Cancer's second genome: Microbial cancer diagnostics and redefining clonal evolution as a multispecies process

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26 ____

27 **SUBTITLE**

28 Humans and their tumors are not aseptic, and the multispecies nature of cancer modulates clinical
29 care and clonal evolution.

30 ____

31 **ABSTRACT**

32 The presence and role of microbes in human cancers has come full circle in the last century.
33 Tumors are no longer considered aseptic, but implications for cancer biology and oncology remain
34 underappreciated. Opportunities to identify and build translational diagnostics, prognostics, and
35 therapeutics that exploit cancer's second genome—the metagenome—are manifold, but require
36 careful consideration of microbial experimental idiosyncrasies that are distinct from host-centric
37 methods. Furthermore, the discoveries of intracellular and intra-metastatic cancer bacteria
38 necessitate fundamental changes in describing clonal evolution and selection, reflecting
39 bidirectional interactions with non-human residents. Reconsidering cancer clonality as a
40 multispecies process similarly holds key implications for understanding metastasis and
41 prognosing therapeutic resistance while providing rational guidance for the next generation of
42 bacterial cancer therapies. Guided by the above opportunities and challenges, this Review
43 describes opportunities to exploit cancer's metagenome in oncology and proposes an
44 evolutionary framework as a first step towards modeling multispecies cancer clonality.

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48 **INTRODUCTION**

49

50 A long and rich history exists between microbes and cancer. As early as 1550 BCE, Egyptian
51 writings suggested a crude therapy for tumors through incision and application of a poultice,
52 thereby inciting an infection.[1–3] Nearly three millennia later, Saint Peregrine Laziosi (c. 1265–
53 1345) documented spontaneous regression of a septic sarcoma on his leg large enough to pierce
54 through skin.[2] Although these accounts predated modern germ theory, they presciently
55 associated acute infections and the retrogression of cancer, which would be independently re-
56 discovered by three physicians between 1868-1893: Wilhelm Busch, Friedrich Fehleisen, and
57 William Coley.[4–6]

58

59 Many spontaneous tumor regressions described by these three physicians were tied to the skin
60 pathogen *Streptococcus pyogenes*, and its concomitant infectious syndrome, erysipelas.
61 However, only Coley seriously considered treating new patients—usually with late-stage or
62 inoperable cancers—by administering live bacteria (which carried serious clinical sequelae), and,
63 later, heat-killed microbial (*Streptococcus* and *Serratia*) toxins. These clinical experiments
64 revealed >10-year disease-free survival in 60 of 210 patients across multiple cancer types,
65 roughly one-third of all patients treated—a statistic only matched by modern immunotherapy.[7]
66 Nonetheless, an unknown mechanism and severe flu-like side effects made ‘Coley’s toxins’
67 unpalatable to oncology, especially when compared to the burgeoning radiotherapy and
68 chemotherapy fields.[8,9] It would take another century for scientists to realize that Coley’s
69 approach comprised the first intentional application of immunotherapy, and accurately predicted
70 a causal relationship between immunotherapy efficacy and an individual’s endogenous or
71 exogenously-administered microbiome.[10–16]

72

73 Viruses have also been crucial for understanding cancer and its genetic material. Peyton Rous's
74 seminal 1911 discovery of his eponymous, transmissible, oncogenic, RNA virus galvanized
75 investigation of the viral origins of cancer, leading to key links between Epstein-Barr, human
76 papilloma (HPV), hepatitis, and most recently Merkel cell polyomavirus and carcinogenesis.[17–19]
77 Although several decades of laborious research led to the conclusion that viruses cause only a
78 minority of cancers, the pursuit of oncogenic viruses indirectly led to the definition of and search
79 for 'oncogenes' capable of transforming benign tissue into malignant tissue.[19] One particularly
80 important oncogene was *src*, a protein kinase identified in transforming-only strains of Rous's
81 Sarcoma Virus (RSV), but found by Michael Bishop and Harold Varmus to exist in cells of non-
82 infected, phylogenetically-divergent birds.[20] Their data suggested a non-viral, cellular origin of
83 *src*: hosts normally contain oncogenes, and transforming strains of RSV had acquired one. This
84 discovery earned them the 1989 Nobel Prize in Medicine.[19,20] Realizing oncogenes were *internal*
85 to cancer motivated characterization of all possible oncogenes in the human cancer genome by
86 sequencing the normal human genome as a reference.[21] Modern cancer genomics thus had its
87 roots in tumor virology.
88
89 The story of RSV and its hijacking of *src* showed how genetic information could transfer between
90 tumors, microbes, and their hosts over evolutionary time and under various selection pressures.
91 After Rous's initial discovery, successive passaging of RSV enabled researchers to evolve the
92 chicken-specific virus to induce tumors in ducks and pigeons, then rats, rabbits, and mice,
93 presumably by activating similar kinase-related oncogenic pathways.[19,22,23] This process
94 represented early examples of intentional transfection and directed evolution, whereby recipient
95 cells received potent genetic cargo capable of being expressed to change cellular fitness.
96 Decades later, a similar ability of bacteria to transfect genetic material, either microbial or human
97 in origin,[24–28] to cells—including cancer cells[29]—with subsequent protein expression would be

98 demonstrated and coined "bactofection."[30] Bactofection was primarily sought after as an
99 alternative to conventional gene therapy or vaccination, but has received little attention.[27,30,31]
100
101 Since Bishop and Varmus's discovery shifted attention to factors internal to the cancer cell, the
102 last 30 years of cancer research has primarily focused on characterizing all major coding,
103 noncoding, structural, and copy number aberrations in the cancer genome.[32–36] Substantial study
104 of the tumor microenvironment (TME) has further elucidated the impacts of heterogeneous tumor
105 architecture, spatial organization, and multifaceted cellular agents (e.g., immune and stromal
106 cells) on cancer evolution, clonality, antitumor immunity, and metastasis.[37–39] Further work has
107 revealed similarities between microbial and cancer evolution. For example, the ubiquitous
108 presence of plasmid-like, extrachromosomal DNA (ecDNA) segments and their unequal
109 segregation during cancer cell division is analogous to unequal segregation of high copy plasmids
110 during bacterial replication.[40–44] Hybrid viral-human sequences on ecDNA segments in HPV-
111 infected cancers even contribute to immune evasion and carcinogenesis.[45,46] Nonetheless, most
112 cancer 'omic' studies have portrayed tumors as sterile entities, and microbial constituents as
113 being unrelated to tumor evolution or clinical care.
114
115 The last five years have persuasively unveiled metabolically-active, immunoreactive, intracellular,
116 cancer type-specific communities of bacteria (and viruses) living within tumor tissues, several of
117 which modulate cancer therapies.[47–60] These microbes may move during metastasis from one
118 bodily location to another and facilitate leaving and/or seeding of metastatic cancer cells.[53,54,61–
119 63] Critically, intratumoral and gut microbes can create chemo-, radio-, and hormonal therapeutic
120 resistance without any genetic or non-genetic changes within the cancer genome.[47,64,65]
121 Conversely, microbiota may render other chemo-, radio-, hormonal, and immunotherapies
122 possible and/or effective without any intervention from cancer cells.[12–14,64,66–68] Trace amounts of
123 cancer type-specific bacterial DNA have also been identified in the circulation of cancer patients,

124 suggesting a novel class of microbial cancer diagnostics.[58,69] Most, if not all, human cancers lack
125 sterility, and their microbes are clinically relevant.
126
127 Towards building a microbially-conscious framework of cancer, we posit cancer-bearing humans
128 as meta-organisms colonized by numerous and diverse microbial constituents (see **Box 1**—
129 "Quantifying the cancer microbiome").[70,71] We propose the clinical utility of microbial information
130 as cancer diagnostics, prognostics, and therapeutics and consider (intracellular) microbes as live,
131 mobile agents within tumors that encounter selection pressures alongside/within cancer cells.
132 Finally, we hypothesize that fundamental ecological rules governing microbial activity and spatial
133 placement (e.g., redox, chemotactic, oxygen gradients)[72] outside tumors also govern them inside
134 tumors. This Review details the study of cancer's "second genome" and its use to advance patient
135 care and models of cancer clonal evolution.
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BOX 1—Quantifying the cancer microbiome

Broadly speaking, the human body microbiota include ~4×10^3 species accounting for ~4×10^{13} total microorganisms, with ~97% of those cells comprising colonic bacteria and ~2-3% comprising extra-colonic bacteria while archaea and eukarya—including fungi—comprise smaller populations around ~0.1-1% of the total microbial abundance.[70,73] Human virus and phage abundance estimates remain undercharacterized but likely have even greater diversity than bacteria.[74] The human gut microbiome contains the largest bodily microbial biomass by far—roughly 0.2 kilograms[70,75]—with substantial effects on host antitumor immunity.[3] Biomass estimates of other body sites or tissues remain unknown.

