory lymphocytes (Treg) and activation of inhibitory signalling pathways, cytokines as well as a selection of certain clones in the thymus gland.

Some cytokines, in the scope of oncogenesis, can cause changes in the tumour microenvironment, thus increasing Treg in the tumour and activating immune checkpoints through the expression of inhibitory molecules. Treg lymphocytes normally comprise about 4% of $CD4^+$ T cells, and in the tumour microenvironment they can reach up to 20 – 30 % of the total population of $CD4^+$ T cells. In several types of malignancies their higher representation correlates with a worse prognosis, but in other types it correlates with a better prognosis. In certain cases, an anti-inflammatory effect may occur; since inflammation can worsen the progression of the tumour, its suppression can be beneficial.

Immune checkpoints can be used by tumour cells as a means of inhibiting the antitumour response of T-lymphocytes.

Until recently, cytokines, antibodies directed against various CD antigens, inhibitors of kinases (for example, Bruton's kinase) and immunomodulating treatment were mainly used with regard to immunotherapy in the treatment of oncologic diseases. In haemato-oncology,

CAR-T therapy with the use of genetically modified T-lymphocytes, with which the chimeric antigen receptor (CAR) has been created, is used.

However, the discovery of immune *checkpoint inhibitors* (CPIs) has caused a breakthrough in the treatment of several types of malignancies, even though, on the other hand, their effectiveness is not sufficient for most patients.

The role of CPI is unblocking, the so-called reactivation of antitumour immunity. In 2018, James Allison and Tasuku Honjo won the Nobel Prize for the discovery of checkpoint inhibition.

Among the most well-known checkpoints are:

- cytotoxic T-lymphocyte antigen 4 (CTLA-4),
- programmed cell death-1 protein PD-1 and its ligand PD-L1.

Even though the CPI receptors PD-1, CTLA-4 and PD-L1 ligand are established in clinical practice, research in recent years has identified new targets for manipulation of the immune system. Potential targets of immunotherapy include the immune checkpoints:

- LAG-3 (CD223) – lymphocyte activation gene 3,
- VISTA V – domain Ig suppressor of T-cell activation,
- TIM-3 – T-cell immunoglobulin 3,
- TIGIT – T-cell immunoreceptor with Ig and ITIM domains.

For the sake of completeness, it needs to be stated that, aside from the enthusiasm over the successes brought by current treatment through CPIs directed against CTLA-4, PD1/PD-L1, the problem of this treatment is their lack of effectiveness in the majority of patients, tumour resistance as well as their unexpected toxicity. Without knowledge of their mechanisms, their therapeutic potential cannot be sufficiently used. For example, there is potentially fatal cardiotoxic effects of PD1/PD-L1 inhibitors, which may be associated with the fact that this signalling has a cardioprotective role in the heart – namely suppressing excessive myocardial inflammation and directly protecting against, for example, myocardial infarction. CTLA-4 knockout mice had autoimmune myocarditis with myocardial infiltration by $CD4^+$ and $CD8^+$ T-lymphocytes, thus confirming the importance of immune checkpoints in the regulation of the T-cell immune response in the heart. If T-cell-mediated responses contribute to the progression of acquired heart disease, inhibitors of immune checkpoint are able to cause acceleration or decompensation of cardiac damage in vulnerable individuals. Thus, mutations of the PTEN and RAS genes contribute to the resistance to PD-1 inhibitors. Mutations in the RAS family may increase PD-L1 expression.

3.5 Ability of angiogenesis and lymphangiogenesis

Angiogenesis represents the formation of new blood vessels from pre-existing vasculature. This new formation of blood vessels from pre-existing vasculature is essential for the growth of solid tumours, but it can also contribute to the progression of haematological malignancies. Tumours with a diameter of just 1 – 2 mm already need their own new formation of blood

vessels for the supply of nutrients. Solid tumours produce both stimulators and inhibitors of angiogenesis. Angiogenesis is influenced by factors produced in tumour cells and in the tumour's microenvironment, but also by the host itself. Among the primary pro-angiogenic factors are VEGF, FGF and EGFR, and the main antiangiogenic factors include thrombospondin-1, angiostatin and endostatin.

Changes in the production of both pro-angiogenic and anti-angiogenic factors occur as a consequence of genetic alterations in tumour and non-tumour cells and as a result of stressful conditions (e.g. hypoxia). *TP53* mutations lead directly to upregulation of VEGF and downregulation of thrombospondin and indirectly cause increased activation of the HIF-1 pathway. In experimental research of acquired resistance to anti-angiogenic treatment with bevacizumab (an anti-VEGF antibody), it was found that most of the gene expression changes occur in the stroma and not in the tumour cells. The tumour stroma can induce increased activation of the FGFR, EGFR, and MET pathways, leading to VEGF independence. These pathways are therefore also the target of modern anticancer treatment. The formation of new capillaries also depends on the local extracellular architecture.

The extracellular matrix (ECM) is a three-dimensional network made up of several biologically active macromolecules. Aside from the supporting function, the ECM also has a role in the transmission of signals through adhesive receptors and is a reservoir of both promoters and inhibitors of angiogenesis. These molecules are released from the ECM by means of matrix metalloproteinases (MMPs). The ECM thus serves:

- as a support network,
- as a source of information needed for new capillaries,
- for the interaction between endothelial cells and ECM molecules in the maturation process of new blood vessels.

The maturation of new blood vessels is a complex process. In consequence of the action of the angiogenic stimulus, the attachment of pericytes and contacts of endothelial cell are disrupted, and the basement membrane is degraded by MMP. Endothelial *tip* cells and *stalk* cells, i.e. "the tip and stalk" cells, grow to an increased extent; they subsequently migrate and proliferate as a result of the action of VEGF and FGF factors. Endothelial cells line up, form chords, and vessels mature. Membrane type 1-matrix metalloproteinase (MT1-MMP), expressed on the surface of endothelial cells, degrades the surrounding matrix. When endothelial cells come into contact with pericytes, MT1-MMP is subject to downregulation. The expression of *tissue inhibitor of metalloproteinases-2* (TIMP-2) is then induced in endothelial cells and TIMP-3 in pericytes, which switch off the proteolytic process. MT1-MMPs are also necessary in the process of the formation of intracellular vacuoles in endothelial cells and lumen in the tubes of the blood vessels. The basement membrane arises as a product of endothelial cells and surrounding cells thanks also to the working of transforming growth factor-β (TGF-β). The basement membrane consists of laminins, collagen IV, XV and XVIII, fibronectin, perlecan, nidogens, fibulins, SPARC (*secreted protein acidic and rich in cysteine*) and heparan sulphate proteoglycans (HSPGs). If any of these proteins is missing, the basement membrane is damaged. During the maturation of endothelial tubes into new vessels, a "recruiting" of pericytes (under the

action of PDGF-BB from endothelial cells) and smooth muscle cells takes place. Pericytes and smooth-muscle vascular cells express angiopoietin-1 (Ang-1) on their surface and are bound through the Tie-2 receptor to the surface of endothelial cells. The subtle interaction between pericytes and endothelial cells ensures blood-vessel stabilisation, but can be antagonized by angiopoietin-2 (Ang-2) expression. An optimal number of pericytes is needed for successful angiogenesis and the stabilisation of new blood vessels. The level of oxygen increases in the newly vascularised area, as a result of which the level of VEGF decreases and the angiogenic cycle is closed. The basement membrane of the blood vessels not only represents a barrier and does not only fulfil a supporting function; it can also modify the behaviour of endothelial cells.

MMP actively influences:

- growth factors,
- receptors for growth factors,
- chemokines,
- adhesive molecules,
- mediators of apoptosis.

Several angiogenesis inhibitors are fragments of larger ECM molecules; e.g. angiostatin is derived from plasminogen, and tumstatin is derived from type IV collagen. MMPs act proangiogenically (e.g. they degrade the ECM, thereby facilitating neovascularisation), but they can also inhibit angiogenesis (e.g. by releasing endothelial cell growth inhibitors). MMP9 can help in angiogenesis by releasing VEGF from the ECM, but it can also work as an inhibitor of angiogenesis by releasing angiogenesis inhibitors from their "parent" molecules. The family of serine proteases belong among another ECM-associated proteases responsible for angiogenesis. ECM is not only a reservoir of vascular growth factors but also plays an important role in tumour angiogenesis through molecules such as collagen I, III, IV, XV, laminin-1 and 8, fibronectin or perlecan. Fibronectin helps the survival and migration of endothelial cells. It also increases the activity of VEGF and controls the retention of other angiogenesis modulators in the ECM, such as, e.g. thrombospondin-1, collagen I and III. The protein osteopontin located in the ECM also has a pro-angiogenic function. Tenascin-C, -R, -X and -W all belong to another family of ECM-adhesive glycoproteins that are usually expressed in branching blood vessels. These proteins interact with integrin receptors. Abnormalities of proto-oncogenes stimulating growth factors and receptors for growth factors play an important role in this process; furthermore, chemokines and other cytokines, adhesive molecules, proteases and their inhibitors, and transcription factors are also present.

Angiogenic factors include:

- growth factors and receptors
- adhesive molecules
- proteases and other proteins of the matrix:
- transcription factors and other molecules
- cytokines (including chemokines)

Excessive production of pro-angiogenic molecules (e.g. VEGF) and downregulation of anti-angiogenic molecules (e.g. thrombospondin) may contribute to the hyperpermeability of tumour vessels. If the balance between pro-angiogenic and anti-angiogenic signalling in endothelial cells is restored, tumour vessels can be structurally and functionally normalised, and this normalisation can reduce tumour cell invasiveness and metastasis to blood and lymphatic vessels. The leakage of fluid into the tumour interstitium as a result of leaky vessels reduces tumour perfusion and its oxygenation. Hypoxia contributes to a worsening of the immune system response and to the reduction of the effectiveness of anticancer therapy. A prerequisite for the forming of new blood vessels is the activation and proliferation of endothelial cells. SDF1 derived from fibroblasts act on endothelial progenitor cells, thus supporting the attraction of vessels to the tumour. Endothelial cells migrate into the remodelled stroma, where they differentiate and organise into new tubular structures. These processes are induced and regulated by a wide spectrum of factors from the VEGF family, then further FGF, angiopoietin 1 and 2, MAPK, adhesive molecules from the group of cadherins and integrins, proteins of the extracellular matrix, a number of transcription factors and regulatory molecules. VEGF also induces the expression of the *delta-like* ligand DLL-4, which binds in the so-called endothelial (*tip and stalk*) cells to Notch 1 and Notch 4 receptors. DLL-4-Notch signalling functions as a preventive mechanism preventing excessive angiogenesis. After the binding of ligands to VEGFR2, the relevant signalling pathways responsible for the following are activated:

- mobilisation of endothelial progenitor cells,
- proliferation, migration, survival of endothelial cells,
- vascular permeability.

VEGF-A (sometimes referred to simply as VEGF), which binds to VEGFR1 and VEGFR2 receptors with TK activity is considered to be the key mediator of angiogenesis. The tumour cells most malignancies (including haematological ones) express it in an increased measure. The role of VEGFR1 (flt-1) has not been fully clarified. Although it binds VEGF1 with 10 times greater affinity than VEGFR2, the subsequent signal transduction is weak. Specific circulating isoforms – VEGF121 and VEGF165 – bind to VEGFR2, which induces the so-called branching angiogenesis. VEGFR2, mediating the cascade of the signal transduction in endothelial cells, is also referred to as flk-1/KDR. The result of activation of multiple signalling pathways is abnormal vascular permeability, proliferation, migration of endothelial cells, and mobilization of endothelial progenitor cells (VEGF2+). Overproduction of VEGF by tumour cells is caused by several genetic and epigenetic factors. For example, changes in proto-oncogenes (*RAS* and *SRC*) and tumour-suppressor genes (*TP53* and *VHL*) contribute to it, and hypoxia is a known important stimulator of VEGF production. Other environmental factors include a low pH, sex hormones (e.g. oestrogens and androgens), growth factors (e.g. FGF, IGF-1, IL-1α and IL-6) released not only from tumour cells, but also from non-tumour cells (e.g. macrophages) and also chemokines, e.g. SDF-1 (stromal-cell-derived factor 1). HIF (hypoxia-inducible transcription factors – HIF-1α and HIF-2α) play a central role in the regulation of VEGF. Under normal normoxic conditions the factor HIF-1α is degraded by a system of enzymes that, together with the tumour suppressor von Hippel-Lindau (VHL) protein, are responsible for preventing

abnormal angiogenesis. More than 70% of patients with renal cell carcinoma are reported as having an inactivating mutation of the VHL protein, which leads to increased levels of HIF-1α and thus an increased risk of the tumour spreading. Under hypoxic conditions, this factor is accumulated and active; it translocates to the nucleus, where it dimerizes with HIF-1β to form the active transcription factor HIF-1. The HIF-1 complex regulates the transcription of many genes responsible for further tumour development, and since HIF also activates specific pathways regulating the multipotency of stem cells, a link between stem cells and hypoxia is hypothesized. An increase in HIF-1α is found in patients with carcinoma of the cervix, ovaries and breast, gastrointestinal stromal tumours as well as other malignancies. Various types of cells – such as endothelial cells, pericytes, smooth muscle cells, progenitor cells, monocytes and leukocytes – participate in angiogenesis. Further, FGF and other factors that stimulate the production of proteolytic enzymes responsible for the degradation of the vascular basement membrane in endothelial cells are also used in angiogenesis. PDGF, which stimulates the migration of supporting cells, is subsequently applied, thereby ensuring the stability of the newly formed blood vessels.

In tumour cells, aside from increased expression of pro-angiogenic factors, the suppression of negative regulators of angiogenesis (which includes angiostatin, endostatin, thrombospondin 1 and 2, interferon α and β, some interleukins, PAI and others) is observed. Anti-angiogenic molecules come from parent molecules of the extracellular matrix. The first known endogenous inhibitor of angiogenesis was thrombospondin-1 (TSP-1). Thrombospondin-1 and -2 are capable of affecting the structure through direct binding to collagen and fibronectin and can modulate protease activity. Despite the fact that most studies have confirmed that thrombospondin-1 is an effective inhibitor of angiogenesis in the tumour microenvironment, some experimental work has demonstrated that TSP-1 may even potentiate angiogenesis through the attraction and stimulation of smooth muscle cells and immune system cells that release proangiogenic factors.

Another endogenous inhibitor of angiogenesis, perhaps the best-studied, is endostatin – a fragment derived from the C-terminal fragment of collagen XVIII. Endostatin inhibits the migration of endothelial cells. Other collagen-derived antiangiogenic molecules include tumstatin, canstatin, and arrestin. Tumstatin suppresses the proliferation of endothelial cells; canstatin induces Fas-dependent endothelial cell apoptosis, and arrestin inhibits endothelial cell migration. Several studies have confirmed that heparan sulphate proteoglycans (HSPGs), which are located in cell membranes or in the ECM, also have anti-angiogenic effects.

Angiostatin is a significant inhibitor of angiogenesis. Under experimental conditions, the primary tumour suppressed the growth of its metastases, and after removal of the primary tumour, metastases began to rapidly grow. Angiostatin was found to be present in the circulation in the presence of a growing primary tumour, and it disappeared a few days after tumour removal. Vessels in tumours are leaky; they do not have a continuous basement membrane, lack a continuous layer of endothelial cells and have a chaotic arrangement of pericytes.

The results of deregulated angiogenesis are:

- increased vascular permeability,
- the leakage of plasma into the interstitia,
- accumulation of fluid in the interstitia,

- increased pressure inside the tumour,
- reduction of blood flow to the tumour,
- reduced oxygenation in the tumour,
- the ability of tumour cells to invade and metastasize is reduced,
- the penetration of anticancer substances into the tumour is reduced,
- immune response is reduced.

Stromal cells also regulate angiogenesis. For example, *cancer associated fibroblasts* (CAFs), also called *tumour associated fibroblasts* (TAFs) release *stroma-derived factor 1* (SDF1) and PDGF-C, thereby promoting the recruitment of endothelial progenitor cells. VEGF can also be produced by fibroblasts. Tumour cells themselves also intervene in angiogenesis in that they produce growth factors, cytokines/chemokines. The resulting attraction of cells of the immune system with the production of additional cytokines/chemokines has a mitogenic effect not only on tumour cells, but also on endothelial cells. High levels of granulocyte colony-stimulating factor (G-CSF), which mobilises endothelial progenitor cells, have been found in tumours resistant to treatment anti-VEGF therapy.

Aside from angiogenesis by budding, so-called mosaic vessels and vasculogenic mimicry can also be created in tumours. In mosaic vessels, the walls of the vessels are also partially formed by tumour cells, while vasculogenic mimicry refers to the channels through which blood flows. The process of angiogenesis is very complex and still poorly understood. It includes:

- activation and survival of endothelial cells,
- degradation of the basement membrane,
- proliferation and migration of endothelial cells,
- creation, elongation and remodelling of tubes,
- maturation of pericytes and smooth muscle cells associated with the vasculature.

Inhibition of angiogenesis has as a goal to prevent the formation of new blood vessels and repair the leaky wall of immature blood vessels so as to eliminate the aforementioned consequences. Overproduction of VEGF is at present blocked in clinical practice with the use of the humanised monoclonal antibody bevacizumab. Bevacizumab does not cause the death of mature formed vessels but prevents the formation of new vessels and causes the modification of immature vessels with a leaky wall so that chemotherapy drugs and oxygen can better penetrate to the tumour.

Pazopanib and other drugs also belong to inhibitors of TK domains of VEGFR 1-3, PDGFR and KIT receptors.

Tumour cells are capable of releasing not only angiogenic factors, but also lymphangiogenic factors.

Lymphangiogenesis

The existence of "neolymphangiogenesis" processes in tumour tissue is currently confirmed, and it appears that the new lymphatic system arises exclusively from the existing lymphatic

capillaries. Growth factors VEGF-C and VEGF-D, which are released by tumour and other cells of the tumour's internal environment, have been found to play an important role in lymphangiogenesis. What's more, a correlation was found between VEGF-C expression and the degree of lymph node metastasis. The well-known factor PDGF-BB is a new factor in the development of lymphatic vessels, and its effect during lymphangiogenesis was shown to not be dependent of the VEGF-C/-D/VEGFR-3 signalling pathway. The effects of other factors in lymphangiogenesis processes, e.g. of integrins and hepatocyte growth factor (HGF), are also currently a subject of study. HGF has been shown to promote the invasiveness of tumour cells by activation of proteases degrading ECM and basement membrane components. Activated lymphatic endothelial cells produce several growth factors and cytokines with a direct stimulatory effect on tumour cells. The lymphatic system also contributes to tumour progression from the viewpoint of active support of angiogenesis.

3.6 Ability to metastasize

The study of metastasis biology at the cellular, molecular, biochemical, physical and systemic levels has undergone dramatic growth over the last 20 years. While the precise pathways are still under investigation, recent research has indicated the new roles of subcellular and cellular mechanisms, such as

- promoting genes with metastasis-driving mutations,
- epigenetic changes
- cancer stem cells,
- circulating tumour cells,
- avoiding of the programmed apoptosis of cells (anoikis),
- cell-cell interactions (between cancer cells, immune cells, and cells in the tumour microenvironment),
- epithelial-to-mesenchymal transition,
- signals from extracellular matrix components,
- extracellular matrix mechanical pressures,
- metastatic dormancy,
- dynamic plasticity of cancer cells.
- self-seeding,
- the gut and intratumoural microbiota.

Moreover at systemic level, inflammation, immune system modulation and immune checkpoint regulation can contribute to this process.

The metastatic cascade includes:

- the detachment of cancer cells from the primary tumour and the gaining of an invasive phenotype,
- local invasion into surrounding tissue,
- intravasation into the circulation,

- systemic transportation,
- extravasation,
- the formation of colonies at distant sites with adaptation and proliferation in secondary organs (Fig. 5).

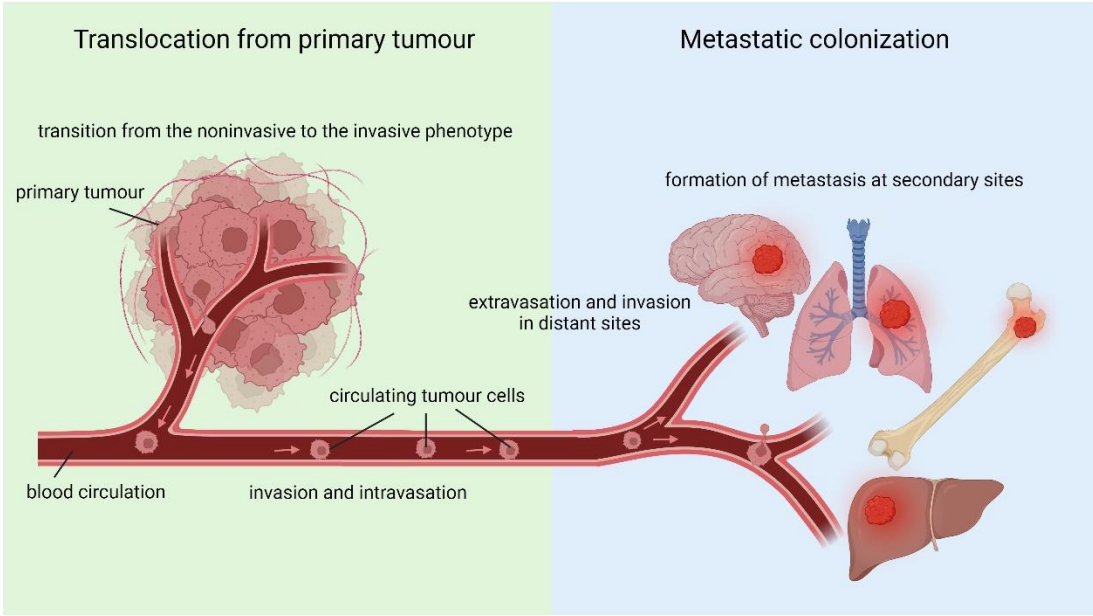

Figure 5
Translocation of tumour cells from the primary tumour to distal organs.
Created with Biorender.com

3.6.1 Detachment and invasiveness

A prerequisite for the metastasizing of a malignant tumour is the acquiring of an invasive phenotype. Tumour cells invade as multicellular units, in clusters or individually, and two programs are applied in this process – either mesenchymal (protease-, stress-, fibro- and integrin-dependent) or amoeboid (Rho/ROCK dependent, integrin- independent) invasion. Aberrant expression of *Rho-associated kinase* (ROCK) is associated with an unfavourable prognosis. Rho-associated kinases also activate matrix metalloproteinases. Tumour cells can "convert" between these two programs; therefore, when suppressing *single-cell* invasiveness, it is necessary to strike at both of these programs. EMT plays a major role in acquiring the ability to invade through the basement membrane and matrix during intravasation.

