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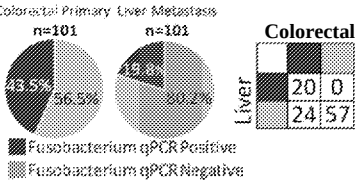
(54) Title: CANCER DIAGNOSTIC AND TREATMENT

(57) Abstract: Bacterialspecies and the associated microbiome persist in tumors and metastases. Antibiotic treatment selectively reduces microbiome-induced tumor growth and can advantageously be included in treatment regimens. Accordingly, the present invention disclosure relates to, for example, the diagnosing cancer in a subject and providing identifying an effective treatment regimen for the subject.

FIG. 1A



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CANCER DIAGNOSTIC AND TREATMENT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/534,672, filed July 19, 2017. The entire contents of the above-identified application are hereby fully incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grant number CA197568 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[0003] The subject matter disclosed herein is generally directed to methods for diagnosing and treating cancers in a subject. More specifically, the disclosure relates to methods of inhibiting bacterial growth for treating neoplasms in a subject, or to methods of diagnosing whether a patient has a bacterial infection in or associated with the neoplasm.

BACKGROUND

[0004] Cancers, like other diseased or healthy tissues, are comprised of a subject's own cells encoded by their own genomes, together with a diverse population of associated microorganisms (the microbiota). The microbiota and host form a complex "super-organism" in which symbiotic relationships confer benefits necessary for human health in addition to pathogenic consequences. Recent studies have demonstrated that perturbations in the composition of the human microbiota, or dysbiosis, can significantly increase the risk of specific cancer types, especially colorectal cancer (CRC). The human colon is the anatomical location with the largest number of microbes; a growing body of evidence demonstrates the role of particular microorganisms in modulating inflammatory environments and promoting tumor growth and metastasis.

[0005] For example, *Fusobacterium* is a genus of obligate anaerobic, Gram-negative bacteria that usually colonize in the oral cavity of nearly all humans, some strains of *Fusobacterium* contribute to the development of dental plaques and periodontal disease. These bacteria are poor colonizers of healthy colon mucosa and cannot breach the intact

colon wall. However, when an inflammation, adenoma or carcinoma develops, the deteriorated microenvironment of the colon wall may allow these microorganisms including *Fusobacterium, Peptostreptococcus,* and *Lactococcus* to access and adhere the basement membrane. One of reasons may be the formation of local anaerobic microenvironment induced by aerobic bacteria such as pseudomonas, which is suitable for these potential pathogens to colonize. Different from *Fusobacterium,* however, *Lactococcus,* which produce a single product-lactic acid, plays a probiotic role in colon. It is not yet clear whether these bacterial passengers merely benefit from the CRC microenvironment or they also play an active part in disease progression.

[0006] Many cancers are known to be caused by infection, such as cervical cancer (caused by human papillomavirus), hepatocellular cancer (caused by hepatitis B virus) and Burkitt's lymphoma (caused by the Epstein-Barr virus). Similarly, some auto-immune diseases are caused by infection, such as rheumatic heart valvular disease caused by a streptococcal infection and gastritis and gastric ulcers, caused by a *Helicobacter pylori* infection. Many other chronic diseases (such as cancers, inflammatory diseases and autoimmune diseases) are suspected to be caused by pathogens, yet the specific microbe, if any, is unknown.

[0007] Computational subtraction methods for pathogen discovery are contemplated for pathogen discovery. The principle behind sequence-based computation subtraction is that the human genome sequence is nearly complete and that infected tissues contain human and microbial RNA and DNA. The method entails generating and sequencing libraries from human tissue and computational subtraction of normal human sequences, wherein the remainder sequences are of non-human origin, thereby allowing disease-specific sequences to be validated experimentally. (see, e.g., Weber, Shendure et al., Nature Genetics, 2002 and Sorek & Safer, Nucleic Acids Research, 2003). Improved genome sequencing technology facilitates pathogen discovery especially since the cost per genome has dropped significantly since 2007 (see, e.g., genome.gov/sequencingcosts).

[0008] Another software for identifying or discovering microbes by deep sequencing of human tissue is PathSeq, which also includes a microbial classification module (see, e.g., Kostic et al., Nature Biotechnology, 201 1). PathSeq has been utilized in pathogen analysis of colorectal cancer/normal genome pairs (see, e.g., Bass et al., Nature Genetics, 201 1 and Kostic et al., Genome Research, 2012). The initial analysis identified tumor-enrichment of *Fusobacterium* and *Streptococcaceae* in colorectal cancer. The analytic method involved counts of bacterial reads from whole genome DNA sequence data from 9 cases and testing

with LEfSe (Segata et al., 2012); linear discriminant analysis (LDA) coupled with effect size measurements, comparing tumor and normal cells. The analysis of 95 cases with 16S PCR data showed enrichment only of *Fusobacteria* (see, e.g., Kostic et al., Genome Research, 2012 and Castellarin et al., Genome Research, 2012, using RNA sequencing).

[0009] Characteristics of *Fusobacterium* species are as follows. It is a Gram (-) , filamentous, anaerobic bacteria, classed as a human pathogen (i.e. a disease causing bacteria), present in the oral cavity and plays a role in periodontal disease and not a common component of the lower gastrointestinal tract (human microbiome project). There is modest promotion of intestinal tumorigenesis by *Fusobacterium* in an *Apc(Min)* model (see, e.g., Kostic et al., Cell Host & Microbe, 2013). *Fusobacterium* is associated with an inflammatory signature in colon cancer (see, e.g., Kostic et al., Cell Host & Microbe, 2013).

[0010] Citation or identification of any document in this application is not an admission that such document is available as prior art to the present invention.

SUMMARY

[0011] The continued association of *Fusobacterium* species with human tumors indicates it is a critical component of the tumor microenvironment. This observation extends beyond *Fusobacterium* to include a range of co-occurring gram-negative anaerobes such as *B.fragilis and S. sputigena. Fusobacterium-associated* colorectal cancers exhibit a distinct microbial signature that is maintained through metastasis and multiple serial passages of xenografts in mice. Antibiotics targeting *Fusobacterium* can retard the growth of *Fusobacterium-emiched* xenografts, demonstrating a key role for *Fusobacterium* infection in the proliferation of colorectal cancer and providing new opportunities for therapeutic intervention.

[0012] Accordingly, the invention provides a method of treating a neoplasm in a subject comprising administering to the subject an agent, compound, or composition that inhibits bacterial growth in the subject. In certain embodiments, the invention further includes diagnosing whether the subject has a bacterial infection.

[0013] According to the invention, a bacterial infection associated with a neoplasm, advantageously a bacterial infection comprising a gram negative bacteria is diagnosed. In certain embodiments, the gram negative bacteria is an Fusobacterium. Fusobacterium is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of Selenomonas, Bacteroides, and Prevotella genera. When diagnosed, the infection is advantageously treated for example with an antibiotic, preferably with an antibiotic selective for the one or more organisms identified.

[0014] The invention is advantageously applied for any neoplasm, tumor, cancer and the like with which the gram negative bacterium, e.g., the *Fusobacterium,* is associated, which can be, without limitation, a gastrointestinal cancer such as a colon cancer, or a metastatic tumor. The infecting bacteria, often having originated from the gut and transported to the location of the neoplasm, can be associated with any neoplasm, tumor, or cancer type, regardless of location. Likewise, neoplasm, tumor or cancer can be metastatic, without regard to the location or tumor type from which it originated.

[0015] The bacterial infection can be detected and identified by any convenient method, and can involve, without limitation, directly detecting and identifying the bacteria or by detecting and identifying any bacterial component. For example, the bacteria or component thereof can be identified in a tumor biopsy, in a stool sample, in circulating in plasma, and the like. Advantageously, the bacteria can be identified in nucleic acids samples from the subject. In preferred embodiments, detection and identification of nucleic acid components is sensitive and rapid, using for example, CRISPR based identification methods.

[0016] In embodiments of the invention, agent, compound, or composition comprises an antibiotic effective against the identified bacteria. Preferably, the agent is selective for the bacteria. For example, in an embodiment of the invention, the bacteria is a *Fusobacterium* and the antibiotic or antibacterial agent selectively targets the *Fusobacterium. Fusobacterium* is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of *Selenomonas, Bacteroides,* and *Prevotella* genera. In certain embodiments, the agent, compound, or composition can target such bacteria instead of or in addition to *Fusobacterium.* In certain embodiments, when the identified bacteria is *Fusobacterium,* the selective agent comprises metronidazole or 5-fluorouracil. In an embodiment of the invention, the treatment method can comprise diet modification. In a non-limiting example, a subject is assigned or prescribed a diet low in fat, low in lipid content, and or low in carbohydrates. According to the invention, the method can comprise coadministration of the antibacterial agent with any suitable antineoplastic treatment.

[0017] In another aspect, the invention provides a method of identifying a cellular component or pathway linked to sensitivity to growth induction by a *Fusobacterium,* which comprises contacting a test cell which comprises a mutation or phenotype linked to the cellular component or pathway with a *Fusobacterium,* contacting a control cell with the *Fusobacterium,* and identifying the cellular component or pathway as linked to sensitivity to induction by *Fusobacterium* if the test cell and control cell display a *Fusobacterium*-dependent phenotypic change. In certain embodiments, the method comprises comparing a

library of test cells to a control cell. Further, the test cell or library can be from any neoplasm-related cell, including but not limited to a tumor cell, a transformed cell, or a model cell, and can be an experimental cell line or a cell from a patient tumor.

[0018] In an aspect of the invention, there is provided a method of identifying a compound or composition that inhibits induction of a bacterial cell associated with a tumor, for example, *Fusobacterium*, which comprises culturing a mammalian cell with a *Fusobacterium* in the presence of a test compound, culturing the mammalian cell with a *Fusobacterium* in the absence of the test compound, and identifying the compound as an induction inhibitor if the test compound inhibits growth of the test culture. The mammalian cell can be a tumor cell, a transformed cell, or a model cell, and can be an experimental cell line or a cell from a subject tumor.

[0019] In an aspect, there is provided a method of identifying a subpopulation of a population of patients having a neoplasm comprising diagnosing and identifying therefrom patients in the population having a bacterial infection in or associated with the neoplasm, whereby those patients having the bacterial infection in or associated with the neoplasm are the subpopulation. The bacterial infection can comprise a gram negative bacterial infection, including but not limited to a Fusobacterium infection. In certain embodiments, the bacterial infection is in or associated with gastrointestinal cancer or metastatic tumor or is in a stool sample. Further, the invention improves therapy by distinguishing patients having certain bacterial infections in or associated with their neoplasm or tumor from and able to benefit from such treatment from those that do not. Accordingly, the invention identifies subjects that might not benefit from the antibacterial treatment and avoids administration of potentially dangerous and or toxic agents and compounds to subjects that would not benefit. For example, whereas 5-fluorouracil is identified for treatment of subjects having Fusobacterium in or associated with neoplasm, the invention avoids administration of 5-fluorouracil in the absence of such bacteria. 5-fluorouracil has been associated with cardiotoxicity during chemotherapy for adenocarcinoma of the small bowel.

[0020] In an aspect of the invention, there is provided a method of identifying a treatment regimen for a patient having a neoplasm comprising diagnosing whether the patient has a bacterial infection in or associated with the neoplasm. In an embodiment of the invention, the infection comprises a gram negative bacterial infection, including but not limited to a *Fusobacterium* infection. *Fusobacterium* is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of *Selenomonas, Bacteroides,* and *Prevotella* genera. In certain embodiments, such bacteria can be diagnosed instead of or in

addition to *Fusobacterium.* The bacterial infection can be associated with a gastrointestinal cancer or metastatic tumor or identified in a stool sample. The method can additionally include administering an agent, compound or composition that inhibits bacterial growth in the subject, optionally wherein the agent, compound or composition is specific to the bacterial infection, and further optionally on a regimen comprising coincident with or sequentially to administration of anti-neoplastic agents.

[0021] The invention also provides a method for removal of a tumor in a subject comprising systemically and/or locally to the tumor testing for the presence of a bacterial infection, optionally wherein the testing comprises a rapid diagnostic that identifies an RNA and/or DNA signature of a bacterial, and further optionally wherein the testing includes measuring bacterial load. In an embodiment of the invention, the bacterial infection comprises a gram negative bacterial infection, including but not limited to a *Fusobacterium* infection. *Fusobacterium* is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of *Selenomonas, Bacteroides,* and *Prevotella* genera. In certain embodiments, such bacteria can be diagnosed instead of or in addition to *Fusobacterium.* The bacterial infection can be in or associate with, without limitation a gastrointestinal cancer or metastatic tumor or is in a stool sample. In certain embodiments, the method additionally includes administering an agent, compound or composition that inhibits bacterial growth in the subject. The administering can be locally to the area of the patient from which the tumor has been or is being removed, and/or can be systemic.

[0022] Accordingly, it is an object of the invention not to encompass within the invention any previously known product, process of making the product, or method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product. It may be advantageous in the practice of the invention to be in compliance with Art. 53(c) EPC and Rule 28(b) and (c) EPC. All rights to explicitly disclaim any embodiments that are the subject of any granted patent(s) of applicant in the lineage of this application or in any

other lineage or in any prior filed application of any third party is explicitly reserved Nothing herein is to be construed as a promise.

[0023] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of and "consists essentially of have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[0024] These and other aspects, objects, features, and advantages of the example embodiments will become apparent to those having ordinary skill in the art upon consideration of the following detailed description of illustrated example embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] An understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention may be utilized, and the accompanying drawings of which:

[0026] **Figs. 1A-1F:** *Fusobacterium* colonizes liver metastases of *Fusobacterium*-associated colorectal primary tumors. **Fig. 1A:** Pie charts represent the overall *Fusobacterium* qPCR status of matched primary colorectal cancers and liver metastases from 101 patients. The two-by-two table represent the number of primary-metastasis pairs (individual patients) that were *Fusobacterium* qPCR positive (dark grey) or negative (light grey). **Fig. IB:** Schematic of *Fusobacterium* culture and *Fusobacterium* targeted qPCR status of paired snap frozen colorectal primary tumors and liver metastases from eleven patients (PI to Pll). **Fig. 1C:** Species level microbial composition of paired colorectal primary (CP) tumors and liver metastases (LM) tumors, assayed by RNA sequencing followed by PathSeq analysis for microbial identification. For simplicity only organisms with >2% relative abundance (RA) in at least one tumor are shown. The colors correspond to bacterial taxonomic class, red: Fusobacteriia, pink: Negativicutes, blue/green: Bacteroidia, orange: Clostridia, yellow: Gamma-proteobacteria, dark brown: Spirochaetes. P=patient. The samples are separated into three groups: *Fusobacterium-positive* primary tumor and metastases (n=6 pairs),

Fusobacterium-positive primary tumor and *Fusobacterium-negative* metastases (n=2 pairs), *Fusobacterium-negative* primary tumor and metastases (n=2 pairs). Patient 7 (P7) had insufficient tissue for RNA sequencing analysis. **Fig. ID:** Box plots represent the Jaccard Index (proportion of shared genera/species) between paired colorectal primary (CP) tumors and liver metastases (LM) at both the genus and species level at 1% RA. The box represents the first and third quartiles, and error bars indicate 95% confidence of the median. Paired samples that were *Fusobacterium-positive* in both the primary tumor and metastasis are compared to paired samples where the metastasis was *Fusobacterium-negative*. P values were determined using Welch Two Sample t-test. **Fig. IE:** Box plots of Fusobacteria relative abundance in primary COAD (n=435) and primary LIHC (n=201) from TCGA and primary-metastasis pairs from ten patients. The box represents the first and third quartiles, and error bars indicate 95% confidence of the median. P values were determined using Welch Two Sample t-test with correction for unequal variances. **Fig. IF:** Identification of bacteria that co-occur with *Fusobacterium* in primary COAD. Primary COAD tumors were subset into two groups, *Fusobacterium* "High" if they had a *Fusobacterium* RA >1% (n=110, median RA=5.72%, mean RA=7.7%) and *Fusobacterium* "Low/Neg" if they had a RA <1% (n=325, median RA=0.06%, mean RA=0.16%). The bar plot demonstrates genera enriched (red) and depleted (green) in COAD with >1% *Fusobacterium* RA. The cladogram lists the respective bacterial phyla and class.

[0027] Figs. 2A-2H: *F. nucleatum* RNA *in situ* hybridization analysis of matched primary colorectal tumors and liver metastases. Representative images of *F. nucleatum* spatial distribution in paired samples from patient 187 (PI 87) primary colorectal tumor (Fig. 2A and Fig. **2B)** and liver metastasis (Fig, **2E** and Fig. 2F), and patient 188 (PI 88) primary colorectal tumor (Fig. 2C and Fig. **2D)** and liver metastasis (Fig. 2G and Fig. **2H)** from the "FFPE paired cohort" are shown. Arrows indicate cells whose histomorphology is consistent with colon cancer cells infected by invasive *F. nucleatum* (red dots) in both primary colorectal tumors (Fig. 2B and Fig. 2D) and matched liver metastases (Fig. 2F and Fig. **2H).** *Fusobacterium* containing biofilm (bf) is highlighted in the colorectal tumor of patient 187 (Fig. 2A). *Fusobacterium* was not detected in normal liver (nl) tissue (Fig. 2E, Fig. 2F). Stroma; s. Magnification, (Fig. **2A,** Fig. 2C, Fig. 2E and Fig. **2G),** inset magnification, (Fig. 2B, Fig. 2D, Fig. 2F and Fig. *IB).* The black boxes represent areas of the images selected for the corresponding inset magnification image. Scale bars are included at the bottom left of each image.

[0028] **Figs. 3A-3C:** *Fusobacterium* and co-occurring anaerobes persist in colon adenocarcinoma patient derived xenografts. **Fig. 3A:** The box plots demonstrate PDX engraftment success rate in relation to *Fusobacterium* culture or qPCR status. P values were determined using the *z* score test for two population proportions. **Fig. 3B:** Assessment of *Fusobacterium* persistence in PDX COCA36 over the period of 204 days. *Fusobacterium* persistence was determined via microbial culture and *Fusobacterium-targeted* qPCR. *Note that there are no negative timepoints. **Fig. 3C:** Species level microbial composition of three patient primary colon adenocarcinomas (COCA36, COCA39 and COCA6) and subsequent PDX's. Unbiased RNA sequencing, followed by PathSeq analysis for microbial identification. For simplicity selected species with >1% relative abundance in the primary tumor and either correspond PDX are show. The colors correspond to bacterial taxonomic class, red: Fusobacteriia, pink: Negativicutes, blue/green: Bacteroidia, orange: Clostridia.

[0029] **Figs. 4A-4D:** Primary colon cancer and derived xenograft *F. nucleatum* isolates are invasive. **Fig. 4A:** Transmission electron microscopy (TEM) of primary colon cancer *F. nucleatum* isolate (Fn-COCA36P-01) adhering to and invading HT-29 colon cancer cells. Black arrows demonstrate the presence of *F. nucleatum* cells. Cyto = cytoplasm, Nucl = nucleus. **Fig. 4B:** TEM of PDX *F. nucleatum* isolate (Fn-COCA36F3-01) adhering to and invading HCT-116 colon cancer cells. **Fig. 4C:** TEM of PDX *F. nucleatum* isolate *(Fn*-COCA36F3-01) adhering to and invading HT-29 colon cancer cells. **Fig. 4D:** TEM of patient derived xenograft (COCA36). Black arrows demonstrate the presence of bacterial cells adhering to and invading the PDX cells.

[0030] **Figs. 5A-5D:** Treatment of *Fusobacterium* colonized PDX with metronidazole reduces tumor growth *in vivo.* **Fig. 5A:** Percentage tumor volume increase of *Fusobacterium*-free xenograft derived from HT-29 cells treated with metronidazole (treated) or with vehicle (untreated). P values, determined using a Welch's Two Sample t-test, were not statistically significant between the two conditions, at any of the tested timepoints ($p>0.05$). Tumors were measured in a blinded fashion on Mondays, Wednesdays and Fridays each week. Error bars represent the mean +/- SEM. **Fig. 5B:** Percentage tumor volume increase in *Fusobacterium*-positive PDX tumors (COCA36) treated with metronidazole (treated) or with vehicle (untreated). P values were determined using the Welch's Two Sample t-test (* $p<0.05$, ** $p<0.01$). Tumor volumes were measured as in **Fig. 5A. Fig. 5C:** Assessment of *Fusobacterium* tissue load. *Fusobacterium-targeted* qPCR on PDX tissue (COCA36) following treatment with metronidazole (treated) or with vehicle (untreated). ND =not

detected. The center bar represents the mean and the error bars are the standard error of the mean (SEM). P values were determined using Welch Two Sample t-test. **Fig. 5D:** BrdU immunohistochemistry of PDX tumors to assess cell proliferation. The 40x images represent BrdU staining (brown) of PDXs following treatment with metronidazole (treated) or with vehicle (untreated). The bar plot represents the percentage of cells with BrdU incorporation in treated and untreated PDXs (n=6 per arm); error bars represent the mean +/- the SEM. The p values were determined using the Welch Two Sample t-test.

[0031] **Fig. 6A-6B:** Identical strains of *Fusobacterium* colonize both the primary colorectal tumor and liver metastasis of respective patients. **Fig. 6A:** Whole genome sequencing and average nucleotide identity (ANI) analysis reveal identical an identical strain of *F. necrophorum* in both the colorectal primary and liver metastasis of patient 1 (PI) despite these tumors being collected two years apart. **Fig. 6B:** Whole genome sequencing and average nucleotide identity (ANI) analysis reveal identical an identical strain of *F. nucleatum* in both the colorectal primary and liver metastasis of patient 2 (P2) despite these tumors being collected three months apart.

[0032] **Fig.** 7: The dominant bacterial genera and species in colorectal primary tumors are also dominant genera and species in corresponding liver metastasis. Box plots represent the Jaccard Index (proportion of shared genera/species) between paired colorectal primary (CP) tumors and liver metastases (LM) at both the genus and species level at 1% relative abundance (RA) and <1% RA of the *Fusobacterium-positive* primary-metastases pairs (n=6). The box represents the first and third quartiles, and error bars indicate 95% confidence of median. P values were determined using the *z* score test for two population proportions (two-tailed), the number of shared organisms between individual patient primary-metastasis pairs were compared at >1% and <1% RA genera and species, in all cases the $p<0.05$.

[0033] **Fig. 8:** *Fusobacteria* are not present in the oral cavity or fecal pellets of Nu/Nu mice in our study. Microbial analysis of murine fecal pellets and oral swabs. Whole genome sequencing and PathSeq analysis of five fecal pellets and two oral swabs from Nu/Nu mice demonstrates that these mice are not carrying *Fusobacterium* in their oral cavity or in their fecal pellets. *Fusobacterium* qPCR also confirmed that the Nu/Nu mice were *Fusobacterium* negative in the lower and upper gastrointestinal tract.

[0034] **Figs. 9A-9C:** Bacterial 16S rRNA gene sequencing demonstrates *Fusobacterium* colonizes liver metastases of *Fusobacterium-associated* colorectal primary tumors (frozen paired cohort). **Fig. 9A:** Genus level (OTU_L7) microbial composition of paired colorectal primary (CP) tumors and liver metastases (LM) tumors from eleven patients (PI to PI 1),

assayed by bacterial 16S rRNA gene sequencing. For simplicity only organisms with >2% relative abundance (RA) in at least one tumor are shown. The samples are separated into three groups: *Fusobacterium-positive* (>1% RA) primary tumor and metastases (n=7 pairs), *Fusobacterium-positive* primary tumor and *Fusobacterium-negative* (<1% RA) metastases (n=l pair), *Fusobacterium-negative* primary tumor and metastases (n=3 pairs). **Fig. 9B:** The box-plot represent the Jaccard Index (proportion of shared genera) between paired colorectal primary (CP) tumors and liver metastases (LM) at 1% RA. The box represents the first and third quartiles, and error bars indicate 95% confidence of the median. Paired samples that were *Fusobacterium-positive* in both the primary tumor and metastasis are compared to paired samples where the metastasis was *Fusobacterium-negative.* The p-values were determined using Welch Two Sample t-test. **Fig. 9C:** Linear regression analysis of *Fusobacterium* RA (%) in paired primary colorectal tumors and liver metastasis from eleven patients (PI to P I 1). The point for P9, P10 and P I 1 are overlap on the x and y axes.

[0035] Figs. 10A-10D: *Fusobacterium* associated cecum/ascending colon tumors. **Fig. 10A:** Pie charts represent the overall *Fusobacterium* qPCR status of matched primary colorectal cancers and liver metastases from 101 patients from the "FFPE paired cohort". The two-by-two table represents the number of primary-metastasis pairs (individual patients) that were *Fusobacterium* qPCR positive (dark grey) or negative (light grey). **Fig. 10B:** The heat-map demonstrates *Fusobacterium* status across the colon and rectum of 101 colorectal adenocarcinoma tumors with liver metastasis from the "FFPE paired cohort". The 101 patients are divided into three groups: those that were *Fusobacterium* qPCR positive in the colorectal primary (CP) and liver metastasis (LM) n=20, those that were *Fusobacterium* qPCR positive in the CP and *Fusobacterium* qPCR negative in the LM n=24, and those that were *Fusobacterium* qPCR negative in the CP and LM n=57. Color scale represents the percentage of tumors from different regions of the colon, within each group. P values in the adjacent table were determined using the *z* score test for two population proportions (two-tailed), comparing the *Fusobacterium* CP-LM double positive tumors (n=20) to the *Fusobacterium* CP-LM double negative tumors. **Fig. IOC:** Kaplan Meier curves of patient overall survival from TCGA colon adenocarcinomas ("TCGA cohort", n=405/430 with survival data) based upon tumor location (cecum/ascending vs. non-cecum ascending) and **Fig. 10D:** *F. nucleatum* load (determined by PathSeq analysis) in cecum/ascending colon adenocarcinomas. RA=relative abundance. P values were determined by the Log-rank Mantel-Cox test. *Fusobacterium* "High" = *Fusobacterium* >1% relative abundance.

[0036] **Figs. 11A-11B:** Analysis of tumor grade from "xenograft cohort" **(Fig. 11A)** Fischer's exact test for patient derived xenograft (PDX) success and tumor grade **(Fig. 11B)** Fischer's exact test for *Fusobacterium* culture and tumor grade

[0037] **Fig. 12:** Bacterial 16s rRNA sequencing demonstrating *Fusobacterium* and co-occurring anaerobes persist in colon adenocarcinoma patient derived xenografts (PDXs). Genus level (OTU_L7) microbial composition of three patient primary colon adenocarcinomas (COCA36, COCA39 and COCA6) and subsequent PDX's from the "xenograft cohort". Bacterial 16s rRNA sequencing was used for microbial identification. For simplicity, only species with >1% relative abundance in the primary tumor and correspond PDX are shown.

[0038] **Figs. 13A-13D:** Representative images of RNA ISH detecting *F. nucleatum* spiked in HCT1 16 cell lines. **Fig. 13A:** *F. nucleatum* was not detected in non-infected HCT1 16 cells, multiplicity of infection (MOI) of bacteria to eukaryotic cells 0:1, **(Fig. 13B-Fig. 13D)** Increasing *F. nucleatum* signals (red dots) were associated with increasing doses of *F. nucleatum* in cell lines. **Fig. 13B:** MOI bacteria to eukaryotic cells 1:1, **Fig. 13C:** MOI bacteria to eukaryotic cells 10:1, **Fig. 13D:** MOI bacteria to eukaryotic cells, 100:1

[0039] **Fig. 14:** Antibiotic sensitivity testing (E-test) of clinical *F. nucleatum* isolates. E-test on *F. nucleatum* strains isolated from COCA36 primary tumor and derived xenografts. All E-tests were carried out in triplicate on both FAA agar. The antibiotics tested were metronidazole (MZ), cefoxitin (FX) and imipenem (IP). All *F. nucleatum* strains tested were sensitive to cefoxitin and metronidazole *in vitro:* test range was 0.016 to 256 ug/ml. No bacterial growth at 0.016ug/ml, MIC <0.016ug/ml. The *F. nucleatum* strains tested were resistant/hetero-resistant to imipenem *in vitro.*

[0040] **Fig. 15:** Metronidazole susceptibility testing with four *Fusobacterium* clinical isolates. Test range: 0.058 -30 μM (0.01 - 5.13 μg/ml). The center bar represents the mean and the error bars are the standard error of the mean (SEM).

[0041] **Fig. 16** depicts determining threshold to identify inhibitor hits. Neutral Control = untreated *F. nucleatum.* Inhibitor Control = *F. nucleatum* treated with metronidazole. 3 SD from mean of neutral controls = -86% inhibition hit cut-off.

[0042] **Fig. 17** depicts identifying compounds that inhibit *Fusobacterium* growth. The CRC drug, 5-FU, is a potent inhibitor of *Fusobacterium* growth. 3 SD from mean of neutral controls = -86% inhibition hit cut-off. 34 inhibitory compounds identified.

[0043] **Fig. 18** depicts validation of inhibitor hits (and related compounds). The 5-FU prodrug Carmofur is a potent inhibitor of *Fusobacterium* growth. Validation/dose plates: 8-

point, 2-fold dilutions, range 30 - 0.23 μM. Dose Response Curves [0.32 - 30 μM]: COCA36F3 *F. nucleatum* (colorectal cancer isolate).

[0044] **Fig. 19** depicts validation of with additional patient CRC isolates of *Fusobacterium.* 5-Fluorouracil inhibits *Fusobacterium* growth at levels comparable to achievable doses in plasma of treated patients. (Takimoto et al., *Clinical cancer research : an official journal of the American Association for Cancer Research* 5, 1347-1352 (1999)).

[0045] **Fig. 20** illustrates a comparison between tumors treated with 5-FU or a 5-FU prodrug (right panel) and tumors with no treatment (left panel). A total of 91 patient colorectal adenocarcinoma tumors with recurrence data were analyzed by *Fusobacterium* culture and bacterial 16s rRNA sequencing. As shown, *Fusobacterium-positive* colorectal tumors treated with 5-FU or a 5-FU prodrug are less likely to develop recurrence/metastasis. * indicates *Fusobacterium-culture* positive and/or *Fusobacterium* >1% relative abundance (microbiome analysis).

DETAILED DESCRIPTION OF THE EXAMPLE EMBODIMENTS

General Definitions

[0046] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure pertains. Definitions of common terms and techniques in molecular biology may be found in Molecular Cloning: A Laboratory Manual, 2nd edition (1989) (Sambrook, Fritsch, and Maniatis); Molecular Cloning: A Laboratory Manual, 4th edition (2012) (Green and Sambrook); Current Protocols in Molecular Biology (1987) (F.M. Ausubel et al. eds.); the series Methods in Enzymology (Academic Press, Inc.): PCR 2: A Practical Approach (1995) (M.J. MacPherson, B.D. Hames, and G.R. Taylor eds.): Antibodies, A Laboraotry Manual (1988) (Harlow and Lane, eds.): Antibodies A Laboraotry Manual, 2nd edition 2013 (E.A. Greenfield ed.); Animal Cell Culture (1987) (R.I. Freshney, ed.); Benjamin Lewin, Genes IX, published by Jones and Bartlet, 2008 (ISBN 0763752223); Kendrew *et al.* (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0632021829); Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 9780471 185710); Singleton *et al.,* Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, N.Y. 1994), March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 4th ed., John Wiley & Sons (New York, N.Y. 1992);

and Marten H. Hofker and Jan van Deursen, Transgenic Mouse Methods and Protocols, 2nd edition (201 1) .

[0047] As used herein, the singular forms "a", "an", and "the" include both singular and plural referents unless the context clearly dictates otherwise.

[0048] The term "optional" or "optionally" means that the subsequent described event, circumstance or substituent may or may not occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

[0049] The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints.

[0050] The terms "about" or "approximately" as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, are meant to encompass variations of and from the specified value, such as variations of +/-10% or less, *+1-5%* or less, +/-1% or less, and +/-0.1% or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier "about" or "approximately" refers is itself also specifically, and preferably, disclosed.

