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The intratumoral microbiome: characterization methods and functional impact

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Abstract

Live-pathogenic bacteria, which were identified inside tumors hundreds year ago, are key elements in modern cancer research. As they have a relatively accessible genome, they offer a multitude of metabolic engineering opportunities, useful in several clinical fields. Better understanding of the tumor microenvironment and its associated microbiome would help conceptualize new metabolically engineered species, triggering efficient therapeutic responses against cancer. Unfortunately, given the low microbial biomass nature of tumors, characterizing the tumor microbiome remains a challenge. Tumors have a high host versus bacterial DNA ratio, making it extremely complex to identify tumor-associated bacteria. Nevertheless, with the improvements in next-generation analytic tools, recent studies demonstrated the existence of intratumor bacteria inside defined tumors. It is now proven that each cancer subtype has a unique microbiome, characterized by bacterial communities with specific metabolic functions. This review provides a brief overview of the main approaches used to characterize the tumor microbiome, and of the recently proposed functions of intracellular bacteria identified in oncological entities. The therapeutic aspects of live-pathogenic microbes are also discussed, regarding the tumor microenvironment of each cancer type.

Introduction

Neoplastic diseases are characterized by the uncontrolled growth and spread of abnormal cells in localized tissues [1]. Despite all the progress made in modern medicine, cancer remains one of the leading causes of death in industrialized countries. In 2018, around 18 million people worldwide were diagnosed with cancer, for approximately 9 million associated deaths [2]. In the absence of new therapeutic approaches, numbers of neoplastic-related deaths are expected to continue increasing, in respect of the increase in life expectancy worldwide [1].

Cancers are multifactorial diseases, involving both intrinsic and extrinsic causative factors. Depending on the host's genetic predispositions and the nature of the extrinsic environmental cues to which it is exposed, cancer development leads to the formation of unique tumors, characterized by distinct physiological profiles [3,4]. The ecosystem of cancers comprises several cell types, including immune and stromal cells, all surrounded by an extracellular matrix, specific to each individual tumor [4]. Mounting evidence defines tumors and their respective microenvironments as key elements for regulating cancer progression. Several studies have demonstrated the strong influence of tumor surroundings on cancer pathogenesis and the associated host's responses to treatments [5,6]. Recently, the tight correlation between tumor microenvironment and cancer onset was refined to the presence of specific bacteria inside tumor-like tissues. Following a large metagenomic study of various solid tumors, Nejman *et al.* revealed the existence of intracellular bacteria, inside both host immune and cancer cells surrounding the tumor microenvironment [7]. Characterizing a specific tumor microbiome consolidates the historical report by Coley, who was the first to propose live pathogenic bacteria as active anti-cancer agents [8]. The cancer microbiota adds an additional level of complexity to the already convoluted tumor microenvironment. Broader understanding of the correlation between tissue microbiomes and their ability to regulate host immune responses would make it possible to develop new personalized medicine, enhancing patients' prognosis [9]. This area of research remains relatively new and consequently requires further investigation for a better overview of the microbiome's impact on tumorigenesis and/or tumor progression.

To explain the presence of bacteria in tumor tissues, a model has been proposed (Figure 1). Tumors have all the necessary requirements for supporting high bacterial prevalence. Their intrinsic properties seem suitable for maintaining a steady bacterial survival rate: the enhanced levels of blood vessels surrounding most tumor tissues (e.g. neo-angiogenesis), combined with their natural ability to hide from host immune surveillance, are perfect niches

for nearby circulating bacteria [10] (Figure 1). Most cancer cells naturally express a variety of mechanisms to avoid being recognized by the surrounding immune system (e.g. expression of checkpoint inhibitors), and this potentially supports the survival of intratumor bacterial species. [10,11]. Tumor necrotic regions also release high levels of nutrients (e.g. purines), which influence bacterial survival and their relative abundance [12]. In addition, bacterial chemotaxis towards high chemoattractant compounds presents in necrotic foci (e.g. aspartate, citrate, serine, ribose and galactose) enhances bacterial prevalence in human tumors. The physiological absence of oxygen within tumors provides a natural advantage for the proliferation of strict anaerobic (e.g. *Clostridia* spp.) and facultative anaerobic bacteria (e.g. *Salmonella* spp., lactic acid *Bifidobacteria*) in human cancers [13]. The hypoxic conditions are derived from the rapid growth of malignancies, ultimately leading to low blood supply. Overall, the tumor microenvironment can form a permissive immune-protected environment, where bacteria can easily escape host immune defenses and proliferate [10].

Although convinced of their existence, questions regarding the origin and precise functions of intratumor bacteria remain. Given the relatively low tumor microbial biomass and undetermined culture methods, combined with the enhanced risk of extrinsic contamination during sample analysis, characterizing the tumor microbiome has, for a long time, been a real challenge [9]. It is admitted that each individual tumor corresponds to a unique microbiome, composed of different bacterial communities. Comparative studies between healthy unaffected tissues and cancer-like tissues confirmed the complete diversion of the tumor microbiome from the standard organ-derived microbial profile (Table 1). To better comprehend the mechanisms of cancer, particularly its association with microbiology, deeper understanding of the bacterial microbiome that makes up each existing tumor is essential. Bacterial communities found at each tumor site may, in correlation to the gut microbiota, play an important role in tumor progression. Complementary studies on the tumor microbiome would provide additional details on the impact of intratumor bacteria on the various hallmarks of cancer. It would help fill in the gaps found in the current literature, as well as provide new insights for the development of new bacterial cancer treatments.

This paper reviews modern bacterial cancer research. It outlines the different methods used to characterize the microbiome in solid tumors, and provides evidence for the importance of such bacteria with regard to a cure for cancer (e.g. bacterial cancer therapies).

1. A route towards a specific tumor microbiome: the different analytic methods available to characterize it

1.1. The human microbiome

Throughout evolution, humans have developed a symbiotic relationship with a variety of microorganisms, including not just bacteria, but also fungi and small viruses. On average, each individual encodes ten times more bacterial genes than human genes in its genome [14]. Found both inside and outside the host's epithelial layers, the commensal microbiota supports most human physiological functions, including both metabolic and immunologic processes [15].

Thanks to the progress made in next-generation sequencing, characterizing the human microbiome has improved considerably [16]. Initially considered entirely sterile, it is now shown that each body organ inhabits a unique microbiota, defined by tissue-specific microbial communities. The recent identification of intracellular bacteria in tumor-like tissues confirmed that the microbiome is not only reserved for surrounding healthy tissues [7]. If conditions allow, bacteria can efficiently colonize altered necrotic tissues, using the intrinsic properties of tumors as an energy source to support bacterial proliferation. Characterizing the tumor microbiome is of great interest as more than 16% of the incidence of cancer is now attributed to an infectious agent [17]. The pathogenic nature of microbes consolidates the established influence that bacteria have on cancer development. The human microbiome has long been known for its role in the onset of cancer. Commensal microbiota dysbiosis, generally due to poor nutrition or other related extrinsic factors, is linked to the subsequent development of cancer [18,19]. The carcinogenic influence of pathogenic microbes brings another facet to this established consortium of factors involved in the formation of cancerous tumors. Studies on intratumor bacteria open new possibilities in the field of cancer research. By characterizing the microbiota inhabiting each individual tumor, it is possible to learn more about the universal carcinogenic processes driving tumor progression.

1.2. The main methods used to characterize the intratumoral microbiome

With the significant improvements made in analytic tools, it is now accepted that the microbiome of each organ is far more complex than previously thought [20]. Earlier assessment of the human microbiome thus needs to be reevaluated to obtain an updated overview of the different bacterial species found in each organ-specific microbiota. By using modern methods of bacterial identification, such as 16S rRNA profiling, followed by next-generation sequencing, it is possible to measure and characterize the intratumor bacterial

profile of each associated organ [9]. 16S microbial profiling is a multi-step process, involving visualization, isolation, amplification and sequencing of target bacterial genomic content [9] (Figure 2).

1.2.1. 16S microbial profiling

Microbiologists have, for decades, actively discussed the relationship between phylogeny and function in microorganisms. Defined by specific morphological features, such as flagella, cell size and shape, phylogenetic studies relied for a long time on all these disparities to classify bacteria into defined species [21]. Following the discovery of horizontal gene transfer between species, comparisons of DNA sequences became the principal strategy used to study bacterial phylogeny and diversity [22]. Prior to the emergence of next-generation sequencing, phylogenetic studies were limited to the bacterial species capable of growing *in vitro* [21]. At the time, it was necessary to culture bacteria in specialized flasks, to investigate them further. As most species are not adapted to cell culture, historical phylogenetic studies characterized only a tiny number of bacteria. Fortunately, next-generation sequencing techniques initiated a new era of microbial analytic research. Deep-sequencing approaches helped identify species that were initially undetectable using culture-based methods, based on their relative genomic differences [21]. 16S rRNA is a 1.5 kb long RNA fragment, making up part of the ribosome of all prokaryotic organisms [23]. Regarding the bacterial kingdom, each individual bacterium has one or more copies of the 16S gene. Conserved between most species, this rRNA molecule was rapidly identified by Woese as an “evolutionary clock”, crucial in the reconstruction of the tree of life [24]. Since then, the 16S sequence has primarily been used as the reference sequence for identifying and clustering bacteria into distinct taxonomic groups [25,26]. Under physiological conditions, a standard 16S rRNA fragment comprises 9 hypervariable regions (V1-V9) of approximately 30-100 bp. The sequences of each region are taxon-specific, meaning that they fluctuate, according to the defined taxa of the bacteria present. The small difference in 16S rRNA sequence is the basis for microbiome profiling, using 16S amplicon sequencing [22].

1.2.2. Microbial profiling of solid tumors

16S sequencing varies widely from shotgun whole-metagenomic profiling. Instead of using all DNA sequences available, 16S sequencing focuses primarily on the 16S gene, in order to confer a restricted but precise amount of information, regarding the bacterial species present in the sample of interest [22]. 16S sequencing has numerous advantages, as well as several

undeniable drawbacks (Table 2). It is recommended that all profiling steps be backed up with multiple negative controls to assure the repeatability and accuracy of the experiment. Sterile working conditions must also be maintained to reduce the risk of external contamination of the sample analyzed [7,9].