This program is induced by increased activation of genes, e.g.: *SNAG, SNAIL, SLUG, TWIST,* and *ZEB1,2,* and at the same time requires active signalling between stromal cells and tumour cells. As a result of this transition, epithelial tumour cells acquire the so-called mesenchymal phenotype (with the expression of, e.g. vimentin, fibronectin and N-cadherin) and change into *cancer stem cell-like* (CSC-like) cells, which resemble stem cells. During EMT, intercellular connections are dissolved; cell polarity is lost; epithelial antigens are lost (e.g. E-cadherin

and cytokeratins are downregulated); cells become resistant to apoptosis, and they acquire the ability to self-renew. Most of the currently used chemotherapy drugs preferentially kill the so-called *nonCSC* (*non-cancer stem cells*). Some miRNAs, e.g. the miR200 family, which causes post-transcriptional suppression of ZEB1 and ZEB2, also play an important role in EMT. Detachment of the tumour cell from the primary tumour requires overcoming:

- mutual adhesion of cells (which is provided by cadherins and catenins – receptors for interactions between cells);
- adhesion to the matrix (which is provided by integrins – receptors for the interaction of cells with the extracellular matrix). The production of adhesive molecules decreases during the phase of detachment of the cell from the primary tumour.

Proteolytic enzymes play a role in the local invasion of the basement membrane, to the surrounding extracellular matrix and also to the layers of stromal cells. These include, for example:

- MMP (gelatinases, stromelysins, collagenases, matrilysin and others),
- serine proteinases (plasmin, urokinase and tissue plasminogen activator).

The interaction of urokinase plasminogen activator (uPA) with the relevant receptor (uPAR) leads to the activation of plasmin. Plasmin degrades fibronectin and laminin and activates MMP. In normal tissue, MMP activity is controlled through transcriptional and post-translational mechanisms, but in tumours their activity is increased; they are products of not only tumour cells, but also stromal cells. The aggressiveness of the stroma increases with the progression of the tumour – the stroma becomes "reactive" and has properties reminiscent of tissues during the healing of a wound or with chronic inflammation. Tumour cells invading into reactive stroma come into contact with immune cells (e.g. macrophages), fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes and bone marrow-derived cells (e.g. mesenchymal stem cells). Aggressive stroma affect tumour cells back and cause them to become more aggressive, e.g. tumour-associated macrophages as a result of activation of EGFR signalling (through epidermal growth factor receptor) on breast cancer cells. Adipocytes, through the release of IL-6, also increase the aggressiveness of malignant cells of this cancer.

3.6.2 Intravasation and transportation

Intravasation means the entry of tumour cells into the lumen of blood or lymphatic vessels. Dissemination through blood vessels represents a more significant mechanism than spread through the lymphatic system. The new lymphatic system arises from the existing lymphatic capillaries. The factors VEGF-C, VEGF-D, platelet-derived growth factor, PDGF-BB, HGF, and others have been found to have a part in lymphangiogenesis. The lymphatic system also contributes to tumour progression by actively supporting angiogenesis. Intravasation can be facilitated by several molecules; e.g. penetration in the microcirculation is increased by the cytokine transforming growth factor β (TGF-β) and EGF produced by tumour-associated

macrophages. Intravasation is also associated with the structure of the vessels in the tumour. The result of deregulated angiogenesis is increased vascular permeability, which facilitates the entry of tumour cells into the vessel lumen.

Circulating tumour cells (CTCs) typically arise from epithelial cells that undergo epithelial-to-mesenchymal transition (EMT), resulting in the loss of cell–cell adhesion and apical–basal polarity, the reorganization of the cytoskeleton, acquiring properties of cancer stem cells, and resistance to therapy. This process is regulated by transcription factors in tumour cells (Snail 1, Slug, ZEB1, Twist, FOXC2, etc.) and signalling pathways from the tumour microenvironment (WNT, Notch, Hedgehog, TGFβ, FGF, EGF, HGF signalling, etc.). Additionally, the hypoxia and activation of signalling pathways, including PI3K, WNT/β-catenin, and MAPK, affect EMT regulation. Many studies focus not only on CTC detection and enumeration but also on CTC biomarkers, among which EMT markers are of great interest.

Cancer cells can induce neutrophils to release neutrophil extracellular traps (NETs), which sequester CTCs and promote the metastatic process.

A certain number of CTCs can be eliminated by the programmed apoptosis of cells – anoikis (known as death from loss of home).

However, some cells can survive by mechanisms, such as:

- via oncogene activation (e.g., *ERBB2* and *RAS*),
- an integrin switch (e.g., the downregulation of αvβ3 integrin expression),
- the constitutive activation of antiapoptotic pathways (e.g., the PI3K/Akt signalling pathway),
- the triggering of EMT,
- microRNAs (e.g., the downregulation of the miR200 family),-
- high oxidative stress (e.g., activated growth factor receptors increase intracellular reactive oxygen species production by enzymes such as NADPH oxidase and lipoxygenase),
- hypoxia,
- the metabolic reprogramming of cancer cells.

Not only anoikis, but also natural killer cells (NK cells), haemodynamic stress, the cytotoxic effect of substances from macrophages (e.g. NO) and other factors are responsible for the death ofCTC..

According to some authors, tumour cells survive in the circulation for several minutes to hours. Metastasizing is a highly inefficient process. It is estimated that only 1 in 10,000 to 1 in 100,000 circulating tumour cells have the ability to establish metastatic colonies.

Multifunctional microvesicles, which, aside from coagulation and immune reactions, are also important in communication (*cross talk*) between tumour cells and other cells (e.g. thrombocytes). L- and P-selectins as well as tissue factor (TF) play an important role in this communication.

A tumour cell's diameter, which is often larger (approximately 20 μm) than the diameter of the capillary lumen (7 μm), can also be responsible for tumour cells becoming stuck in the capillaries. The most important, and for metastasis the most risky, subpopulation of circulating tumour cells are cells with properties similar to stem cells (more details in chapter 4).

3.6.3 Extravasation and nidation in secondary localisations

Those circulating tumour cells that survive the transit in the circulation become attached to the microvasculature of the receptive organs. During extravasation, tumour cells migrate by:

- paracellular transport (via junctions between endothelial cells),
- transendothelial transport (through the body of endothelial cells).

Some tumour cells produce factors that cause retraction of endothelial cells. Others divide in the vessel lumen until the vessel ruptures as a result of the growing mass. It is still not been shown whether "tissue tropism" is only a passive process in which the diameter and structure of the capillary are decisive, or whether it is an active process in which genetically determined interactions apply. Some ligand- and receptor-based tumours form secondary foci in characteristic locations; other tumours are able to metastasize to many organs. The localities of metastases are determined by a set of mechanisms and substances that take part in the process of extravasation and attachment of cells.

Cancer cells can attach to specific distant organs/tissues and form colonies through distinct adhesion molecules, including proteoglycans (e.g., CD44), mucins (e.g., MUC16), integrins (e.g., $\alpha 2\beta 1$), and the members of the immunoglobulin superfamily (e.g., ICAM1, VCAM1, and L1CAM).

Tumour cells are probably drawn to the host tissue by certain factors, which include the chemokine gradient in the blood and in the tumour microenvironment, the premetastatic niche, and others. Nidation of tumour cells released from primary prostate and breast carcinomas in secondary locations is mainly influenced by *stroma-derived growth factor 1* (SDF-1/CXCL12), which binds to the chemokine receptor CXCR4. Expression of the CXCR4 receptor (for the factor SDF1) on tumour cells (e.g. breast and prostate cancer) is a typical determinant of bone metastases. Activation of this receptor increases the affinity of tumour cells to the endothelial cells of the microvasculature. The structure of the endothelium in the target organs is also an important factor in extravasation. In the case of metastases to the lungs, similar as with the metastases to the brain, it is difficult to overcome the continuous layer of the endothelium, whereas in the bones and liver, where the endothelium is fenestrated, this problem of overcoming the barrier does not exist. Some proteins (e.g. epiregulin, angiopoietin like 4 protein – ANGPTL4 and MMP1,2) can help disrupt the endothelium in the lungs, thereby facilitating the extravasation of breast cancer cells. Other products of primary tumours (ANGPT2, VEGF, PIF and MMP3,10) cause hyperpermeability of the pulmonary capillaries even before the arrival of circulating tumour cells. Similarly, as before the formation of primary tumours, inflammatory changes, remodelling of the extracellular matrix, increased action of reactive oxygen metabolites and other bioactive molecules are found, and even before the establishment of metastases, it is possible to observe changes in the so-called pre-metastatic niche, which is important for the formation of organ-specific metastases.

Several changes at the cellular and molecular level precede the formation of metastases in distant tissues.

For example, with the formation of lung metastases, even prior to the arrival of tumour cells from the primary site to the lungs, haematopoietic progenitor cells (VEGFR1+) travel from the bone marrow into circulation and settle in the lung tissue, where they adhere to fibronectin produced by fibroblasts and fibroblast-like cells. Adherence of these progenitor cells to fibronectin is mediated by the integrin VLA-4 (α4β1), which the haematopoietic progenitor cells express. Fibronectin is a ligand for haematopoietic progenitor cells (VEGFR1+). In the lungs, these cells form cell clusters also due to the enzyme MMP-9, which they produce, and this facilitates the further extravasation of progenitor cells into the premetastatic niche after the degradation of the basement membrane. In coordination with the factor ID1 and in connection with other stromal cells, other integrins and chemokines (e.g. SDF-1) are activated, which help the attachment of tumour cells in the new location. Subsequently, endothelial cells (VEGFR2+), which are responsible for vascularisation, get to the new focus. The administration of monoclonal antibodies against VEGFR1+ progenitor cells eliminated the formation of the premetastatic niche, and the administration of antibodies against VEGFR2+ endothelial cells led to the formation of only micrometastases without vascularisation. Also, for example, the expression of metadherin on breast cancer cells predisposed them for the development of lung metastases. Bone metastases are among the best-studied secondary locations of tumours. The genes CXCR4, RANKL and others, for example, are responsible for the organ specificity of metastasis in bones. RANKL (*receptor activator of nuclear factor kappa B ligand*), which is a member of the TNF ligand family, binds to RANK – a receptor on the surface of osteoclasts and their precursors, thereby stimulating their proliferation, and this pathway contributes to bone destruction in the process of metastasis. Tumours that form metastases in bones also produce factors, e.g. endothelin-1, interleukin-1, TNF α and parathyroid hormone-associated protein PTHrP, which directly stimulate osteoclasts through upregulation of RANKL. The increased activity of osteoclasts causes the release of calcium and growth factors from the bone matrix, such as TGFβ, IGF, FGF, PDGF and BMPs (*bone morphogenic proteins*), which can further potentiate the metastatic process. Abnormal expression of osteopontin and downregulation of osteoprotegerin (an inhibitor of bone resorption) also play a part in this process. An imbalance in the expression of RANKL and OPG is one of the mechanisms responsible for bone metastases. With their origin, the so-called *circulus viciosus* occurs – factors released from tumour cells stimulate osteoclasts in the bone, which leads to the degradation of the bone matrix, which after degradation releases factors that stimulate tumour growth. After extravasation, the mesenchymal-to-epithelial (MET) process can begin.

Cells that have leaked from the vessels by extravasation can remain in a dormant state, forming micrometastases or, if they sufficiently supplied with blood and escape the host's defence mechanism, they form macrometastases. Awakening from the state of dormancy can occur even many years after the completion of anticancer treatment. Genetic or epigenetic factors, cytokines, chemokines and changes in the microenvironment (e.g. during inflammation) are likely responsible for this.

At present, several metastasis models are among the attractive areas of metastasis research.

According to the thus far accepted "linear" model of metastasis, cells capable of metastasizing are those cells that arise from the gradual accumulation of mutations in specific genes (proto-

-oncogenes, tumour-suppressor and repair genes), i.e. malignant cells with an aggressive phenotype, are the cells that are capable of metastasizing.

Attention is currently being paid to the so-called "parallel" metastasis model. In the parallel model of metastasis, the independent progression of metastases from cells released from a tumour in the early stages of tumour development is also described.

Metastasizing as a two-way street also ranks among the latest areas of research: According to experimental models, a large tumour does not always mean aggressiveness and the risk of metastasis. A large mass may not only be the result of progression, but also of *self-seeding* by circulating cells. Some tumours are excellent "self-seeders" and incompetent "distant seeders", and vice versa. The most aggressive CTCs are related to the infiltration of the primary tumour or established metastasis in a process of "self-seeding". Self-seeding in metastasis is the recruitment of cancer cells and the re-seeding of primary tumours and existing metastases by aggressive cancer cell clones.

This is not always possible - since the stroma in the original location may already be altered by previous treatment, e.g. irradiation (more details about CTCs in chapter 4).

Cancer cells can attach to specific distant organs/tissues and form colonies through distinct adhesion molecules, including proteoglycans (e.g., CD44), mucins (e.g., MUC16), integrins (e.g., α2β1), and the members of the immunoglobulin superfamily (e.g., ICAM1, VCAM1, and L1CAM).

References

Adrover JM, McDowell SAC, He XY, et al. NETworking with cancer: The bidirectional interplay between cancer and neutrophil extracellular traps. Cancer Cell 2023; 41: 505–526.

Comen E, Norton L. Self-seeding in cancer. Recent Results Cancer Res 2012; 195: 13–23.

Fares J, Fares MY, Khachfe HH, et al. Molecular principles of metastasis: a hallmark of cancer revisited. Signal Transduct Target Ther 2020 Mar 12; 5(1):28.

Gerstberger S, Jiang Q, Ganesh K. Metastasis. Cell 2023; 186: 1564-1579.

Giuliano M, Giordano A, Jackson S, et al. Circulating tumor cells as prognostic and predictive markers in metastatic breast cancer patients receiving first-line systemic treatment. Breast Cancer Res **2011;** 13: R67.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144(5): 646-74.

Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Disc 2022; 12(1):31- 46.

Hiratsuka S, Duda DG, Huang Y, et al. C-X-C receptor type 4 promotes metastasis by activating p38 mitogen-activated protein kinase in myeloid differentiation antigen (Gr-1)- positive cells. Proc Natl Acad Sci USA 2011; 108(1):302-7.

Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science 2011; 331(6024):1559-64.

Kaplan RN, Rafii S, Lyden D. Preparing the „soil": the premetastatic niche. Cancer Res 2006; 66(23):11089-93.

Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 2005; 438: 820–827.

Massague J, Ganesh K. Metastasis-Initiating Cells and Ecosystems.Cancer Discov 2021 Apr;11(4):971-994.

Mego M, Karaba M, Minarik G, et al. Circulating Tumor Cells with Epithelial-to-mesenchymal Transition Phenotypes Associated with Inferior Outcomes in Primary Breast Cancer. Anticancer Res 2019; 39: 1829–1837.

Nepali PR, Kyprianou N. Anoikis in phenotypic reprogramming of the prostate tumor microenvironment. Front Endocrinol. 2023; 14: 1160267.

Peinado H, Zhang H, Matei IR, et al. Pre-metastatic niches: Organ-specific homes for metastases. Nat Rev Cancer 2017; 17, 302–317.

Pretzsch E, Bosch F, Neumann J, et al. Mechanisms of Metastasis in Colorectal Cancer and Metastatic Organotropism: Hematogenous versus Peritoneal Spread. J Oncol. 2019 Sep 19;2019:7407190.

Sevcikova A, Mladosievicova B, Mego M, Ciernikova S. Exploring the Role of the Gut and Intratumoral Microbiomes in Tumor Progression and Metastasis. Int J Mol Sci 2023 Dec 6;24(24):17199.

Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002, 2, 442–454.

Thiery JP, Acloque H, Huang RYJ, et al. Epithelial-mesenchymal transtitions in development and disease. Cell 2009; 139:871-89.

Valastyan S, Reinhardt F, Benaich N, et al. A pleiotropically acting mikroRNA, miR-31, inhibits breast cancer metastasis. Cell 2009; 137:1032-104.

Zhong W, Wang Q, Shen X, Du J. The emerging role of neutrophil extracellular traps in cancer: From lab to ward. Front. Oncol 2023, 13, 1163802.

4 Circulating and disseminated tumour cells

Metastatic disease is a major cause of morbidity and mortality in cancer patients. With a large portion of tumours, the first site of metastasis is the regional lymph nodes, but even at an early stage, tumours can metastasize via the haematogenous route without causing clinically detectable distant metastases. Approximately 30 – 40% of patients with breast cancer, but also with other solid (non-haematological) tumours, who present clinically without metastases, have occult (hidden) metastases present in the bone marrow, blood or lymph nodes. These occult metastases are a part of so-called micrometastatic disease. In the bone marrow, we call them *disseminated tumour cells* (DTC), and in the peripheral blood we call them *circulating tumour cells* (CTC), even though, in a broader sense, CTCs are also disseminated tumour cells. The effort to suppress micrometastatic disease is specifically the reason why for some types of tumours adjuvant (augmentation) therapy is provided. Finding the presence of micrometastatic disease can have prognostic significance, and its characterisation from the viewpoint of the presence of treatment targets can be applied in the individualisation of the treatment of oncology patients.

4.1 Circulating tumour cells

Circulating tumour cells (CTCs) are cells involved in the metastatic cascade and tumour spread. It is estimated that approximately 3 million tumour cells are released into the circulation within 24 hours from one gram of tumour tissue. Circulation represents an unfavourable environment for CTCs, due mainly to the action of physical forces, cells of the immune system, as well as the absence of contact with other cells (*anoikis* – apoptosis due to loss of contact with other cells). It is estimated that 99% of CTCs die with a biological half-life of 1 – 2.5 h. Perhaps 1% of the cells survive the stay in circulation, and 0.1% of them are able to form metastases, indicating considerable inefficiency of the metastatic cascade. Cells can enter the bloodstream either passively, e.g. when growing into blood vessels, or actively, due to their increased motility. It is believed that tumour cells that enter the blood passively probably have less biological significance, undergo apoptosis more quickly and are unable to complete the metastatic cascade. In contrast, cells that are capable of actively travelling are cells with the

properties of tumour stem cells (see below), and it is these cells that play a key role in the formation of metastases.

4.1.1 Methods of CTC detection

CTCs are currently detected using several methods (Fig. 6):

1. immunofluorescent methods,
2. molecular genetic methods based on the detection of the expression of tumour-associated antigens using RT-PCR,
3. filtration methods using the different sizes of tumour and haematopoietic cells,
4. platforms utilising microfluidic technology,
5. methods based on the detection of proteins secreted by CTCs into the surroundings (EPISPOT assay).

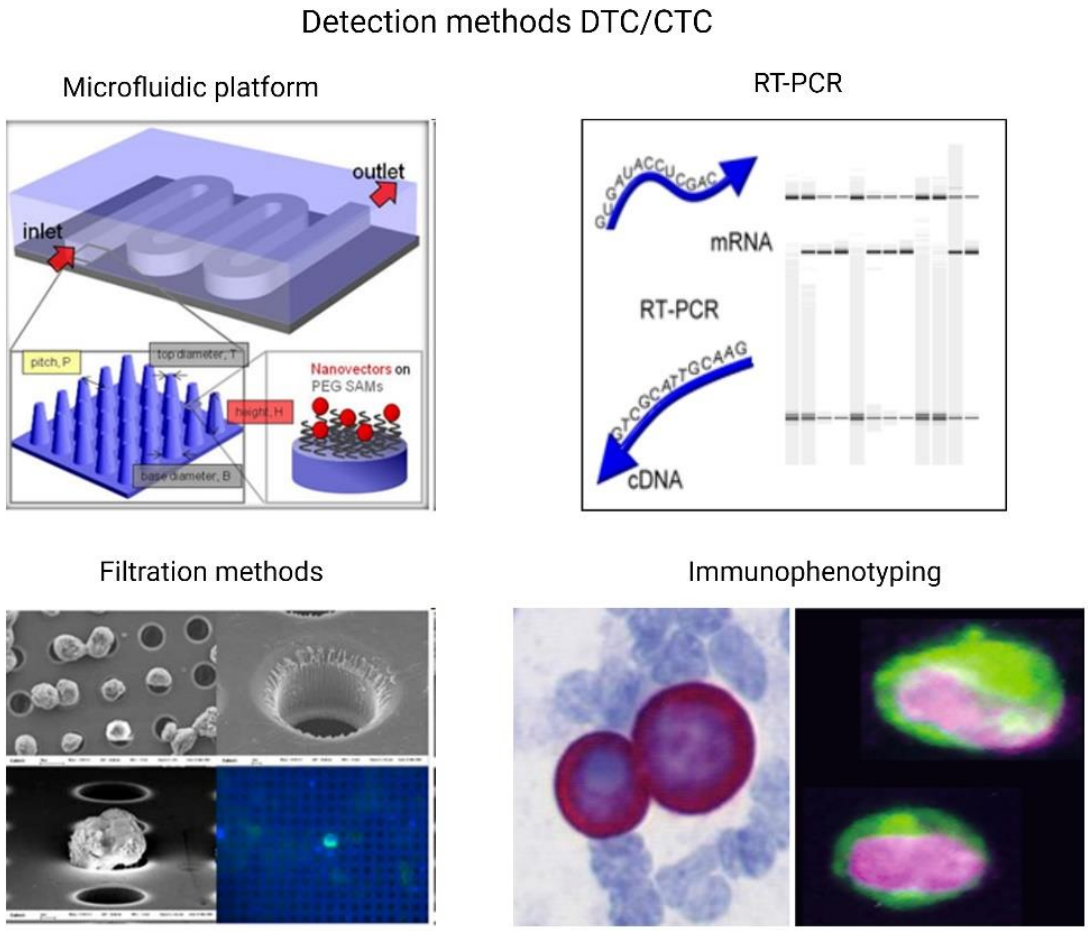

Figure 6
Options for the detection of circulating and disseminated tumour cells.
Created with Biorender.com

CTCs represent a heterogeneous group of cells, including viable cells, apoptotic cells, cells capable of establishing metastasis, as well as dormant cells. Individual methodologies detect different subpopulations of these cells, and CTCs detected by one methodology are not identical with CTCs detected by another method. At present, probably none of the methods is capable of detecting all CTCs; on the other hand, this is not actually the goal. From a prognostic point of view, it is necessary to detect the most biologically (and clinically) significant subpopulation, which should be represented by cells with the properties of tumour stem cells.