Intratumoral microbiome diversity estimates with stringent decontamination controls (~1:2

control to sample ratio) suggest that at least 500 distinct bacterial species inhabit tumors.[57] Intratumoral microbiome abundance estimates have been inferred using shotgun read quantification and quantitative polymerase chain reaction (qPCR) of 16*S* rRNA.[57,58] Microbial profiling of all whole genome and transcriptome studies from The Cancer Genome Atlas (TCGA) revealed an average of 2.5% of total sequencing reads to be microbial and an average of 0.9% of total reads that were resolvable at the genus-level.[58] Given the difference between typical microbial and human genome sizes—often 10^3-fold smaller—it is possible that these percentages underestimate true microbial density. To quantitate abundance, bootstrapping 16*S* rRNA qPCR data by Nejman *et al*. revealed a heterogeneous average number of bacteria per cancer type, ranging from ~13 to ~70 per 40 nanograms (ng) of DNA, among seven major human cancers (**Table 1**).[57] The pan-cancer average was 34.19 bacteria per 40 ng of DNA (**Table 1**). To translate these values to percent tumor composition, it is necessary to first estimate the number of tumor cells per 40 ng of DNA. One way to estimate this for haploid cells is as follows:

$$DNA\ mass\ (haploid) \approx (3.2 \times 10^9 bp/cell)\left(\frac{1\ mole}{6.022 \times 10^{23} bp}\right)\left(\frac{660\ g}{1\ mole\ base\ pair}\right)$$

$$\approx 3.5\ picograms\ /\ haploid\ cell$$

To translate from haploid cell to tumor cell, an estimate of ploidy is needed, which can be derived from the most recent Pan-Cancer Analysis of Whole Genomes (PCAWG) dataset.[32] The mean estimated ploidy in PCAWG across all human cancers is 2.36 and ranges from a low of 1.28 to a high of 6.22. If we assume average cancer ploidy, the average DNA mass per cancer cell is thus:

$$DNA\ mass\ (cancer\ cell) \approx (3.5\ picograms\ /\ haploid\ cell) \times (2.36\ avg.ploidy)$$

$$\approx \mathbf{8.26\ pg\ /\ cancer\ cell}$$

Similarly, the range of DNA masses per cancer cell based on ploidy would be 4.48 pg to 21.77 pg. For simplicity, one can round the average mass value to 8 pg/cancer cell. Assuming that the DNA mass of microbes is negligible compared to that of the host, since its genome is roughly 10^3-fold smaller and there are fewer of them expected, then the estimated percent composition is as follows:

$$Pure\ tumor\ bacterial\ composition \approx \left(\frac{34.19\ bacteria}{40\ ng\ DNA}\right)\left(\frac{0.008\ ng}{1\ cancer\ cell}\right)(100\%)$$

$$= 0.68\%\ bacterial$$

This estimate, however, assumes 100% tumor purity. Fortunately, PCAWG estimated tumor purity across the same samples, showing an average tumor purity of 63.8%.[32] Instead of 5000 cancer cells per 40 ng of DNA, assuming 8 pg per cancer cell, average tumor purity suggests 3190 cancer cells with the remaining cells comprising the TME. While this does not change the percent bacterial composition of the tumor, it does change the ratio of bacteria to cancer cells to approximately ~1:100 or ~1% (i.e. 34.19 bacteria:3190 cancer cells; **Table 1**). Using the 95% confidence interval bounds of the pan-cancer mean number of bacteria per tumor (**Table 1**) gives a range of 0.75% to 1.46% bacterial.

In the case of high tumor ploidy and low tumor purity, it may become important to weigh the contributions between tumor (aneuploid) and stroma (diploid) to the number of cells within 40

ng of DNA. This may be done as follows, for example using a tumor ploidy of 6.0 and 20% purity:

$$Composition \approx \left(\frac{34.19\ bacteria}{40\ ng\ DNA}\right)\left[\frac{20}{100}\left(\frac{0.02177\ ng\ DNA}{1\ cancer\ cell}\right)+\frac{80}{100}\left(\frac{0.007\ ng\ DNA}{1\ stromal\ cell}\right)\right](100\%)$$
$$= 0.85\%\ bacterial$$

whereas a tumor of 100% purity at a ploidy of 6.0 would provide an average tumor bacterial composition of 1.86%. It is noted that cases with high ploidy and high purity will maximize this percentage value, in addition to when there is more observed bacteria.

To compare these bacterial abundances to intratumor immune cell populations, which are usually reported as densities of immune cell counts per square millimeter, it is necessary to first estimate the total number of cells per square millimeter in a tumor. While a handful of density estimates exist in the literature, such as a mean of 5,558 cells (SD 1,980) per mm^2 in metastatic melanoma,[76] it can be inferred directly from circle packing theory.[77] Specifically, given the average diameter of cells in a tissue, then the number of possible cells within the 1 mm^2 square can be calculated. In one way, this can be interpreted as a conservative estimate since cells are often compressed and non-circular in real tissues; conversely, it may overestimate cell density in regions with dense blood or lymphatic vessels. The typical diameter of lymphocytes approximates 6-7 μm in diameter[78] while the diameter of cancer cells vary by type and are approximately ~20 μm in diameter across many cancer cell lines.[79] Using average cell diameters of 12 μm, 15 μm, and 18 μm, circle packing theory predicts the following total cell abundances per 1 mm^2: 8213 cells, 5208 cells, and 3589 cells.

Then, using the previously calculated average pan-cancer tumor bacterial composition of 0.68% (assuming tumor homogeneity), the estimated number of bacteria inferred as the following: 56, 35, 24 bacteria/mm^2 (assuming 12 μm, 15 μm, and 18 μm average diameter cells, respectively). Notably, these bacterial abundance estimates are similar to the proportion of PD1$^+$ cells identified in a recent pan-cancer imaging dataset (~22 PD1$^+$ cells/mm^2) and roughly one-tenth of CD8$^+$ T-cell density (~385 cells/mm^2).[80] Overall, the values reflected in this analysis may vary from tumor to tumor, depending on the assumptions made above—tumor ploidy, purity, homogeneity—but the analysis provides a rough approximation and analogy of intratumor bacterial abundances to immune cell abundances.

To summarize, these calculations estimate an average pan-cancer bacterial composition of ~0.68% with two- and three-dimensional estimates of ~35 bacteria/mm^2 (assuming 5200 cells/mm^2) and approximately 6×10^5 to 6×10^6 bacteria per palpable 1 cm^3 tumor (assuming 10^8-10^9 cells/cm^3).[81] Notably, these estimates can vary between patients by three orders of magnitude and require further examination in additional cohorts.

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143 **TABLE 1.** Abundance estimates of intratumoral bacteria among seven major human cancers
144 profiled by Nejman *et al*. (data shared via private communication with Ravid Straussman).[57] One
145 thousand iteration-bootstraps of the mean approximated the average number of bacteria per 40
146 nanograms of DNA on a per-cancer and pan-cancer basis. Conversions and assumptions of
147 tumor ploidy, purity, and homogeneity are detailed in **Box 1**. Area density estimates assume 5200
148 total cells/mm^2 and volume density estimates assume 10^9 total cells/cm^3.