(a) **Characterizing bacteria with immunohistochemistry**

Intratumor bacteria can be detected with immunohistochemistry (IHC), using specific antibodies targeting lipoteichoic acid (LTA) or polysaccharide binding protein (LBP), or with RNA fluorescence *in situ* hybridization (FISH) [27] (Figure 3). Recently, Nejman *et al.* used these approaches to confirm the prevalent presence of intracellular bacteria inside tumors [7]. Bacterial LPS and 16S rRNA, observed in all samples analyzed, were found in the same distribution between tumors of different types. The 16S rRNA was predominantly discerned in the cytoplasm of cancer cells whereas LPS were spotted in both the cell cytosol and cell nucleus [7]. Despite the prevalence of bacterial LPS inside tumors, Nejman *et al.* did not detect any LTA in the cytoplasm of cancer cells. The inability to detect LTA in tumor-like tissues seems to be in support of significant precarity for Gram-positive intracellular bacteria inside tumors. However, as shown in the same study, Gram-positive 16S bacterial DNA can, in complement to Gram-negative genomic content, be measured in the microbiome of the different solid tumors, totally contradicting the above statement [7]. This discrepancy could be explained by the fact that intratumor bacteria alter their cell envelope when present inside tumor cells by losing their bacterial cell wall (peptidoglycan), rendering direct detection of LTA impossible [28]. Although absent from cancer cell cytosols, bacterial LTA was observed, in combination with LPS, in host macrophages colocalizing in the microenvironment of the tumors [7]. The abundant presence of LTA/LPS in these phagocytic cells suggest two possible hypotheses. First, it is possible that when recognizing foreign bacteria, host macrophages prefer to ingest only bacterial components instead of whole live bacteria. Inversely, high levels of bacterial debris may be a simple reflection of the early accumulation of bacteria inside macrophages [7]. Forestier *et al.* previously showed, using *in vitro* assays and *in vivo* mucin models, intracellular accumulation of LPS inside macrophages for a period of more than 3 months, supporting the aggregation of bacterial cell wall components over time [29].

IHC and FISH staining are generally complemented by correlative light and electron microscopic (CLEM) analysis that confirmed the abundance of microbiome in close proximity to the nuclear membrane of cancer cells [7]. However, microscopic detection

methods provide no data on the metabolic activity of intratumor bacteria. Additional methods, measuring the metabolic activity of the different bacteria identified are then essential for functionally characterizing the intratumoral microbiome [30]. This can be done with active fluorescence analysis of peptidoglycan synthesis, using labeled D-alanine or dimethyl sulfoxide (DMSO) as the carrier control. This approach has the advantage of detecting the presence of metabolically-active bacteria, while keeping apart all dead bacteria found nearby [30,31]. Several studies have demonstrated that cancer cells often display intracellular D-alanine labeling, suggesting the presence of live intracellular bacteria inside tumors [7].

(b) DNA analysis-based methods

Bacterial DNA extraction, PCR amplification and read duplicate high-throughput sequencing are the main elements of all microbiome studies. Given the low microbial biomass nature of tumors, in-depth analysis of defined cancer microbiomes is generally complex. High prevalence of host DNA in tumor-like tissues, combined with the constant risk of external contamination, often leads to inaccurate and barely reproducible results [9]. Efficient microbial profiling relies on a preliminary host DNA depletion strategy, used to focus the final analysis only on isolated bacteria DNA [32]. Depending on the type of tissue, the ratio of host versus bacteria DNA is highly variable. Nevertheless, by using quantitative PCR of 16S ribosomal RNA, Nejman *et al.* estimated the amount of bacteria DNA at around 40 ng per tissue section of various cancers (melanoma, lung, ovarian, glioblastoma, breast, pancreatic and bone sarcomas) which would correspond to 8 pg of DNA per cancer cell [7]. Subsequent PCR implies using defined bacterial primers, targeting highly conserved regions in the 16S gene. Over the years, all hypervariable regions of the 16S rRNA gene have been assessed for their ability to characterize and classify bacterial species [33–36]. Accumulative data tend towards the use of the V3-V4 16S segment, which emerges as the amplicon most commonly employed in 16S sequencing studies [37]. Recently, a new multiplexed 16S rDNA sequencing protocol (the “Short Multiple Region Framework” approach) was proposed as an infringing approach, revolutionizing microbial profiling [39,40]. 16S sequencing reads are generally compared to an available database, using free-access software, such as QIIME and MOTHUR [39]. These programs are useful for performing efficient 16S analysis from end to end. The “quantitative insights into microbial ecology” analytic program, also known as QIIME, is an open-source pipeline used to convert raw data into understandable results [40]. This software comprises a variety of high-throughput microbial analytic tools (e.g. network analysis, sample adaptive measures), providing efficient insights into the different microbial

sequences obtained following PCR amplification [40]. During data analysis, reads are clustered into bins called “Operational Taxonomic Units” (OTUs), based on their similarity threshold. All sequence reads with a similarity threshold of more than 97% are clustered together in the same OTUs [41,42]. Each OTU is characterized by one read, defined as the reference 16S sequence of the group. The selected sequence is subsequently annotated, following the 16S classification method [43]. Based on an active machine learning principal, the 16S classifier rapidly selects sequence reads, by associating them with specific taxonomic groups [43]. The main benefit is the significant reduction in the number of sequences to be analyzed [41]. However, OTU classification also has several limitations [33,44,45]. For instance, the 97% similarity threshold used for most 16S rRNA alignment studies likely provides an uneven estimation of the true similarity there is between two sequence reads. Computational analysis wrongly estimates the number of substitutions present between two confronting sequences. Consequently, paired alignments tend to over-evaluate their likeness, classing the sequences in the same OTUs [46]. Multiple substitutions, occurring at the same time, are also often missed by the analytic system [47]. Similarly, species defined as 97% similar may in reality possess 99% of identical similarity sequences (e.g. *Bacillus psychrophilus*) [48]. The OTU methodology has progressively been replaced by technical approaches using the exact sequences identified that better consider sequence quality. The new approaches make it possible to sequence variants that can be distinguished by a single base, giving rise to amplicon sequence variants (ASV). While OTUs were brought together based on pairwise similarity levels but nonidentical sequences, ASVs correspond to exact sequence of variants making it possible to remove contaminating sequences without significantly affecting relevant reads [49]. ASVs also determine how many time each exact sequence is read. The Divisive Amplicon Denoising Algorithm 2-based pipeline (DADA2) is a widely-used taxonomy classifier based on ASV analysis [50] and was recently used to characterize the microbiome of breast, oral or pancreatic cancers [51–53]. However, in certain cases, using a 16S rRNA gene sequence analysis pipeline with formation of OTUs is more reliable than the new ASV techniques. Depending on the degree of DNA damage present in the sample of interest, generating ASV can appear unsuitable. The formation of single nucleotide polymorphism from possible DNA damage may lead to artificially wrong recognition by next-generation ASV programs [54]. Two identical strains, unfortunately damaged during fixation or paraffin embedding for instance, would be recognized as two distinct strains.

Shotgun metagenomics are an untargeted high-throughput sequencing approach for the entire microbial genome accessible in a biological sample, such as tumor tissue. By recovering all the genome sequences, the shotgun sequencing method is used to profile taxonomic composition and functional properties of the intratumoral microbiome [55]. This technique has been used extensively to characterize the gut metagenome associated with cancer, including colorectal cancer, breast cancer, or melanoma [56–58].

2. Characterization and functional aspects of the tumor microbiome

Since the first identification of intratumor bacteria in solid tumors, the tumor microbiome has been the center of interest in the field of cancer therapy [9]. Recent studies, characterizing the microbiota of the most common cancer types, are summarized in Table 1. Results were obtained from various sample types, using different analytic approaches. Given the difficulties encountered while characterizing tumors, all samples analyzed were most likely altered during the investigation. Any metabarcoding and metagenomic results obtained from analyzing tumor samples thus need to be reassessed or taken into consideration with extreme caution, before drawing any hasty conclusions regarding the association of specific bacterial species with carcinogenesis [9].

2.1. Human colonizing bacteria: a perfect mix between commensal, opportunistic and pathogenic organisms

Over the years, numerous bacterial species have been linked to primary cancers and metastases. These include microbes associated with gynecological cancers [59–63], prostatic cancer [64–66], colorectal cancer [67,68], pancreatic cancer [69,70], or even respiratory-based cancers [71,72]. One of the first bacterium to be characterized as a drastic causative cancer agent was the Gram-negative bacterium *Helicobacter Pylori* and its direct association with gastric adenocarcinoma [67] (Figure 4). With multiple flagella, it can penetrate smoothly into the gastric epithelial cells and alter numerous host mucosal metabolic functions (e.g. altered proliferation of epithelial cells, degradation of intercellular junctions) [73]. *H. pylori* relies on two important cytotoxins to ensure host colonization (cytotoxin-associated gene A and vacuolating cytotoxin A). Cag A was the first bacterial protein proved to be involved in carcinogenesis [74]. This protein leads to the proteasome-mediated degradation of p53, an important tumor-suppressor gene, in gastric epithelial cells, by interfering with the host's AKT signaling pathway [75]. The onset of gastric adenocarcinoma derives from the overstimulation of the local inflammatory response, induced consequently to *H. pylori* bacterial

infection (Figure 4). Gastric bacterium infiltration induces an increased release in pro-inflammatory cytokines (e.g. IL-6 and TNF- α), which favors the recruitment of white blood cells and the production of free radicals (e.g. reactive oxygen species), responsible for specific DNA damage [76].