CTCs are identified on the basis of detecting epithelial markers, such as cytokeratins and/or epithelial common antigen (EpCAM). A whole spectrum of methods is used to detect them; the most data is with the use of methods based on the principle of quantitative RT-PCR (qRT-PCR) and on the principle of immunofluorescence. The main disadvantage of qRT-PCR is the impossibility of enumeration (detecting the number) of CTCs and the reproducibility of results between different workplaces. The most recognised method at present is the CellSearch™ system (Veridex Corporation, Warren, NJ), which is the only one approved by the US Food and Drug Administration (FDA). This system identifies CTCs as nucleated tumour cells, morphologically intact, expressing cytokeratin 8, 18, or 19 and not expressing haematopoietic antigens (CD45). The method is based on the principle of immunofluorescence and enables the enumeration of CTCs. The development of the method currently focuses on the possibility of identifying other antigens on the surface of CTCs, e.g. HER-2/neu protein for the purpose of identifying therapeutic targets and the possibility of individualising treatment for patients.

Growing knowledge indicates the existence of an additional population of CTCs that have a reduced expression of epithelial and increased expression of mesenchymal antigens with properties of tumour stem cells.

4.1.2 Factors affecting the number of CTCs

The prognostic role of CTCs is closely linked to their number, and several factors may affect the number of CTCs (Fig. 7).

4.1.2.1 Signalling pathways within a tumour

CTCs are released into the bloodstream from the tumour mass; therefore, some biological characteristics of the tumour could affect the number of CTCs. In the case of metastatic breast cancer no association has to date been found between primary tumour characteristics and the CTC count. On the other hand, it needs to be noted that in previous studies, only a limited spectrum of breast tumour characteristics was monitored (e.g. histological type, degree of differentiation – grading, hormone receptor status, and HER-2 status, lymphovascular invasion or proliferation index. Other biologically significant factors, such as, e.g. the activation of pathways that play a significant part in the biology of breast cancer and that may be involved in the release of CTCs,

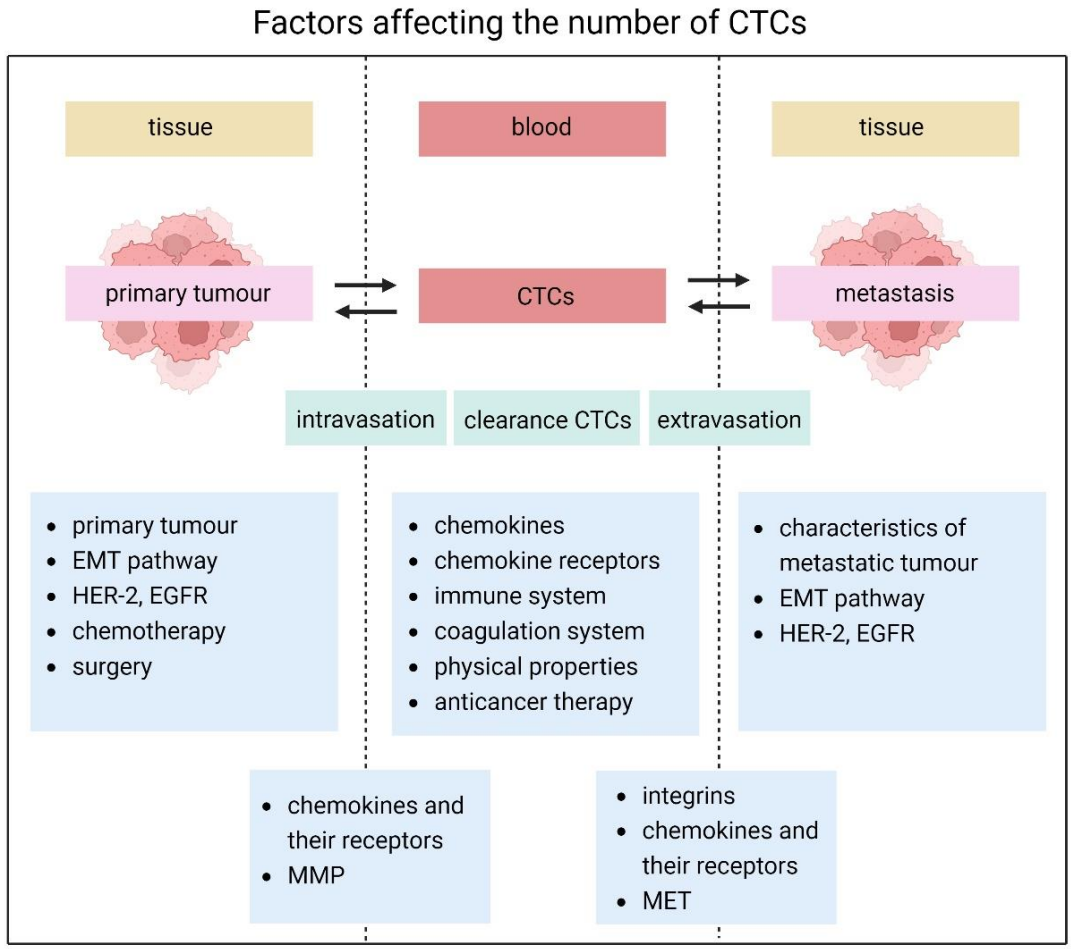

Figure 7
Factors affecting the number of CTCs.
Created with Biorender.com

were not monitored in terms of their association with the presence of CTCs (e.g. EGFR, PI3K/Akt, TGF-beta, the IGF pathway). In early breast cancer, overexpression of the HER-2/neu oncogene was accompanied by an increased number of CTCs. Currently, a study is underway that tracks the association between the gene profile of the tumour and the number of CTCs. The results of this study may contribute to the finding of an association between some signalling pathway and CTCs.

4.1.2.2 Epithelial-mesenchymal transition (EMT)

Several studies indicate that reactivation of the transdifferentiation program, the so-called epithelial-mesenchymal transition, which allows tumour cells to carry out individual steps of the metastatic cascade, plays an important role in the pathogenesis of tumours. EMT is a process that is physiologically applied during embryogenesis and that is applied under pathological circumstances in end-stage renal failure, pulmonary fibrosis or in carcinogenesis. During EMT,

epithelial cells lose intercellular contacts and cell polarity, decrease the expression of epithelial antigens (e.g. cytokeratins and E-cadherin) and increase the expression of mesenchymal antigens (vimentin) (Fig. 8).

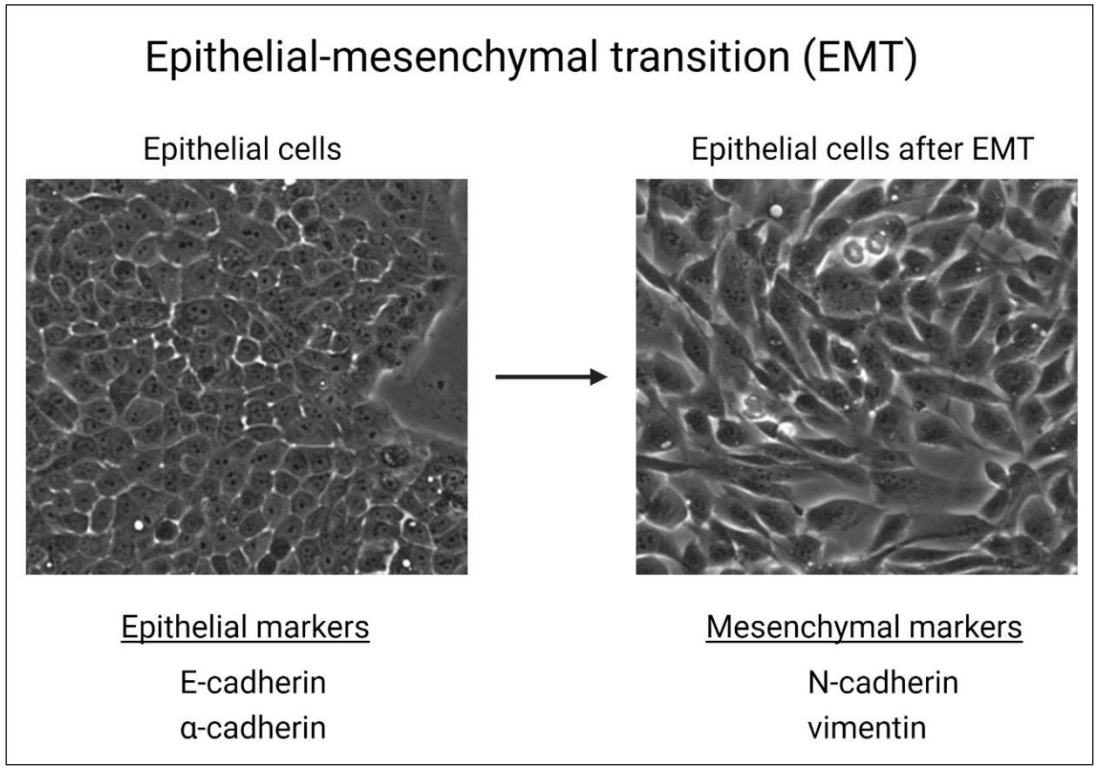

Figure 8
Cellular changes during epithelial-mesenchymal transition

EMT is accompanied by notable changes in the cytoskeleton, which enables them to acquire the mesenchymal phenotype with increased mobility and invasiveness. In consequence of EMT, the cell acquires the properties of a tumour stem cell and resistance to chemotherapy or radiotherapy. EMT also plays a key role in the progression of cancers by facilitating the spread of tumour cells. During EMT, the cell acquires a mesenchymal phenotype and has higher motility, which promotes active intravasation and the release of CTCs. In one experiment, the blocking of EMT in the highly metastatic 4T1 cell line led to a reduction in the number of CTCs. Activation of several pathways that play an important role in tumour biology, such as Hedgehog, EGF, HGF, IGF, TGF-beta or HER2/neu, leads to the induction of EMT. Data on the association between the Recent work has shown the expression of genes involved in the induction of EMT in a portion of circulating tumour cells, which supports the importance of EMT in the metastatic cascade. At the same time, it was shown that CTCs with the so-called partial phenotype – those that have both epithelial and mesenchymal features – are the most biologically aggressive.

4.1.2.3 Other factors affecting CTCs

Along with the characteristics of the primary tumour, the number of CTCs can also be influenced by other factors that allow active intravasation of tumour cells. These are mainly MMPs, urokinase activator of the plasminogen system (uPA) and its receptor (uPAR) and chemokines. Activation of the MMP and uPA system is connected with a poor prognosis in breast cancer and other tumours. The activation of uPA leads to the degradation of the ECM with subsequent activation of many MMPs, which then destroy it. The ECM is the main physical barrier for tumour cells and at the same time is an essential step that the cell must manage before intravasation. Proteolytic degradation of the ECM assists cell migration and makes the formation of metastases easier. Analysis of individual CTCs showed that an increased expression of uPAR is present in a significant proportion of CTCs in patients with advanced disease.

The number of CTCs can also be influenced by their destruction in the bloodstream and by their extravasation. In addition to physical factors, such as shearing forces, the immune system is another factor that affects the clearance of CTCs. One of the possible explanations for the increased number of CTCs in patients with poor prognosis may be a dysfunction of the immune system, which is unable to destroy CTCs or control the growth of a solid tumour mass. In an experimental model, a reduction in the number of lung metastases occurred when CTCs were pre-exposed to NK cells. EMT accelerates the initiation of metastases not only through enhanced invasion, but also in consequence of the induction of immunosuppression. Experimental works indicate that inhibition of EMT, aside from the direct effect on the metastatic cascade, should lead to a weakening of immunosuppression induced by cancer.

4.1.2.4 Coagulation and CTCs

Coagulation factors may play a role in the clearance of CTC. These factors have an important role in the metastatic cascade. By binding to coagulation factors, including TF, fibrinogen, fibrin and thrombin, tumour cells form an embolus that facilitates their entrapment in the capillary bed and subsequent extravasation.

CTCs may be directly involved in the activation of coagulation. The surface of cancer cells shows alterations in glycosylation and often contains very branched or sialylated oligosaccharides. The presence of these oligosaccharides on the surface of tumour cells correlates directly with a poor prognosis in cancer patients, with progression and metastatic spread. Furthermore, the procoagulant activity of tumour cells which express SLE (a) and SLE (x) antigens can be inhibited by anti-SLE (a) or anti-SLE (x) antibodies, which further supports the role of these glycoproteins in tumour-associated thrombosis. CTCs can activate the coagulation cascade through the expression and release of TF or the generation of TF (+) microparticles. The expression of TF is higher in cells with a tumour stem cell phenotype and can be induced through EMT. Since a portion of CTCs are tumour stem cells involved in tumour dissemination, CTCs may represent an important source of TF and thereby may be directly involved in the activation of coagulation. A connection between the presence of

CTCs and the risk of venous thromboembolism (VTE) was observed in one retrospective study, and an association between CTCs and haemostasis activation was likewise observed in two prospective studies. The results of the meta-analysis show a favourable effect of anticoagulant therapy on the survival of patients with non-metastatic breast cancer, in which the influence of the metastatic cascade by heparin may play a part. The antitumour effect of heparin not only reflects its anticoagulant activity, but is rather associated to the ability of heparin to inhibit the interaction of some oligosaccharides present on the surface of tumour cells and P-selectin on platelets, which may affect the clearance of CTCs.

4.1.3 Prognostic and clinical significance of CTC detection

Malignancies represent a heterogeneous group of diseases, and the prognosis of patients depends on various host and tumour-related factors. The presence of CTCs in peripheral blood is among the new and promising prognostic factors in cancer patients.

The potential clinical use of CTC detection can be applied in:

1. prediction of the prognosis,
2. monitoring the effect of treatment,
3. as a mininvasive biopsy of a tumour in real time, enabling individualisation of treatment,
4. identification of therapeutic goals.

CTCs are being shown to a promising prognostic factor in several tumour types. The prognostic value of CTCs detected using the CellSearch™ method has been repeatedly demonstrated in metastatic breast, prostate and colorectal cancers. The original work of Cristofanilli et al. showed that number of CTCs before treatment is an independent predictor of progression-free survival and overall survival in patients with metastatic breast cancer. Improved survival was observed in patients with a CTC count of less than 5 in 7.5 ml of blood regardless of histological subtype, hormone receptor and HER-2/neu receptor expression, site of metastasis, or whether recurrent or *de novo* diagnosed metastatic disease was involved. The prognostic value and number of CTCs did not correlate with the overall tumour mass or level of serum tumour markers, which points to the special biological value of CTCs detected by the CellSearch™ methodology or to the fact that these CTCs represent a biologically significant subpopulation of CTCs. The prognostic value of CTCs is shown to be superior even in comparison with conventional (computed tomography) and functional (PET) imaging examinations, and CTCs also enable a monitoring of the effectiveness of treatment. These data indicate the possibility that CTCs detected in this way represent a population of tumour cells with tumour-initiating properties, or tumour stem cells, and that these cells may play an important role in tumour spread.

The real challenge of DTC/CTC detection technologies is monitoring minimal residual disease in patients with no detectable metastases. Since most DTCs and CTCs are in a non-proliferative state at the time of primary diagnosis, adjuvant chemotherapy has a relatively limited effect on these cells. The use of targeted therapy along with chemotherapy and radiotherapy could be a promising approach to get around this problem. HER-2/neu represents

one of the most important biological targets for a specific therapy, and remarkable results have been observed in clinical trials using the monoclonal antibody against HER-2 – trastuzumab. Detection of HER-2/neu expression in CTCs would allow the monitoring of HER-2 status during the clinical course of the disease and adjusting the treatment accordingly. Several studies have shown differences in HER-2/neu expression between primary tumours, lymph nodes and distant metastases. Therefore, assessing HER-2 status in DTCs and CTCs could add important information for choosing a treatment without the need for repeated biopsies of the primary tumour, or metastases.

Despite the clear prognostic value, at present there is a shortage of data on the basis of which CTC detection would have an impact on patient treatment, and the use of CTC in routine clinical practice remains a topic of debate. The prognostic importance of CTCs is relatively well documented in the case of metastatic breast, prostate and colon cancer, and incorporation of the number of CTCs as a staging parameter in these tumours is currently under considered. On the other hand, there are only limited data on the biological and phenotypic properties of CTCs. A better functional and morphological characterisation of CTCs can be reflected not only in the treatment of the patient but may also lead to a better understanding of the pathogenesis of metastasis.

4.2 Disseminated tumour cells in bone marrow

Disseminated tumour cells (DTC) are isolated tumour cells that are detected in the bone marrow of patients. The methods used for their detection are on a similar principle as in the case of CTC.

Several clinical studies have provided evidence of a connection between the presence of DTCs detected in the bone marrow at the time of resection of the primary tumour and the risk of recurrence in patients with breast, prostate, lung, and digestive tract tumours. The most data was related to breast cancer and followed from an international study that included 4,703 patients with early (non-metastatic) breast cancer. DTCs were found in the bone marrow in approximately 30% of the patients. When followed for more than 10 years, patients with DTCs had an increased risk of recurrence and shorter overall survival compared to patients without DTCs. Not only DTCs at the time of resection of the primary tumour, but also DTCs at the time of completion of adjuvant (augmentation) treatment have been shown to be a prognostic factor. In contrast to this, the prognostic importance of DTCs in the bone marrow is lost in patients with manifest metastatic disease (i.e. metastases can be detected by imaging studies, CT, X-ray, etc.).

There are at present only a limited number of studies that have compared the presence of DTCs in bone marrow with the presence of CTCs in peripheral blood. All the studies showed, however, that in repeated sampling from the same patient, DTCs were detected more frequently than CTCs. This observation is explained by the fact that the bone marrow is the place where DTCs accumulate and thus serves as a kind of reservoir. In contrast to this, with respect to the short half-life of CTCs in the blood (1 – 2.5 h), these show only a short moment of tumour

dissemination, while DTCs accumulate in the bone marrow over a long period, which is also confirmed by the considerable heterogeneity of DTCs, as shown by their genetic analysis.

A number of studies have shown that the bone marrow is a common site for DTC homing in various epithelial tumours, including breast, prostate, lung, and colon cancer. Although DTCs can be present in other organs, bone marrow can serve as a reservoir of DTCs from which they can eventually metastasize to other tissues such as lung, liver and others. The observed correlation between the presence of DTCs in bone marrow and the risk of locoregional recurrence suggests that DTCs can "travel" back to the site of the primary tumour. It is possible that additional research will show whether DTCs in bone marrow persist in the same locations, the so-called niches, such as haematopoietic stem cells.

Disseminated tumour cells in the bone marrow, as well as in other tissues, may not manifest in the form of growth with the subsequent formation of metastasis, but instead may persist in a quiescent state (in the G0 phase of the cell cycle) for many years. We are speaking here of tumour dormancy. Sometimes, under the influence of not yet fully known stimuli, these cells leave this state and begin to proliferate, forming a metastasis, but in a portion of patients, these cells will not be reactivated until the end of their lives. For example, in breast cancer patients recurrences may occur more than 10 years after the removal of the primary tumour. TCTCs have likewise been observed in cured breast cancer patients despite the absence of distant metastases even 22 years after removal of the primary tumour, which suggests the possibility that dormant DTCs and CTCs may be present in many cured patients. The processes that "snap" a tumour cell out of a state of dormancy are not exactly known. The origin of additional mutations, epigenetic changes (e.g. methylation of promoters) regulating gene expression, or changes in the microenvironment around the tumour cell have all been considered. For example, with inflammation or wound healing, a large number of cytokines are released, and some of them can induce the migration and growth of epithelial tumour cells. It is likewise possible that chemotherapy or radiotherapy do not necessarily eliminate tumour cells but only induce a state of dormancy in them, which is clinically manifested as a cure.

4.3 CTCs and DTCs as tumour stem cells

Recent advances in cell biology suggest that tumour dissemination and resistance to therapy is mediated by the existence of so-called tumour stem cells. Tumour stem cells form a minority population of cells within the tumour mass; however, they have the ability for self-renewal; they can divide indefinitely and retain the ability to differentiate. It is thought that these cells are responsible for tumour dissemination from the primary site and only these cells are capable of successfully completing the metastatic cascade. Tumour stem cells are at the same time resistant to chemotherapy and radiotherapy, and it is thought that this subpopulation of tumour cells is responsible for tumour recurrence, or for treatment failure. Intensive research aimed at therapeutically influencing these cells is currently underway.