[0051] As used herein, a "biological sample" may contain whole cells and/or live cells and/or cell debris. The biological sample may contain (or be derived from) a "bodily fluid". The present invention encompasses embodiments wherein the bodily fluid is selected from amniotic fluid, aqueous humour, vitreous humour, bile, blood serum, breast milk, cerebrospinal fluid, cerumen (earwax), chyle, chyme, endolymph, perilymph, exudates, feces, female ejaculate, gastric acid, gastric juice, lymph, mucus (including nasal drainage and phlegm), pericardial fluid, peritoneal fluid, pleural fluid, pus, rheum, saliva, sebum (skin oil), semen, sputum, synovial fluid, sweat, tears, urine, vaginal secretion, vomit and mixtures of one or more thereof. Biological samples include cell cultures, bodily fluids, cell cultures from bodily fluids. Bodily fluids may be obtained from a mammal organism, for example by puncture, or other collecting or sampling procedures.

[0052] The terms "subject," "individual," and "patient" are used interchangeably herein to refer to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets. Tissues, cells and their progeny of a biological entity obtained in vivo or cultured in vitro are also encompassed.

[0053] Various embodiments are described hereinafter. It should be noted that the specific embodiments are not intended as an exhaustive description or as a limitation to the

broader aspects discussed herein. One aspect described in conjunction with a particular embodiment is not necessarily limited to that embodiment and can be practiced with any other embodiment(s). Reference throughout this specification to "one embodiment", "an embodiment," "an example embodiment," means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases "in one embodiment," "in an embodiment," or "an example embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention. For example, in the appended claims, any of the claimed embodiments can be used in any combination.

[0054] All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

Overview

[0055] Associations between different bacteria and various tumors have been reported, and growth of bacteria specifically within tumos following deliberate systemic administration has been demonstrated for numerous bacterial species at preclinical and clinical levels. In some cases, such bacteria are thought to be causative agents of malignancy, but in other cases, bacteria within tumors may arise from spontaneous infection.

[0056] The invention provides a method of treating a neoplasm in a subject comprising administering to the subject an agent, compound, or composition that inhibits bacterial growth in the subject. In certain embodiments, the invention further includes diagnosing whether the subject has a bacterial infection.

[0057] According to the invention, a bacterial infection associated with a neoplasm, advantageously a bacterial infection comprising a gram negative bacteria is diagnosed. In certain embodiments, the gram negative bacteria is an *Fusobacterium.* *Fusobacterium* is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of *Selenomonas, Bacteroides,* and *Prevotella* genera. When diagnosed, the

infection is advantageously treated for example with an antibiotic, preferably with an antibiotic selective for the one or more organisms identified.

[0058] The invention is advantageously applied for any neoplasm, tumor, cancer and the like with which the gram negative bacterium, e.g., the *Fusobacterium,* is associated, which can be, without limitation, a gastrointestinal cancer such as a colon cancer, or a metastatic tumor. The infecting bacteria, often having originated from the gut and transported to the location of the neoplasm, can be associated with any neoplasm, tumor, or cancer type, regardless of location. Likewise, neoplasm, tumor or cancer can be metastatic, without regard to the location or tumor type from which it originated.

[0059] The bacterial infection can be detected and identified by any convenient method, and can involve, without limitation, directly detecting and identifying the bacteria or by detecting and identifying any bacterial component. For example, the bacteria or component thereof can be identified in a tumor biopsy, in a stool sample, in circulating in plasma, and the like. Advantageously, the bacteria can be identified in nucleic acids samples from the subject. In preferred embodiments, detection and identification of nucleic acid components is sensitive and rapid, using for example, CRISPR based identification methods.

[0060] In embodiments of the invention, agent, compound, or composition comprises an antibiotic effective against the identified bacteria. Preferably, the agent is selective for the bacteria. For example, in an embodiment of the invention, the bacteria is a *Fusobacterium* and the antibiotic or antibacterial agent selectively targets the *Fusobacterium. Fusobacterium* is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of *Selenomonas, Bacteroides,* and *Prevotella* genera. In certain embodiments, the agent, compound, or composition can target such bacteria instead of or in addition to *Fusobacterium.* In certain embodiments, when the identified bacteria is *Fusobacterium,* the selective agent comprises metronidazole or 5-fluorouracil. In an embodiment of the invention, the treatment method can comprise diet modification. In a non-limiting example, a subject is assigned or prescribed a diet low in fat, low in lipid content, and or low in carbohydrates. According to the invention, the method can comprise coadministration of the antibacterial agent with any suitable antineoplastic treatment.

[0061] In another aspect, the invention provides a method of identifying a cellular component or pathway linked to sensitivity to growth induction by a *Fusobacterium,* which comprises contacting a test cell which comprises a mutation or phenotype linked to the cellular component or pathway with a *Fusobacterium,* contacting a control cell with the *Fusobacterium,* and identifying the cellular component or pathway as linked to sensitivity to

induction by *Fusobacterium* if the test cell and control cell display a *Fusobacterium*-dependent phenotypic change. In certain embodiments, the method comprises comparing a library of test cells to a control cell. Further, the test cell or library can be from any neoplasm-related cell, including but not limited to a tumor cell, a transformed cell, or a model cell, and can be an experimental cell line or a cell from a patient tumor.

[0062] In an aspect of the invention, there is provided a method of identifying a compound or composition that inhibits induction of a bacterial cell associated with a tumor, for example, *Fusobacterium*, which comprises culturing a mammalian cell with a *Fusobacterium* in the presence of a test compound, culturing the mammalian cell with a *Fusobacterium* in the absence of the test compound, and identifying the compound as an induction inhibitor if the test compound inhibits growth of the test culture. The mammalian cell can be a tumor cell, a transformed cell, or a model cell, and can be an experimental cell line or a cell from a subject tumor.

[0063] In an aspect, there is provided a method of identifying a subpopulation of a population of patients having a neoplasm comprising diagnosing and identifying therefrom patients in the population having a bacterial infection in or associated with the neoplasm, whereby those patients having the bacterial infection in or associated with the neoplasm are the subpopulation. The bacterial infection can comprise a gram negative bacterial infection, including but not limited to a Fusobacterium infection. In certain embodiments, the bacterial infection is in or associated with gastrointestinal cancer or metastatic tumor or is in a stool sample.

[0064] In an aspect of the invention, there is provided a method of identifying a treatment regimen for a patient having a neoplasm comprising diagnosing whether the patient has a bacterial infection in or associated with the neoplasm. In an embodiment of the invention, the infection comprises a gram negative bacterial infection, including but not limited to a *Fusobacterium* infection. *Fusobacterium* is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of *Selenomonas, Bacteroides,* and *Prevotella* genera. In certain embodiments, such bacteria can be diagnosed instead of or in addition to *Fusobacterium.* The bacterial infection can be associated with a gastrointestinal cancer or metastatic tumor or identified in a stool sample. The method can additionally include administering an agent, compound or composition that inhibits bacterial growth in the subject, optionally wherein the agent, compound or composition is specific to the bacterial infection, and further optionally on a regimen comprising coincident with or sequentially to administration of anti-neoplastic agents.

[0065] The invention also provides a method for removal of a tumor in a subject comprising systemically and/or locally to the tumor testing for the presence of a bacterial infection, optionally wherein the testing comprises a rapid diagnostic that identifies an RNA and/or DNA signature of a bacterial, and further optionally wherein the testing includes measuring bacterial load. In an embodiment of the invention, the bacterial infection comprises a gram negative bacterial infection, including but not limited to a *Fusobacterium* infection. *Fusobacterium* is often found accompanied by other bacteria, often anaerobic bacteria including but not limited to one or more of *Selenomonas, Bacteroides,* and *Prevotella* genera. In certain embodiments, such bacteria can be diagnosed instead of or in addition to *Fusobacterium.* The bacterial infection can be in or associate with, without limitation a gastrointestinal cancer or metastatic tumor or is in a stool sample. In certain embodiments, the method additionally includes administering an agent, compound or composition that inhibits bacterial growth in the subject. The administering can be locally to the area of the patient from which the tumor has been or is being removed, and/or can be systemic.

***Fusobacterium* and detection**

[0066] *Fusobacterium* organisms are anaerobic, non-motile, gram-negative bacilli and include F. necrophorum, F. nucleatum, F. mortiferu, F. varium, F. gonidaformans, F. alocis, F. pseudonecrophorum, F. salci, and F. ulcerans. Microscopically, they are characterized by slender or fusiform rods with tapered ends, though some species may be pleomorphic. *Fusobacterium* is included in the genera of anaerobic, gram-negative, non-spore-forming bacteria, which include *Bacteroides*, *Prevotella,* and *Porphyromonas. Fusobacterium* can be differentiated from these other gram-negative, obligate anaerobes by its ability to produce significant amounts of butyric acid from glucose, giving cultured colonies a characteristic odor. Identification in the laboratory is made by morphology and the following biochemical assays:

[0067] Identification of *F. necrophorum* species:

Indole	Positive
Lipase	Positive
Hydrogen sulfide	Negative
Catalase	Negative
Esculin	Negative
Catalase	Negative

[0068] *Fusobacterium* species are normal inhabitants of all mucosal surfaces, including the mouth, upper respiratory tract, gastrointestinal tract, and urogenital tract. Worldwide, *F. nucleatum* is the most common *Fusobacterium* species found in clinical infections, while *F. necrophorum* is the most virulent. The species is generally susceptible to penicillin, clindamycin, and chloramphenicol and resistant to erythromycin and macrolides.

[0069] Though part of the normal flora of human tissues, *Fusobacterium* can invade tissues after surgical or accidental trauma, edema, anoxia, and/or tissue destruction. *F. necrophorum* contains particulary powerful endotoxic lipopolysaccharides in its cell wall and produces a coagulase enzyme that encourages clot formation. Additionally, it produces a variety of exotoxins, including leukocidin, hemolysin, lipase, and cytoplasmic toxin, all of which likely contribute to its pathogenicity.

Antibiotics and dosing

[0070] Anti-gram negative, and gram-negative antibiotic are used interchangeably to refer to antibiotic active agents (and formulations comprising such active agents) which have effectiveness against gram negative bacteria.

[0071] As an overview, in one or more embodiments, an aqueous composition comprises anti-gram-negative antibiotic or salt thereof being present at an amount ranging from about 100 mg/ml to about 200 mg/ml.

[0072] In one or more embodiments, an aqueous composition comprises an antibiotic or salt thereof being present at a concentration ranging from about 0.6 to about 0.9 of the water solubility limit, at 25 C and 1.0 atmosphere, of the antibiotic or salt thereof.

[0073] In one or more embodiments, a unit dose comprises a container and an aqueous composition, comprising an anti-gram-negative antibiotic or salt thereof at a concentration ranging from about 100 mg/ml to about 200 mg/ml.

[0074] In one or more embodiments, a unit dose comprises a container and a powder comprising an antibiotic or salt thereof, wherein the powder is present in an amount ranging from about 550 mg to about 900 mg.

[0075] In one or more embodiments, a unit dose comprises a container; and a powder comprising an antibiotic or salt thereof, wherein the powder is present in an amount ranging from about 150 mg to about 450 mg.

[0076] In one or more embodiments, a method of administering an antibiotic formulation to a patient in need thereof comprises aerosolizing an antibiotic formulation to administer the antibiotic formulation to the pulmonary system of the patient. The antibiotic formulation has

a concentration of anti-gram-negative antibiotic or salt thereof ranging from about 100 mg/ml to about 200 mg/ml.

[0077] In one or more embodiments, a method of administering an antibiotic formulation to a patient in need thereof comprises inserting a tube into a trachea of a patient. The method also comprises aerosolizing an antibiotic formulation to administer the antibiotic formulation to the pulmonary system of the patient. The antibiotic formulation consists essentially of an anti-gram-negative antibiotic or salt thereof and water.

[0078] In one or more embodiments, a method of administering an antibiotic formulation to a patient in need thereof comprises aerosolizing an antibiotic formulation to administer the antibiotic formulation to the pulmonary system of the patient. The antibiotic formulation has a concentration of anti-gram-negative antibiotic or salt thereof ranging from about 100 mg/ml to about 200 mg/ml.

[0079] In one or more embodiments, a method of administering an antibiotic formulation to a patient in need thereof comprises inserting a tube into a trachea of a patient. The method also comprises aerosolizing an antibiotic formulation to administer the antibiotic formulation to the pulmonary system of the patient The antibiotic formulation consists essentially of an anti-gram-negative antibiotic or salt thereof and water.

[0080] The concentration of the antibiotic, corrected for potency, in one or more embodiments, may range from about 40 mg/ml to about 200 mg/ml, such as about 60 mg/ml to about 140 mg/ml, or about 80 mg/ml to about 120 mg/ml. For example, in the case of anti-gram-negative antibiotics or salts thereof, the concentration as corrected for potency may range from about 40 mg/ml to about 200 mg/ml, such as from about 90 mg/ml to about 200 mg/ml, about 110 mg/ml to about 150 mg/ml, or about 120 mg/ml to about 140 mg/ml.

[0081] The aqueous compositions typically have a pH that is compatible with physiological administration, such as pulmonary administration. For example, the aqueous composition may have a pH ranging from about 3 to about **7,** such as about 4 to about 6.

[0082] In addition, the aqueous compositions typically have an osmolality that is compatible with physiological administration, such as pulmonary administration. In one or more embodiments, the aqueous composition may have an osmolality ranging from about 90 mOsmol/kg to about 500 mOsmol/kg, such as 120 mOsmol/kg to about 500 mOsmol/kg, or about 150 mOsmol/kg to about 300 mOsmol/kg.

[0083] In one or more embodiments, the aqueous compositions are stable. For instance, in some cases, no precipitate forms in the aqueous composition when the aqueous composition is stored for 1 year, or even 2 years, at 25 C.

[0084] The potency of the antibiotic or salt thereof may range from about 500 micrograms/mg to about 1100 .mu.g/mg. In one or more embodiments, the potency of anti-gram-negative antibiotics or salts thereof, such as gentamicin, typically ranges from about 500 micrograms /mg to about 1100 micrograms /mg, such as about 600 micrograms /mg to about 1000 micrograms /mg, or about 700 micrograms/mg to about 800 micrograms/mg.

[0085] The chromatographic purity level of the antibiotic or salt thereof typically greater than about 80%, such as greater than about 85%, greater than about 90%, or greater than about 95%. In this regard, there is generally no major impurity greater than about 10%, such as no greater than about 5% or no greater than about *2%.* For instance, the amount of heavy metals is typically less than about 0.005 wt %, such as less than about 0.004 wt %, less than about 0.003 wt %, less than about 0.002 wt %, or less than about 0.001 wt %.

[0086] The amount of antibiotic or other active agent in the pharmaceutical formulation will be that amount necessary to deliver a therapeutically or prophylactically effective amount of the active agent per unit dose to achieve the desired result. In practice, this will vary widely depending upon the particular agent, its activity, the severity of the condition to be treated, the patient population, dosing requirements, and the desired therapeutic effect. The composition will generally contain anywhere from about 1 wt % to about 99 wt %, such as from about 2 wt % to about 95 wt %, or from about 5 wt % to 85 wt %, of the active agent, and will also depend upon the relative amounts of additives contained in the composition. The compositions of the invention are particularly useful for active agents that are delivered in doses of from 0.001 mg/day to 100 mg/day, such as in doses from 0.01, mg/day to 75 mg/day, or in doses from 0.10 mg/day to 50 mg/day. It is to be understood that more than one active agent may be incorporated into the formulations described herein and that the use of the term "agent" in no way excludes the use of two or more such agents.

[0087] The pharmaceutical formulation may also comprise a buffer or a pH adjusting agent, typically a salt prepared from an organic acid or base. Representative buffers comprise organic acid salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid, Tris, tromethamine hydrochloride, or phosphate buffers.

[0088] The pharmaceutical formulation may also include polymeric excipients/additives, e.g., polyvinylpyrrolidones, celluloses and derivatized celluloses such as hydroxymethylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose, Ficolls (a polymeric sugar), hydroxyethylstarch, dextrates (e.g., cyclodextrins, such as 2-

hydroxypropyl-.beta.-cyclodextrin and sulfobutylether-.beta.-cyclodextrin), polyethylene glycols, and pectin.

[0089] The pharmaceutical formulation may further include flavoring agents, taste-masking agents, inorganic salts (for example sodium chloride), antimicrobial agents (for example benzalkonium chloride), sweeteners, antioxidants, antistatic agents, surfactants (for example polvsorbates such as "TWEEN 20" and "TWEEN 80"), sorbitan esters, lipids (for example phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines), fatty acids and fatty esters, steroids (for example cholesterol), and chelating agents (for example EDTA, zinc and other such suitable cations). Other pharmaceutical excipients and/or additives suitable for use in the compositions according to the invention are listed in "Remington: The Science & Practice of Pharmacy", 19.sup.th ed., Williams & Williams, (1995), and in the "Physician's Desk Reference", 52nd ed., Medical Economics, Montvale, N.J. (1998), both of which are incorporated herein by reference in their entireties.

[0090] A skilled person can adjust dosing depending on factors taken into consideration by medical or veterinary practitioners, such as gender, age, general condition or overall health, weight, allergies to antibiotics or structurally similar compounds, and the like.

Nucleic acid detection and diagnostics

[0091] Nucleic acids are a universal signature of biological information. The ability to rapidly detect nucleic acids with high sensitivity and single-base specificity provides a useful tool for diagnosis and monitoring of disease.

Rapid Diagnostics

[0092] Sensitive and rapid diagnostics provide real time detection and adjustment of therapeutic methods. For example, when a tumor is removed, diagnostics can be used to identify bacterial load of one or more bacteria, locally at the site where a tumor is identified and/or removed, as well as systemically. Further, such site directed and rapid diagnostics aid in the systemic and local administration of therapeutics, for example antibacterial agents, preferably antibacterial agents selective or specific to the content of a tumor identified and optionally removed. Likewise, if a target bacteria is not identified, broad based therapy would be adopted.

Bacteria

[0093] The following provides an example list of the types of microbes that might be detected using the embodiments disclosed herein. In certain example embodiments, the microbe is a bacterium. Examples of bacteria that can be detected in accordance with the

disclosed methods include without limitation any one or more of (or any combination of) *Acinetobacter baumanii, Actinobacillus sp., Actinomycetes, Actinomyces sp.* (such as *Actinomyces israelii* and *Actinomyces naeslundii), Aeromonas sp.* (such as *Aeromonas hydrophila, Aeromonas veronii biovar sobria (Aeromonas sobrid),* and *Aeromonas caviae), Anaplasma phagocytophilum, Anaplasma marginale Alcaligenes xylosoxidans, Acinetobacter baumanii, Actinobacillus actinomycetemcomitans, Bacillus sp.* (such as *Bacillus anthracis, Bacillus cereus, Bacillus subtilis, Bacillus thuringiensis,* and *Bacillus stearothermophilus), Bacteroides sp.* (such as *Bacteroides fragilis), Bartonella sp.* (such as *Bartonella bacilliformis* and *Bartonella henselae, Bifidobacterium sp., Bordetella sp.* (such as *Bordetella pertussis, Bordetella parapertussis,* and *Bordetella bronchiseptica), Borrelia sp.* (such as *Borrelia recurrentis,* and *Borrelia burgdorferi), Brucella sp.* (such as *Brucella abortus, Brucella canis, Brucella melintensis* and *Brucella suis), Burkholderia sp.* (such as *Burkholderia pseudomallei* and *Burkholderia cepacia), Campylobacter sp.* (such as *Campylobacter jejuni, Campylobacter coli, Campylobacter lari* and *Campylobacter fetus), Capnocytophaga sp., Cardiobacterium hominis, Chlamydia trachomatis, Chlamydophila pneumoniae, Chlamydophila psittaci, Citrobacter sp. Coxiella burnetii, Corynebacterium sp.* (such as, *Corynebacterium diphtheriae, Corynebacterium jeikeum* and *Corynebacterium), Clostridium sp.* (such as *Clostridium perfringens, Clostridium difficile, Clostridium botulinum* and *Clostridium tetani), Eikenella corrodens, Enterobacter sp.* (such as *Enterobacter aerogenes, Enterobacter agglomerans, Enterobacter cloacae* and *Escherichia coli, including opportunistic Escherichia coli,* such as *enterotoxigenic E. coli, enteroinvasive E. coli, enteropathogenic E. coli, enterohemorrhagic E. coli, enteroaggregative E. coli* and *uropathogenic E. coli) Enterococcus sp.* (such as *Enterococcus faecalis* and *Enterococcus faecium) Ehrlichia sp.* (such as *Ehrlichia chafeensia* and *Ehrlichia canis), Epidermophyton floccosum, Erysipelothrix rhusiopathiae, Eubacterium sp., Francisella tularensis, Fusobacterium nucleatum, Gardnerella vaginalis, Gemella morbillorum, Haemophilus sp.* (such as *Haemophilus influenzae, Haemophilus ducreyi, Haemophilus aegyptius, Haemophilus parainfluenzae, Haemophilus haemolyticus* and *Haemophilus parahaemolyticus, Helicobacter sp.* (such as *Helicobacter pylori, Helicobacter cinaedi* and *Helicobacter fennelliae), Kingella kingii, Klebsiella sp.* (such as *Klebsiella pneumoniae, Klebsiella granulomatis* and *Klebsiella oxytoca), Lactobacillus sp., Listeria monocytogenes, Leptospira interrogans, Legionella pneumophila, Leptospira interrogans, Peptostreptococcus sp., Mannheimia hemolytica, Microsporum canis, Moraxella catarrhalis, Morganella sp., Mobiluncus sp., Micrococcus sp., Mycobacterium sp.* (such as *Mycobacterium leprae,*

Mycobacterium tuberculosis, Mycobacterium paratuberculosis, Mycobacterium intracellular, Mycobacterium avium, Mycobacterium bovis, and *Mycobacterium marinum), Mycoplasm sp.* (such as *Mycoplasma pneumoniae, Mycoplasma hominis,* and *Mycoplasma genitalium), Nocardia sp.* (such as *Nocardia asteroides, Nocardia cyriacigeorgica* and *Nocardia brasiliensis), Neisseria sp.* (such as *Neisseria gonorrhoeae* and *Neisseria meningitidis), Pasteurella multocida, Pityrosporum orbiculare* (Malassezia furfur), *Plesiomonas shigelloides. Prevotella sp., Porphyromonas sp., Prevotella melaninogenica, Selenomonas sp., Proteus sp.* (such as *Proteus vulgaris* and *Proteus mirabilis), Providencia sp.* (such as *Providencia alcalifaciens, Providencia rettgeri* and *Providencia stuartii), Pseudomonas aeruginosa, Propionibacterium acnes, Rhodococcus equi, Rickettsia sp.* (such as *Rickettsia rickettsii, Rickettsia akari* and *Rickettsia prowazekii, Orientia tsutsugamushi (formerly: Rickettsia tsutsugamushi)* and *Rickettsia typhi), Rhodococcus sp., Serratia marcescens, Stenotrophomonas maltophilia, Salmonella sp.* (such as *Salmonella enterica, Salmonella typhi, Salmonella paratyphi, Salmonella enteritidis, Salmonella cholerasuis* and *Salmonella typhimurium), Serratia sp.* (such as *Serratia marcesans* and *Serratia liquifaciens), Shigella sp.* (such as *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei), Staphylococcus sp.* (such as *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hemolyticus, Staphylococcus saprophyticus), Streptococcus sp.* (such as *Streptococcus pneumoniae* (for example *chloramphenicol-resistant serotype 4 Streptococcus pneumoniae, spectinomycin-resistant serotype 6B Streptococcus pneumoniae, streptomycin-resistant serotype 9V Streptococcus pneumoniae, erythromycin-resistant serotype 14 Streptococcus pneumoniae, optochin-resistant serotype 14 Streptococcus pneumoniae, rifampicin-resistant serotype 18C Streptococcus pneumoniae, tetracycline-resistant serotype 19F Streptococcus pneumoniae, penicillin-resistant serotype 19F Streptococcus pneumoniae,* and *trimethoprim-resistant serotype 23F Streptococcus pneumoniae, chloramphenicol-resistant serotype 4 Streptococcus pneumoniae, spectinomycin-resistant serotype 6B Streptococcus pneumoniae, streptomycin-resistant serotype 9V Streptococcus pneumoniae, optochin-resistant serotype 14 Streptococcus pneumoniae, rifampicin-resistant serotype 18C Streptococcus pneumoniae, penicillin-resistant serotype 19F Streptococcus pneumoniae, or trimethoprim-resistant serotype 23F Streptococcus pneumoniae), Streptococcus agalactiae, Streptococcus mutans, Streptococcus pyogenes, Group A streptococci, Streptococcus pyogenes, Group B streptococci, Streptococcus agalactiae, Group C streptococci, Streptococcus anginosus, Streptococcus equismilis, Group D streptococci, Streptococcus bovis, Group F streptococci,* and

Streptococcus anginosus Group G streptococci), Spirillum minus, Streptobacillus moniliformi, *Treponema sp.* (such as *Treponema carateum, Treponema petenue, Treponema pallidum* and *Treponema endemicum, Trichophyton rubrum, T. mentagrophytes, Tropheryma whippelii, Ureaplasma urealyticum, Veillonella sp., Vibrio sp.* (such as *Vibrio cholerae, Vibrio parahemolyticus, Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio alginolyticus, Vibrio mimicus, Vibrio hollisae, Vibrio fluvialis, Vibrio metchnikovii, Vibrio damsela* and *Vibrio furnisii), Yersinia sp.* (such as *Yersinia enterocolitica, Yersinia pestis,* and *Yersinia pseudotuberculosis)* and *Xanthomonas maltophilia* among others.

Nucleic acid detection systems

[0094] Nucleic acid detection systems comprising CRISPR systems offer rapid and robust signal detection and offer the option to detect DNA or RNA. The CRISPR system effector protein can be an RNA-targeting effector protein or a DNA-targeting effector protein. Exemplary DNA-targeting effector proteins include, without limitation, Cas9 and Cpfl. Exemplary RNA-targeting proteins include, without limitation, Casl3b and C2c2 (now known as Casl3a). The effector protein can be from an organism of a genus selected from: Leptotrichia, Listeria, Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma, Campylobacter, and Lachnospira, for example from an organism selected from Leptotrichia shahii, Leptotrichia. wadei, Listeria seeligeri, Clostridium aminophilum, Carnobacterium gallinarum, Paludibacter propionicigenes, Listeria weihenstephanensis, L. wadei F0279 or L. wadei F0279 (Lw2) C2C2

[0095] In certain embodiments, the system may further comprise nucleic acid amplification reagents. An example of such a system is termed SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) can rapidly detect DNA or RNA with attomolar sensitivity and single-base mismatch specificity. (Gootenberg et al., 2017, Nucleic acid detection with CRISPR-Casl3a/C2c2, Science 10.1 126/science.aam9321).

[0096] SHERLOCK (coupled with reverse transcription as needed) achieves single molecule sensitivity for both RNA and DNA, as verified by digital-droplet PCR (ddPCR) and attomolar sensitivity can be attained in a single reaction, demonstrating the viability of this platform as a point-of-care (POC) diagnostic. SHERLOCK has similar levels of sensitivity as ddPCR and quantitative PCR (qPCR), two established sensitive nucleic acid detection approaches, whereas RPA alone was not sensitive enough to detect low levels of target.

Moreover, SHERLOCK shows less variation than ddPCR, qPCR, and RPA, as measured by the coefficient of variation across replicates.

[0097] SHERLOCK is effective in infectious disease applications that require high sensitivity, including detecting viral particles down to 2 aM and discriminating between similar viruses such as between ZIKV and DENV. The method is also able to detect virus in clinical isolates (serum, urine, or saliva) where titers can be as low as 2×10^3 copies/mL (3.2 aM).

[0098] Another important application is the identification of bacterial pathogens and detection of specific bacterial genes. In a panel of five possible targeting crRNAs for different pathogenic strains and gDNA isolated from E. coli and Pseudomonas aeruginosa, SHERLOCK correctly genotyped strains and showed low cross-reactivity.

[0099] SHERLOCK can detect low frequency cancer mutations in cell free (cf) DNA fragments, which is challenging because of the high levels of wild-type DNA in patient blood For example, SHERLOCK can detect ssDNA 1 at attomolar concentrations diluted in a background of genomic DNA and can detect single nucleotide polymorphism (SNP)-containing alleles at levels as low as 0.1% of background DNA, which is in the clinically relevant range.

[0100] The nucleic acid amplification reagents may comprise a primer comprising an RNA polymerase promoter. In certain embodiments, sample nucleic acids are amplified to obtain a DNA template comprising an RNA polymerase promoter, whereby a target RNA molecule may be generated by transcription. The nucleic acid may be DNA and amplified by any method described herein. The nucleic acid may be RNA and amplified by a reverse transcription method as described herein. The aptamer sequence may be amplified upon unmasking of the primer binding site, whereby a trigger RNA is transcribed from the amplified DNA product. The target molecule may be a target DNA and the system may further comprises a primer that binds the target DNA and comprises a RNA polymerase promoter.

[0101] The systems feature one or more guide nucleic acids which are designed to bind to one or more target molecules that are diagnostic for a disease state. A disease state includes, without limitation, an infection, an organ disease, a blood disease, an immune system disease, a cancer, a brain and nervous system disease, an endocrine disease, a pregnancy or childbirth-related disease, an inherited disease, or an environmentally-acquired disease. In still further embodiments, the disease state is cancer or an autoimmune disease or an infection.

[0102] RNA sequencing (RNA-Seq) is a powerful tool for transcriptome profiling, but is hampered by sequence-dependent bias and inaccuracy at low copy numbers intrinsic to exponential PCR amplification. To mitigate these complications to allow truly digital RNA-Seq, a large set of barcode sequences is added in excess, and nearly every cDNA molecule is uniquely labeled by random attachment of barcode sequences to both ends (Shiroguchi K, et al. Proc Natl Acad Sci U S A. 2012 Jan 24; 109(4): 1347-52). After PCR, paired-end deep sequencing is applied to read the two barcodes and cDNA sequences. Rather than counting the number of reads, RNA abundance is measured based on the number of unique barcode sequences observed for a given cDNA sequence (Shiroguchi K, et al. Proc Natl Acad Sci U S A. 2012 Jan 24; 109(4): 1347-52). The barcodes may be optimized to be unambiguously identifiable, even in the presence of multiple sequencing errors. This method allows counting with single-copy resolution despite sequence-dependent bias and PCR-amplification noise, and is analogous to digital PCR but amendable to quantifying a whole transcriptome (Shiroguchi K, et al. Proc Natl Acad Sci U S A. 2012 Jan 24; 109(4): 1347-52).

[0103] Fixation of cells or tissue may involve the use of cross-linking agents, such as formaldehyde, and may involve embedding cells or tissue in a paraffin wax or polyacrylamide support matrix (Chung K, et al. Nature. 2013 May 16; 497(7449): 322-7).

[0104] Amplification may involve thermocycling or isothermal amplification (such as through the methods RPA or LAMP). Cross-linking may involve overlap-extension PCR or use of ligase to associate multiple amplification products with each other.

[0105] For purpose of this invention, amplification means any method employing a primer and a polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA polymerases such as TaqGold™, T7 DNA polymerase, Klenow fragment of E.coli DNA polymerase, and reverse transcriptase. A preferred amplification method is PCR. In particular, the isolated RNA can be subjected to a reverse transcription assay that is coupled with a quantitative polymerase chain reaction (RT-PCR) in order to quantify the expression level of a sequence associated with a signaling biochemical pathway.

[0106] Detection of the gene expression level can be conducted in real time in an amplification assay. In one aspect, the amplified products can be directly visualized with fluorescent DNA-binding agents including but not limited to DNA intercalators and DNA groove binders. Because the amount of the intercalators incorporated into the double-stranded DNA molecules is typically proportional to the amount of the amplified DNA products, one can conveniently determine the amount of the amplified products by quantifying the

fluorescence of the intercalated dye using conventional optical systems in the art. DNA-binding dye suitable for this application include SYBR green, SYBR blue, DAPI, propidium iodine, Hoeste, SYBR gold, ethidium bromide, acridines, proflavine, acridine orange, acriflavine, fluorcoumanin, ellipticine, daunomycin, chloroquine, distamycin D, chromomycin, homidium, mithramycin, ruthenium polypyridyls, anthramycin, and the like.