A variety of other species have been found in high prevalence in specific tumors. In most cases, the presence of these bacteria coincides with observable patterns of cancer onset, reasserting their primary role in carcinogenesis. Hieken *et al.* explored the microbiota in breast tissue samples collected from benign and cancer patients [77]. The microbiota was formed mostly by Bacteroidetes and Firmicutes in both tissue types, however a higher Firmicutes to Bacteroidetes ratio was detected in malignant compared to benign tumors, highlighting the potential role of the microbiota in the tumorigenic process [77]. Similarly, by comparing the microbiome of colorectal adenocarcinoma, adenoma and paired non-cancerous tissues (healthy tissues), Budgaard-Nielsen *et al.* demonstrated that the bacterial composition of cancerous tissues and adenoma overlapped with the microbiome of paired normal tissue and was enriched in *Fusobacterium nucleatum* [78]. The authors hypothesized that there was a potential role for the tumor microbiome in adenoma-carcinoma transition. *F. nucleatum* was detected in breast and pancreatic cancers [7,79] but was prevalently identified in colorectal cancer (CRC) patients [80]. Naturally encountered in several oral diseases (e.g. periodontitis [81] and gingivitis [82]), this Gram-negative obligate anaerobe was recently found in abundance, along with *Clostridium difficile*, in several cases of human colorectal adenomas and carcinomas [83]. Additional studies confirmed that *F. nucleatum* may, when associated with other Gram-negative species (e.g. *Streptococcus*, *Campylobacter* spp. and *Leptotrichia*) favor the development of CRC [67,84]. High prevalence of *F. nucleatum* in CRC was rapidly linked to a shorter survival time [85]. The role of *F. nucleatum* in CRC development relies on a series of tumor molecular events, including microsatellite instability, induction of specific genetic mutations (e.g. in the TP53 gene, leading to high production of the tumor protein P53) and acquisition of a CpG island methylator phenotype [85,86]. *In vitro* studies, using different human colon specimens, showed that *F. nucleatum* stimulates CRC tumor growth, by activating the host β -catenin signaling pathway and upregulating oncogenic gene expression through FadA adhesion virulence factor [87]. It is thought that the Gram-negative bacterium adheres to endothelial and epithelial cells via FadA, a unique virulent factor expressed on the surface of the bacteria (e.g. attachment of FadA to host E-cadherin receptors, activating the β -catenin signaling pathway) [88]. The defined mutation of FadA confirmed the relative

importance of surface-exposed adhesion in cellular invasion and placental colonization by *F. nucleatum*. Recently, Shao *et al.* demonstrated that *F. nucleatum* could promote invasion of oral squamous cell carcinoma cells by inducing a partial epithelial mesenchymal transition of cancer cells, revealing a new facet of this bacterium [89]. Identical effects on the host's cellular proliferative and pro-survival pathways were observed with *Bacteroides fragilis* metalloproteinase toxin (MP). Like *F. nucleatum* FadA, high release of MP on systemic *B. fragilis* infection can interact with the host's epithelial E-cadherin, which may disrupt intercellular junctions and activate β -catenin signaling [89]. This thus leads to enhanced cell proliferation, influencing the carcinogenic potential of affected host's cells. Similarly, *Salmonella enterica* effector avirulence protein A (AvrA) can translocate into host cells and upregulate the β -catenin pathway via its intrinsic de-ubiquitinase activity [90]. Similarly, other studies on pancreatic cancer suggest that the microbiome may also promote oncogenesis through the induction of an innate and adaptive immune response [91].

Likewise, several studies associated mycoplasma infections with increased risks of lung cancer infections [92]. Jiang *et al.* worked on the impact of mycoplasma species on the physiological developmental patterns of different subgroups of cell lines. By contaminating several cell lines (mesenchymal, epithelial and myeloid), the authors looked at the role of mycoplasma in the production of bone morphogenetic protein-2 (BMP-2) *in situ* [92]. BMP-2 plays a crucial role in maintaining metabolic homeostasis and physiological developmental patterns in cells. BMP-2 RNA production was significantly increased on systemic mycoplasma infection. Bacterial contamination even induced a strong expression of mature secreted BMP2 in immortalized human bronchial epithelial cells (BEAS-2B), which usually do not express BMP2. Mycoplasma infection also increased BMP2 production in specific A549 lung adenocarcinoma cells [92]. The authors associated the overall increase in BMP2 production with diverse post-transcriptional mechanisms (e.g. RNA stability). Later on, the enhanced BMP-2 production observed in BEAS-2B cells was proved to stimulate the proliferative potential of infected cells. When treated with Noggin (a BMP-2 antagonist), a lower cell number was detected compared to the untreated affected cells. Characterizing the impact of mycoplasma on the BMP2-regulated process is of great interest, as this might significantly influence the rate of tumor growth (e.g. alter cell proliferation, differentiation and apoptosis) [92]. *Chlamydia pneumoniae* is a Gram-negative bacillus responsible for more than 50% of respiratory infections measured in adults. For more than 20 years, several teams have tried to associate this obligate intracellular parasite with an increased risk of lung cancer. In 2011, a large meta-analytic study revealed the existence of a dose-response effect

in which a high risk of lung cancer was linked to an increased IgA antibody titer [93]. Risk of lung cancer was subsequently graded, based on the respective antibody titer. The higher the titer, the higher the risk of developing cancer [93]. Several studies associated *C. pneumoniae* with increased risk of lung cancers in young children and men, as well as former smokers [94–96].

Promptly categorized as cancer causative agents, improvements in analytic techniques have, since then, provided new information about the carcinogenic nature of bacteria. Live-pathogenic microbes can, under certain circumstances, confer cancer protection. Well-known examples of good anticancer agents remain gut metabolites, generally defined as being of bacterial origin. Short-chain fatty acids (SCFAs), such as butyrate or propionate, are both components obtained from the bacterial fermentation of our diet [97]. These small metabolites, produced by the commensal gut bacteria, have the ability to inhibit tumor cell histone deacetylases, efficiently regulating tumor progression [97]. Tumor surveillance is also increased in the presence of pyridoxine, another bacterially-derived metabolite, known to directly modulate the host immune system [98]. Regardless of their ability to induce or repress cancer progression, bacteria may also colonize tumors as simple opportunistic pathogens [99,100]. In harmony with the physiological characteristics of tumors, bacterial growth is supported at high levels in tumor-like tissues. The main model for bacterial occupation includes circulating bacteria, travelling through the blood stream, embedding into the leaky vasculature surrounding the tumor and rapidly colonizing the tissues [9] (Figure 1). In addition to tumor colonization by blood circulating bacteria, several studies proposed the existence of a bacterial gut axis, from which commensal gut bacteria could originate before colonizing tumors found in nearby organs [101]. The exact origin of the bacteria found outside the gastrointestinal tract still needs to be clarified. Several mechanisms may relate bacteria residing within tumors to the disease itself.

2.2 Metabolic specificities of the tumor microbiomes

For many years, people have tried to associate specific bacterial species with defined tumor subtypes. Characterizing a precise tumor microbiome would provide useful insights for the development of tumor-specific treatments [102]. The comparative analysis of several tumor types confirmed the existence of tumor-specific microbiomes. Each individual tumor comprises a unique microbiota, characterized by a specific metabolic profile [7]. The microbiome-derived metabolome is a new area under investigation that leads to the description of the metabolites produced in a defined biological system. It is developed by

using mass spectrometry-based approaches and can identify metabolic products of cancer-associated bacteria (e.g. production of SCFAs) [103]. Previous comparative studies described an enrichment of certain bacterial pathways in specific tumor subtypes [7]. In most cases, these pathways were combined with enhanced degradation of defined metabolites in the tissues concerned. For example, most bone tumors were shown to be composed of bacteria, expressing metabolic pathways, involved in the degradation of hydroxyproline [7]. Derived from the abundant levels of collagen in bone tissues, this hydroxylated amino acid is naturally found in bone-associated pathologies, such as bone cancers. The physiological presence of high hydroxyproline levels in bone cancers may explain the likelihood of hydroxyproline-degrading bacteria in their respective tumor microbiomes [104]. Similar associations were subsequently made between metabolites derived from cigarette smoke and the specific presence of defined bacterial species involved in their degradation inside the lungs [7]. Most bacteria making up the microbiomes of lung tumors displayed a significant increase in their ability to degrade chemicals such as toluene, aminobenzoates and acrylonitrile, all found abundantly in cigarette smoke [105]. Each individual microbiota seems capable of adapting to their surrounding microenvironment. High levels of metabolites inside tumors promote the creation of specific niches, supporting the survival of bacteria adequately skilled in using the metabolite present (e.g. hydroxyproline-degrading bacteria in bone cancer).

Recently, Poore *et al.* introduced microbiome-derived metabolome as a good cancer diagnostic approach (e.g. reliable predictive biomarker of response) [106]. Indeed, end-product metabolites released by bacterial metabolic activity (e.g. SCFAs) may influence the likelihood of patients to respond to different treatments. Metabolomic data can therefore help practitioners determining who is susceptible to require early immunotherapy [106]. Overall, studying tumor metabolome is of important interest as it contributes to supply quicker medical care, which is important to improve patient prognosis. Nomura *et al.* confirmed the role played by microbiome-derived metabolome in patient immunotherapeutic responses [107]. By assessing the serum/fecal metabolites present in 52 cancer patients, the authors showed that good responders displayed higher SCFA levels compared to patients suffering from early progressive diseases. Specific end-products, including high concentration of acetic, propionic, butyric and valeric acid, were associated with longer survival following immunotherapy [107]. Similar results were found by Botticelli *et al.*, who established the metabolomic profile of gut microbiome in 11 non-small cell lung cancer patients (NSCLC) treated with nivolumab immunotherapy [108]. They found that NSCLC patients with lower levels of SCFAs (e.g. acetic, propionic, butyric and valeric acid) had higher risks of early

disease progression after the start of treatment. This contrasted with long-term responders, who displayed high levels of SCFAs in their feces [108]. In contrast to both studies, Coutzac *et al.* associated high concentration of SCFAs with disease progression [109]. Reduced concentrations of butyrate and propionate were this time seen as biomarkers of longer progression free survival time. In addition to preliminary assessment of tumor-associated metabolome, metabolomic studies can also be useful during treatment to measure the levels of SCFAs present in the microbiome analyzed. In doing so, the metabolome becomes an important guide to immunological responses, allowing real-time assessment of the patient's response to treatment [110]. Theoretically, microbiome-derived metabolome opens several therapeutic possibilities. Metabolomic data seems easier to manipulate, compared to complex bacterial ecosystems. This may provide opportunities for manipulating the host immune responses to enhance the anti-carcinogenic effects of treatments (e.g. preliminary test of this approach with cases of autoimmune bowel disease) [110].

To confirm the nascent correlation between bacterial metabolic functions and their respective tumor microenvironment, a precise comparison of tissues derived from healthy individuals and cancer patients is required. The tumor-specific nature of cancer microbiomes can be illustrated at different levels. At the phylotypic level, each tumor comprises a different ratio of bacterial communities, belonging to specific bacterial phyla. For instance, colorectal tumors are principally composed of *Firmicutes* and *Fusobacteria* bacteria [111], whereas the microbiome of pancreatic cancers is predominantly made up of *Proteobacteria* [91].

By comparing primary tumors in colorectal cancer and paired metastases, Bullman *et al.* observed that the *Fusobacterium* species (*F. nucleatum* and *F. necrophorum*) were predominantly present in the intratumoral microbiome and they found a similar pattern in the paired-primary metastatic foci in the liver [112]. These data are in favor of a co-migration process for tumor cells and bacteria from the primary to the metastatic site. *Fusobacterium* co-occurred with other Gram-negative anaerobes such as *Bacteroidetes*, *Selenomonas*, and *Prevotella* species, in primary and matched metastatic tumors. Interestingly, there was little similarity between the microbiome in the primary cancer and liver metastases where *Fusobacterium* was present in the primary site but not detected in the metastases, or where the *Fusobacterium* load was low or absent in the primary cancer [112]. More recently, Kalaora *et al.* provided new evidence of the host immune system through intracellular bacteria [113]. The immune system became active against tumor cells through peptide presentation. These authors identified HLA bacterial peptides shared among multiple metastases from a single patient that suggest the presence of metastatic bacterial hitchhikers traveling with migrating

cancer cells. Interestingly, they observed dissimilar microbiomes in different metastatic sites. Overall, this work demonstrated the key impact of the intratumoral microbiome in both the tumor microenvironment and the tumor progression.