Cancer type in Nejman *et*	qPCR sample size (*n*)	Absolute range (bacteria/40ng) (min, max)	Bootstrapped estimate of average bacteria per 40 ng	Area density estimate	Volume density estimate

***al*. 2020**[57]			**DNA (mean, 95% CI) (1000 iterations)**	**(bacteria /mm^2)**	**(bacteria/ cm^3)**
Melanoma	200	(0.85, 3023)	**31.69** (9.71, 71.20)	~33	~6.3×10^6
Lung	274	(1.2, 3663)	**22.50** (7.90, 50.35)	~23	~4.5×10^6
Ovarian	57	(1.84, 73.2)	**12.72** (10.25, 16.00)	~13	~2.5×10^6
GBM	37	(3.41, 77.4)	**15.55** (10.89, 20.85)	~16	~3.1×10^6
Pancreatic	66	(3.82, 2147)	**70.43** (26.19, 147.78)	~73	~14×10^6
Bone	30	(1.62, 76.4)	**19.33** (13.97, 25.51)	~20	~3.9×10^6
Breast	352	(0.765, 1723)	**44.63** (31.41, 59.83)	~46	~8.9×10^6
Pan-cancer	1016	(0.765, 3663)	**34.19** (24.04, 46.56)	~35	~6.8×10^6

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154 **CANCER MICROBIOME DIAGNOSTICS AND PROGNOSTICS**

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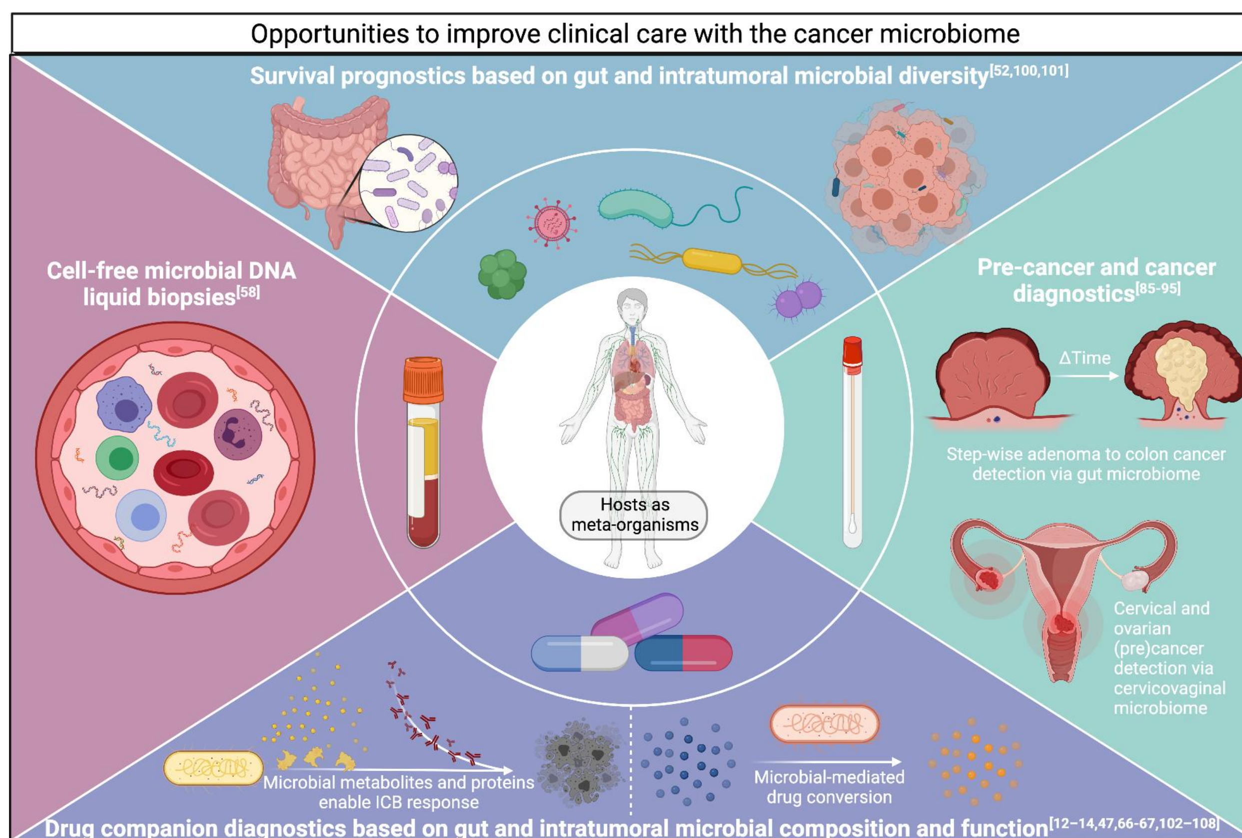
156 The concept of "strength in numbers" applies to cancer diagnostics, especially for low-biomass
157 material. For instance, liquid biopsies in cancer rely on detecting minute quantities of analytes
158 (DNA, RNA, proteins, or modifications thereof) shed from the tumor to diagnose the presence
159 and/or type of cancer.[82] The low-biomass, limited unique number, and limits of detection of these
160 analytes usually restricts utility of liquid biopsies to tumors on the scale of multiple cubic
161 centimeters, corresponding to later stage cancers.[82,83] Critically, more analytes or modifications,
162 even if rare, increase the overall sensitivity of the test sigmoidally.[84] Cristiano *et al*. demonstrated
163 this principle using Monte Carlo simulations of liquid biopsies, showing that a test examining DNA
164 modifications comprising ≤0.001% of total plasma material could still have near-perfect sensitivity
165 if at least 256 alterations were interrogated.[84]

166

167 These conclusions from cancer genomics suggest that the inherent diversity of the intratumoral
168 microbiome (≥500 unique bacterial species)[57] and the gut microbiome (~4×10^3 bacterial
169 species)[73] provide strong rationale for creating microbiome-focused cancer diagnostics, even if
170 any individual microbe is rare or lowly abundant. Two alternative ways of phrasing this idea is that
171 (i) high microbial diversity provides “many shots on goal” for making a single diagnosis and (ii),
172 using machine learning syntax, interrogating the microbiome is analogous to employing an
173 ensemble of many weak learners that collectively provide strong prediction performance (i.e., the
174 conceptual basis of boosting). We further note that for diagnostic purposes detected microbes do
175 not need to be causally associated with carcinogenesis but only consistently correlated with
176 cancer presence, absence, and/or growth. These microbial-informed or augmented diagnostics
177 and prognostics hold much potential to improve clinical cancer care (**Figure 1**).
178



179

180 **FIGURE 1**. Illustration of opportunities to enhance clinical cancer diagnostics and prognostics
181 using the cancer microbiome. Relevant references are listed in the title of each quadrant.

182

183

184 **Pre-cancer and cancer microbiome diagnostics**

185

186 Pre-cancer diagnostics identify lesions that are likely to progress to cancer but otherwise do not
187 meet the criteria for malignant tissue, most commonly including cervical and colorectal cancer
188 (CRC) precursors. With a focus on the gut microbiota, metagenomic studies have identified
189 distinct fecal microbial compositions between colonic adenoma-bearing hosts and healthy
190 individuals, often but not always with increases in *Proteobacteria* abundance.[85–88] Yachida *et al*.
191 further characterized shotgun metagenomic and metabolomic shifts in the guts of healthy
192 individuals, those with polypoid adenomas, and those with stage 0 to stage IV CRCs, revealing

193 distinct stage-wise microbial and metabolic compositions sufficient to build fecal stage-specific
194 classifiers.[85] Other studies of the vaginal microbiome have revealed distinguishable microbial
195 compositions and functions between healthy patients, those with cervical intraepithelial neoplasia
196 or cervical cancer, and modifying effects of HPV or HIV status.[89–91] In a longitudinal trial, Usyk *et*
197 *al*. found that women presenting for high-risk HPV infection with abundant vaginal *Lactobacillus*
198 were more likely to clear the infection by their second visit (average 1.5 years later); conversely,
199 those with abundant vaginal *Gardnerella* upon presentation were much more likely to show
200 disease progression by the second visit.[89] These studies suggest the opportunity for minimally-
201 invasive, swab-based stool and vaginal microbiome diagnostics that detect precursor cancer
202 lesions and/or forecast risk of cancer conversion.
203
204 Pre-cancerous syndromes are also pertinent for microbiome diagnostics, such as genetically-
205 driven familial adenomatous polyposis (FAP), pre-leukaemic myeloproliferation (PMP), and
206 *BRCA1* status, for they augur subsequent carcinogenesis in ways not fully predicted by host
207 genomics. For example, PMP is highly associated with *Tet2* mutations, but only a fraction of
208 people with germline *Tet2* mutations develop PMP or bona fide myeloid malignancies.[92]
209 Comparing gut microbiota from patients with and without FAP, Dejea *et al*. elucidated that FAP
210 encourages biofilm formation comprising genotoxic strains of *Escherichia coli* and *Bacteroides*
211 *fragilis* with greater expression of their colibactin and *B. fragilis* toxins, thereby increasing IL-17-
212 dependent inflammation, DNA damage, and faster cancer conversion.[93] Meisel and colleagues
213 then demonstrated that microbial translocation from the gut to systemic circulation with resultant
214 IL-6 production mechanistically drives conversion from predisposing *Tet2* germline mutations to
215 PMP.[94] Nené *et al*. also reported significant cervicovaginal microbiome changes—absence of
216 *Lactobacillus* spp.—among *BRCA1*-positive, non-cancer-carrying women that were shared
217 among women with ovarian cancer, suggesting that germline mutations can affect microbial
218 composition and may show continuity with subsequent cancer conversion.[95] Collectively, these

studies argue that pre-cancerous syndromes indeed modify and interact with microbiota, suggesting an opportunity to develop diagnostic tools tracking their presence, and interventions that reduce cancer conversion rates.