[0107] In another aspect, other fluorescent labels such as sequence specific probes can be employed in the amplification reaction to facilitate the detection and quantification of the amplified products. Probe-based quantitative amplification relies on the sequence-specific detection of a desired amplified product. It utilizes fluorescent, target-specific probes (e.g., TaqMan® probes) resulting in increased specificity and sensitivity. Methods for performing probe-based quantitative amplification are well established in the art and are taught in U.S. Patent No. 5,210,015.

[0108] Sequencing may be performed on any high-throughput platform with read-length (either single- or paired-end) sufficient to cover both template and cross-linking event UID's. Methods of sequencing oligonucleotides and nucleic acids are well known in the art (see, e.g., W093/23564, WO98/28440 and W098/13523; U.S. Pat. Nos. 5,525,464; 5,202,231; 5,695,940; 4,971,903; 5,902,723; 5,795,782; 5,547,839 and 5,403,708; Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977); Drmanac et al., Genomics 4:1 14 (1989); Koster et al., Nature Biotechnology 14:1 123 (1996); Hyman, Anal. Biochem. 174:423 (1988); Rosenthal, International Patent Application Publication 761 107 (1989); Metzker et al., Nucl. Acids Res. 22:4259 (1994); Jones, Biotechniques 22:938 (1997); Ronaghi et al., Anal. Biochem. 242:84 (1996); Ronaghi et al., Science 281:363 (1998); Nyren et al., Anal. Biochem. 151:504 (1985); Canard and Arzumanov, Gene 11:1 (1994); Dyatkina and Arzumanov, Nucleic Acids Symp Ser 18:1 17 (1987); Johnson et al., Anal. Biochem. 136: 192 (1984); and Elgen and Rigler, Proc. Natl. Acad. Sci. USA 91(13):5740 (1994), all of which are expressly incorporated by reference).

[0109] The present invention may be applied to (1) single-cell transcriptomics: cDNA synthesized from mRNA is barcoded and cross-linked during in situ amplification, (2) single-cell proteomics: cDNA or DNA synthesized from RNA- or DNA-tagged antibodies of one or multiple specificities maps the abundance and distributions of different protein-antigens and (3) whole-tissue transcriptomic/proteomic mapping (molecular microscopy or VIPUR microscopy): using the frequency of cross-contamination between cells to determine their physical proximity, and via applications (1) single-cell transcriptomics and (2) single-cell proteomics, determining the global spatial distribution of mRNA, protein, or other

biomolecules in a biological sample. This may be used, for example, to screen for anti-cancer/pathogen immunoglobulins (by analyzing co-localization of B-cells and T-cells within affected tissue) for immunotherapy.

[0110] The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. The term also encompasses nucleic-acid-like structures with synthetic backbones, see, e.g., Eckstein, 1991; Baserga et al., 1992; Milligan, 1993; WO 97/0321 1; WO 96/39154; Mata, 1997; Strauss-Soukup, 1997; and Samstag, 1996. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[0111] As used herein the term "variant" should be taken to mean the exhibition of qualities that differ, such as, but not limited to, genetic variations including SNPs, insertion deletion events, and the like.

[0112] The terms "non-naturally occurring" or "engineered" are used interchangeably and indicate the involvement of the hand of man. The terms, when referring to nucleic acid molecules or polypeptides mean that the nucleic acid molecule or the polypeptide is at least substantially free from at least one other component with which they are naturally associated in nature and as found in nature.

[0113] "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%,

90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions.

[0114] As used herein, "stringent conditions" for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), Laboratory Techniques In Biochemistry And Molecular Biology-Hybridization With Nucleic Acid Probes Part I, Second Chapter "Overview of principles of hybridization and the strategy of nucleic acid probe assay", Elsevier, N.Y.

[0115] "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence.

[0116] As used herein, the term "genomic locus" or "locus" (plural loci) is the specific location of a gene or DNA sequence on a chromosome. A "gene" refers to stretches of DNA or RNA that encode a polypeptide or an RNA chain that has functional role to play in an organism and hence is the molecular unit of heredity in living organisms. For the purpose of this invention it may be considered that genes include regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding

sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0117] As used herein, "expression of a genomic locus" or "gene expression" is the process by which information from a gene is used in the synthesis of a functional gene product. The products of gene expression are often proteins, but in non-protein coding genes such as rRNA genes or tRNA genes, the product is functional RNA. The process of gene expression is used by all known life - eukaryotes (including multicellular organisms), prokaryotes (bacteria and archaea) and viruses to generate functional products to survive. As used herein "expression" of a gene or nucleic acid encompasses not only cellular gene expression, but also the transcription and translation of nucleic acid(s) in cloning systems and in any other context. As used herein, "expression" also refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as "gene product." If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0118] As described in aspects of the invention, sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences.

[0119] Sequence homologies may be generated by any of a number of computer programs known in the art, for example BLAST or FASTA, etc. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, Nucleic Acids Research 12:387). Examples of other software than may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 ibid - Chapter 18), FASTA (Atschul et al., 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999 ibid, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program. % homology may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid or nucleotide in one sequence is directly compared with the corresponding amino acid or nucleotide in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed

only over a relatively short number of residues. Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion may cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without unduly penalizing the overall homology or identity score. This is achieved by inserting "gaps" in the sequence alignment to try to maximize local homology or identity. However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - may achieve a higher score than one with many gaps. "Affinity gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties may, of course, produce optimized alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example, when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension. Calculation of maximum % homology therefore first requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux et al., 1984 Nuc. Acids Research 12 p387). Examples of other software than may perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 Short Protocols in Molecular Biology, 4th Ed. - Chapter 18), FASTA (Altschul et al., 1990 J. Mol. Biol. 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999, Short Protocols in Molecular Biology, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequences (see FEMS Microbiol Lett. 1999 174(2): 247-50; FEMS Microbiol Lett. 1999 177(1): 187-8 and the website of the National Center for Biotechnology information at the website of the National Institutes for Health). Although the final % homology may be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pair-wise

comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table, if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

[0120] Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASISTM (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), Gene 73(1), 237-244). Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[0121] Embodiments of the invention include sequences (both polynucleotide or polypeptide) which may comprise homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue or nucleotide, with an alternative residue or nucleotide) that may occur i.e., like-for-like substitution in the case of amino acids such as basic for basic, acidic for acidic, polar for polar, etc. Non-homologous substitution may also occur i.e., from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

[0122] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

[0123] Hybridization can be performed under conditions of various stringency. Suitable hybridization conditions for the practice of the present invention are such that the recognition interaction between the probe and sequences associated with a signaling biochemical

pathway is both sufficiently specific and sufficiently stable. Conditions that increase the stringency of a hybridization reaction are widely known and published in the art. See, for example, (Sambrook, et al., (1989); Nonradioactive In Situ Hybridization Application Manual, Boehringer Mannheim, second edition). The hybridization assay can be formed using probes immobilized on any solid support, including but are not limited to nitrocellulose, glass, silicon, and a variety of gene arrays. A preferred hybridization assay is conducted on high-density gene chips as described in U.S. Patent No. 5,445,934.

[0124] For a convenient detection of the probe-target complexes formed during the hybridization assay, the nucleotide probes are conjugated to a detectable label. Detectable labels suitable for use in the present invention include any composition detectable by photochemical, biochemical, spectroscopic, immunochemical, electrical, optical or chemical means. A wide variety of appropriate detectable labels are known in the art, which include fluorescent or chemiluminescent labels, radioactive isotope labels, enzymatic or other ligands. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as digoxigenin, β-galactosidase, urease, alkaline phosphatase or peroxidase, avidin/biotin complex.

[0125] The detection methods used to detect or quantify the hybridization intensity will typically depend upon the label selected above. For example, radiolabels may be detected using photographic film or a phosphoimager. Fluorescent markers may be detected and quantified using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and measuring the reaction product produced by the action of the enzyme on the substrate; and finally colorimetric labels are detected by simply visualizing the colored label.

[0126] Examples of the labeling substance which may be employed include labeling substances known to those skilled in the art, such as fluorescent dyes, enzymes, coenzymes, chemiluminescent substances, and radioactive substances. Specific examples include radioisotopes (e.g., 32P, 14C, 1251, 3H, and 1311), fluorescein, rhodamine, dansyl chloride, umbelliferone, luciferase, peroxidase, alkaline phosphatase, β-galactosidase, β-glucosidase, horseradish peroxidase, glucoamylase, lysozyme, saccharide oxidase, microperoxidase, biotin, and ruthenium. In the case where biotin is employed as a labeling substance, preferably, after addition of a biotin-labeled antibody, streptavidin bound to an enzyme (e.g., peroxidase) is further added.

[0127] Advantageously, the label is a fluorescent label. Examples of fluorescent labels include, but are not limited to, Atto dyes, 4-acetamido-4'-isothiocyanatostilbene-

2,2'disulfonic acid; acridine and derivatives: acridine, acridine isothiocyanate; 5-(2'-aminoethyl)aminonaphthalene-l -sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate; N-(4-anilino-l -naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives; coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumaran 151); cyanine dyes; cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5'5''-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-l-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives; eosin, eosin isothiocyanate, erythrosin and derivatives; erythrosin B, erythrosin, isothiocyanate; ethidium; fluorescein and derivatives; 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2',7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, QFITC, (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferoneortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives: pyrene, pyrene butyrate, succinimidyl 1-pyrene; butyrate quantum dots; Reactive Red 4 (Cibacron.TM. Brilliant Red 3B-A) rhodamine and derivatives: 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N' tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid; terbium chelate derivatives; Cy3; Cy5; Cy5.5; Cy7; IRD 700; IRD 800; La Jolta Blue; phthalo cyanine; and naphthalo cyanine

[0128] The fluorescent label may be a fluorescent protein, such as blue fluorescent protein, cyan fluorescent protein, green fluorescent protein, red fluorescent protein, yellow fluorescent protein or any photoconvertible protein. Colormetric labeling, bioluminescent labeling and/or chemiluminescent labeling may further accomplish labeling. Labeling further may include energy transfer between molecules in the hybridization complex by perturbation analysis, quenching, or electron transport between donor and acceptor molecules, the latter of which may be facilitated by double stranded match hybridization complexes. The fluorescent label may be a perylene or a terrylen. In the alternative, the fluorescent label may be a fluorescent bar code.

[0129] In an advantageous embodiment, the label may be light sensitive, wherein the label is light-activated and/or light cleaves the one or more linkers to release the molecular cargo. The light-activated molecular cargo may be a major light-harvesting complex (LHCII). In another embodiment, the fluorescent label may induce free radical formation.

[0130] In an advantageous embodiment, agents may be uniquely labeled in a dynamic manner (see, e.g., international patent application serial no. PCT/US20 13/6 1182 filed September 23, 2012). The unique labels are, at least in part, nucleic acid in nature, and may be generated by sequentially attaching two or more detectable oligonucleotide tags to each other and each unique label may be associated with a separate agent. A detectable oligonucleotide tag may be an oligonucleotide that may be detected by sequencing of its nucleotide sequence and/or by detecting non-nucleic acid detectable moieties to which it may be attached.

[0131] The oligonucleotide tags may be detectable by virtue of their nucleotide sequence, or by virtue of a non-nucleic acid detectable moiety that is attached to the oligonucleotide such as but not limited to a fluorophore, or by virtue of a combination of their nucleotide sequence and the nonnucleic acid detectable moiety.

[0132] In some embodiments, a detectable oligonucleotide tag may comprise one or more nonoligonucleotide detectable moieties. Examples of detectable moieties may include, but are not limited to, fluorophores, microparticles including quantum dots (Empodocles, et al., Nature 399:126-130, 1999), gold nanoparticles (Reichert et al., Anal. Chem. 72:6025-6029, 2000), biotin, DNP (dinitrophenyl), fucose, digoxigenin, haptens, and other detectable moieties known to those skilled in the art. In some embodiments, the detectable moieties may be quantum dots. Methods for detecting such moieties are described herein and/or are known in the art.

[0133] Thus, detectable oligonucleotide tags may be, but are not limited to, oligonucleotides which may comprise unique nucleotide sequences, oligonucleotides which may comprise detectable moieties, and oligonucleotides which may comprise both unique nucleotide sequences and detectable moieties.

[0134] A unique label may be produced by sequentially attaching two or more detectable oligonucleotide tags to each other. The detectable tags may be present or provided in a plurality of detectable tags. The same or a different plurality of tags may be used as the source of each detectable tag may be part of a unique label. In other words, a plurality of tags may be subdivided into subsets and single subsets may be used as the source for each tag.

[0135] In some embodiments, a detectable oligonucleotide tag may comprise one or more non-oligonucleotide detectable moieties. Examples of detectable moieties include, but are not limited to, fluorophores, microparticles including quantum dots (Empodocles, et al., Nature 399:126-130, 1999), gold nanoparticles (Reichert et al., Anal. Chem. 72:6025-6029, 2000), biotin, DNP (dinitrophenyl), fucose, digoxigenin, haptens, and other detectable moieties known to those skilled in the art. In some embodiments, the detectable moieties are quantum dots. Methods for detecting such moieties are described herein and/or are known in the art.

[0136] Thus, detectable oligonucleotide tags may be, but are not limited to, oligonucleotides which may comprise unique nucleotide sequences, oligonucleotides which may comprise detectable moieties, and oligonucleotides which may comprise both unique nucleotide sequences and detectable moieties.

[0137] A unique nucleotide sequence may be a nucleotide sequence that is different (and thus distinguishable) from the sequence of each detectable oligonucleotide tag in a plurality of detectable oligonucleotide tags. A unique nucleotide sequence may also be a nucleotide sequence that is different (and thus distinguishable) from the sequence of each detectable oligonucleotide tag in a first plurality of detectable oligonucleotide tags but identical to the sequence of at least one detectable oligonucleotide tag in a second plurality of detectable oligonucleotide tags. A unique sequence may differ from other sequences by multiple bases (or base pairs). The multiple bases may be contiguous or non-contiguous. Methods for obtaining nucleotide sequences (e.g., sequencing methods) are described herein and/or are known in the art.

[0138] In some embodiments, detectable oligonucleotide tags comprise one or more of a ligation sequence, a priming sequence, a capture sequence, and a unique sequence (optionally referred to herein as an index sequence). A ligation sequence is a sequence complementary to a second nucleotide sequence which allows for ligation of the detectable oligonucleotide tag to another entity which may comprise the second nucleotide sequence, e.g., another detectable oligonucleotide tag or an oligonucleotide adapter. A priming sequence is a sequence complementary to a primer, e.g., an oligonucleotide primer used for an amplification reaction such as but not limited to PCR. A capture sequence is a sequence capable of being bound by a capture entity. A capture entity may be an oligonucleotide which may comprise a nucleotide sequence complementary to a capture sequence, e.g. a second detectable oligonucleotide tag. A capture entity may also be any other entity capable of binding to the capture sequence, e.g. an antibody, hapten or peptide. An index sequence is a

sequence which may comprise a unique nucleotide sequence and/or a detectable moiety as described above.

[0139] "Complementary" is a term which is used to indicate a sufficient degree of complementarity between two nucleotide sequences such that stable and specific binding occurs between one and preferably more bases (or nucleotides, as the terms are used interchangeably herein) of the two sequences. For example, if a nucleotide in a first nucleotide sequence is capable of hydrogen bonding with a nucleotide in second nucleotide sequence, then the bases are considered to be complementary to each other. Complete (i.e., 100%) complementarity between a first nucleotide sequence and a second nucleotide is preferable, but not required for ligation, priming, or capture sequences.

[0140] The present invention also relates to a computer system involved in carrying out the methods of the invention relating to both computations and sequencing.

[0141] A computer system (or digital device) may be used to receive, transmit, display and/or store results, analyze the results, and/or produce a report of the results and analysis. A computer system may be understood as a logical apparatus that can read instructions from media (e.g. software) and/or network port (e.g. from the internet), which can optionally be connected to a server having fixed media. A computer system may comprise one or more of a CPU, disk drives, input devices such as keyboard and/or mouse, and a display (e.g. a monitor). Data communication, such as transmission of instructions or reports, can be achieved through a communication medium to a server at a local or a remote location. The communication medium can include any means of transmitting and/or receiving data. For example, the communication medium can be a network connection, a wireless connection, or an internet connection. Such a connection can provide for communication over the World Wide Web. It is envisioned that data relating to the present invention can be transmitted over such networks or connections (or any other suitable means for transmitting information, including but not limited to mailing a physical report, such as a print-out) for reception and/or for review by a receiver. The receiver can be but is not limited to an individual, or electronic system (e.g. one or more computers, and/or one or more servers).

[0142] In some embodiments, the computer system may comprise one or more processors. Processors may be associated with one or more controllers, calculation units, and/or other units of a computer system, or implanted in firmware as desired. If implemented in software, the routines may be stored in any computer readable memory such as in RAM, ROM, flash memory, a magnetic disk, a laser disk, or other suitable storage medium. Likewise, this software may be delivered to a computing device via any known delivery

method including, for example, over a communication channel such as a telephone line, the internet, a wireless connection, etc., or via a transportable medium, such as a computer readable disk, flash drive, etc. The various steps may be implemented as various blocks, operations, tools, modules and techniques which, in turn, may be implemented in hardware, firmware, software, or any combination of hardware, firmware, and/or software. When implemented in hardware, some or all of the blocks, operations, techniques, etc. may be implemented in, for example, a custom integrated circuit (IC), an application specific integrated circuit (ASIC), a field programmable logic array (FPGA), a programmable logic array (PLA), etc.

[0143] A client-server, relational database architecture can be used in embodiments of the invention. A client-server architecture is a network architecture in which each computer or process on the network is either a client or a server. Server computers are typically powerful computers dedicated to managing disk drives (file servers), printers (print servers), or network traffic (network servers). Client computers include PCs (personal computers) or workstations on which users run applications, as well as example output devices as disclosed herein. Client computers rely on server computers for resources, such as files, devices, and even processing power. In some embodiments of the invention, the server computer handles all of the database functionality. The client computer can have software that handles all the front-end data management and can also receive data input from users.

[0144] A machine readable medium which may comprise computer-executable code may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, etc. shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire and fiber optics, including the wires that comprise a bus within a computer system. Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD or DVD-ROM, any other optical medium, punch cards paper tape, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables or

links transporting such a carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

[0145] The subject computer-executable code can be executed on any suitable device which may comprise a processor, including a server, a PC, or a mobile device such as a smartphone or tablet. Any controller or computer optionally includes a monitor, which can be a cathode ray tube ("CRT") display, a flat panel display (e.g., active matrix liquid crystal display, liquid crystal display, etc.), or others. Computer circuitry is often placed in a box, which includes numerous integrated circuit chips, such as a microprocessor, memory, interface circuits, and others. The box also optionally includes a hard disk drive, a floppy disk drive, a high capacity removable drive such as a writeable CD-ROM, and other common peripheral elements. Inputting devices such as a keyboard, mouse, or touch-sensitive screen, optionally provide for input from a user. The computer can include appropriate software for receiving user instructions, either in the form of user input into a set of parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations.

[0146] The present invention also contemplates multiplex assays. The present invention is especially well suited for multiplex assays. For example, the invention encompasses use of a SureSelect$^{XT'}$ SureSelect^{XT2} and SureSelect^{QXT} Target Enrichment System for Illumina Multiplexed Sequencing developed by Agilent Technologies (see, e.g., www.agilent.com/genomics/protocolvideos), a SeqCap EZ kit developed by Roche NimbleGen, a TruSeq® Enrichment Kit developed by Illumina and other hybridization-based target enrichment methods and kits that add sample-specific sequence tags either before or after the enrichment step.as well as Illumina HiSeq, MiSeq and NexSeq,, Life Technology Ion Torrent. Pacific Biosciences PacBio RSII, Oxford Nanopore Minion, Promethlon and Gridlon and other massively parallel Multiplexed Sequencing Platforms.

[0147] Usable methods for hybrid selection are described in Melnikov, *et al,* Genome Biology 12:R73, 201 1; Geniez, *et al,* Symbiosis 58:201-207, 2012; and Matranga, *et al,* Genome Biology 15:519, 2014). Bait design and hybrid selection was done similarly to a previously published method (see, e.g., Gnirke, *et al,* Nature biotechnology 27:182-189, 2009, US patent publications No. US 2010/0029498, US 2013/0230857, US 2014/0200163, US 2014/0228223, and US 2015/0126377 and international patent publication No. WO 2009/099602). Briefly, baits may be designed by first concatenating all consensus sequences

(such as LASV) into two single bait sets (such as one for Nigerian clades and another for the Sierra Leone clade). Duplicate probes, defined as a DNA sequence with 0 mismatches, were removed. The baits sequences were tiled across the genome (such as LASV) creating a probe every 50 bases. Two sets of adapters were used for each bait set. Adapters alternated with each 50 base probe to improve the efficiency of PCR amplification of probes. The oligo array was synthesized on a CustomArray B3 Synthesizer, as recommended by the manufacturer. The oligonucleotides were cleaved-off the array and amplified by PCR with primers containing T7 RNA polymerase promoters. Biotinylated baits were then prepared through in vitro transcription (MEGAshortscript, Ambion). RNA baits for each clade were prepared separately and mixed at the equal RNA concentration prior to hybridization. Libraries of the genome (such as LASV) were added to the baits and hybridized over a 72 hrs. After capture and washing, libraries were amplified by PCR using the Illumina adapter sequences. Libraries were then pooled and sequenced on the MiSeq platform.

[0148] In one aspect of the invention, a method for analyzing a pathogen sequence, such as a bacterial or viral sequence, is provided. The method may comprise sequencing the pathogen sequence according to the method for analyzing a sample which may comprise a target sequence as described above, wherein the target sequence is the pathogen sequence. Preferably the pathogen sequence is a genome of the pathogen or a fragment thereof. The method further may comprise determining the evolution of the pathogen from the sequenced pathogen sequence. Determining the evolution of the pathogen may comprise identification of pathogen mutations in the sequenced pathogen sequence, *e.g.* nucleotide deletion, nucleotide insertion, nucleotide substitution. Amongst the latter, there are nonsynonymous, synonymous, and noncoding substitutions. Mutations are more frequently nonsynonymous during an outbreak. The method may further comprise determining the substitution rate between two pathogen sequences analyzed as described above. Whether the mutations are deleterious or even adaptive would require functional analysis, however, the rate of nonsynonymous mutations suggests that continued progression of this epidemic could afford an opportunity for viral adaptation, underscoring the need for rapid containment. Thus, the method may further comprise assessing the risk of viral adaptation, wherein the number nonsynonymous mutations is determined. (Gire, *etal.*, Science 345, 1369, 2014).

Screening of compounds

[0149] Accordingly, the invention involves a non-human eukaryote, animal, mammal, primate, rodent, etc or cell thereof or tissue thereof that may be used as a disease model. As used herein, "disease" refers to a disease, disorder, or indication in a subject. For example, a

method of the invention may be used to create a non-human eukaryote, e.g., an animal, mammal, primate, rodent or cell that comprises a modification, e.g., 3-50 modifications, in one or more nucleic acid sequences associated or correlated with a disease caused by a *Fusobacterium* infection. Such a mutated nucleic acid sequence be associated or correlated with a disease caused by a *Fusobacterium* infection and may encode a disease associated protein sequence or may be a disease associated or correlated control sequence. The cell may be *in vivo* or *ex vivo* in the cases of multicellular organisms. In the instance where the cell is in cultured, a cell line may be established if appropriate culturing conditions are met and preferably if the cell is suitably adapted for this purpose (for instance a stem cell). Hence, cell lines are also envisaged. In some methods, the disease model can be used to study the effects of mutations on the animal or cell and development and/or progression of the disease using measures commonly used in the study of the disease. Alternatively, such a disease model is useful for studying the effect of a putatively pharmaceutically active compound or gene therapy on the disease. A disease-associated gene or polynucleotide can be modified to give rise to the disease in the model, and then putatively pharmaceutically active compound and/or gene therapy can be administered so as to observe whether disease development and/or progression is inhibited or reduced. In particular, the method comprises modifying so as to produce, one or more, advantageously 3-50 or more disease-associated or correlated gene(s) or polynucleotide(s). Accordingly, in some methods, a genetically modified animal may be compared with an animal predisposed to development of the disease, such that administering putative gene therapy, or pharmaceutically acceptable compound(s), or any combination thereof can be performed to assess how such putative therapy(ies) or treatment(s) may perform in a human.

[0150] Screening of such putative pharmaceutically active compound(s) and/or gene therapy(ies) can be by cellular function change and/or intracellular signaling or extracellular signaling change. Such screening can involve evaluating for dosages or dose curves, as well as combinations of potential drugs and/or therapies. An altered expression of one or more genome sequences associated with a signaling biochemical pathway can be determined by assaying for a difference in the mRNA levels of the corresponding genes between the disease model eukaryote or animal or cell or tissue thereof and a normal eukaryote, animal, tissue or cell, and to ascertain whether when the disease model is administered or contacted with a candidate chemical agent or gene therapy it reverts to or towards normal. An assay can be for mutation(s)-induced alteration in the level of mRNA transcripts or corresponding polynucleotides in comparison with such level(s) in a normal eukaryote or animal and

whether such level(s) are placed towards or to normal when a therapy or treatment or agent is employed.

[0151] With respect to general information on CRISPR-Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, AAV, and making and using thereof, including as to amounts and formulations, all useful in the practice of the instant invention, reference is made to: US Patents Nos. 8,999,641, 8,993,233, 8,945,839, 8,932,814, 8,906,616, 8,895,308, 8,889,418, 8,889,356, 8,871,445, 8,865,406, 8,795,965, 8,771,945 and 8,697,359; US Patent Publications US 2014-0310830 (US APP. Ser. No. 14/105,031), US 2014-0287938 A1 (U.S. App. Ser. No. 14/213,991), US 2014-0273234 A1 (U.S. App. Ser. No. 14/293,674), US2014-0273232 A1 (U.S. App. Ser. No. 14/290,575), US 2014-0273231 (U.S. App. Ser. No. 14/259,420), US 2014-0256046 A1 (U.S. App. Ser. No. 14/226,274), US 2014-0248702 A1 (U.S. App. Ser. No. 14/258,458), US 2014-0242700 A1 (U.S. App. Ser. No. 14/222,930), US 2014-0242699 A1 (U.S. App. Ser. No. 14/183,512), US 2014-0242664 A1 (U.S. App. Ser. No. 14/104,990), US 2014-0234972 A1 (U.S. App. Ser. No. 14/183,471), US 2014-0227787 A1 (U.S. App. Ser. No. 14/256,912), US 2014-0189896 A1 (U.S. App. Ser. No. 14/105,035), US 2014-0186958 (U.S. App. Ser. No. 14/105,017), US 2014-0186919 A1 (U.S. App. Ser. No. 14/104,977), US 2014-0186843 A1 (U.S. App. Ser. No. 14/104,900), US 2014-0179770 A1 (U.S. App. Ser. No. 14/104,837) and US 2014-0179006 A1 (U.S. App. Ser. No. 14/183,486), US 2014-0170753 (US App Ser No 14/183,429); European Patents EP 2 784 162 B1 and EP 2 771 468 B1; European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP 13824232.6), and EP 2 784 162 (EP 14170383.5); and PCT Patent Publications PCT Patent Publications WO 2014/093661 (PCT/US20 13/074743), WO 2014/093694 (PCT/US20 13/074790), **WO** 2014/093595 (PCT/US20 13/074611), **WO** 2014/093718 (PCT/US20 13/074825), **WO** 2014/093709 (PCT/US20 13/074812), **WO** 2014/093622 (PCT/US20 13/074667), **WO** 2014/093635 (PCT/US20 13/074691), **WO** 2014/093655 (PCT/US20 13/074736), **WO** 2014/093712 (PCT/US20 13/074819), **WO** 2014/093701 (PCT/US20 13/074800), **WO** 2014/018423 (PCT/US2013/051418), **WO** 2014/204723 (PCT/US20 14/041790), **WO** 2014/204724 (PCT/US20 14/041800), **WO** 2014/204725 (PCT/US2014/041803), **WO** 2014/204726 (PCT/US20 14/041804), **WO** 2014/204727 (PCT/US20 14/041806), **WO** 2014/204728 (PCT/US20 14/041808), **WO** 2014/204729 (PCT/US20 14/041809). Reference is also made to US provisional patent applications 61/758,468; 61/802,174; 61/806,375; 61/814,263; 61/819,803 and 61/828,130, filed on January 30, 2013; March 15, 2013; March 28, 2013; April 20, 2013; May 6, 2013

and May 28, 2013 respectively. Reference is also made to US provisional patent application 61/836,123, filed on June 17, 2013. Reference is additionally made to US provisional patent applications 61/835,931, 61/835,936, 61/836,127, 61/836, 101, 61/836,080 and 61/835,973, each filed June 17, 2013. Further reference is made to US provisional patent applications 61/862,468 and 61/862,355 filed on August 5, 2013; 61/871,301 filed on August 28, 2013; 61/960,777 filed on September 25, 2013 and 61/961,980 filed on October 28, 2013. Reference is yet further made to: PCT Patent applications Nos: PCT/US20 14/04 1803, PCT/US20 14/04 1800, PCT/US20 14/04 1809, PCT/US20 14/04 1804 and PCT/US20 14/04 1806, each filed June 10, 2014 6/10/14; PCT/US20 14/04 1808 filed June 11, 2014; and PCT/US2014/62558 filed October 28, 2014, and US Provisional Patent Applications Serial Nos.: 61/915,150, 61/915,301, 61/915,267 and 61/915,260, each filed December 12, 2013; 61/757,972 and 61/768,959, filed on January 29, 2013 and February 25, 2013; 61/835,936, 61/836,127, 61/836,101, 61/836,080, 61/835,973, and 61/835,931, filed June 17, 2013; 62/010,888 and 62/010,879, both filed June 11, 2014; 62/010,329 and 62/010,441, each filed June 10, 2014; 61/939,228 and 61/939,242, each filed February 12, 2014; 61/980,012, filed April 15,2014; 62/038,358, filed August 17, 2014; 62/054,490, 62/055,484, 62/055,460 and 62/055,487, each filed September 25, 2014; and 62/069,243, filed October 27, 2014. Reference is also made to US provisional patent applications Nos. 62/055,484, 62/055,460, and 62/055,487, filed September 25, 2014; US provisional patent application 61/980,012, filed April 15, 2014; and US provisional patent application 61/939,242 filed February 12, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US14/41806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013. Reference is made to US provisional patent application USSN 61/980,012 filed April 15, 2014. Reference is made to PCT application designating, inter alia, the United States, application No. PCT/US 14/4 1806, filed June 10, 2014. Reference is made to US provisional patent application 61/930,214 filed on January 22, 2014. Reference is made to US provisional patent applications 61/915,251; 61/915,260 and 61/915,267, each filed on December 12, 2013.