The disparity between microbiota making up tumors from different subtypes reinforces the idea of a tumor-specific profile for cancer microbiomes [7]. The unique nature of the individual tumor microbiome is also apparent at the species level. Any tumor subtype is characterized by a unique community of specific bacterial species (e.g. *Fusobacterium nucleatum* in colorectal tumors [80], breast and pancreatic tumors [7]). Likewise, the tumor microbiome can vary between subtypes of the same tumor type. Depending on the class of breast cancer (e.g. defined based on their estrogen, progesterone receptors and HER2 status), bacterial taxa are shown to significantly differ between tumors [114]. In most cases, high levels of *Proteobacteria* and *Firmicutes* are measured in mammary tumors and their enhanced proportion may rely on the higher amount of breast tissue fatty acids compared to other tissues [60,61]. Interestingly, Urbaniak *et al.* found differential breast microbial communities between Canadian and Irish women highlighting a potential ethnic specificity in the intratumoral microbiome [60]. This observation was confirmed more recently by Thyagarajan *et al.* [115]. In a 16s rRNA sequencing study carried out on both breast tumors and matched normal adjacent tumor tissues, the respective mammary tumor microbiome of black non-Hispanic (BNH) and white non-Hispanic (WNH) women revealed significant differences in the relative abundance of specific bacterial taxa [115]. This remained true at both phylum and genus levels. In fact, microbial diversity, as expressed by the Shannon index, was lower in BNH triple negative breast tumors compared to matched non tumoral tissue, while an inverse pattern was observed in the WNH cohort [115].

3. The therapeutic potential of intratumor bacteria

Characterizing the tumor microbiome is required for better understanding of the correlation between bacteria, cancer onset, and tumor progression. However, additional knowledge of tumor bacterial composition may also provide critical insight for the development of new therapies, with the ability to overcome conventional cancer treatments. To date, the main cancer therapies are based on surgery, radiotherapy and chemotherapy [116]. Although efficient against most defined tumors, all have several drawbacks, outweighing their respective qualities as anticancer agents. These methods rely on long, tedious procedures, acting non-specifically against tumors [116]. They are generally unable to distinguish malignant from healthy tissues. Instead, these therapeutic approaches act on a

broad spectrum, preferring rapidity rather than efficacy of treatment. The lack of specificity towards tumor-like regions allows certain cancer cells to survive and colonize nearby tissues, leading to potential cancer relapse [116]. Likewise, unexpected side effects can potentially emerge from the non-specific targeting of healthy tissues, inducing severe carcinogenic DNA damage. All these disadvantages, combined with the constant risk of developing resistance to treatment, are associated with an increased rate of cancer mortality and morbidity [116]. The development of new cancer therapies, capable of handling any given tumor with the same degree of efficacy, would revolutionize current cancer immunotherapies. Ideally, a perfect cancer therapeutic candidate should (i) be able to target any specific tumors, (ii) respond to external stimuli, (iii) sense its local environment, (iv) produce external signals to other cells, while (v) releasing adequate cytotoxic molecules, as well as (vi) being self-sufficient inside the host (e.g. freedom of movements) [102]. If appropriately engineered, live pathogenic bacteria have all the qualities needed to be good anticancer agents. Over the last ten years, cancer research has focused on developing new therapeutic approaches, based on the pathogenic nature of bacteria. With a relatively accessible genome, live-therapeutic bacteria offer a multitude of different possibilities for rapid metabolic engineering (e.g. sense chemotactic proteins and extrinsic molecular signals, express reporter and anticancer proteins) [102]. So far, more than ten pathogenic species have been tested as antitumor vectors, used to release specific cytotoxic drugs in the hypoxic regions of defined tumors [117].

3.1. Intratumoral microbiome as biomarkers in cancer

As bacteria are part of the tumor microenvironment and can be affected by both the carcinogenesis process and exogenous therapies (e.g. conventional chemotherapy, immunotherapy), the intratumoral microbiome provides an opportunity to benefit from a new prognostic/diagnostic tool [118]. The specific composition of this microbiome with prevalent bacteria according to the host sites could be a snapshot of the tumor's properties at a given time [7,106]. Poore *et al.* have recently re-examined whole-genome and whole-transcriptome sequencing studies of various types of cancer from treatment-naive patients for microbial reads. From 18,116 samples analyzed, the authors found unique microbial signatures in most types of cancer. Overall, the works of Nejman *et al.* [7] and Poore *et al.* [106] highlight distinct tumor microbiotes among more than 30 cancer types and blood, and proposed the potential use of the intratumoral microbiome as a blood-based diagnostic tool. Gnanasekar *et al.* showed that specific intratumoral microbes were associated with thyroid carcinoma and correlated with higher mutation expression and methylation of tumor suppressors [119].

Frankia sp. and uncultured *Gammaproteobacteria* bacterium were then abundant in all papillary thyroid carcinomas analyzed and intratumoral microbe dysbiosis may be related to tumor progression and pathogenesis. Correlations between intratumor bacteria and the response to immune checkpoint inhibitors was also noted in melanoma [7]. In esophageal squamous cell carcinoma, an intratumoral microbiome enriched in *F. nucleatum* may have prognostic significance for predicting poor recurrence-free survival [120]. These studies highlight the potential use of intratumoral microbiomes as prognostic biomarkers.

3.2. Impact on the host immune system

Following the first characterization of commensal bacteria in the human body, the impact of bacteria on the host immune system has been widely studied, especially in cancer immunotherapeutic research [5,6]. Although little is known about the role played by commensal bacteria in the host immune system, these prokaryotic organisms can influence, either positively or negatively, host immune responses. As shown in previous studies, gut microbiota are key elements in the monitoring of host responses to treatments [58,121,122]. Depending on the bacterial communities found inside the gut, responses to certain therapies can vary significantly from one individual to another. In addition, in the case of generic dysbiosis, the gut microbiome can also be associated with microbially-driven carcinogenesis. Broader understanding of the correlation between defined tissue-microbiomes and alterations in host immune responses would favor the development of new, personalized medicine, enhancing patient prognosis [9]. Once determined, the composition of the human microbiome can be easily manipulated for the patient's own benefit (e.g. enhancement of anticancer bacterial species over pro-pathogenic species) [123,124]. So far, most research carried out on the impact of bacteria on the host immune response to cancer therapy was carried out in patients with pancreatic cancer. Pushalkar *et al.* demonstrated, using pancreatic ductal adenocarcinoma (PDA) mouse models, that certain bacteria found inside pancreatic tumors were likely to suppress host innate and adaptive immune responses, promoting cancer onset [91]. More specifically, the authors showed that *Bifidobacterium pseudolongum* was differentially abundant in the pancreatic tumor microbiome compared to that found in the gastric digestive system. Similarly, an increase in bacterial diversity was measured in the microbiome of both human and mice PDA when contrasted with that found in healthy pancreas (e.g. higher levels of *B. pseudolongum* in PDA mice) [91]. The increased activation of Pathogen-recognition receptors has long been known to accelerate oncogenesis, by inducing innate and adaptive immune suppression [125–127]. Based on this statement,

Pushalkar *et al.* proposed that the enhanced immune tolerance observed in PDA mice was caused by peritumoral immune suppression induced by the presence of a specific microbiome [91]. The PDA microbiome would then be responsible for higher TLR expression on the surface of nearby macrophages. The enhanced TLR signaling subsequently programs tumor-associated macrophages (TAM) to increase the overall immune tolerance of the tumor (e.g. higher expression of TAM- M2, upregulation of tolerogenic cytokines such as IL-10). By performing microbial ablation, Pushalkar *et al.* showed that a reduced pancreatic microbiota can lead to rapid immunogenic reprogramming of mouse models [91]. In the presence of a reduced PDA microbiome, mice displayed higher levels of intratumoral T cells, as well as lower levels of myeloid-derived suppressor cells. Likewise, ablated PDA tumors appeared rich in TAM M1 macrophages instead of TAM M2, inducing increased expression of MHC-II (e.g. Th1 differentiation of CD4⁺ T cells and CD8⁺ T cell activation) and pro-inflammatory cytokines around the tumor (e.g. TNF- α and IL-6) [91]. The reduction in pancreatic microbiome diversity ultimately slowed down cancer progression, confirming the role of the PDA microbiome in PDA tumor development. In addition, bacterial ablation was also shown to increase PAD mice's responsiveness to checkpoint-targeted immunotherapy, by upregulating programmed cell death 1 protein (PD-1) expression [91].

By sequencing the 16S ribosomal RNA, Sivan *et al.* identified *Bifidobacterium* as being associated with antitumor effects, which is contrary to the data found in the previous study, where high levels of *Bifidobacterium pseudolongum* were responsible for an increase in pancreatic oncogenesis [128]. In addition, oral administration of *Bifidobacterium* alone improved tumor control to the same degree as PD- ligand 1-specific antibody therapy, and combination treatment almost abolished tumor outgrowth. These results show that different species may have diverging effects in the tumor microenvironment [128]. Similarly, Riquelme *et al.* supported the presence of a distinct microbial profile within tumors of long pancreatic ductal adenocarcinoma survival patients, compared to tumors isolated from patients diagnosed with a reduced survival rate [129]. The latter paper claims that the existence of a specific pancreatic microbiome found in long-term survivors promotes cancer regression, as well as significantly increasing the chance of remission. The tumor microenvironment of long-term survivors exhibited high quantity and quality of neoantigens, combined with an enhanced ratio of active CD8⁺ T cells [130]. By inducing robust effects on tumor growth and tumor immune infiltration, modulation of the tumor microbiome can have a significant influence on the host immune response.

3.3. Intratumor bacteria identified as efficient *in vivo* bio-transformers

Bacteria have, for a long time, been known for their ability to transform organic matter (e.g. organic chemicals), using specific enzymatic activity. Recently, numerous species have been associated with specific alterations observed in the microenvironment of distinct solid tumors. These have been shown to influence, both negatively and positively, the use of certain chemotherapeutic approaches [121].