In the case of breast cancer, tumour stem cell markers have been identified based on their ability to initiate xenografts in immunocompromised mice. Al-Hajj et al. found that cells with

a $CD44^{+}/CD24^{-/low}$ phenotype, isolated from human breast carcinoma, have an increased ability to form tumours when put into the mammary adipose tissue of immunocompromised NOD/SCID mice. Their work showed that fewer than 100 $CD44^{+}/CD24^{-/low}$ cells were sufficient for tumour formation, and the administration of more than 10,000 cells of a different phenotype failed to create tumours. The tumour-initiating ability of this cell population was then refined in transplantation studies by Ginestier et al. Their study examined the activity of aldehyde dehydrogenase 1 (ALDH1) in breast cancer cells. Using flow cytometry, they found that the tumour-initiating population of $CD44^{+}/CD24^{-/low}$ cells is a committed subpopulation that showed intense ALDH1 activity. ALDH1 activity was also recently detected in the CTC fraction using RT-PCR. The tumour-initiating ability of CTC is also indirectly supported by the observation in the case of metastatic lung cancer, where mutations responsible for resistance to EGFR inhibitors were detected in CTC. Since patients typically have metastatic carcinoma progression in several localities and these metastases mostly having the same type of mutation, it is likely that CTCs are responsible for the transfer of this resistance.

It likewise appears that most DTCs in the bone marrow have the tumour stem cell phenotype. This is pointed to by the correlation between DTC and the risk of tumour recurrence, the resistance of DTC to chemotherapy, and also the DTC phenotype characteristic of tumour stem cells.

Monitoring DTC/CTC provides a new glimpse into the identification of tumour cells and the selection of appropriate biological treatment. Additional biological characterisation of these cells may lead to the identification of new therapeutic targets for these cells, with the subsequent possibility of eradicating micrometastatic disease or at least inducing a state of permanent dormancy, thus turning the tumour into at least a curable chronic disease.

4.4 The role of CTCs in tumour growth and progression

The dissemination of a tumour is traditionally seen as a sequential process. The arising of distant metastases is usually a late event in the course of cancer, and the risk of metastases grows with the size of the primary tumour. Based on this, we could assume that only advanced tumours release CTCs into the bloodstream. Increasing knowledge, however, shows that this is not the case, and that the dissemination of tumour cells occurs relatively early. In an experimental transgenic mouse model of breast cancer, dissemination was found to have occurred as early as at the stage of atypical hyperplasia, when individual tumour cells began to penetrate through the basement membrane. In another experimental model, untransformed cells were put into the systemic circulation of mice, and tumour transformation was induced only after extravasation in the lungs. These data showed that CTCs can persist for longer periods in distant organs, that tumour transformation may not occur only at the site of the primary tumour, and transformation induced outside the primary tumour may be enough for metastatic growth.

Furthermore, CTCs also play an important role in tumour growth in a process called *self-seeding*. This is a process in which the primary tumour as well as existing metastases are colonized by more aggressive clones of tumour cells. In practice, this occurs such that

tumour cells with more biologically aggressive properties are released into the bloodstream and subsequently settle in the place of already existing metastases, where they actively divide and thus share in the growth of the metastatic focus. Analogously, this may lead to the reverse colonization of the primary deposit by cells from metastases. This phenomenon also explains the frequently observed coincidence between the primary tumour and metastases as well as the dissemination of resistance to anticancer therapy, where tumour cells in different metastatic foci often show the same type of resistance.

In an experimental model of "self-seeding" in breast, colon, and melanoma tumours in mice, *self-seeding* was preferentially mediated by more aggressive CTCs, above all those with metastatic tropism towards bone, lung or brain. Tumour-produced cytokines IL-6 and IL-8 have likewise been found to act as CTC attractants, while MMP-1 and collagenase 1, as well as the cytoskeletal component fascin 1, are mediators of tumour infiltration by CTCs. Even more importantly is the fact that self-seeding CTCs taking part in "self-seeding" only colonize existing tumour foci and therefore require only a small adaptation to grow after extravasation at a new site. On the other hand, they do not create new metastatic foci in unaffected places, and thus the "self-seeding" process does not mediate the creation of new metastatic foci, but the growth of already existing ones, including the primary tumour. *Tumour self-seeding* explains the relationship between tumour size, prognosis as well as local recurrence after complete excision of the primary tumour, which is mediated by cells that have disseminated at earlier stages of tumour development. Enderling et al. used an experimental mathematical model and its results indicate that the tumour's ability to activate *self-seeding* is the most important factor in tumour progression and is influenced by the balance between cell proliferation, cell migration and cell death. Recent observations by Ginestier et al. demonstrated that inhibition of the overexpression of CXCR1 chemokine receptor in tumour stem cells is associated with a lower incidence of systemic metastases.

It seems that not all CTCs are equally capable of metastasizing. Important insights have come from observing patients with malignant ascites. In the past, peritoneo-venous shunts, with which some billion tumour cells per week were delivered from the ascites to the *vena jugularis*, were used for the symptomatic relief of ascites. These patients typically had such a shunt in place for several months; therefore, the number of tumour cells that entered into the bloodstream in this way was huge. A follow-up autopsy revealed that some of the patients had no metastases in other previously unaffected organs, while some had a larger number of small foci in different organs, indicating successful tumour colonization. The remaining group of patients had additional metastases present in the same organs in which metastases were already present. Still more importantly, none of these patients had symptoms from these tumour foci, and all died as a result of progressive growth of the primary tumour.

These data support the concept of CTC heterogeneity, where different CTC subclones have different abilities to colonize metastatic sites and form metastases. At the same time, the microenvironment at the distant site influences the ability of CTCs to grow after extravasation.

References

Aceto N, Toner M, Maheswaran S, Haber DA. En Route to Metastasis: Circulating Tumor Cell Clusters and Epithelial-to-Mesenchymal Transition. Trends Cancer 2015; 1(1):44-52.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells.Proc Natl Acad Sci USA 2003; 100:3983-3988.

Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004; 351:781-791.

Enderling H, Andeson ARA, Chaplain MAJ, et al. Paradoxical Dependencies of Tumor Dormancy and Progression on Basic Cell Kinetics. Cancer Res 2009; 69:(22).8814-8820.

Fehm T, Müller V, Alix-Panabières C, Pantel K. Micrometastatic spread in breast cancer: detection, molecular characterization and clinical relevance. Breast Cancer Res 2008; 10 Suppl 1: S1.

Fridrichova I, Kalinkova L, Ciernikova S. Clinical Relevancy of Circulating Tumor Cells in Breast Cancer: Epithelial or Mesenchymal Characteristics, Single Cells or Clusters? Int J Mol Sci 2022; 23(20):12141.

Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 Is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome. Cell Stem Cell 2007; 15:555-567.

Kim, MY et al. Tumor self-seeding by circulating cancer cells. Cell 2009; 139:1315-1326.

Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133:704-715.

Mego M, De Giorgi U, Broglio K, et al. Andreopoulou E, Valero V, Dawood S, Handy B, Reuben JM, Cristofanilli, M. Circulating tumor cells and risk of venous thromboembolism in metastatic breast cancer patients Br J Cancer 2009; 101:1813-1816.

Mego M, Karaba M, Minarik G, et al. Circulating Tumor Cells With Epithelial-to mesenchymal Transition Phenotypes Associated With Inferior Outcomes in Primary Breast Cancer. Anticancer Res 2019; 39(4):1829-1837.

Mego M, Mani SA, Cristofanilli M. Molecular mechanisms of metastasis in breast cancer – clinical applications. Nature Reviews Clinical Oncology 2010;:7: 693-701.

Norton, L. Cancer stem cells, self-seeding, and decremented exponential growth: theoretical and clinical implications. Breast Dis 2008; 29:27-36.

Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. Nat Rev Cancer 2004; 4:448-456.

Reuben JM, B. Lee, A. Lucci, et al. Disseminated tumor cells in primary breast cancer: Evaluation of the percentage of breast cancer stem cells in bone marrow aspirates of patients receiving neoadjuvant chemotherapy. J Clin Oncol 2009; 27:15s, (suppl; abstr 505).

Riethdorf S, Fritsche H, Müller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch™ system. Clin Cancer Res 2007; 13:920-928.

Yang J, Weinberg RA. Epithelial-Mesenchymal Transition: At the Crossroads of Development and Tumor Metastasis. Developmental Cell 2008; 14:818-829.

5 The role of the gut microbiome in carcinogenesis

Man exists in mutual symbiosis with the microorganisms that colonize his skin and mucous membranes, and maintaining the microbial balance is essential from the point of view of human health. The study of the gut microbiome is nowadays coming to the forefront of oncology research, from several aspects. Uncovering the role of the microbiome in carcinogenesis is important not only in terms of prevention, but also in the treatment of certain forms of cancer. Growing evidence has confirmed the ability of gut bacteria to increase the immune response against tumour cells by mobilising the immune system not only in the, gut but also throughout the body. Experimental as well as many clinical studies have pointed to the effect the microbiome has on the effectiveness of anticancer therapy, especially immunotherapy and chemotherapy. These observations transmit a signal to the modulating gut microbiota to increase treatment efficacy while reducing its toxicity. Mounting evidence also indicates that the microbiome is one of the important factors influencing the development of late effects of anticancer therapy in cancer patients.

A comprehensive analysis of microbial studies carried out on samples from patients with 33 different types of oncologic diseases pointed to the presence of unique microbial biomarkers in the tissues and blood of most of the most common types of malignancies. These data indicate the potential use of microbial DNA from blood in the diagnosis of oncologic diseases. Furthermore, *The Cancer Microbiome Atlas*, made up of a set of microbial profiles of gastrointestinal tumour tissues that were identified by analysis of sequencing data from *The Cancer Genome Atlas* project, was introduced in 2021. These findings led to the discovery of numerous species and potential microbial biomarkers associated with mucosal barrier damage, a common feature of colorectal cancer as well as inflammatory bowel disease. Importantly, a novel concept of the Gut OncoMicrobiome Signatures (GOMS) outlines different GOMS patterns observed in cancer patients compared to healthy individuals, highlighting their potential clinical use as predictive and prognostic biomarkers in immuno-oncology.

5.1 Analysis of the human microbiome

The skin and mucosal surfaces of the human body are inhabited by approximately 100 trillion microorganisms, which are collectively known as the microbiota. The number of microorganisms is several times greater than the number of human cells themselves, and their presence is essential for the proper functioning of the human body.

Identification and characterisation of the microbial composition were traditionally based particularly on the use of cultivation and isolation methods. As early as the 17th century, Dutch merchant and naturalist Antonie van Leeuwenhoek observed differences in the microbial makeup of samples obtained from the oral cavity and stool samples. Later, in 1881, Robert Koch managed to introduce the cultivation of microorganisms on culture dishes and their subsequent identification. This approach greatly misrepresented the real situation, since most of the microorganisms in the human body are species that cannot be cultivated under laboratory conditions. Significant progress in sequencing methods and bioinformatics tools has led to the emergence of metagenomics. This branch of science deals with the study of communities of microbial organisms directly in their natural environment, i.e. without the need to isolate them and laboratory cultivation. Genetic and molecular-biological methods are used in metagenomic analyses, and they make it possible to understand the dynamics of the microbial community.

Determination of the microbial repertoire is done by amplicon sequencing of the 16S rRNA gene or by whole genome "shotgun" sequencing. The bacterial gene for 16S rRNA, with a size of 1,500 bp, serves as the standard for taxonomic distinction between the selected bacterial communities. The gene encoding the 16S rRNA subunit is made up of sections that are conserved for individual bacterial taxa, but it also contains 9 hypervariable regions, and these are unique to individual bacterial species; we refer to them as the V1 – V9 regions. Whole genome metagenomic sequencing brings a wider spectrum of information about microbial diversity and the presence of functional genes; it thus also maps the functional potential of microbial communities.

After the completion of the *Human Genome Project* (HGP) in 2003 and the determination of the complete nucleotide sequence of human DNA, the National Health Institute (NHI) in the USA launched the *Human Microbiome Project* (HMP) in 2007. The latter focused on identifying the microbial genes and genomes present in the human body. The project used a complex metagenomic approach, and the primary goal of the first phase was the comprehensive characterisation of the microbial community of mucous membranes and surfaces in a healthy person (nose, mouth, skin, gastrointestinal and urogenital tract). The second phase of the project was focused on identifying correlations between changes in the human microbiome and health, and the output consists of reference sequences that enable comparisons of the microbiome within the population.

5.2 Gut microbiota and gut microbiome

Metagenomic analyses confirmed that the vast majority of commensal, symbiotic and pathogenic microorganisms inhabit the gastrointestinal tract. Both the number of microorganisms and their taxonomic representation increase along the entire gastrointestinal tract, from 10^1 to 10^3 microorganisms/g in the stomach and duodenum up to the highest number in the intestine, where the population density is in the range of $10^{11} – 10^{12}$ microorganisms/g. Gut microbiota defines the set of all microorganisms present in the intestinal tract, including bacteria, archaea, viruses, fungi, and protozoa. The aggregate genome of all microorganisms is labelled the metagenome. The gut microbiome forms a complex ecosystem, i.e. a set of all microorganisms together with their genes, metabolic activities and ecological functions. It thus creates a dynamic microecosystem, whose importance for human health is constantly being confirmed. Based on observations, it can be stated that the number of genes in an individual's gut microbiome is a hundred-fold greater than the number of genes in the human genome. This is why the gut microbiome is often referred to as a person's "second genome".

5.2.1 Composition of the gut microbiome

The human microbiota begins to develop immediately after birth. Aside from genetic determinants and method of birth, breastfeeding is also crucial for microbial community profiling and the health of the child. Mother's milk was long considered sterile, but in reality, it is the main source of probiotic bacteria for the baby, representing the genera *Lactobacillus*, *Enterococcus,* and *Bifidobacterium*. Moreover, human milk oligosaccharides are one of the crucial components of breast milk, which have many beneficial properties for health due to their role in the proper development of the microbiome and immune system.

The building of the microbiota becomes fixed by the third year of life. With growing age, a person's microbial composition changes and is affected by a number of other factors, such as nutrition, age, sex, environment, lifestyle, eating habits, physical activity, use of antibiotics, and other treatments. The diversity and abundance of bacteria gradually increase and reach the greatest complexity in adulthood (Fig. 9).

The result of the mutual interaction of determining factors is the unique composition of the gut microbiome in each person. The composition of the gut microbiome, however, shows considerable diversity not only between individuals, but also between the studied populations. Therefore, not only the American HMP project, but also the European *Metagenomics of the Human Intestinal Tract* (MetaHIT) project and the *Asian Microbiome Project* (AMP) focused on determining the healthy bacterial composition. Advances in sequencing methods have made it possible to acquire data on the composition of the healthy gut microbiome, with the dominating bacterial phyla being *Firmicutes* and *Bacteroidetes*, followed by *Proteobacteria*, *Actinobacteria*, *Fusobacteria,* and *Verrucomicrobia*. The most abundant phylum *Firmicutes* is composed of more than 200 different genera, among them *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus,* and *Ruminicoccus*. The predominant representatives of *Bacteroidetes* are

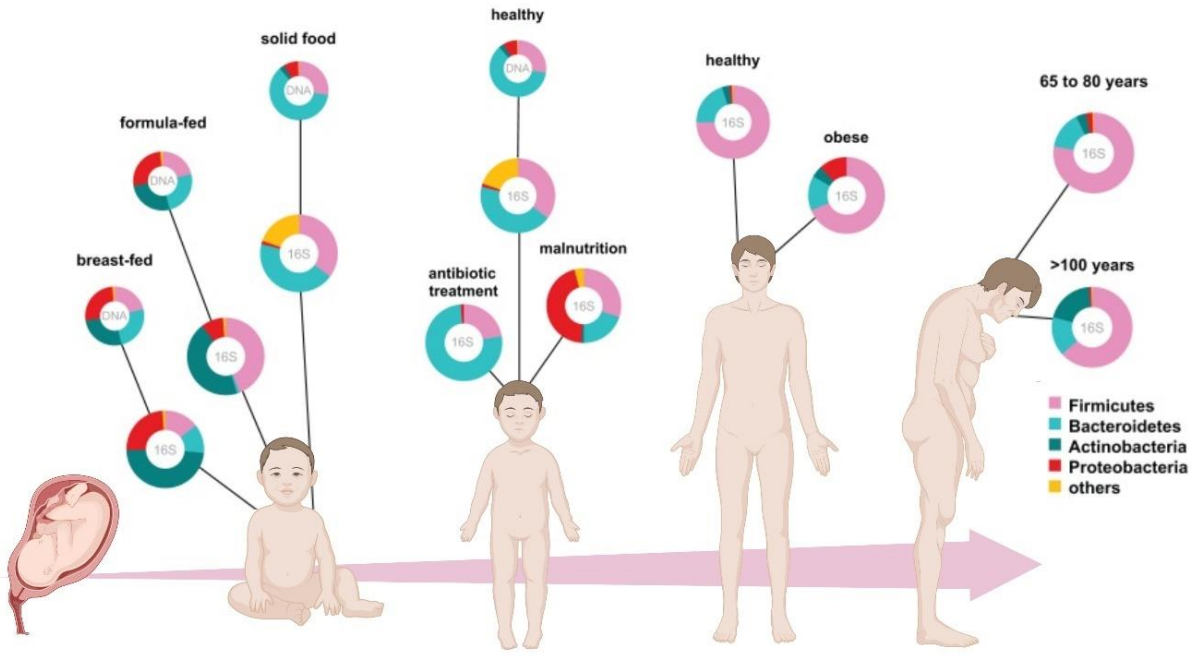

Figure 9
Changes in gut microbiome composition with an increasing age.
The graphical visualisation offers a comprehensive overview on the abundance of major bacterial phyla within the human microbiome across various life stages, as determined through either 16S RNA or metagenomic sequencing **(**modified according to Ottman et al., 2012). Created with Biorender.com

especially the genera *Bacteroides* and *Prevotella*. The phylum *Actinobacteria*, which is mainly represented by the genus *Bifidobacterium*, forms a smaller percentage.

A comprehensive metagenomic approach provides information on how individual bacterial species affect the health of the host. The human intestine is known to be a producer of intestinal metabolites that can enter into the bloodstream and affect the functions of important internal organs such as the brain and liver. As a result of constant interactions with microorganisms, the intestinal epithelium plays a major role in recognising specific bacterial ligands (lipopolysaccharides, lipoproteins), thus enabling the tolerance of commensal bacteria that form a symbiotic intestinal microecosystem. In contrast, the recognition of *pathogen-associated molecular patterns* (PAMPs) from translocated bacteria activates the signalling pathway through *Toll-like* receptors (TLR) and leads to the triggering of oxidative stress and inflammatory processes. A disturbed balance of the intestinal microsystem, called dysbiosis, is associated with the development of many serious diseases, including cancer.

5.2.2 Functions of the gut microbiome

Maintaining a healthy intestinal homeostasis is critical to host health. The gut microbiome helps strengthen the integrity of the mucosal barrier, provides protection against pathogens, influences a whole range of metabolic processes and significantly shares in the formation and

modulation of the innate and adaptive immune response. Among the metabolites produced by intestinal microorganisms are amino acids, bile acids, dopamine, histamine, para-cresol, and serotonin, as well as vitamins B, K, riboflavin, pyridoxine, and folic acid. However, among the main metabolites of the gut microbiome are *short-chain fatty acids* (SCFA), which are produced by anaerobic fermentation of indigestible food residues, such as cellulose, hemicellulose, starch, oligosaccharides, and lignin. The most represented SCFAs are acetate, propionate, and butyrate, and studies have shown that their concentration in the stool is directly proportional to the concentration in the gut. SCFAs have a positive effect on human health and are engaged in immunomodulating and anti-inflammatory processes. Several results have confirmed their protective effect in the case of serious diseases, such as *diabetes mellitus* and cancer. Butyrate serves as an important source of energy for enterocytes, and the in particular bacteria *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii,* and *Coprococcus* spp. from the *Firmicutes* and *Bacteroidetes* phyla share in its production. It has also been shown to induce apoptosis of colorectal cancer cells and to activate intestinal gluconeogenesis. Acetate is the most represented SCFA and plays an important role in lipogenesis and cholesterol metabolism. Propionate is likewise responsible for the regulation of gluconeogenesis and enters the liver via the portal vein.

Growing knowledge points to the important role of the gut microbiome in the aetiology of oncologic diseases; therefore, it is essential to understand the mechanisms of mutual interactions between representatives of the microbiota and the host. A correctly functioning immune system of the intestinal mucosa recognises and tolerates beneficial commensal bacteria, but also eliminates the excessive growth of pathogenic microorganisms. The integrity of the cells of the intestinal epithelium depends in part on the intensity of the tight intercellular connection, but also on the coordinated regulation of the amount of mucus and the adaptive immune response of the host. Goblet cells secrete a layer of mucus on the surface of the epithelium, which protects it from damage. Its main component is mucin, formed from large glycoproteins connected by oligosaccharide chains. Intestinal microorganisms and bacterial metabolites are recognised by the sensory system of the intestinal epithelial and immune cells, and these activate the mechanisms of innate immunity. Disruption of intestinal homeostasis and damage to the mucosal barrier leads to the permeability of the intestine, and inflammation of the intestinal mucosa and can cause the induction of signalling pathways associated with tumourigenesis.

One important role of the gut microbiome is the regulation of the intestinal immune system. Immune system CD4+ T cells are mainly present in the *lamina propria* of the intestine, and their differentiation into Th1, Th2, Th17, and Treg cells depends on the bacterial composition of the gut microbiome. While *Bacteroides fragilis* promotes differentiation into Th1 cells, members of the genus *Clostridium* (cluster IV and XIVa) induce Treg differentiation. From the point of view of the host, the gut microbiome is irreplaceable, since certain commensal bacteria are responsible for the synthesis of essential micronutrients, such as vitamin K, riboflavin, biotin, thiamine, pantothenic acid, and pyridoxine. Bifidobacteria produce folic acid, which is important for DNA synthesis and repair. Representatives of *Bacteroides* (*Bacteroides inneris* and to a certain extent also *Bacteroides fragilis*) are capable of deconjugating primary bile acids to form secondary bile acid deoxycholic acid. What's more, many intestinal commensals can modify small amino acids into signalling molecules, such as histidine to histamine or glutamate to γ-aminobutyric acid (GABA).

5.3 Changes in the gut microbiome of an oncology patient

A healthy intestinal microbiota is characterised by rich microbial diversity with a dominance of symbiotic commensal species, a high secretion of immunoglobulin A, and colonization resistance, which prevents the proliferation of pathogens. The intestinal membrane is compact; the enterocytes are connected by tight junctions, which prevents bacterial translocation.