[0152] Mention is also made of US application 62/091,455, filed, 12-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US application 62/096,708, 24-Dec-14, PROTECTED GUIDE RNAS (PGRNAS); US application 62/091,462, 12-Dec-14, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/096,324, 23-Dec-

14, DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS; US application 62/091,456, 12-Dec-14, ESCORTED AND FUNCTIONALIZED GUIDES FOR CRISPR-CAS SYSTEMS; US application 62/091,461, 12-Dec-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR GENOME EDITING AS TO HEMATOPOETIC STEM CELLS (HSCs); US application 62/094,903, 19-Dec-14, UNBIASED IDENTIFICATION OF DOUBLE-STRAND BREAKS AND GENOMIC REARRANGEMENT BY GENOME-WISE INSERT CAPTURE SEQUENCING; US application 62/096,761, 24-Dec-14, ENGINEERING OF SYSTEMS, METHODS AND OPTIMIZED ENZYME AND GUIDE SCAFFOLDS FOR SEQUENCE MANIPULATION; US application 62/098,059, 30-Dec-14, RNA-TARGETING SYSTEM; US application 62/096,656, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH DESTABILIZATION DOMAINS; US application 62/096,697, 24-Dec-14, CRISPR HAVING OR ASSOCIATED WITH AAV; US application 62/098,158, 30-Dec-14, ENGINEERED CRISPR COMPLEX INSERTIONAL TARGETING SYSTEMS; US application 62/151,052, 22-Apr-15, CELLULAR TARGETING FOR EXTRACELLULAR EXOSOMAL REPORTING; US application 62/054,490, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING PARTICLE DELIVERY COMPONENTS; US application 62/055,484, 25-Sep-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,537, 4-Dec-14, SYSTEMS, METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/054,651, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/067,886, 23-Oct-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR MODELING COMPETITION OF MULTIPLE CANCER MUTATIONS IN VIVO; US application 62/054,675, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN NEURONAL CELLS/TISSUES; US application 62/054,528, 24-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS IN IMMUNE DISEASES OR DISORDERS; US application 62/055,454,

25-Sep-14, DELIVERY, USE AND THERAPEUTIC APPLICATIONS OF THE CRISPR-CAS SYSTEMS AND COMPOSITIONS FOR TARGETING DISORDERS AND DISEASES USING CELL PENETRATION PEPTIDES (CPP); US application 62/055,460, 25-Sep-14, MULTIFUNCTIONAL-CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; US application 62/087,475, 4-Dec-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/055,487, 25-Sep-14, FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS; US application 62/087,546, 4-Dec-14, MULTIFUNCTIONAL CRISPR COMPLEXES AND/OR OPTIMIZED ENZYME LINKED FUNCTIONAL-CRISPR COMPLEXES; and US application 62/098,285, 30-Dec-14, CRISPR MEDIATED IN VIVO MODELING AND GENETIC SCREENING OF TUMOR GROWTH AND METASTASIS.

[0153] Each of these patents, patent publications, and applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, together with any instructions, descriptions, product specifications, and product sheets for any products mentioned therein or in any document therein and incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. All documents (e.g., these patents, patent publications and applications and the appln cited documents) are incorporated herein by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

[0154] Also with respect to general information on CRISPR-Cas Systems, mention is made of the following (also hereby incorporated herein by reference):

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- RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Jiang W., Bikard D., Cox D., Zhang F, Marraffini LA. Nat Biotechnol Mar;31(3):233-9 (2013);
- One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering. Wang H., Yang H., Shivalila CS., Dawlaty MM., Cheng AW., Zhang F., Jaenisch R. Cell May 9;153(4):910-8 (2013);
- Optical control of mammalian endogenous transcription and epigenetic states. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Piatt RJ,

Scott DA, Church GM, Zhang F. Nature. Aug 22;500(7463):472-6. doi: 10.1038/Naturel2466. Epub 2013 Aug 23 (2013);

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> DNA targeting specificity of RNA-guided Cas9 nucleases. Hsu, P., Scott, D., Weinstein, J., Ran, FA., Konermann, S., Agarwala, V., Li, Y., Fine, E., Wu, X., Shalem, O., Cradick, TJ., Marraffini, LA., Bao, G., & Zhang, F. Nat Biotechnol doi:10.1038/nbt.2647 (2013);

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> Genetic screens in human cells using the CRISPR/Cas9 system, Wang T, Wei JJ, Sabatini DM, Lander ES., Science. January 3; 343(6166): 80-84. doi: 10. 1126/science. 1246981 (2014);

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- Genome-wide CRISPR Screen in a Mouse Model of Tumor Growth and Metastasis, Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, Shi X, Scott DA, Song J, Pan JQ, Weissleder R, Lee H, Zhang F, Sharp PA. Cell 160, 1246-1260, March 12, 2015 (multiplex screen in mouse), and
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- Gao *et al,* "Engineered Cpfl Enzymes with Altered PAM Specificities," bioRxiv 09161 1; doi: http://dx.doi.org/10.1 101/09161 1 Epub Dec. 4, 2016
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each of which is incorporated herein by reference, may be considered in the practice of the instant invention, and discussed briefly below:

- Cong *et al.* engineered type II CRISPR-Cas systems for use in eukaryotic cells based on both *Streptococcus thermophilics* Cas9 and also *Streptococcus pyogenes* Cas9 and demonstrated that Cas9 nucleases can be directed by short RNAs to induce precise cleavage of DNA in human and mouse cells. Their study further showed that Cas9 as converted into a nicking enzyme can be used to facilitate homology-directed repair in eukaryotic cells with minimal mutagenic activity. Additionally, their study demonstrated that multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several at endogenous genomic loci sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology. This ability to use RNA to program sequence specific DNA cleavage in cells defined a new class of genome engineering tools. These studies further showed that other CRISPR loci are likely to be transplantable into mammalian cells and can also mediate mammalian genome cleavage. Importantly, it can be envisaged that several aspects of the CRISPR-Cas system can be further improved to increase its efficiency and versatility.
- Jiang *et al.* used the clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated Cas9 endonuclease complexed with dual-RNAs to introduce precise mutations in the genomes of *Streptococcus pneumoniae* and *Escherichia coli.* The approach relied on dual-RNA:Cas9-directed cleavage at the targeted genomic site to kill unmutated cells and circumvents the need for selectable markers or counter-selection systems. The study reported reprogramming dual-RNA:Cas9 specificity by

changing the sequence of short CRISPR RNA (crRNA) to make single- and multinucleotide changes carried on editing templates. The study showed that simultaneous use of two crRNAs enabled multiplex mutagenesis. Furthermore, when the approach was used in combination with recombineering, in *S. pneumoniae,* nearly 100% of cells that were recovered using the described approach contained the desired mutation, and in *E. coli,* 65% that were recovered contained the mutation.

- Wang *et al.* (2013) used the CRISPR/Cas system for the one-step generation of mice carrying mutations in multiple genes which were traditionally generated in multiple steps by sequential recombination in embryonic stem cells and/or time-consuming intercrossing of mice with a single mutation. The CRISPR/Cas system will greatly accelerate the *in vivo* study of functionally redundant genes and of epistatic gene interactions.
- Konermann *et al.* (2013) addressed the need in the art for versatile and robust technologies that enable optical and chemical modulation of DNA-binding domains based CRISPR Cas9 enzyme and also Transcriptional Activator Like Effectors
- Ran *et al.* (2013-A) described an approach that combined a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. This addresses the issue of the Cas9 nuclease from the microbial CRISPR-Cas system being targeted to specific genomic loci by a guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking *via* appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. The authors demonstrated that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.
- Hsu *et al.* (2013) characterized SpCas9 targeting specificity in human cells to inform the selection of target sites and avoid off-target effects. The study evaluated >700 guide RNA variants and SpCas9-induced indel mutation levels at >100 predicted genomic off-target loci in 293T and 293FT cells. The authors that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner, sensitive to the number, position and distribution of mismatches. The authors further showed that SpCas9-mediated cleavage is unaffected by DNA

methylation and that the dosage of SpCas9 and sgRNA can be titrated to minimize off-target modification. Additionally, to facilitate mammalian genome engineering applications, the authors reported providing a web-based software tool to guide the selection and validation of target sequences as well as off-target analyses.

- Ran *et al.* (2013-B) described a set of tools for Cas9-mediated genome editing *via* non-homologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, as well as generation of modified cell lines for downstream functional studies. To minimize off-target cleavage, the authors further described a double-nicking strategy using the Cas9 nickase mutant with paired guide RNAs. The protocol provided by the authors experimentally derived guidelines for the selection of target sites, evaluation of cleavage efficiency and analysis of off-target activity. The studies showed that beginning with target design, gene modifications can be achieved within as little as 1-2 weeks, and modified clonal cell lines can be derived within 2-3 weeks.
- Shalem *et al.* described a new way to interrogate gene function on a genome-wide scale. Their studies showed that delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeted 18,080 genes with 64,751 unique guide sequences enabled both negative and positive selection screening in human cells. First, the authors showed use of the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, the authors screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF. Their studies showed that the highest-ranking candidates included previously validated genes NF1 and MED 12 as well as novel hits NF2, CUL3, TADA2B, and TADA1. The authors observed a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, and thus demonstrated the promise of genome-scale screening with Cas9.
- Nishimasu *et al.* reported the crystal structure of *Streptococcus pyogenes* Cas9 in complex with sgRNA and its target DNA at 2.5 A° resolution. The structure revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the sgRNA:DNA heteroduplex in a positively charged groove at their interface. Whereas the recognition lobe is essential for binding sgRNA and DNA, the nuclease lobe contains the HNH and RuvC nuclease domains, which are properly positioned for cleavage of the complementary and non-complementary strands of the

target DNA, respectively. The nuclease lobe also contains a carboxyl-terminal domain responsible for the interaction with the protospacer adjacent motif (PAM). This high-resolution structure and accompanying functional analyses have revealed the molecular mechanism of RNA-guided DNA targeting by Cas9, thus paving the way for the rational design of new, versatile genome-editing technologies.

- Wu *et al.* mapped genome-wide binding sites of a catalytically inactive Cas9 (dCas9) from *Streptococcus pyogenes* loaded with single guide RNAs (sgRNAs) in mouse embryonic stem cells (mESCs). The authors showed that each of the four sgRNAs tested targets dCas9 to between tens and thousands of genomic sites, frequently characterized by a 5-nucleotide seed region in the sgRNA and an NGG protospacer adjacent motif (PAM). Chromatin inaccessibility decreases dCas9 binding to other sites with matching seed sequences; thus 70% of off-target sites are associated with genes. The authors showed that targeted sequencing of 295 dCas9 binding sites in mESCs transfected with catalytically active Cas9 identified only one site mutated above background levels. The authors proposed a two-state model for Cas9 binding and cleavage, in which a seed match triggers binding but extensive pairing with target DNA is required for cleavage.
- Piatt *et al.* established a Cre-dependent Cas9 knockin mouse. The authors demonstrated *in vivo* as well as *ex vivo* genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells.
- Hsu *et al.* (2014) is a review article that discusses generally CRISPR-Cas9 history from yogurt to genome editing, including genetic screening of cells.
- Wang *et al.* (2014) relates to a pooled, loss-of-function genetic screening approach suitable for both positive and negative selection that uses a genome-scale lentiviral single guide RNA (sgRNA) library.
- Doench *et al.* created a pool of sgRNAs, tiling across all possible target sites of a panel of six endogenous mouse and three endogenous human genes and quantitatively assessed their ability to produce null alleles of their target gene by antibody staining and flow cytometry. The authors showed that optimization of the PAM improved activity and also provided an on-line tool for designing sgRNAs.
- Swiech *et al.* demonstrate that AAV-mediated SpCas9 genome editing can enable reverse genetic studies of gene function in the brain.

- Konermann *et al.* (2015) discusses the ability to attach multiple effector domains, e.g., transcriptional activator, functional and epigenomic regulators at appropriate positions on the guide such as stem or tetraloop with and without linkers.
- Zetsche *et al.* demonstrates that the Cas9 enzyme can be split into two and hence the assembly of Cas9 for activation can be controlled.
- Chen *et al.* relates to multiplex screening by demonstrating that a genome-wide *in vivo* CRISPR-Cas9 screen in mice reveals genes regulating lung metastasis.
- Ran *et al.* (2015) relates to SaCas9 and its ability to edit genomes and demonstrates that one cannot extrapolate from biochemical assays. Shalem *et al.* (2015) described ways in which catalytically inactive Cas9 (dCas9) fusions are used to synthetically repress (CRISPRi) or activate (CRISPRa) expression, showing. advances using Cas9 for genome-scale screens, including arrayed and pooled screens, knockout approaches that inactivate genomic loci and strategies that modulate transcriptional activity.
- Shalem *et al.* (2015) described ways in which catalytically inactive Cas9 (dCas9) fusions are used to synthetically repress (CRISPRi) or activate (CRISPRa) expression, showing. advances using Cas9 for genome-scale screens, including arrayed and pooled screens, knockout approaches that inactivate genomic loci and strategies that modulate transcriptional activity.
- Xu *et al.* (2015) assessed the DNA sequence features that contribute to single guide RNA (sgRNA) efficiency in CRISPR-based screens. The authors explored efficiency of CRISPR/Cas9 knockout and nucleotide preference at the cleavage site. The authors also found that the sequence preference for CRISPRi/a is substantially different from that for CRISPR/Cas9 knockout.
- Parnas *et al.* (2015) introduced genome-wide pooled CRISPR-Cas9 libraries into dendritic cells (DCs) to identify genes that control the induction of tumor necrosis factor (Tnf) by bacterial lipopolysaccharide (LPS). Known regulators of Tlr4 signaling and previously unknown candidates were identified and classified into three functional modules with distinct effects on the canonical responses to LPS.
- Ramanan *et al* (2015) demonstrated cleavage of viral episomal DNA (cccDNA) in infected cells. The HBV genome exists in the nuclei of infected hepatocytes as a 3.2kb double-stranded episomal DNA species called covalently closed circular DNA (cccDNA), which is a key component in the HBV life cycle whose replication is not inhibited by current therapies. The authors showed that sgRNAs specifically

targeting highly conserved regions of HBV robustly suppresses viral replication and depleted cccDNA.

- Nishimasu *et al.* (2015) reported the crystal structures of SaCas9 in complex with a single guide RNA (sgRNA) and its double-stranded DNA targets, containing the 5'-TTGAAT-3' PAM and the 5'-TTGGGT-3' PAM. A structural comparison of SaCas9 with SpCas9 highlighted both structural conservation and divergence, explaining their distinct PAM specificities and orthologous sgRNA recognition.
- Zetsche *et al.* (2015) reported the characterization of Cpfl, a putative class 2 CRISPR effector. It was demonstrated that Cpfl mediates robust DNA interference with features distinct from Cas9. Identifying this mechanism of interference broadens our understanding of CRISPR-Cas systems and advances their genome editing applications.
- Shmakov *et al.* (2015) reported the characterization of three distinct Class 2 CRISPR-Cas systems. The effectors of two of the identified systems, C2cl and C2c3, contain RuvC like endonuclease domains distantly related to Cpfl. The third system, C2c2, contains an effector with two predicted HEPN RNase domains.

[0155] Gao et *al.* (2016) reported using a structure- guided saturation mutagenesis screen to increase the targeting range of Cpfl . AsCpf 1 variants were engineered with the mutations S542R/K607R and S542R/K548V/N552R that can cleave target sites with TYCV/CCCC and TATV PAMs, respectively, with enhanced activities *in vitro* and in human cells.

[0156] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined in the appended claims.

[0157] The present invention will be further illustrated in the following Examples which are given for illustration purposes only and are not intended to limit the invention in any way.

EXAMPLES

Example 1: Analysis of Fusobacterium persistence and antibiotic response in human colorectal cancers

[0158] In colorectal cancer, malignant cells are surrounded by a complex microenvironment encompassing a range of non-transformed cells, but also a diverse collection of microorganisms. Increasing evidence links alterations in the composition of the

gut microbiota with **colorectal cancer.** Applicants demonstrate that *Fusobacterium* is persistently associated with colorectal cancer in liver metastases and in multiple generations of patient derived xenografts in mice. *Fusobacterium* persistence is associated with other gram-negative anaerobes including *Bacteroides fragilis* and *Selenomonas sputigena.* Colorectal cancer-derived *Fusobacterium nucleatum* isolates are invasive in cell culture and there is evidence of bacterial invasion in respective patient derived xenografts. Finally, treatment of mice bearing xenografts of human colorectal cancers with the *Fusobacterium*-killing antibiotic, metronidazole, reduces tumor growth rate and cancer cell proliferation. Applicants' observations that *Fusobacterium-associated* colorectal cancers exhibit a distinct microbial signature; that this signature is maintained through metastasis and multiple serial passages of xenografts in mice; and that antibiotics targeting *Fusobacterium* can retard the growth of *Fusobacterium-enriched* xenografts, strongly support a key role for *Fusobacterium* infection in the proliferation of colorectal cancer and suggest new opportunities for therapeutic intervention.

[0159] *Fusobacterium* species **and the** associated microbiome persist **in** liver metastases and mouse xenografts of human colon cancer; antibiotic treatment reduces xenograft growth.

[0160] Cancers, like other diseased or healthy tissues, are comprised of Applicants' own eukaryotic cells encoded by our own genomes, together with a diverse population of associated microorganisms (the microbiota). The microbiota and host form a complex 'super-organism' in which symbiotic relationships confer benefits necessary for human health (Schwabe et al., *Nat Rev Cancer* **13,** 800-812 (2013)) in addition to pathogenic consequences. Recent studies have demonstrated that perturbations in the composition of the human microbiota, or dysbiosis, can significantly increase the risk of specific cancer types, especially colorectal cancer (CRC) (Hope et al., *FEMS Microbiol Lett* **244,** 1-7 (2005); Rowland et al., *Curr Pharm Des* **15,** 1524-1527 (2009); Yang et al., *World J Gastroenterol* **12,** 6741-6746 (2006)). The human colon is the anatomical location with the largest number of microbes; a growing body of evidence demonstrates the role of particular microorganisms in modulating inflammatory environments and promoting tumor growth and metastasis. Such organisms include the pro-inflammatory enterotoxigenic *Bacteroides fragilis* (ETBF) (Wu *et al., Nat Med* **15,** 1016-1022 (2009)) and colibactin-producing *E. coli* (Arthur *et al., Science* **338,** 120-123 (2012)).

[0161] Recent evidence has proposed an association of *Fusobacterium nucleatum* with human colon cancers (Kostic *et al., Genome research* **22,** 292-298 (2012); Castellarin *et al.,*

Genome research **22,** 299-306 (2012)). Subsequent metagenomic and molecular analyses across North America (Tahara *et al., Cancer research* **74,** 1311-1318 (2014); McCoy *et al., PloS one* **8,** e53653 (2013)), Europe (Flanagan *et al., European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **33,** 1381-1390 (2014)), Asia (Li *et al., World journal of gastroenterology* **22,** 3227-3233 (2016); Ito *et al., International journal of cancer* **137,** 1258-1268 (2015)) and Africa (Viljoen et al., *PloS one* **10,** eOl 19462 (2015)) have confirmed a consistent enrichment of *Fusobacterium* in human colorectal carcinomas and adenomas compared with adjacent normal tissue. Increased tumor levels of *F. nucleatum* correlates with a lower T-cell response, poorer patient survival and advanced disease stage (Flanagan *et al., European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **33,** 1381-1390 (2014); Ito *et al., International journal of cancer* **137,** 1258-1268 (2015); Mima *et al., Gut,* (2015); Mima *et al., JAMA oncology* **1,** 653-661 (2015)) along with specific clinical and molecular subsets of CRC including right-sided anatomic location, *BRAF* mutation, and hypermutation with microsatellite instability (Tahara *et al., Cancer research* **74,** 1311-1318 (2014); Li *et al., World journal of gastroenterology* **22,** 3227-3233 (2016); Mima *et al., Gut,* (2015)).

[0162] Although these studies provide compelling evidence for the association of *Fusobacterium* with CRC, a basic question remains; does *Fusobacterium* enrichment cause colon cancer or is it predominantly a consequence of the cancer itself? In attempts to address this question Applicants previously demonstrated that exposure of $Apc^{Min/+}$ mice to *F. nucleatum* was sufficient to accelerate murine small intestinal adenocarcinoma development (Kostic *et al., Cell host & microbe* **14,** 207-215 (2013)). Also, *F. nucleatum* has been shown to modulate human and murine tumor immune infiltrates, potentially providing colonized tumors with an immune evasion mechanism (Kostic *et al., Cell host & microbe* **14,** 207-215 (2013); Gur *et al., Immunity* **42,** 344-355 (2015)). Although these findings support a role for *Fusobacterium* in colorectal cancer beyond an innocent bystander, such approaches have been limited to externally-induced single organism infections of murine cancers as well as *in vitro* assays, which do not (and cannot) account for the complexity of the microbial mileu in regulating the growth and proliferation of human tumors (Kostic *et al., Cell host & microbe* **14,** 207-215 (2013); Gur *et al., Immunity* **42,** 344-355 (2015)).

[0163] To investigate the relationship of *Fusobacterium* with human colon cancers *in vivo* Applicants set out to determine if *Fusobacterium* could persist in distant site metastases

and in patient-derived xenografts (PDXs), which could provide a more discriminative model for functional analysis and intervention.

[0164] First, Applicants asked whether *Fusobacterium* observed in colorectal tumors persists in the setting of distant metastasis. Preliminary observations from Applicants (Kostic *et al., Genome research* **22,** 292-298 (2012)) and others (Abed *et al., Cell host & microbe* **20,** 215-225 (2016)) have demonstrated the presence of *Fusobacterium* DNA in hepatic and lymph node metastases of colon cancer using quantitative PCR (qPCR) measurements. However these samples were not paired with primary colon cancers to evaluate the persistence of the bacteria.

[0165] To assess whether *Fusobacterium* and other tumor-associated bacteria are present in corresponding metastases from *Fusobacterium-colonized* primary CRCs, Applicants began by testing formalin-fixed, paraffin-embedded sections of primary colon tumors and liver metastases, similar to previous studies (REFs), but now in paired samples and from a larger cohort of 101 patients. 43% (n=44/101) of primary colon cancers tested positive for *Fusobacterium* by qPCR, and 45% (n=20/44) of liver metastases from these *Fusobacterium*-positive primary tumors were *Fusobacterium* qPCR positive **(Figure 1A).** None of the 57 *Fusobacterium-negative* primary colon tumors were associated with a *Fusobacterium*-positive liver metastasis (n=0/57; p=0) **(Figure 1A, Figure 10A).** Additionally, the presence of *Fusobacterium* in paired primary tumors and corresponding metastasis was enriched in metastatic cecum/ascending colon cancers (n=10/20, p=0.002), while cancers that were *Fusobacterium-negative* in both primary and metastasis were more likely to be rectal cancers (n=29/57 of the *Fusobacterium-negative* primary-metastasis pairs, p=0.016).

[0166] To assess the relationship between *Fusobacterium* presence in the primary cecum/ascending colon and patient survival, Applicants carried out PathSeq analysis (Kostic *et al., Nat Biotechnol* **29,** 393-396 (2011)) on RNA sequencing data from the 430 primary colon adenocarcinomas in the "TCGA cohort". Patients with cecum/ascending colon cancer exhibited worse overall survival than patients with non-cecum/ascending colon cancer in the TCGA cohort (p=0.01) **(Figure** IOC). Among patients with cecum/ascending colon tumors, we observed significantly poorer overall survival in correlation with tumor Fusobacterium load **(Figure** IOC, p=0.004).

[0167] Next, Applicants wanted to determine whether they could culture viable *Fusobacterium* strains from primary CRC and corresponding liver metastases. Of eleven snap-frozen primary-metastasis tumor pairs that were suitable for microbial culture,

Applicants could successfully culture *Fusobacterium* species from primary tumors and liver metastases from two patients (PI and P2), although seven (64%) pairs tested positive for *Fusobacterium* by qPCR **(Figure IB).** Whole genome sequencing analysis of tumors from patients P I and P2 revealed identical strains of *Fusobacterium* in both the primary colorectal tumors and their associated liver metastases, despite the tissue being collected up to two years apart. Applicants cultured *F. necrophorum* subsp. *funduliforme* from the primary colorectal tumor and liver metastases of patient P I and *F. nucleatum* subsp. *animalis* from the primary colorectal tumor and liver metastases of patient P2 **(Figure 6A-6B).** In addition to culturing *Fusobacterium* species from the primary-metastasis pairs, Applicants have cultured a range of other co-occurring anaerobes, including *Bacteroides* species **(Table 1).** Interestingly, Applicants noted that a higher fraction of colorectal adenocarcinomas with metastases (n=8/l l or 73%) were *Fusobacterium* culture-positive compared to an independent set of surgically resected colorectal adenocarcinomas for which metastatic outcome was unknown (n=15/53, or 28%) (p = 0.01, non-parametric Fisher's exact test) **(Table 2).**

[0168] **Table 1** - Selected bacterial species isolated from colorectal primary tumors and/or liver metastasis.

Bacterial species cultured from both primary-metastasis pairs
Patient 1 (P1) colorectal primary and liver metastasis
Fusobacterium necrophorum
Bacteroides fragilis
Escherichia coli
Streptococcus sanguinis
Patient 2 (P2) colorectal primary and liver metastasis
Fusobacterium nucleatum
Gemella morbillorum
Bacteroides uniformis
Clostridium Hathaway
Streptococcus sanguinis
Patient 4 (P4) colorectal primary and liver metastasis
Parvimonas micra

Bacteroides thetaiotaomicron
Solobacterium moorei
Bacteroides salyersiae
Bacteroides ovatus
Selected species isolated from either primary or metastasis tumors
Patient 1 (P1) liver metastasis
Gemella morbillorum
Eggerthella lenta
Patient 2 (P2) colorectal primary
Campylobacter ureolyticus
Porphyromonas asaccharolytica
Patient 3 (P3) colorectal primary
Fusobacterium nucleatum
Campylobacter ureolyticus
Porphyromonas asaccharolytica
Patient 4 (P4) colorectal primary
Fusobacterium nucleatum
Campylobacter gracilis
Prevotella intermedia
Mogibacterium timidum
Parvimonas micra
Patient 4 (P4) liver metastasis
Olsenella uli
Ruminococcus gnavus
Eggerthella species

Patient 5 (P5) colorectal primary
Fusobacterium nucleatum
Campylobacter ureolyticus
Alistipes shahii
Akkermansia muciniphila
Patient 6 (P6) colorectal primary
Fusobacterium necrophorum
Porphyromonas asaccharolytica
Patient 7 (P7) colorectal primary
Fusobacterium nucleatum
Parvimonas micra
Patient 8 (P8) colorectal primary
Fusobacterium nucleatum
Bacteroides fragilis
Patient 9 (P9) colorectal primary
Campylobacter ureolyticus
Patient 10 (P10) colorectal primary
Veillonella parvula
Odoribacter splanchnicus
Patient 11 (P11) colorectal primary
Odoribacter splanchnicus

[0169] **Table 2** - Metastatic colorectal tumors have a higher proportion of *Fusobacterium* culture positives. Fisher's exact test, $p = 0.01$.

CRC	Fusobacterium Culture	
	Positive	Negative
With Metastases	8	3
Metastases Unknown	15	38

[0170] To quantitate Fusobacterium abundance and to evaluate the broader microbiome, Applicants performed RNA sequencing analysis of ten primary cancers and their matched liver metastases (patients P1-P6 and P8-P11). PathSeq analysis (Kostic et al., Nat Biotechnol 29, 393-396 (2011)) of the resulting RNA sequencing data shows that the same Fusobacterium species are present, at a similarly relative abundance, in paired primary-metastasis pairs **(Figure 1C,** samples P1-P6). Furthermore, the overall dominant microbiome is qualitatively similar; persistent microbes in the liver metastases include F. nucleatum and F. necrophorum, a range of Bacteroides species including B. fragilis and B. thetaiotaomicron, and several typically oral anaerobes such as Prevotella intermedia and Selenomonas sputigena. In contrast, there was little similarity between the primary colorectal cancer and liver metastasis in the lone sample where Fusobacterium was present in the primary cancer but not detected in the metastasis **(Figure 1C,** sample P8) or in the three samples with low or undetectable levels of Fusobacterium in the primary cancer **(Figure 1C,** samples P9-P11). Furthermore, the Jaccard index revealed high correlation between the dominant bacteria in the primary tumor and metastasis for Fusobacterium-positive pairs but low correlation for Fusobacterium-negative pairs **(Figures ID** and **Figures 6A-6B).** Targeted bacterial 16S rRNA gene sequencing on DNA from the 11 frozen paired samples confirmed that (i) Fusobacterium species are present in paired primary-metastastic tumors, (ii) the relative abundance of Fusobacterium is correlated between primary tumors and metastases, and (iii) the dominant microbial genera of the liver metastases are correlated with the dominant genera of the primary tumors, demonstrating microbiome stability between paired primary-metastatic tumors harboring Fusobacterium (p=0.01), **(Figures 9A-9C).** As a control for liver metastases of colorectal carcinoma, Applicants asked whether Fusobacteria are associated with primary liver hepatocellular carcinoma (LIHC) by PathSeq analysis (Kostic et al., Nat Biotechnol 29, 393-396 (2011)) of RNA sequencing data from 201 primary LIHC tumors from The Cancer Genome Atlas (TCGA). This analysis demonstrated that Fusobacteria are rare in LIHC and that the relative abundance of Fusobacteria is significantly different between TCGA primary LIHC and liver metastases arising from colorectal cancers (p=0.01) **(Figure IE)**.

[0171] To assess whether the microbes that persist in liver metastases of *Fusobacterium*-positive CRC are similar to those associated with *Fusobacterium* in primary CRC, Applicants carried out PathSeq analysis (Kostic *et al., Nat Biotechnol* 29, 393-396 (2011)) on RNA sequencing data from 435 primary colon adenocarcinoma (COAD) from TCGA (N. Cancer Genome Atlas, Comprehensive molecular characterization of human colon and rectal cancer. *Nature* **487,** 330-337 (2012)). COAD tumors were divided into two groups based upon *Fusobacterium* abundance. The most abundant anaerobic bacteria in the primary colon cancer and liver metastases, including *Selenomonas, Bacteroides* and *Prevotella* genera, showed a correlation with *Fusobacterium* in primary COAD [false discovery rate (FDR) < 0.05, nonparametric *t* test] **(Figure IF, Table** 3). These results demonstrate that *Fusobacterium* and co-occurring tumor anaerobes are maintained and remain viable in distant site metastases.

[0172] **Table** 3 - Comparative analysis of bacterial genera **(A)** and species **(B)** that are significantly enriched in *F. nucleatum* "High" COAD. The top 50 genera or species are shown. False Discovery Rate (FDR).