Intratumor *Escherichia coli* remains one of the best examples of bio-transformers known so far. This bacterium negatively impacts the use of gemcitabine, a cytotoxic antimetabolite, commonly employed in chemotherapy [131]. Mostly delivered intravenously, this drug targets cancer cells undergoing DNA synthesis. Gemcitabine specifically inhibits ribonucleotide reductase, an enzyme important in the process of DNA replication *in vivo* [132]. The negative influence of intratumor *E. coli* was previously shown in several pre-clinical trials [131]. Using bacteria (e.g. *E. coli* and *Listeria welshimeri*) and different cancer cell lines (e.g. Lewis lung, mammary and colorectal carcinomas), Lehouritis *et al.* carried out an in-depth analysis of the effects of the tumor microbiome on the cell's killing capacity, mediated by several chemotherapeutic agents (e.g. gemcitabine and CB₁₉₅₄). Tumor microbiota from both healthy and affected cancerous mice were compared to draw possible conclusions, regarding bacterial influence on cancer resistance [131]. Out of 30 chemicals tested *in vitro*, the efficacy of 10 was reduced by the presence of defined bacterial species (e.g. gemcitabine in the presence of *E. coli*). The same bacteria positively influenced 6 other chemicals, by drastically increasing their efficacy with regard to cancer cells (e.g. AQ4N in the presence of *E. coli*). Complementary *in vitro* assays, including high performance liquid chromatography and mass spectrometry, helped characterize the reasons behind the different chemoresistant and cytotoxicity patterns previously observed. The authors showed that bacterial exposure was responsible for inducing changes in drug chemical structures, known as biotransformation [131]. All drugs tested presented new chromatogram peaks, in the presence of bacteria, illustrating a change in their physiological biochemical structure (e.g. acetylation of gemcitabine). All *in vitro* observations made above were then confirmed in *in vivo* subcutaneous tumor models. Lehouritis *et al.* replicated the same effects on drug efficacy, when tested on different mouse models. Most resistant animals displayed an enhanced level of intratumor Gammaproteobacteria (e.g. *Escherichia coli*) in the colon tumor microbiome [131]. Species found in resistant mouse models were found to express the bacterial enzyme cytidine deaminase, which has a primary function of catalyzing the degradation of gemcitabine. Overall, findings suggest that, on systemic or local infection,

bacteria (e.g. *E. coli* sp found in the colon cancer microbiome) can alter tumor responses to established chemotherapeutic treatments [131]. Similar results were found by Geller *et al.*, who confirmed the likelihood of Gammaproteobacteria mediating tumor resistance to the chemotherapeutic drug gemcitabine. Using colon cancer mouse models, the authors found 86 tumors out of 113 (76%) positive for bacteria, mainly Gammaproteobacteria [133].

Considering the influence of bacteria on host responses to chemotherapy, improved therapeutic approaches can be proposed to favor the regulation of the onset of cancer. By characterizing the composition of each respective tumor microbiota before treatment, it is possible to remove, through successive antibiotic exposure, all bacteria with the ability to repress chemotherapeutic effects (e.g. Gammaproteobacteria in colon cancer) [123,124]. Likewise, bacteria, which enhance chemotherapeutic outcomes, can inversely be added to the tumor microenvironment to improve the host response to treatment. It is important to consider all possibilities before using antibiotics to manually alter the microbiome's composition. Probiotics have been suggested as an alternative, to positively balance the diversity of any given microbiota. Despite the fact that they may prevent the unnecessary use of antibiotics, recent findings have refuted their efficacy in cancer patients treated with chemotherapy (e.g. treatment with immune checkpoint blockade). Probiotics have effectively been shown to decrease the microbial diversity of patient tumor microbiomes, impacting their ability to respond efficiently to treatment. A more promising approach would be to use fecal microbiota transplantation (FMT) from healthy donors. This strategy has shown its efficacy in various diseases, including *Clostridioides difficile* infections [134], inflammatory bowel disease [135], as well as graft-versus-host disease [136].

Based on the local administration of fecal matter from a healthy donor into the gastrointestinal tract of a recipient, FMT is an efficient method used to directly modify the recipient's microbial composition [137]. It helps the recipient reacquire a stable microbiome. This strategy could be applied in cancer patients, but the lack of understanding of the active ingredients and potential risks of such therapies raises questions. Strategies based on defined microbial components will probably enhance the potential of this therapy but we still need to identify the effector microbes directly associated with a given phenotype [138].

3.4. Engineered therapeutic bacteria – the future of bacterial cancer therapy

Regarding the enhanced likelihood of bacteria surviving within tumors, bacterial engineering offers the possibility of using bacteria to produce local therapeutics in defined regions of the body. It also provides the opportunity to increase the efficacy of a given drug by local

transformation [139–141]. By specifically modifying live pathogenic bacteria, it is possible to develop unique anticancer vectors, capable of curing any tumor-like tissues in a personalized manner [102]. In addition, active regulation of tumor microbiomes can help reduce unexpected effects of already-used therapies. Overall, the development of engineered microorganisms would revolutionize current cancer bacterial therapies, by further increasing their therapeutic and diagnostic functions. To demonstrate the application of bacterial engineering as a tool for reducing cancer progression, Murphy *et al.* introduced a model of an engineered non-pathogenic *E. coli* MG1655 strain, capable of releasing TNF- α in the locality of the tumor [140]. As a highly potent anticancer therapeutic, TNF- α cytotoxic functions must imperatively be constrained within the tumor to prevent the emergence of serious side effects. In essence, the *E. coli* TNF α -producing construct was injected into mouse models via either intratumoral or intravenous administration. *In vivo* bioluminescence imaging studies, followed by *ex vivo* immunofluorescent analyses, supported the long-life survival and high proliferation ratio of the constructs in the various murine tumor models tested (e.g. CT26 colon, RENCA renal, and TRAMP prostate) [140]. High levels of TNF- α were subsequently measured in treated mice tumors compared to controls (e.g. proved by ELISA of tumor extracts).

Likewise, Zhen *et al.* proposed a two-step enhanced cancer immunotherapeutic approach, using an engineered *Salmonella typhimurium* strain, capable of secreting *Vibrio vulnificus* flagellin B (FlaB) into tumor tissues [142]. The engineered bacteria effectively suppressed tumor growth and metastases in mouse models, by over-activating TLR receptors present at the surface of the cancer cells. Longer survival was also recorded. By using Toll-like receptor 5 (TLR5)-negative colon cancer cell lines, the authors showed that this immunotherapeutic effect was in fact caused by a TLR5-mediated host reaction in the tumor microenvironment [142]. Following exposure to different murine models, all therapeutic effects were entirely absent in TLR4 and MyD88 knockout mice but remained partly conserved in TLR5 knockout mice. This confirmed that TLR4 signaling is crucial for maintaining tumor suppressor functions by FlaB-secreting bacteria. On the contrary, TLR5 only seems responsible for increasing the tumor-suppressive host reaction [142]. The progressive degradation of tumors was here explained by increased inflation of host immune cells, on delivery of engineered bacteria into the tumor microenvironment. The presence of an attenuated modified *S. typhi* strain induced abundant recruitment of monocytes, macrophages and neutrophils via TLR4 signaling from cancer cells. FlaB secretion from *S. typhi* bacteria led to changes in the

intratumoral macrophage phenotypes, with high levels of M1-like macrophages observed in treated mice. Overall, this paper demonstrates the use of non-virulent tumor targeting bacteria as cancer immunotherapy [142]. Abundant release of TLR ligands appeared beneficial for progressively reducing tumor size and increasing survival in mouse models.

Complementary to the studies focusing directly on tumors, Byrne *et al.* looked at the feasibility of engineering bacteria-mediated DNA constructs for drug delivery to tumor-associated phagocytic cells [143]. Host immune cells, including macrophages, dendritic cells, and neutrophils, are known to play a negative role in many diseases, such as cancer. For instance, macrophages exhibit a strong pathophysiological role in several pathologies and are often targeted by diverse therapeutics. Knowing that non-pathogenic bacteria cannot enter host cells, except for phagocytes, the authors used that specificity to engineer a non-invasive *E. coli* strain, capable of passively transfecting specific genomic content inside tumor-associated phagocytic cells [143]. Using an *in vitro* differentiated human monocyte cell line, as well as two *in vivo* mouse models (an ovarian cancer ascites and a solid colon tumor model), Byrne *et al.* efficiently delivered transgene-loaded bacteria to tumor regions. Interestingly, a higher proportion of phagocytic cells was brought to the tumor, following transfection of the engineered bacterial construct. Positive delivery was thus proved, by measuring the levels of bacteria-carrying reporter constructs [143].

In addition to being useful delivery vectors, certain therapeutic bacteria can be engineered in such a way that they become active sensors of their environment. In doing so, they actively act in accordance with the current status of the patient [102]. Live-pathogenic bacteria are preponderantly found in environments harboring disease. They provide natural platforms for the development of “live-acting” engineered therapies. [144] In 2016, Din *et al.* engineered a specific bacteriophage strain programmed to lyse synchronously at a threshold population density and to release genetically encoded cargo. After quorum sensing (= regulation of gene expression in response to fluctuation in cell population density), the engineered species could automatically reseed the growing population when the latter reached a certain level, leading to continuous pulsatile delivery cycles [144]. The authors confirmed the potential of this engineered strain as a drug delivery carrier via co-cultures with human cancer cells (e.g. HeLa cells) *in vivo*. The strain was administered with either motile or non-motile *S. typhimurium*, SL1344. The engineered bacterial constructs were administered orally, either alone or in combination with a chemotherapeutic drug, to a syngeneic transplantation model of hepatic colorectal metastases. This combination led to a notable reduction in tumor activity, with a marked survival benefit [144].