The results of the studies have pointed to changes in the gut microbiome in patients with different types of cancer. The composition of the gut microbiome in an oncology patient is affected by several factors – aside from the disease itself and the influence of genetics, also diet, lifestyle, the use of antibiotics and immunosuppressants, but especially any anticancer treatment administered. When using laxatives and prokinetics, the diversity of the natural gut microbiome is reduced. Taking medicines that reduce the production of stomach acid creates a better breeding ground for the bacteria of the stomach and small intestine, which worsens the digestion of food. Antibiotic treatment, in turn, suppresses the natural microbiota, which lowers its resistance to colonization by pathogens and increases the risk of proliferation of pathogenic and potentially pathogenic microorganisms, which, in combination with the reduced immunity of an oncology patient, can represent a threat. Chemotherapy, like radiotherapy, weakens the immune system, causes disruption of the mucosal barrier, and interferes with the balance of the microbial environment. Dysbiosis and proliferation of opportunistic pathogens can then lead to a massive pro-inflammatory immune response. Damage to the mucous membrane allows the translocation of pathogens into the intestinal mucosa, and their entrance into the bloodstream causes serious infectious complications. The altered microbiome of patients may cause gastrointestinal toxicity, cardiotoxicity, a decrease in colonization resistance, changes in mood and cognitive functions, or cause severe bacteraemia (Fig. 10).

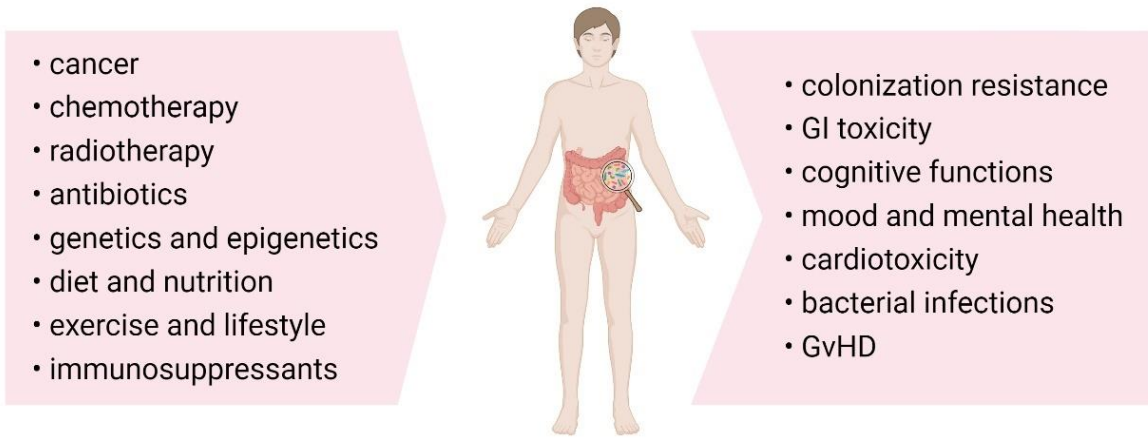

Figure 10
Factors influencing the composition of the microbiome in an oncology patient and implications for treatment course and late toxicity.
Created with Biorender.com. *Explanations: GI toxicity – gastrointestinal toxicity; GvHD – graft-versus-host disease.*

According to the recent research, the microbiome is deemed to be an important factor affecting carcinogenesis in all stages of its development, especially by modulation of the immune system. One of the mechanisms is the production of SCFAs, which improve the maturation and differentiation of T-lymphocytes. Tumour cells activate T-lymphocytes, which then have an anticancer effect, eliminating them. The administration of antibiotics to experimental animals caused a significant reduction in the efficiency of the acquired T-cell anticancer activity. Other mechanisms of action of the microbiome are the activation of the so-called myeloid cells, which are found in the inflammatory infiltrate. In the next phase of carcinogenesis, tumour cells show a tendency to change their characteristics due to their mutagenic potential. This process is often linked with local suppression of the immune system by means of T-regulatory lymphocytes. In this stage, the microbiome can enhance the immune response and block unwanted immune suppression.

Insufficient vascular supply of rapidly growing tumour tissue and high demand for oxygen lead to the formation of necrotic, oxygen-poor areas in which bacteria often proliferate. Excessive bacteria affect the immune system, as well as the effect of anticancer therapy. In colorectal cancer, we often encounter the overgrowth of the bacteria *Fusobacterium nucleatum*, which reduces the activity of the immune system by inhibiting NK cells and T-lymphocytes.

5.4 The role of the gut microbiome in the aetiology of gastrointestinal malignancies

Approximately 15 – 20% of all cancers are assumed to be linked to microorganisms, either of viral or bacterial origin and representatives of protozoa. One of the first documented cases of the participation of a bacterial taxon in the aetiology of cancer was *Helicobacter pylori*, in which a direct association with the development of gastric adenocarcinoma, or gastric lymphoma of the type *mucosa-associated lymphatic tissue* (MALT), was clinically proven. This bacterium produces the *cytotoxin-associated gene A* protein (CagA), which directly affects the signalling pathways of the host cell, thereby inducing the process of carcinogenesis. What's more, the CagA protein causes damage to the cells of the gastric mucosa, leading to inflammatory processes in the tissue.

The results of pre-clinical and clinical studies have indicated correlations between changes in the intestinal microbiota and various types of malignancies, with the mechanisms of influence of the gut microbiome on the development of colorectal tumourigenesis being the most studied. Mechanisms through which gut microorganisms contribute to the initiation or development of colorectal cancer include the induction of chronic inflammation, biosynthesis of genotoxic substances that interfere with the regulatory mechanisms of the cell cycle, production of toxic metabolites and the activation of carcinogenic food components. The composition of microorganisms changes according to the anatomical location in the colorectum, which may be related to epidemiological, microbial, and molecular differences in the pathogenesis of colorectal cancer.

Chronic inflammation is associated with an increased risk of developing colorectal malignancies, namely through mutations, inhibition of apoptosis or stimulation of angiogenesis and proliferation of intestinal epithelial cells. Inflammatory processes occur due to dysbiosis, increased permeability of the intestinal membrane, translocation of bacteria and activation of the innate and acquired immunity systems. Some pathogenic bacteria are capable of inhibiting the host's immune response or intensifying existing inflammation, thus promoting tumour formation. Activation of the mechanisms of innate immunity leads to excessive release of pro-inflammatory cytokines, such as IL-12, IL-23, TNF-α, and INF-γ, with subsequent activation of cells of the innate immune system (T- and B-lymphocytes) and various inflammatory mediators. One of the main consequences of such an inflammatory response is the activation of the transcription factors of specific key signalling pathways, such as NF-κB and STAT3, in intestinal epithelial cells. Chronic inflammation also supports the formation of reactive forms of oxygen and nitrogen, which in turn leads to oxidative stress, DNA damage, excessive proliferation, and ultimately to the formation of colorectal adenomas and carcinomas.

From the point of view of colorectal tumourigenesis, Gram-negative anaerobic bacterial species, including *Bacteroides fragilis* and *Fusobacterium nucleautum*, which are directly linked with the tumour microenvironment and colorectal tumourigenesis, are the most studied. The enterotoxic *Bacteroides fragilis* is among the *driver* bacteria due to the genotoxic effect of its toxin on intestinal epithelial cells, which causes DNA damage, increased cell proliferation and pro-inflammatory processes. This toxin stimulates the release of epithelial cells as a result of the cleavage of E-cadherin, by which previously bound β-catenin is released into the cytoplasm, thus activating the Wnt signalling pathway. Along with disrupting of the intestinal barrier by damaging intercellular adherent junctions, excessive proliferation is also triggered. Furthermore, *Bacteroides fragilis* toxin activates the transcription factor NF-κB, which results in the release of cytokines sharing in the inflammatory response of the intestinal mucosa. *Fusobacterium nucleatum* acts as a driving force of tumourigenesis while suppressing the immune response and inducing chronic inflammatory processes. Recent research shows that, in addition to *Bacteroides fragilis* toxin, toxins produced by the bacteria *Escherichia coli*, *Salmonella enterica,* and *Shigella flexneri* are also highly expressed in the tumour tissues of patients. Some commensal strains of *Escherichia coli* are capable of producing colibactin, which causes breaks in the DNA double-strand in colonocytes. Mechanisms of the repair system fail to repair these lesions, which cause an accumulation of chromosomal abnormalities and thus mutations leading to colorectal tumourigenesis. Several analyses have shown that *Helicobacter pylori*, *Bacteroides fragilis,* and *Enterococcus faecalis* can affect tumourigenesis in an indirect way, e.g. by generating *reactive oxygen species* (ROS), which causes damage in the DNA.

Maintaining the integrity of the intestinal epithelium is enabled by the existence of tight-adherent junctions between cells, which are regulated through a signalling pathway involving the E-cadherin/β-catenin complex. Damage to the intestinal mucosa and increased permeability have as a consequence the translocation of pathogens into the lamina propria, which can lead to local or systemic inflammation and often to the triggering of colorectal tumourigenesis. Proteins produced by certain bacterial species, such as protein CagA (*Helicobacter pylori*), virulence

factor *Fusobacterium adhesin A* (FadA, *Fusobacterium nucleatum*), or metalloproteinase toxin (*Bacteroides fragilis*), disrupt tight junctions through their interactions and can thereby take part in the malignant transformation of intestinal epithelial cells. An increased presence of *Fusobacterium nucleatum* correlated with reduced survival of patients with colorectal cancer.

Aside from toxins, the products of protein fermentation can also be involved in tumour formation processes in colorectal cancer. If an increased intake of dietary proteins occurs, an excess of sulphides, nitrates, amines, ammonia, and branched-chain amino acids occurs in the large intestine. These products of protein fermentation can stimulate the growth of sulphate-reducing bacteria, such as Deltaproteobacteria and Firmicutes. It is specifically the production of sulphane that can cause damage to the intestinal mucosa and cause serious damage to DNA in human cells.

The intestinal microbiota is also involved in the metabolism of bile acids. Secondary bile acids are considered to be genotoxic and support tumourigenesis. Deoxycholic acid damages the mucosa of the intestinal tract, takes part in increasing oxidative stress and DNA damage and promotes tumour growth.

5.5 Study of the gut microbiome in anticancer therapy

Growing evidence emphasises the significant role of the symbiotic composition of the gut microbiome in the treatment of oncologic diseases. Some bacterial species are essential for getting a response to administered anticancer therapy, while others reduce the effects of multiple medicines. Detailed examination of the relationships between the host, the gut microbiome and the effectiveness of oncology treatment helps reveal the mechanisms through which bacteria modulate patients' responses to treatment, as well as associations with clinical outcomes.

5.5.1 The microbiome and chemotherapy

The composition of the gut microbiome is associated with the efficacy of chemotherapy treatment based on cisplatin, 5-fluorouracil, irinotecan, cyclophosphamide, methotrexate, gemcitabine and other chemotherapy drugs. The "TIMER concept" describes the key mechanisms through which the microbiota modulates the effects of chemotherapeutics, namely using Translocation, Immunomodulation, Metabolism, Enzymatic degradation and Reduced diversity. Treatment with the alkylating drug cyclophosphamide led to a change in the microbiome and subsequent permeability of the intestinal barrier and translocation of *Lactobacillus johnsonii*, *Lactobacillus murinus,* and *Enterococcus hirae* to the lymphatic organs in mice bearing subcutaneous sarcomas or melanomas. The treatment caused a decrease in the genera *Clostridium* cluster *XIVa*, *Roseburia*, *unclassified Lachnospiraceae*, and *Coprococcus.* In patients with lung cancer, it led to microbiome changes during chemotherapy treatment, with *Firmicutes* levels significantly increased after treatment. Patients with

faecal *Faecalibacterium*, *Klebsiella*, *Coprococcus*, *Roseburia*, *Lactobacillus*, *Streptococcus*, *Prevotella*, *Dorea,* and *Collinsella* had a higher risk of disease progression than patients with *Veillonella*, *Ruminococcus*, *Paraprevotella*, *Lachnoclostridium*, *Akkermansia,* and *Clostridium.* The increased or the reduced presence of *Roseburia faecis* in the microbiome of patients with gastrointestinal malignancies represents a potential biomarker of the efficacy of chemotherapy based on oxaliplatin and 5-fluorouracil. Growth in *Roseburia faecis* was observed in individuals responding to chemotherapy, and the level of this bacterium decreased in non-respondents to this treatment. Samples from healthy individuals showed a dominance of *Faecalibacterium prausnitzii*, *Roseburia faecis*, *Clostridium clostridioforme*, *Blautia producta*, *Bifidobacterium adolescent,* and *Butyricicoccus pullicaecorum*. An increase in *Bacteroides* and a decrease in *Lactobacillus* and *Turicibacter* in the gut microbiome were recorded in an animal model of epithelial ovarian cancer after cisplatin treatment. Mice with lung tumours that were treated with antibiotics and cisplatin showed disrupted gut microbiome composition, an insufficient response to cisplatin and reduced survival.

5.5.2 The microbiome and radiotherapy

The resistance of germ-free mice to radiation-induced enteritis, as well as changes in microbial diversity after radiotherapy, indicate a correlation between the composition of the gut microbiome, the efficacy of radiotherapy and the repair of radiation damage. Rectal irradiation caused dysbiosis and increased expression of IL-1β and TNFα in the animals. Germ-free mice colonized with irradiated microbiota were predisposed to both radiation damage and colitis. Since tissue damage induced by microbiota radiation was at least partially mediated by IL-1β, modulation of the microbiome or direct inhibition of IL-1 may represent a potential therapeutic approach with the aim of reducing radiation-induced intestinal mucositis.

A shift in the intestinal microbiota makeup induced by irradiation was confirmed not only in mouse models, but also in clinical studies. A prospective observational study of a set of patients with gynaecological malignancies showed significant differences in the overall microbial composition of the intestine between patients and healthy individuals from the control group. Radiotherapy of the pelvis significantly reduced the number of bacterial species, and the most notable changes occurred in the case of representatives of the bacterial phylum *Firmicutes*. A pyrosequencing analysis of the 16S rRNA gene in 11 oncology patients also revealed a notable decrease in the diversity and richness of the gut microbiota after a five-week cycle of pelvic radiotherapy. In addition to this, the results showed that the microbial composition was not influenced only by radiotherapy, but existing changes in the *Firmicutes*/*Bacteroidetes* ratio could serve as a predictive tool for identifying patients who later developed diarrhoea.

A recent study of stool samples from 18 patients with cervical cancer reported changes in gut microbial profiles after radiotherapy. In patients with radiation enteritis, a significantly reduced diversity of the microbiome was observed, with a relatively higher representation of *Proteobacteria* and *Gammaproteobacteria* and a lower representation of *Bacteroides* bacteria. The results of one of the largest clinical trials, in which 134 patients undergoing radiotherapy

were included, showed that low bacterial diversity and higher representation of *Clostridium IV*, *Roseburia* and *Phascolarctobacterium* were significantly associated with late radiation enteropathy.

5.5.3 The microbiome and immunotherapy

A pilot study from 2013 pointed out that mice treated with antibiotics or sterile mice showed a reduced response to immunotherapy, while administration of bacterial lipopolysaccharides was sufficient to increase the anticancer response. Two years later, the results of experiments revealed that mice with malignant melanoma showed different effectiveness of treatment depending on the composition of their gut microbiome. The conclusions reached suggest that intestinal bacteria of the genus *Bifidobacterium* activate immune dendritic cells, thereby increasing the effectiveness of immunotherapy and contributing to tumour regression. Faecal microbiota transplantation (FMT) from mice with a better response to treatment to mice without a treatment response led to tumour regression in previously non-responding animals. Another experimental study also pointed to the positive effect of immunotherapy in mice with sarcomas, which had a standard microbial colonization of the gut. In contrast, mice treated with antibiotics as well as sterile mice showed no treatment effect. One finding of interest was the increased activation of dendritic cells in the tumour and its regression in the case that bacteria of the genera *Bacteroides* and *Burkholderia* were administered to sterile animals before the immunotherapy itself.

The results of clinical studies also confirmed the important influence of the microbiome on the effectiveness of immunotherapy using immune checkpoint inhibitors. The species *Enterococcus faecium*, *Collinsella aerofaciens*, *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Klebsiella pneumoniae*, *Veillonella parvula,* and *Parabacteroides merdae* were more prominent in stool samples from patients with metastatic melanoma who responded to immunotherapy with PD-1 antibodies. In contrast to this, *Ruminococcus obeum* and *Roseburia intestinalis* were predominant in those who did not respond to the treatment. Another study on a sample of patients with melanoma showed differences in the gut microbiome of patients who responded versus those who did not respond to immunotherapeutic treatment with PD-1 blockade. Sequencing of stool samples revealed that the microbiome of those responding to treatment was dominated by members of the family *Ruminococcaceae* and *Faecalibacterium*, while the microbiome of those not responding was represented by *Bacteroides thetaiotaomicron*, *Escherichia coli,* and *Anaerotruncus colihominis.* The presence of the beneficial genus *Faecalibacterium* correlated positively with effector $CD4^+$ and $CD8^+$ T cells in the circulation, ensuring an enhanced anticancer and systemic immune response. Increased levels of commensal *Akkermansia muciniphila* correlated with better survival in patients with advanced non-small cell lung cancer and renal cell carcinoma treated with immunotherapy. A higher treatment efficiency and the absence of disease progression were observed in patients with a richer representation of the gut microbiome.

5.5.4 The microbiome and haematopoietic stem cell transplantation

Before haematopoietic stem cell transplantation (HSCT), haemato-oncology patients are given the so-called a preparatory regimen, which serves for the removal of tumour cells and inducing a state of immunosuppression. It includes high-dose chemotherapy or radiotherapy, which causes major changes in the composition of the gut microbiome. Several clinical studies comparing the composition of the faecal microbiota before and after allogeneic HSCT pointed to a significant loss of bacterial diversity after treatment and an association with an increased risk of *graft-versus-host disease* (GvHD). This serious post-transplantation complication is among the most common causes of death in patients after HSCT. The microbial analyses focuse on the search for microbial markers that would help identify patients at higher risk of serious complications after HSCT. Modulation of the gut microbiome of patients towards beneficial bacterial species could thus represent a new trend in the care of oncology patients undergoing transplantation in the future. A large retrospective study of stool samples from 541 patients admitted for allogeneic HSCT showed an association between a higher incidence of *Eubacterium limosum* and a reduced risk of disease relapse. Some studies, in turn, have shown that reduced GvHD-related mortality and improved overall survival in patients after allogeneic HSCT may be associated with the presence of anaerobic *Blautia* species. A comparison of microbiome changes in patients with autologous HSCT, in which the patient's own haematopoietic stem cells are administered, and allogeneic HSCT pointed to a faster restoration of the gut microbiota in patients after autologous transplantation.

5.5.5 The "microbiota-gut-brain" axis

The human brain is very sensitive to microbial disharmony. Animal studies have shown that changes in the microbiota-host relationship affect the enteric nervous system (ENS) and activate neuroimmune signalling pathways that affect brain development and function. Mice raised under germ-free conditions or mice treated with broad-spectrum antibiotics showed disrupted maturation of immune microglial cells present in the brain.

The gut microbiota communicates with the human brain through the "microbiota-gut-brain" axis, which represents a two-way communication link between the gut microbiome, the ENS, and the central nervous system (CNS). Signals sent by the brain through the sympathetic and parasympathetic arms of the autonomic nervous system control the motility of the gastrointestinal tract, and sensory and secretory functions through endocrine and nervous mechanisms. Conversely, endocrine, neurocrine, and inflammatory signals generated by gut microorganisms and specialised cells in the gut can profoundly affect brain function as well as mood, psyche and behaviour. The effects on the brain include both systemic communication via the bloodstream as well as signalling through the *vagus nerve*, which anatomically connects the gut and the brain (Fig. 11).

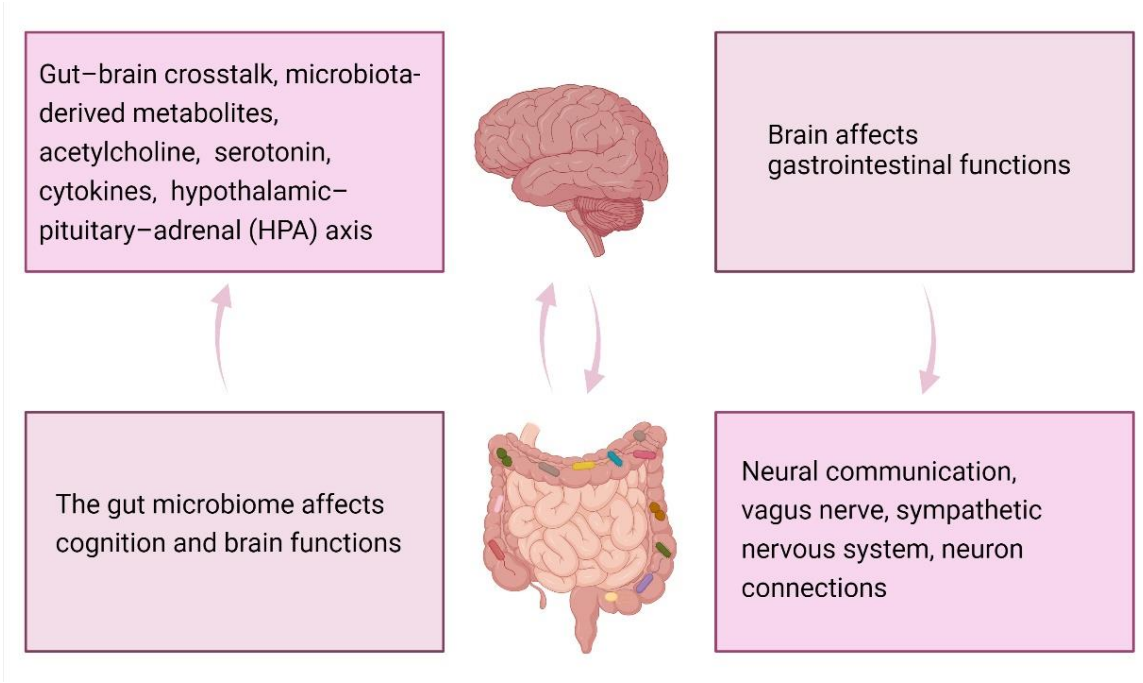

Figure 11
The "microbiota-gut-brain" axis.
A two-way communication link connects the gut microbiome, the ENS and the CNS.
Created with Biorender.com. *Explanations: CNS – central nervous system, ENS – enteric nervous system.*

The study of these mechanisms revealed the signalling pathways through which communication takes place along the "microbiota-gut-brain" axis. Microorganisms present in the gastrointestinal tract produce metabolites, such as SCFA, trimethylamine (TMA) and endotoxins, that circulate through the blood to the brain and can thus affect nerve function. Certain bacteria secrete neurotransmitters, such as acetylcholine, GABA and serotonin (a derivative of tryptophan). Some strains can metabolise tryptophan to 5-hydroxytryptamine (5HT), a lack of which leads to long-term changes and neurodevelopmental disorders. GABA is a neurotransmitter that is important for a healthy brain and nervous system. As much as 90% of serotonin in the body is estimated to be made in the digestive tract, and this neurotransmitter plays an important role in mood, learning, appetite control and sleep. The CNS is intricately connected with the ENS, through which an exchange of neurotransmitters can take place. What's more, the increased immune response to intestinal dysbiosis leads to the release of pro-inflammatory cytokines, which are transported through the bloodstream to the brain, where they can trigger inflammatory processes.