(A)

Bacterial Genera	Rank	p-value	FDR	Fold Change
FUSOBACTERIUM	1	0.0020	0.0140	47.1133
ILYOBACTER	2	0.0020	0.0140	28.8882
SEBALDELLA	3	0.0020	0.0140	15.4157
STREPTOBACILLUS	4	0.0020	0.0140	13.7608
BACTEROIDES	5	0.0020	0.0140	3.4281
ALKALIPHILUS	6	0.0020	0.0140	3.0667
SYNTROPHOTHERMUS	7	0.0020	0.0140	2.9430
BUTYRIVIBRIO	8	0.0020	0.0140	2.7807
LACHNOCLOSTRIDIUM	9	0.0020	0.0140	2.6687
DICTYOGLOMUS	10	0.0020	0.0140	5.4044
SELENOMONAS	11	0.0020	0.0140	11.1374
ROSEBURIA	12	0.0020	0.0140	2.6685

CELLULOSILYTICUM	13	0.0020	0.0140	2.4087
PEPTOCLOSTRIDIUM	14	0.0020	0.0140	2.9072
LAWSONIA	15	0.0020	0.0140	5.0552
EUBACTERIUM	16	0.0020	0.0140	2.5455
CAMPYLOBACTER	17	0.0020	0.0140	7.3743
RUMINICLOSTRIDIUM	18	0.0020	0.0140	2.3232
ACIDAMINOCOCCUS	19	0.0020	0.0140	2.7733
ACETOBACTERIUM	20	0.0020	0.0140	2.4387
DESULFOVIBRIO	21	0.0020	0.0140	2.5469
CANDIDATUS AZOBACTEROIDES	22	0.0020	0.0140	2.5707
CLOSTRIDIOIDES	23	0.0020	0.0140	3.8920
RHODOTHERMUS	24	0.0020	0.0140	2.8532
PREVOTELLA	25	0.0020	0.0140	3.1566
HALANAEROBIUM	26	0.0020	0.0140	2.3960
PORPHYROMONAS	27	0.0020	0.0140	3.8514
TANNERELLA	28	0.0020	0.0140	2.2752
AMINOBACTERIUM	29	0.0020	0.0140	5.5475
DESULFOTOMACULUM	30	0.0020	0.0140	1.9935
THERMOANAEROBACTERIUM	31	0.0020	0.0140	2.3354
SLACKIA	32	0.0020	0.0140	2.9141
THERMANAEROVIBRIO	33	0.0020	0.0140	10.4379
THERMINCOLA	34	0.0020	0.0140	2.3776
THERMOANAEROBACTER	35	0.0020	0.0140	1.8895
CHLAMYDIA	36	0.0020	0.0140	2.9236

CANDIDATUS DESULFORUDIS	37	0.0020	0.0140	2.9047
LEPTOTRICHIA	38	0.0020	0.0140	9.9322
DESULFITOBACTERIUM	39	0.0020	0.0140	2.2703
ACHOLEPLASMA	40	0.0020	0.0140	4.3440
OSCILLIBACTER	41	0.0020	0.0140	2.5028
DESULFOSPOROSINUS	42	0.0020	0.0140	2.0085
CANDIDATUS ARTHROMITUS	43	0.0020	0.0140	1.9736
THERMOVIRGA	44	0.0020	0.0140	4.6990
SPHAEROCHAETA	45	0.0020	0.0140	3.5031
HYDROGENOBACULUM	46	0.0020	0.0140	3.0195
CORIOBACTERIUM	47	0.0020	0.0140	2.7495
SYNTROPHOBOTULUS	48	0.0020	0.0140	2.1824
ACETOHALOBIUM	49	0.0040	0.0240	2.5122
RUMINOCOCCUS	50	0.0020	0.0140	2.1276

(B)

Bacterial Species	Rank	p-value	FDR	Fold Change
*FUSOBACTERIUM*_NUCLEATUM	1	0.0019	0.016	47.0
ILYOBACTER_POLYTROPUS	2	0.0019	0.016	28.9
SEBALDELLA_TERMITIDIS	3	0.0019	0.016	15.4
STREPTOBACILLUS_MONILIFORMIS	4	0.0019	0.016	13.7
BACTEROIDES_FRAGILIS	5	0.0019	0.016	5.0
CAMPYLOBACTER_CURVUS	6	0.0019	0.016	11.5
DICTYOGLOMUS_TURGIDUM	7	0.0019	0.016	5.4
BACTEROIDES_HELCOGENES	8	0.0019	0.016	2.9

ALKALIPHILUS_METALLIREDIGENS	9	0.0019	0.016	3.3
SYNTROPHOTHERMUS_LIPOCALIDUS	10	0.0019	0.016	2.9
ALKALIPHILUS_OREMLANDII	11	0.0019	0.016	2.9
CLOSTRIDIUM_SP._SY8519	12	0.0019	0.016	2.7
BUTYRIVIBRIO_PROTEOCLASTICUS	13	0.0019	0.016	2.8
LACHNOCLOSTRIDIUM_PHYTOFERMENTANS	14	0.0019	0.016	2.6
[CLOSTRIDIUM]_SACCHAROLYTICUM	15	0.0019	0.016	2.7
ROSEBURIA_HOMINIS	16	0.0019	0.016	2.7
CELLULOSILYTICUM_LENTOCELLUM	17	0.0019	0.016	2.4
[EUBACTERIUM]_ELIGENS	18	0.0019	0.016	2.6
SELENOMONAS_SPUTIGENA	19	0.0019	0.016	16.5
[EUBACTERIUM]_RECTALE	20	0.0019	0.016	2.5
[CLOSTRIDIUM]_STICKLANDII	21	0.0019	0.016	2.9
LAWSONIA_INTRACELLULARIS	22	0.0019	0.016	5.0
SELENOMONAS_RUMINANTIUM	23	0.0019	0.016	4.5
[CLOSTRIDIUM]_CELLULOLYTICUM	24	0.0019	0.016	2.5
DESULFOVIBRIO_VULGARIS	25	0.0019	0.016	3.4
ACIDAMINOCOCCUS_FERMENTANS	26	0.0019	0.016	2.9
BACTEROIDES_SALANITRONIS	27	0.0019	0.016	2.3
PORPHYROMONAS_GINGIVALIS	28	0.0019	0.016	2.2
ACETOBACTERIUM_WOODII	29	0.0019	0.016	2.4
PREVOTELLA_DENTICOLA	30	0.0019	0.016	3.0
CAMPYLOBACTER_CONCISUS	31	0.0019	0.016	8.6
[CLOSTRIDIUM]_CLARIFLAVUM	32	0.0019	0.016	2.4

CLOSTRIDIUM_SP._BNL1100	33	0.0019	0.016	2.2
CLOSTRIDIOIDES_DIFFICILE	34	0.0019	0.016	3.9
DICTYOGLOMUS_THERMOPHILUM	35	0.0019	0.016	5.4
RUMINICLOSTRIDIUM_THERMOCELLUM	36	0.0019	0.016	2.2
RHODOTHERMUS_MARINUS	37	0.0019	0.016	2.8
ACIDAMINOCOCCUS_INTESTINI	38	0.0019	0.016	2.6
TANNERELLA_FORSYTHIA	39	0.0019	0.016	2.3
HALANAEROBIUM_HYDROGENIFORMANS	40	0.0019	0.016	3.4
THERMOANAEROBACTERIUM_THERMOSACCHAROLYTICUM	41	0.0019	0.016	2.5
CHLAMYDIA_TRACHOMATIS	42	0.0019	0.016	3.2
AMINOBACTERIUM_COLOMBIENSE	43	0.0019	0.016	5.5
DESULFOTOMACULUM_ACETOXIDANS	44	0.0019	0.016	2.2
SLACKIA_HELIOTRINIREDUCENS	45	0.0019	0.016	2.9
DESULFOTOMACULUM_NIGRIFICANS	46	0.0019	0.016	2.4
THERMANAEROVIBRIO_ACIDAMINOVORANS	47	0.0019	0.016	10.5
CAMPYLOBACTER_FETUS	48	0.0019	0.016	7.2
LEPTOTRICHIA_BUCCALIS	49	0.0019	0.016	9.9
PREVOTELLA_INTERMEDIA	50	0.0019	0.016	4.3

[0173] Next, to determine the spatial distribution of *Fusobacterium* in these tumors, Applicants performed *Fusobacterium* RNA *in-situ* hybridization (ISH) analysis was performed on five qPCR-positive primary/metastasis pairs from this cohort **(Figures 2A-2H and Figures 13A-13D).** Both biofilm and invasive *F. nucleatum* were observed in primary colorectal cancer (Figures. 2A-2D). Invasive *F. nucleatum* distribution was highly heterogeneous and focal, found in isolated or small groups of cells with morphology consistent with malignant cells, located close to the lumen and ulcerated regions. *F. nucleatum* was also observed in glandular structures present in the tumor center and invasive

margins, but to a lesser extent. In adjacent normal mucosa (when present) *F. nucleatum* was exclusively located in the biofilm. In liver metastasis, *F. nucleatum* was predominantly localized in isolated cells whose histomorphology is consistent with colon cancer cells (Figures **2E-2H),** although occasional stromal *F. nucleatum* could be observed as well. No *F. nucleatum* was detected in the adjacent residual liver parenchyma.

[0174] Given the persistence of viable Fusobacterium species in metastatic CRC, Applicants next asked if Fusobacterium could persist in colorectal cancer xenografts. To address this question, fresh primary human CRC tumors were subjected to microbial analysis (culture and qPCR) to assess the presence of Fusobacterium and were implanted subcutaneously into Nu/Nu mice, to establish patient-derived xenografts (PDXs). All Fusobacterium-culture positive CRCs resulted in successful xenografts (Figure 3A), while all attempts to generate PDXs from Fusobacterium-culture negative tumors were unsuccessful (p=0.003). Tumor grade did not appear to significantly influence successful xenograft formation (p=0.1) (Figure 11A), although we noted a modest association between Fusobacterium-cultivability and high-grade tumors in this cohort (n=4/5, p=0.03) (Figure **11B).**

[0175] Next, Applicants sought to determine whether *Fusobacterium* would remain viably associated with colorectal cancer xenografts when propagated through multiple generations of mice. A PDX derived from an i7. *nucleatum* culture positive CRC (COCA36) was passaged to F8, allowing us to test for the presence of *F. nucleatum* over a period of 29 weeks *in vivo.* Applicants cultured *F. nucleatum* from this PDX for up to four generations and 124 days *in vivo.* All xenograft generations, from F1 through F8, were *Fusobacterium* qPCR positive **(Figure 3B).** Additionally, Applicants cultured other anaerobic bacteria, including *B. fragilis and B. thetaiotaomicron,* from both the primary tumor and PDX. Applicants further cultured *Fusobacterium* from PDXs of two additional patients **(Table 4).** *Fusobacterium* targeting qPCR and microbiome analysis of fecal pellets and oral swabs from these animals were negative for *Fusobacterium* species **(Figure 8),** arguing against the possibility that *Fusobacterium* was arising from the endogenous murine microbiome.

[0176] **Table 4 -** *Fusobacterium-targeted* qPCR and culture of primary colorectal tumors, metastases and derived xenografts. **Fusobacterium* culture positive.

Tumor	**Source**	***Fusobacterium* qPCR**
COCA6 Primary	Colorectal adenocarcinoma	Positive*
COCA6 F3	Colorectal adenocarcinoma PDX	Positive*
COCA31 Primary	Colorectal adenocarcinoma	Positive
COCA31 F1	Colorectal adenocarcinoma PDX	Positive

COCA38 Primary	Colorectal adenocarcinoma	Positive
COCA38 F2	Colorectal adenocarcinoma PDX	Positive*
COCA38 F3	Colorectal adenocarcinoma PDX	Positive
COCA39 Primary	Colorectal adenocarcinoma	Positive*
COCA39 F2	Colorectal adenocarcinoma PDX	Positive
COCA39 F3	Colorectal adenocarcinoma PDX	Positive
COCA39 F4	Colorectal adenocarcinoma PDX	Positive
COCA45 Primary	Colorectal adenocarcinoma	Positive*
COCA45 F2	Colorectal adenocarcinoma PDX	Positive
Retrospective Analysis of Human Metastases from Colorectal Adenocarcinoma		
COCA32 Metastases	Abdominal wall metastases	Positive*
COCA32F1	Abdominal wall metastases PDX	Positive*
COCA43 Metastases	Abdominal wall metastases	Positive
COCA43F1	Abdominal wall metastases PDX	Negative
COCA68 Metastases	Liver metastases	Positive
COCA68 F0	Liver metastases PDX	Positive
COCA74 Metastases	Liver metastases	Positive

[0177] To identify bacteria that are persistently associated with the primary colorectal tumor and derivative xenografts, Applicants carried out unbiased total RNA sequencing followed by PathSeq analysis (Kostic *et al., Nat Biotechnol* **29,** 393-396 (2011)), which revealed that *F. nucleatum* and other gram-negative anaerobes, including *B. fragilis* and *Selenomonas sputigena,* persist in these PDX models for multiple generations **(Figure 3C).** Remarkably, the bacteria that persist within the PDX include the genera that Applicants report to persist in distant site metastases to the liver **(Figure 1C)** and are enriched in *Fusobacterium-associated* colorectal cancer from analysis of TCGA data **(Figure IE).** Bacterial 16S rRNA gene sequencing further confirmed the persistence of *Fusobacterium* and co-occurring anaerobes in these primary colorectal tumors and derived xenografts **(Figure 12).**

[0178] Applicants then assessed if the primary tumor and PDX *F. nucleatum* isolates were capable of invading human colon cancer cells. Transmission electron microscopy (TEM) showed that *F. nucleatum* isolates from both primary CRCs and PDXs were invasive when incubated with HT-29 and HCT-116 colon cancer cell lines. Upon infection with *F. nucleatum,* Applicants saw evidence of bacterial cells within vesicle-like structures in the cancer cell **(Figures 4A-4C).** Interestingly, Applicants also see evidence of bacterial adhesion and invasion in the respective patient xenograft tissue **(Figure 4D).**

[0179] Finally, Applicants asked whether treatment of *Fusobacterium-positive* colorectal cancer xenografts with an antibiotic that targets *Fusobacterium* species would affect tumor

growth. A recent study reported that infection with *F. nucleatum* increased the growth rate of tumor xenografts derived from CRC cell lines *in vivo (Y*ang *et al., Gastroenterology,* (2016)). Similar to previous findings from Applicants' group demonstrating an NF-κB-driven pro-inflammatory response in *Fusobacterium* associated human colorectal cancers (Kostic *et al., Cell host & microbe* **14,** 207-215 (2013)), this group reports that *F. nucleatum* initially activates TLR-4 signaling, promoting subsequent NF-κB activation (Yang *et al., Gastroenterology,* (2016)). Fusobacteria are reported to be highly sensitive to metronidazole (Lofmark et al., *Clinical Infectious Diseases* **50,** S16-S23 (2010)). Applicants confirmed that *F. nucleatum* isolates from the PDX are sensitive to metronidazole (minimum inhibitory concentration <0.016 μg/ml) **(Figures 14 and 15).** To assess whether metronidazole is generally toxic to colorectal carcinomas, Applicants treated *Fusobacterium-free* xenografts, derived from HT-29 colon adenocarcinoma cells, with metronidazole and observed no significant decrease in tumor growth ($p>0.05$ at all timepoints measured) **(Figure 5A).** Ideally, a *Fusobacterium-negative* PDX would have been included as a control. However, none of the *Fusobacterium-negative* CRC tumors tested generated successful PDXs **(Figure 3A),** which prevented this comparison.

[0180] Upon oral administration of metronidazole to mice bearing *Fusobacterium*-positive PDXs, Applicants observed a statistically significant decrease in tumor growth, compared to PDXs in mice treated with vehicle ($p=0.0005$) **(Figure 5B).** Treatment with metronidazole was associated with a significant decrease in *Fusobacterium* load in the tumor tissue ($p=0.002$) **(Figure 5C),** along with a significant reduction in tumor cell proliferation ($p=0.0015$) **(Figure 5D).**

[0181] In summary, Applicants have shown that 1) *Fusobacterium* is persistently associated with colorectal cancer in distant metastases from human cancers, and survives through multiple generations of xenografts in mice, 2) *Fusobacterium* is associated with other co-occurring anaerobes, including *B. fragilis* and *S. sputigena,* 3) CRC-derived *Fusobacterium* can invade colon cancer cell lines, and Applicants observe evidence of bacterial invasion in xenografts, 4) Treatment of *Fusobacterium-enriched* PDXs with the *Fusobacterium-killing* antibiotic, metronidazole, decreases *Fusobacterium* load, leads to decreased cancer cell proliferation, and reduces tumor growth.

[0182] The persistence of *Fusobacterium* in distant sites and xenografts provides a distinct view of the cancer population in metastasis. Applicants hypothesize that invasive *Fusobacterium* strains travel with primary tumor cells to distant sites and establish

colonization in metastases. This observation makes metastasis a property of a cancer community including the microbiome rather than a property of a single cancer cell.

[0183] The continued association of *Fusobacterium* species with human tumors appears to be a critical component of the tumor microenvironment. This observation extends beyond *Fusobacterium* to include a range of co-occurring gram-negative anaerobes such as *B. fragilis and S. sputigena,* suggesting that the tumor microbiome is a persistent feature of a given cancer, rather than a property of its anatomical location. Noting *F. nucleatum's* role as a bridging organism in biofilm formation as shown within the oral cavity (Kolenbrander et al., *Infection and immunity* **57,** 3194-3203 (1989); Kolenbrander et al., *Journal of bacteriology* **175,** 3247-3252 (1993)), it is plausible that Fusobacteria provide a physical and/or metabolic scaffold for the broader microbial community in colorectal cancer evolution (Warren *et al., Microbiome* **1,** 16 (2013)).

[0184] Applicants' results suggest the potential for further studies of modulating the microbiome in the treatment of colorectal carcinomas. Given the long experience with and relative safety of antibiotics, it is worth evaluating what further experimental data are required to initiate clinical trials of antibiotic treatment in conjunction with chemotherapeutic treatment for *Fusobacterium-positive* colorectal cancers. Supporting the potential for targeting tumor associated microbes in colon cancer treatment, Sears and colleagues (DeStefano Shields *et al., The Journal of infectious diseases* **214,** 122-129 (2016)) recently demonstrated a reduction in tumorigenesis by treating ETBF induced murine colonic tumors with the antibiotic cefoxitin. Some concerns could include the impact of broad spectrum antibiotics on intestinal microbes and the possibility of developing opportunistic infections.

[0185] In the long run, given that metronidazole targets a range of anaerobic bacteria including co-occurring anaerobes that persist with *Fusobacterium,* it could be valuable to develop and test a *Fusobacterium-speciiic* antimicrobial agent to determine if the growth effect of metronidazole treatment is dependent primarily on *Fusobacterium,* or due to a range of co-occurring anaerobes within the tumor. Applicants' results provide a foundation for developing targeted approaches, perhaps with probiotic bacterial strains or selective small molecules, against key microbes associated with CRC.

[0186] Finally, Applicants ask in conclusion, does *Fusobacterium* cause cancer, is it an innocent bystander, or is its presence a consequence of cancer? Applicants' view at present is that *Fusobacterium* could be liable for causing colorectal cancer in a civil suit, which requires a preponderance of the evidence. However, Applicants believe that *Fusobacterium*

would currently be found not guilty of causing colorectal cancer in a criminal case, which requires evidence beyond a reasonable doubt.

Methods

Patient Specimen Collection

[0187] For the analysis of *Fusobacterium* 's persistence in distant site metastasis Applicants analyzed formalin fixed, paraffin embedded (FFPE) sections of primary colorectal tumors and matched liver metastasis from 101 patients, and snap frozen tissue of primary colorectal tumors and matched liver metastasis from an additional eleven patients from Vail d'Hebron University Hospital, Barcelona, Catalonia, Spain.

[0188] For the analysis of *Fusobacterium* 's persistence in patient derived xenografts Applicants collected 13 fresh colorectal tissues from Brigham and Women's Hospital, Boston, MA, 021 15, USA. Retrospectively, colorectal metastasis (abdominal wall n=2 and liver n=2), collected from Brigham and Women's Hospital from four patients that established patient derived xenografts, were tested for the presence of *Fusobacterium.* An additional 40 fresh colorectal tissues were collected from the Brigham and Women's Hospital, Boston, MA, 021 15, USA, for microbial analysis but were not implanted into mice for xenograft studies. Written informed consent was obtained from all patients, and the study was approved by the Institutional Review Boards of all participating institutions.

DNA And RNA Extraction

[0189] DNA was extracted from FFPE samples using the QIAamp DNA FFPE Tissue Kit (Qiagen Inc., USA), from fresh or snap frozen tissues and oral swabs using the DNeasy Blood & Tissue Kit (Qiagen Inc., USA), from bacterial cells using the QIAamp DNA Mini Kit (Qiagen Inc., USA) and RNA was extracted from tissue using the RNeasy Mini Kit (Qiagen Inc., USA), all according to manufactures instructions. DNA was extracted from murine fecal samples using an isopropanol DNA precipitation method as described previously (https://www.qiagen.com/us/resources/molecular-biology-methods/dna/) .

Quantitative PCR (qPCR)

[0190] A custom TaqMan primer/probe set was used to amplify *Fusobacterium* species DNA (Integrated DNA technologies, CA) as previously described *(28).* The cycle threshold (Ct) values for *Fusobacterium* species were normalized to the amounts of human genomic DNA in each reaction by using a primer and probe set for the prostaglandin transporter *(PGT)* reference gene, and the fold difference ($2^{-\Delta Ct}$) in *Fusobacterium* load in tumor tissue was calculated as described before *(8).* Each reaction contained lOOng of genomic DNA and was assayed in triplicate in 20 μL, reactions containing 1× final concentration TaqMan Universal

Master Mix (Applied Biosystems) and each TaqMan Gene Expression Assay (Applied Biosystems), in a 96-well optical PCR plate. Amplification and detection of DNA was performed with the ABI 7300 Real-Time PCR System (Applied Biosystems) using the following reaction conditions: 10min at 95°C and 42 cycles of 15 s at 95°C and 1min at 60°C. Cycle thresholding (Ct) was calculated using the automated settings (Applied Biosystems). The primer and probe sequences for each TaqMan Gene Expression Assay were as follows (SEQ ID. 1-6): *Fusobacterium* species forward primer, 5'-AAGCGCGTCT AGGTGGTT ATGT-3'; *Fusobacterium* species reverse primer, 5'-TGT AGTTCCGCTT ACCTCTCC AG-3'; *Fusobacterium* species FAM probe, 5'-CAACGC AAT ACAGAGTTGAG-3'. PGT forward primer, 5'-ATCCCCAAAGCACCTGGTTT-3'; PGT reverse primer, 5'-AGAGGCC AAGAT AGTCCTGGT AA-3'; PGT FAM probe, 5'-CCATCCATGTCCTCATCTC-3'.

DNA And RNA Library Construction For Next-Generation Sequencing

[0191] DNA and RNA from specimens were quantified using the NanoDrop apparatus along with the 2100 Bioanalyzer system (Agilent) prior to library construction. DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and total RNA sequencing libraries were prepared using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs) per manufacturer's instructions. Samples were pooled following library quantification using the KAPA Library Quantification Kits for Next-Generation Sequencing (KAPA Biosystems) and library insert size visualization using the 2100 Bioanalyzer system (Agilent). RNAseq libraries from tissue samples were sequenced on the Illumina HiSeq2500 platform in "Rapid Run" mode using 100 bp paired end sequencing, with 7-8 samples per lane, with an average of 41 million reads per sample. DNA libraries from murine oral swabs and fecal pellets were sequenced on the Illumina HiSeq2500 platform in "Rapid Run" mode using 100 bp paired end sequencing, with an average of 10.2 million reads per sample. Bacterial whole genomes were sequenced using the MiSeq platform with MiSeq Reagent Kit v2 (300 cycles), 150 bp paired end sequencing with an average of 60X coverage.

Bacterial Culture From Tissue

[0192] Snap frozen or fresh tumor sections (ranging from 1-5mm ^{3}) were placed in to 500 µL of pre-warmed tryptic soya broth (TSB), the tissue was agitated and gently broken up using a sterile 16G1/2 needle followed by a 22G1/2 needle (PrecisionGlide, BD).

[0193] Fifty microliter aliquots of this suspension were directly spread onto fastidious anaerobe agar (FAA) plates (Neogen Acumedia, Fisher Scientific, USA) supplemented with

7% defibrinated horse blood (DHB) (Lampire Biological Laboratories, Fisher Scientific, USA). If the tissue was resected from the colon or rectum the 500 μL suspension was split in half and fifty microliter aliquots of this suspension were also spread on selective agar (JVN), FAA + 7% DFIB plates with josamycin, vancomycin and norfloxacin, at 3, 4 and 1 μg/ml, respectively (Sigma Aldrich, USA). Plates were incubated at 37 °C for up to 14 days under anaerobic conditions (AnaeroGen Gas Generating Systems, Oxoid), with a positive and negative control plate.

[0194] Plates were inspected every 2 days for growth and AnaeroGen™ gas packs (Oxoid), were changed. Colonies were picked and streak-purified on FAA + 7% DFIB plates. Single colonies were examined by Gram stain under a light microscope, looking for gram-negative slender rods or needle- shaped cells characteristic of F. nucleatum. Colony PCR was also carried out on selected bacterial colonies. Briefly, pure colonies of interest were picked using a 1μL sterile loop and inoculated in to 30μL of sterile water, the bacterial suspension was then boiled for 20 minutes at 98 °C and cooled to 4 °C. Two microliters of the resulting colony DNA was used in a 20 μL PCR reaction (REDTaq Readymix, Sigma Aldrich, USA) with the universal bacterial primers, 342F and 1492R to amplify approximately 1.15kb of the bacterial 16s rRNA gene as described previously (29). These colony PCR products were sent for Sanger sequencing and BLASTn analysis of obtained traces was used to confirm bacterial species identity. Bacterial species of interest were suspended in TSB with 40% glycerol and stored at -80°C.

Antibiotic Sensitivity Testing (E-Test) For Fusobacterium Strains

[0195] A 1 McFarland solution, in TSB, was prepared from Fusobacterium strains grown for 24-48hours on FAA under anaerobic conditions. Using a sterile cotton swab, each Fusobacterium strain were inoculated onto antibiotic free blood agar (FAA) and E-test strip (bioMerieux) for Metronidazole (MZ), Clindamycin (CM), Cefoxitin (FX) and Imipenem (IP) were incubated for up to 72 hours and sensitivity checked after 48 hours. Each sensitivity assay was conducted in triplicate. An FAA blood plate inoculated with each Fusobacterium strain, but without the E-test strip, was used as a growth control.

Establishment Of Patient Derived Xenografts

[0196] This analysis was conducted on tissue for which signed informed consent was previously obtained from the patient undergoing surgery, according to an Institutional Review Board-approved research protocol. Fresh primary colon cancer biopsies were first incubated in an antibiotic cocktail of peniciilin/streptornycin/arnphotericin B/Ciprofloxacin for 1-2 hours to prevent abscess formation in vivo and then tumors are cut into approximately

8 mm^3 pieces of tissue and implanted into the flanks of 5 weeks old, female nude mice (Nu/Nu; Taconic). Mice are then housed in a pathogen free environment for up to 6 months to determine if primary PDX are established. When the xenografts reached -200 mm^3 mice were sacrificed, tumors harvested and serially passaged as subcutaneous implants of tumor fragments approximately 2-3 mm in diameter. At each passage tissues were cryopreserved in Bambanker cell freezing media (Wako Chemicals USA) and/or snap frozen in liquid nitrogen and stored at -80 °C. Snap frozen tissue was used for RNA and DNA extraction. When possible fresh tissue was used for microbial culture attempts.

In Vivo Metronidazole Trial

[0197] Animals bearing tumors (with volumes <250 mm^3) were randomized into statistically identical cohorts and treated with 100 mg/kg/day of metronidazole (Sigma Aldrich, USA), (suspension in 0.5% methylcellulose) or with the vehicle alone (0.5% methylcellulose) by oral gavage, daily, for approximately 3 weeks.

[0198] For HT-29 cell line xenograft experiments, approximately 1 x 10^6 HT29 cells were suspended in PBS, mixed 1:1 with Matrigel (BD Biosciences), and injected subcutaneously into the flanks of nude mice (Nu/Nu; Taconic) in a final volume of 100 µl. Following 10-14 days post implantation, mice were randomized into statistically identical cohorts and treated with 100 mg/kg/day of metronidazole (suspension in 0.5% methylcellulose) or with vehicle alone by oral gavage daily for approximately 3 weeks.

[0199] Nu/nu mice were raised in the specific-pathogen-free (SPF) conditions with autoclaved food and water. For both patient and HT-29 cell derived xenografts, tumor size was measured by caliper every Monday, Wednesday and Friday each week. Tumor volume was calculated using the formula: Volume = 0.5xL><W^2 and growth rate was measured by calculating the percentage increase in volume. Tumor measurements were carried out in a blinded approach. Mouse body weight was recorded every 3 to 7 days. Mice were euthanized when the tumor diameter reached 2 cm or following the 3 weeks of drug treatment. Tissues were cryopreserved in Bambanker cell freezing media (Wako Chemicals USA) or snap frozen in liquid nitrogen and stored at -80 °C. PDX experiments were performed with 22 (untreated group) and 24 (treated group) mice at timepoint zero, over three independent trials. HT-29 derived xenograft experiments were performed with 20 (untreated group) and 19 (treated group) mice at timepoint zero, over two independent trials. All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees of the Dana-Farber Cancer Institute.

In Vivo Bromodeoxyuridine (Brdu) Incorporation Assay

[0200] Bromodeoxyuridine (BrdUrd) solution (10 mg/mL, 0.2 mL/mouse) was administered via intraperitoneal injection 16 hours after the last dose of metronidazole or vehicle. After 2 additional hours, mice were sacrificed and tumors were fixed in 10% formalin for immunohistochemistry (IHC) analysis. A total of 6 animals per arm, that were treated with metronidazole or the vehicle for three weeks were randomly selected, over two independent trials, for BrdU incorporation analysis.

IHC Analysis

[0201] IHC was performed on 4-μπι sections of formalin-fixed paraffin-embedded samples. Tissue sections were deparaffinized and rehydrated, and antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0) in a 750 W microwave oven at 199°C for 30 minutes for BrdU staining, BrdU primary antibody (BD Biosciences, #347580) was added at a dilution of 1:100 and incubated for 1 hour at room temperature. Sections were further processed with horseradish peroxidase-conjugated secondary antibody. The reaction was detected by 3,3-diaminobenzidine and hematoxylin staining. Images were obtained with an Olympus CX41 microscope and QCapture software (Qlmaging).

Statistical Analysis

[0202] Comparisons between groups were made using the Welch two-tailed unpaired *t* test (unequal variances *t*-test) in R.

[0203] Comparisons between group proportions were made using the *z* score test for two population proportions (two-tailed).

[0204] The relationships between *Fusobacterium* culture and metastatic colorectal tumors was analyzed using Fisher's exact test.

[0205] In all cases, P values were two-tailed and P values less than 0.05 were designated as significantly different.

Cell Culture

[0206] The CRC cell lines (HCT1 16 and HT29) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All of the cell lines were cultured in appropriate conditions as recommended by ATCC.

Epithelial Cell Invasion Transmission Electron Micrograph (Tern) Analysis

[0207] The *F. nucleatum* isolates from primary colon tumor (COCA36) and derived xenograft (COCA36F3) were grown on JVN agar for 30 hours under anaerobic conditions as described above. Bacterial culture suspensions were normalized for cell number using predetermined McFarland standards. HT-29 and HCT-1 16 cells, at 80% confluency in 6 well plates, were infected with *F. nucleatum* at a multiplicity of infection (MOI) of 100:1

(bacterial cells: epithelial cells). Following infection, cells were maintained for 2.5 h at 37°C, 5% $C0_2$, after which cell media was removed, the cells were gently washed twice with PBS and were then trypsinized and again gently washed twice with PBS. The pellet was collected and stored in 3% EM grade glutaraldehyde (Fisher Scientific) at 4°C overnight. Sections were prepared for TEM analysis.

Bacterial Genome Assembly and Analysis

[0208] Bacterial genomes were assembled using the Velvet assembly tool *(30)* and a range of k-mer values from 39 to 99 (which determine the minimum read overlap) was tested to find the optimum hash length for assembly of the data. Assembled bacterial genomes were annotated and compared using both RAST *(31)* and IMG/ER *(32).* Genomes were compared using the Average Nucleotide Identity (ANI) program in IMG/ER and Kostas Lab online ANI tool (http://enve-omics.ce.gatech.edu). The parameters for the Kostas Lab online ANI tool were set to default (alignment options: minimum length 700bp, minimum identity 70%, minimum alignment 50. Fragment options: step size 200bp) with the exception of the window size which was set to 300bp. The CGView Server was used for a comparative analysis and to generate circular genomes of bacterial strains *(33).*

[0209] Microbial Detection in Whole Genome Sequencing (WGS) and RNA Sequencing Datasets.

[0210] The *PathSeq (20, 37)* algorithm was used to perform computational subtraction of human reads, followed by alignments of residual reads to human reference genomes / transcriptomes and microbial reference genomes (which include bacterial, viral, archaeal, and fungal sequences - downloaded from NCBI in October, 2015). These alignments resulted in taxonomical classification of reads into bacterial, viral, archaeal, and fungal sequences in whole genome sequencing (WGS) and RNA sequencing (RNASeq) data.

[0211] Briefly, *PathSeq* is used to remove low quality reads followed by subtraction of human reads by mapping reads to a database of human genomes (downloaded from NCBI in November 201 1) using BWA *(34)* (Release 0.6.1, default settings), MegaBLAST (Release 2.2.25, cut-off E-value 10^{-7}, word size 16) and BLASTN *(35)* (Release 2.2.25, cut-off E-value 10^{-7}, word size 7, nucleotide match reward 1, nucleotide mismatch score -3, gap open cost 5, gap extension cost 2). Only sequences with perfect or near perfect matches to the human genome were removed in the subtraction process. In addition, low complexity and highly repetitive reads were removed using *Repeat* Masker (version open-3.3.0, libraries dated 201 1-04-19).

[0212] Taxonomic classification is performed by residual reads alignment using MegaBLAST (Release 2.2.25, cut-off E-value 10^{-7}, word size 16) to a database of bacterial sequences and followed by BLASTN (Release 2.2.25, cut-off E-value 10^{-7}, word size 7) to human reference gnome and microbial reference genomes.

[0213] Following the taxonomic classification of non-human DNA sequencing reads the relative abundance value for each bacterial organism is calculated as follows by using reads that maps with >=90% sequence identity and >=90% query coverage. Classifications were performed at the domain, then phylum, then genus, then species level, requiring unique alignments (i.e., reads with equivalent E-values to multiple taxa were removed from analysis).