3.5. Virus: part of the intratumoral microbiome and therapeutic value

The therapeutic outcomes of engineered bacteria can be improved by combining multiple microorganisms [145]. Interestingly, both oncolytic viruses (OVs) and bacteria replicate specifically inside tumors, after systematic administration [146]. Concerning non-pathogenic bacteria, tumor selectivity relies on the likelihood of these species growing extracellularly within tumor stroma, which ultimately constrains, if produced, bacterial-derived therapeutic agents to the tumors. On the other hand, OVs' tumor-selective replication relates to the primary functions of these viruses. Vesicular stomatitis viruses (VSV) are known for their strong interferon antagonist functions. On infection, they release a matrix M protein inside the host, which prevents interferon production. The M protein blocks the transport of interferon mRNA from the nucleus of affected cells [147]. Knowing that most cancer cells release enhanced levels of INF- α on development, the likelihood of finding colonizing VSV inside tumors is relatively high. Overall, VSV viruses exhibit a broad cancer cell tropism and can efficiently target tumors, when delivered intravenously in mouse models. They emerge as promising oncolytic virotherapies to slow down tumor growth and prevent carcinogenesis [146,148] Although neurotoxic in its wild-type forms, attenuated versions of the virus (e.g. VSV Δ 51) have shown a capacity for retaining all oncolytic functions, while remaining harmless against the treated host [149]. Promising at first, VSV viral therapy rapidly revealed certain limitations. Tumors are composed of cells, which generally exhibit different levels of antiviral responses. Unpredictable intra/inter tumor heterogeneity, in terms of treatment efficacy, may lead to incomplete oncolysis with VSV therapy (e.g. low VSV Δ 51 replication in HT29 colon carcinoma [146]. Cronin *et al.* demonstrated the capacity of the type I interferon antagonist B18R to increase the viral replication and survival of vesicular stomatitis virus (VSV) inside tumors [145]. Derived from *Vaccinia virus*, B18R is a gene encoding a secreted decoy receptor with high antagonist effects against type 1 interferons. The localized expression of this specific gene was shown to increase the efficacy of attenuated VSV Δ 51 for growing and killing tumors [150,151]. The non-pathogenic *E. coli* strain, expressing the type 1 interferon antagonist B18R facilitated tumor-specific production of B18R, resulting in a microenvironment depleted of bioactive antiviral cytokine, thus conditioning the tumor to enhance subsequent tumor destruction by means of an oncolytic virus. Both *in vitro* and *in vivo*, infection by VSV Δ 51, an attenuated version of the vesicular stomatitis virus (VSV), was greatly enhanced by B18R produced from *E. coli*. A significant

increase in therapeutic efficacy resulted from intravenous injection of bacteria to tumor-bearing mice 5 days prior to VSV Δ 51 administration. This resulted in a significant reduction in tumor growth and increased survival in mice [145]. Characterizing the tumor microbiome would prevent any unnecessary genetic modifications made to these bacteria. By defining the most prevalent species in tumors beforehand, metabolic engineering studies can focus on these organisms, while leaving the other bacterial subtypes out of the equation. Preliminary selection of bacterial species reduces risks of genetic misconception, while increasing the rapidity of metabolic development [102]. Although promising, the field of engineered bacterial therapy is only in its very early stages. Issues regarding the overall safety of the approach, and the feasibility of using such bacteria in any individual, are still under investigation.

Conclusion

Despite increasing interests from the scientific community about the tumor microbiome, the mechanisms by which bacteria colonize tumors are only just starting to be characterized. In order to better understand the strategies used by circulating bacteria to get into tumors and rapidly adapt to their respective microenvironments, the development of new analytic tools have been proposed (e.g. shotgun-based approaches, metabolome, DADA2 taxonomy classifier), providing greater microbial profiling of cancer. To date, each tumor is thought to comprise a unique microbiota, made up of specific bacterial species. Intratumor bacteria have distinctive metabolic profiles, in harmony with the metabolites present in the associated tumor even if a relative overlap between primary tumors and normal tissues was observed. Since the first clinical trial proposing bacteria as therapeutic agents, the intratumoral microbiome has paved the way for therapeutic opportunities in addition to their use as biomarkers. After appropriate metabolic engineering, live-pathogenic bacteria may be well-suited as anticancer agents, acting as efficient intracellular anticancer vectors. Clinical trials are essential for confirming the therapeutic potential of the bacterial species identified in pre-clinical animal models. In-depth characterization of the different tumor microbiomes may make it possible, in the foreseeable future, to manipulate these bacterial communities for the benefits of patients. Live-therapeutic bacteria have all the tools necessary for rapidly providing outstanding personalized medicine. Although important, tumor microbiome not only comprises bacteria, but is also rich in all sorts of viruses. Additional studies on the tumor viral microbiota would potentially open new doors for promising anti-cancer therapeutics.

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Figure Legends

Figure 1. Model for bacterial invasion of tumor tissue. Based on the intrinsic properties of tumor tissues, tumor foci may be a perfect permissive environment for supporting bacterial invasion, survival, and growth. (1) Tumor masses are frequently a hypoxic environment in which restricted levels of oxygen favor the growth of specific bacterial species (e.g. facultative and strict anaerobic bacteria); (2) Necrotic tissue is generally full of nutrients (e.g. purines), benefiting both the survival of bacteria and rapid cell proliferation. Likewise, several chemoattractant signals emerge from tumor necrotic regions and facilitate the extravasation of bacteria; (3) As tumors develop, new blood vessels emerge and surround the growing tissues. This angiogenic process leads to the formation of a leaky vasculature around tumor cells, subsequently used by circulating bacteria to enter the tissues. Bacteria can proliferate in the extracellular or intracellular compartments; (4) Generally surrounded by immune cells that preventing the tumor necrotic regions from expanding, tumors are protected from host immune surveillance. Colonizing bacteria take advantage of the immune privileged status of tumors to proliferate at ease, away from any disruption by the host immune system.

Figure 2. Characterizing the solid tumor microbiome. Whether or not healthy tissues were initially considered to be deprived of microbiota, intracellular bacteria were recently observed in solid tumors. Identifying the tumor microbiome opens new opportunities in the field of cancer research. Better characterization of the intratumoral microbiome may lead to the

development of new therapeutic approaches, overcoming conventional cancer treatments. Next generation sequencing methods, including 16S amplicon sequencing, make it possible, after tissue extraction and fixation in paraffin, to precisely cluster intratumor bacteria in defined bacterial subgroups. Bacterial DNA in FFPE samples is generally extremely limited, due to its reduced biomass compared to host DNA. To assure the proper isolation of bacterial genomic content, it is recommended to add as many negative controls as possible.

Figure 3. Detecting intratumoral Gram-negative bacteria in FFPE breast cancers. The Gram-negative bacterial surface is characterized by a thin peptidoglycan surrounded by a thick outer membrane, whereas Gram-positive displays a thick peptidoglycan on top of a thin inner membrane. Both cell walls are composed of different associated components, lying all over their apparent membrane. Gram-negative species have high levels of lipopolysaccharides (LPS) bound to their outer membrane which are crucial for interacting with neighboring bacteria and their microenvironment. The presence of Gram-negative bacteria in breast tumor formalin-fixed paraffin-embedded tissue can be assessed with immunohistochemistry (IHC), using an anti-LPS primary antibody. After antigen retrieval, FFPE sections were incubated with anti-Lipopolysaccharide binding protein primary antibody (1/100 dilution – BioLegend #863801) for 1h in a humidified chamber. HRP coloration was then performed, followed by hematoxylin counterstaining. Original magnification: X100 (insert: X200).

Figure 4. *Helicobacter pylori*, a classic example of gastric colonizing bacterium. *H. pylori*, also known as *Campylobacter pyloridis*, is a Gram-negative bacterium, abundantly found in human gastric mucus. *H. pylori* bacteria are generally 2.5-5.0 μm long, 0.5-1.0 μm wide and possess one to six unipolar-sheathed flagella. Prevalent in developing countries, where lack of hygiene combined with high population levels favor bacterial spread, *H. pylori* enters the body through the mouth, travels through the gastrointestinal tract and colonizes the human stomach. Thanks to their numerous flagella, *H. pylori* bacteria easily pass through the gastric epithelial layers. By secreting urease, a strong enzymatic catalyzer, these spiral-shaped microaerophilic bacteria can survive within the gastric mucus. Infections by *H. pylori* induce several changes in the homeostatic profile of the gastric epithelial layers (e.g. alteration of epithelial cell junctions, recruitment of pro-inflammatory immune cells, etc.). To do so, the bacterium expresses multiple virulence factors, important for maintaining bacterial survival (e.g. release of urease), enhancing bacterial colonization (e.g. flagella), and favorizing bacterial adhesion to gastric epithelial cells (e.g. bacterial adhesins). The pathogenic nature of

H. pylori also relies on the specific expression of the oncoprotein cagA, subsequently released off the bacterial cell wall into the gastric mucus via a Type IV secretion system. CagA induces strong conformational changes on the gastric mucosal surface (e.g. generic disruption of the membrane's intercellular junctions, loss of cell polarity). Similarly, the bacterial toxin vacA, expressed by most *H. pylori* strains, regulates expression of a variety of different cell types, including epithelial cells, phagocytic cells, and lymphocytes. It stimulates specific cellular signaling pathways, reduces mitochondrial membrane permeability, as well as altering final endocytic compartmentalization.

Table 1: Recent studies on the microbiota associated to the most common cancer types

Oncologic entities	Microbiota Detection and OTU picking method	Sampling material, size and site	Main Findings	References
<p>Breast cancer</p>	<p>Real-time qPCR and pyrosequencing</p>	<p>Tumor tissues from 20 patients with estrogen receptor-(ER) positive breast cancer compared with associated paired control samples.</p>	<p>-Enhanced ratio Proteobacteria in breast cancer tissues. -High levels of <i>Methylobacterium radiotolerans</i> in breast cancer samples. -High levels of <i>Shingomonas yanoikuyae</i> in paired normal tissues. -Commensal skin bacteria (e.g. <i>Staphylococcus</i>, <i>Corynebacterium</i>, etc.) found in equal quantity in both paired normal and tumor tissues.</p>	<p>[59]</p>
	<p>Ion Torrent V6 16S rRNA sequencing</p>	<p>81 Breast tissue samples from Canadian and Irish women with or without cancer.</p>	<p>- Higher bacterial richness observed in tumor tissues, despite the reduction in bacterial load in advanced stages of breast tumors.</p>	<p>[60]</p>
	<p>16S V6 rRNA gene sequencing. OTUs were binned with FASTTREE based on an OTU sequence alignment made with MUSLCE.</p>	<p>Fresh breast tissues collected from 71 women undergoing breast surgery (58 underwent lumpectomies or mastectomies for being/malignant tumors, 23 were free of disease and underwent surgery for either breast reduction or enhancement)</p>	<p>-Different bacterial communities, according to the tissue collection site. -Reduced expression of antibacterial response genes in tumor samples. -Enhanced ratio Proteobacteria in breast cancer tissues. -Different bacterial profile identified in breast tissues and associated normal paired tissues. -High levels of <i>Bacillus</i>, <i>Enterobacteriaceae</i> <i>Staphylococcus</i>, <i>Comamondaceae</i> <i>Bacteroidetes</i> in breast cancer samples. -Enhanced levels of <i>Prevotella</i>, <i>Lactococcus</i>, <i>Streptococcus</i>, <i>Corynebacterium</i> and <i>Micrococcus</i> in healthy non-cancerous samples.</p>	<p>[61]</p>
<p>Ovarian cancer</p>	<p>Quantitative PCR followed by 16S rRNA high-throughput sequencing. OTUs were binned using a Mothur software package.</p>	<p>25 ovarian cancer tissues compared to 25 normal distal fallopian tube tissues.</p>	<p>-Enhanced ratio of <i>Proteobacteria</i> and <i>Firmicutes</i> in ovarian cancer samples. -High levels of <i>Actinobacteria</i>, <i>Sphingomonas</i> and <i>Methylobacterium</i> in cancer tissues -High levels of <i>Lactococcus</i> and <i>Methylobacterium</i> in healthy controls. -Decreased microbial diversity and reduced bacterial</p>	<p>[62]</p>

