Old Herborn University Seminar Monograph

36. THE MICROBIOME AND CANCER

A summary of the lectures given during the 36th Old Herborn University Seminar

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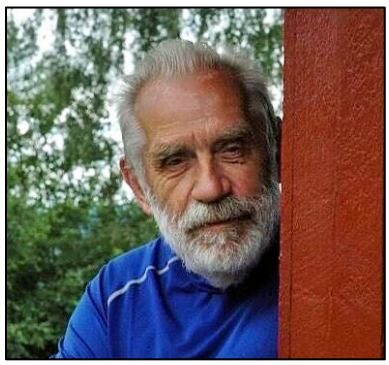
In Memoriam



Tore Midtvedt
1934-2025

Tore Midtvedt, who has been a highly valued member of the Science Advisory Committee of the Old Herborn University Foundation since 1992, passed away on December 2, 2025.

Tore was born on February 24, 1934 in Horten, Norway. In 2019, he said during an interview that it was his childhood dream to become a professional cyclist. That did not work out. After finishing school he studied medicine at the University of Oslo, Norway (1952-1956) and the University of Bergen, Norway (1956-1958). He received his license to practice medicine from the Norwegian Board of Health in 1959. He earned his Ph.D. degree (Doctor of Medicine) at the Karolinska Institute, Sweden, in 1968. In 2010 he was promoted to Doctor of Veterinary Medicine Honoris Causa by the Faculty of Veterinary Medicine of the Norwegian University of Life Sciences.

Tore held different positions at the Faculty of Medicine of the University of Oslo until he was appointed Professor of Medical Microbiology in 1982. In 1983 he was appointed Professor and Chairman of the Department of Medical Microbial Ecology, Cell and Molecular Biology at the Karolinska Institute in Stockholm, Sweden. He held this position until his retirement in 1999.

He was a member of several national and international organisations, was Editor-in-Chief of the journal 'Microbial Ecology in Health and Disease' and member of the Editorial Board of 3 international journals. He published more than 900 publications on antibiotics, ecology, gnotobiology, infectious diseases, microbiology, and pharmacology.

In 2018, Tore was appointed Knight of the 1st Class of the Order of St. Olav, a high Norwegian decoration, part of the highest order of chivalry of Norway. This decoration is awarded for exceptional services to Norway and humanity, such as in science, art or public service.

After he became a member of the Science Advisory Committee of the Old Herborn University Foundation, Tore attended all seminars from 1992 onwards and was co-editor of 10 Old Herborn University Seminar Monographs.

We will miss him greatly and will remember him as a dear friend and as a driven scientist whose ideas and discoveries will continue to have great influence on basic research as well as on the medical field.

We express our condolences to his wife Kari and his family.

Peter Heidt

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MICROBES, ONCOGENES AND CANCER

Introduction

The subject of the presentation of *Prof. Dr. med. Andreas Neubauer* (Philipps-Universität Marburg and University Hospital Gießen and Marburg, Department of Haematology, Oncology and Immunology, Baldingerstraße 1, 35043 Marburg, Germany) was “microbes, oncogenes and cancer”.

Cancer is a readout of chronic inflammation. Normally, cancer diagnosis is an easy task: the pathologists will tell if it is cancer tissue or if is inflammatory tissue, but sometimes it is not that easy. When it comes to the hallmarks of cancer, one does not think of inflammation but of proliferation, of the loss of the capacity to differentiate. There is a bidirectional relationship between cancer and chronic inflammation. Chronic inflammation can lead to cancer by damaging cell
DNA and promoting tumour growth, and cancer itself can trigger or perpetuate chronic inflammation, creating a cycle that fuels the disease's development, progression, and spread. This complex interaction occurs at almost every stage of cancer, involving processes like immune suppression, tissue remodelling, DNA damage, and increased cell proliferation. That is the normal view of how a cell transforms to a cancer cell, which is due to mutagenic changes in the genome. The classical hallmarks of cancer are proliferation, oncogenic changes, and the absence of differentiation.

The 14-18 translocation

Dr. Neubauer presented a 50-year-old female patient who came to the emergency room because of chest pain.

The CT-scan in Figure 1 shows a huge tumour which is infiltrating the ribs and the chest wall and the tumour is also infiltrating the heart. The patient was a non-smoker. The diagnosis of the

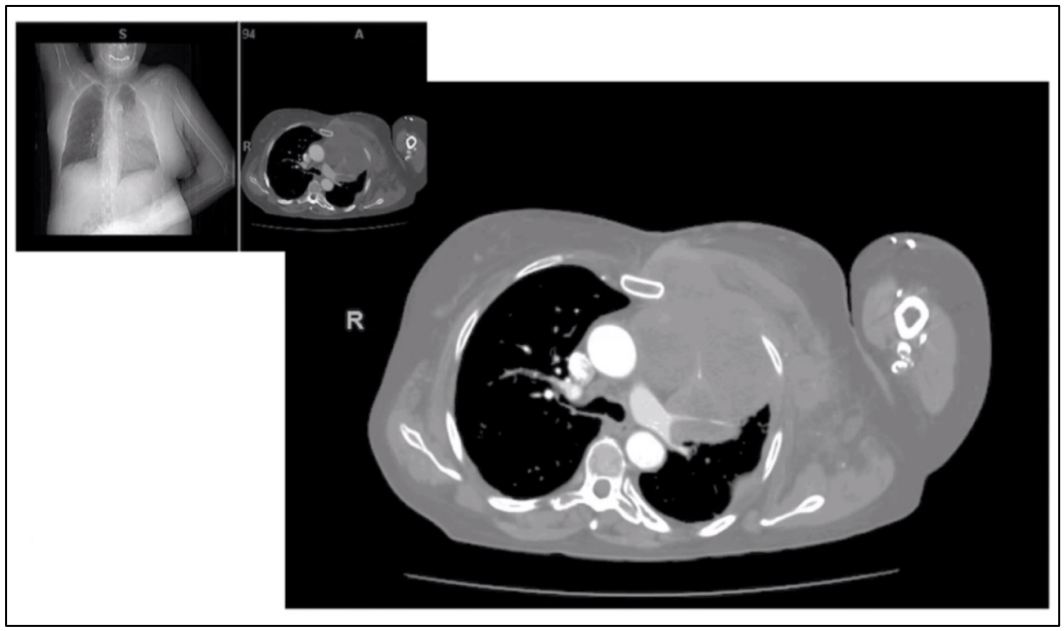

Figure 1: CT-scan of a 59-years old female with chest pain showing an massive tumour infiltrating the chest wall and probably the heart. Later, histology showed that this was a follicular B-cell lymphoma.

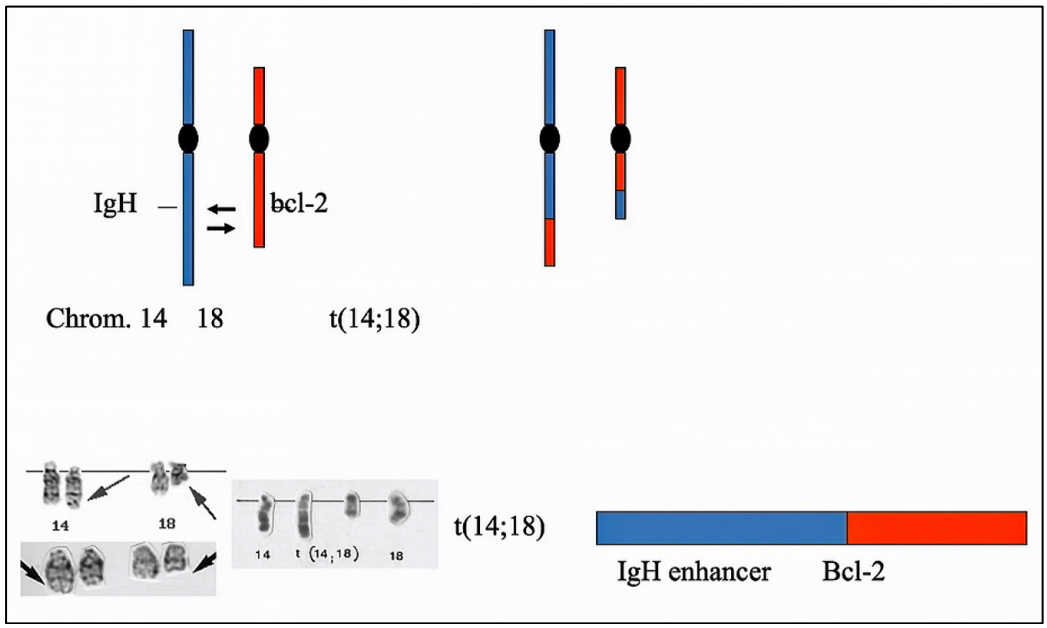

Figure 2: 14;18 translocation leading to promotor / enhancer exchange.
(Composition figure from A.N. and Atlas of cytogenetics [lower left panel]).

pathologist was "indolent" follicular B-cell lymphoma (according to the American classification). "*dolere*" in Latin means "to suffer pain", so indolent means that there is no pain to suffer. Lymphomas do not typically cause pain itself, but symptoms like pain can arise when swollen lymph nodes press on nearby nerves and tissues and that is what is happening in this patient. Histology showed an increase of follicles in the biopsy with quite regular appearance, but finally the diagnosis of a follicular lymphoma was made.

What was also found in this patient with the follicular B-cell lymphoma was a 14;18 translocation. The 14;18 translocation (or t(14;18)) is a specific genetic abnormality that involves the joining of the IgH promotor and enhancer on chromosome 14 to the Bcl-2 gene on chromosome 18 (which is the most important anti-apoptotic protein which prevents Caspase-9 to be recruited to the nucleus and start apoptosis), leading to the over-expression of the Bcl-2 gene (Figure 2).

This translocation is considered the hallmark genetic event in follicular lymphoma and is found in about 80-90% of cases. It can also be present in healthy individuals at low levels and is associated with an increased risk of developing follicular lymphoma, especially with increasing age. However, when performing a very sensitive PCR on normal tonsils from regular people, this translocation can be found without the presence of cancer.

Philadelphia chromosome

Prof. Neubauer presented another patient. She was a 19-year-old patient with leukaemia. She had a blood count of 500,000 leukocytes and died. The post-mortem histology showed a bone marrow full with white cells and a left-shift, which indicates the presence of immature white blood cells (like band neutrophils, myelocytes, and meta-myelocytes) in the peripheral blood, which is characteristic of chronic myeloid leukaemia (CML). In CML, this signifies abnormal myeloid cell

production, where the bone marrow releases these immature cells prematurely due to the clonal proliferation of myeloid cells caused by the Philadelphia chromosome.

The Philadelphia chromosome is a genetic abnormality found in the bone marrow cells of almost all CML patients. This abnormality occurs when a part of chromosome 9 breaks off and fuses with chromosome 22, creating a new gene (BCR::ABL1) that causes immature white blood cells to grow uncontrollably. The term highlights the strong diagnostic link between CML and the Philadelphia chromosome, named for the city where it was discovered in 1960. But here again, if you do a sensitive PCR in normal bone marrow, you find this translocation also in a certain percentage of healthy people.

These are 2 cases where an oncogene is involved in the development of cancer, but these oncogenes can also be found in healthy persons without any signs of disease. Apparently, just carrying an oncogene is not enough to develop cancer, so the question is: What drives cancer? In addition, in both cases, the cells under the microscopy did not look transformed at all, the cells appeared rather regular, or inflamed. These cases were presented to open up a discussion as to whether there may be a border after which one could call a tissue a cancer-tissue, but that there may be a gradual, stepwise progression to cancer where one sometimes cannot define the correct border.

Oncologists focus on oncogenes and on some tumours, but forget all the other things. Persons suffering from primary or secondary immunodeficiency have a more probability of developing cancer in their lifetime. Another important (and long-time neglected) influence on the development of cancers is “infection”.

In 2020 the WHO calculated 19,2 million new cases of cancer and 9.9 million mortalities from cancer. Increasingly, apart from lifestyle, diet, and genetic predisposition, infections caused by pathogenic microorganisms and parasites have also been linked to cancer (14% of all cancers worldwide).

The Axl gene

In the 1990-ies, prof Neubauer worked in the laboratories of Dr. E.T. Liu (Lineberger Comprehensive Cancer Center, University of North Carolina in Chapel Hill, USA). There, together with Drs. John O'Brien and Patty Coxwell, he worked on leukaemia with the key question: what actually turns the chronic phase to the acute phase, to the plastic phase, where all the patients at that time died from?

After almost 2 years of hard work, using a sensitive transfection-tumourigenicity assay, they isolated a novel transforming gene from the DNA of two patients with chronic myelogenous leukaemia (*O’Bryan* et al., 1991). This gene, that they named Axl, is a tyrosine kinase receptor that promotes cancer development by increasing proliferation, survival, invasion, and migration in cancer cells. Axl also contributes to the development of resistance to chemo-, radio-, immune- and targeted therapy in many cancer types. They showed that Axl is expressed in cells of the myeloid lineage in both normal and malignant states, and that in normal haematopoietic cells Axl is expressed predominantly in myeloid precursors and in stromal cells (*O’Bryan* et al., 1995). What was also found is that Axl can transform CML into a more aggressive blast phase (or blast crisis), which is a type of acute leukaemia (*Neubauer* et al., 1994).

Axl is a gene also causing inflammation and recently it was shown in mice by Takehiko Shibata and colleagues that Axl/GAS6 prevents immunity against *Streptococcus pneumoniae* and that blocking the Axl/GAS6 fully restored

the antibacterial immunity (*Shibata* et al., 2020). Axl/GAS6 is preventing immunity because it polarizes macrophages.

The AXL gene encodes for a receptor tyrosine kinase (RTK) that, when activated by its ligand Gas6, promotes cell survival, proliferation, and invasion, playing a significant role in cancer progression and metastasis. In the context of *Helicobacter pylori* (*H. pylori*) infection, studies have shown that elevated levels of Gas6 and Axl are associated with increased gastric cancer survival and invasiveness, with Axl contributing to the malignant phenotype. Therefore, Axl represents a potential therapeutic target for *H. pylori*-associated gastric cancers.

With regard to the relation between inflammation and cancer, Neubauer and colleagues were the first to prove that curing the *H. pylori* infection in humans can lead to complete regression of gastric MALT-lymphomas (*Bayerdörffer* et al., 1994; *Bayerdörffer* et al., 1995; *Neubauer* et al., 1997; *Thiede* et al., 1997; *Thiede* et al., 2001; *Wündisch* et al., 2003; *Wundisch* et al., 2005). They later performed the largest phase II trial that firmly established this antibiotic therapy as first line therapy (*Wündisch* et al., 2012). They observed, using B-cell clonality PCR, that there were patients where lymphoma could not be detected histologically, but that PCR showed monoclonal disease. So they called this a monoclonal gastritis.
Vice versa, in a follow-up study they found that 14 of 52 analysed lymphoma patients reaching complete histologic remission showed ongoing B-cell monoclonality which was associated with a higher risk of relapse (*Wundisch* et al., 2005).

Consequently, the WHO called infection with *H. pylori* in 1994 the "carcinogen" for gastric cancer (*IARC Monograph on the Evaluation of Carcinogenic Risks to Humans Volume 61*, 1994).

The Plcg2 gene

Prof. Neubauer and colleagues used BALB/c mice with a gain-of-function mutation in the Plcg2 gene (Ali5) to analyse its role in the development of gastric MALT lymphoma.

Heterozygous BALB/c Plcg2Ali5/+ and wildtype (WT) mice were infected with *Helicobacter felis* (*H. felis*) and observed up to 16 months for development of gastric MALT lymphomas. Plcg2Ali5/+ mice developed MALT lymphomas less frequently than their WT littermates after long-term infection of 16 months (*Gossmann* et al., 2016).
Infected Plcg2Ali5/+ mice showed downregulation of proinflammatory cytokines and decreased *H. felis*-specific IgG1 and IgG2a antibody responses. Plcg2Ali5/+ mice harboured higher numbers of CD73 expressing regulatory T cells (Tregs), possibly responsible for impaired immune response towards *Helicobacter* infection. Plcg2Ali5/+ mice may be protected from developing gastric MALT lymphomas as a result of elevated Treg numbers, reduced response to *H. felis* and decrease of proinflammatory cytokines.

TET-1 and TET-2 genes

TET-1 and TET-2 are genes that code for Ten-eleven translocation (TET) proteins, which are enzymes that catalyse DNA demethylation by converting 5-methylcytosine to 5-hydroxymethylcytosine, playing a crucial role in epigenetic regulation. Different mutations in TET-1 and TET-2 can lead to distinct phenotypes, such as TET-2 loss promoting increased stem cell self-renewal and myeloid transformation, and TET-1 loss being associated with B-cell lymphoma.

While frequent in haematological malignancies like leukaemia, their

functions are also emerging in solid tumours. Combined TET-1 and TET-2 loss can promote B-cell malignancies (*Zhao* et al., 2015).

t(10;11) translocation

A t(10;11) translocation is a rare chromosomal abnormality where parts of chromosomes 10 and 11 are exchanged, most commonly seen in paediatric and young adult acute myeloid leukaemia (AML). This translocation creates an MLL-TET-1 gene fusion, which can occur in various cancers like T-cell lymphoblastic lymphoma and AML.

The specific breakpoints are often written as t(10;11)(p12;q23), indicating the locations on the short (p) arm of chromosome 10 and the long (q) arm of chromosome 11.

It is associated with distinct clinical features and can lead to complications like diffuse intravascular coagulation and tumour lysis syndrome. Patients with t(10;11) are often classified into high-risk groups for treatment protocols and have an unfavourable prognosis.
The t(10;11) translocation, specifically t(10;11)(p12;q23), occurs in about 8-9% of AML cases and is particularly relevant in childhood AML.