[0214] In the case of WGS data, species level relative abundance (RA) for each organism was calculated as follows: relative abundance of a given organism in a sample= (number of unique alignment positions in genome x 1,000,000)/ (number of total aligned bacterial reads x genome size). The RA values were then per-sample normalized such that the total relative abundance for each sample sums to one.

[0215] In the case of RNA sequencing data, species level relative abundance (RA) for each organism was calculated as follows: relative abundance of a given organism in a sample= ((number of reads aligned to a given organism/number of human reads in the sample) x 1,000,000). The RA values were then per-sample normalized such that the total relative abundance for each sample sums to one.

Comparative Microbial Analysis

[0216] Following *PathSeq* analysis of colon adenocarcinoma (COAD) samples from TCGA, samples were subset into *Fusobacterium* 'High' (>1% RA, n=1 10, median RA=5.72%, mean RA=7.7%) and *Fusobacterium* 'Low/Negative' (RA <1%, n=325, median RA=0.06%, mean RA=0.16%) tumors based upon *F. nucleatum* relative abundance. Comparative analysis was carried out between these two groups using LEfSe *(36)* (online version available on August 2016), using default parameters, except the linear discriminant score (LDA score) was increased to >=3.5 to identify significant bacterial organisms enriched or depleted in the *F. nucleatum* "High" COAD group. The GENE-E analysis tool (https://software.broadinstitute.org/GENE-E/), marker selection analysis, was used to further identify co-occurring microbes in the *F. nucleatum* "High" v's *F. nucleatum* "Low/Negative" COAD samples and to confirm LEfSe results **(Table 3).**

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Example 2: Compound Screen to Identify Compounds that Inhibit the Growth of Fusobacterium

[0217] A goal is to identify compounds that selectively inhibit the growth of patient colorectcal cancer isolates of *Fusobacterium.* A pilot screen containing 1,846 compounds (in duplicate), at [32 μM] was carried out. Approximately 1-2 x 10^6 *Fusobacterium nucleatum* cells/ml was seeded and incubated anaerobically for 48 hours. The read out was by the addition of BacTiter Glow and luminescence read out.

[0218] A method for determining the threshold to identify inhibitor hits was established and carried out **(Figure 16).** Thirty-four compound inhibitors were identified to significantly inhibit *F. nucleatum* growth, including CRC drug, 5-FU, and metronidazole **(Figure 17).** Validation of inhibitor hits and related compounds was carried out and dose response was determined for 5-FU, carmofur, and tegafur **(Figure 18).** Validation of inhibitor hits was also carried out with additional patient CRC isolates of *Fusobacterium* **(Figure 19).** Applicants

found that 5-FU inhibits *Fusobacterium* growth at levels comparable to achievable doses in plasma of treated patients. Applicants also investigated whether the presence of *Fusobacterium* in colorectal tumors can explain patient response to 5-FU-based therapies. Data of 91 patient colorectal adenocarcinoma tumors with recurrence were obtained by *Fusobacterium* culture and bacterial 16s rRNA sequencing analysis. Applicants found that *Fusobacterium-positive* colorectal tumors treated with 5-FU or a 5-FU prodrug are less likely to develop recurrence/metastasis **(Figure 20).**

[0219] In summary, Applicants' data demonstrates: (1) 5-FU (and prodrugs) are potent inhibitors of *Fusobacterium* growth (Applicants have tested patient colorectal cancer isolates of *Fusobacterium* nucleatum and *Fusobacterium* necrophorum); (2) 5-FU inhibition of *Fusobacterium* occurs within a range comparable to patient plasma levels when receiving 5-fluorouracil infusion; (3) the 5-FU analog, carmofur (contains a hexylcarbomoyl tail), is even more potent in killing *Fusobacterium* than the active drug 5-FU; and (4) *Fusobacterium*-positive colorectal tumors treated with 5-FU or a 5-FU prodrug are less likely to develop recurrence/metastasis ($p<0.05$) based on the determined *Fusobacterium* status of 43 primary colecteral adenocarcinoma patients (with recurrence information) that were treated with 5-FU or a 5-FU prodrug.

Example 3: Association Of Dietary Patterns With Risk Of Colorectal Cancer Subtypes Classified By Fusobacterium Nucleatum In Tumor Tissue

[0220] Accumulating evidence suggests that the human gut microbiome is linked to colorectal cancer development. (Ahn et al., J. *Natl. Cancer Inst.* 2013; 105(24): 1907-191 1; Dejea et al., *Proc. Natl. Acad. Sci. USA* 2014; 111(51): 18321-18326; Nakatsu et al., *Nat. Commun.* 2015; 6:8727; Flemer et al., *Gut* [published online March 18, 2016], doi:10.1 136/gutnl-2015-309595). *Fusobacterium nucleatum* has been found to be enriched in colorectal cancer tissue relative to normal adjacent colonic tissue and is detected at higher levels in stool among individuals with colorectal cancer compared with those without cancer. (Ahn et al., J. *Natl. Cancer Inst.* 2013; 105(24): 1907-191 1; Kostic et al., Genome Res. 2012; 22(2):292-298; Castellarin et al., Genome Res. 2012; 22(2):299-306; McCoy et al., PLoS One 2013; 8(l):e53653; Warren et al., Microbiome 2013; 1(1): 16; Mima et al., JAMA Oncol. 2015; 1(5):653-661; Sinha et al., PLoS One 2016; 1l(3):e0152126). Recent experimental data suggest that *F nucleatum* may contribute to colorectal carcinogenesis through modulation of host immunity and activation of pathways associated with cellular proliferation. (Mima et al., JAMA Oncol. 2015; 1(5):653-661; Rubinstein et al., Cell Host Microbe 2013; 14(2): 195-206; Gur et al., Immunity 2015; 42(2):344-355). Furthermore, a

higher amount of *F nucleatum* in colorectal cancer tissue has been linked to shorter survival, proximal tumor location, and specific tumor molecular features, such as high level CpG island methylator phenotype and microsatellite instability. (Ito et al., Int. J. Cancer 2015; 137(6); 1258-1268; Mima et al., Gut 2016; 65(12)1973-1980; Tahara et al., Cancer Res. 2014 74(5):131 1-1318).

[0221] Prudent dietary patterns—rich in fruits, vegetables, and whole grains—have been associated with a lower risk of colorectal cancer and adenoma (Fung et al., Arch Intern Med. 2003; 163(3):309-314; Terry et al., Am J Epidemiol 2001; 154(12): 1143-1 149; Kim et al., Int. J. Cancer 2005; 115(5):790-798; Cottet et al., Eur J Cancer Prev 2005; 14(l):21-29; Flood et al., Am J Clin Nutr 2008; 88(1): 176-184; Mizoue et al., Am J Epidemiol 2005; 1161(4):338-345) as reviewed in a recent systematic meta-analysis. (Magalhaes et al., Eur J Cancer Prev 2012; 21(1): 15-23). In contrast, Western dietary patterns— dominated by red and processed meats—have been linked with colorectal carcinogenesis. (Fung et al., Arch Intern Med. 2003; 163(3):309-314; Magalhaes et al., Eur J Cancer Prev 2012; 21(1): 15-23). Although mechanisms underlying these diet-cancer associations remain unclear, it is postulated that the gut microbiota may play a mediating role. (Song et al., Gastroenterology 2015;148(6):1244-60.el6). Recently, in a dietary intervention study, stool *F nucleatum* levels markedly increased after participants were switched from a prudent-style, high-fiber, low-fat diet to a low-fiber, high-fat diet. (**O**'Keefe et al., Nat. Commn. 2015;6:6342). In addition, accumulating data suggest that low fiber consumption and high meat intake may be associated with altered bacterial and metagenomic profiles as well as an inflammatory phenotype determined by serum levels of metabolites. (Sonnenburg et al., Nature 2016;529(7585):212-215; Cotillard et al., Nature 2013;500(7464):585-588; Le Chatelier et al., Nature 2013;500(7464):541-546; Koeth et al., Nat. Med. 2013;19(5):576-585).

[0222] Based on these findings, Applicants hypothesized that the inverse association between prudent diets and risk of colorectal cancer might be more evident for a cancer subgroup enriched with tissue *F nucleatum* than for a subgroup without detectable tissue *Fnucleatum.* To test this hypothesis, Applicants used 2 US nationwide, prospective cohort studies: the Nurses'Health Study (NHS) (June 1, 1980, to June 1, 2012) and the Health Professional Follow-up Study (HPFS). These 2 studies offered a unique opportunity to integrate prospectively collected, regularly updated dietary intake data with tissue microbial features in incident colorectal cancers that occurred over longterm follow-up.

Summary

[0223] *Fusobacterium nucleatum* appears to play a role in colorectal carcinogenesis through suppression of the hosts' immune response to tumor. Evidence also suggests that diet influences intestinal *F nucleatum.* However, the role of *F nucleatum* in mediating the relationship between diet and the risk of colorectal cancer is unknown.

[0224] The objective was to test the hypothesis that the associations of prudent diets (rich in whole grains and dietary fiber) and Western diets (rich in red and processed meat, refined grains, and desserts) with colorectal cancer risk may differ according to the presence of *F nucleatum* in tumor tissue.

[0225] A prospective cohort study was conducted using data from the Nurses' Health Study (June 1, 1980, to June 1, 2012) and the Health Professionals Follow-up Study (June 1, 1986, to June 1, 2012) on a total of 121 700 US female nurses and 51 529 US male health professionals aged 30 to 55 years and 40 to 75 years, respectively (both predominantly white individuals), at enrollment. Data analysis was performed from March 15, 2015, to August 10, 2016. Subjects were exposed to prudent and western diets.

[0226] Main outcomes and measures were incidence of colorectal carcinoma subclassified by *F. nucleatum* status in tumor tissue, determined by quantitative polymerase chain reaction. Of the 173 229 individuals considered for the study, 137 217 were included in the analysis, 47 449 were male (34.6%), and mean (SD) baseline age for men was 54.0 (9.8) years and for women, 46.3 (7.2) years. A total of 1019 incident colon and rectal cancer cases with available *F nucleatum* data were documented over 26 to 32 years of follow-up, encompassing 3 643 562 person-years. The association of prudent diet with colorectal cancer significantly differed by tissue *F nucleatum* status (*P* = .01 for heterogeneity); prudent diet score was associated with a lower risk of *F nucleatum–positive* cancers (*P* = .003 for trend; multivariable hazard ratio of 0.43; 95% CI, 0.25-0.72, for the highest vs the lowest prudent score quartile) but not with *F nucleatum–negative* cancers (*P* = .47 for trend, the corresponding multivariable hazard ratio of 0.95; 95% CI, 0.77-1.17). There was no significant heterogeneity between the subgroups in relation toWestern dietary pattern scores.

[0227] Applicants concluded that Prudent diets rich in whole grains and dietary fiber are associated with a lower risk for *F nucleatum-positive* colorectal cancer but not *F nucleatum*-negative cancer, supporting a potential role for intestinal microbiota in mediating the association between diet and colorectal neoplasms.

Methods

Detailed Methodology

[0228] Prior to analysis, a prospective, written protocol was submitted and approved by the research groups that oversaw the Nurses' Health Study and the Health Professionals Follow-up Study.

[0229] At baseline in the NHS, Applicants excluded 1,001 participants with a prior history of cancer, 1,044 participants with a history of ulcerative colitis, and 430 participants with recorded intakes less than 600 calories per day. At baseline in the HPFS, Applicants excluded 1,997 participants with a prior history of cancer and 475 participants with a prior history of ulcerative colitis.

[0230] Normal and tumor sections from all colorectal carcinoma cases in this study were reviewed by a pathologist (S.O.). For various characteristics, colorectal cancer patients with available tumor tissue data (n = 1,019) were generally similar to patients without available tumor tissue data (n = 2,241) (median age 66.8 vs 67.1; current smoker, 15% vs. 15%; mean body-mass index, 26.3 vs. 26.2 kg/m2; previous lower gastrointestinal endoscopy, 34% vs. 36%; mean red meat intake [servings per day], 0.55 vs. 0.54; mean dietary fiber intake, 20.0 vs 19.7 g per day; mean alcohol intake, 8.5 vs. 8.2 g per day). Tumors with and without available tissue were also similar by clinical factors, including grade and stage [proportion of poorly differentiated tumors, 16% vs. 17%; mean pT stage, 2.7 vs. 2.5; proportion with no regional lymph node metastases, 59% vs. 49%; proportion of distant metastasis (M stage), 11% vs. 17%].

[0231] Food items from the FFQ were classified into approximately 40 food groups. Factor analysis was performed using an orthogonal rotation procedure to produce two maximally uncorrected factors, selected based upon the largest eigenvalues. All analyses were adjusted for total caloric intake (kcal per day) and stratified by age (in months), year of questionnaire return and sex (in the analysis using combined cohorts). In multivariable analysis, Applicants adjusted for potential confounders including body mass index (kg/m2), pack-years of smoking (never, 0 to 4 pack-years, 5 to 19 pack-years, 20 to 39 pack-years, or >40 years), family history of colorectal cancer in any first-degree relative (yes or no), previous lower gastrointestinal endoscopy (yes or no), postmenopausal hormone use (for women only; never, past, or current), physical activity [quintiles of metabolic-equivalent task (MET)-hours per week], and regular aspirin or NSAID use (>2 tablets per week; yes or no). Prior to pooling data from the two cohorts, Applicants examined the possible heterogeneity between cohorts, using the Q statistic for the association between the prudent (or Western) dietary score and overall incidence of colorectal cancer.

[0232] Participants whose *F. nucleatum* tumor status was unknown and those who died of causes other than colorectal cancer were censored during the 26-32 years of follow-up. In addition, in the incidence analysis of one subtype, incidences of other tumor subtypes were treated as censored data. Applicants assessed the proportional hazards assumption by including the product term between age and each covariable (including the exposure of interest, prudent dietary pattern scores) into the Cox model, and testing the statistical significance of the term by Wald test. No deviation from proportional hazards assumption was detected at the a of 0.05 level. Applicants did not conduct a formal power calculation prior to the current analysis. Applicants recognized that participants might have varied their diets over the study period. Thus, Applicants utilized time-varying covariates such that each individual participant contributed multiple person-times with differing dietary data provided on each questionnaire.

Study population

[0233] Applicants used data drawn from 2 ongoing prospective cohort studies, the NHS and the HPFS. The NHS began in 1976 among 121 700 US female nurses aged 30 to 55 years at enrollment. The HPFS began in 1986 among 51 529 US male health professionals aged 40 to 75 years at enrollment. In both cohorts, participants have returned questionnaires every 2 years, with follow-up rates exceeding 90%, to provide information about lifestyle and dietary factors, medication use, and diagnoses of colorectal cancer and other diseases. The institutional review board at the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health approved this study, and informed consent was obtained from all participants. The study was conducted from June 1, 1980, to June 1, 2012.

[0234] Of 173 229 individuals considered for the study, a total of 137 217 individuals (47 449 men and 89 768 women) were included in this analysis. Applicants excluded participants with implausibly high or low caloric intakes (i.e., <600 or >3500 kcal/d for women and <800 or >4200 kcal/d for men), missing dietary pattern data, or those with a history of ulcerative colitis or cancer (except for nonmelanoma skin cancer) before baseline (1980 for the NHS and 1986 for the HPFS) (Methods described herein).

Assessment of Diet

[0235] Participants reported average food intake over the preceding year (of each questionnaire return) through semiquantitative food frequency questionnaires, which have been previously validated and described. (Rimm et al., Am J Epidemiol 1992; 135(10): 1114-1126). Total nutrient intake was calculated by summing intakes from all foods and adjusted for total energy intake by the residual method. As previously described, total dietary fiber

was calculated according to methods from the Association of Official Analytic Chemists. (Ananthakrishnan et al., Gastroenterology 2013; 145(5):970-977). For this analysis, Applicants used information from food frequency questionnaires administered in the following years: 1980, 1984, 1986, 1990, 1994, 1998, 2002, 2006, and 2010 for the NHS and 1986, 1990, 1994, 1998, 2002, 2006, and 2010 for the HPFS.

Assessment of Colorectal Cancer Cases

[0236] In both cohorts, incident cases of colorectal cancer were reported by participants through the 2012 follow-up for the HPFS and NHS. Applicants identified and confirmed lethal colorectal cancer cases through information from various sources including next of kin, the National Death Index, death certificates, and medical records. A study physician (including J.A.M. and C.S.F.), blinded to exposure information, reviewed records and extracted data on histologic type, anatomical location, and stage. The cohort study groups attempted to collect formalin-fixed, paraffin-embedded (FFPE) tissue specimens from hospitals throughout the United States as previously detailed. (Mima et al., JAMA Oncol. 2015; 1(5):653-661). Cases with available tissue data (n = 1019) for the present study were similar to those without tissue data (n = 2241) regarding patient and clinical characteristics (Methods described herein).

F nucleatum Analysis

[0237] Applicants extracted DNA from colorectal cancer tissue obtained from sections of FFPE tumor blocks (QIAamp DNA FFPE tissue kits; Qiagen). Applicants performed a real-time polymerase chain reaction (PCR) assay using custom TaqMan primer/probe sets (Applied Biosystems) for the *nusG* gene of *F nucleatum.* (Mima et al., JAMA Oncol. 2015; 1(5):653-661). The interassay coefficient of variation of cycle threshold values from each of 5 selected specimens in 5 different batches was less than 1% for all targets in the validation study. (Mima et al., Gut 2016;65(12): 1973-1980). *Fusobacterium nucleatum* positivity was defined as a detectable level of *F nucleatum* DNA within 45 PCR cycles, and *F nucleatum* negativity was defined as an undetectable level with a proper amplification of human reference gene *SLC02A1* (HGNC: 10955).

Statistical Analysis

[0238] All statistical tests were 2-sided. To account for multiple testing for the 2 primary hypotheses (related to prudent and Western dietary scores) associated with the 2 tumor subtype variables, Applicants adjusted the 2-sided a level to .01 (approximately .05/4) by simple Bonferroni correction in our primary and secondary analysis.

[0239] Two maximally uncorrelated dietary patterns—one named *prudent* and another named *Western*—were derived by principal component analysis, as previously described and validated with good reproducibility. (Fung et al., Arch Intern Med. 2003;163(3):309-314; Hu et al., Am J Clin Nutr 2000;72(4):912-921). Factor loadings were derived based on the correlations between food groups and the 2 derived factors. Each participant was assigned a factor score, determined by adding the reported frequencies of food group intakes weighted by the factor loadings. These factor scores were then standardized to have a mean (SD) of 0 (1). To capture long-term habitual consumption, Applicants calculated the cumulative mean of the prudent (or Western) dietary pattern scores from preceding food frequency questionnaires up to each questionnaire cycle. Then, the cumulative average score was categorized into sex-specific quartiles and used as the primary exposure variable.

[0240] Using Cox proportional hazards regression models, Applicants computed hazard ratios (HRs) to examine the association of the prudent orWestern dietary score with incidence of colorectal cancer. To test for trend with the Wald test, participants were assigned to the median score of their sex-specific dietary pattern quartile, and then this variable was entered into the models as a continuous term. The covariates included in the multivariable models are described in **Table 5** and the method described herein. All analyses were adjusted for total caloric intake (kilocalories per day) and stratified by age (in months), year of questionnaire return, and sex (in the analysis using combined cohorts). In multivariable analysis, Applicants adjusted for potential confounders, including body mass index (calculated as weight in kilograms divided by height in meters squared), pack-years of smoking (never, 0-4 pack-years, 5-19 pack-years, 20-39 pack-years, or >40 pack-years), family history of colorectal cancer in any first-degree relative (yes or no), previous lower gastrointestinal endoscopy (yes or no), postmenopausal hormone use (for women only: never, past, or current), physical activity (quintiles of metabolic equivalent task hours per week), and regular use of aspirin or nonsteroidal anti-inflammatory agents (>2 tablets per week: yes or no).

[0241] **Table 5** - Hazard Ratios of Incident Colorectal Cancer Overall and by *Fusobacterium* nucleatum Status

Characteristic	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P* Value	
					Trend[b]	Heterogeneity[c]
Prudent Dietary Pattern						
Overall colorectal cancer						
Person-years	913 589	907 676	912 395	909 922	NA	NA
No. of cases (n = 1019), No. (%)	250 (24.5)	248 (24.3)	268 (26.3)	253 (24.8)		NA
Age-adjusted HR (95% CI)[a]	1 [Reference]	0.93 (0.77-1.11)	0.98 (0.75-1.08)	0.79 (0.65-0.95)	.01	NA
Multivariable HR (95% CI)[a]	1 [Reference]	0.95 (0.80-1.14)	0.95 (0.79-1.14)	0.85 (0.69-1.03)	.08	NA
F nucleatum–positive colorectal cancer						
No. of cases (n = 125), No. (%)	43 (34.4)	26 (20.8)	34 (27.2)	22 (17.6)	NA	NA
Age-adjusted HR (95% CI)[a]	1 [Reference]	0.54 (0.33-0.89)	0.67 (0.42-1.05)	0.40 (0.24-0.67)	<.001	NA
Multivariable HR (95% CI)[a]	1 [Reference]	0.56 (0.34-0.92)	0.70 (0.44-1.10)	0.43 (0.25-0.72)	.003	NA
F nucleatum–negative colorectal cancer					NA	.01
No. of cases (n = 894), No. (%)	207 (23.2)	222 (24.8)	234 (26.2)	231 (25.8)	NA	NA
Age-adjusted HR (95% CI)[a]	1 [Reference]	1.01 (0.83-1.22)	0.96 (0.79-1.16)	0.88 (0.72-1.08)	.15	NA
Multivariable HR (95% CI)[a]	1 [Reference]	1.04 (0.86-1.26)	1.00 (0.83-1.22)	0.95 (0.77-1.17)	.47	NA
Western Dietary Pattern						
Overall colorectal cancer						
Person-years	910 656	910 525	910 465	911 916	NA	NA
No. of cases (n = 1019), No. (%)	244 (23.9)	275 (27.0)	243 (23.8)	257 (25.2)	NA	NA
Age-adjusted HR (95% CI)[a]	1 [Reference]	1.24 (1.04-1.48)	1.21 (1.00-1.46)	1.46 (1.18-1.82)	.001	NA
Multivariable HR (95% CI)[a]	1 [Reference]	1.19 (1.00-1.43)	1.12 (0.92-1.36)	1.29 (1.03-1.62)	.05	NA
F nucleatum–positive colorectal cancer						NA
No. of cases (n = 125), No. (%)	25 (20.0)	33 (26.4)	33 (26.4)	34 (27.2)	NA	NA
Age-adjusted HR (95% CI)[a]	1 [Reference]	1.42 (0.84-2.40)	1.59 (0.94-2.69)	1.92 (1.12-3.29)	.01	NA
Multivariable HR (95% CI)[a]	1 [Reference]	1.37 (0.81-2.31)	1.49 (0.88-2.53)	1.69 (0.98-2.90)	.05	NA
F nucleatum–negative colorectal cancer					NA	.23
No. of cases (n = 894), No. (%)	219 (24.5)	242 (27.1)	210 (23.5)	223 (24.9)	NA	NA
Age-adjusted HR (95% CI)[a]	1 [Reference]	1.25 (1.03-1.50)	1.16 (0.95-1.42)	1.42 (1.13-1.78)	.006	NA
Multivariable HR (95% CI)[a]	1 [Reference]	1.20 (0.99-1.44)	1.08 (0.88-1.33)	1.25 (0.99-1.58)	.12	NA

Abbreviations: HR, hazard ratio; NA, not applicable.

[a] According to prudent or Western dietary score quartiles in the combined cohort of the Health Professionals Follow-up Study (1986-2012) and the Nurses' Health Study (1980-2012).

[b] Tests for trend were conducted using the median value of each quartile category as a continuous variable.

[c] We tested for heterogeneity by using a likelihood ratio test comparing a model that allows separate associations for the 2 colorectal cancer subgroups (ie, *F nucleatum*–positive and *F nucleatum*–negative subgroups) with a model that assumes a common association.

[d] Stratified by age, calendar year, and sex and adjusted for total caloric intake (kilocalories per day).

[e] Stratified as listed in the above footnote and additionally adjusted for family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0-4, 5-19, 20-39, or ≥40), body mass index, physical activity (metabolic-equivalent task hours per week), and regular aspirin or nonsteroidal anti-inflammatory drug use (≥2 tablets/wk).

[0242] To examine whether the association between dietary patterns and incidence of colorectal cancer subgroups differed according to tissue *F nucleatum* status, Applicants used Cox proportional hazards regression models with a duplication method for competing risks data. As our primary hypothesis testing, Applicants tested for heterogeneity by using a likelihood ratio test, comparing amodel that allows for separate associations of dietary patterns and risk of cancer subgroups according to *F nucleatum* status with a model that assumes a common association. (Wang et al., Stat Med 2016;35(5):782-800). In secondary analyses, Applicants examined heterogeneity of the associations with cancer subgroups in

relation to dominant factor loadings for the prudent dietary pattern using cumulative average intakes of fruits, vegetables, legumes, and whole grains as well as energy-adjusted intakes of fat, fiber, and protein, all of which were categorized into quartiles. Applicants used SAS software, version 9.3 (SAS Institute Inc) for all statistical analyses. Data analysis was performed from March 15, 2015, to August 10, 2016.

Results

[0243] Of the 137 217 individuals included in the analysis, 47 449 were male (34.6%); mean (SD) baseline age for men was 54.0 (9.8) years and for women, 46.3 (7.2) years. Two major, uncorrelated dietary patterns were identified by factor analysis. The prudent dietary pattern was characterized by high intake of vegetables, fruits, whole grains, and legumes, and the Western dietary pattern was characterized by red and processed meats, refined grains, and desserts **(Table 6).** Consistent with prior analyses, (Fung et al., Arch Intern Med 2003;163(3):309-314) participants with high prudent scores in the HPFS and NHS tended to smoke less, exercise more, and have greater rates of lower gastrointestinal endoscopy, whereas Western pattern scores were associated with behaviors typically considered unhealthy **(Table** 7).

[0244] **Table 6** - Factor Loading Matrix for Dietary Patterns in the Health Professionals Follow-up Study (HPFS) and the Nurses' Health Study (NHS). ([1]) Only items with correlation coefficients >0.30 are presented. With the orthogonal rotation used, correlations are identical to factor loading matrix. ([2]) Other vegetables include corn, onion, eggplant, celery, green peppers, and mixed vegetables. ([3]) Desserts include chocolate, candy bars, cookies, brownies, cake, pie, and pastries. ([4]) Condiments include soy sauce, non-dairy creamer, Worcestershire sauce, red chili sauce, and pepper. ([5]) Snacks include chips, popcorn, and crackers.

Food Item[1]	HPFS		NHS	
	Prudent pattern	Western pattern	Prudent pattern	Western pattern
Other vegetables[2]	.69		.68	
Yellow vegetables	.65		.65	
Leafy green vegetables	.63		.64	
Cruciferous vegetables	.63		.61	
Fruits	.58		.58	
Legumes	.62		.57	
Tomatoes	.52		.48	

Fish	.46		.51	
Whole grains	.38		.41	
Potatoes	.33			.34
Poultry	.32		.41	
Water			.32	
Salad dressing			.33	
Low-fat dairy			.32	
Red meat		.66		.61
Processed meat		.61		.58
High fat dairy		.51		.50
French fries		.49		.46
Eggs		.47		.41
Desserts[3]		.43		.45
Condiments[4]		.39		.36
Refined grains		.38		.38
Butter		.38		.50
Mayonaise		.36		.34
Margarine		.34		.32
Snacks5		.34		
Pizza		.33		.36
Creamy soups		.31		.32
Sugar-sweetened beverages		.31		.33

[0245] **Table** 7 - Age-Standardized Characteristics of Participants in the Health Professionals Follow-up Study (HPFS, in 1994) and the Nurses' Health Study (NHS, in 1990) According to Prudent and Western Dietary Score Quartiles (Ql to Q4). ([1]) Continuous variables are described as means. ([2]) Regular users are defined as ≥2 standard (325-mg) tablets of aspirin or ≥2 tablets of non-steroidal anti-inflammatory drugs (NSAIDs) per week. ([3]) The percentages of postmenopausal participants as well as menopausal hormone use are among women only. ([4]) Physical activity is represented by the product sum of the metabolic equivalent task (MET) score of each specific recreational activity and hours spent on that activity per week. MET-hours/week values were assessed in 1988 for the NHS.

Characteristic[1]	Health Professionals Follow-up Study	Nurses' Health Study

	Prudent		Western		Prudent		Western	
	Q1	Q4	Q1	Q4	Q1	Q4	Q1	Q4
Age (mean)	61.1	61.3	61.4	61.0	56.2	56.4	56.4	56.2
Pack-years smoked (mean)	16.1	11.0	9.2	18.3	17.8	10.6	12.1	15.5
Current smoker, %	12	5	3	13	27	12	13	23
Family history of colorectal cancer, %	13	14	14	13	16	17	16	17
History of previous endoscopy, %	17	21	22	16	23	40	28	22
Current multivitamin use, %	39	47	47	39	33	44	44	32
Regular aspirin/NSAID use, %[2]	42	45	42	46	48	48	45	51
Postmenopause, %[3]					78	79	79	77
Monopausal hormone use, %[3]					32	36	38	30
Body mass index, kg/m^2 (mean)	26.1	25.8	25.4	26.5	25.5	26.0	25.3	26.3
Physical activity, MET-hours/week[3] (mean)	26.4	44.8	37.7	34.4	9.9	18.3	17.6	10.9
Dietary intake (means)								
Unprocessed red meat, svg/day	.59	.52	.24	.94	.53	.50	.31	.76
Processed red meat, svg/day	.33	.20	.07	.54	.26	.17	.09	.40
Poultry, svg/day	.30	.52	.40	.42	.27	.51	.42	.36
Fruit, svg/day	1.64	3.86	3.08	2.42	1.52	3.28	2.44	2.29
Vegetable, svg/day	1.84	5.54	3.65	3.48	1.95	5.10	3.49	3.34
Alcohol, g/day	11.2	10.6	7.4	14.2	5.9	4.8	5.6	5.0
Folate, µg/day	450	617	627	449	376	490	499	367
Calcium, mg/day	864	975	1024	831	900	1062	1151	835
Vitamin D, IU/day	408	498	541	378	309	376	420	271
Dietary fiber, g/day	18	29	28	19	15	22	21	19

[0246] After 26 years (in HPFS) and 32 years (in NHS) of follow-up encompassing 3 643 562 person-years, Applicants documented 1019 incident colorectal cancers with available data on tissue *F nucleatum* status. Among these cancer cases, there were 125 (12.3%) *F nucleatum* - positive tumors and 894 (87.7%) *F nucleatum-negative* tumors. Applicants

examined the association of prudent and Western dietary pattern scores with the incidence of overall colorectal cancer. Western dietary pattern scores showed a trend toward associations with overall risk of colorectal cancer in the HPFS **(Table 8)** and the combined cohort **(Table 5);** however, statistical significance was not reached with the adjusted a level of .01. Applicants did not observe significant heterogeneity in the associations of the dietary scores with colorectal cancer risk between the 2 cohorts *(P* ≥ .21). To maximize statistical power, Applicants used the combined cohort for further analyses.