5.6 Modulation of the gut microbiome

The great advantage of the human microbiome over the human genome is the fact that it can be relatively easily modulated. Dysbiosis in the gastrointestinal tract can be improved in several ways – the simplest way is to change a lifestyle, dietary habits, and physical

activity. However, mounting evidence from the clinical studies is focused on modulating the composition of the gut microbiome in cancer patients through the administration of probiotics, prebiotics, postbiotics, or faecal microbiota transplantation (FMT). One of the main reasons for probiotic supplementation in cancer patients is to reduce the gastrointestinal toxicity caused by chemotherapy or radiotherapy. On the other hand, FMT is considered a promising tool to increase the efficacy of antitumour therapy in patients resistant to the applied treatment regimes. In the case of immunocompromised oncological patients, the issue of the safety of microbiota modulation with probiotics and FMT still arises, given the possible risk of infections from administered bacterial species. In this context, increasing evidence from clinical trials highlights the safety of probiotics, showing very infrequent cases of probiotic-related sepsis, bacteremia, or infections. These findings are also supported by several comprehensive meta-analyses comprising a large number of cancer patients, which indicated that incorporating probiotics into treatment regimens could potentially serve as a cost-effective and safe microbiome-based approach.

5.6.1 Probiotic supplementation in cancer patients

Probiotics have attracted the attention of the scientific community since the early twentieth century. Over a century ago, Nobel Prize laureate Professor Metchnikoff first introduced the concept, describing how the gut microbiota could be modulated, with pathogenic microorganisms being replaced by species beneficial to health. Metchnikoff proposed that drinking fermented milk containing *Bacillus bulgaricus* would create a layer of beneficial bacteria, lowering the intestinal pH and suppressing the growth of proteolytic bacteria.

According to the latest definition by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), probiotics are characterized as live microorganisms that confer a benefit to the host when administered in adequate amounts. Based on data, it is assumed that consuming probiotic preparations in an approximate amount of 100 grams per day is necessary for their beneficial effect to deliver at least 10^9 live cells to the intestine. The most commonly represented probiotic bacterial genera are *Lactobacillus* and *Bifidobacterium*, with the most well-known probiotic species, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus longum, Lactobacillus plantarum, Bifidobacterium bifidum, Bifidobacterium breve*, and *Bifidobacterium infantis*. *Lactobacillus* is a known producer of antioxidants (superoxide dismutase, glutathione, catalase, and anti-angiogenic factors) that reduce DNA damage and inflammatory processes, leading to a downregulated expression of tumour-specific proteins. Representatives of the *Bacillus, Enterococcus,* and *Streptococcus* genera also have probiotic features. The yeast *Saccharomyces boulardii* is also often used in gastrointestinal complications. Probiotic bacteria produce antimicrobial substances such as bacteriocins, lactic acid, reuterin, hydrogen peroxide, and deconjugated bile acids, resulting in the inhibition of pathogenic bacteria. Some probiotic bacteria can compete with pathogens for nutrients or adhere to intestinal epithelial cells, thus physically blocking the adhesion of pathogens.

Clinical studies indicate the safety of probiotics, as infections caused by probiotic species are very rare. A study on the use of probiotics in oncological patients undergoing chemotherapy or chemoradiotherapy documented that almost a third of patients (28.5%) take probiotic supplementation. Only 8.5% of supplemented patients reported side effects such as diarrhoea, vomiting, allergy, infection, constipation, and flatulence. The results of a large meta-analysis involving 2.982 patients suggested that probiotic administration could be a safe tool for reducing infectious complications and gastrointestinal toxicity in cancer patients.

Chemotherapy-induced gastrointestinal mucositis often has very severe consequences and can lead to a reduction in drug dosage or a complete interruption of therapy. Administration of the probiotic preparation Colon Dophilus™ reduced the incidence of grade 3 and 4 diarrhoea in the probiotic group of patients with colorectal cancer treated with irinotecan compared to the placebo group. Similarly, a meta-analysis involving 1.557 participants in 11 studies confirmed a positive effect of probiotics on the severity and incidence of diarrhoea in cancer patients. As a result, the need for antidiarrhoeal medications also decreased. A study focusing on the development of oral mucositis after chemoradiotherapy showed that administration of lozenges containing *Lactobacillus brevis* CD2 reduced the incidence of grade 3 and 4 oral mucositis in patients with head and neck cancer after the treatment. On the other hand, a recently published meta-analysis did not find a mitigating incidence of severe diarrhoea after oral administration of probiotics in a group of patients with colorectal cancer, oesophagal cancer, and gynaecological malignancies undergoing chemotherapy or chemoradiotherapy.

So far, only a few studies have published the results concerning probiotic administration to alleviate chemotherapy-induced cognitive effects. According to the findings, supplementation with *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* alleviated symptoms of depression, anxiety, and fatigue in surviving patients with colorectal cancer. A randomized, double-blind, controlled study involving 120 elderly patients with colorectal cancer or orthopaedic disorders found a correlation between perioperative application of orally administered probiotics and postoperative reduction in cognitive impairments. The probiotic group of patients showed increased bacterial diversity and decreased plasma levels of interleukin IL-6 and cortisol, suggesting a possible mechanism via the reduction of peripheral inflammation and stress response. Probiotic intervention in patients with laryngeal cancer reduced preoperative clinical anxiety by suppressing levels of corticotropin-releasing factors in the serum and also prevented preoperative increases in heart rate in patients.

Neuroprotective effects of probiotics have been observed in many experimental models and clinical studies dealing with behavioural disorders and neurodegenerative disorders. The proposed underlying mechanisms include the ability of probiotic strains to produce SCFAs, leading to increased expression of claudin and occludin, which help to maintain the integrity of the blood-brain barrier. The production of tryptophan metabolites, blocking pro-inflammatory NF-kB, VEGF-B together with astrocyte and microglial cell activation represent other possible mechanisms. Long-term administration of probiotic strains in mice reduces anxiety and depressive states, normalizes immune response, induces changes in GABA production, reduces markers of oxidative stress in the brain, and restores basal levels of norepinephrine in the brainstem. In clinical settings, *Lactobacillus helveticus* and *Bifidobacterium longum*

have shown a positive impact on mood and memory improvement in healthy volunteers. Oral administration of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175V brought benefits to anxiety and depression-related behaviour in healthy volunteers.

Besides their neuroprotective effects, the probiotics also possess cardioprotective properties. Particularly, representatives of the genus *Lactobacillus* exhibited reduced apoptosis of cardiomyocytes, protective effects against myocardial damage, enhancement in cardiac function, and increased survival of post-infarction animals in mouse and rat models. Consumption of probiotics in patients with heart failure led to improvement in disease-related parameters. Moreover, patients with metabolic syndrome exhibited a reduced risk of cardiovascular diseases after probiotic treatment.

In conclusion, increasing evidence supports the positive impact and clinical utility of probiotics in cancer patients. However, not all findings are consistent, and further research in this area is much needed. Patients undergoing cancer therapy should always consult the possibility of probiotic supplementation with their oncologist.

5.6.2 Faecal microbiota transplantation

Faecal microbiota transplantation (FMT) is the method of transferring processed stool from donors to recipients, aiming to restore a healthy gut microbiota in the recipients. This method appears to be a promising trend in oncology, as it quantitatively and qualitatively surpasses the administration of probiotics alone. Currently, it is mainly used in the treatment of severe dysbioses, such as infections caused by *Clostridium difficile*, which can lead to diarrhoea and life-threatening colitis with secondary sepsis.

Several delivery ways for transferring faecal microbiota exist, each with advantages and disadvantages. FMT can be administered orally via nasogastric, nasoduodenal and nasojejunal tubes, or through oral intake of frozen capsule forms. However, in most cases, patients undergo the transfer of a small amount of liquefied, filtered stool through colonoscopy into the last part of the small/large intestine. Although colonoscopic administration of FMT appears to be the most effective method for patients, it may cause discomfort, possible gastrointestinal bleeding, and the risk of intestinal perforation. Due to the potential risk of transmitting infectious microorganisms or multidrug-resistant strains, stool donors are strictly selected based on stringent criteria. Before stool collection, donors undergo a series of tests to exclude syphilis, hepatitis A, B, C, HIV, AIDS, atopic, and autoimmune diseases. Patients with obesity, gastrointestinal malignancies, chronic tract diseases, or gastric bypass are excluded from donation. Additionally, donors cannot be supplemented with antibiotics, probiotics, or immunosuppressants for at least three months before stool collection due to possible changes in microbial composition. Stool samples from donors are also tested for the presence of worm eggs and toxins produced by pathogenic bacterial species such as *Helicobacter pylori, Clostridium difficile, Staphylococcus aureus*, and *Shigella* spp. All recipients should be informed about the potential adverse effects of FMT, with immunodeficient patients being at the highest risk.

Experimental studies have indicated the possibility of using FMT to prevent adverse effects of anticancer therapy and to increase treatment efficacy. The development of intestinal mucositis represents an example of acute toxicity induced by the treatment of colorectal cancer using the FOLFOX regimen, based on a combination of 5-fluorouracil, leucovorin, and oxaliplatin. *In vivo* study focusing on FMT from healthy mice to recipient colorectal tumour-bearing animals undergoing FOLFOX therapy uncovered that faecal transfer restored the gut microbial composition in the transplant recipient mice. As shown, FMT led to reduced occurrence of diarrhoea and intestinal damage compared to animals treated only with chemotherapy.

Recently, clinical studies have emphasized the significant relationship between favourable intestinal microbiome composition and treatment efficacy, especially immunotherapy and chemotherapy. Important insights in this area have come from experiments where faecal microbiota from cancer patients responding to immunotherapy was transferred to experimental mice. The results documented improved response to anticancer therapy in recipient animals. Conversely, transferring stool from non-responding patients led to tumour progression and treatment resistance. Gut bacteria associated with a better treatment response may influence innate and acquired immunity. These findings are consistent with pilot analyses obtained from cohorts of cancer patients, where FMT from responders increased the efficacy of immunotherapy in initially refractory individuals. The safety of this method in cancer patients with high levels of immunosuppression is of major concern due to the unknown complex composition of microbial communities in donor stool. Further research on large patient cohorts, detailed recipient stratification, along with continuous improvement of criteria for transplant donors and comprehensive testing according to evidence-based medicine, will facilitate the potential trend of FMT in routine clinical practice.

The study of the gut microbiome in cancer patients represents a new trend in the era of personalized medicine. The application of individual microbiome profiles as early diagnostic and prognostic biomarkers for treatment efficacy is a significant challenge for cancer research. Increasing evidence confirms the key role of the microbiome in tumourigenesis and its undeniable impact on the outcomes of anticancer treatment and its adverse effects. Gut microbiome modulation through probiotics, prebiotics, or FMT represents an attractive and feasible adjuvant to existing treatment modalities.

However, the complexity of interactions between the host and intestinal microecosystem and underlying mechanisms are far from adequately understood. Although the number of studies aiming to identify microbiomes is increasing, a crucial question for understanding the functional impact of the microbiome remains the limitations in taxonomic resolutions. Therefore, a comprehensive approach involving metagenomics, metatranscriptomics, metabolomics, and metaproteomics is strongly recommended for the most precise evaluation of the role of the microbiome in cancer. Results obtained from animal models and clinical studies suggest that anticancer and supportive therapy modulates the microbiome composition in cancer patients, and conversely, the gut microbiome modulates the response to treatment, especially chemotherapy and immunotherapy. A deeper understanding of relevant associations and advances in therapeutic approaches for microbiota modulation is important for cancer prevention, but also for increasing treatment efficacy and improving outcomes in patients with an oncologic diagnosis.

References

Alexander JL, Wilson ID, Teare J, et al. Gut microbiota modulation of chemotherapy efficacy and toxicity.Nature reviews. Gastroenterology & hepatology 2017; 14(6):356-365.

Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. Nature 2011; 473(7346):174-180.

Bajic JE, Johnston IN, Howarth GS, et al. From the Bottom-Up: Chemotherapy and GutBrain Axis Dysregulation. Front Behav Neuroscience 2018; 12:104.

Ciernikova S, Kasperova B, Drgona L, et al. Targeting the gut microbiome: An emerging trend in hematopoietic stem cell transplantation. Blood reviews 2021; 48:100790.

Ciernikova S, Mego M, Hainova K, et al. Modification of microflora imbalance: future directions for prevention and treatment of colorectal cancer? Neoplasma 2015; 62(3):345-352.

Ciernikova S, Mego M, Chovanec M. Exploring the Potential Role of the Gut Microbiome in Chemotherapy-Induced Neurocognitive Disorders and Cardiovascular Toxicity. Cancers 2021; 13(4):782.

Ciernikova S, Sevcikova A, Drgona L, Mego M. Modulating the gut microbiota by probiotics, prebiotics, postbiotics, and fecal microbiota transplantation: An emerging trend in cancer patient care. Biochim Biophys Acta Rev Cancer 2023;1878(6):188990.

Dohlman AB, Arguijo Mendoza D, Ding S, et al. The cancer microbiome atlas: a pan-cancer comparative analysis to distinguish tissue-resident microbiota from contaminants. Cell host & microbe 2021; 29(2):281-298.e5.

Gopalakrishnan V, Spencer CN, Nezi L, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science 2018; 359(6371):97-103.

Gur C, Ibrahim Y, Isaacson B, et al. mBinding of the Fap2 protein of Fusobacterium nucleatum to human inhibitory receptor TIGIT protects tumors from immune cell attack. Immunity 2015; 42(2):344-355.

Huang K, Liu Y, Tang H, et al. Glabridin Prevents Doxorubicin-Induced Cardiotoxicity Through Gut Microbiota Modulation and Colonic Macrophage Polarization in Mice. Front Pharmacol. 2019; 10:107.

Chovanec M, Vasilkova L, Setteyova L, et al. Long-Term Cognitive Functioning in Testicular Germ-Cell Tumor Survivors. Oncologist 2018; 23(5):617-623.

Iida N, Dzutsev A, Stewart CA, et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science 2013; 342: 967-970.

Integrative HMP (iHMP) Research Network Consortium. The Integrative Human Microbiome Project. Nature 2019; 569(7758):641-648.

Krautkramer KA, Fan J, Bäckhed F. Gut microbial metabolites as multi-kingdom intermediates. Nature reviews. Microbiology 2021; 19(2):77-94.

Loman BR, Jordan KR, Haynes B, et al. Chemotherapy-induced neuroinflammation is associated with disrupted colonic and bacterial homeostasis in female mice. Sci Rep. 2019; 9(1):16490.

Mego M, Holec V, Drgona L, et al. Probiotic bacteria in cancer patients undergoing chemotherapy and radiation therapy. Complementary therapies in medicine 2013; 21(6): 712-723.

Okubo R, Kinoshita T, Katsumata N, et al. Impact of chemotherapy on the association between

fear of cancer recurrence and the gut microbiota in breast cancer survivors. Brain Behav Immun. 2020; 85:186-191.

Ottman N, Smidt H, de Vos WM, et al. The function of our microbiota: who is out there and what do they do? Front Cell Infect Microbiol 2012; 2:104.

Peled JU, Devlin SM, Staffas A, et al. Intestinal Microbiota and Relapse After Hematopoietic-Cell Transplantation. J Clin Oncol 2017; 35(15):1650-1659.

Poore GD, Kopylova E, Zhu Q, et al. Microbiome analyses of blood and tissues suggest cancer diagnostic approach. Nature 2020; 579(7800):567-574.

Qin J, Li R, Raes J,Arumugam M, et al.Ahuman gut microbial gene catalogue established by metagenomic sequencing. Nature 2010; 464(7285):59-65.

Routy B, Le Chatelier E, Derosa L, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science 2018; 359(6371):91-97.

Sevcikova A, Izoldova N, Stevurkova V, et al. The Impact of the Microbiome on Resistance to Cancer Treatment with Chemotherapeutic Agents and Immunotherapy. International journal of molecular sciences 2022; 23(1):488.

Sivan A, Corrales L, Hubert N, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Science 2015; 350(6264):1084-1089.

Subramaniam CB, Bowen JM, Gladman MA, et al. The microbiota-gut-brain axis: An emerging therapeutic target in chemotherapy-induced cognitive impairment. Neurosci Biobehav Rev. 2020; 116:470-479.

Taur Y, Coyte K, Schluter J, et al. Reconstitution of the gut microbiota of antibiotic-treated patients by autologous fecal microbiota transplant. Science Translational Medicine 2018; 10(460):eaap9489.

The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature 2012; 486(7402):207-214.

Thomas AM, Fidelle M, Routy B, et al. Gut OncoMicrobiome Signatures (GOMS) as next-generation biomarkers for cancer immunotherapy. Nat Rev Clin Oncol 2023; 20(9):583-603.

Vétizou M, Pitt JM, Daillère R, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science 2015; 350(6264):1079-1084.

Viaud S, Saccheri F, Mignot G, et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science 2013; 342(6161):971-976.

Wang L, Chen Q, Qi H, et al. Doxorubicin-Induced Systemic Inflammation Is Driven by Upregulation of Toll-Like Receptor TLR4 and Endotoxin Leakage. Cancer Res 2016; 76(22): 6631-6642.

Zhao LY, Mei JX, Yu G, et al. Role of the gut microbiota in anticancer therapy: from molecular mechanisms to clinical applications. Signal Transduct Target Ther 2023; 8(1):201.

6 The tumour microenvironment and tumour microbiome

The microenvironment is a complex communication network formed by cellular components and molecules. Growth factors, enzymes, cytokines (and their component chemokines) as well as other molecules are used in the molecular signalling system of the microenvironment. In the microenvironment, there is less heterogeneity of cells with better genetic stability in comparison with tumour cells.

6.1 Composition of the tumour microenvironment

The tumour microenvironment generally consists of non-malignant components, including immune cells, non-cellular extracellular matrix (ECM), stromal cells and blood vessels. It has a very dynamic composition that differs for individual types of tumours; however, the mentioned basic components are characteristic of all of them. Through cellular interactions, secreted proteins, cytokines, and metabolites, it provides malignant cells with signals for survival and proliferation and also mediates immunomodulating signals.

The constant interactions between malignant and non-malignant cells within the tumour microenvironment affect the development and progression of oncologic diseases. Non-malignant cells often have a pro-tumourigenic function in all stages of carcinogenesis and stimulate uncontrolled cell proliferation. The tumour microenvironment contributes to the support of angiogenesis with the aim of overcoming hypoxic conditions and restoring oxygen and nutrient supplementation. Tumours interact with the surrounding microenvironment and other host organs through the circulatory system for blood and lymph.

The tumour microenvironment consists of:

- immune cells: such as, e.g. T-cells, B-cells, NK cells, neutrophils, basophils, mast cells, and macrophages. These are an important component of the tumour microenvironment, and they can have either tumour-promoting or tumour-inhibiting properties;
- endothelial cells: they line blood vessels and support the formation of new blood vessels (angiogenesis), which supply the tumour with nutrients and oxygen;

- fibroblasts: belong among the connective tissue cells and provide structural support to the tumour. They produce collagen and other components of the extracellular matrix;
- pericytes: they help regulate blood flow in vessels and can contribute to angiogenesis;
- mesenchymal stem cells: they can differentiate into many different cell types including fibroblasts, endothelial cells, and immune cells. They contribute to tumour growth by supporting angiogenesis and suppressing the immune response;
- tumour-associated fibroblasts: provide tumour cells with growth factors and cytokines to support their survival and proliferation. They can, however, also inhibit tumourigenesis;
- dendritic cells: they can capture antigens released from tumour cells and then present them on their surface to T-lymphocytes, which results in the stimulation of adaptive T-cell immunity in interaction with cytokines and interferons;
- adipocytes: they influence the tumour microenvironment through the secretion of metabolites, enzymes, hormones, growth factors and cytokines;
- stellate cells: after activation, they modify the extracellular matrix and stimulate pro-angiogenic factors;
- senescent cells: they can support the growth and progression of tumours by producing substances that are part of the secretory phenotype associated with senescence;
- non-cellular extracellular matrix.