IDH mutation

An IDH mutation is a genetic change in the isocitrate dehydrogenase (IDH) gene that leads to the production of an abnormal substance called 2-hydroxyglutarate (2-HG). This process can promote cancer by disrupting the function of other enzymes involved in DNA and histone methylation, which affects cell growth and development (*Prensner* and *Chinnaiyan*, 2011). These mutations are common in brain tumours, such as gliomas, and also in other cancers, including AML.

There is no leukaemia with a t(10;11) translocation as well as an IDH mutation. These mutations run the same epigenetic pathway. The IDH mutations, IDH-1 or IDH-2, can either be in mitochondria or in the cell cytoplasm. Both mutations actually create the wrong metabolism. The 2-hydroxy glutamate (2-HG) inhibits TET-1 and TET-2, so it wouldn't make any sense for a cancer cell to have a mutation in TET as well as a strong epigenetic mutation.
When over 60 persons are being sequenced, 3-5% clonal haematopoietic cells carrying mutations are being found. They don't play a big role, except maybe that these persons have a higher frequency of myocardial infarctions and strokes.

Prof. Dr. Andreas Burchert (University Hospital Giessen and Marburg) published an article about pulmonary inflammation, which is now called inflammageing, because this inflammation is increasing by age (*Burchert*, 2022).

In a normal situation there are millions of different progenitor cells. Mice can be transplanted with one single bone marrow stem cell. They are then reconstituted and can live a normal life. But we have millions of blood-forming stem cells of which a small number can acquire mutations and expand in number. “CHIP" refers to Clonal Haematopoiesis of Indeterminate Potential, a condition where a small number of blood-forming stem cells acquire mutations, get a growth advantage and expand in number, increasing the risk of developing blood cancers or cardiovascular disease (Figure 3).

TET-2 deficiency, bacterial translocation and preleukaemic proliferation

Meisel and colleagues (*Meisel* et al., 2018) used TET-2 deficient mice. TET-2 deficiency ($TET^{-/-}$) leads to severe myeloproliferation, extramedullary haematopoiesis and splenomegaly that

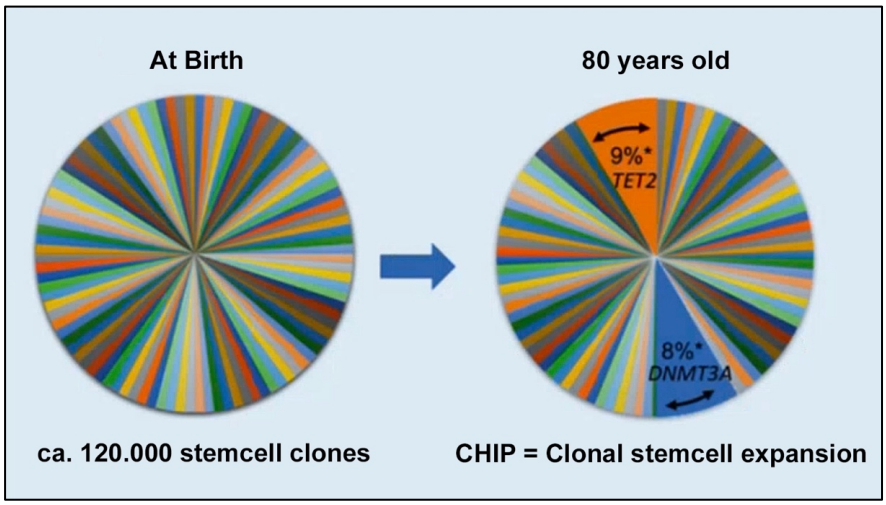

Figure 3: Clonal haematopoiesis and epigenetics are closely linked with age-related epigenetic changes. (Figure from *Burchert*, 2022).

mimic preleukaemic myeloproliferative disorders. Figure 4 shows the difference in self-renewal of haemopoietic stem cells (HSC) between $TET2^{+/+}$ mice and $TET2^{-/-}$ mice. However, preleukaemic myeloproliferation (PMP) occurs in only a fraction of $TET^{-/-}$ mice. This suggest that extrinsic non-cell autonomous factors are required for disease onset. Meisel and colleagues showed that bacterial translocation from the small intestines is critical for the development of PMP in TET2-/- mice (*Meisel* et al., 2018). This translocation is the result of dysfunction of the small intestine barrier. In symptom-free $TET2^{-/-}$ mice, PMP can be induced by disrupting the intestinal barrier integrity, or in response to systemic bacterial stimuli such as the toll-like receptor 2 antagonist. PMP was reversed by antibiotic treatment and did not develop in germfree $TRT2^{-/-}$ mice, illustrating the importance of microbial signals in the development of PMP.

Antibiotics, microbiota diversity and allogeneic stem cell transplantation

Six or seven years ago the clinic in Marburg stopped giving antibiotics as infection prevention during allogeneic stem cell transplantation, based on studies from four centres in the USA that reported a reduction in microbiota diversity after allogeneic hematopoietic stem cell transplantation for leukaemia and found that lower microbiota diversity was associated with higher mortality after transplantation. Higher diversity of intestinal microbiota at the time of neutrophil engraftment was associated with lower mortality (*Peled* et al., 2020).

When haematopoietic stem cell transplantations were started in Marburg in 1985 it was common use to eradicate the colonization microbiota in order to prevent infections in those immunocompromised patients. At present, based on the publication of Peled and colleagues, the Marburg transplantation team tries to prevent antibiotic treatment as much as possible in order to preserve the microbiome of the patient.

Therapeutic role in cancer of bacteria and bacterial products

The concept that bacteria or their products play a therapeutic role in cancer is not new; in 1891, Coley used the toxins from *Streptococcus erysipelas* and

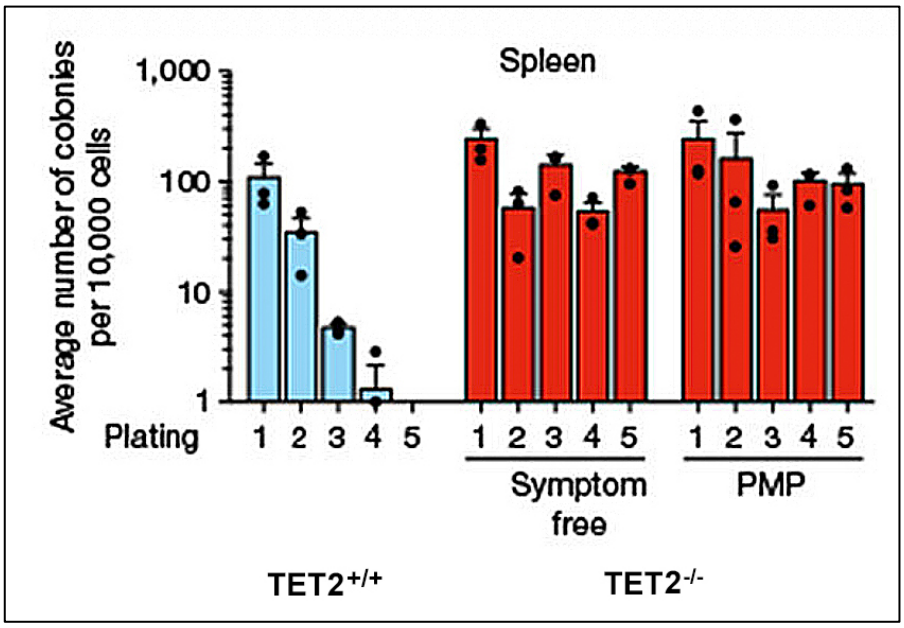

Figure 4: *In vitro* HSC self-renewal colony forming assay of haematopoietic progenitors in mice. Mean ± s.e.m. (Figure adapted from *Meisel* et al., 2018).

Serratia marascens to treat inoperable sarcoma (*Hoption Cann* et al., 2003).

Cancer prevention by vaccination

Another important issue is vaccination. A Study among 1,672,983 girls and women who were 10 to 30 years of age from 2006 through 2017 were included in a study to he efficacy and effectiveness of the quadrivalent human papillomavirus (HPV) vaccine in preventing high-grade cervical lesions. The conclusion was that among Swedish girls and women 10 to 30 years old, quadrivalent HPV vaccination was associated with a substantially reduced risk of invasive cervical cancer at the population level (*Lei* et al., 2020).

Summary

Cancer is not a box that opens up and then you have cancer, yet cancer develops over years, sometimes decades. One important reason may be infections, such as *H. pylori*. That curing the infection can clear malignant lymphomas remains a wonderful example of the gradual and still "reactive" process of malignant transformation.

This paper was reviewed Prof. Dr. med. Andreas Neubauer before publishing.

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MICROBIOME AND THE HALLMARKS OF CANCER: COLIBACTIN-PRODUCING PKS-POSITIVE *ESCHERICHIA COLI*

Introduction

Dr. Janelle Arthur (Department of Microbiology and Immunology, Center for Gastrointestinal Biology and Disease, University of North Carolina at Chapel Hill, USA) presented data on the microbiome and the hallmarks of cancer, with special focus on colibactin-producing PKS-positive *Escherichia coli*. She started her presentation with the general statement that it is unlikely that resident microbes alone can cause colorectal cancer. Genetic susceptibility, mutagens, diet, and other environmental factors are also involved in this process. It is known that this disease is associated with dysbiosis, a disruption of the microbiome where the balance between 'good' and 'bad' microorganisms is disturbed, having an impact on health

Hallmarks of cancer

Multiple microbial mechanisms influence cancer, and it is not just "who is there?", but "what are they doing?". It is important to understand what those bacteria are actually doing. A few "bad bugs" have been identified as associated with and driving various aspects of colorectal cancer. These include enterotoxigenic *Bacteroides fragilis*, *Fusobacterium nucleatum*, and colibactin producing, also called PKS-positive, *E. coli*. PKS-positive *E. coli* bacteria carry the polyketide synthase (PKS) gene cluster. This gene cluster enables the production of colibactin, a small molecule genotoxin that can cause DNA damage to intestinal cells and plays a role in the development of colorectal cancer.

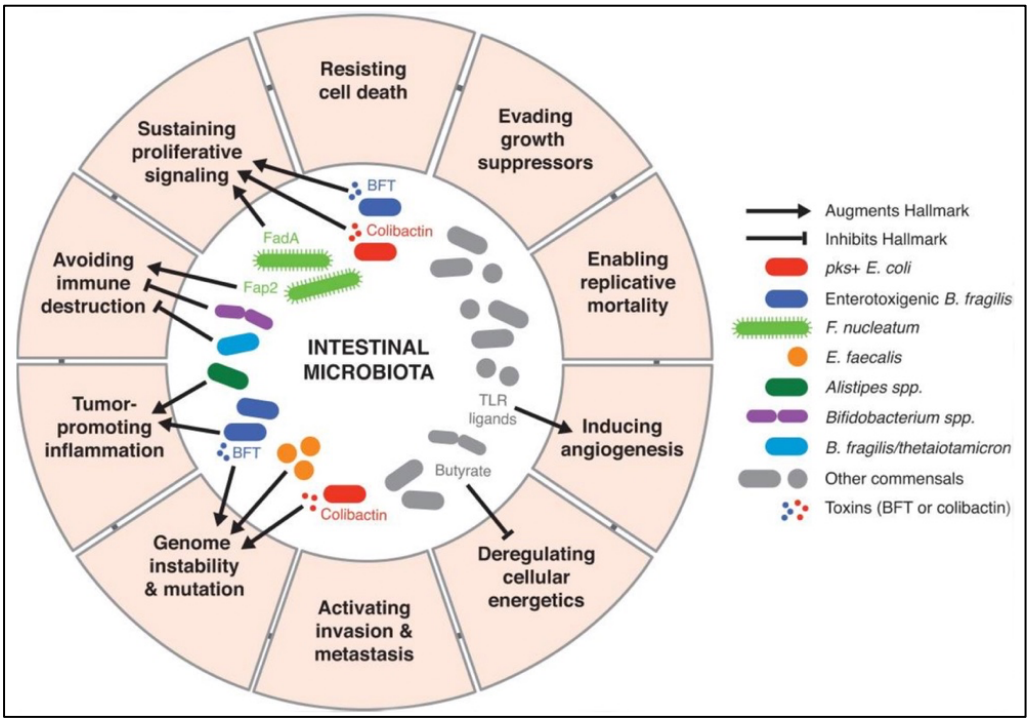

Figure 1: Microbial-derived signals modulate numerous hallmarks of cancer through diverse mechanisms. (Figure from *Fulbright* et al., 2017).

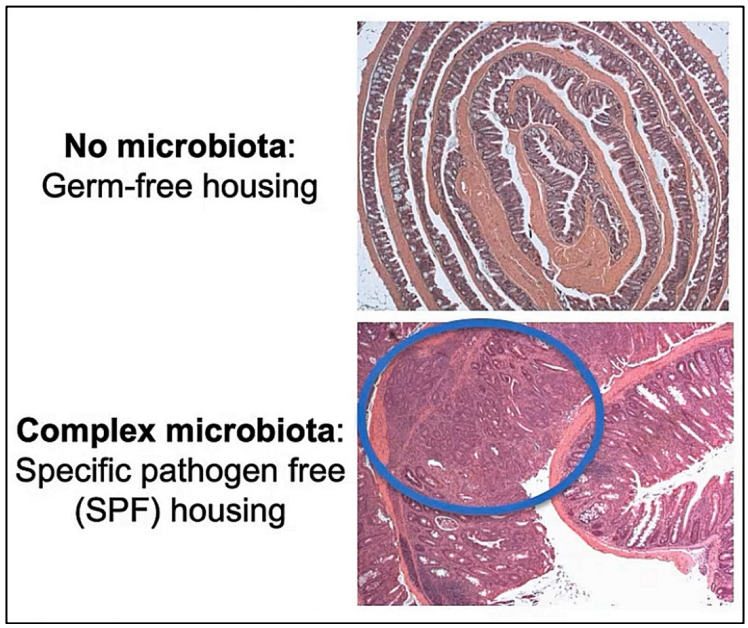

Figure 2: Histology of the colon (swiss roll technique) of IL-10$^{-/-}$ germfree (upper photo) and SPF (lower photo) mice.

Dr. Arthur and colleagues published in 2017 a drawing of how the microbiota influenced the hallmarks of cancer (Figure 1; *Fulbright* et al., 2017). The microbiota influences many of these hallmarks. In figure 1 there is at the 11 o'clock position a sustaining proliferative signalling influenced by the FadA adhesin from *Fusobacterium nucleatum* while the *B. fragilis* toxin and colibactin from PKS-positive *E. coli* influences proliferative signalling. Of the TLR ligands, inducing angiogenesis, and butyrate deregulating cellular energetics (at the 4 and 5 o'clock position) it can reasonably be assumed that they may influence cancer.

Microbiome, IL-10 and inflammation-associated colorectal cancer

Dr. Arthur presented a figure from a study performed in 2009, showing that resident microbes are required for inflammation-associated cancer in interleukin 10 (IL-10)-deficient (*Il10*$^{-/-}$) mice. (*Uronis* et al., 2009). IL-10 is an immunoregulatory cytokine that is required to dampen inflammation. When IL-10-deficient mice are raised under germ-free conditions they do not develop inflammation, but when they are colonized with a complex microbiota they develop robust inflammation in the gut, which is shown in figure 2. This figure shows “swiss rolls” of the colon. By this swiss roll technique (*Moolenbeek* and *Ruitenberg*, 1981), the entire colon of the mice is removed, flushed, and opened longitudinally and rolled with the mucosa inwards. After histological processing, microscopical examination of the entire length of the colon is then possible from the distal to the proximal colon. By cutting through there, a snapshot is obtained. But it is possible to look at the entirety of colon under microscopy to assess all inflammation and tumorigenesis. In figure 2, no inflammation can be seen in the upper histology image from germ-free mice, but robust inflammation (intestinal hyperplasia and thickening of the colon) and inflammatory infiltrates can be seen in the bottom histology image.

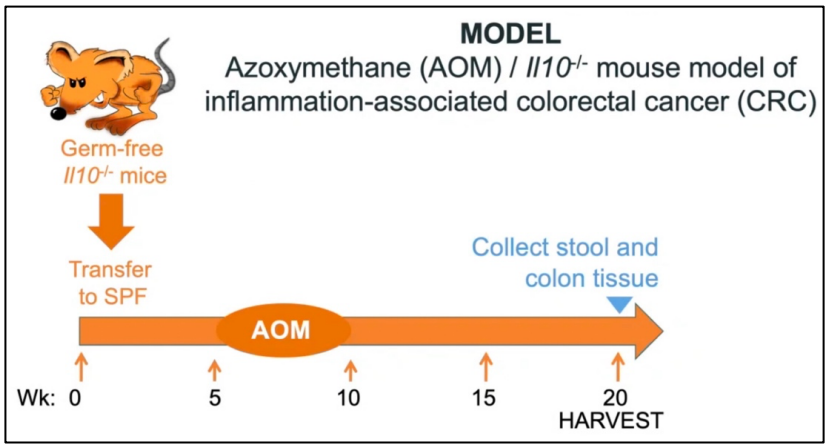

Figure 3: Mouse model of inflammation-associated colorectal cancer induction by azoxymethane in SPF IL-10 deficient mice.