[0247] **Table 8** - Hazard Ratios (HRs) of Incident Colorectal Cancer, Overall, According to Prudent or Western Dietary Pattern Score Quartiles in the Health Professionals Follow-up Study and the Nurses' Health Study. ([1]) Stratified by age and calendar year and adjusted for total caloric intake. ([2]) As above, and additionally adjusted for family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0-4, 5-19, 20-39, or >40), body mass index (kg/m^2), physical activity (MET-hours/week), regular aspirin or NSAID use (>2 tablets/week), menopausal hormone therapy status (never, past current) (women only), and total caloric intake (kcal/day). ([3]) Tests for trend were conducted using the mediam value of each category as a continuous variable. Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[3]
Prudent dietary pattern					
Health Professionals Follow-up Study					
Person-years	260,140	258,863	260,286	258,342	
No. of cases (n=388)	85	105	112	86	
Age-adjusted[1] HR (95% CI)[1]	1 (referent)	1.08 (0.81-1.44)	1.03 (0.77-1.398	0.74 (0.53-1.02)	0.04
Multivariable HR (95% CI)[2]	1 (referent)	1.11 (0.83-1.49)	1.07 (0.80-1.45)	0.80 (0.56-1.11)	0.10
Nurses' Health Study					
Person-years	653,429	648,813	652,109	651,380	
No. of cases (n=631)	165	143	156	167	
Age-adjusted HR (95% CI)[1]	1 (referent)	0.84 (0.67-1.06)	0.84 (0.67-1.05)	0.83 (0.66-1.05)	0.17
Multivariable HR (95% CI)[2]	1 (referent)	0.88 (0.70-1.10)	0.89 (0.71-1.12)	0.91 (0.71-1.16)	0.52
Western dietary pattern					
Health Professionals Follow-up Study					
Person-years	259,064	259,719	259,567	259,480	
No. of cases (n=388)	83	104	96	105	
Age-adjusted HR (95% CI)[1]	1 (referent)	1.42 (1.06-1.91)	1.45 (1.05-1.98)	1.80 (1.25-2.59)	0.003
Multivariable HR (95% CI)[2]	1 (referent)	1.37 (1.01-1.84)	1.35 (0.98-1.86)	1.62 (1.11-2.37)	0.02
Nurses' Health Study					
Person-years	651,592	650,806	650,898	652,436	
No. of cases (n=631)	161	171	147	152	
Age-adjusted HR (95% CI)[1]	1 (referent)	1.18 (0.95-1.47)	1.09 (0.86-1.40)	1.31 (1.00-1.73)	0.09
Multivariable HR (95% CI)[2]	1 (referent)	1.13 (0.91-1.41)	1.01 (0.79-1.29)	1.13 (0.85-1.51)	0.55

[0248] Applicants then tested our primary hypothesis that the association of prudent and Western diets with colorectal cancer incidence might differ according to the presence of *F nucleatumm* tumor tissue. Notably, the association between prudent dietary pattern and risk

of colorectal cancer significantly differed by tumor *F nucleatum* status (*P* = .01 for heterogeneity) **(Table 5).** Applicants found a significant inverse association of prudent dietary scores with *F nucleatum–positive* cancer risk (*P* = .003 for trend) but not with *F nucleatum–negative* cancer risk (*P* = .47 for trend). Comparing participants in the highest prudent dietary score quartile with those in the lowest quartile, the multivariable HR for *F nucleatum–positive* tumors was 0.43 (95% CI, 0.25-0.72); in contrast, the corresponding HR for *F nucleatum-negative* tumors was 0.95 (95%CI, 0.77-1.17). Applicants found similar differential associations by *F nucleatum* status in men (HPFS) and women (NHS), although statistical power was limited **(Table 9).** In addition, although statistical power was limited, Applicants found similar results when lev levels of *F nucleatum* were categorized as low or high on the basis of the median cutoff point among *F nucleatum-posihwe* cases as performed in our previous analyses **(Table 10).** (Mima et al., JAMA Oncol 2015; 1(5):653-661). Because Applicants observed that the fraction of colorectal cancers enriched with *F nucleatum* gradually decreased from cecum to rectum, (Mima et al., Clin Transl Gastroenterol 2016;7(l l):e200) Applicants conducted exploratory analyses stratified by tumor location **(Table 11).** The differential association of prudent diet score with colorectal cancer by tissue *F nucleatum* status appeared to be consistent in both proximal and distal cancer strata.

[0249] When Applicants examined the association of the Western dietary pattern with colorectal cancer subgroups according to tumor *F nucleatum* status, although Western dietary pattern scores appeared to be more strongly associated with *F nucleatum–posihwe* cancer risk, there was no significant heterogeneity between the subgroups (*P* = .23 for heterogeneity) **(Table 5)**.

[0250] **Table 9** - Cohort-Specific Hazard Ratios (HRs) of Incident Colorectal Cancer Subgroups by *F. nucleatum* status According to Prudent Dietary Score Quartiles in the Health Professionals Follow-up Study (1986-2012) and the Nurses' Health Study (1980-2012). ([1]) Stratified by age and calendar year and adjusted for total caloric intake, family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0-4, 5-19, 20-39, or >40), body mass index (kg/m^2), physical activity (MET- hours/week), regular aspirin or NSAID use (>2 tablets/week), menopausal hormone therapy status (never, past current) (women only), and total caloric intake (kcal/day). ([2]) Tests for trend were conducted using the median value of each category as a continuous variable. ([3]) We tested for heterogeneity by using a likelihood ratio test, comparing a model that allows separate associations for the two

colorectal cancer subgroups (i.e., *F. nucleatum-positive* and negative subgroups) with a model that assumes a common association. Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.

Prudent dietary score	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[2]	$P_{heterogeneity}$[3]
Health Professionals Follow-up Study						
F. nucleatum (+) colorectal cancer (n=39)	12	11	11	5		
Multivariable HR (95% CI)[1]	1 (referent)	0.82 (0.32-1.73)	0.74 (0.32-1.73)	0.31 (0.11-0.90)	0.02	
F. nucleatum (-) colorectal cancer (n=349)	73	94	101	81		0.07
Multivariable HR (95% CI)[1]	1 (referent)	1.16 (0.85-1.59)	1.13 (0.82-1.55)	0.87 (0.61-1.24)	0.29	
Nurses' Health Study						
F. nucleatum (+) colorectal cancer (n=86)	31	15	23	17		
Multivariable HR (95% CI)[1]	1 (referent)	0.47 (0.25-0.87)	0.68 (0.4-1.18)	0.49 (0.27-0.89)	0.05	
F. nucleatum (-) colorectal cancer (n=545)	134	128	133	150		0.05
Multivariable HR (95% CI)[1]	1 (referent)	0.97 (0.76-1.25)	0.94 (0.73-1.2)	1.01 (0.78-1.3)	0.97	

[0251] **Table 10** - Hazard ratios (HRs) of Incident Colorectal Cancer—by Low, High, or No Detectable Levels of *F. Nucleatum* in Tumor Tissue—According to Prudent or Western Dietary Score Quartiles in the Combined Cohort of the Health Professionals Follow-up Study (1986-2012) and the Nurses' Health Study (1980-2012). ([1]) Stratified by age, calendar year, and gender and adjusted for total caloric intake (kcal/day), family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0-4, 5-19, 20-39, or >40), body mass index (kg/m^2), physical activity (MET-hours/week), and regular aspirin or NSAID use (>2 tablets/week). ([2]) Tests for trend were conducted using the median value of each quartile category as a continuous variable. ([3]) We tested for heterogeneity by using a likelihood ratio test, comparing a model that allows separate associations for the two colorectal cancer subgroups (i.e., *F. nucleatum-positive* and negative subgroups) with a model that assumes a common association. Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[3]	$P_{heterogeneity}$[3]
Prudent dietary pattern						
F. nucleatum-high colorectal cancer						
No. of cases (n=60)	20	10	20	10		
Multivariable HR (95% CI)[1]	1 (referent)	0.47 (0.22-1.00)	0.92 (0.49-1.73)	0.43 (0.20-0.92)	0.08	
F. nucleatum-low colorectal cancer						
No. of cases (n=65)	23	16	14	12		0.03
Multivariable HR (95% CI)[1]	1 (referent)	0.65 (0.34-1.23)	0.52 (0.26-1.01)	0.43 (0.22-0.87)	0.02	
F. nucleatum-negative colorectal cancer						
No. of cases (n=894)	207	222	234	231		
Multivariable HR (95% CI)[1]	1 (referent)	1.04 (0.86-1.26)	1.00 (0.83-1.22)	0.95 (0.77-1.17)	0.47	
Western dietary pattern						
F. nucleatum-high colorectal cancer						
No. of cases (n=60)	13	18	17	12		
Multivariable HR (95% CI)[1]	1 (referent)	1.44 (0.70-2.95)	1.49 (0.70-3.09)	1.16 (0.52-2.60)	0.72	
F. nucleatum-low colorectal cancer						
No. of cases (n=65)	13	15	16	22		0.19
Multivariable HR (95% CI)[1]	1 (referent)	1.29 (0.60-2.78)	1.50 (0.70-3.20)	2.25 (1.08-4.66)	0.02	
F. nucleatum-negative colorectal cancer						
No. of cases (n=894)	219	242	210	223		
Multivariable HR (95% CI)[1]	1 (referent)	1.20 (0.99-1.44)	1.08 (0.88-1.33)	1.25 (0.99-1.58)	0.12	

[0252] **Table 11 -** Relative Risks (RRs) of Incident Colorectal Cancer Subgroups, Jointly Classified by *F. Nucleatum* Status and Anatomic Subsite, According to Prudent Dietary Pattern Scores Quartiles in the Combined Cohort of the Health Professionals Follow-up Study (1986-2012) and the Nurses' Health Study (1980-2012). ([1]) Tumors were classified as proximal if they were removed from the cecum to the transverse colon, distal if they were removed from the splenic flexure to the sigmoid colon, and rectal if they were removed from the rectosigmoid junction to the anal canal (excluding anal squamous cell carcinoma). ([2]) Stratified by age, calendar year, and gender and adjusted for family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0-4, 5-19, 20-39, or >40), body mass index (kg/m^2), physical activity (MET-hours/week), regular aspirin or NSAID use (>2 tablets/week), and total caloric intake (kcal/day). ([3]) Tests for trend were conducted using the median value of each category as a continuous variable. Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.

Anatomic subsite[1]	*F. nucleatum* status	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[2]
Proximal colon cancer (n=496)	*F. nucleatum* (+) colorectal cancer					
	No. of cases (n=79)	28	12	23	16	
	Multivariable HR (95% CI)[3]	1 (referent)	0.38 (0.19-0.76)	0.70 (0.40-1.23)	0.46 (0.24-0.85)	0.05
	F. nucleatum (-) colorectal cancer					
	No. of cases (n=417)	89	98	110	120	
	Multivariable HR (95% CI)[3]	1 (referent)	1.05 (0.78-1.40)	1.06 (0.80-1.41)	1.10 (0.82-1.47)	0.47
Distal colon and rectal cancer (n=515)	*F. nucleatum* (+) colorectal cancer					
	No. of cases (n=44)	14	14	10	6	
	Multivariable HR (95% CI)[3]	1 (referent)	0.98 (0.46-2.06)	0.65 (0.28-1.48)	0.38 (0.14-1.00)	0.03
	F. nucleatum (-) colorectal cancer					
	No. of cases (n=471)	117	123	123	108	
	Multivariable HR (95% CI)[3]	1 (referent)	1.03 (0.80-1.33)	0.96 (0.74-1.24)	0.81 (0.61-1.07)	0.07

[0253] In a secondary analysis, Applicants sought to determine whether specific food groups might explain the observed differential associations between prudent dietary patterns and risk of colorectal cancer according to *F. nucleatum* status. Applicants examined the top 4 dominantly contributing food groups to the prudent diet pattern (vegetables, fruits, legumes, and wholegrains) in relation to the risk of colorectal cancer according to *F. nucleatum* status (Table 12). Applicants observed no significant heterogeneity (with the adjusted a of .01).

[0254] Table 12 - Hazard Ratios (HRs) of Incident Colorectal Cancer by *F. nucleatum* Status According to The Top Four Food Items (by Factor Loadings) in the Prudent Dietary Pattern in the Combined Cohort. (1) Stratified by age, calendar year, and gender and adjusted for family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack- years of smoking (never, 0-4, 5-19, 20-39, or >40), body mass index (kg/m^2), physical activity (MET-hours/week), regular aspirin or NSAID use (>2 tablets/week), and total caloric intake (kcal/day). (2) Tests for trend were conducted using the median value of each quartile category as a continuous variable. (3) We tested for heterogeneity by using a likelihood ratio test, comparing a model that allows separate associations for the two colorectal cancer subgroups (i.e., *F. nucleatum-positive* and negative subgroups) with a model that assumes a common association.

Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[2]	$P_{heterogeneity}$[2]
Vegetables						
F. nucleatum (+) colorectal cancer (n=125)	29	34	31	31		
Multivariable HR (95% CI)[1]	1 (referent)	0.95 (0.58-1.57)	0.80 (0.47-1.33)	0.70 (0.42-1.18)	0.12	
F. nucleatum (-) colorectal cancer (n=894)	156	216	261	261		0.03
Multivariable HR (95% CI)[1]	1 (referent)	1.20 (0.97-1.48)	1.33 (1.08-1.64)	1.25 (1.01-1.56)	0.08	
Fruits						
F. nucleatum (+) colorectal cancer (n=125)	31	31	37	26		
Multivariable HR (95% CI)[1]	1 (referent)	0.77 (0.47-1.28)	0.84 (0.52-1.37)	0.55 (0.32-0.95)	0.04	
F. nucleatum (-) colorectal cancer (n=894)	192	234	231	237		0.22
Multivariable HR (95% CI)[1]	1 (referent)	1.03 (0.85-1.25)	0.91 (0.74-1.11)	0.85 (0.68-1.05)	0.04	
Legumes						
F. nucleatum (+) colorectal cancer (n=125)	23	40	38	24		
Multivariable HR (95% CI)[1]	1 (referent)	1.15 (0.68-1.96)	0.94 (0.55-1.60)	0.98 (0.54-1.79)	0.83	
F. nucleatum (-) colorectal cancer (n=894)	151	265	283	195		0.60
Multivariable HR (95% CI)[1]	1 (referent)	1.36 (1.11-1.67)	1.26 (1.02-1.55)	1.16 (0.92-1.47)	0.39	
Whole grains						
F. nucleatum (+) colorectal cancer (n=125)	26	42	37	20		
Multivariable HR (95% CI)[1]	1 (referent)	1.07 (0.60-1.76)	0.95 (0.57-1.58)	0.56 (0.31-1.03)	0.06	
F. nucleatum (-) colorectal cancer (n=894)	151	264	247	232		0.05
Multivariable HR (95% CI)[1]	1 (referent)	1.31 (1.06-1.61)	1.23 (0.99-1.52)	1.17 (0.94-1.45)	0.56	

[0255] Finally, to further determine whether any specific macronutrient components of the prudent dietary pattern might explain the observed differential associations according to *F nucleatum* status, Applicants explored associations of fiber, fat, and protein intake with colorectal cancer subgroups **(Table 13).** There appeared to be heterogeneity in the differential association of fiber intake with cancer subgroups classified by *F nucleatum* status (*P* = .02 for heterogeneity), similar to the findings for prudent dietary pattern scores. Comparing participants in the highest quartile of fiber intake (>26 g/d for men and >19 g/d for women) with those in the lowest quartile (<18 g/d for men and <13 g/d for women), the multivariable HR for *F nucleatum–positive* tumors was 0.54 (95% CI, 0.32-0.92); in contrast, the corresponding HR for *F nucleatum-negative* tumors was 1.13 (95% CI, 0.92-1.40). In further exploratory analyses, Applicants found that intakes of cereal-derived fiber might be differentially associated with colorectal cancer according to *F nucleatum* status (*P* = .01 for heterogeneity) **(Table 14).** Applicants did not observe such heterogeneity for fat or protein.

[0256] **Table 13** - Hazard Ratios (HRs) of Incident Colorectal Cancer by *F. nucleatum* Status According to Three Major Macronutrients in the Combined Cohort. ([1]) Stratified by age, calendar year, and gender and adjusted for family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0-4, 5-19, 20-39, or >40), body mass index (kg/m^2), physical activity (MET- hours/week), regular aspirin or NSAID use (>2 tablets/week), and total caloric intake (kcal/day). ([2]) Tests for trend were conducted using the

median value of each quartile category as a continuous variable. ([3]) We tested for heterogeneity by using a likelihood ratio test, comparing a model that allows separate associations for the two colorectal cancer subgroups (i.e., *F. nucleatum-positive* and negative subgroups) with a model that assumes a common association. Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[2]	$P_{heterogeneity}$[3]
Fiber						
F. nucleatum (+) colorectal cancer (n=125)	30	27	41	27		
Multivariable HR (95% CI)[1]	1 (referent)	0.68 (0.40-1.16)	0.87 (0.53-1.41)	0.54 (0.32-0.92)	0.04	
F. nucleatum (-) colorectal cancer (n=894)	164	230	236	264		0.02
Multivariable HR (95% CI)[1]	1 (referent)	1.14 (0.93-1.40)	1.08 (0.87-1.32)	1.13 (0.92-1.40)	0.40	
Fat						
F. nucleatum (+) colorectal cancer (n=125)	28	28	31	28		
Multivariable HR (95% CI)[1]	1 (referent)	1.41 (0.86-2.30)	1.27 (0.75-2.12)	1.63 (0.96-2.78)	0.10	0.29
F. nucleatum (-) colorectal cancer (n=894)	237	234	230	193		
Multivariable HR (95% CI)[1]	1 (referent)	1.05 (0.87-1.26)	1.12 (0.92-1.35)	1.13 (0.92-1.37)	0.17	
Protein						
F. nucleatum (+) colorectal cancer (n=125)	32	36	37	20		
Multivariable HR (95% CI)[1]	1 (referent)	1.01 (0.62-1.63)	1.08 (0.67-1.73)	0.68 (0.39-1.18)	0.27	0.72
F. nucleatum (-) colorectal cancer (n=894)	225	243	246	180		
Multivariable HR (95% CI)[1]	1 (referent)	1.06 (0.89-1.28)	1.08 (0.90-1.30)	0.81 (0.66-0.99)	0.05	

[0257] **Table 14** - Hazard Ratios (HRs) of Incident Colorectal Cancer Subgroups by F. nucleatum Status According to Intakes of Three Major Subclasses of Dietary Fiber in the Combined Cohort. ([1]) Stratified by age, calendar year, and gender and adjusted for family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0-4, 5-19, 20-39, or >40), body mass index (kg/m^2), physical activity (MET- hours/week), regular aspirin or NSAID use (>2 tablets/week), and total caloric intake (kcal/day). ([2]) Tests for trend were conducted using the median value of each category as a continuous variable. ([3]) We tested for heterogeneity by using a likelihood ratio test, comparing a model that allows separate associations for the two colorectal cancer subgroups (i.e., F. nucleatum-positive and negative subgroups) with a model that assumes a common association. Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.

Discussion

[0258] In the 2 US nationwide prospective cohorts, Applicants found that participants with higher long-term prudent dietary pattern scores were associated with a lower risk of *F. nucleatum-positive* colorectal cancers but not *F. nucleatum–negative* cancers. Our data also suggest that higher intakes of dietary fiber, one of the components of the prudent diet, may be

associated with a lower risk of *F nucleatum–positive* colorectal cancer but not *F nucleatum*–negative cancer. These findings support the hypothesis that the possible cancer preventive effects of prudent diets rich in dietary fiber may be mediated by modulation of specific species in the gut microbiota and subsequent alteration of the amount of *F nucleatum* in local colonic tissue. To our knowledge, our study represents the first to examine the intersection of diet and incidence of colorectal cancer subgroups according to microbial status in human tumor tissue.

[0259] The potential role of diet in modulating the risk of a variety of diseases, including colorectal cancer, has been widely recognized. (Song et al., Gastroenterol oty 2015;148(6):1244-60.el6; Tuddenham et al., Curr Opin Infect Dis 2015;28(5):464-470). According to the World Cancer Research Fund and American Institute for Cancer Research, foods with fiber including whole grains are one of the strongest factors linked to decreasing the risk of colorectal cancer. (World Cancer Research Fund/ American Institute for Cancer Research. Continuous update projectkeeping the science current. Colorectal Cancer 201 1 Report: food, nutrition, physical activity, and the prevention of colorectal cancer. http://wcrf.org/int/research-we-fund/our-cancer-prevention-recommendations.Accessed April 21, 2016). However, there has been considerable heterogeneity in the epidemiologic data associating prudent dietary patterns and the major components of the prudent diet with colorectal cancer. (Aune et al., BMJ 201 1;343:d6617). Our results here suggest that the inconsistency in the association of prudent dietary patterns (and components of the diet) with lower colorectal cancer risk may be in part attributable to differential associations with cancer subgroups according to *F nucleatum* in tumor tissue. In addition, given recent findings between increasing amounts of *F nucleatum* DNA in colorectal cancer tissue and worsened survival, (Mima et al., Gut 2016;65(12): 1973-1980) our data lend additional support to the promotion of healthy diets to reduce mortality from colorectal cancer.

[0260] The precise mechanism by which prudent diets rich in dietary fiber may lower *F nucleatum-enriched* cancer incidence remains unclear. Accumulating evidence suggests that long term dietary fiber intake has a profound effect on the gut microbiome, specifically through promotion of microbial diversity and by lowering levels of inflammatory metabolites. (Sonnenburg et al., Nature 2016;529(7585):212-215; Wu et al., Science 201 1;334(6052): 105-108; Filippis et al., Gut 2016;65(1 1): 1812-1821; Ou et al., Am J Clin Nutr 2013;98(1): 111-120; Claesson et al., Nature 2012;488(7410): 178-184). A recent study showed that a 2-week feeding intervention switching rural-dwelling South Africans from a high-fiber, low-fat diet to a low-fiber, high-fat diet was associated with an increase in *F*

nucleatum measured by PCR in the stool. (O'Keefe et al., Nat. Commun. 2015;6:6342). In addition, some have hypothesized that the variation observed in *F nucleatum* levels in colorectal cancers collected from Spain, Vietnam, Japan, and the United States may be attributable to differences in dietary practices in these countries. (Kostic et al., Genome Res. 2012;22(2):292-298; Nosho et al., World J Gastroenterol 2016;22(2):557-566). Furthermore, in a cross-sectional study, participants with advanced adenoma were associated with lower dietary fiber intakes as well as distinct fecal microbiome communities compared with healthy controls. (Chen et al., Am J Clin Nutr. 2013;97(5): 1044-1052). It is plausible that an abundance of microbiota-accessible carbohydrates from prudent diets may influence bacterial fermentation of dietary fiber, resulting in altered levels of short-chain fatty acids. These changes may alter pH, increase transit time of gut contents, or lead to differences in local immune surveillance, which are less hospitable for nonnative species, such as *F nucleatum*, to establish themselves in the colonic niche and potentiate colorectal carcinogenesis. (O'Keefe et al., Nat. Commun. 2015;6:6342; Sonnenburg et al., Nature 2016;529(7585):212-215; Garrett et al., Science 2015;348(6230):80-86; Smith et al., Science 2013;341(6145):569-573). Taken together, these data provide evidence of substantial influences of diet on the gut microbiome, which may in turn influence tumorigenesis.

[0261] There are several strengths in this study. First, our dietary data were prospectively collected and have been well validated. (Rimm et al., Am J Epidemiol 1992; 135(10): 1114-1126). Second, our data were detailed and updated such that Applicants could examine long-term effects of overall dietary patterns, specific food groups, and macronutrients in relation to colorectal cancer risk. Third, Applicants collected detailed data on multiple potential confounders, although residual confounding cannot be excluded. Finally, our molecular pathological epidemiology (MPE) research (Ogino et al., Gut 201 1;60(3):397-41 1) provides refined risk estimates for specific cancer subgroups, such as *F nucleatum-positive* cancer, and thereby offers insights into pathogenesis and causality. Molecular subtyping in the MPE approach can gather pathogenetically similar cases, and thus can enhance statistical inference (even with a relatively small number of cases). (Ogino et al., Epidemiology 2016;27(4):602-6 11). The present study represents emerging unique microbial MPE research (Hamada et al., Molecular pathological epidemiology: new developing frontiers of big data science to study etiologies and pathogenesis [published online October 13, 2016], J. Gastroenterol.) in which the microbial feature in tumor tissue can serve as a pathogenic signature.

Limitations

[0262] Applicants acknowledge limitations of this study. First, this study was observational, and residual confounding may be an issue. Nevertheless, adjustment for a variety of known risk factors for colorectal cancer showed no substantial effect on the results. Second, the diet data were derived from food frequency questionnaires and subject to measurement errors. Nonetheless, studies have shown that food frequency questionnaires can better capture long term dietary intakes than detailed diet diaries in a limited period. (Willett et al., Nutritional Epidemiology, New York, NY: Oxford University Press; 2012). Third, with the use of FFPE tissue specimens, routine histopathologic procedures might have influenced performance characteristics of our PCR assay to detect *F nucleatum.* Nonetheless, Applicants conducted a rigorous validation study that showed high precision of our PCR assay to detect *F nucleatum.* (Mima et al., JAMA Oncol. 2015; 1(5):653-661). Moreover, our assay has previously been shown to have high specificity for *F nucleatum.* (Castellarin et al., Genome Res. 2012;22(2):299-306). Fourth, Applicants could not collect FFPE blocks from all colorectal cancer cases in the cohorts; nonetheless, cases with available tissue were generally similar to those without tissue with regard to patient characteristics. Fifth, because our participants were all health professionals and most were white, generalizability of the findings to other populations needs to be examined in future studies.

Conclusions

[0263] This study has shown that a prudent diet is associated with a lower risk of *F nucleatum–positive* colorectal cancer but not *F nucleatum–negative* cancer. Our data generate new hypotheses about how the intestinal microbiotamay mediate the association between diet and colorectal neoplasms. Further studies are needed to confirm these findings and determine the potential utility of characterization of *F nucleatum* in colonic mucosa, tumor, or stool as a biomarker for personalized nutritional, probiotic, or antibiotic interventions. In addition, our findings underscore the importance of future large-scale, prospective studies that examine the gut microbiota to understand the complex intersection of diet, the gut microbiome, and carcinogenesis. (Fu et al., *Ann Epidemiol.* 2016;26(5):373-379).

* * *

[00264] Various modifications and variations of the described methods, pharmaceutical compositions, and kits of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it will be understood that it is capable of

further modifications and that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the invention. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known customary practice within the art to which the invention pertains and may be applied to the essential features herein before set forth.

CLAIMS

What is claimed is:

1. A method of treating a neoplasm in a subject comprising administering to the subject an agent, compound, or composition that inhibits bacterial growth in the subject.

2. The method of claim 1, which further comprises diagnosing whether the subject has a bacterial infection.

3. The method of any one of claims 1 or 2, wherein the bacterial infection comprises a gram negative bacterial infection.

4. The method of any one of claims 1 or 2, wherein the bacterial infection comprises *Fusobacterium* infection.

5. The method of claim 1, wherein the neoplasm is a gastrointestinal cancer.

6. The method of claim 5, wherein the gastrointestinal cancer is colon cancer.

7. The method of claim 1, wherein the neoplasm is a metastatic tumor.

8. The method of any one of claims 1 to 7, wherein the subject has a detectable bacteria or *Fusobacterium* infection.

9. The method of claim 8, which comprises detecting bacteria or *Fusobacterium* in or associated with the gastrointestinal cancer or metastatic tumor.

10. The method of claim 8, which comprises detecting bacteria or *Fusobacterium* in a stool sample from the subject.

11. The method of claim 8, which comprises detecting circulating bacteria or *Fusobacterium* nucleic acids.

12. The method of any one of claims 8 to 11, which comprises detecting bacteria or *Fusobacterium* nucleic acids in the subject.

13. The method of claim 12, wherein the detection method comprises use of a CRISPR effector system.

14. The method of claim 1, wherein the agent, compound, or composition comprises a *Fusobacterium-selective* antimicrobial agent.

15. The method of claim 1, wherein the agent, compound, or composition comprises metronidazole or 5-fluorouracil.

16. The method of claim 1, wherein the subject is put on a low fat or low lipid or low carbohydrate diet.

17. The method of any one of claims 1 to 15, which comprises coadministering the compound or composition that inhibits *Fusobacterium* with an anti-neoplastic agent.

18. A method of identifying a cellular component or pathway linked to sensitivity to growth induction by a *Fusobacterium,* which comprises contacting a test cell which comprises a mutation or phenotype linked to the cellular component or pathway with a *Fusobacterium,* contacting a control cell with the *Fusobacterium,* and identifying the cellular component or pathway as linked to sensitivity to induction by *Fusobacterium* if the test cell and control cell display a *Fusobacterium-dependent* phenotypic change.

19. The method of claim 18, wherein the method comprises comparing a library of test cells to a control cell.

20. The method of any one of claims 18 or 19, wherein the test cell or library is from a tumor cell.

21. The method of any one of claims 18 or 19, wherein the test cell or library is from transformed cell.

22. The method of any one of claims 18 or 19, wherein the cell or library is from a tumor cell from a subject.

23. A method of identifying a compound or composition that inhibits induction of a cell by *Fusobacterium*, which comprises culturing a mammalian cell with a *Fusobacterium* in the presence of a test compound, culturing the mammalian cell with a *Fusobacterium* in the absence of the test compound, and identifying the compound as an induction inhibitor if the test compound inhibits growth of the test culture.

24. The method of claim 23, wherein the mammalian cell is from a tumor cell.

25. The method of claim 23, wherein the test cell or library is from transformed cell.

26. The method of claim 23, wherein the cell or library is from a tumor cell from a subject.

27. A method of identifying a subpopulation of a population of patients having a neoplasm comprising diagnosing and identifying therefrom patients in the population having a bacterial infection in or associated with the neoplasm, whereby those patients having the bacterial infection in or associated with the neoplasm are the subpopulation.

28. The method of claim 27 wherein the bacterial infection comprises a gram negative bacterial infection.

29. The method of claim 28 wherein the bacterial infection comprises a *Fusobacterium* infection.

30. The method of any one of claims 27 to 29 wherein the bacterial infection is in or associated with gastrointestinal cancer or metastatic tumor or is in a stool sample.

31. A method of identifying a treatment regimen for a patient having a neoplasm comprising diagnosing whether the patient has a bacterial infection in or associated with the neoplasm.

32. The method of claim 31 wherein the bacterial infection comprises a gram negative bacterial infection.

33. The method of claim 32 wherein the bacterial infection comprises a *Fusobacterium* infection.

34. The method of any one of claims 31 to 33 wherein the bacterial infection is in or associated with gastrointestinal cancer or metastatic tumor or is in a stool sample.

35. The method of any one of claims 31 to 34 additionally comprising administering an agent, compound or composition that inhibits bacterial growth in the subject, optionally wherein the agent, compound or composition is specific to the bacterial infection, and further optionally on a regimen comprising coincident with or sequentially to administration of anti-neoplastic agents.

36. A method for removal of a tumor in a subject comprising systemically and/or locally to the tumor testing for the presence of a bacterial infection, optionally wherein the testing comprises a rapid diagnostic that identifies an RNA and/or DNA signature of a bacterial, and further optionally wherein the testing includes measuring bacterial load.

37. The method of claim 36 wherein the bacterial infection comprises a gram negative bacterial infection.

38. The method of claim 37 wherein the bacterial infection comprises a *Fusobacterium* infection.

39. The method of any one of claims 36 to 38 wherein the bacterial infection is in or associated with gastrointestinal cancer or metastatic tumor or is in a stool sample.

40. The method of any one of claims 36 to 39 additionally comprising administering an agent, compound or composition that inhibits bacterial growth in the subject.

41. The method of claim 40 wherein the administering is locally to the area of the patient from which the tumor has been or is being removed.