For solid tumor and blood microbiome diagnostics, Nejman *et al*. and Poore *et al*. provide the most comprehensive analyses to date, demonstrating cancer type-specific microbial signatures among >30 cancer types, showing their diagnostic applicability to human plasma samples, and providing evidence of intracellular microbial localization in tumors.[57,58] Nejman and colleagues combined imaging, cultivation, qPCR, and a multi-region 16*S* rRNA sequencing strategy to thoroughly characterize intratumoral bacteria among breast, bone, pancreas, brain, ovarian, lung, melanoma, and colon cancers. Inclusion of 811 experimental contamination controls (i.e., DNA extraction controls, no-template PCR controls, paraffin controls) for 1010 tumor samples enabled stringent decontamination that removed 94.5% of detected bacterial species, leaving 528 confident species-level calls. Poore and colleagues used an alternative approach by mining all whole genome and transcriptome sequencing data in TCGA (n=18,116 samples) and using shotgun metagenomic strategies to derive ~2000 genus-level calls.[58] *In silico* decontamination based on sample DNA or RNA concentrations[96] removed up to 92.3% of microbial information, but machine learning performance to distinguish between cancer types and tumor versus adjacent normal tissue remained strong. Based on historical and epidemiological data associating bacteremias with subsequent CRC diagnosis,[69,97] they then tested whether blood-derived, genus-level microbes in TCGA were capable of distinguishing CRC from other cancer types. Finding this to be true, they next tested whether blood-derived microbiomes could discriminate between ~20 other cancer types, as well as when restricting samples to early cancer stages (stages 1-2) and tumors without any canonical mutations on two commercial cell-free tumor DNA (ctDNA) panels. Application of the same approach to 100 plasma samples from three cancer types (lung, prostate, melanoma) and 69 HIV-negative, non-cancer controls suggested that cell-

245 free microbial DNA (cf-mbDNA) was capable of distinguishing between healthy and cancer
246 patients and between cancer types.[58] Although the origin of cf-mbDNA remains unknown, we
247 speculate based on the literature a multiplicity of sources including the oral, gut, and intratumoral
248 microbiomes.[53,61,62,98,99] We also speculate that the strength of the cf-mbDNA test derives from
249 the quantity of microbial biomarkers assayed rather than the absolute amount of microbial DNA
250 present in plasma, as analogously shown in fragmentomic-based liquid biopsies.[84] Both of these
251 studies lay the foundation for multiple cancer detection tests using the cancer microbiome.
252
253 **Prognostics and companion diagnostics**
254
255 The impact of gut and intratumoral microbiomes on local and systemic immune tone and host
256 metabolites makes them versatile prognostics and companion diagnostics.[3] Higher alpha
257 diversity of intratumoral or gut microbiomes prognoses long-term survivors in pancreatic and
258 cervical cancers, as well as in patients undergoing hematopoietic stem cell transplantation for
259 cancer therapy.[52,100,101] Additionally, colorectal cancer stages reflect successive microbial
260 changes in the fecal microbiome,[85,88] and early versus late stage lung cancer can be
261 distinguished through lower airway microbiota compositions.[51] Intratumoral microbiomes can
262 similarly distinguish stage I from stage IV tumors in multiple gastrointestinal cancers (stomach,
263 colon, rectal) and renal cell cancer.[58]
264
265 Therapeutically, numerous studies demonstrate how the efficacies of anti-CTLA-4 and anti-PD-
266 (L)1 immune checkpoint blockade (ICB) are predicted by and mechanistically tied to gut
267 microbiome composition and function,[12–14,67,102–106] and recently the intratumoral microbiome has
268 shown a similar capacity.[49,57] Similarly, the efficacy and host toxicity of cyclophosphamide,[66,107]
269 gemcitabine[47], and platinum-based[67,108] chemotherapy depend on the composition and
270 metabolic capacity of gut and intratumoral microbiota.[109] In specific cases, bacterial enzymes

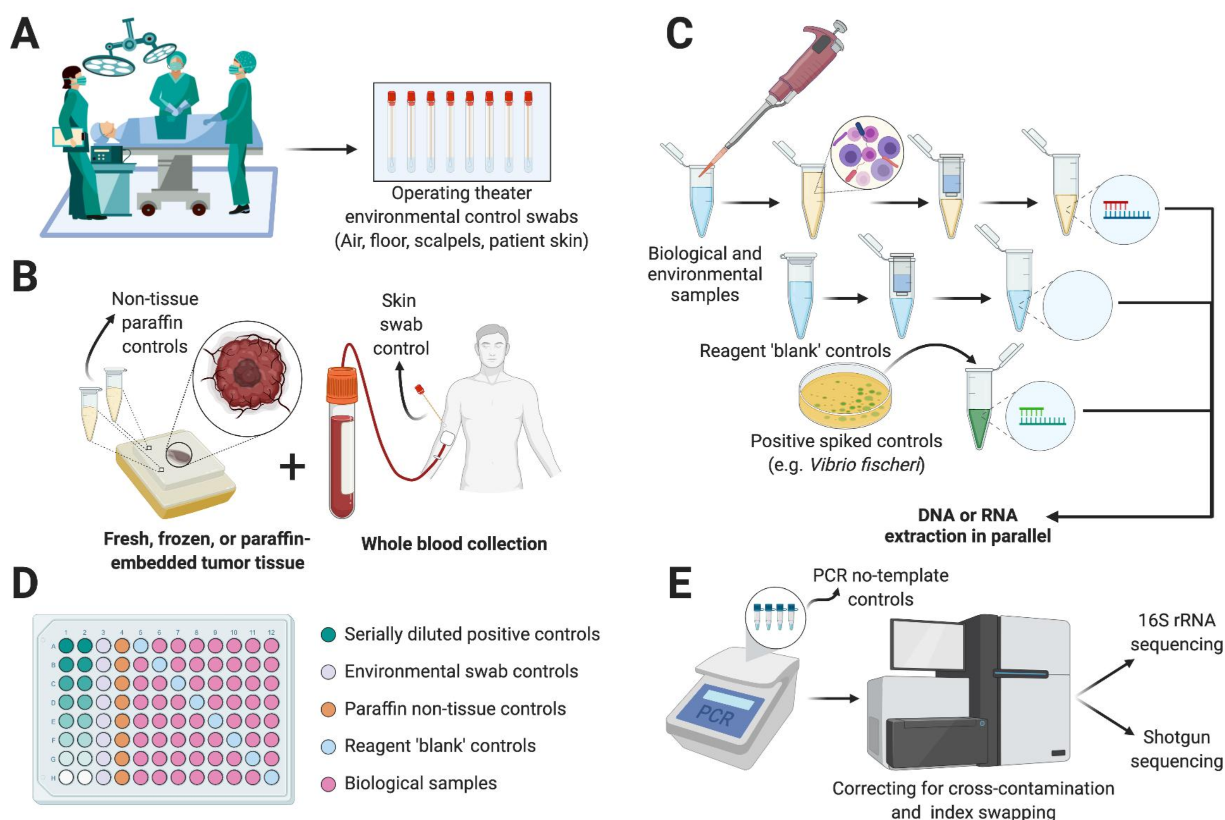
directly degrade chemotherapy compounds into non-functional byproducts (e.g., gemcitabine degradation by cytidine deaminase),[47] suggesting colonized patients would have no drug response or quickly develop therapeutic resistance. In HER2-positive breast cancer, antibiotic administration impairs trastuzumab efficacy and fecal microbiota transplant from non-responders to responders improves outcomes, implicating gut microbiota as critical agents for therapeutic response.[68]

Gut microbiota also affect hormonal therapies. Administration of abiraterone acetate (AA) in the setting of castrate-resistant prostate cancer promoted outgrowth of *Akkermansia muciniphila* and appeared to aid overall AA therapeutic efficacy.[110] However, androgen deprivation therapy also increases gut-residing *Ruminococcus* species containing CYP17A1-like enzymes that catalyze pregnenolone conversion to the sex hormone precursor dehydroepiandrosterone (DHEA) and testosterone, thereby enhancing progression to castration-resistant prostate cancer.[65] Thus, targeted longitudinal profiling of implicated gut microbes may provide an early indicator of failing androgen deprivation therapy while also substantiating their timed targeted removal. It has been speculated, albeit unproven, that estrogen-receptor-positive breast cancer may similarly be affected by microbial hormone metabolism.[111,112] It further remains unknown if or how intratumoral microbes affect hormonal metabolism. Altogether, the myriad of gut and intratumoral microbiome effects on virtually every domain of cancer therapy and predictive associations with patient survival enforce their clinical utility as prognostic indicators and companion diagnostics.