	Pan-Pathogen array using a PathoChip screening design. Next-generation Illumina sequencing	99 ovarian cancer samples compared with 20 matched paired healthy tissues and 20 unmatched control samples.	richness in ovarian cancer tissues. -High ratio of Proteobacteria and Firmicutes observed in ovarian tumor microbiome. -Enhanced levels of <i>Brucella</i> , <i>Chlamydia</i> and <i>Mycoplasma</i> detected in ovarian tumor samples.	[63]
Prostate cancer	Organism-specific PCR combined with 16S sequencing.	170 prostate tissue core samples isolated from 30 different patients.	-Identification of 83 distinct bacterial species, following PCR amplification of prostate tumor samples.	[64]
	PCR and microarray analysis performed as dual-color hybridization.	12 intra-epithelial prostatic neoplasia, 59 prostate adenocarcinoma and 9 Benign hyperplasia.	-High levels of <i>Propionibacterium acnes</i> (e.g. found in > 80% of tumor samples) in the microbiome of prostate cancer patients.	[65]
	Ultradeep pyrosequencing	Prostate specimens from 16 White Caucasian, nondiabetic, nonobese prostatic cancer patients.	-Variation of prostate cancer microbiome (e.g. different bacterial community, with different pathophysiology), according to the nature of the analyzed tissue. - Differences in microbial populations among tumor, peri-tumor and nontumor prostate samples. - High levels of <i>Propionibacterium</i> spp. in prostate cancer samples. - Reduced levels of Lactobacillales and <i>Streptococcaceae</i> in control samples compared to tumor/peri-tumor tissues. - High levels of Actinobacteria, Firmicutes and Proteobacteria observed in prostatic tumor tissues.	[66]
Colorectal cancer	Illumina sequencing	65 tumor samples, compared to 65 paired control tissues.	-High levels of bacteria belonging to the oral microbiome, in colorectal tumor tissues. -Over-representation of <i>Fusobacterium</i> , <i>Leptotrichia</i> and <i>Campylobacter</i> species in colorectal tumor samples.	[67]
	16S rRNA amplicon sequencing and Reverse-transcription real-time qPCR	Fecal and mucosal samples from 59 patients undergoing surgery for colorectal cancer, 21 individuals with polyps and 56 healthy controls.	- High contrast between colorectal cancer microbiome and healthy control microbiome. - Variation between proximal and distal colorectal cancer microbiomes. - Colorectal cancer tissues have a microbial signature similar to surrounding tissues. - High levels of <i>Bacteroides</i> , <i>Roseburia</i> , <i>Oscillobacter</i> , as well as oral pathogens (e.g. <i>Fusobacterium</i> spp.) observed in	[68]

Pancreatic cancer	PCR sequencing and MiSeq sequencing. OTUs binned using UPARSE pipeline.	Stool samples from 167 patients were obtained. Out of 167, 85 were derived from pancreatic cancer. Those were compared with 57 matched healthy controls.	colorectal cancer patients. -Abundant levels of <i>Bacteroidetes</i> , <i>Firmicutes</i> and <i>Proteobacteria</i> in stools from both pancreatic cancer and healthy control patients. - Higher ratio of Bacteroidetes in pancreatic cancer samples. - Higher ratio of Firmicutes and Proteobacteria in control stool samples. -Unique microbial profile observed in stools from pancreatic cancer (<i>Prevotella</i> , <i>Veillonella</i> , <i>Klebsiella</i> , <i>Selenomonas</i> , <i>Enterobacter</i> ...).	[69]
	16S rRNA amplicon sequencing. OTUs binned using UPARSE pipeline.	Stool samples from 30 patients with pancreatic adenocarcinoma, 6 patients with pre-cancerous lesions, 13 healthy subjects (control 1) and 16 patients with non-alcoholic fatty liver disease (control 2).	-Under-representation of bacterial, families and genera belonging to the phylum of Firmicutes in stools derived from pancreatic cancer patients. -Existence of a specific pancreatic cancer-associated gut microbiome signature (e.g. <i>Veillonellaceae</i> , <i>Akkermansia</i> and <i>Odoribacter</i>).	[70]
Lung cancer	Quantitative PCR, followed by Illumina sequencing	Saliva samples from 20 patients with lung cancer, including 10 squamous cell carcinoma and 10 lung adenocarcinomas. Results compared to 10 samples obtained from control patients	-High levels of salivary <i>Capnocytophaga</i> , <i>Selenomonas</i> , <i>Veillonella</i> and <i>Neisseria</i> measured in saliva samples from lung cancer patients. -Abundant levels of <i>Capnocytophaga</i> and <i>Veillonella</i> in saliva from lung cancer patient (Possibility to use these species as biomarkers for improved disease detection). -Association between the oral microbiome, severe respiratory conditions and the onset of lung cancer.	[71]
	16S rRNA amplicon sequencing, following by Illumina sequencing	40 Bronchial washing fluid and 52 sputum samples from newly diagnosed lung cancer patients.	-High variability of the airway microbiome in lung cancer. -Different microbial profile, according to the clinical status of the disease. -Variation of the microbiome, according to the metastatic status of lung cancers. -High ratio of Proteobacteria measured in Bronchial washing fluid samples	[72]

Table 2. Microbial profiling of FFPE samples: advantages and drawbacks

Step of microbial profiling	Advantages	Disadvantages
Sample collection (e.g. Direct tissue excision)	<ul style="list-style-type: none"> -Efficient localized extraction process. -Rapid fixation and embedding of sample in FFPE blocks, assuring the preservation of the extracted tumor tissue. 	<ul style="list-style-type: none"> -High risk of environmental contamination. Possible sources: <ul style="list-style-type: none"> -From other sites of the host body. -From the surgery room, while handling different objects. -During the paraffin-embedding of sample. -From the laboratory, involved in the analysis of the final data.
Sample preparation	<p>FFPE samples:</p> <ul style="list-style-type: none"> -Valuable resource for cancer microbiome research. -Natural preservation of the tissue morphology, cellular details, as well as all the DNA contained within the sample. -Stable at room temperature. <p>Fresh samples:</p> <ul style="list-style-type: none"> -Lower risk of tissue alteration, compared to FFPE samples. -Exact representation of the tumor tissues observed in the patient, providing highly reliable results 	<p>FFPE samples:</p> <ul style="list-style-type: none"> -Formalin fixation induces DNA cross-linking of histone-like protein to DNA present in the sample. - Fixing process is often associated with severe DNA degradation, as well as unexpected mutation of the initial genomic content observed in FFPE samples. -Formalin fixing and paraffin embedding are both non-sterile processes. <p>Fresh samples:</p> <ul style="list-style-type: none"> -Shorter preservation time.
Sample visualization (e.g. Immunohistochemistry)	<ul style="list-style-type: none"> - Immunohistochemistry labelling provides an interesting insight on the bacteria present in a given sample. -Possibility to test multiple different antibodies, staining distinct target epitopes. 	<ul style="list-style-type: none"> -Labelling can vary from one sample to another. -Preliminary antigen retrieval, antibody dilution, as well as time of incubation can significantly influence the obtained results.
DNA extraction	<ul style="list-style-type: none"> - Variety of different DNA extraction protocols available. - Rapid process, providing high levels of exploitable DNA for PCR amplification and further sequencing analysis. -Microbial enrichment, by establishing an appropriate host DNA depletion strategy. 	<ul style="list-style-type: none"> -Method of DNA extraction should be optimized, according to the sample of interest. -Risk of contamination during extraction from external contaminants or reagents used during the process itself. -DNA purity ratio might be lower than expected, when DNA is extracted from FFPE samples (e.g. dewaxing difficulty). - In the absence of a DNA repair process (used to repair fixation-associated DNA damages), it is difficult to produce reproducible results. -DNA repair process must be carefully monitored to prevent the emergence of contamination.

<p>DNA amplification and Sequencing library preparation</p>	<ul style="list-style-type: none"> -Prevalent presence of 16S rRNA gene in all bacterial species, offering the possibility to instore an effective host DNA depletion strategy, prior 16S sequencing. -Amplification of defined read-outs from characterized bacterial species, following the use of appropriate primers. -Direct assessment of bacterial DNA amplification, using labelled enzymatic marker (e.g. SYBR Green). 	<ul style="list-style-type: none"> -High host vs bacterial DNA ratio, providing less reliable data. -PCR impairments, explained by the high levels of host DNA in the analyzed samples. -Difficulties encountered, while preparing reads of the sequencing library. DNA damages induced during the preparation of FFPE derived sample reduce PCR amplification of bacterial DNA present in tumor tissues.
<p>16S sequencing and data analysis</p>	<ul style="list-style-type: none"> - Specific selection of sequence reads from a genome of interest, by 16S amplicon sequencing strategy. -Amplification of defined read-outs from characterized bacterial species, using appropriate primers -Suitable for the metagenomic analysis of low-biomass samples (e.g. tumors). -Cost-effective approach, compared to WGS. -Method affected to a lesser extent by the reduced bacterial biomass levels, compared to WGS. -Rapid classification of species into OTU clusters, based on their similarity levels. 	<ul style="list-style-type: none"> -No information about the metabolic functions of the identified bacterial species with 16S sequencing. -Analysis limitation to species recognized by available 16S primers (Lower sequencing richness compared to WGS). -Lower strain level resolution and no possibility of targeting species from other kingdoms of the tree of life. -OTU clustering provides only a broad idea of the actual similarity levels (e.g. 97% similarity threshold), existing between different bacterial species.