After transfer of germfree *Il10*$^{-/-}$ mice to a specific pathogen free (SPF) environment or colonising them with the SPF microbiome by oral gavage of faecal material, they were injected i.p. with the colon specific carcinogen Azoxymethane (AOM) (Figure 3). AOM induces invasive colorectal tumours in the setting of *Il10*$^{-/-}$ inflammation. These tumours are flat, which is very similar to the colorectal cancers that inflammatory bowel disease (IBD) patients develop. IBD patients are at a very high risk for developing colorectal cancer and these colorectal cancers are difficult to spot by endoscope because they are flat. This mouse model is a very good model for inflammation-associated colorectal cancer and is very reproducible (*Arthur* et al., 2012).

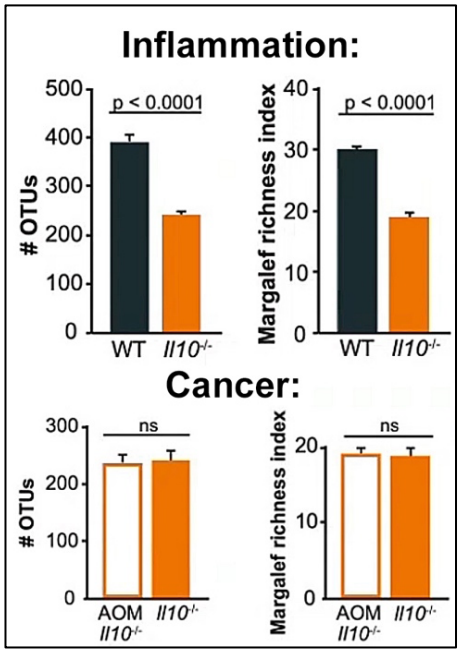

Figure 4: Alpha diversity in wild-type and *Il10*$^{-/-}$ mice. (OTU = operational taxonomic unit).

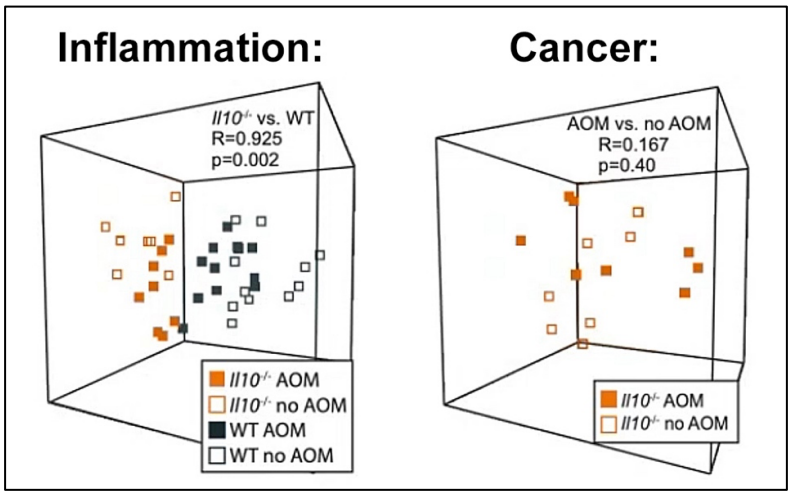

Figure 5: Beta diversity in the different groups of mice

After 18 to 20 weeks, stool samples and colon tissue were collected and 16S rRNA sequencing of the microbiome by Illumina HiSeq 2000 sequencing was used to determine what bacterial groups were present and which changed as inflammation and cancer developed. A major finding was that that chronic inflammation alters microbial community composition in the colon. Figure 4 shows that alpha diversity, a simple metric of how many bacterial groups are present, is reduced in the inflamed *Il10*$^{-/-}$ mice. Whether the *Il10*$^{-/-}$ mice received AOM (having robust inflammation and cancer) or had not (having robust inflammation and no cancer), the number of bacterial strains or species estimated was significantly reduced, which is similar to what is seen in human IBD patients.

Figure 5 shows the beta diversity, which measures dissimilarity in the composition of two communities. In this 3-dimensional plot the symbols represent the microbiota of an individual mouse. The distance between the symbols show the difference between these communities. One can clearly see that the symbols separate by mouse genotypes, the inflamed *Il10*$^{-/-}$ mice vs. WT uninflamed mice. From the figure it can be concluded that cancer is not driving the changes in the microbiome, but that inflammation is causing the robust changes.

From the experiments using this model it can be concluded that the microbiome is an important factor in the development of inflammation-associated colorectal cancer. The question is whether inflammation alters the microbiota and in this way select microbes that are associated with cancer. If this is true, even mild inflammation of the gut can alter the intestinal microbiome. The microbiome will become dysbiotic, more pro-inflammatory and thus more pro-carcinogenic which will act back and so induce more inflammation.

When looking at taxonomy in the colon microbiome, a hundredfold expansion of *E. coli* was observed in the *Il10*$^{-/-}$ mice. To find out whether *E. coli* could alter or even induce tumorigenesis, germfree wild-type and *Il10*$^{-/-}$ mice were mono-associated with either the Gram-negative *E. coli* NC101 or with the Gram-positive *Enterococcus faecalis* and injected with AOM to induce tumorigenesis in the *Il10*$^{-/-}$ mice. Both bacteria induce inflammation, but with different kinetics and different localization (*Kim et al, 2005).* As expected, both

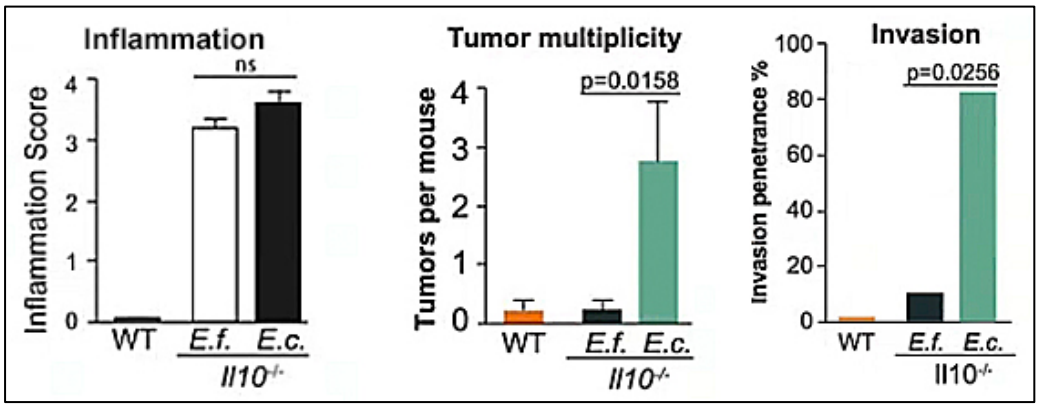

Figure 6: Inflammation, induction of tumours and invasion of tumours due to *Enterococcus faecalis* and *E. coli* NC101 in Il-10$^{-/-}$ and wild-type mic

bacteria induced robust inflammation in the *Il10*$^{-/-}$ mice, while the wild-type mice did not develop inflammation. Due to the absence of inflammation in the wild-type mice, these mono-associated wild-type mice do not develop tumours. Surprisingly though, the *Il10*$^{-/-}$ mice that were mono-associated with *Enterococcus faecalis* and developed robust inflammation did not develop tumours. In contrast, the *E. coli* mono-associated *Il10*$^{-/-}$ mice developed tumours, many of which were invasive (Figure 6). These data showed that inflammation and tumorigenesis are not always directly correlated and additional bacterial factors may influence the development of cancer.

As Figure 6 showed an increase of invasive tumours in the *E. coli* mono-associated *Il10*$^{-/-}$ mice, a potential bacterial driver of tumorigenesis was searched for. Searching the literature revealed a potential explanation in the "PKS pathogenicity island", a cluster of genes found in some *E. coli* strains that encodes enzymes for the biosynthesis of the small molecule genotoxin colibactin **(***Nougayrède* et al., 2006).

Colibactin is a polyketide-peptide that can cause double-strand breaks in DNA, and its presence is associated with colorectal cancer (CRC). This PKS pathogenicity island was present in the used *E. coli* NC101 strain, but not in a reference *E. coli* strain (*E. coli* K12) and also not in the used *Enterococcus faecalis* strain. Deletion of the PKS from the *E. coli* NC101 abrogated its ability to induce DNA damage in intestinal epithelial cells *in vitro.* Specially, flow cytometry analysis revealed that PKS+ *E. coli* induced cell cycle arrest, which indicates that the cells have stopped to repair their DNA damage, but PKS-deficient *E. coli* did not. An assay measuring γH2AX foci in the nucleas that form upon DNA damage repair also indicated that the PKS island is responsible for these phenotypes. When these strains are put back into the mice and ran through the same colitis-associated cancer model, the PKS positive *E. coli* enhanced tumorigenesis without impacting inflammation (Figure 7).

On the right-hand side of figure 7 it is shown that the inflammation score in mice mono-associated with either of these strains is the same. However, the tumour multiplicity (left on the figure) and importantly, the tumour invasion (centre of the figure) is significantly reduced when the PKS island is deleted from the strain. This suggests that the DNA damage from the product of this PKS island, the genotoxin colibactin, is driving the majority of the tumorigenesis in this model. Indeed, a higher

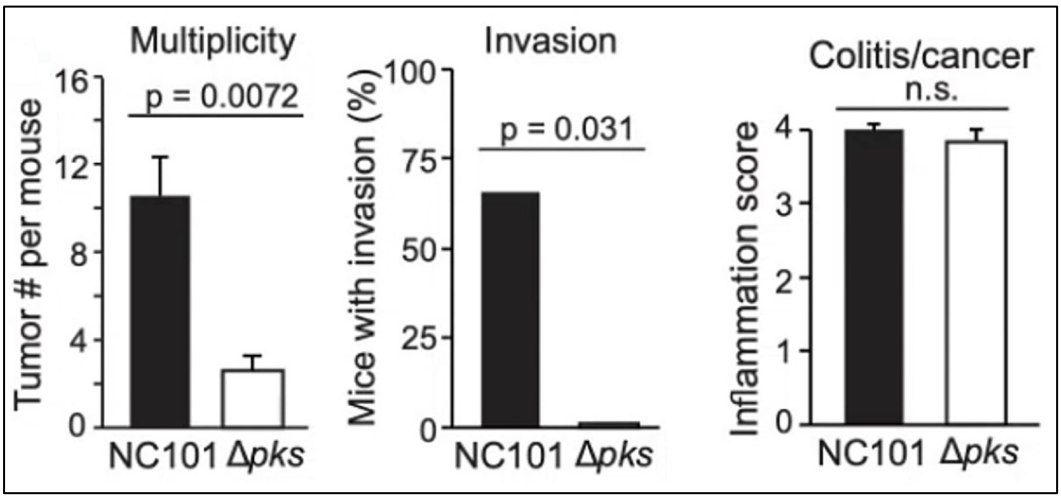

Figure 7: number of tumours, invasion and inflammation score in mice colonised with E. coli NC101 and the same strain with the PKS deletion.

proportion of patients suffering from colorectal cancer (CRC) or Inflammatory Bowel Disease (IBD) harboured PKS positive *E. coli*. From the CRC patients 67% harboured *E. coli* strains with the PKS island, 40% of the IBD patients did, and only 21% of healthy control persons harboured these *E. coli* strains (Table 1).

Colibactin and colorectal cancer

As mentioned earlier, colibactin is a genotoxic toxin that can cause DNA damage to intestinal cells and plays a role in the development of colorectal cancer. Colibactin is a small molecule, made as an inactive precursor (precolibactin) inside the *E. coli* cell. This is important because in bacteria, genetic material is not restricted to the nucleus as in eukaryotic cells; producing an active genotoxin in the bacterial cell cytoplasm would damage its own DNA. Instead, pre-colibactin is transported into the periplasm (the space in between the inner and outer membranes) and activated via cleavage of a pro-drug motif by the ClbP peptidase. Now in its active form, colibactin is released from the bacteria. It is not known how active colibactin is released from *E. coli*, how it enters the mammalian cell and reaches the nucleus, but cell-cell contact is required.

While the work of Dr. Arthur and that of others showed that *E. coli*-produced colibactin can promote colorectal cancer, chemists were focused on how enzymes of the PKS island synthesized colibactin, identified its active chemical form, and revealed details about its interactions with mammalian DNA and

Table 1: Human IBD and CRC patients harbour an abundance of PKS positive *E. coli*

Disease	Number of patients	% PKS positive	P value
CRC	21	66.87	< 0.001
IBD	35	40.0	<0.05
Control	24	20.8	

the specific types of DNA damage it inflicted (*Addington, Sandalli and Roe*, 2024). Most carcinogens have a known mutagenic signature – specific base pair substitutions or insertions/deletions – that results from exposure to the carcinogen. To link colibactin exposure to human cancer, researchers would have to determine colibactin's mutagenic signature. Two groups identified this mutagenic signature and found it in human colorectyal tumours and metatstases. The group led by Hans Clevers used human intestinal organoids (*Pleguezuelos-Manzano* et al., 2020). They exposed them repeatedly to *E. coli* with an intact PKS island or to an isogenic strain of which the PKS island was deleted. After this repeated exposure, they performed whole genome sequencing to identify carcinogenic signatures unique to the organoids exposed to PKS+ *E. coli*. They found a unique single base pair substitution and an insertion-deletion. These are called the SBS-PKS or the ID-PKS. They then looked in cancer genome databases and could find them in human colorectal tumours and metastases, often with SBS-PKS and ID-PKS evident in the same patient. This suggests that colibactin is inducing a carcinogenic signature that is found in human colorectal tumours and metastases. Its presence here demonstrates exposure to colibactin, and suggests colibactin may be contributing to colorectal cancer. In another study direct evidence was shown that colibactin contributes to mutational processes in humans, supporting its causal role in colorectal cancer (*Dziubańska-Kusibab* et al., 2020). Further work has revealed that 12% of colorectal cancers displayed a colibactin-induced mutational signature. Mutations were present in the adenomatous polyposis coli (APC) gene, a tumour suppressor gene that is one of the key mutations in colorectal cancer (*Rosendahl Huber* et al., 2024). Even more recently, evidence of colibactin exposure (presence of SBS-PKS and/or ID-PKS) was found in 21.1% of colorectal cancers across a global population (*Diaz-Gay*, 2025). Colibactin mutagenesis may be the cause of cancer or can contribute to causing cancer by stressing the DNA damage repair machinery at some point, most probably at the initiation stage. In sporadic cancer models it is shown that colibactin-producing *E. coli* can promote cancer. Thus, inflammation is not necessary, but inflammation (such as in IBD patients) alters the microbiota, including the bloom of potentially pro-carcinogenic microbes like PKS positive *E. coli*. Several questions remain that are currently investigated by the research team of Dr. Arthur. Since cell-cell contact is necessary for colibactin to induce its genotoxic effects, we must understand what features of intestinal *E. coli* allow it to stably colonize patients. This would permit it to reside in the mucosal microbiome and potentially adhere to epithelial cells and deliver colibactin to cause DNA damage. In addition, the molecular control of colibactin production is yet not well understood. We must understand what conditions in the gut induce colibactin production, especially those conserved across a broad range of PKS positive *E. coli* strains. Finally, future epidemiological studies will be needed for the research field to demonstrate if there is sufficient evidence to name colibactin as a carcinogen driving human colorectal cancer.

This paper was reviewed by Dr. Janelle Arthur before publishing.

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THE IMPACT OF GUT MICROBIOTA-DERIVED METABOLITES ON THE TUMOUR IMMUNE MICROENVIRONMENT

Introduction

The topic of the presentation of *Prof. Dr. Alexander Visekruna* (Institute for Medical Microbiology and Hospital Hygiene, Philipps-University, Marburg, Germany) was "the impact of gut microbiota-derived metabolites on the tumour immune microenvironment".

Under homeostatic conditions, intestinal microbes produce and modify many small substances collectively termed "microbial metabolites", which serve as a very efficient means of communication between intestinal bacteria and the host. These small substances might profoundly affect human health, but many of them are poorly characterized and some of them are even unknown.

The Human Microbiome Project

At the moment, there is a lot of research performed on the microbiome with many publications as result. Twenty years ago it was different, at that time only a few researchers worked on the microbiome. This changed in 2006, when new powerful technologies were developed, like next-generation sequencing, mass spectrometry, metabolomics, etc. In 2007, the National Institutes of Health (NIH) initiated one of the biggest and most interesting research projects that would run until 2016: The Human Microbiome Project. It had many collaborators worldwide. The aim was to improve understanding of the microbiota involved in human health and disease. Launched in 2007, the first phase (HMP1) focused on identifying and characterizing human microbiota. The second phase, known as the Integrative Human Microbiome Project (iHMP), was launched in 2014 with the aim of generating resources to characterize the microbiome and elucidating the roles of microbes in health and disease states.

Gut microbiota-derived metabolites

In his presentation, special attention was given by Prof. Visekruna to "gut microbiota-derived metabolites". These small molecules are very interesting targets for clinical translation. In the gut there is only one thin layer of epithelial cells between the host and the harsh environment with all kind of bacterial metabolites. Bacteria should not cross the epithelial barrier, but the small molecules produced by the bacteria can diffuse across the epithelial barrier and reach the lamina propria (Figure 1).

These small bacterial metabolites can even enter the circulation and can impact on remote organs. The main group of microbial metabolites is called short-chain fatty acids. these are products of bacterial fermentation of non-digestible carbohydrates. In 2013, three publications showed the importance of microbial metabolites for mucosal immunity (*Furosawa* et al., 2013; *Arpaia* et al., 20013, *Smith* et al., 2013). Furosawa and colleagues found that gut commensal microbes, especially *Clostridia*, influence mucosal immunity by promoting differentiation and expansion of various T-cell types. Butyrate, a microbial fermentation product in the large intestine, induces Treg-cell differentiation in mice. Among short-chain fatty acids, butyrate is the most effective inducer of Treg-cell differentiation, both *in vitro* and *in vivo*. Their findings illuminate how microbial metabolites help maintain immune homeostasis in the gut by promoting regulatory T-cell development (Figure 2; *Furosawa* et al., 2013).