Challenges for cancer microbiome diagnostics and prognostics

Low-biomass microbial sampling creates analysis challenges that necessitate careful consideration and removal of contamination.[113,114] While less impactful in gut microbiome studies or large-scale meta-analyses, external (e.g., environmental) and internal (e.g., cross-seeding

297 between samples) contamination can skew small-to-moderate scale profiling of the cancer
298 microbiome.[113,114] Standardized experimental contamination controls (**Figure 2**) alongside *in*
299 *silico* decontamination methods[96,114] can enable more robust and reproducible results, especially
300 for assaying intratumoral and blood-borne microbes, thereby enabling better microbiome-
301 augmented cancer diagnostics and prognostics. Notably, very few cancer genomics studies
302 implement any of these contamination controls and basic usage thereof would allow broad
303 utilization of "cancer-specific" data for simultaneous interrogation of microbial analytes, although
304 this may be overcomable by integrating many thousands of samples.
305
306 Other challenges with microbiome studies include (i) the degree to which results vary with sample
307 and bioinformatics processing choices;[115] (ii) fundamental differences in data properties and
308 appropriate statistics when using relative abundances compared to host 'omic data;[116–118] and
309 (iii) compositional differences as a function of geography and ethnicity, particularly when assaying
310 gut microbiota.[119–121] One or more of these factors have, for example, resulted in three major
311 microbiome studies[12–14] concluding that different gut microbes predict anti-PD(L)1
312 immunotherapy response—a fact that has remained irreconcilable despite analyses that
313 reprocessed all the data equally or instead examined their microbial functions.[104] Large meta-
314 analyses can surmount some of these problems, with two key studies identifying conserved gut
315 microbial signatures predictive of colorectal cancer across diverse cohorts and
316 geographies.[122,123]
317
318



319

320 **FIGURE 2**. Extracting and analyzing low-biomass microbiomes requires special care to control
321 external and internal contamination.[96,113,114] (**A**) Collection of environmental controls ideally
322 begins in the operating room to account for non-patient environmental sources. (**B**) Post-operative
323 tissues, if paraffin embedded, can have non-tissue paraffin controls taken to ensure the
324 embedding process is not contaminated. Whole blood should ideally be collected with a skin swab
325 to account for peri-needle contamination. (**C**) Negative reagent-only 'blank' controls and positive
326 titrated controls should be processed simultaneously alongside nucleic acid extraction from
327 biological and environmental samples. (**D**) Plating strategies should be considered to reduce
328 cross-contamination; controls may include up to 40% of total samples. (**E**) Amplification steps
329 may include PCR no-template controls and sequencing may include correction for cross-
330 contamination or index swapping, although the latter remains challenging.

331

332

333 **REDEFINING CANCER CLONALITY AS MULTISPECIES**

334

335 **Redefining traditional meaning of clonal evolution and selection in cancer**