Immune cells, which are an important component of the tumour microenvironment, influence the growth and progression of tumours. We can divide them into two specific categories: immune cells of innate immunity (dendritic cells, lymphoid cells, macrophages, myeloid-derived suppressor cells, NK cells, and neutrophils) and immune cells of adaptive immunity (B-cells and T-cells). The presence of infiltrated innate and adaptive cells in tumours can support either an anti- or a pro-tumourigenic processes in patients. The ECM is a highly dynamic non-cellular component of the tumour microenvironment, composed mainly of collagen, fibronectin, elastin, and laminin. Its key role is to direct cell migration and proliferation through its topography and physical properties. According to the findings, tumour cells, as well as cancer-associated fibroblasts (CAF), are the main source of ECM molecules. In many solid tumours, the ECM makes up nearly 60% of the tumour mass and changes in its composition take place during progression and metastatic spread. Stromal cells, including CAFs, form most of the tumour stroma and support not only tumour initiation but also angiogenesis and tumour progression. Studies have confirmed that CAFs contribute to therapeutic resistance in breast cancer and directly suppress anticancer T-cells within the tumour microenvironment. The tumour vasculature, unlike the normal vasculature of ordered and differentiated arteries, arterioles, capillaries, and veins, is irregular and chaotic. Blood vessels are more abundant at the tumour interface with reduced vascularity towards the central region, which leads to zones of ischemia as well as necrotic areas. Imbalanced secretion of pro-angiogenic vascular endothelial growth factor-A (VEGF-A) results in the formation of blood vessels.

The proliferation and metastatic spread of tumour cells are associated with the reprogramming of the tumour microenvironment, the goal of which is to support the supplementation of

tumour cells with nutrients. Complex interactions are determined by structural and biochemical properties along with mutual communication. The mutual dialogue in the microenvironment is mediated by a mixture of cytokines, chemokines, growth factors, and inflammatory mediators together with remodelling enzymes of the ECM. At present, new methods of communication via extracellular vesicles (EVs), including exosomes and apoptotic bodies, as well as miRNAs, are also being explored.

Data is constantly growing that documents the significant influence of the host's gut microbiome on shaping the tumour microenvironment. The microbiota regulates immune and hormonal signalling, which influences the components of the microenvironment due to the modulation of processes involved in the promotion of tumour growth. In addition to this, microbial metabolites can enter the tumour microenvironment through circulation and thus become part of it. More and more data have recently been accumulated confirming the presence of bacteria in the tumour microenvironment, which indicates their involvement in several pathways related to tumourigenesis and tumour progression (Fig. 12).

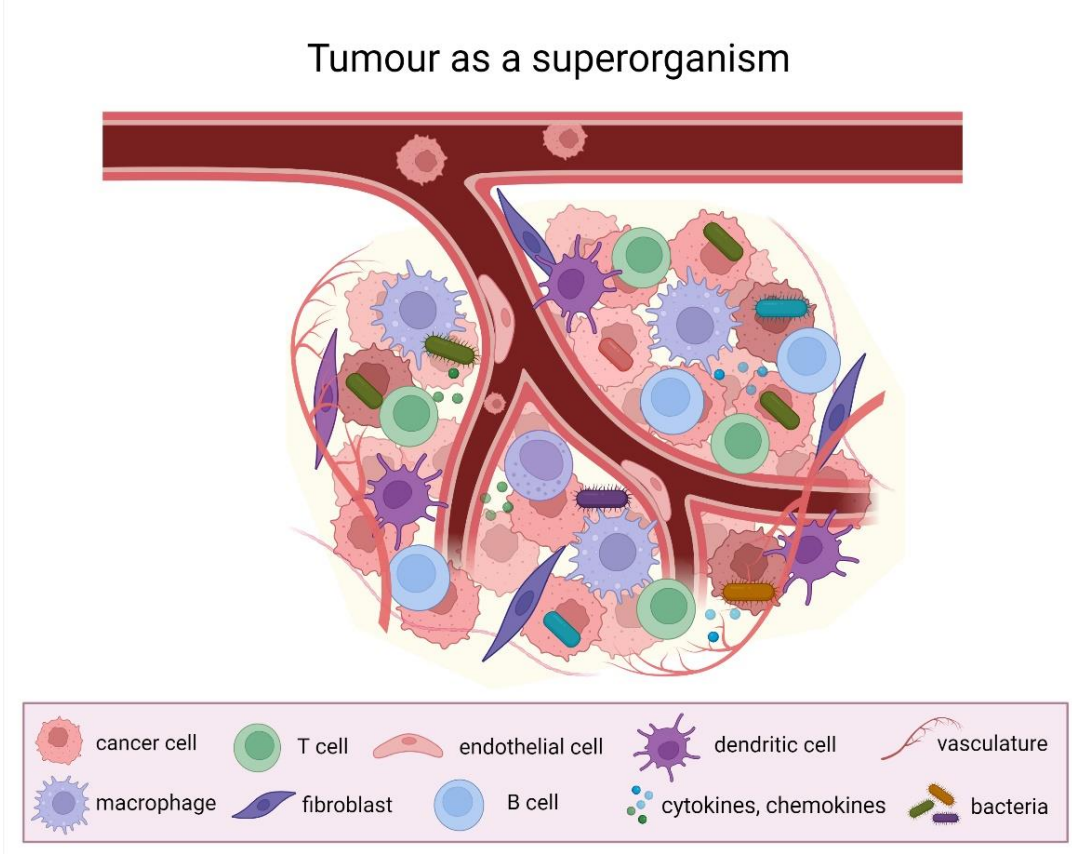

Figure 12
The tumour as a superorganism.
A tumour is a multicellular and multisignal system. The microenvironment is an ecosystem that provides a protective environment for the tumour. Tumour cells are surrounded by stromal cells and immune cells, which together build a regulatory network that supports tumour growth by creating a specific environment to ensure tumour protection from the immune system and subsequent destruction. More recent data show the presence of bacteria in the tumour microenvironment. Created with Biorender.com

6.2 The tumour microbiome

The study of the tumour microbiome is an area that is currently expanding our understanding of the origin of tumour-associated microorganisms and their functions. Bacteria were found to be located in tumours more than 100 years ago, but only advances in sequencing methods have enabled a more detailed examination of the tumour microbiome and its role in tumourigenesis and progression. Techniques used to investigate the tumour microbiome must be sufficiently sensitive and specific to allow adequate characterisation. Present methods include microscopy as well as methods based on genomics and microbial culture. The combination of these technological approaches and their improvement for the study of microorganisms directly in the tumour microenvironment enable us to obtain the most complete view possible. The use of the necessary controls is necessary to avoid biasing the interpretation due to contaminants.

Recent knowledge is based on the study of bacteria associated with the tumour, which can play a role as an immune activator, inhibitor or both. It has been shown that in some types of malignancies, they can also affect the progression and prognosis of the oncologic disease. The tumour microbiome represents an important source of signals and antigens for starting the body's immune responses, which can potentially influence the response to cancer immunotherapy. The significant role of the microbiome in tumourigenesis, differentiation and malignant progression is also underlined by the fact that in 2022 polymorphic microbiomes were added to a complex integrative concept summarising the characteristic key features of malignancies called *The Hallmarks of Cancer*. The microbiome directly interacts, either positively or negatively, with other hallmarks of cancer, such as inflammation, immune impairment, genomic instability and resistance to anticancer therapy. The most significant evidence for this integrated role comes from studies of the gut microbiome, but the role of microorganisms in other tissues/organs, and especially microbial communities that are localised in tumours (intratumoural or tumour microbiome), has recently been increasingly emphasised.

6.3 Study of the tumour microbiome in various malignancies

Analyses focused on identifying the tumour microbiome are part of a transdisciplinary approach, which includes microbiological, immunological and oncological concepts. While the associations between the gut microbiome, carcinogenesis and response to anticancer treatment have been intensively studied, the influence of the tumour microbiome requires further comprehensive research. Among the very important findings is that recent studies have revealed a correlation between the tumour microbiome and the clinical characteristics of the corresponding tumours, which points to the possibility of using the identification of intratumoural bacteria and metabolites to benefit the clinical status of patients (Fig. 13).

Recent research aimed at determining the presence of bacteria in the tumour microenvironment, characterising the microbial composition in individual types of tumours and monitoring the impact of the tumour microbiome on the response of patients to anticancer therapy is a significant

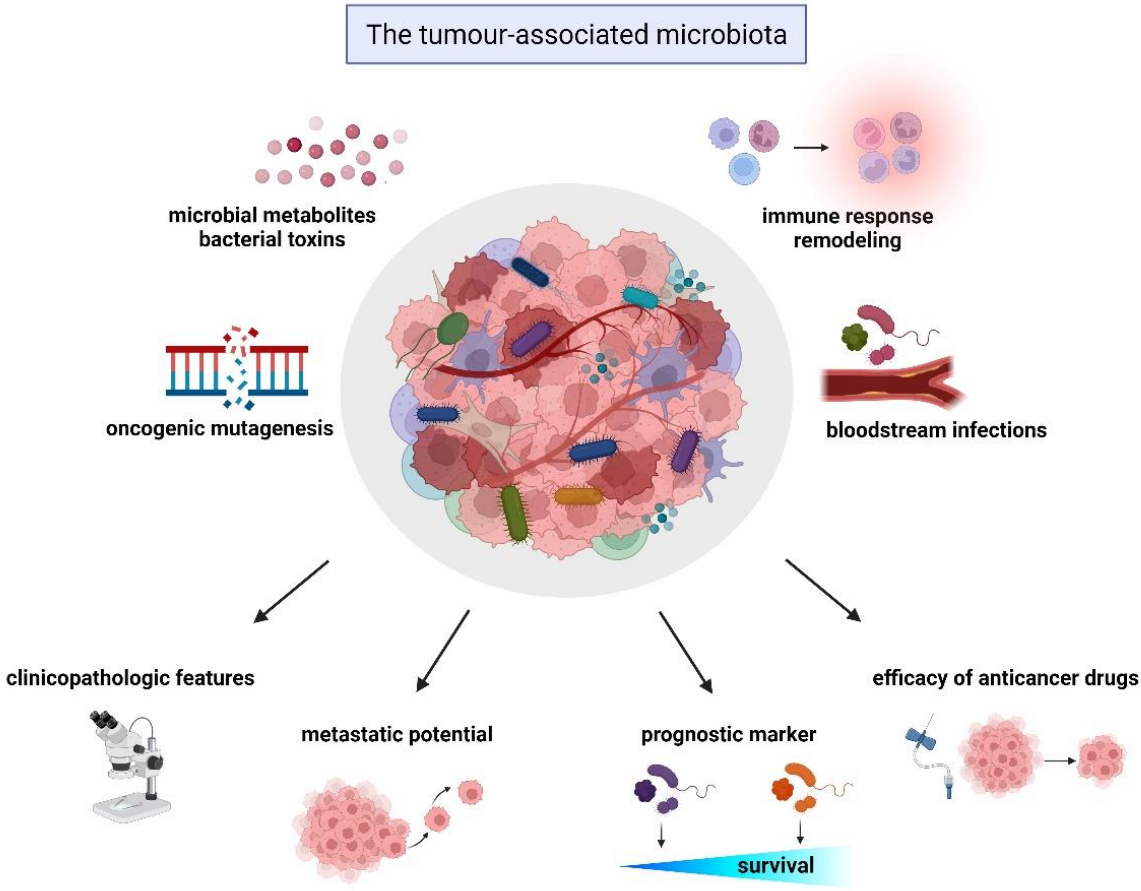

Figure 13
The tumour-associated microbiota.
The tumour microbiome is important in the progression and treatment of oncologic diseases. The proposed mechanisms by which intratumoural microbiota influence tumourigenesis, progression and response to therapeutics include increased mutagenesis, regulation of oncogenes and oncogenic pathways, modulation of host immune response pathways, metabolism of anticancer drugs and the production of bacterial toxins and metabolites. Increasing evidence from both animal models and clinical studies has revealed the association of the tumour microbiome with clinicopathological features, efficacy of anticancer therapy, metastatic potential and the survival of cancer patients.
(Adapted and translated from Ciernikova et al. 2022, created with Biorender.com)

contribution to the issue of the tumour microbiome. A comprehensive tumour microbiome analysis of more than 1,500 samples from different types of malignancies, including breast, lung, ovarian, pancreatic, bone, brain, and melanomas as well as adjacent healthy tissues, revealed that each tumour type has a distinct microbial composition. *Proteobacteria* and *Firmicutes* were most represented in all types of tumours, while the phylum *Actinobacteria*, including the families *Corynebacteriaceae* and *Micrococcaceae*, dominated in non-gastrointestinal tumours. Based on the obtained results, biopsy samples from breast tumours were characterised by having the richest representation of bacteria and the highest bacterial diversity. In the majority of samples, these were intracellularly localised bacteria present in tumour and immune cells. To confirm the presence of live bacteria, sections from freshly resected breast tumours were cultured *ex vivo* with fluorescently labelled d-alanine, which is used in the formation of peptidoglycan,

a key component of the bacterial cell wall. Intracellular staining in breast tumours confirmed the presence of living, metabolically active bacteria. The correlation between specific bacterial species present in the tumour microenvironment and tumour types, smoking status and response to immunotherapy was also a very important finding. The authors of the study, however, could not determine whether the bacteria present in tumour tissues play a causal role in the development of malignancies, or whether their presence in tumours is of secondary origin and reflects infections in already established tumours.

6.3.1 Colorectal cancer

Based on more than 50 years of research, colorectal cancer represents the malignancy with the most prominent association between the microbiome and tumourigenesis. In 1998, the presence of intracellular *Escherichia coli* was documented in the colonic mucosa of 87% of patients with colorectal cancer; it was not observed in healthy individuals. The results of several studies have shown a higher representation of *Fusobacterium nucleatum* in colorectal adenomas and carcinomas compared to control tissues. A positive correlation between the presence of *Fusobacterium* and the development of metastases in the lymph nodes was a very important finding. What's more, DNA from *Fusobacterium* was also detected in 181 liver metastases of colorectal cancer. A lower density of $CD8^+$ cytotoxic T-cells and a higher density of myeloid-derived suppressor cells were observed in colorectal cancer liver metastases positive for *Fusobacterium.*

Along with the presence of *Fusobacterium* spp., some studies have revealed an increased abundance of pathogenic *Providencia* in colon tumours. *Fusobacterium* is known to be involved in colorectal carcinogenesis, while *Providencia* has demonstrated a role in infections. Both bacterial taxa share similar phenotypic characteristics, including the ability to affect and damage colorectal tissues. Patient-matched normal and colon tumour samples showed differences in the amounts of *Firmicutes*, *Bacteroidetes,* and *Proteobacteria*. While *Proteobacteria* predominated, representatives of *Firmicutes* and *Bacteroidetes* showed reduced levels in colon tumours. The colorectal tumour microbiome was also characterised by reduced amounts of *Lachnospiraceae*, *Ruminococcaceae*, *Faecalibacterium prausnitzii*, *Bacteroides*, *Rikenellaceae,* and *Bacteroides uniformis*.

Colorectal tumours also showed a higher abundance of *Lactococcus*, *Bacteroides*, *Fusobacterium*, *Prevotella,* and *Streptococcus*, while *Pseudomonas* was present around the healthy tissue surrounding the tumours. A recent analysis of intracellular bacteria recorded an increased incidence of intracellular *Escherichia coli* in adenoma and carcinoma specimens compared with control tissues obtained during routine colonoscopy. Further, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Bacillus cereus* were present in biopsies from patients with colorectal adenomas. Another series of studies revealed lower abundances of *Lanchnospiraceae* and *Ruminococcaceae* in colorectal tumour biopsies in comparison with polyps, adenomas, or control samples. In contrast, a significantly higher level of *Prevotella* and *Porphyromonas* was found in the tumour tissue.

Research has revealed that *Fusobacterium nucleatum* and *Escherichia coli* 17 are able to translocate into tumours via the circulatory system, suggesting an influence of the gut microbiome on the tumour microbiome. As a beneficial bacterial taxon, *Bifidobacterium* can stimulate the anticancer effect of immunotherapy through its translocation into the tumour. Systemic administration of *Bifidobacterium* resulted in increased response to immunotherapy in mice with colon tumours that had not responded to previous treatment. Intratumoural injection of the antibiotic cocktail, however, resulted in the ineffectiveness of the immunotherapy in responding mice showing the presence of *Bifidobacterium.*

6.3.2 Gastric cancer

Infection with *Helicobacter pylori* is a known risk factor for the development of gastric cancer. The presence of this bacterium is linked with changes in gastric acidity, which leads to differences in the composition of bacterial taxa. Other biological factors that contribute to maintaining the microenvironment of malignant tumours also play a role in the aetiology of gastric cancer. The data has shown that microbial diversity was higher in the tumour tissues of patients older than 60 compared to younger patients.

Microbial analysis of samples from normal, peritumoural and tumour tissues revealed an increased abundance of *Proteobacteria* in peritumoural samples, while elevated levels of *Firmicutes* and *Fusobacteria* were detected in tumour samples. Closer specification of the peritumoural samples found that there was an increase in *Halomonas*, *Shewanella*, *Enterococcus*, and *Brevundimonas* and a decrease in *Legionella. Streptococcus*, *Peptostreptococcus*, *Lactobacillus*, *Bifidobacterium*, *Neisseria*, *Veillonella,* and *Shewanella* dominated in tumour samples compared with normal gastric mucosa tissues. As the results showed, downregulation of the immune system was correlated with increased levels of immunosuppressive cells $BDCA2^{+}$pDCs and $Foxp3^{+}$Tregs within the tumour microenvironment. A study including biopsies from 33 participants with chronic gastritis associated with *Helicobacter pylori*, gastric intestinal metaplasia and gastric adenocarcinoma showed the presence of *Flavobacterium*, *Klebsiella*, *Serratia marcescens*, *Stenotrophomnonas*, *Achromobacter*, and *Pseudomonas* in tumour samples versus controls. As was predicted, *Helicobacter pylori* was the dominant species in samples from *Helicobacter pylori*-positive individuals, while *Haemophilus*, *Serratia*, *Neisseria,* and *Stenotrophomonas* were more abundant in *Helicobacter pylori*-negative samples.

Data from a clinical trial in patients with chronic gastritis and gastric cancer revealed that a specific nitrosating bacterial community within gastric cancer had genotoxic potential. In the case of tumour samples, a lowered diversity with dysbiotic potential and the dominance of *Phyllobacterium*, *Achromobacter*, *Xanthomonadaceae,* and *Enterobacteriaceae* as well as *Helicobacter* and *Neisseria* were observed. *Proteobacteria* dominated in almost 90% of malignant samples obtained by subtotal gastrectomy in 62 patients with gastric cancer. *Peptostreptococcus*, *Streptococcus,* and *Fusobacterium* were found in tumour samples. On the other hand, samples from adjacent non-tumour tissue contained lactic acid-producing bacteria.

It has been shown that specific differences in the microbial composition of the stomach can serve as a potential predictor of disease stages. Comprehensive metabolomic profiling of samples from gastric tumours documented higher bacterial diversity together with increased levels of amino acids, carbohydrates, carbohydrate conjugates, glycerophospholipids, and nucleosides compared to control samples. Several metabolites can be considered as biomarkers pointing to the difference between tumour and non-tumour tissues, including 1-methyl nicotinamide and N-acetyl-D-glucosamine-6-phosphate.

6.3.3 Pancreatic cancer

Several studies have confirmed the significant role of the intratumoural microbiome in the progression of pancreatic cancer and the modulation of responses to anticancer therapy. Higher microbial diversity was observed in the tumour microbiome of patients with pancreatic adenocarcinoma who showed long-term survival, and patients with a higher incidence of intratumoural taxa *Saccharopolyspora*, *Pseudoxanthomonas,* and *Streptomyces* showed better results. In patients with short-term survival, no predominant intratumoural bacterial genus was observed. Likewise, a higher density of $CD3^+$, $CD8^+$ cells and granzyme B was detected in patients with higher survival. Based on these results, it can be hypothesised that higher microbial diversity influences the anticancer immune response through the activation of immune cells. Up to 76% of pancreatic adenocarcinoma patient samples were positive for the presence of *Gammaproteobacteria.* The data obtained indicate that tumours contain bacteria that are able to influence the sensitivity of the tumour to the anticancer drug. What's more, published data indicate that the bacterium *Fusobacterium nucleatum* was specifically present in pancreatic tumours.

The potential role of *Helicobacter pylori* was also confirmed in the case of pancreatic cancer, since the DNA of this bacterium was detected in pancreatic tumours as well as in surrounding tissue samples, but not in controls. Co-cultivation of pancreatic cells with *Helicobacter pylori* increased their malignant potential, which led to higher NF-κB and AP-1 activity with subsequent dysregulation of cellular processes. *Fusobacterium nucleatum* was described especially in colorectal tumours, but levels of this bacterium were also elevated in pancreatic tumours. Furthermore, data have shown a correlation of *Fusobacterium nucleatum* with shorter survival rates in cancer patients. Microbial analysis of samples of the basal subtype of pancreatic adenocarcinoma showed an enrichment of *Acinetobacter*, *Pseudomonas* and *Sphingopyxis.* The presence of these three bacterial genera was positively correlated with DNA replication and the K-ras signalling pathway, but negatively correlated with bile acid metabolism. On the other hand, some studies did not confirm statistically significant differences in the pancreatic microbiome between normal pancreatic tissues compared to tumour samples.

Some data indicate the ability of the gut and tumour microbiome to promote the growth of pancreatic tumours. The gut microbiome of cancer patients showed a higher occurrence of *Proteobacteria*, *Actinobacteria*, *Fusobacteria,* and *Verrucomicrobia* compared with healthy controls. Of interest is that an analysis of the tumour microbiome showed a potential

translocation of *Proteobacteria* into tumours, and this bacterial phylum being represented in tumour tissue at a level of up to 50%.