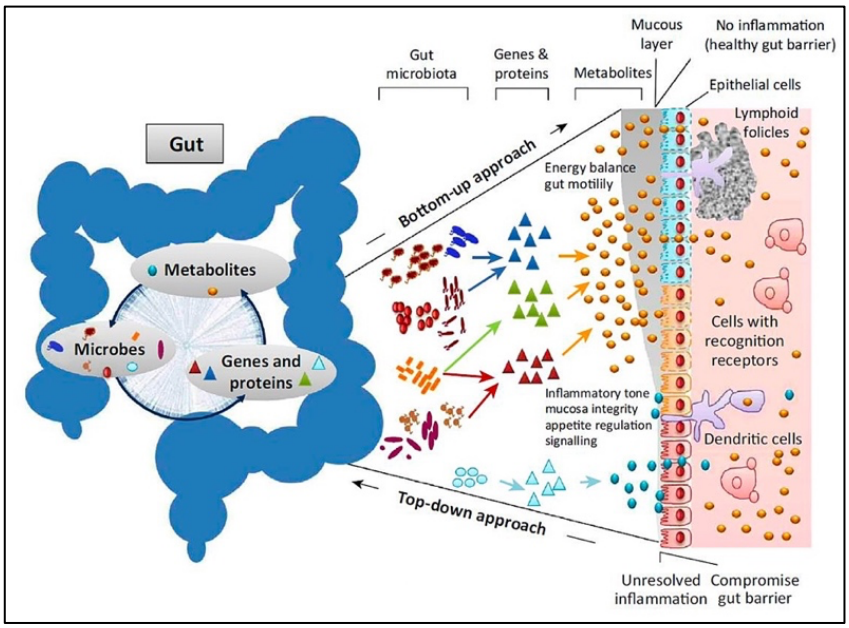

Figure 1: Bacterial metabolites can cross the epithelial gut barrier. (Figure from *Moya* and *Ferrer*, 2016).

Pentanoate is a microbiota-derived metabolite, it is present (0.4 - 0.6 µMol/g) in the coecum and the colon of germ-free mice (but is absent in the small intestine). The amount is 2.8% of all the short-chain fatty acids that can be found in the colon. Although the amount of pentanoate is very low when compared to acetate, propionate or butyrate, Prof. Visekruna's research was directed at

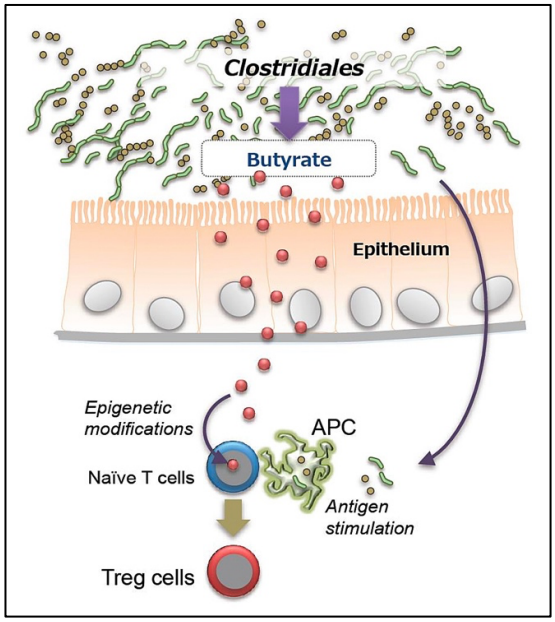

Figure 2: Butyrate induces differentiation of colonic Treg cells in mice. (Figure from *Furusawa* et al., 2013).

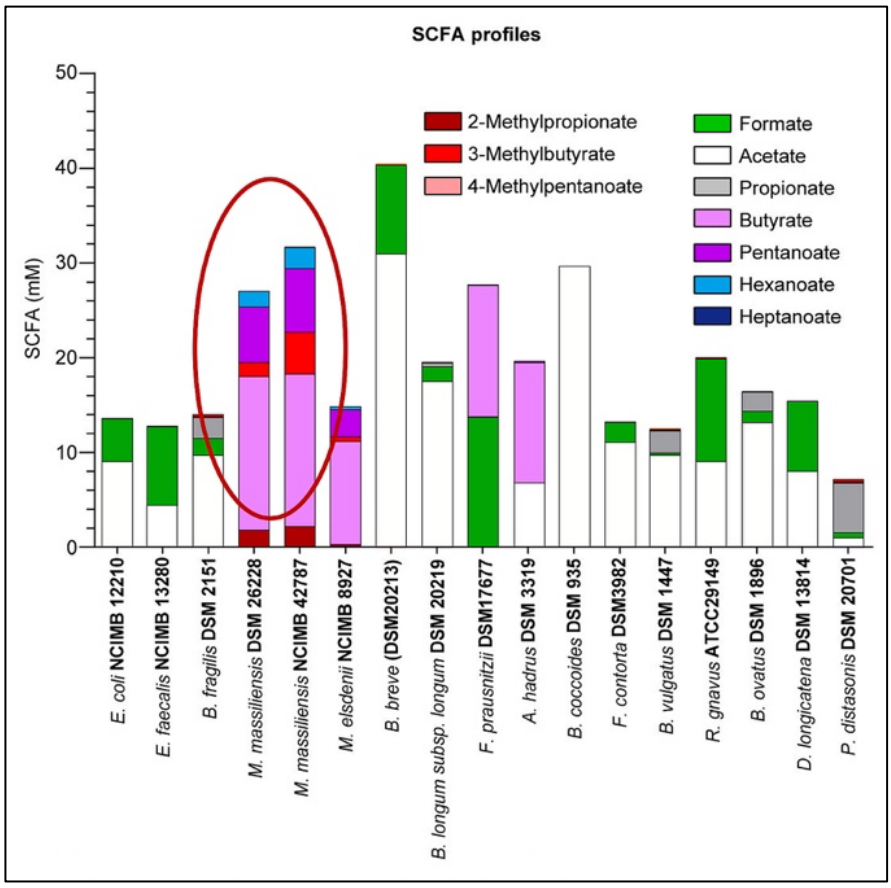

Figure 3: Microbiota-derived short-chain fatty acids from human gut bacteria. (Figure from *Luu* et al., 2021).

pentanoate, a short-chain fatty acid that has not received much attention in the literature. They concentrated on this molecule because they had the idea that this molecule could have therapeutic properties. They started a cooperation with the University of Aberdeen in Scotland which are experts in cultivating intestinal bacteria.

All together they screened 67 intestinal bacteria from humans, and only one, *Megasphaera massiliensis*, turned out to be able to produce pentanoate (Figure 3). This bacterium also produces high amounts of butyrate (*Luu* et al., 2021).

Histone acetylation, catalysed by histone acetyltransferases (HATs), adds an acetyl group to lysine residues, neutralizing the positive charge and loosening DNA-histone interactions to regulate gene expression.

Microbiota-derived short-chain fatty acids

Microbiota-derived short-chain fatty acids are strong class I histone deacetylase (HDAC) inhibitors. Pentanoate strongly increase the acetylation of histone 3 and histone 4, which modifies epigenetic regulation. This effect was confirmed in an *in vitro* assay by testing more than nine histone deacetylases.

Short-chain fatty acids have immuno-modulatory effects, but there is limited knowledge about the role of pentanoate in regulating immune cell functions. Th17 cells are pro-inflammatory immune cells that are implicated in the

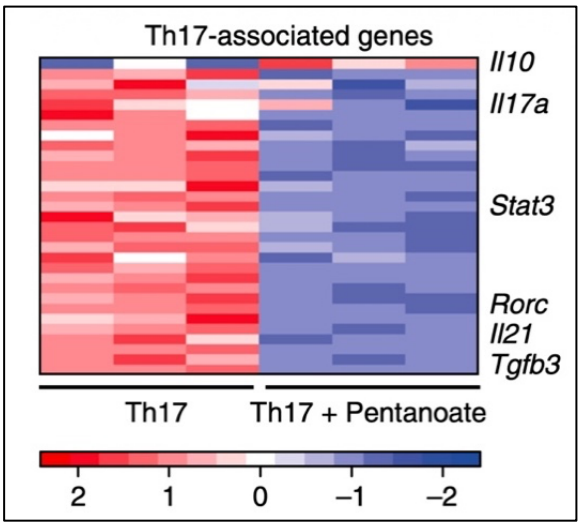

Figure 4: RNA-seq analysis of pathogenic Th17 cells in the presence or absence of pentanoate. (Figure from *Luu* et al., 2019).

pathology of many auto-immune diseases, such as multiple sclerosis and Crohn's disease. The effect of pentanoate from the supernatant of *Megasphaera* cultures on Th17 cells was tested. The global RNA-seq analysis revealed that pentanoate upregulated IL-10 expression and downregulated most of the Th17-associated genes including Rorc, IL-21, Stat3, and predominantly Transforming Growth Factor beta-3 (TGFß-3), which is endogenously produced by pathogenic Th17 cells (Figure 4).

Fax analysis of Th17 cells in the absence or presence of pentanoate showed a cytokine switch, treatment of the Th17 cells with pentanoate caused the loss of the pro-inflammatory cytokine IL-17A (Figure 5).

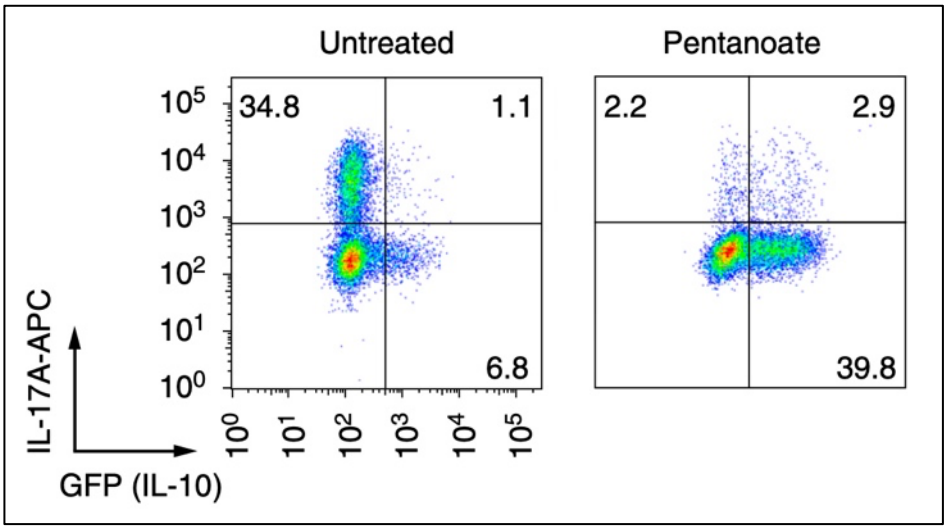

Figure 5: Flow-cytometry results of analysing CD4+ T cells isolated from FIR x tiger mice, polarized under Th17-inducing conditions and treated with pentanoate (5 mM). (Figure from *Luu* et al., 2019)

Not the presence of intestinal bacteria, but the presence of certain strains in the intestinal microbiome is important for the defence against intestinal pathogens. By using mono-contaminated germ-free mice it was shown that the presence of segmented filamentous bacteria (SFB) in the small intestine is sufficient to trigger the development of Th17 cells which are crucial for defence against intestinal pathogens (*Ivanov* et al., 2009).

Effect of pentanoate on the immune system

To investigate whether pentanoate could modulate inflammation of the central nervous system (CNS), experimental autoimmune encephalomyelitis (EAE) was induced in FIR × tiger reporter mice. The treatment of mice with pentanoate ameliorated EAE severity and reduced the number of infiltrating CD4+ and CD8+ T-cells in the CNS. Treatment with pentanoate of SFB-mono-colonized germ-free mice also significantly ameliorated EAE and led to reduced cell numbers and frequencies of Th17 cells in the CNS (*Luu* et al., 2019).

Thus, the microbial product pentanoate has a drastic effect on $CD4_+$ and $CD8_+$ T-cells. CD4+ and CD8+ T-cells are crucial components of the adaptive immune system; CD4+ T-cells (helper T-cells) coordinate the immune response, while CD8+ T-cells (cytotoxic T-cells) directly kill infected or cancerous cells. Both cell types are used in immunotherapy to fight cancer.

Extracts from the contents of the ileum, coecum and colon of SPF and germ-free mice were added to cytotoxic (CD8+) T-cells and incubated for 3 days. The extracts from SPF mice, in contrast to those from germ-free mice, stimulated the CD8+ T-cells to differentiate into cytotoxic T lymphocytes (CTLs), showing the role of bacterial metabolites in this process. CTLs produce interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and granzyme B (GrB), all involved in killing virus infected cells and tumour cells. The extracts of the coecum and the colon of SPF mice significantly increased the amount of measured IFN-γ and TNF-α when compared to extracts obtained from germ-free mice, while the extracts form the ileum did not significantly differ in measured IFN-γ and TNF-α between SPF and germ-free mice. Short-chain fatty acids could only be detected in the extracts from the coecum and colon of SPF mice, not in the ileum of SPF and germfree mice.

Having shown the effect of short-chain fatty acids *in vitro*, a mouse model was used to test the effectivity of the CTLs obtained by stimulation of CD8+ T-cells by pentanoate compared to CTLs from CD8+ T-cells that had not been stimulated by pentanoate. B16-OVA mouse melanoma cells were injected subcutaneously in wild-type mice and after 5 days of tumour growth the animals were i.p. injected with both types of CTL's. The tumour volume was measured every second day during 15 days after tumour transplantation, starting at day 5 after tumour transplantation.

The tumour growth after injection with the untreated CTLs was lower than the growth in animals that had not received CTL's, but the tumour growth in the mice injected with the CTL's that were obtained after stimulation with pentanoate was significantly lower than the growth in both the untreated mice and the mice injected with the untreated CTLs (Figure 6).

This experiment was repeated by transplanting "Panc OVA" cells into the mice. Panc OVA is a murine pancreatic cancer cell line. The modified cell line, Panc-OVA, is used in cancer research to study immune responses to tumours

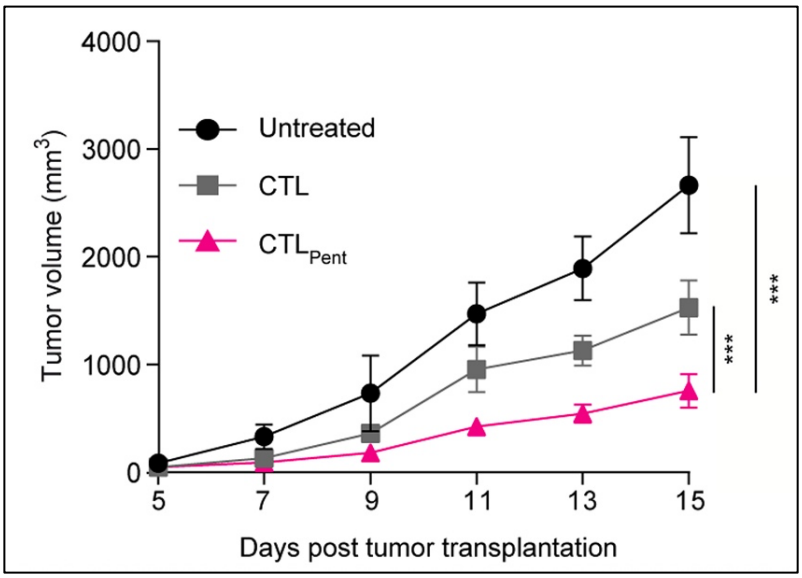

Figure 6: Growth of B16-OVA mouse melanoma in mice injected with untreated CTLs and CTLs obtained after stimulation with pentanoate, compared with tumour growth in untreated mice. (Figure from *Luu* et al., 2021).

because it allows researchers to track specific T-cell responses against the OVA antigen. In this second experiment, the results were even better, showing complete tumour suppression after injection with the CTL obtained from CD8+ T-cells that had been stimulated by pentanoate (Figure 7).

These promising data show that the short-chain fatty acid pentanoate can be a candidate to be used for cancer treatment, possibly in combination therapy.

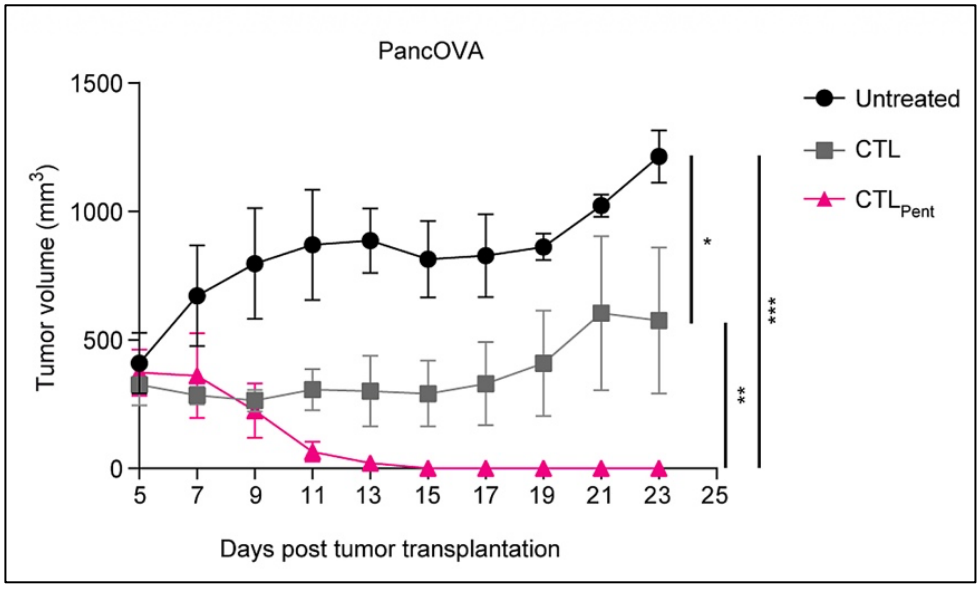

Figure 7: Growth of Panc OVA pancreatic tumour cells in mice injected with untreated CTLs and CTLs obtained after stimulation with pentanoate, compared with tumour growth in untreated mice. (Figure from *Luu* et al., 2021).