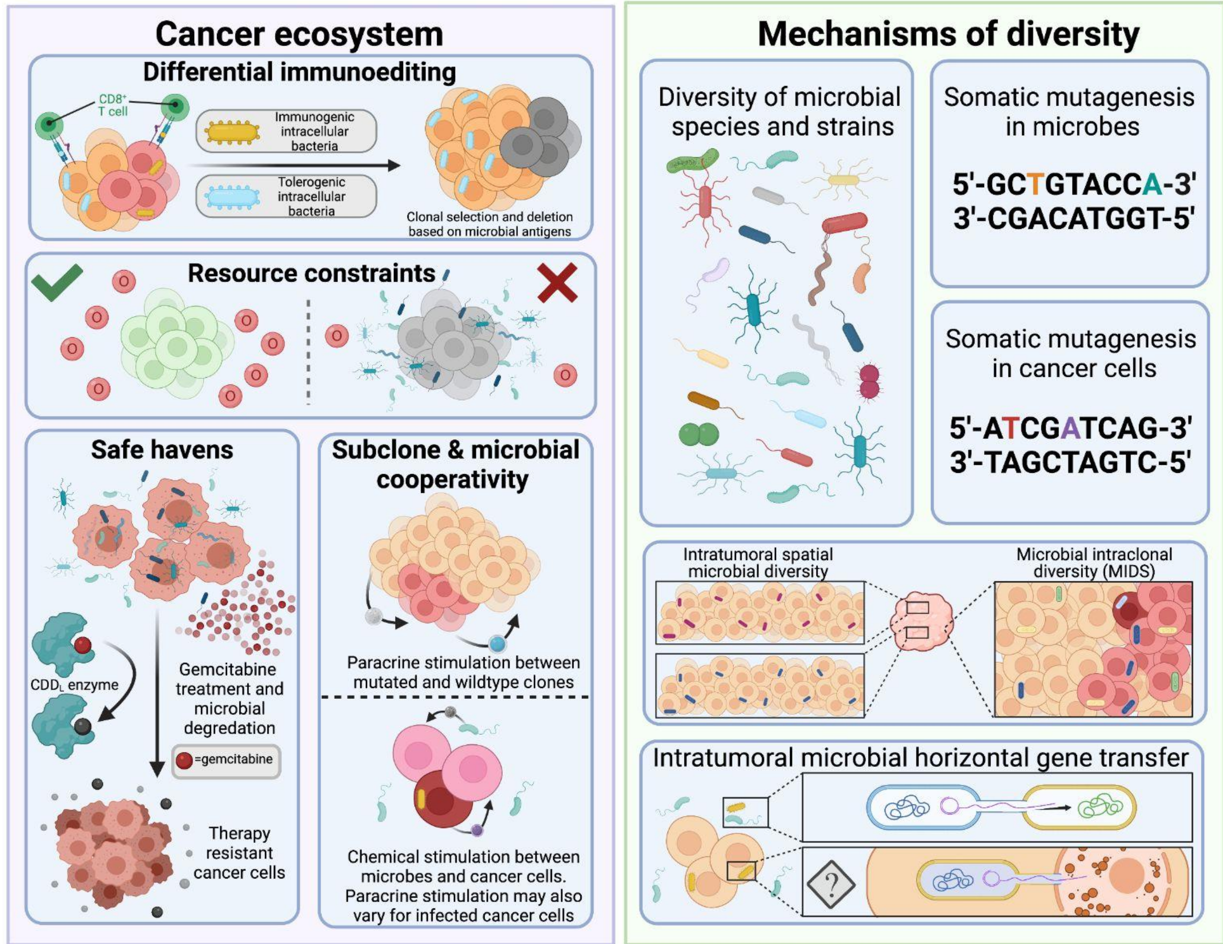
336

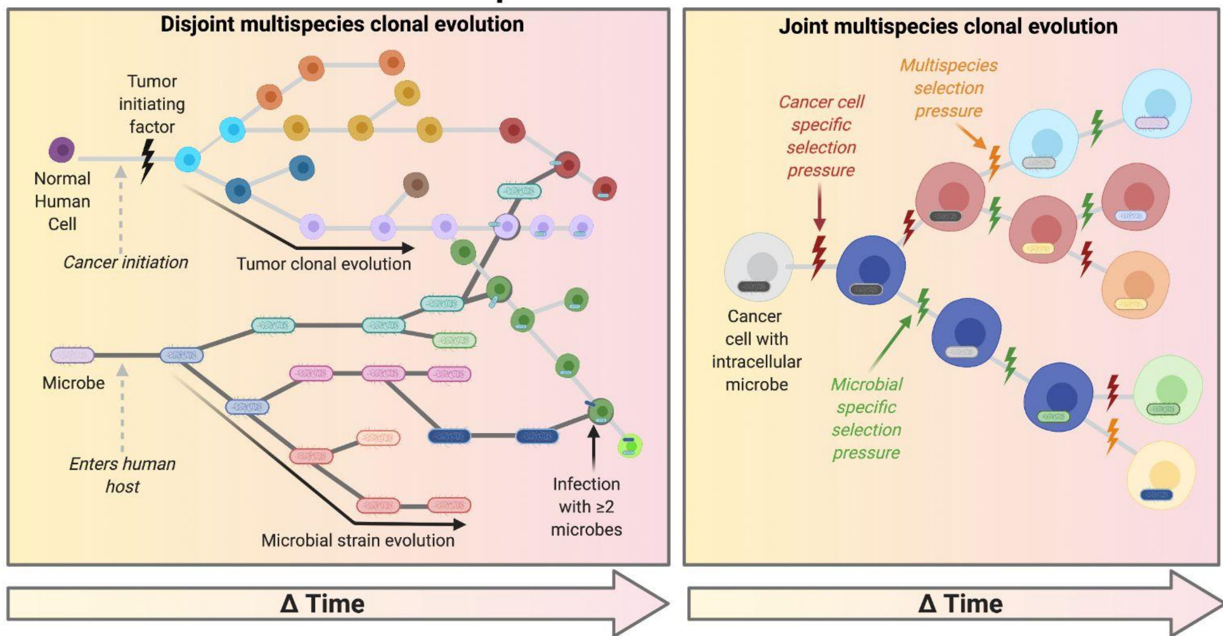
337 Cancer cells evolve through space and time. Although the traditional view of clonal evolution has
338 historically centered on genetic alterations,[37,124,125] it is increasingly recognized that non-genetic
339 alterations such as epimutations also contribute.[126–128] The emergence of single-cell multi-omics
340 and longitudinal studies offers opportunities for a more inclusive, multi-analyte view of intratumor
341 heterogeneity and clonal evolution.[129,130] Recognition of the role of multi-omics in functional clonal
342 diversity advocates for broader definitions beyond cancer genomics.[38]

343

344 Research demonstrating effects of extracellular and intracellular microbes on the cancer cell
345 genome,[93,131] transcriptome,[48,50,51,132] proteome,[49] and metabolome[47,65] strongly justify their
346 inclusion in any multi-omic model of clonal evolution (**Figure 3**). Additional microbial functions
347 that enable or abolish chemo-, radio-, and/or immunotherapy efficacy without *any* interventional
348 cancer genomic changes provide further rationale for their inclusion.[3,11,133] Intracellular
349 localization of metabolically active, immunogenic cancer microbes that shape cancer
350 immunoediting—evolutionary processes and selection pressures previously privileged to cancer
351 clonal selection—also provides justification.[49,57,63] Identification of hybrid microbial-human reads
352 involved in carcinogenesis on plasmid-like ecDNA segments intimately links cancer and microbial
353 fitness.[45,46] Microbial mechanisms that modify immunosurveillance also impact when and where
354 tumors grow and/or metastasize.[49,53,54,61,62,134] Negatively, ignorance of microbial information in
355 clonal evolution precludes accurate identification of cancer dynamics, therapeutic resistance, and
356 metastasis. However, as distinct agents from cancer cells with separate genetic material that is,
357 at times, under discordant selection pressure(s) from the cancer genome (e.g., antibiotic therapy
358 for bacteria, targeted kinase therapies for cancer cells), there must be nuance. Cancer microbes

359 cannot merely be added as another "-ome." Simultaneously, studies examining the roles of cancer
360 microbiota have not seriously considered the clonality of these microbes or their impacts on
361 cancer cell clonality. Thus, there is a persisting theoretical gap between the microbiota in cancer
362 and clonal evolution modelling that we propose bridging.
363





364

365 **FIGURE 3**. Impacts of intratumoral microbes on cancer evolution and arguments for multispecies
366 clonal evolution modelling. Effects are summarized into three major categories: modulation
367 ecosystem effects, mechanisms of clonal diversity, and example disjoint and joint phylogenetic
368 clonal evolution.

369

370

371

372

373 **Key evidence that argues cancer clonality is multispecies**

374

375 ***Decoupling of therapeutic efficacy from host and cancer cell genetic changes***

376

377 The genetic model of clonal evolution provides explanations for relapses caused by mutagenesis.
378 For example, mutations in cancer cell epidermal growth factor receptor (EGFR) induce resistance
379 to various generations of EGFR tyrosine kinase inhibitors while simultaneously creating favorable
380 selection pressures for mutated cells over non-mutated counterparts.[135,136] As a result, EGFR-
381 mutated cells outcompete their neighbors and clonally expand.

382

383 However, this model fails to always explain treatment efficacy or failure, both for conventional
384 cancer and microbial-modulated therapies. For example, isocitrate dehydrogenase (IDH1/IDH2)-
385 mutated acute myeloid leukemia patients treated with IDH1/2 inhibitors can show complete and
386 sustainable responses to treatment without eliminating mutated cells.[137–139] The same is
387 observed in chronic myelomonocytic leukemia treated with hypomethylating agents. Patient
388 responses, even when complete, demonstrate no decrease in the mutational load and no specific
389 selection events explaining secondary escape.[140] Moreover, despite a clear reduction in cancer
390 cell burden, thereby generating a selective bottleneck, relapse can occur without genetic

selection. For example, in childhood B-cell precursor acute lymphoblastic leukemia, a recent study by Turati *et al*. demonstrated how treatment with vincristine and dexamethasone drastically reduced the leukemic burden but induced very little change, if any, in clonal composition.[141] Conversely, a transcriptional bottleneck was observed in single-cell RNA-Seq, with a major loss in transcriptomic intratumor heterogeneity. A similar resistant transcriptomic profile was found in the leukemic cells before treatment, suggesting positive selection of these rare pre-existing resistant cells rather than induction of that phenotype under treatment exposure. These resistant cells comprised a subfraction of low cycling cells and have been associated with a distinct metabolic program.[141,142] Several hypotheses are currently discussed with regard to this non-genetic resistance to therapies,[143] which mostly focus on transcriptomic and epigenetic properties.

Classic genetic clonal evolution also fails to account for microbial-mediated treatment efficacy or failure of (i) cyclophosphamide,[66,107] gemcitabine[47], and platinum-based[67,108] chemotherapy; (ii) anti-CTLA-4 and anti-PD-(L)1 ICB efficacy;[12–14,102–106] (iii) androgen deprivation therapy in prostate cancer;[65] and (iv) trastuzumab in HER-2-positive breast cancer.[68] Notably, some of these examples (e.g., gemcitabine resistance) rely on *microbial* genetic content (e.g., cytidine deaminase long (CDD_L) isoforms),[47] which further may be shared among multiple species through conventional horizontal gene transfer and may also be intracellular. Similarly, cancer clonal selection may entirely occur on CDD_L-containing microbes by providing growth advantages to those that can metabolize it as a concentrated carbon source, and cancer cell survival is tied to CDD_L^+-microbe proximity. Yet, cancer genome-centric evolutionary models miss all of these effects and fail to accurately forecast evolutionary changes.

Impact of intracellular bacteria on cancer cell properties and fitness

417 Immunohistochemistry, immunofluorescence, and electron microscopy data document the
418 intracellular localization of intratumoral bacteria.[49,57,61,63] Bacteria inside cancer cells modify their
419 properties—transcriptional state,[63] proteome,[49] and metabolic repertoire[47,57]—in ways that are
420 intrinsically tied to clonal evolution. Extracellular bacteria also modulate these properties and
421 cause cancer cell genomic mutations.[93,131,144] Key affected clonal aspects comprise cancer cell
422 metabolism, immunoediting, clonal expansion and metastasis, and mutagenesis.
423

424 First, intracellular microbes change host cell metabolism, including degradation of exogenous
425 chemotherapy[47] and xenobiotic D-alanine.[57] Geller *et al*. originally identified microbial
426 gemcitabine resistance through incidentally discovering *Mycoplasma* contamination of cell
427 cultures and concomitant drug resistance.[47] Isolation of the responsible enzyme and its drug-
428 degrading isoform (CDD_L) followed by bioinformatic searches revealed >300 CDD_L^+ species,
429 98.4% within *Gammaproteobacteria*. Imaging, sequencing, and cultivation from gemcitabine-
430 associated pancreatic cancer patient biopsies indeed revealed CDD_L^+ bacteria in most samples
431 that conferred gemcitabine resistance in subsequent co-cultures with cancer cell lines.[47]
432