6.3.4 Breast cancer

According to GLOBOCAN 2020 global statistics, breast cancer is the most frequently diagnosed cancer and one of the leading causes of cancer-related deaths in women worldwide. Data are increasing every day on the association of the microbial composition in breast tissue with physiological, but also pathological conditions. The modified composition of the gut and breast tumour microbiome promotes disease progression. Changes in metabolic pathways induced by a shift in the composition of microbial communities are associated with the heterogeneity of breast cancer. In this context, a different microbiome was observed in the breast of women with benign and malignant disease. Breast tissues from women with invasive breast cancer showed increased amounts of *Fusobacterium*, *Atopobium*, *Hydrogenophaga*, *Gluconacetobacter,* and *Lactobacillus*. Increased metabolism of cysteine, methionine, and fatty-acid synthesis were observed in benign tissues, while tumour tissues showed a decrease in the metabolism of inositol phosphate. Activation of invariant natural killer T-cells (iNKT) by certain bacterial taxa was correlated with protective function and promotion of anticancer immunity in the regulation of metastatic spread of breast cancer.

Comparing the healthy and breast tumour microbiomes showed that *Bacillus*, *Staphylococcus*, *Enterobacteriaceae*, *Comamondaceae,* and *Bacteroidetes* were more abundant in breast tumours, while *Prevotella*, *Lactococcus*, *Streptococcus*, *Corynebacterium*, and *Micrococcus* dominated in normal tissues of healthy women. RNA-seq analysis of breast tumour and normal tissue samples from Slovak patients compared with samples obtained from the SRA database (originating in China) revealed that *Proteobacteria*, *Firmicutes,* and *Actinobacteria* were the dominant bacterial phyla in both cohorts. Differences were noted, however, in the levels of *Bacteroides* and *Cyanobacteria*.

Chemotherapy changes the composition of the microbiome in breast tumours and causes shifts towards specific microorganisms. Based on the results, the presence of tumour-specific bacteria may correlate with the recurrence of malignancy. Neoadjuvant chemotherapy regimens, such as a combination of anthracycline, alkylating agents and taxanes, are often used to shrink a breast tumour prior to surgery. The results of the studies have shown that neoadjuvant chemotherapy led to increased levels of *Pseudomonas* and decreased *Prevotella* in breast tumours. *Pseudomonas aeruginosa* was identified in 56% of primary breast tumours and 20% of normal surrounding breast tissues. *Pseudomonas aeruginosa* has been shown to modulate tumour cell proliferation and doxorubicin-mediated cell death.

Analysis using 16S rRNA sequencing of the makeup of the microbiome in normal breast tissue and breast tumour tissues as well as in metastases located in the lymph nodes showed that tumour tissue samples contained significantly higher amounts of *Enterococcus* and *Streptococcus* than samples of surrounding normal breast tissue. Of interest is that lymph node metastases were closely connected with the breast tumour microbiome, which supports the

idea that the microorganisms in the metastases originate from the primary tumour. This study indicated that bacteria can travel through the circulatory system together with tumour cells and settle in distal organs, and this idea was supported by another study, showing that the bacteria spread to the liver and induced the formation of a premetastatic niche.

6.3.5 Lung cancer

Lung cancer is one of the most common malignancies worldwide. As a result, the scientific and clinical communities are making intensive efforts to find the most effective therapeutic strategies. Although the lung in healthy humans has long been considered to be a sterile organ, new methods of metagenomic analysis and advances in molecular techniques have revealed the presence of the lung microbiome. Studies have likewise demonstrated a complex two-way connection between the gut and lung microbiota via both the lymphatic and circulatory systems.

A rich and diverse microbiome has been identified in the lung tissue of healthy subjects, with a high abundance of *Bacteroidetes*, *Firmicutes,* and *Proteobacteria* and *Prevotella*, *Veillomella*, *Streptococcus*, *Neisseria*, *Haemophilus,* and *Fusobacterium* as prominent genera. Based on the findings, the lung microbiome may serve as a biomarker for both lung cancer prognosis and patient survival. A study focusing on differences in the specific microbiome of squamous lung tumours between smokers and non-smokers revealed a higher incidence of *Acidovorax*, *Ruminococcus*, *Oscillospira*, *Duganella*, *Ensifer,* and *Rhizobium* in samples from smokers. The presence of the given taxa was positively correlated with mutations in TP53 in tumour cells. Sequencing of the 16S rRNA gene revealed the dominance of *Actinobacteria*, *Proteobacteria*,*Firmicutes,* and *Bacteroidetes* in tumour and adjacent tissue samples from patients with non-small cell lung cancer (NSCLC). In the later stages of the disease, lower levels of *Pseudomonas*, *Burkholderia,* and *Aquabacterium* and higher levels of *Corynebacterium*, *Sphingomonas*, *Streptococcus*, *Neisseria*, *Halomonas*, *Kocuria*, *Parvimonas,* and *Rothia* were recorded. A recent study on a sample of lung cancer patients showed reduced levels of *Corynebacterium*, *Lachnoanaerobaculum,* and *Halomonas* in samples of lung tumours and at the phyla level, *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, *Acidobacteria,* and *Chloroflexi* were more abundant in the lung tumour microbiome. Some data suggest that *Streptococcus* may be more present in lung tumour tissue samples and *Staphylococcus* in lung samples from control patients who consented to bronchoscopy.

Interesting results were brought by one animal study in which the authors showed that the change of the lung microbiome after the application of antibiotic and probiotic aerosol therapy can prevent lung metastases in female C57BL/6 mice with melanoma and increase the response to anticancer therapy. Aerosol therapy with vancomycin/neomycin reduced the number of regulatory T-cells and increased the activation of NK cells, thus leading to a decrease in bacterial load and a significant reduction in lung metastases. At the same time, aerosol application of *Lactobacillus rhamnosus* GG and *Bifidobacterium bifidum* MIMBb23sg significantly increased the anticancer effect of the standard chemotherapeutic drug dacarbazine in animals. Additional studies are needed to clarify the molecular mechanisms of the immunomodulatory effects.

6.3.6 Urogenital malignancies

From a long-term perspective, the bladder and also the urine itself in healthy individuals were considered sterile. Despite the assumptions, the results of recent studies have confirmed that the urine of a healthy person contains bacterial communities that can contribute to health or the development of diseases. Bacterial dysbiosis of the urinary tract may affect the pathogenesis of prostate and bladder cancer. Sequences of bacterial DNA were detected in samples from patients with cancer and chronic prostatitis as well as prostate cancer. A higher abundance of *Escherichia*, *Propionibacterium*, *Acinetobacter,* and *Pseudomonas* was detected in samples of prostate tumours and the surrounding benign tissue. *Propionibacterium acnes* was present in the tested tumour biopsies, though it was absent in healthy tissues. *Propionibacterium acnes* has been linked with the origin of prostate cancer due to its high presence in the urinary tract of men after puberty, when this bacterium can cause infections. An *in vitro* experiment demonstrated that its presence stimulated the secretion of IL-6 and IL-8 from infected cells, thus triggering a strong immune response. Through the use of the pyrosequencing method, the differences in the bacterial population between samples of prostate tumours, peritumoural tissue and normal prostate tissue were successfully clarified. Species of *Staphylococcus* were observed in both prostate tumours and peritumoural samples, while *Streptococcus* species were detected exclusively in normal tissue. A newer study reported the dominance of *Akkermansia*, *Bacteroides*, *Clostridium*, *Enterobacter,* and *Klebsiella* in bladder tumour samples and urine analysis in patients showed a high presence of *Lactobacillus*, *Corynebacterium*, *Streptococcus,* and *Staphylococcus.* A different study also described modifications in the urinary microbiome in patients with prostate cancer compared to samples from healthy individuals. Oncology patients showed higher levels of *Veillonella*, *Streptococcus,* and *Bacteroides* with a lower occurrence of *Faecalibacterium*, *Lactobacillus,* and *Acinetobacter*.

SCFA-producing bacteria, including *Alistipes* and *Lachnospira*, were more abundant in men with prostate cancer compared to individuals without cancer. Patients showed increased activation of the metabolic pathways involved in starch and sucrose metabolism, phenylpropanoid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, cyanoamino acid metabolism, and histidine metabolism. A study comparing the metabolic profiles of prostate cancer patients versus healthy subjects found 28 prostate cancer-specific metabolites, including two nucleosides – pseudouridine and uridine.

The risk of bladder cancer is lower in women in comparison with men. The different representation of microbial communities in the bladder may be why the disease affects fewer women than men. An analysis of 32 bladder tumour samples taken during resection identified lower levels of *Actinobacteria* in tumour tissues. After determining that the higher bacterial diversity indicates a healthy microbiome, increased diversity was observed in non-tumour tissues. Tumour-associated mucosa in 13 patients with bladder cancer was enriched by *Barnesiella*, *Parabacteroides*, *Prevotella*, *Alistipes,* and *Lachnospiracea incertae sedis.* Based on the observations, it can be assumed that the detected microorganisms play a role in the initial development of bladder cancer, since, e.g. *Enterococcus* was more abundant in tumour samples with a lower stage of disease.

6.3.7 Gynaecological malignancies

Among gynaecological malignancies, we assign a group of tumours of the female genital organs, such as endometrial, ovarian and cervical cancer. Studies have revealed an association of gynaecological tumours with changes in the microbial composition of tumour tissues and the potential involvement of bacterial toxins and metabolites produced by the tumour microbiome in the development of gynaecological malignancies. Due to its anatomy, the female genital tract is exposed to the external environment, and this provides an opportunity for bacteria to enter the reproductive organs. Bacteria can also colonize ovarian tissue through the pelvic cavity, particularly in patients suffering from chronic pelvic inflammatory disease.

In the majority of studies of healthy endometrium, *Lactobacillus* is the most commonly presented gram-positive genus. It has been shown that selected bacterial taxa may contribute to the pathogenesis of endometrial carcinoma. A comparison of microbial profiles at the genus level revealed that microbial diversity is greater in tumour tissue versus benign endometrium. Increased levels of *Micrococcus* were recorded in the endometrial microbiota of women with endometrial tumours undergoing hysterectomy, while *Pseudoramibacter*, *Eubacterium*, *Rhodobacter*, *Vogesella*, *Bilophila*, *Rheinheimera,* and *Megamonas* were more abundant in the endometrial microbiota of women with benign tumours of the uterus. The results of several analyses revealed an association between *Porphyromonas* spp. and the development of endometrial cancer. *Porphyromonas somerae* occurred in all patients with type II endometrial malignancy compared with 57% of patients with endometrial hyperplasia. Analysis *in vitro* on HEC-1A human endometrial adenocarcinoma cells confirmed that co-cultivation of cells with *Atopobium vaginae* and *Porphyromons somerae* led to the release of inflammatory cytokines and chemokines by endometrial cells. Another study in patients with endometrial carcinoma documented that *Acidorovax*, *Bradyrhizobium*, *Flavobacterium*, *Hyphomicrobium*, *Pelomonas,* and *Pseudomonas* distinguished tumour tissue samples from samples of benign uterus tissue.

Ovarian cancer, which is usually diagnosed only at a late stage of the disease, is the most common type of gynaecological malignancy. A different bacterial composition was identified in tumour and non-tumour ovarian samples. Analysis of the ovarian microbiome from tumour tissues using 16S rRNA sequencing showed reduced diversity in tumour samples compared with samples from healthy control individuals. Some results indicated a higher level of *Proteobacteria* and a reduced presence of *Firmicutes* and *Acidobacteria* in tumours. At the genus level, a high presence of *Acinetobacter*, *Sphingomonas,* and *Methylobacterium* was found in the tumour samples, while the presence of *Lactococcus* was significantly reduced. The cervicovaginal microbiota of patients with ovarian cancer and carriers of the BRCA1/2 gene mutation younger than 50 years old was not dominated by representatives of *Lactobacillus* spp. According to the findings, the specific vaginal microbiota with less than 50% representation of *Lactobacillus* spp. may be the cause of ovarian cancer. The authors of a recent study report a correlation between the microbial composition of the upper reproductive tract and the development of ovarian cancer. *Bosea, Mesorhizobium*, *Mycobacterium*, *Ralstonia,* and *Variovorax* occurred more frequently, while *Acidovorax*, *Acinetobacter*, *Aeromonas*,

Cloacibacterium, *Conexibacter*, *Mariomonas*, *Methylobacterium*, *Propionibacterium*, *Pseudoalteromonas*, *Vibrio,* and *Xanthomonas* spp. had reduced levels in ovarian tumours compared with control tissues. A study relating to the oncobiotic peritoneal microbiome demonstrated that patients with ovarian cancer exhibited a unique peritoneal microbial profile when compared with patients with benign tumours.

References

Anderson NM, Simon MC. Tumor microenvironment. Current Biology 2020; 30:R921-R925.

Bertocchi A, Carloni S, Ravenda PS, et al. Gut vascular barrier impairment leads to intestinal bacteria dissemination and colorectal cancer metastasis to liver. Cancer Cell 2021; 39(5): 708-724.e11.

Carbone C, Piro G, Di Noia V, et al. Lung and Gut Microbiota as Potential Hidden Driver of Immunotherapy Efficacy in Lung Cancer. Mediators Inflamm 2019; 2019:7652014.

Caselli E, Soffritti I, D'Accolti M, et al. Atopobium Vaginae and Porphyromonas Somerae Induce Proinflammatory Cytokines Expression in Endometrial Cells: A Possible Implication for Endometrial Cancer? Cancer Manag Res 2019; 11:8571-5.

Cavarretta I, Ferrarese R, Cazzaniga W, et al. The Microbiome of the Prostate Tumor Microenvironment. Eur Urol 2017; 72(4):625-31.

Ciernikova S, Novisedlakova M, Cholujova D, et al. The Emerging Role of Microbiota and Microbiome in Pancreatic Ductal Adenocarcinoma. Biomedicines 2020; 8(12):565.

Ciernikova S, Sevcikova A, Drgona L, Mego M. Modulating the gut microbiota by probiotics, prebiotics, postbiotics, and fecal microbiota transplantation: An emerging trend in cancer patient care. Biochim Biophys Acta Rev Cancer. 2023;1878(6):188990.

Ciernikova S, Sevcikova A, Mladosievicova B, Mego, M. Microbiome in Cancer Development and Treatment. Microorganisms. 2023; 12(1):24.

Ciernikova S, Sevcikova A, Stevurkova V, et al. Tumor microbiome – an integral part of the tumor microenvironment. Frontiers in oncology 2022; 12:1063100.

Feng Y, Ramnarine VR, Bell R, et al. Metagenomic and Metatranscriptomic Analysis of Human Prostate Microbiota from Patients with Prostate Cancer. BMC Genomics 2019; 20(1):146.

Galeano Niño JL, Wu H, LaCourse KD, et al. Effect of the intratumoral microbiota on spatial and cellular heterogeneity in cancer. Nature 2022; 611(7937):810-817.

Gao Z, Guo B, Gao R, et al. Microbiota Disbiosis Is Associated with Colorectal Cancer. Front Microbiol 2015; 6:20.

Geller LT, Barzily-Rokni M, Danino T, et al. Potential Role of Intratumor Bacteria in Mediating Tumor Resistance to the Chemotherapeutic Drug Gemcitabine. Science 2017; 357(6356): 1156-60.

Goubet AG. Could the tumor-associated microbiota be the new multi-faceted player in the tumor microenvironment? Front Oncol 2023; 13:1185163.

Greathouse KL, White JR, Vargas AJ, et al. Author Correction: Interaction between the Microbiome and Tp53 in Human Lung Cancer. Genome Biol 2020; 21(1):41.
Hadzega D, Minarik G, Karaba M, et al. Uncovering Microbial Composition in Human Breast Cancer Primary Tumour Tissue Using Transcriptomic RNA-seq. International journal of molecular sciences 2021; 22(16):9058.
Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer discovery 2022; 12(1):31-46.
Huang D, Su X, Yuan M, et al. The Characterization of Lung Microbiome in Lung Cancer Patients with Different Clinicopathology. Am J Cancer Res 2019; 9(9):2047-63.
Cheng H, Wang Z, Cui L, et al. Opportunities and Challenges of the Human Microbiome in Ovarian Cancer. Front Oncol 2020; 10:163.
Chiba A, Bawaneh A, Velazquez C, et al. Neoadjuvant Chemotherapy Shifts Breast Tumor Microbiota Populations to Regulate Drug Responsiveness and the Development of Metastasis. Mol Cancer Res 2020; 18(1):130-9.
Le Noci V, Guglielmetti S, Arioli S, et al. Modulation of Pulmonary Microbiota by Antibiotic or Probiotic Aerosol Therapy: A Strategy to Promote Immunosurveillance against Lung Metastases. Cell reports 2018; 24(13):3528-3538.
Mansour B, Monyok A, Makra N, et al. Bladder Cancer-Related Microbiota: Examining Differences in Urine and Tissue Samples. Sci Rep 2020; 10(1):11042.
Mego M, Danis R, Chovanec J, et al. Randomized double-blind, placebo-controlled multicenter phase III study of prevention of irinotecan-induced diarrhea by a probiotic mixture containing Bifidobacterium BB-12®Lactobacillus rhamnosus LGG® in colorectal cancer patients. Front Oncol 2023; 13:1168654.
Mego M, Chovanec J, Vochyanova-Andrezalova I, et al. Prevention of irinotecan induced diarrhea by probiotics: A randomized double blind, placebo controlled pilot study. Complementary therapies in medicine 2015; 23(3):356-362.
Meng YF, Fan ZY, Zhou B, Zhan HX. Role of the intratumoral microbiome in tumor progression and therapeutics implications. Biochim Biophys Acta Rev Cancer 2023; 1878(6):189014.
Najafi S, Abedini F, Azimzadeh Jamalkandi S, et al. The Composition of Lung Microbiome in Lung Cancer: A Systematic Review and Meta-Analysis. BMC Microbiol 2021; 21(1):315.
Nejman D, Livyatan I, Fuks G, et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. Science 2020; 368(6494):973-980.
Nene NR, Reisel D, Leimbach A, et al. Association between the Cervicovaginal Microbiome, Brca1 Mutation Status, and Risk of Ovarian Cancer: A Case-Control Study. Lancet Oncol 2019; 20(8):1171-82.
Peters BA, Hayes RB, Goparaju C, et al. The Microbiome in Lung Cancer Tissue and Recurrence-Free Survival. Cancer Epidemiol Biomarkers Prev 2019; 28(4):731-40.
Rao Malla R, Marni R, Kumari S, Chakraborty A, et al. Microbiome Assisted Tumor Microenvironment: Emerging Target of Breast Cancer. Clin Breast Cancer 2022; 22(3): 200-211.
Riquelme E, Zhang Y, Zhang L, et al. Tumor Microbiome Diversity and Composition Influence Pancreatic Cancer Outcomes. Cell 2019; 178(4):795-806.e12.
Sevcikova A, Mladosievicova B, Mego M, Ciernikova S. Exploring the Role of the Gut and

Intratumoral Microbiomes in Tumor Progression and Metastasis. Int J Mol Sci. 2023; 24(24):17199.

Swidsinski A, Khilkin M, Kerjaschki D, et al. Association between intraepithelial Escherichia coli and colorectal cancer. Gastroenterology 1998; 115(2):281-286.

Urbaniak C, Gloor GB, Brackstone M, et al. The Microbiota of Breast Tissue and Its Association with Breast Cancer. Appl Environ Microbiol 2016; 82(16):5039-48.

Wachsmannova L, Majek J, Zajac V, et al. The Study of Bacteria in Biopsies from Slovak Colorectal Adenoma and Carcinoma Patients. Neoplasma 2018; 65(4):644-8.

Wang Z, Gao X, Zeng R, et al. Changes of the Gastric Mucosal Microbiome Associated with Histological Stages of Gastric Carcinogenesis. Front Microbiol 2020; 11:997.

Wang Q, Zhao L, Han L, et al. The Differential Distribution of Bacteria between Cancerous and Noncancerous Ovarian Tissues in Situ. J Ovarian Res 2020; 13(1):8.

Xiao Y, Yu D. Tumor microenvironment as a therapeutic target in cancer. Pharmacol Ther 2021; 221:107753.

Summary

Different microbial communities which show a tumour-specific character are found in the tumour microenvironment. Increasing evidence has described differences in microbiome composition between tumour and non-tumour tissues. A detailed understanding of the complex interactions between the tumour microbiome, tumour cells, and other components of the tumour microenvironment can provide clinically relevant information. Furthermore, comprehensive studies on large cohorts of tumour samples can identify microbial biomarkers predictive for metastatic spread. An exact characterisation of tumour microbiome profiles could be used to stratify cancer patients and develop more effective, individualised and tumour-specific therapies. However, we must also take into consideration the significant differences in microbiome composition between patients, the existence of confounding factors as well as heterogeneity in host genetic susceptibility.

One of the most serious limitations of tumour microbiome studies is the risk of microbial contamination during sample collection, storage and processing. The processes guiding the development of 3D imaging methods may in the future enable the analysis of direct interactions between the microbial community and other components of the host tumour microenvironment. Recent studies have focused mainly on the relative abundance of bacterial taxa in each sample, and this represents a certain kind of limitation, since changes in overall abundance may bring more accurate information about the true impact of microbiota and bacterial metabolites on host physiology. Data from recent studies describe the occurrence of intratumoural bacteria, but do not distinguish whether their presence is causal from the point of view of the development of malignancy, or whether they coexist in the tumour microenvironment due to leaky vasculature and an immunosuppressed microenvironment.

The co-cultivation of specific bacterial taxa with organoids prepared from pluripotent stem cells or tissues may present a powerful tool for studying host-microbiome interactions and evaluating the potential and mechanisms behind them. Given the interaction between the intestinal and tumour microbiomes, modulations of the intestinal microbiota may also affect the composition of the tumour microbiome. Research in the coming years will show how to use and modulate the tumour microbiome in order to improve the clinical benefit in oncology patients. Therefore, personalised determination of a patient's gut and tumour microbiome composition may represent a potential diagnostic and prognostic tool, and rebalancing the microbial community may improve treatment efficacy and patient outcomes.

Molecular Mechanisms of Cancer Pathogenesis

Beata Mladosievičová
Soňa Čierniková
Michal Mego

Published by Comenius University Bratislava
Technical editor: Kristína Vozáková

Edited by authors

Manuscript 96 pages, 8,03 AH, 1st edition
Published as electronic publication

ISBN 978-80-223-5881-1 (online)

ISBN 978-80-223-5881-1 (online)