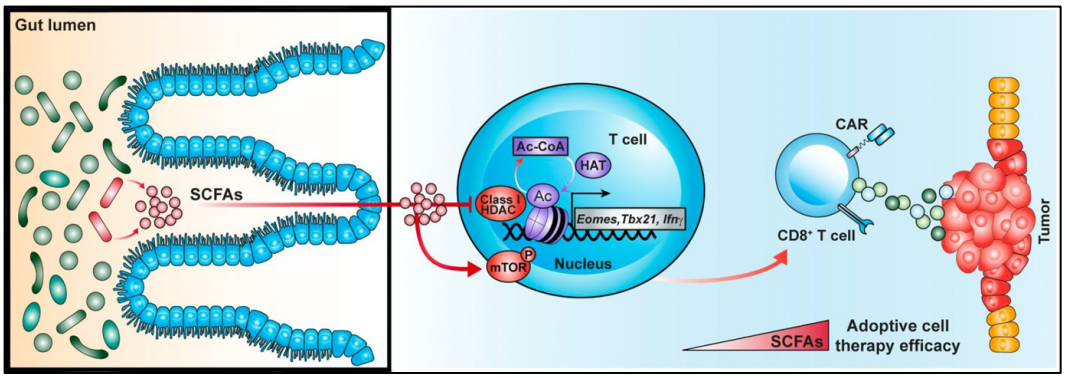

Figure 8: The crosstalk between microbial metabolites and T cells improves cellular immunotherapy. (Figure from *Luu* and *Visekruna*, 2021)

Immunotherapy for cancer

Mocetinostat is a histone deacetylase (HDAC) inhibitor that targets the class I HDACs, and it is being investigated for cancer therapy. TMP-195 is a selective class IIa histone HDAC inhibitor being also investigated for its potential in cancer therapy and other diseases. Both HDAC inhibitors were investigated for their impact on cytotoxic T lymphocytes (CTLs); they can *in vitro* activate these cells. Recent data show that pentanoate is a class I HDAC inhibitor and is more effective in activating CTLs than mocetinostat. Since there are indications that mocetinostat (and TMP-195) may play a role in treating Alzheimer's disease by reducing the production of amyloid-ß and tau protein, there possibly might also be a role for pentanoate in treating this neurodegenerating disease.

ROR1-specific CAR (Chimeric Antigen Receptor) T-cell therapy is a type of immunotherapy that genetically engineers a patient's T-cells to recognize and attack cancer cells that express the ROR1 protein. ROR1 is highly expressed in various cancers like leukaemia, lymphoma, and some solid tumours, but is minimally expressed in normal adult tissues, making it an attractive target.

Pentanoate improves the efficacy of human CAR T-cells by enhancing their anti-tumour activity through metabolic and epigenetic reprogramming. It increases the function of the mTOR pathway and inhibits histone deacetylases (HDACs), leading to increased production of effector molecules like CD25, IFN-γ, and TNF-α. This results in better performance against tumours.

A publication of Luu and Visekruna in 2021 summarizes how the molecular interaction of microbial metabolites (SCFAs) with T-cells improves immunotherapy for cancer (Figure 8; *Luu* and *Visekruna*, 2021).

In the human intestinal tract the low-abundant human bacterium *Megasphaera massiliensis* is able to produce high amounts of the SCFAs butyrate and pentanoate. Several CTL-associated effector molecules are upregulated following pentanoate- or butyrate-mediated inhibition of HDACs, which results in augmented anti-cancer potency of CTLs and CAR T-cells. This is a promising example of the therapeutic potential of microbial metabolites to improve immunotherapy by reprogramming the metabolic and epigenetic status of CTLs.

This paper was reviewed by Prof. Dr. Alexander Visekruna before publishing.

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A MICROBIOTA-MODULATED CHECKPOINT DIRECTS IMMUNOSUPPRESSIVE INTESTINAL T CELLS INTO CANCERS

Introduction

The topic of the presentation of *Dr. Marine Fidelle* (Gustave Roussy Cancer Campus, Villejuif Cedex, France) was "*A microbiota-modulated checkpoint directs immunosuppressive intestinal T cells into cancers*". She started with pointing at an European consortium (*PREVALUNG EU, N°101095604*) that is aiming at finding biomarkers based on multi-omics and associated with cancer and microbiome for cancer prevention and early diagnosis.

Chemotherapy and the recent advent of immunotherapies have improved the results of the treatment of cancer, and the role of the microbiome is increasingly recognised in oncology. Immune checkpoint inhibitors (ICB) is a type of immunotherapy that reinvigorate the immune system to eliminate cancer cells, but for many patients, the treatment is ineffective from the start (primary resistance) or becomes ineffective over time (acquired resistance). These patients have a significant unmet clinical need for other treatments or combinations to overcome this resistance. Overcoming resistance to immunotherapy remains a challenge for patients and society. During the last decade, several studies have shown that a "favourable" gut microbiome composition can have an impact on the outcome of anticancer treatment (*Viaud* et al., 2013; *Vetizou* et al., 2015; *Daillère* et al., 2016; *Routy* et al., 2018; *Roberti* et al., 2020; *Derosa* et al., 2020; *Fluckiger* et al., 2020; *Dart*, 2020; *Hanahan*, 2022).

Cancer-associated gut microbiota deviation (dysbiosis)

Fifty years ago, some papers already showed that malignant diseases could be associated with a change in the intestinal barrier (*Deller* et al., 1967; *Gilat* et al., 1972). Recently, Laurence Zitvogel's team discovered that the intestine can sense cancer development. It is linked to the balance between the sympathetic and parasympathetic signalling in the intestine and involved enteroendocrine cells. Pharmacologic blockade of beta-adrenergic receptors by betablockers limited the growth of extra-intestinal tumours, in this way preventing cancer-induced ileopathy characterized by an alteration of the crypt and villus ratio (*Yonekura* et al., 2022). This change of the gut epithelial barrier fitness and permeability culminated in a long-lasting dysbiosis (*Yonekura* et al. 2022). Indeed, three to seven days after a tumour is subcutaneously implanted in mice, the gut microbiota is affected with a change of composition, resulting in a dominance of Gram-positive *Enterocloster* species (formerly, *Clostridium* species). This is also observed in patients as shown in figure 1. This figure shows, through metagenomic analysis, the deviation of the gut microbiota composition in patients with cancer across several cancer types in comparison to healthy volunteers (HV).

The gut onco-microbiota signatures (GOMS)

Dr. Fidelle discussed a rationale for an impact of the gut microbiome in cancer immuno-modulation. Primary resistance to immune checkpoint inhibitors (ICBs) is a phenomenon in which cancer treatments become ineffective, notably due to immune evasion mechanisms.

Resident gut bacteria can affect patient responses to cancer immunotherapy. Thomas and colleagues have shown that the composition of the gut microbiota can predict clinical outcomes in patients treated with ICB and have defined the gut onco-microbiota

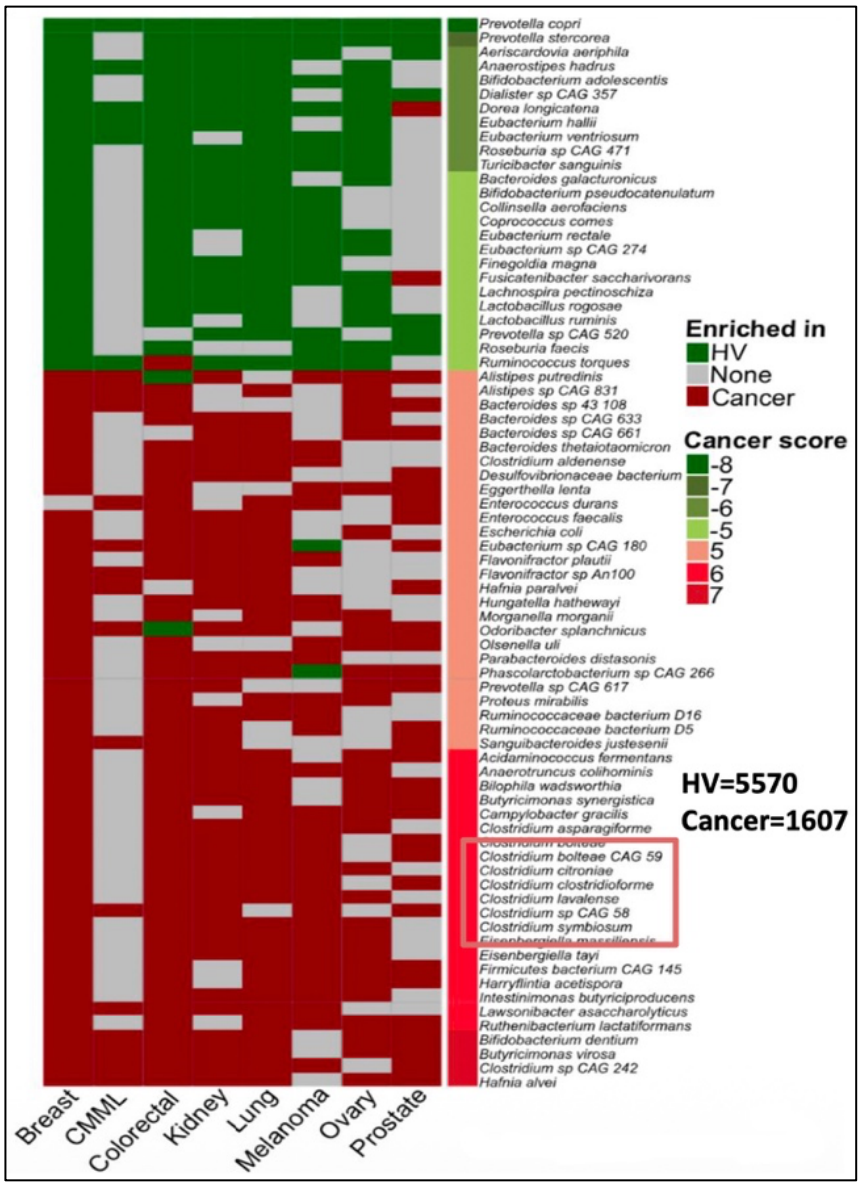

Figure 1: Heatmap showing the dominance of *Clostridium* spp. In five out of eight cancer categories. (Figure from *Yonekura*, 2022)

signatures (GOMS) (*Thomas* et al., 2023). They found that non-responding patients with lung or kidney cancer had low levels of the bacterium *Akkermansia muciniphila*. Likewise, two other studies in melanoma patients receiving ICB found a greater abundance of other "favourable" bacteria in the guts of responding patients. Non-responders had an imbalance in gut flora composition, which correlated with impaired immune cell activity. Finally, metanalysis found common patterns between cancer-driven dysbiosis and non-response to ICB, such as an overrepresentation of bacteria from *Enterocloster* spp. (*Park* et al., 2022) Thus, maintaining healthy gut flora could help

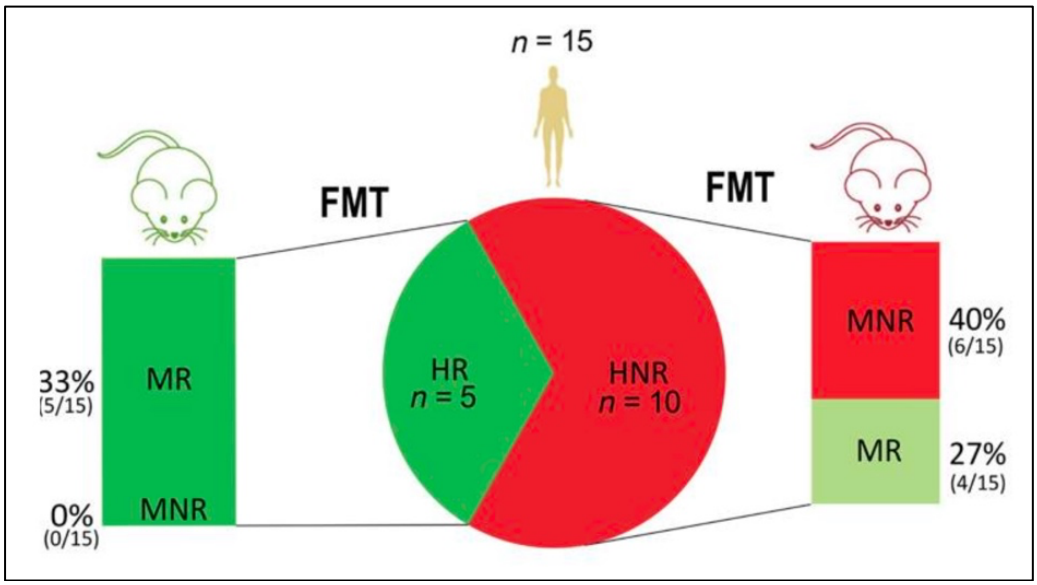

Figure 2: Proportion of the response in mice that matches the clinical response of the corresponding patient, based on the 15 FMT donors (human responders [HR] and non-responders [HNR]) and their outcomes in mice (mouse responders [MR] and non-responders [MNR]).(Figure from *Derosa* et al., 2020).

patients combat cancer (*Routy* et al., 2018; *Matson* et al., 2018; *Gopalakrishnan* et al., 2018; *Park* et al., 2022).

Dr. Fidelle explained their use of an avatar mice model to demonstrate the causal relationship between patient gut microbiota and ICB efficacy. Avatar mice refer to an experimental method employed to transplant the gut microbiota of a donor into mice. Here, stool samples were collected from patients before they started ICB and transplanted into the intestines of mice via faecal microbiota transplantation (FMT). Then they inoculated the tumour subcutaneously and treated the mice with ICB. The effectiveness of the treatment in mice actually corresponded to the patients' response to their immunotherapy, meaning that the composition of the patient's gut microbiota at the initiation of the treatment has an impact on the response to immunotherapy (*Routy* et al., 2018). (Figure 2).

They also have shown that antibiotics known to alter the gut microbiota composition ("dysbiosis") were associated with poor response to ICB as PD-1/PD-L1 blockade in patients with lung or kidney cancers. A retrospective meta-analysis done by Dr. Derosa and colleagues on multiple clinical studies comprising about 12 000 patients, confirmed that antibiotics has a negative impact on the success of immunotherapy with reduced overall survival (*Derosa* et al., 2020). Antibiotics facilitated the dominance of distinct species such as *Enterocloster spp.*, which were also preferentially over-represented in stools from patients with a cancer compared to healthy volunteers or non-responder patients (*Park* et al., 2022; *Fidelle* et al. 2023).

Development of tools to diagnose gut microbiota dysbiosis in patients

Given the importance of gut microbiota composition on the response to immune checkpoint blockers (ICB), it is essential to be able to diagnose dysbiosis in patients who will receive this treatment. This requires the development of diagnostic tools that are easy to use routinely.

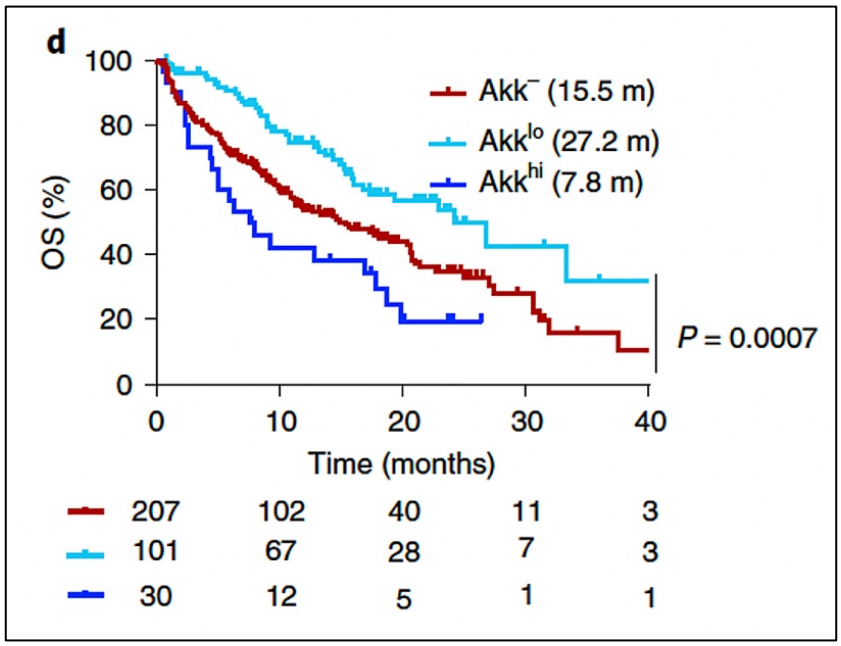

Figure 3: Overall survival (percentage) of non-small cell lung cancer patients according to *Akkermansia muciniphila* (Akk) relative abundance, segregated in 3 groups (Akk^{-}, Akk^{low} and Akk^{high}). (Figure from *Derosa* et al., 2022)

Routy, and colleagues have shown that patients having the bacterium *Akkermansia muciniphila* in their stools benefited from ICB in lung and renal cell cancers (*Routy* et al., 2018; *Derosa* et al 2022). Furthermore, oral supplementation of the bacterium *Akkermansia muciniphila*, in antibiotic-treated mice colonized with flora from non-responder patients restored the response to immunotherapy (*Routy* et al., 2018). Surprisingly, patients with high levels of *Akkermansia muciniphila* (Akk^{hi}) showed a lower overall survival in comparison to the ones with lower levels (Akk^{lo}), as depicted in Figure 3.