433 Second, intratumoral microbes modulate the immune response, favoring immune escape, or,
434 conversely, recognition. *Fusobacterium nucleatum* inhibits natural killer cell (NK)-dependent
435 tumor killing through Fap2 interaction with TIGIT, constituting a bacterium-dependent mechanism
436 of tumor-immune evasion.[145] Pancreatic cancer bacteria also induce innate and adaptive immune
437 suppression, including via selective Toll-like receptor ligation leading to T-cell anergy.[48] Another
438 metastatic melanoma study elucidated immunogenic, MHC I and II-bound bacterial peptides
439 presented on cancer and immune cells that putatively shape cancer immunoediting and posit gut-
440 tumor antigenic overlap.[49] Moreover, an uneven partitioning of microbes among cancer cells can
441 result in the differential elimination or maintenance thereof. Such a perspective enriches the

442 traditional “3Es” of elimination, equilibrium, and escape[146] and documents how cancer cell fitness
443 is decoupled from its own genome.
444
445 Third, intratumoral microbes can favor cancer cell expansion and metastases. Bullman *et al*.
446 demonstrated *Fusobacterium* persistence in colorectal cancers through successive mouse
447 xenografts and similar bacterial compositions in matched primary-metastasis (colorectal-liver)
448 patient samples.[61] Metronidazole treatment reduced tumor growth, implying greater fitness
449 conferred by *Fusobacterium* colonization.[61] Bertocchi *et al*. later showed that colorectal bacteria
450 stepwise enter tumor tissue, modify the gut vascular barrier, migrate to the liver, and foster the
451 formation of a premetastatic niche favoring metachronous metastasis.[62] Parhi *et al*. noted how
452 *Fusobacterium*-seeded breast cancers metastasized earlier. Hence, intratumoral bacteria
453 enhance metastatic formation and seeding.
454
455 Fourth, microbes cause genotoxin-mediated mutagenesis.[93,131] Pleguezuelos-Manzano *et al*.
456 showed how *pks*$^{+}$ *E. coli* generates mutational signatures in head and neck, colorectal, and
457 urinary tract cancers. Moreover, various gut-residing *Proteobacteria* species produce cytolethal
458 distending toxin (CDT) capable of inducing single- and double-stranded DNA breaks.[144]
459 Collectively, all of these mechanisms shape cancer cell properties and fitness.
460
461 **Implications and hypotheses if cancer clonality is multispecies**
462
463 Imaging data portray intracellular bacteria as unevenly distributed among cancer cells and tumor
464 regions,[57,61] suggesting differential fitness at the single cell level that may not correspond with
465 mutational status. This challenges the definition of cancer clones as private lineages of mutated
466 cells stemming from common ancestors and violates modelling assumptions whereby clonal
467 lineages comprise homogeneous cell populations. Although no two cancer cells are equal in every

468 respect, the primary assertion of clonality is that individual differences between two cancer cells
469 of the same clone are negligible.[147] However, if intracellular bacteria alter phenotypes, behaviors,
470 and fitness of spatially-adjacent cancer cells, then they create major intraclonal heterogeneity,
471 which we define as "microbial intraclonal diversity" (MIDS). MIDS questions clonal lineage
472 homogeneity and motivates revising clonal boundaries, most simplistically through further
473 subsetting (e.g., *KRAS*-mutated, *Fusobacterium*-infected cells versus *KRAS*-mutated uninfected
474 cells) or more accurately through revised modelling approaches that account for discordant
475 microbe-cancer selection pressures. MIDS also includes mimicry between microbial and cancer
476 antigens.[148,149] Should genetic cargo be shared between intracellular bacteria and host cells, as
477 biotechnology already shows is possible[24] and cancer virology affirms,[45,46] MIDS must account
478 for DNA and RNA from multiple species.
479
480 Beyond challenging clonal boundaries, intracellular bacteria may require revision of the
481 evolutionary tree. Typical clonal evolution model depicts an evolutionary tree with one trunk and
482 several branches, relying on the assumption of vertically transmitted traits from mother cells to
483 daughter cells at each round of cell division. If future research affirms horizontal/lateral gene
484 transfers between intracellular bacteria and host cancer cells, multiple tree trunks and connexions
485 between branches would be required. A similar debate has taken place in evolutionary biology,
486 challenging the traditional Darwinian view about "tree of life."[150–152] Clonal evolution may then be
487 better articulated as a case of "reticulated evolution," wherein horizontal/lateral transfers change
488 the fitness, function, or/or phenotype of host cancer cells.
489
490 ***Considerations for cancer microbiome therapeutics under multispecies clonality***
491
492 Multispecies cancer clonality offers new therapeutic strategies that neither human nor microbial
493 clonality alone propose. For instance, Byndloss *et al*. demonstrated an interplay between

494 fastidious anaerobic gut bacteria and butyrate-mediated, PPAR-γ-dependent host signaling that
495 maintained low oxygen levels in the gut and prevented outgrowth of facultative pathogens.[153]
496 Conversely, antibiotics increased gut oxygen concentration and pathogen outgrowth.[153]
497 Analogously, there may be opportunities in cancer to target host processes that facilitate microbial
498 homeostasis as a means to mitigate microbial-mediated carcinogenesis in favor of blunted
499 antibiotics. Butler *et al*. provide another example whereby administration of a bacterial protease
500 depleted cellular MYC in colon and bladder cancers.[154] Similarly, identification of anticancer
501 microbial enzymes or metabolites may provide effective host-modulating cancer therapies or
502 improve the efficacy of existing therapies—a strategy that several groups have already taken with
503 immunotherapy.[105,106]
504

505 **EVOLUTIONARY MODELING OF THE CANCER MICROBIOME**
506

507 **Example of *Helicobacter pylori***
508

509 Incorporating intratumoral microbes into evolutionary models requires nuance because selection
510 pressures may be discordant with those experienced by cancer cells. A long-standing and well-
511 studied example of microbes in the cancer environment is *Helicobacter pylori,*[155] which has
512 adapted to thrive in more than half of the human population long enough to trace human migration
513 events.[156] *H. pylori* has co-evolved protective and pathogenic roles within humans: protective in
514 gastric cardia and esophageal adenocarcinoma[157,158] and pathogenic in noncardiac gastric
515 cancer.[155] Most *H. pylori*-positive patients carry multiple strains, including at least one strain
516 unique to their body along with more common strains such as VacA, CagA, and BabA.[156] This
517 extreme genetic diversity stems from slipped-strand mispairing in multiple genes and *H. pylori's*
518 lack of DNA repair genes unlike most bacteria.[156] High strain diversity across individual human
519 hosts also enhances *H. pylori*'s population-wide resilience, allowing it to adapt to many diverse

520 environments by expanding upon the strain with the highest fitness in that setting. Collectively,
521 high diversity and concomitant mutagenesis of *H. pylori* combined with human immune selection
522 pressures and pathological impacts on noncardiac gastric carcinogenesis help portray an
523 exemplary “big picture” of multispecies cancer evolution. Building on this idea, we describe how
524 existing clonal evolution modeling may take intratumoral microbes into account.
525
526 **Common constraints of the tumor microenvironment**
527
528 As detailed above, the TME contains intracellular and extracellular microbes that affect cancer
529 clonality and comprise independent clonal agents. Importantly, the TME contexture applies
530 simultaneous, shared selective pressures and environmental constraints on co-located cancer
531 cells and microbes. Shared resources necessitate cooperative use and/or competition, which may
532 further limit their abundance. For instance, oxygen availability drives spatial organization and
533 metabolic capacities of cancer cells[159] and is known to similarly affect microbes in environmental
534 contexts and model systems (e.g., Winogradsky columns).[160–162] Common selection pressures
535 may in turn drive common evolutionary solutions, such as metabolic symbiosis between cancer
536 cells[163] or between microbes positioned along the oxygen gradient.[162] Gradients of pH are tied
537 to oxygen and common in tumors,[159] and they shape microbial compositions in environmental
538 contexts.[164] Hence, multispecies evolutionary models should take into account joint
539 environmental constraints.
540
541 Anderson and colleagues have presented compatible multiscale mathematical models of cancer
542 growth that take into account both cellular biophysical properties and TME factors.[165–167] Their
543 model determined that aggressive cancer clones were established under the harshest TME
544 conditions (e.g., hypoxia, heterogenous extracellular matrix) but that their impact on overall tumor
545 invasiveness was blunted under milder TME conditions. Hence, microenvironmentally harsh