Figure 1

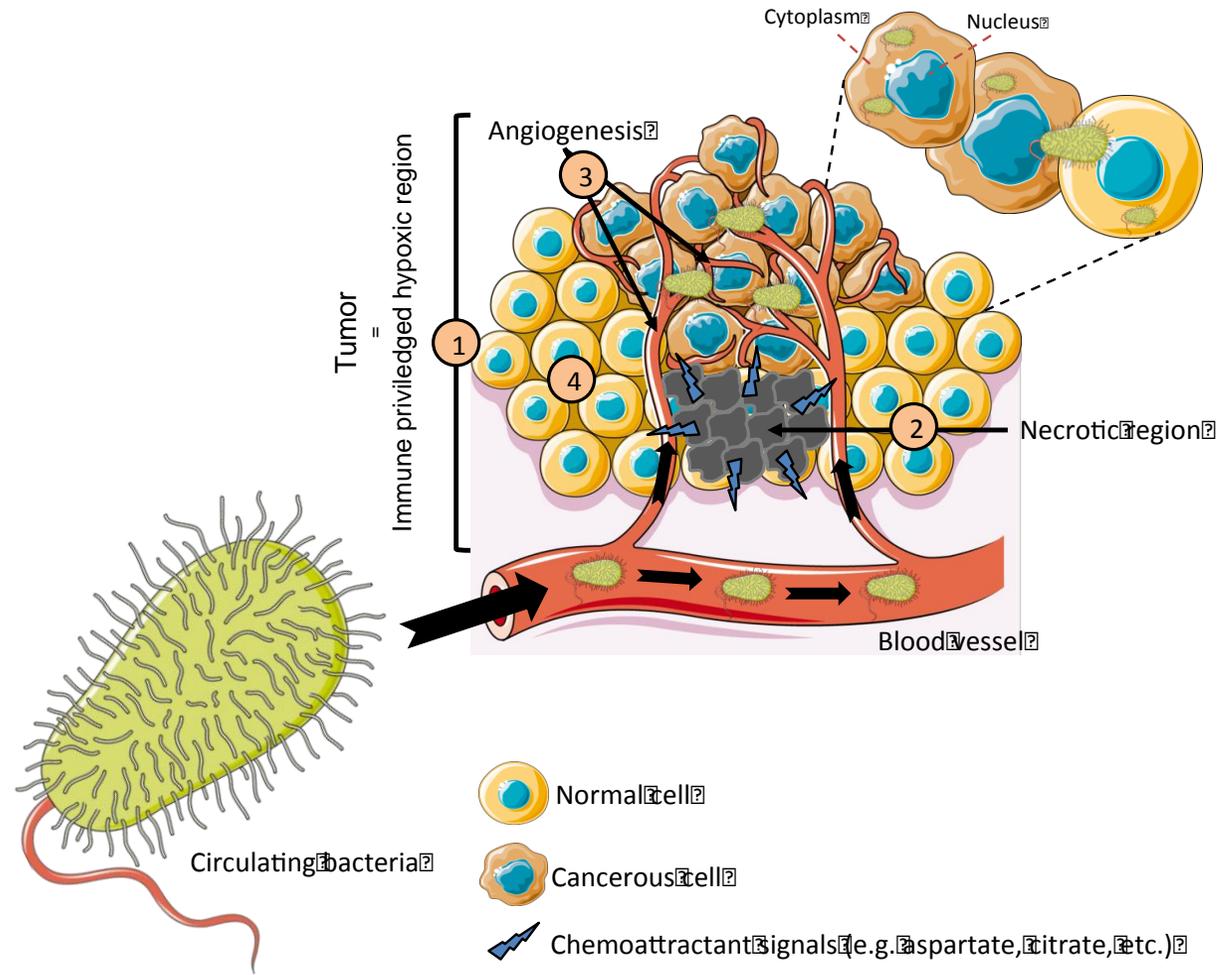


Figure 2

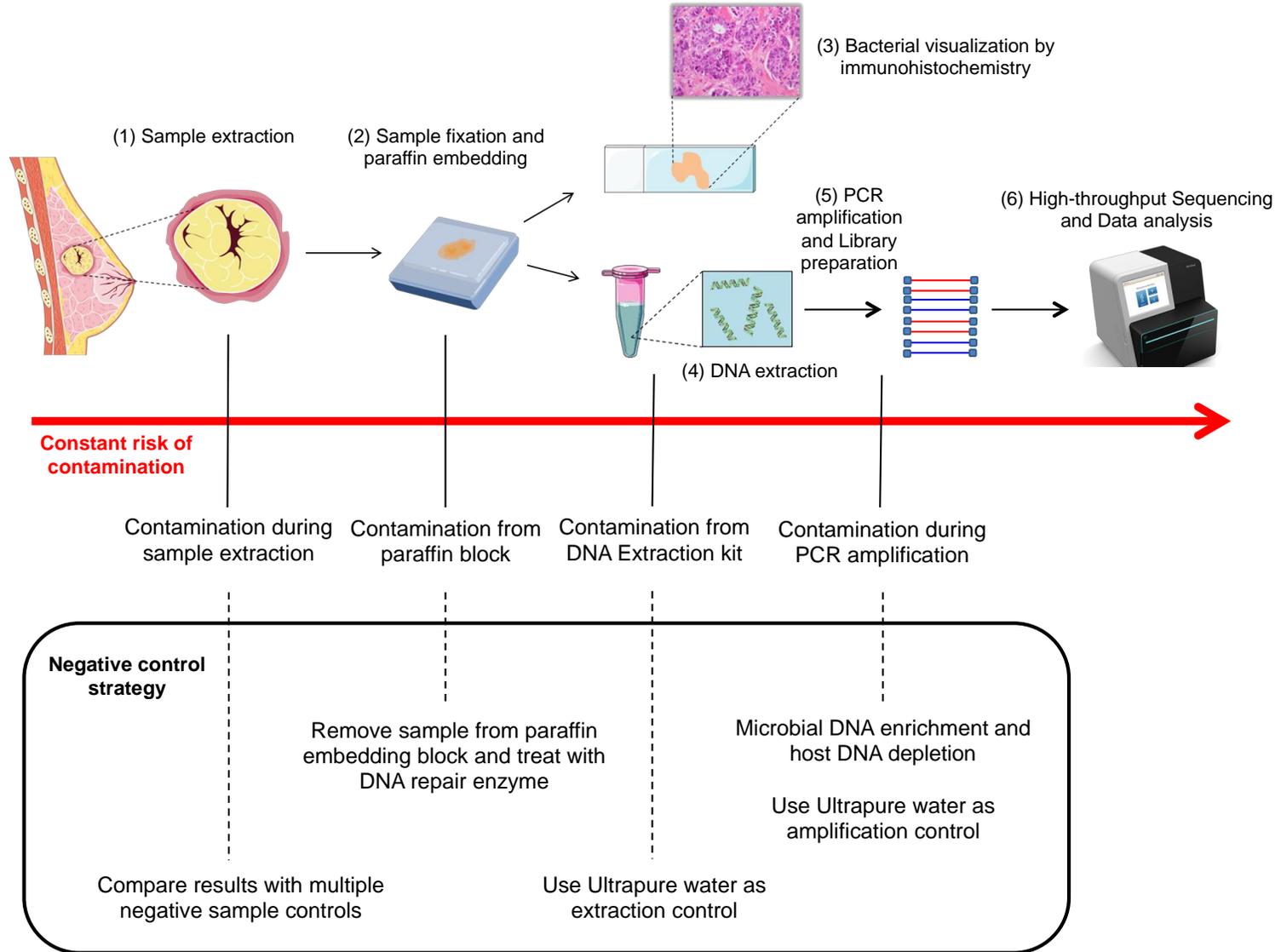


Figure 3

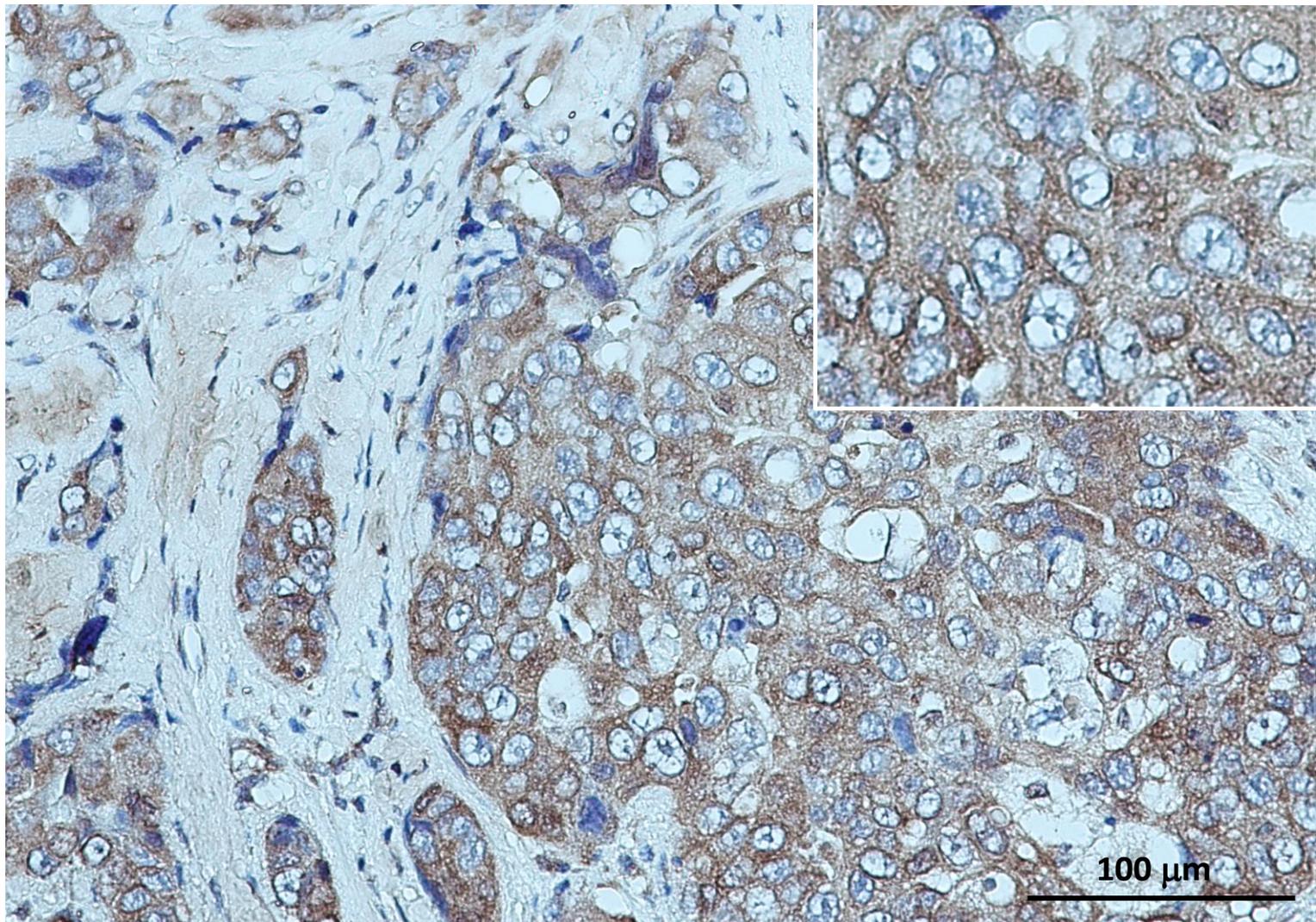


Figure 4