To diagnose dysbiosis, Derosa and colleagues developed a score based on the composition of patients' stools (*Derosa* et al., 2024). This was done by the construction of species-level co-abundance networks, based on metagenomics (MG) sequencing of 245 NSCLC patient faeces. The network analysis clustered bacteria into species-interacting groups (SIGs) correlating with overall survival. Thirty-seven and forty-five MG species (MGSs) were associated with poor (SIG1) or favourable (SIG2) clinical outcomes, respectively, in patients treated with ICB. Quantification of SIG1 and SIG2 bacteria yields a continuous score (from 0 to 1) where the extremes, <0.5 or >0.8, are associated with an unfavourable or favourable composition, respectively, for clinical response. However, a "grey zone" remains and when combining the quantification of *Akkermansia muciniphila*, this procedure allowed a person-based calculation of a topological score (TOPOSCORE). This score was validated in an additional 254 NSCLC patients and in 216 genito-urinary cancer patients. Finally, this TOPOSCORE was translated into a 21-bacterial probe set-based quantitative real-time PCR (qPCR) scoring that was validated in a prospective cohort of NSCLC patients as well as in colorectal and melanoma patients. This approach could represent a dynamic diagnosis

tool for intestinal dysbiosis to guide personalized microbiota-centred interventions (*Derosa* et al., 2024).

As previously reported, antibiotics can be harmful when taken close to the initiation of immunotherapy (*Routy* et al., 2018; *Derosa* et al., 2020). Fidelle and colleagues investigated whether bacteria that recolonise after discontinuation of antibiotic treatment may affect the treatment response. She has shown that *Enterocloster* species, that recolonized the guts of mice or patients treated with antibiotics, down-regulated the expression of the mucosal addressin cell adhesion molecule 1 (MAdCAM-1), the ligand for integrin α4β7 that helps to retain an enterotropic subset of T cells within the gut. In mice, the downregulation of MAdCAM-1 expression on intestinal high endothelial venules leads to a recirculation of immunosuppressive IL-17 secreting Treg cells (Tr17 cells) to tumours and tumour-draining lymph nodes, where they compromise immune checkpoint blockade therapy (*Fidelle* et al., 2023).

In cancer patients undergoing immunotherapy, low levels of serum-soluble MAdCAM-1 (sMAdCAM-1) correlated with intestinal dysbiosis, defined by a decrease of bacterial diversity and an overabundance of *Enterocloster* spp. or *Veillonella* spp. in their gut microbiota and poor clinical outcomes for renal, bladder, and lung tumours (*Fidelle* et al., 2023). Therefore, sMAdCAM-1 may be an easy-to-measure biomarker in the blood for dysbiosis and response to immunotherapy in patients.

These two scores pave the way for the rapid routine diagnosis of intestinal dysbiosis, and open the door to a microbiota-centred intervention (MCI), such as the use of faecal microbiota transplantation (FMT), pre- and pro-biotics and also the administration of *Akkermansia* spp. to correct the dysbiosis.

Microbiota-centred interventions (MCI)

Given the accumulating evidence on the importance of the gut microbiota in the efficacy of ICB, MCI could be a way to overcome primary resistance to ICB in patients. Therefore, two faecal microbiota transplantation (FMT) studies were conducted in melanoma patients with primary resistance to ICB.

Dr. Baruch and colleagues performed a phase 1 clinical trial to assess the safety and feasibility of FMT and reinduction of anti-PD-1 immunotherapy in 10 patients with anti-PD-1-refractory metastatic melanoma. They observed clinical responses in a third of patients, including two partial responses and one complete response, indicating a role of the gut microbiota in cancer treatment (*Baruch* et al., 2021).

Similar results were obtained by Dr. Davar and colleagues in a clinical trial evaluating the safety and efficacy of responder-derived FMT together with anti-PD-1 in patients with PD-1-refractory melanoma. This combination was well tolerated, provided clinical benefit in 6 of 15 patients, and induced rapid and durable microbiota modification (*Davar* et al., 2021). They concluded that FMT and anti-PD-1 changed the gut microbiome and reprogrammed the tumour microenvironment to overcome resistance to anti-PD-1 in a subset of PD-1 advanced melanoma.

Conclusion

The study of the microbiota in oncology has made it possible to determine its impact on the antitumor immune system and the response to treatments. Mechanisms such as the loss of MAdCAM-1 expression, or the understanding of its composition (GOMS), have led to the development of dysbiosis scores (Toposcore, sMAdCAM-1 assay) that can be easily used routinely by oncologists. These advances pave the way for

the use of microbiota-centred interventions (MCI), such as FMT, to correct dysbiosis in patients and enhance their chances of responding to immunotherapies.

This paper was reviewed by Dr. Marine Fidelle before publishing.

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MICROBIOTA-CENTRED INTERVENTIONS IN IMMUNO-ONCOLOGY

Introduction

The topic of the presentation given by *Dr. Meriem Messaoudene* (Hospital Research Centre (CRCHUM), University of Montreal, 1560 Rue Sherbrooke E, Montreal, Canada) was "Microbiota–centred interventions in immuno-oncology".

She started with the statement that the gut microbiome has emerged as one of the hallmarks of cancer in addition to the already recognised hallmarks (Figure 1).

The polymorphic gut microbiome can harbour both beneficial and harmful bacteria. Its composition and function can be modulated by genetics, lifestyle and therapeutic measures including diet, probiotics, prebiotics, and in some cases faecal microbiota transplantation (FMT).

Negative influences on the microbiome

However, some interventions can also have a negative impact on the gut microbiome. In particular, the use of antibiotics can eliminate beneficial bacteria, thereby allowing harmful bacteria the possibility to expand. It was shown in patients with advanced non-small-cell lung cancer (NSCLC) treated with immune checkpoint inhibitors (ICI) for their cancer, that antibiotic exposure has been associated with altered gut microbiota composition and reduced effectiveness of ICI treatment (Figure 2; *Derosa* et al., 2018).

Routy and colleagues showed also that antibiotics inhibited the clinical benefit of ICIs in patients with advanced cancer. In functional transfer experi-

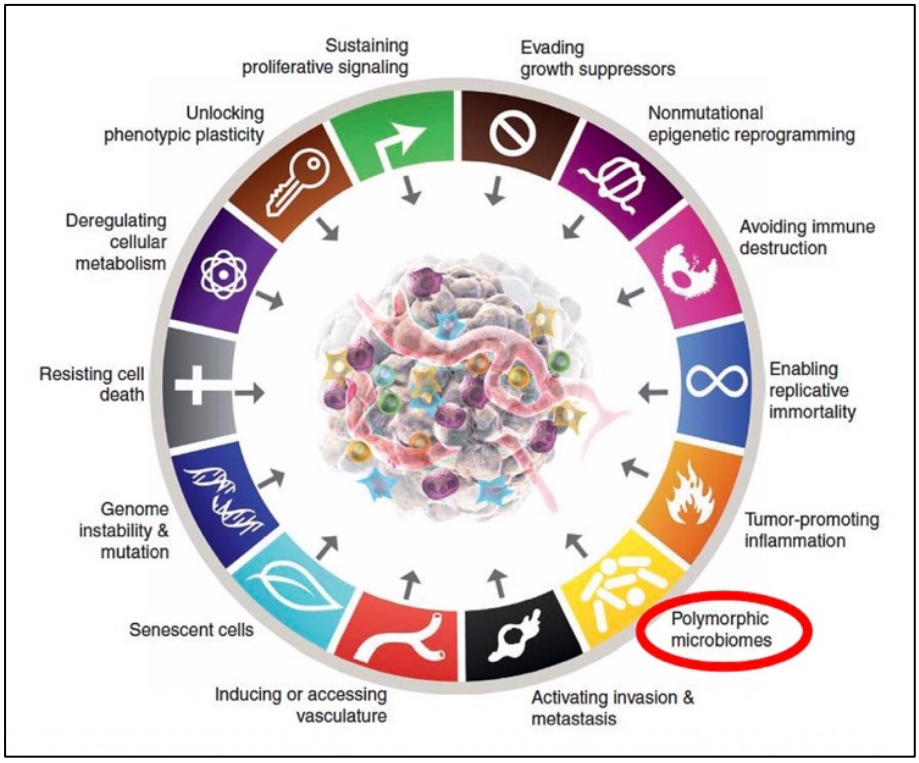

Figure 1: The hallmarks of cancer include the polymorphic microbiome. (Figure adapted from *Hanahan*, 2022).

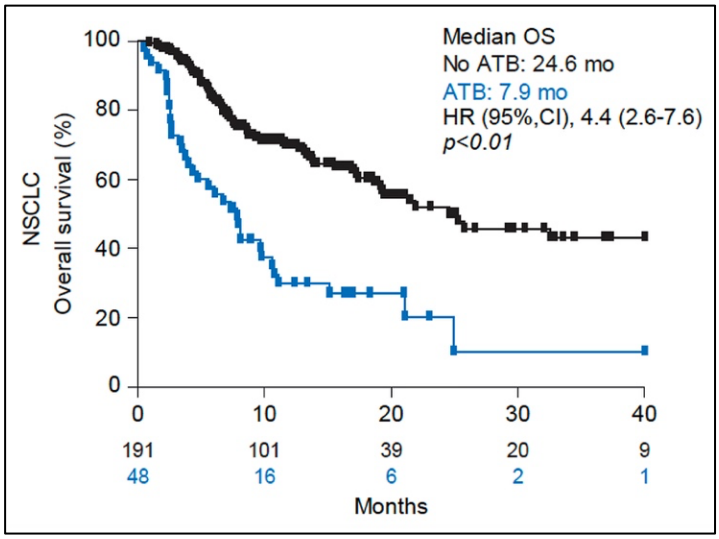

Figure 2: Overall survival in patients with NSCLC treated with ICI, stratified by use of antibiotics within 30 days of initiating ICI. (Figure from *Derosa* et al., 2018).

ments, faecal microbiota transplantation (FMT) from cancer patients who responded to ICIs into germ-free or antibiotic-treated mice ameliorated the anti-tumour effects of PD-1 blockade, whereas FMT from non-responding patients failed to do so (*Routy* et al., 2018). They further reported correlations between clinical responses to ICIs and the relative abundance of *Akkermansia muciniphila*, and showed that oral supplementation with *A. muciniphila* after FMT with nonresponder faeces restored the efficacy of PD-1 blockade.

When antibiotics are clinically unavoidable, a pragmatic approach is to use the narrowest effective spectrum for the shortest necessary duration, to minimize disruption of the microbiome and potential negative impact on immunotherapy.

The microbiome-protective effect of the antibiotic absorbent DAV132

Dr. Messaoudene reported on a study that was ongoing at the time of the seminar, and has since been published in Nature Communications (*Messaoudene* et al., 2024). In this study the authors tested a colon targeted absorbent (DAV132) given orally, which sequester antibiotics in the distal intestine and thereby limit microbiota damage. DAV132 is charcoal-coated formulated for ileo-caecum delivery, enabling adsorption of residual antibiotics before they reach the colon in active form, reducing the risk of dysbiosis.

The DAV132 colon-targeted absorbent was tested in a randomised phase I clinical trial with 72 healthy volunteers. Two doses of DAV132 were tested. The study consisted of three different arms. The first arm included a group of healthy volunteers who did not receive antibiotics with or without DAV132. The volunteers in the second arm were administered ceftazidime-avibactam (CZA), which is one of the most common antibiotics administered to patients, with or without DAV132. And finally, the third arm consisted of healthy volunteers treated with piperacillin-tazobactam (PTZ) alone, or in combination with DAV132 (Figure 3).

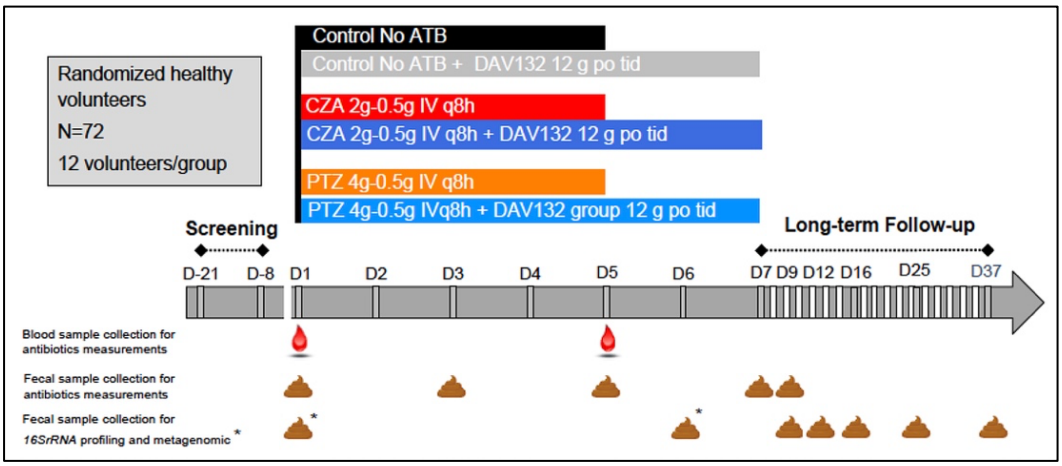

Figure 3: Clinical trial design and pharmacokinetics of antibiotics in the plasma and faeces of healthy volunteers (HV). (Figure from *Messaoudene* et al., 2024).

Antibiotics were administered intravenously for 5 days, while DAV132 was administered orally 12 g three times a day for 7 days. Blood samples were collected at 2 timepoints to measure antibiotic concentration in the blood and faecal samples were collected at different timepoints to measure the antibiotic concentration and to perform 16SrRNA sequencing and metagenomics microbiome profiling. They found that DAV132 led to significant decrease in CZA or PTZ faeces concentration. When co-administered with antibiotics, DAV132 preserved microbiome diversity, accelerated recovery to baseline composition and protected key commensals. The authors concluded that DAV132 represents a promising strategy to mitigate antibiotic-associated dysbiosis and warrants evaluation in patients, including those receiving cancer immunotherapy.

The study also explored functional consequences for anti-PD-1 efficacy using donor-to-mouse transfer experiments. For this, they transplanted the stools from healthy volunteers from the clinical study treated with antibiotics alone or with antibiotics plus DAV132 into germ-free C57BL/6 mice. Two weeks after FMT, mice were implanted subcutaneously with 0.8×10^6 MCA-205 (mouse fibrosarcoma cell line) cells. When the tumours reached 25 to 35 mm^2 in size, mice were treated four times intraperitoneally every three days with anti-PD-1 monoclonal antibody (250 µg/mouse). Tumours were harvested 11 days after the first injection of anti-PD-1. The transplanted faeces from healthy volunteers treated with CZA or PTZ alone inhibited the anti-PD-1 response, whereas FMT from volunteers treated with antibiotics plus DAV132 preserved sensitivity to anti-PD-1. This supports the concept that microbiome protection during antibiotic exposure can maintain ICI responsiveness in recipient mice.

Flow cytometry analysis on the tumour of the different groups of mice suggested that the mice receiving FMT from the group CZA plus DAV132 after six days of treatment maintain a high ratio $CD8^+$ T cells compared to the mice receiving stools from the group treated only with CZA during six days. It was also observed that the mice receiving faecal transplants from the volunteers treated with PTZ plus DAV132 showed an increase of $CD8^+$ T cell population after treatment with anti-PD-1.

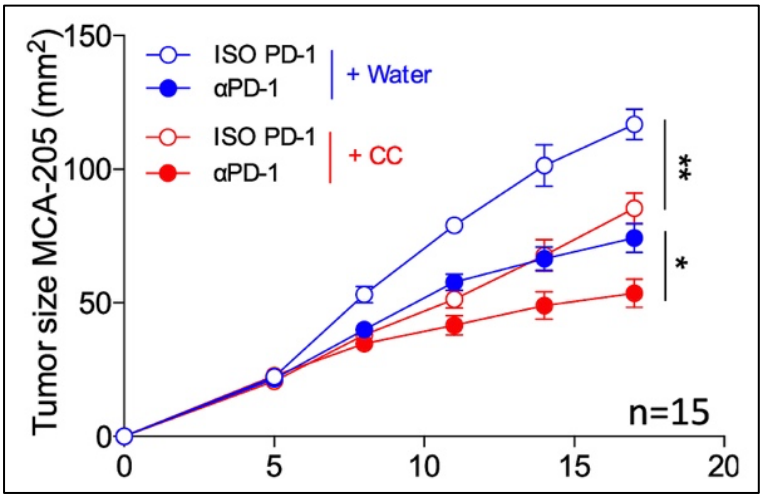

Figure 4: Tumour growth kinetics in SPF C57BL/6 mice after sequential injections of anti-PD-1 or iso-PD-1 and daily oral gavage with camu-camu or water in a MCA-205 sarcoma tumour model (n=15 mice/group). (Figure from: *Messaoudene* et al., 2022).

Overall, the study confirms that antibiotic-related dysbiosis is associated with a decrease of alpha diversity, a decrease of the "good bacteria" (like *Ruminococcus*) of which it is known that they are associated with a good response to immunotherapy. The antibiotic-related dysbiosis can result in an increase of the "bad bacteria" (like *Hungatella* and *Akkermansia*). When the microbiome is protected using DAV132, the alpha diversity is partially protected and can preserve the good bacteria such as *Ruminococcus*, *Eubacterium*, *Alistipes*, and *Faecalibacterium prausnitzii*. The protection of these bacteria seems to be associated with maintaining the immunotherapy response in mice.