546 chemotherapy may worsen long-term cancer invasiveness. Incorporating microbes into their
547 multiscale model equations—their reliance and impact on TME chemical gradients and cancer
548 metabolism—could inform multispecies clonal dynamics and ideal TME conditions that in turn
549 would inform multispecies therapeutic strategies.
550
551 **Microbes affect clonal fitness**
552
553 As detailed above, intratumoral microbes affect cancer cell fitness, justifying their inclusion to
554 accurately model clonal evolution. Current models typically account for factors like probabilities
555 of cell division and cell death alongside inferred mutation rates and human driver genes. However,
556 intracellular microbes likely need to be included in these equations as well in certain scenarios,
557 particularly their mutational, division, and death rates. In circumstances of microbial enzymatic
558 degradation, such as CDD_L-mediated gemcitabine metabolism,[47] transcriptional rates and
559 enzymatic efficiencies may be useful variables to include. In circumstances of genotoxin-mediated
560 mutagenesis, such as from *pks*$^+$ *E. coli*,[131] the base-pair motif and rate of mutations may also be
561 instructive to include.
562
563 Likelihood of clone development, treatment resistance, and fitness are all major parts of clonal
564 evolution models and are related to extracellular and intracellular microbes, but these have not
565 been typically considered in models of cancer evolution thus far. In common population genetics
566 models of clonal evolution, including Wright-Fisher diffusion type models[168] and Moran type
567 models,[169] it may be helpful to consider clonal fitness as a function of time-dependent fluctuations
568 in microbial abundances or presence/absence of particular species. Additionally, branching
569 process stochastic models of tumor growth that parameterize evolution in terms of proliferation
570 and mutation rates[170–172] may also benefit from considering microbial colonization rates and
571 species-specific transcriptional effects. Furthermore, cancer microbes individually (and likely

572 jointly) undergo somatic clonal evolution, as described in the case of *H. pylori*. Phylogenetic tree
573 reconstructions of clonal evolution[173] may thus need to include multispecies lineages, but
574 specialized methods likely need to be created for this purpose. To summarize, we have created
575 a table of major clonal evolution models and suggested strategies for incorporating microbial
576 information into them (**Table 2**).
577
578 **CONCLUSIONS**
579
580 Rigorous studies provide extensive evidence for the existence and functionality of cancer-
581 associated gut and intratumoral microbes while echoing ancient historical narratives of microbial-
582 mediated recovery. Drawing from cancer genomics, the inherently high diversity of the cancer
583 microbiome substantiates its strong predictive power, even when any individual microbe is rare
584 or lowly abundant. Cancer microbiota can distinguish healthy, pre-cancer, and cancerous tissues
585 across multiple cancer and sample types, although most diagnostics remain unvalidated in large,
586 multi-national, prospective cohorts. Cancer microbiota also demonstrate stage-specific
587 differences that may enable simultaneous identification and prognostication of tumors.
588 Nonetheless, contamination challenges in low-biomass settings and analytic idiosyncrasies of
589 microbiomic data have hitherto complicated routine clinical application of cancer microbial
590 diagnostics or prognostics.
591
592 Numerous microbial mechanisms affect the cancer genome, transcriptome, proteome, and
593 metabolome, advocating for their inclusion in models of cancer evolution. Extracellular and
594 intracellular microbes affect virtually every cancer medication class and may drive therapeutic
595 efficacy or resistance without any cancer cell(s) interventions. Negatively, it is not possible to
596 accurately model cancer-drug dynamics, clonality, or fitness without accounting for microbes.
597 Serious consideration of multispecies clonality, however, is complex and necessitates reworking

598 cancer evolution models since microbes carry distinct, although plausibly shareable, genetic
599 cargo that may undergo discordant selection pressures from the cancer genome. Flexible
600 evolutionary models treating intratumoral microbes as independent, albeit rule-abiding agents
601 within the TME may be appropriate. Multispecies clonality also informs treatments, such that
602 intentional modifications of cancer pathways may comprise more effective ways to restore healthy
603 microbial ecologies than targeted antimicrobials, and vice versa. Multispecies treatment
604 strategies may further benefit from target selectivity, for targeting microbial genes generally
605 carries fewer side effects than targeting the host's. Altogether, understanding cancer's
606 metagenome carries key ramifications for cancer care and clonal evolution for the benefit of
607 patients worldwide.
608
609
610 **TABLE 2. Microbial integration into mathematical models of evolution.** Overview of each
611 model's characteristics and references provided with modeling examples, as well as suggested
612 ways that microbes could be potentially incorporated into model structure. Hybrid models that
613 include aspects of more than one model type are also utilized in practice.
614

Model Type	Overview of Model	Examples of Potential Incorporation of Microbes	Literature
Agent-based model	● Define 'agents' : individuals or members of the microenvironment with specific properties and actions on a structured grid or 3-D space ● Can have stochastic and deterministic components with spatial constraints ● Define environmental rules and presence of factors in space such as signalling proteins like VEGF ● Define agent-agent interaction rules	● Create microbe as one agent type and cancer cell as another agent type ● Allow clonal evolution of cancer cells and separate evolution of microbes in equations ● Create biophysical rules accounting for spatial movement of microbes and effect of microbes on evolutionary rates such as proliferation and survival of cancer cells ● Introduce complexity with microbes as agents within the	[174,175]

		microenvironment	
Wright-Fisher type model	● Population size remains constant over time (can be extended to growing populations) ● Considers finite number of population species/k-alleles ● To create the next non-overlapping generation, alleles are randomly sampled with replacement ● Allele frequency in new generation is combination of random sampling of population and the fitness of alleles ● Captures genetic drift and natural selection if included	● Microbes will likely not be directly considered in the population species, but instead the effects of microbes will be interwoven into fitness ● Fitness parameter of certain genotypes may depend on metabolites, proteins, and antigens from intracellular bacteria, which in certain cases may drive differential immunoediting between cancer cell-bearing bacteria	[168,176]
Moran-type model	● Two or more species considered in a population ● Asexual reproduction, overlapping generations ● Simultaneous birth and death events occur ● As in the Wright-Fisher model, can be formulated as a diffusion approximation	● Similar to the Wright-Fisher type model, microbes will likely not be directly considered in the population species, but instead the effects of microbes will be interwoven into fitness ● Fitness parameter of certain genotypes may depend on metabolites, proteins, and antigens from intracellular bacteria, which in certain cases may drive differential immunoediting between cancer cell-bearing bacteria	[169,177]
Birth-death stochastic process	● Continuous time Markov model (branching process) where ‘birth’ or ‘death’ events can change the state/population size ● A ‘birth’ increases the state by one ● A ‘death’ decreases the state by one ● Allows for multiple cell types (e.g., with/without driver mutations), fluctuations in total population size, stochastic extinction of cells, and mutation to other types	● Option 1 ○ Birth and death defined in terms of human cancer cells with: ■ Probability of birth and/or death affected by microenvironment ■ Probability of birth and/or death dependent on a function of the population of intratumoral microbes present ● Option 2	[178,179]

		○ Birth and death events defined in terms of both human cancer cells and microbial populations	
Evolutionary game theory model	● Includes density-dependent fitness with cell-cell interactions ● Models cooperation, e.g., between tumor and stromal cells ● Fitness landscapes in non-cancer models have been central to understanding microbial evolution such as *E. coli*	● Include microbes as a type of "player" in the modeled ecosystem including tumor cells for limited chemicals and nutrients (e.g., oxygen, sugars, etc.) ● "Public good" produced by tumor cells, such as glycolytic acid and vascular endothelial growth factor, included in a game as competing resources with microbial populations	[180–183]

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1368

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1382

1383 **CONFLICTS OF INTEREST**

1384

1385 G.D.S.-P. and R.K. are inventors on a US patent application (PCT/US2019/059647) submitted by

The Regents of the University of California and licensed by Micronoma; that application covers methods of diagnosing and treating cancer using microbial biomarkers in blood and cancer tissues. G.D.S.-P. and R.K. are founders of and report stock interest in Micronoma. G.D.S.-P. has filed several additional US patent applications on cancer microbiome diagnostics that are owned by The Regents of the University of California. R.K. additionally is a member of the scientific advisory board for GenCirq, holds an equity interest in GenCirq, and can receive reimbursements for expenses up to US $5,000 per year.