These results are promising, but further studies are needed to evaluate the possible role of DAV132 in the treatment of cancer patients.

The influence of camu-camu on the gut microbiome

Dr. Messaoudene reported on a second study. Camu-camu, also known as *Myrciaria dubia*, is an Amazonian berry rich in phytochemicals and has been shown to exert protective prebiotic effects against obesity and related metabolic disorders in mice through increasing the abundance of *Akkermansia muciniphila* and *Bifidobacterium* in the gut (*Anhê* et al., 2019). In this second study, Messaoudene and colleagues evaluated whether the prebiotic action of camu-camu could also shift the gut microbiome in a way that improves antitumour immunity and responsiveness to immune checkpoint blockade. In the MCA-205 tumour model, known to be sensitive to anti-PD-1, they evaluated whether camu-camu could enhance anti-PD-1 efficacy. Figure 4 shows the tumour growth in mice treated with isotype control (iso-PD-1, the open blue dots) or with anti-PD-1 (filled blue dots), with (the open red dots) or without 1 (filled red dots) daily camu-camu. In addition, camu-camu alone showed antitumour activity comparable to anti-PD-1 in this setting. Importantly, combining camu-camu with anti-PD-1 further improved tumour control, with smaller tumour volumes over time.

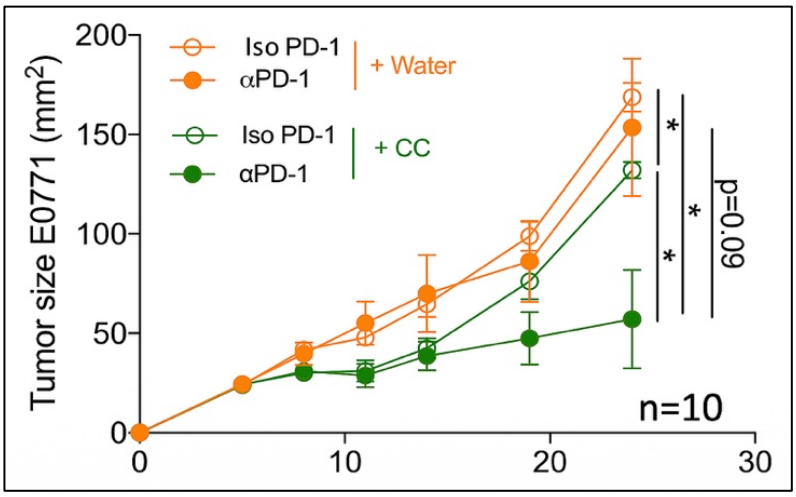

Figure 5: Tumour growth kinetics in SPF C57BL/6 mice after sequential injections of anti-PD-1or iso-PD-1 and daily oral gavage with camu-camu or water in a E0771 breast cancer model (n=10 mice/group). (Figure from: *Messaoudene* et al., 2022).

They tested camu-camu also in the anti-PD-1-resistant E0771 breast cancer model (n = 10 mice/group) and confirmed that the anti-PD-1 alone did not exhibit any anti-tumour activity in this model (Figure 5). Camu-camu alone had only a minimal effect, whereas camu-camu combined with anti-PD-1 transformed this resistant tumour model to a sensitive model. Camu-camu/iso-PD-1 demonstrated minimal anti-tumour activity, whereas the combination of camu-camu/anti-PD-1 increased the anti-PD-1 activity.

To identify the bio-active compound(s), camu-camu, was fractionated by High Performance Liquid Chromatography (HPLC), yielding 49 compounds. All the compounds were tested individually and only one compound, castalagin, showed the same effect as the complete camu-camu raw extract. Castalagin is a polyphenol also present in oak wood and is known to have anti-inflammatory properties.

The influence of castalagin on the gut microbiome

To test whether castalagin has the same effect as camu-camu, they performed FMT into germ-free mice, using faeces from non-small cell lung cancer patients known to be resistant to anti-PD-1. These patients were non-responders and performing FMT using faeces from these non-responder patients induces a resistance to anti-PD-1. After subcutaneous implantation of MCA-205 cells, they treated the mice with either the anti-PD-1 or the iso-PD-1 combined with castalagin administration by oral gavage or combined with water administration (controls). In the mice that were given anti-PD-1 and castalagin, a decrease in tumour size was observed when compared to the group that was treated with iso-PD-1 plus castalagin or the water control group (Figure 6).

The following step was performing a microbiome profiling of the mice faeces and they observed that the mice receiving castalagin had an increase in their faeces of *Ruminococcus*, *Akkermansia muciniphila*, *Ruminococcus*, *Blaucia*, *Alistipes* and other so called "good" bacteria, all being associated with a good response in patients treated with immunotherapy.

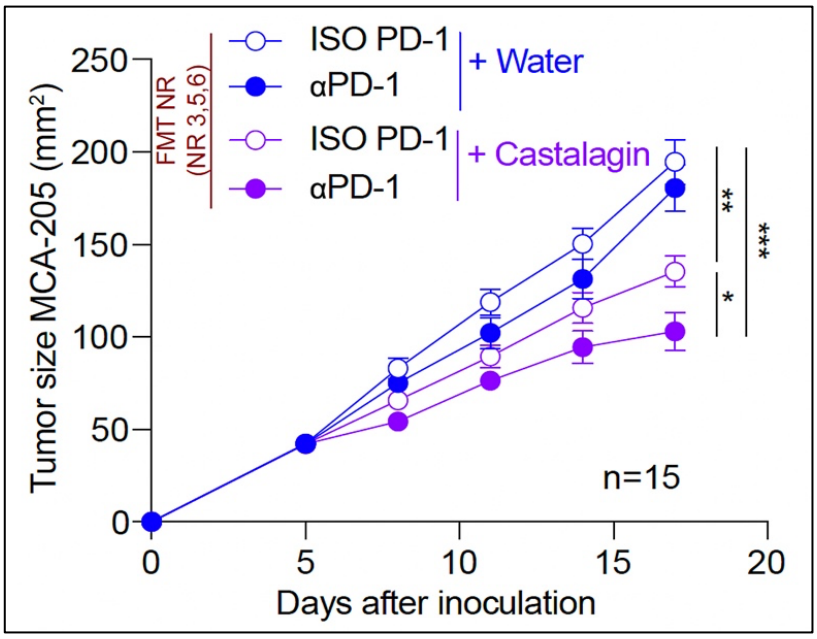

Figure 6: MCA-205 tumour growth kinetics in germ-free C57BL/6 mice after FMT from three non-responder patients with daily oral supplementation with castalagin or water in combination with anti-PD-1 or iso-PD-1.

They also performed an immune profiling using multiple technics including flow cytometry and immunofluorescence to test whether the response associated with the combination of castalagin with anti-PD-1 is also associated with a change of the immune system. They observed an increase of immune surrogate markers in the mice receiving castalagin including central memory $CD8^+$ T cells in the mice receiving castalagin. The percentage killing of CD8+ T OT-1 cells from the draining lymph nodes in mice treated with castalagin or water and immunized with CpG/OVA was also increased.

Dr. Messaoudene reported an ongoing clinical trial assessing camu-camu as a prebiotic adjunct to immune checkpoint inhibition in patients with non-small cell lung cancer and melanoma with very encouraging preliminary results.

In addition, preclinical results suggest that modulation of the gut microbiome by antibiotics is associated with ICI resistance. The faeces of two non-cancer patients enrolled in a clinical trial addressing the safety of oral administration of camu-camu were analysed. Preliminary faecal metagenomics revealed a positive trend in diversity and toward enrichment of *Ruminococcus bromii*, consistent with the results obtained in camu-camu-treated mice.

Faecal microbiome transplantation in the treatment of advanced cutaneous melanoma and advanced non-small cell lung cancer

The final part of the presentation focused on clinical FMT trials in advanced melanoma. Two independent phase I studies (*Davar* et al., 2021; *Baruch* et al., 2021) administered stool from complete responder melanoma patients treated with anti-PD-1 to patients resistant to anti-PD-1, resulting in clinical responses in approximately 25% of treated patients.

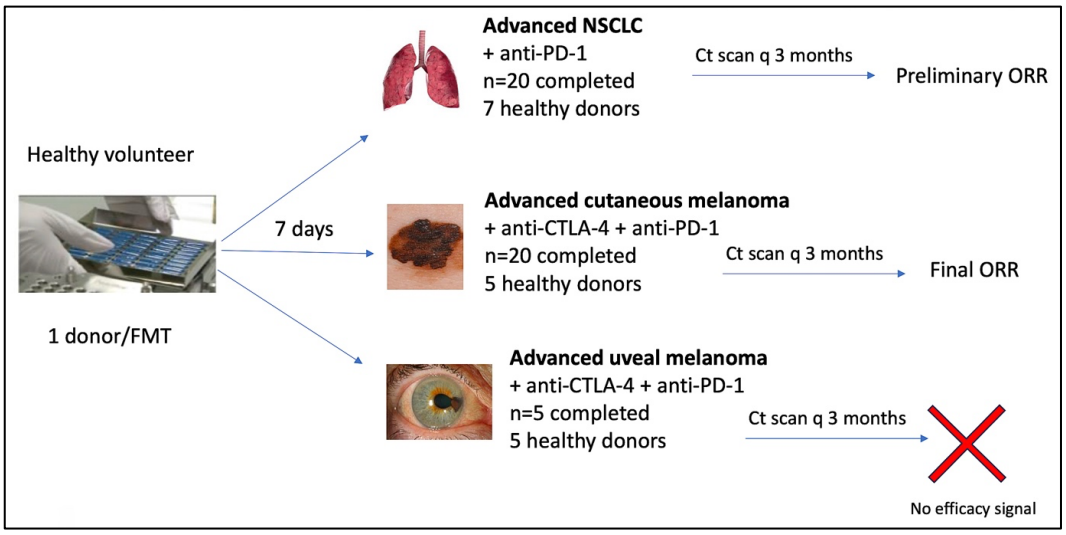

Figure 7: Clinical design of the FMT phase 1 study.

Based on these results, Dr. Messaoudene described a third phase I clinical trial (MIMICs) that they conducted in their lab at the Centre hospitalier de l'Université de Montréal (CHUM) (*Routy* et al., 2023). They did not use stools of complete responder patients, but stools of healthy volunteers. They enrolled 20 patients with cutaneous melanoma in this trial. They observed a 65% response compared to the 45% response that was obtained in the classical randomized phase 3 clinical trials. This was an increase in response of 20% in the patients treated with FMT plus anti-PD-1. Longitudinal microbiome profiling revealed that all patients engrafted strains from their respective donors. However, the acquired similarity between donor and patient microbiomes was only maintained over time in responders. Responders experienced an enrichment of immunogenic bacteria and a loss of deleterious bacteria following FMT. The responders maintained the microbiome of their donors, while in the non-responders the microbiome returned after one week to its original composition. The immune system of these patients was also analysed and they observed an increase of $ICOS^+$ $CD8^+$ T cells in the responders compared to the non-responders, consistent with enhanced anti-tumour immune activation.

Following the results of this MIMICs clinical trial, they started a second FMT clinical trial (FMT Luminate). Three different arms were included in this study (Figure 7). The first arm included advanced non-small cell lung cancer patients treated with anti-PD-1. The second arm included advanced cutaneous melanoma patients that were treated with anti-CTLA-4 plus anti-PD-1. This arm was also already completed with the enrolment of 20 patients. The third arm included advanced uveal melanoma patients treated with anti-CTLA-4 and anti-PD-1. However, after including five patients, this arm was closed because no signs of efficacy were observed in these patients.

Preliminary results in the non-small cell lung cancer patients, using FMT in combination with immunotherapy, showed an response rate of 72.2 %, an increase of about 30% compared to the

30% immunotherapy response in these patients.

The results in the advanced cutaneous melanoma cohort were also very good. They observed an increase in the objective rate response in these patients with about 20% compared to the classical randomized clinical trial using exactly the same clinical criteria.

In this study they observed one month after FMT in the responder group a decrease of the "bad bacteria", such as *Enterocluster*, and an increase of the "good bacteria", such as *Ruminococcus*, *Eubacterium*, and *Prevotella copri*.

The preliminary results of the last study at the time of the presentation of Dr. Messaoudene were:

- FMT alone has no safety signal and is acceptable for patients,
- Uveal melanoma is resistant to FMT + anti-PD-1 + anti-CLTA-4,
- Very strong preliminary results of FMT + anti-PD-1 +/- CTLA-4 in non-small cell lung cancer and metastatic cutaneous melanoma.

This paper was reviewed by Dr. Meriem Messaoudene before publishing.

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RELATIONSHIP BETWEEN GUT MICROBIOTA AND SYSTEMIC CHEMOTHERAPY

Introduction

Dr. Aadra Bhatt (Division of Gastroenterology & Hepatology, University of North Carolina at Chapel Hill, USA) presented data about the relationship between gut bacteria and systemic chemotherapy.

Medications can influence intestinal bacteria

Over the last years there is an increase in research that describes the reciprocal relationship between medications and the intestinal bacteria. It is known that medications can influence the microbiota by altering intestinal pH and the osmotic balance, and many medications can have an impact on barrier integrity. Medications can also affect nutrient availability for the microbiota and many drugs, including a class of antipsychotics, have been recently described to have bacteriostatic side-effects. So even drugs of which we do not think as being antibiotic can influence the vitality of our microbiome.

Intestinal bacteria can influence medications

However, another important observation is that microbiota can alter medications. Microbiota can alter nearly every aspect of drug metabolism that includes absorption, metabolism, distribution and excretion, which are basically the cornerstones of pharmacokinetics.

A number of host factors can regulate how we respond to medications. These include our age, our biological sex, physiological states such as pregnancy, our environment, our underlying genetics and ethnicity. These factors are unmodifiable. Ultimately these contributors to drug response are fixed and not changeable. However, intestinal bacteria are major contributors to drug response and this is important because microbiota are modifiable. They are one of the few modifiable contributors to drug response. This is a field that requires intense investigation because ultimately it can improve drug usage, drug tolerability, and drug access.

Antibiotics do attack our bacteria while those same bacteria are really important for homeostasis of the whole biont, for maintaining our health and for who we are as people. The goal of Dr. Bhatt's research is to identify the mechanisms by which microbiota alter drug metabolism and selectively target this with the purpose to preserve the integrity of the entire microbiome.

Pharmaco-microbiomics

Pharmaco-microbiomics is the study of microbiota and drug interactions. This is a very new and upcoming field. Dr. Bhatt's laboratory has studied drug microbiota interactions as to improve precision medicine by targeting bacteria and in particular specific bacterial functions. Dr. Bhatt gave a few specific examples of how bacteria influence drug metabolism and she started with two medications that are very widely used but they are not used in the context of cancer (Figure 1). One is digoxin, a cardio-protective drug that is converted by an enzyme expressed by a bacterium called *Eggerthella lenta*. There is a reduction of the one double bond in digoxin which converts digoxin into its inactive form called dihydrodigoxin. This is an example by which this bacterial function can be selectively targeted to preserve the efficacy of digoxin. A converse example is the activation of a compound called sulfasalazine which is used to treat ulcerative colitis. There is a class of bacterial enzymes called azoreductases that convert the azo-bond

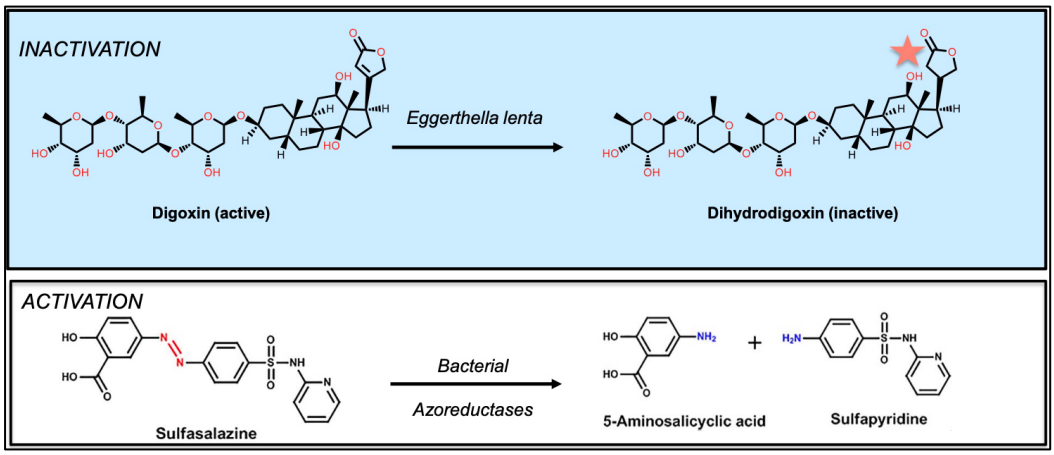

Figure 1: Gut bacterial enzymes directly alter drug efficacy. (Figure adapted from *Ervin* et al., 2020).

and release the protective compound called 5-aminosalicyclic acid which is an immunomodulatory compound that exerts the beneficial effects of sulfasalazine for people with ulcerative colitis.

Dr Bhatt recently published that patients suffering from ulcerative colitis that don't have this class of bacterial azoreductases in their gut are not actually going to derive any benefit from sulfasalazine treatment.

Another example of bacterial modification of a medication is levodopa converted by *Helicobacter pylori* into dopamine. Dopamine is unable to cross the blood-brain-barrier and patients who have Parkinson's disease early stage derive no benefit from levopoda treatment if they also have a concurrent *Helicobacter pylori* infection.

Bacterial enzymes can also increase the gastro-intestinal toxicity of 5-fluorouracil (5FU), which is a very widely used cancer drug which can also increase the nephrotoxicity of acetaminophen (paracetamol). Bacterial conversion of paracetamol into para-aminophenol results in a toxic metabolite that can cause kidney damage.

Bacterial enzymes can also convert molecules like para-cresol, which is generated during bacterial fermentation of protein in the human large intestine into the molecule called para-cresol sulphate. Para-cresol sulphate compete for the same detoxification enzymes that our body uses to detoxify paracetamol. This competition causes accumulation of toxic metabolites of paracetamol that again can exert nephrotoxicity.

It is also known from studies in germfree mice that germfree mice have a high expression of the constitutive androstane receptor (CAR) which is important for drug metabolism and this actually causes different responses to anaesthetics that are used for surgery.

One of the most exciting examples of how drug metabolism can affect host disease is that of choline. Choline is a dietary compound found very highly concentrated in red meat and eggs. Bacteria convert choline into trimethylamine (TMA) which is then subsequently oxidised by a bacterial enzyme responsible for drug metabolism called flavin mononucleotide (FMN) and generates the molecule called trimethylamine N-oxide (TMAO) which is cardiotoxic and linked to cardiovascular disease. TMAO has been very strongly associated with atherosclerosis.

Those are examples by which bacterial metabolism directly generates

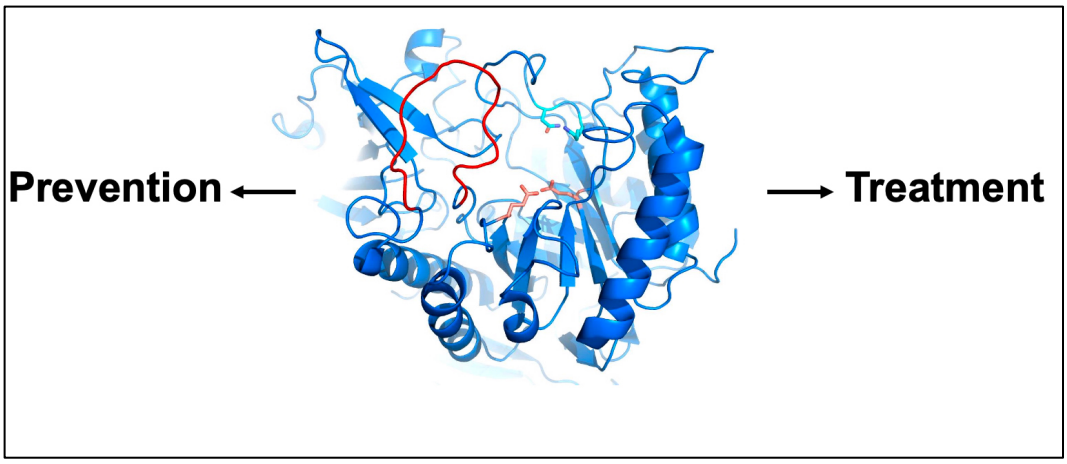

Figure 2: Monomer of *E. coli* ß-glucuronidase. (Figure from *Walace* et al., Science 2010)

molecules that can exert various kinds of toxicity or altered drug responses. There is an additional mechanism how bacterial enzymes interact with phase II conjugates that our body generates in response to detoxifying compounds. Whenever we take a medication that is hydrophobic, these hydrophobic molecules are conjugated in the liver by a class of enzymes called uridine-diphosphate-glucuronosyltransferases (UGT's) with a small 6-carbon sugar: glucuronic acid. This conjugation generates a hydrophilic molecule called hydrophilic glucuronide conjugated compound, making it easier for the body to excrete through urine or bile. There are additional Phase II conjugation reactions including sulfation.It are not just xenobiotics that are being recycled in this way. Antibiotics and substances that we make, such as hormones and transmitters like serotonin are also recycled by the same mechanisms. So these conjugated molecules are inactive and they are unable to exert their chemical effects and are considered to be inactive. The generation of these hydrophilic compounds allows them to be easily eliminated through urine or faeces. When they are eliminated through the faeces they encounter a class of bacterial enzymes called ß-glucuronidases which, as the name suggests, hydrolyses the glucuronide conjugate from these inactive molecules and convert them into active molecules in the gut. This is because glucuronic acid is a source of carbon in the highly competitive environment in the gut. Bacterial ß-glucuronidase or GUS is a non-essential carbon scavenging enzyme that is essential in humans because its deficiency causes a type of lysosomal storage disease called Sly syndrome. However, in bacteria is GUS a non-essential enzyme that is involved in carbon scavenging. It is not essential because when it is knocked-out of a lab strain of *E. coli*, the "knock-out GUS" shows the same sort of fitness and growth as the wild-type strain.

The loop

Figure 2 shows a monomer of *E. coli* ß-glucuronidase and deep within the enzyme is this catalytic site which is able to actually bind to a glucuronic acid molecule and adjacent to the catalytic site is this red floppy motif which is called "the loop". The loop is like a molecular clamp that holds the glucuronide conjugate really close to the active site so that the hydrolysis reaction can occur very efficiently. The catalytic site of ß-glucuronidase is highly

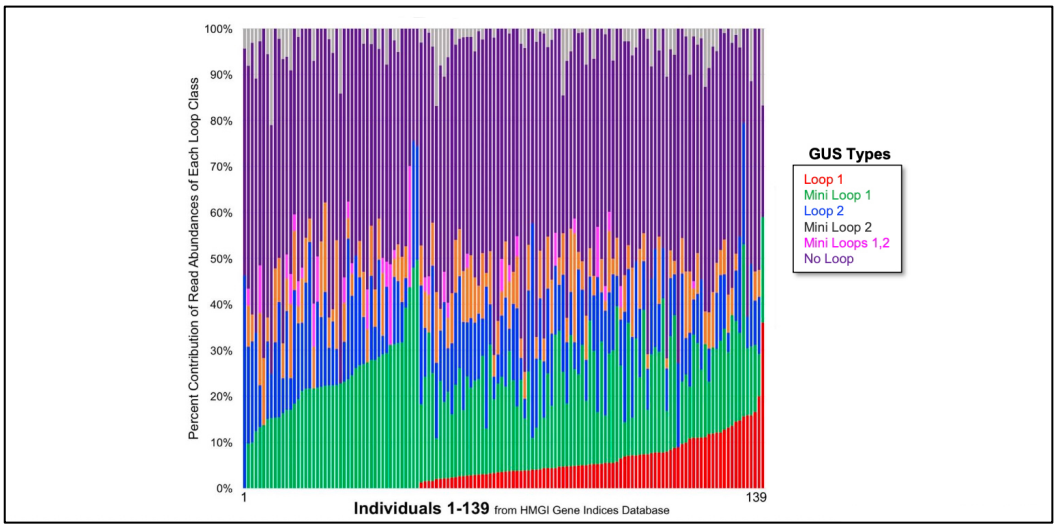

Figure 3: GUS types in 139 healthy individuals in the Human Microbiome Project catalogue. (Figure from *Pollet* et al., Structure 2017).

conserved among all bacteria and this catalytic site is used almost as a "bait" to delf through the Human Microbiome Project. When looking through the faecal database of the Human Microbiome Project, using this catalytic site as "bait", a number of structural features of ß-glucuronidase can be identified that cluster into six specific types, which are termed "Loop 1" such as those expressed by *E. coli*, "Loop 2" such as those expressed by sero-Bacteroides, "No Loop", "Mini-Loop 1" and "Mini-Loop 2" and bacteria that have both "Mini-Loop 1" and "Mini-Loop 2". Each one of these loop structures is essential for determining the substrate specificity. The specific loop motive directs the type of substrate that each of these bacterial enzymes has a specificity towards.

Over the last few years we have been really delving deeply into what are the specific substrates for each of these loop types. Every bacterium in every phylum have their unique GUS, so we know this is a very widely expressed bacterial enzyme. What all the structural and molecular work has helped to understand is that these features are absent in the million ortholog ß-glucuronidases and this is important because it allows to selectively target the bacterial isoforms while leaving the million enzymes unperturbed. This is very important because GUSes are essential for how we process various polysaccharides.

Each one of the bars in Figure 3 is an individual from the Human Microbiome Project. There are 139 bars and the different colours represent a specific loop type. Only about 2/3 of all individuals express "Loop 1" GUSes. This is really important because "Loop 1" GUSes, are the specific bacterial enzymes that are involved in deconjugating drug glucuronide-conjugates.

Cancer drugs and gastrointestinal toxicity

Table 1 shows a list of FDA approved cancer medications that are all detoxified in the liver by conjugation with glucuronic acid. Important is that all of these drugs are causing gastrointestinal toxicities, specifically diarrhoea. Irinotecan, as an example, is used for treating colorectal cancer and also sometimes pancreatic cancer; either alone but most usually in combination with other

Table 1: Cancer drugs detoxified by glucuronic acid conjugation via Phase II metabolism cause gastrointestinal toxicity

Dasatanib	Bicalutamide	Mycophenolate	Vandetanib	Daunorubicin
Irinotecan	Sorafenib	Epirubicin	Olaparenib	Cyclophosphamide
5-Fluorouracil	Bevacizumab	Vorinostat	Etoposide	Bortazomib
Anastrozole	Panobinostat	Afatanib	Axitinib	Fulvestrant
Bexarotene	Regorafenib	Capecitabine		

compounds. Irinotecan is administered intravenously. It is first converted into a molecule called SN38 by plasma carboxylesterases. SN38 has almost a 10,000 fold higher affinity than irinotecan to bind its cellular target which is topo-isomerase which is an enzyme that is important for unwinding DNA during DNA replication. Irinotecan or SN38 can selectively target highly proliferative cells such as cancer cells. The gut is a highly proliferative organ that turns over once every five days which is a high rate of proliferation. Irinotecan is known to cause severe diarrhoea of which the only way to resolve it is to suspend therapy. Stopping treatment with their anti-cancer drug is of course for someone undergoing treatment for cancer not a really good idea.

SN-38 is detoxified in the liver by an enzyme called UGT which binds glucuronic acid to SN-38 generating SN38-glucuronide which is inactive and unable to bind to the topoisomerase I enzyme. SN-38 is excreted via faeces where bacterial ß-glucuronidases encounter this glucuronic conjugate and hydrolyse it forming an SN38 molecule in a site where it probably should not be.

Several years ago, selective inhibitors of bacterial ß-glucuronidases were developed and when they were administered in concert with irinotecan to naïve mice, the weight loss and bleeding and diarrhoea that mice experience with irinotecan treatment could be stopped with co-administration of a GUS inhibitor.

When irinotecan is administered to mice and the activity of bacterial ß-glucuronidases is examined in the faeces (*in fimo*), an increase in total gut activity after administration is observed. As soon as a substrate is put into the mix, activation of the bacterial enzyme is a result. This was replicated using gnotobiotic facilities at UNC. Germfree wild-type C57 black six (C57BL6) mice were colonised with either a wild type *E. coli* strain or the isogenic mutant that lacks a functional ß-glucuronidase. Mice were colonised for a week and treated with a single dose of irinotecan, after which the proliferative pool of intestinal stem cells in the colon as well as ileum were examined (figure 4).

In these mice proliferation was qualified using *in vivo* BrdU (5-bromo-2'-deoxyuridine) labelling. In mice that were colonised with the wild type strain of *E. coli* and subsequently treated with this proliferation inhibitor, a reduced number of proliferative cells was observed (the dark spots in the lower micrographs of figure 4). Fewer or hardly any dark spots are seen in the colon of mice colonised with the wild type *E. coli* strain compared to the mice that were colonised with the isogenic ß-glucuronidase mutant (upper panel of the micrographs in figure 4). This also demonstrates the importance of bacterial ß-glucuronidase in exerting the toxicity in the gut of irinotecan.

Because there are very few ways to treat triple negative breast cancer (TNBC), two different mouse models of triple negative breast cancer were used: one was an xenograft model in which immunodeficient mice were injected

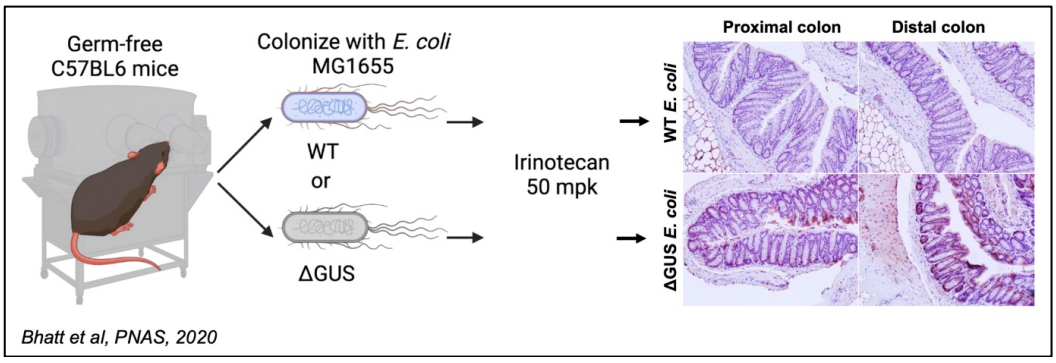

Figure 4: Germfree wild-type mice colonised with GUS-deficient *E. coli* are protected from irinotecan-mediated injury

with TNBC cell line, and the other model was a genetically engineered mouse model (GEMM) for TNBC in which the SV40 large T antigen drives mammary epithelial cell specific expression of the T antigen. In both models, tumours were allowed to develop to hundred cubic millimetres after which the study was initiated. The mice were randomised into 4 groups in which mice received irinotecan alone or with a next generation GUS-inhibitor in combination and of course the respective control groups (GUS-inhibitor alone or vehicle). In both models it was found that irinotecan alone was able to reduce tumour growth and the co-administration of the GUS-inhibitor did not change tumour volumes when measured serially (figure 5).

At the end of the study the tumours were dissected out of these mice. The total weight of the tumours also didn't differ in either group and in the GEMM it was found that the tumours were practically undetectable. This protection might be largely due to the prevention of diarrhoea. The diarrhoea that resulted even from irinotecan treatment in the mouse model was so severe that mice did lose up to 20% of their body weight, which is the humane cut off in the protocol. Co-administration of the GUS-inhibitor allowed a majority of the mice to remain diarrhoea-free for a longer time which resulted in preserved body

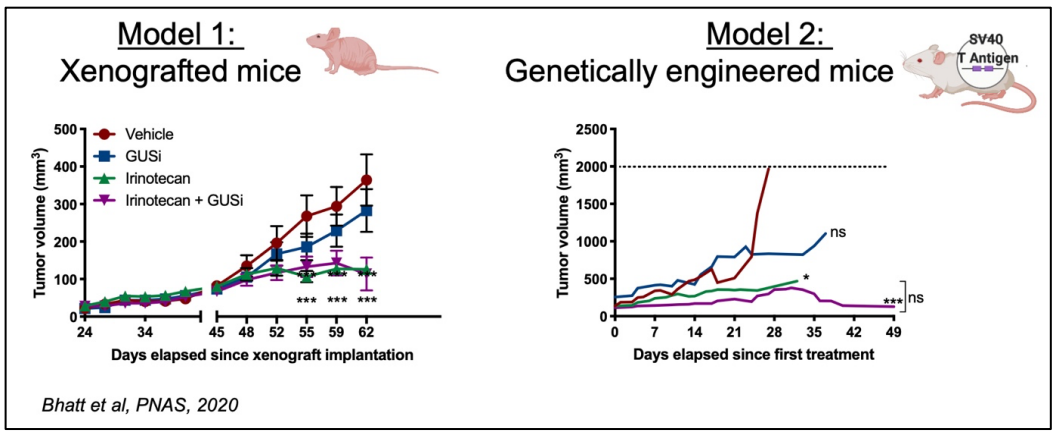

Figure 5: GUS-inhibitor co-treatment does not impede irinotecan's anti-tumour effects in murine models

weight, which again resulted in the mice to be able to withstand higher or larger number of doses of irinotecan. This is a big win because very often people fail treatment because of the side-effect and not because of the actual treatment on its own.

Personalised chemotherapy

The described experiments serve as a proof of concept that selectively modifying specific microbiota function, as opposed to wiping out entire classes of bacteria, might be a good way to improve drug response by reducing the toxic side effects that are exerted by the microbiota. This is an example of how we can use pharmaco-microbiomics to improve drug responses.

The long-term goal is to personalise chemotherapy to improve drug responses. *In fimo* drug reactivation rates may serve as a prognosticator of adverse drug responses. By quantifying the rate of turnover of glucuronides it will be possible to stratify individuals to be at high, medium or low risk of developing intestinal side-effects.

As mentioned earlier, multiple chemotherapy therapeutic drugs are detoxified by conjugation with glucuronic acid. But it is not just chemotherapy that is detoxified in this way. This might also be true for more drugs that, for instance, are being used for gout, and drugs like raloxifene that is being used for treating osteoporosis, or metformin that is being used for treatment of diabetes type 2. These are compounds that are detoxified by conjugation with glucuronic acid and many of them also have often diarrhoea side-effects.

Summary

- Bacterial drug metabolism can explain the inter-individual variability in drug responses.
- ß-glucuronidases can reactivate conjugated drug metabolites in the gut.
- Selective and non-lethal GUS-inhibitors can be a useful strategy to block drug-glucuronide activation in the gut.
- "Drugging the bug" can be an effective strategy to improve on drug response.
- Microbiome targeting can improve precision medicine.

This paper was reviewed by Dr. Aadra Bhatt before publishing.

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