42. The method of claim 40 or 41 wherein the administering is systemic.

FIG. 1A

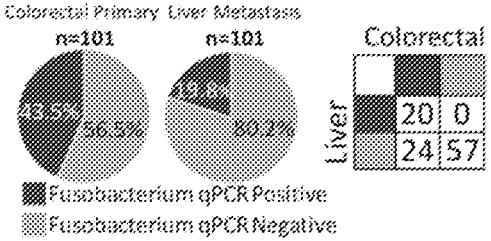


FIG. 1B

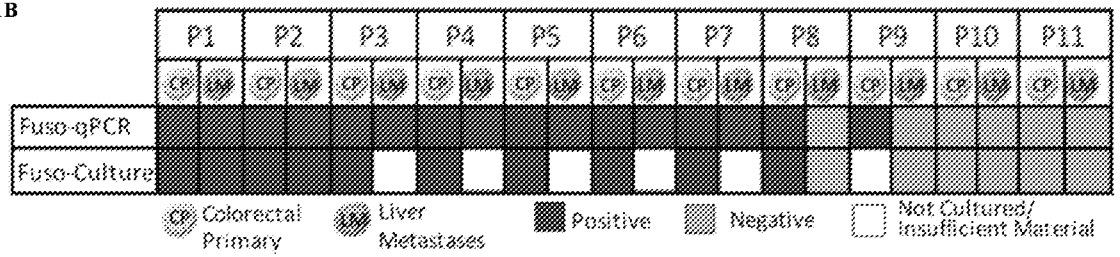


FIG. 1C

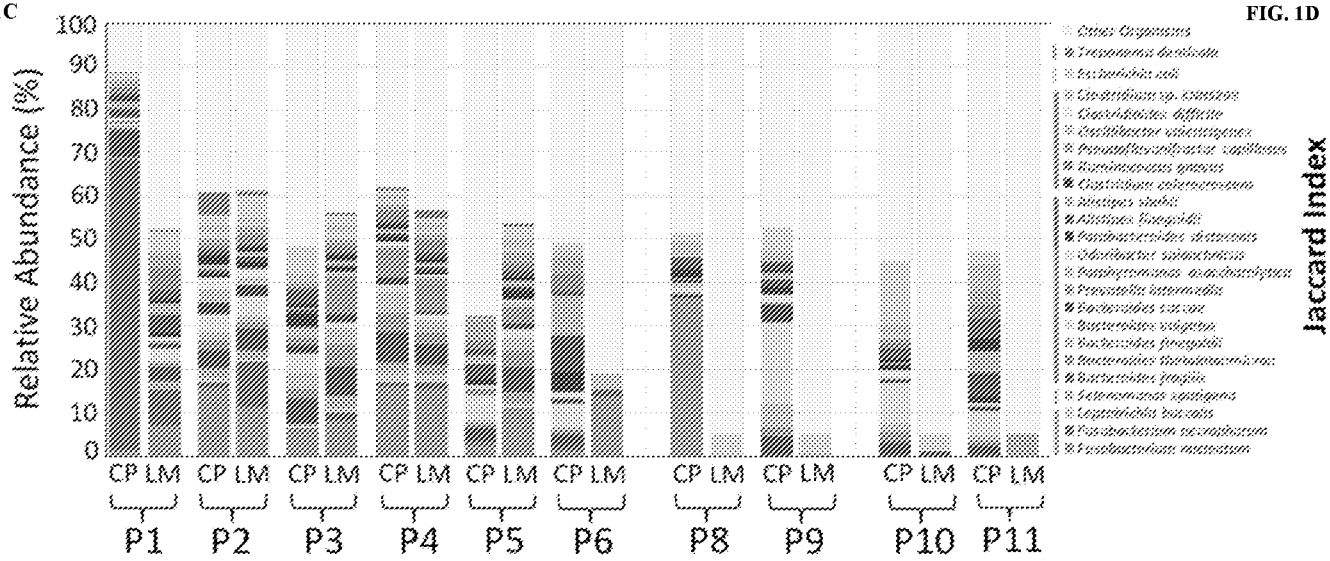


FIG. 1D

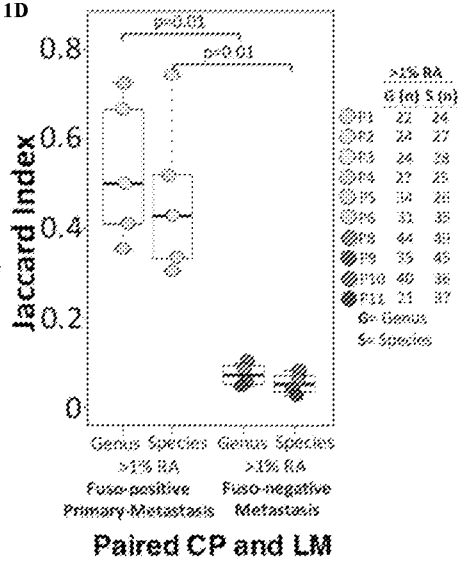


FIG. 1E

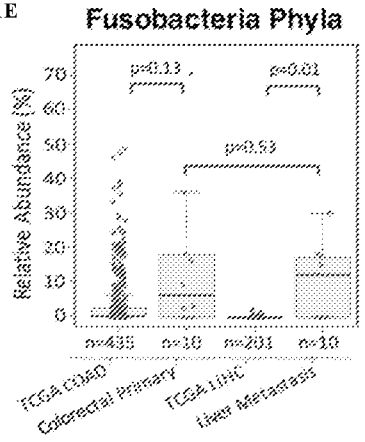
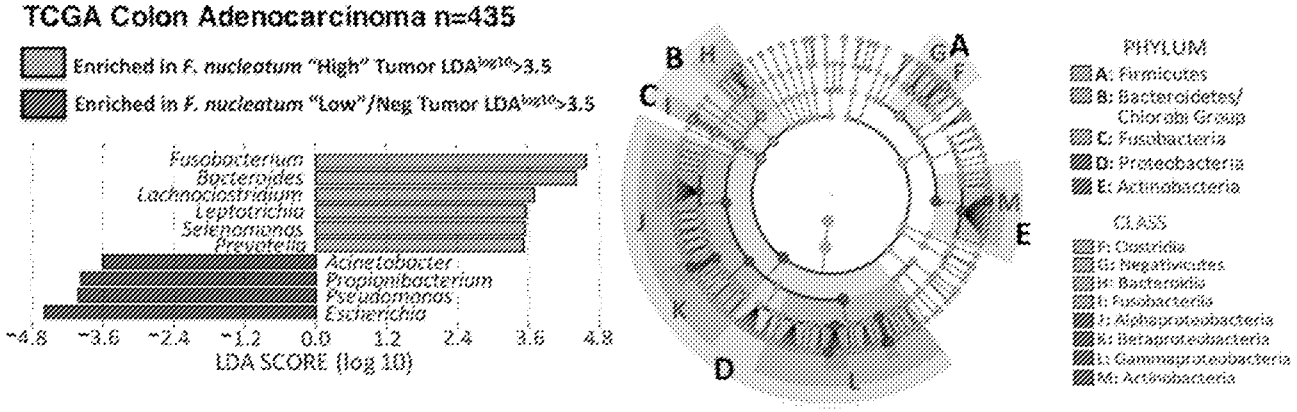


FIG. 1F



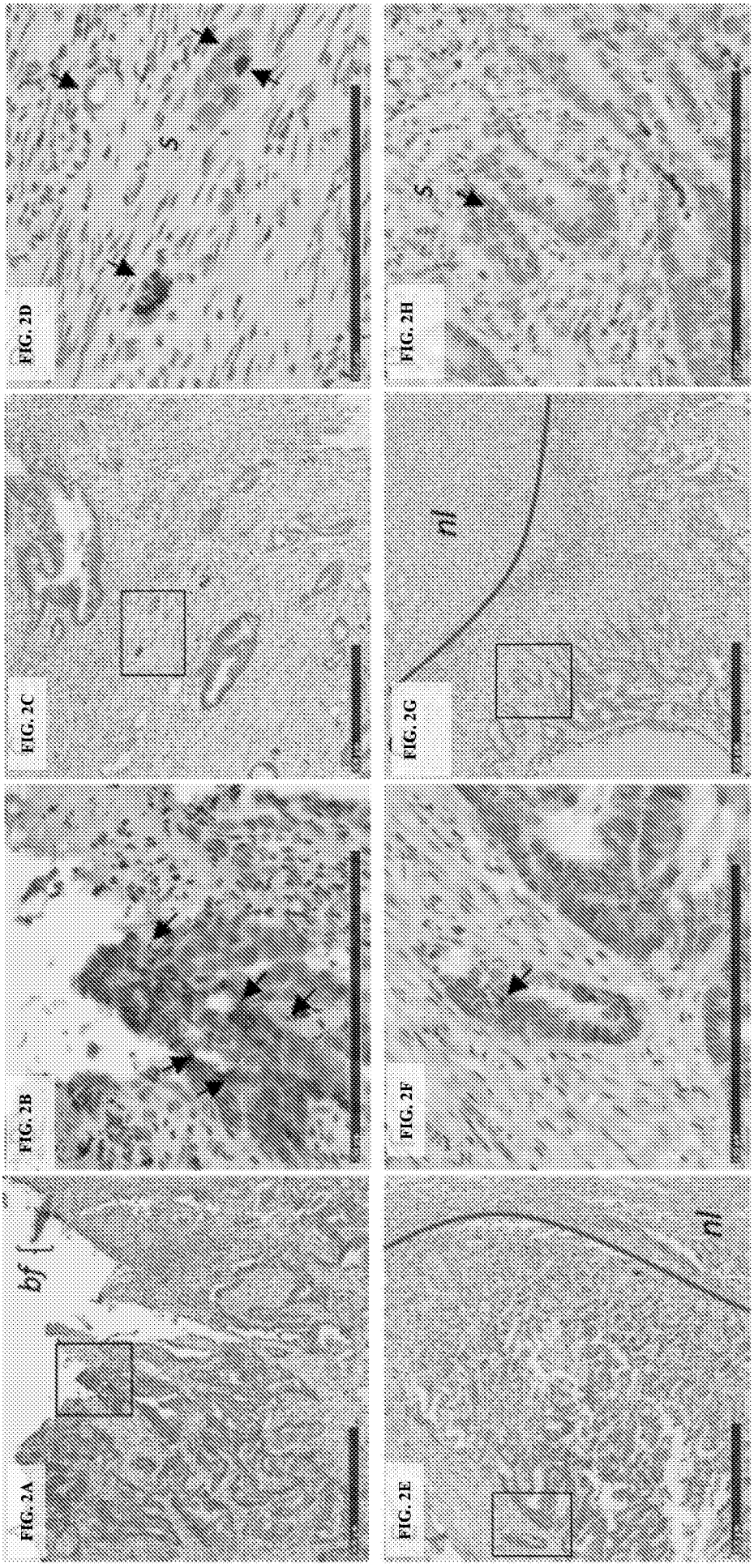


FIG. 2A
bf
FIG. 2B
FIG. 2C
FIG. 2D
S
FIG. 2E
nl
FIG. 2F
FIG. 2G
nl
FIG. 2H
S

FIG. 3A
Establishing Patient Derived Xenografts
p=0.0027
p=0.017
Unsuccessful PDX
Successful PDX
Count
Culture and qPCR Positive
qPCR Positive Only
Negative
Fusobacterium Status of Primary Tumor
FIG. 3B
1° Colon
F0
F1
F2
F3
F4
F5
F6
F7
F8
0
44
64
84
104
124
144
164
184
204
DAYS
Fuso-qPCR
Fuso-Culture
COCA36
Positive
Negative*
Not Cultured/ Insufficient Material
FIG. 3C
Relative Abundance (%)
Other Organisms
Eubacterium eligens
Clostridium sp. KNHs205
Roseburia hominis
Ruminococcus gnavus
Porphyromonas asaccharolytica
Prevotella sp.
Bacteroides caccae
Bacteroides vulgatus
Bacteroides fragilis
Bacteroides thetaiotaomicron
Bacteroides finegoldii
Subdoligranulum sp.
Fusobacterium nucleatum
Primary Tumor
PDX F1
PDX F3
COCA36 Primary & PDX Culture Positive
Primary Tumor
PDX F2
PDX F4
COCA39 Primary Culture Positive
Primary Tumor
PDX F3
COCA6 Primary & PDX Culture Positive

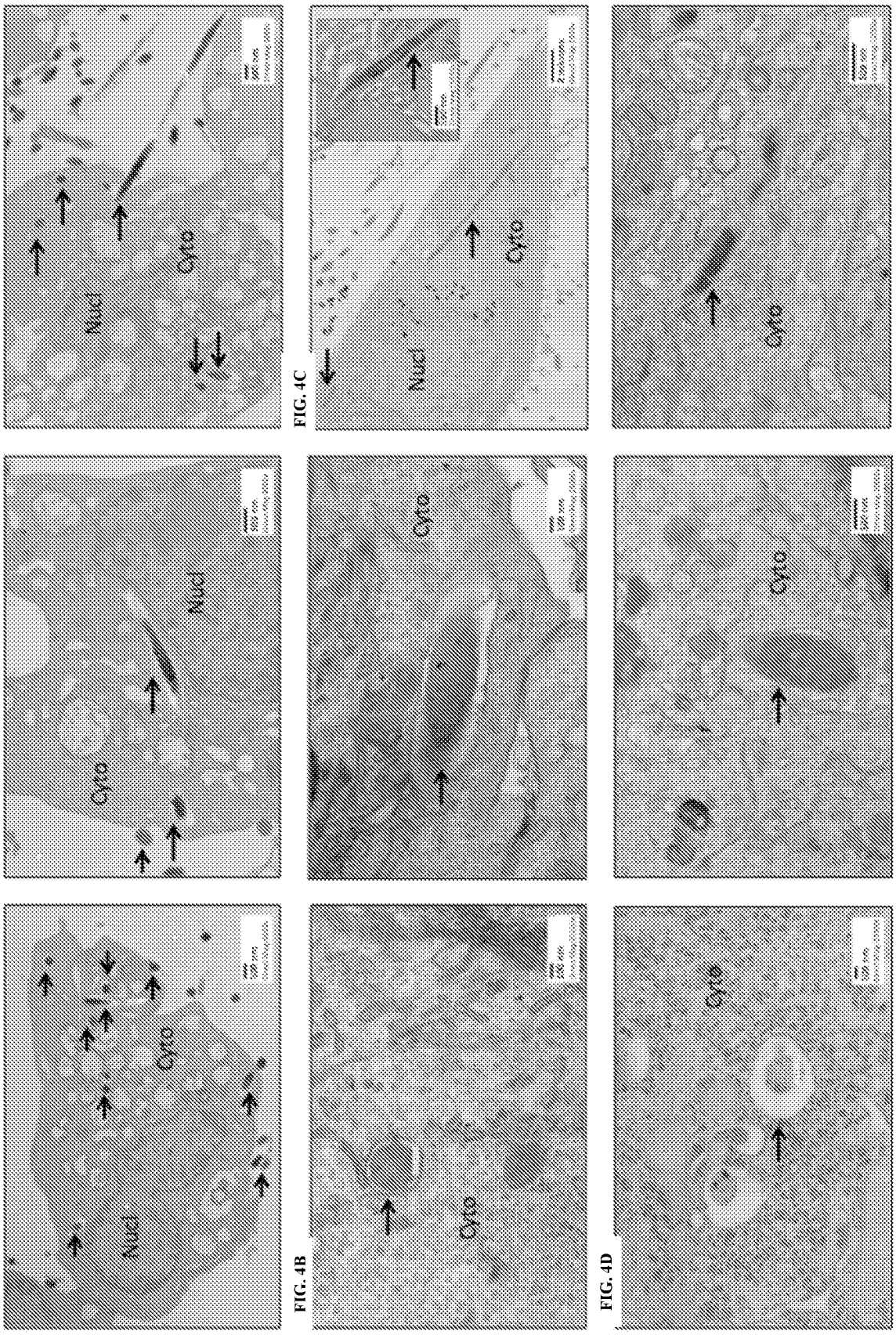


FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

FIG. 5A

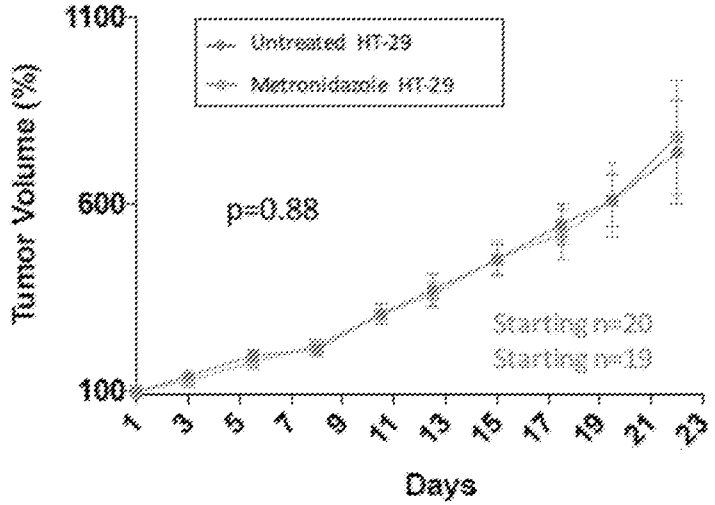


FIG. 5B

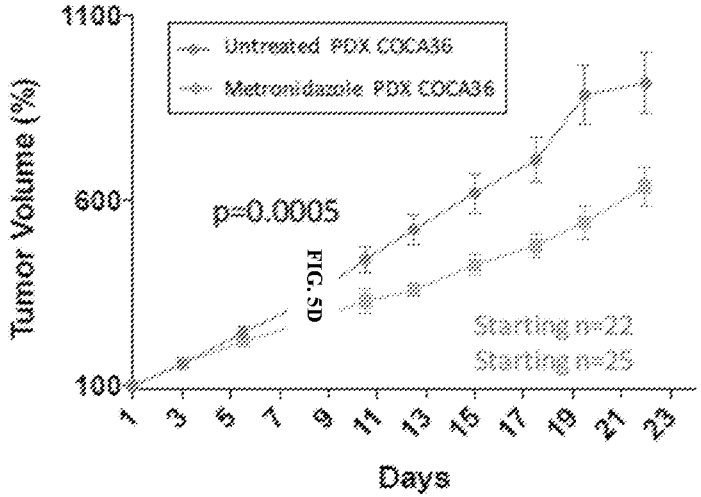


FIG. 5C

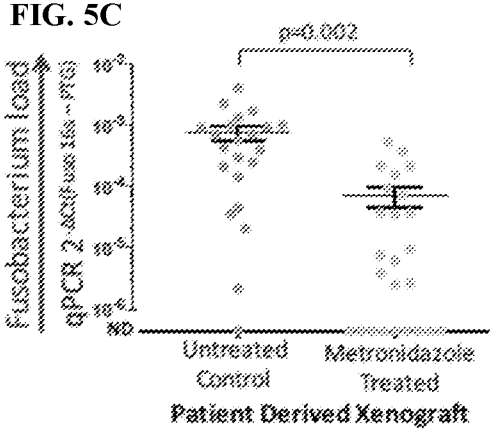


FIG. 5D

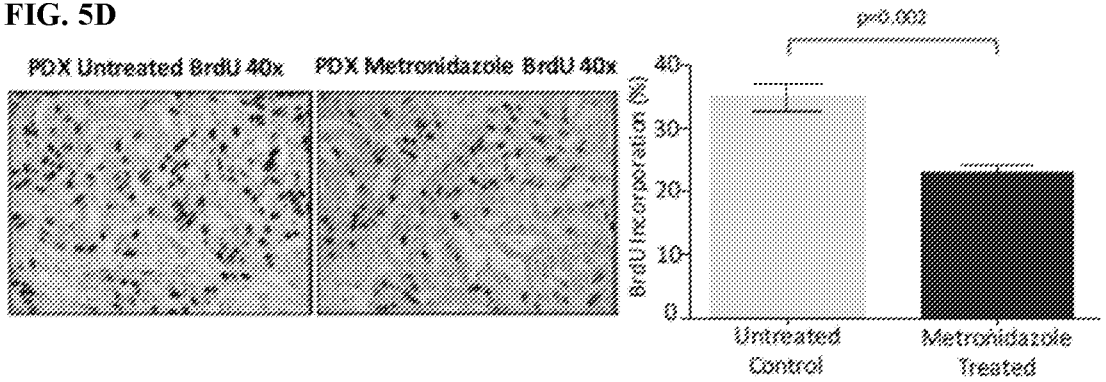
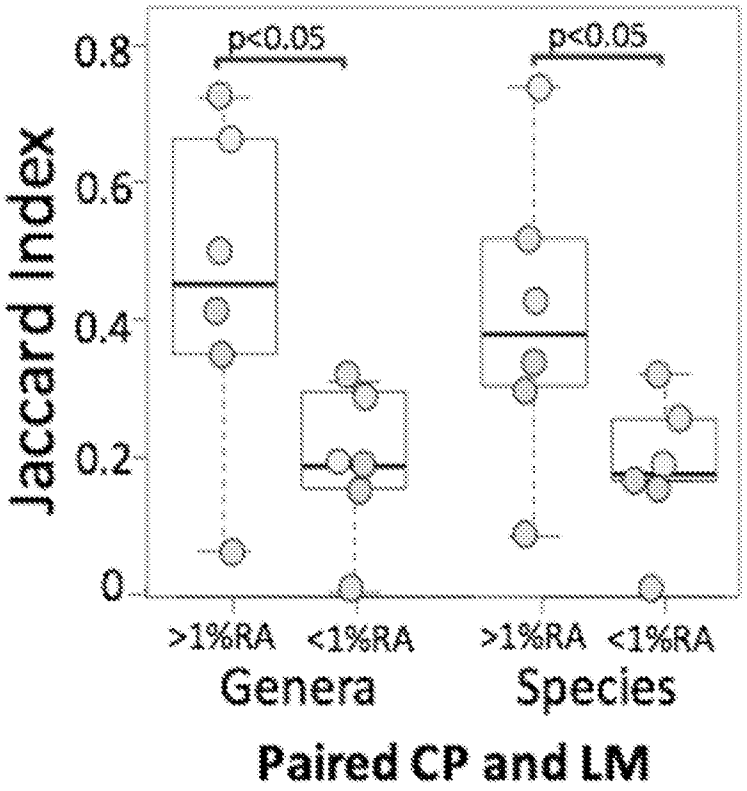


FIG. 5D

FIG. 6A Average nucleotide identity (ANI) of *F. necrophorum* colon tumor and liver metastases isolates from **Patient 1**

FIG. 6B Average nucleotide identity (ANI) of *F. nucleatum* colon tumor and liver metastases isolates from **Patient 2**



	Genera (n)		Species (n)	
RA	>1%	<1%	>1%	<1%
P1	22	159	24	370
P2	24	373	27	885
P3	24	211	28	435
P4	22	262	25	370
P5	34	133	26	293
P6	31	249	35	513

FIG. 7

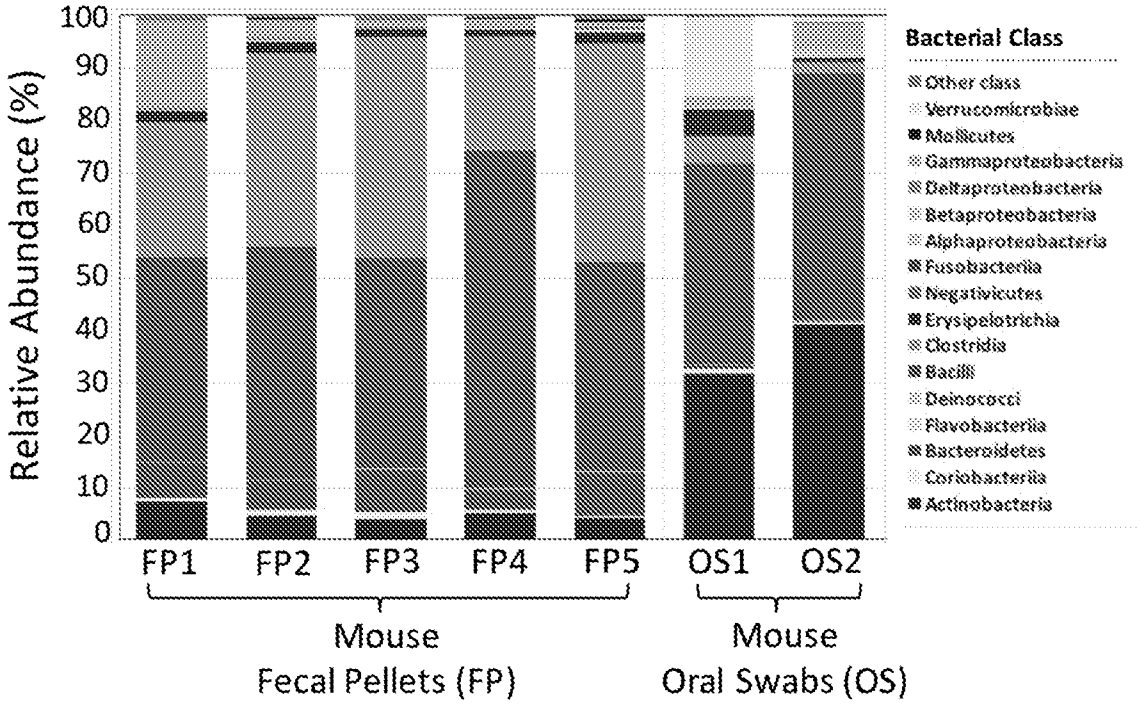

Relative Abundance (%)
100
90
80
70
60
50
40
30
20
10
0
FP1
FP2
FP3
FP4
FP5
OS1
OS2
Mouse
Fecal Pellets (FP)
Mouse
Oral Swabs (OS)
Bacterial Class
Other class
Verrucomicrobiae
Mollicutes
Gammaproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Fusobacteriia
Negativicutes
Erysipelotrichia
Clostridia
Bacilli
Deinococci
Flavobacteriia
Bacteroidetes
Coriobacteriia
Actinobacteria

FIG. 8

FIG. 9A

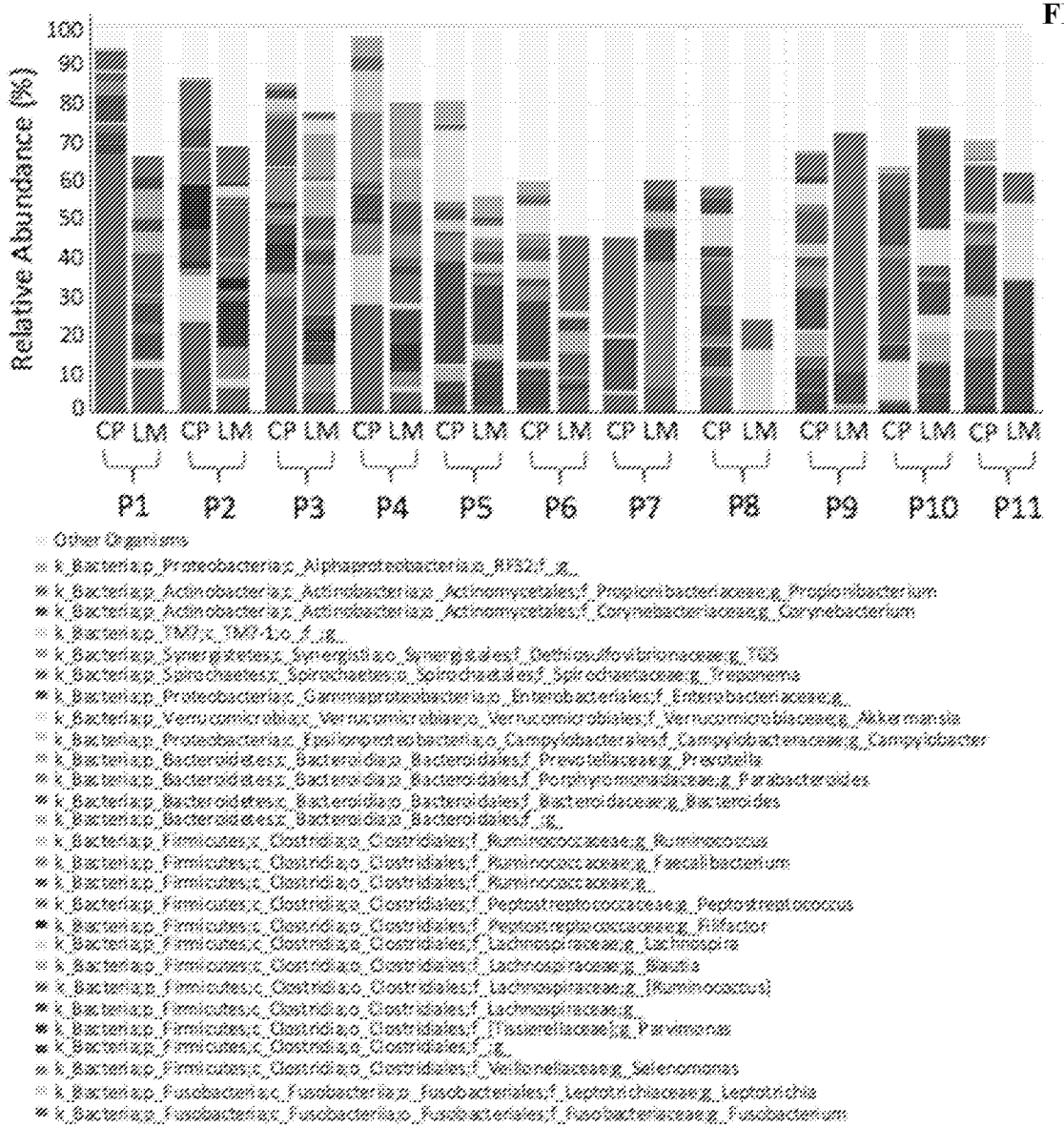


FIG. 9B

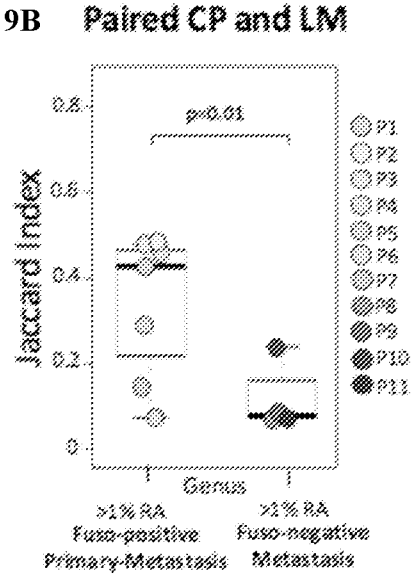


FIG. 9C

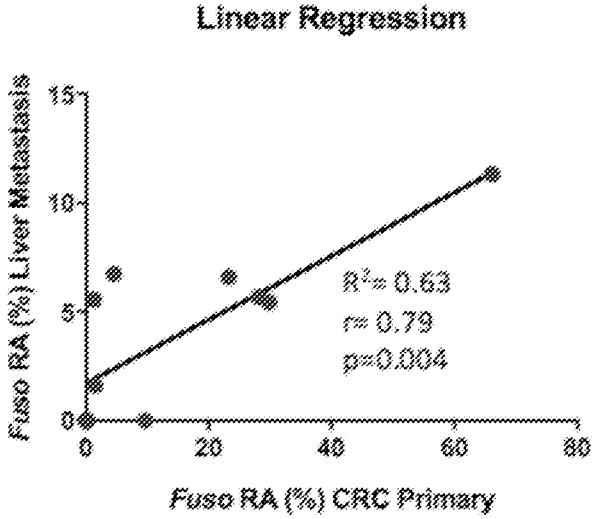


FIG. 10A

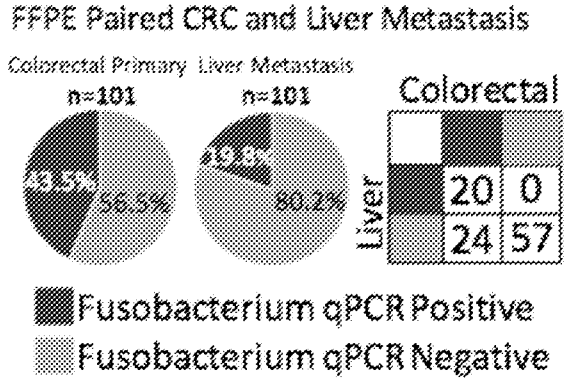
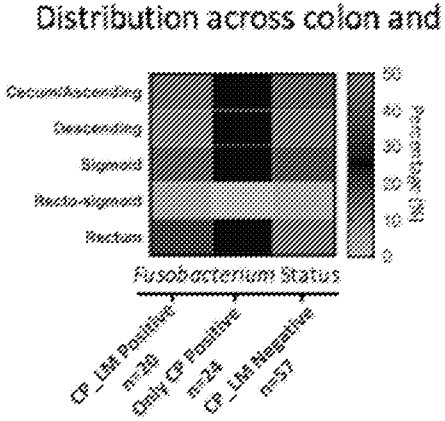


FIG. 10B



	CP_LM Fuso-Pos (n=20)	CP_LM Fuso-Neg (n=57)	P-value
Cecum/Ascending	10 (50%)	9 (15.8%)	0.0023
Descending	2 (10%)	7 (12.3%)	0.73
Sigmoid	3 (15%)	10 (17.5%)	0.56
Recto-sigmoid	1 (5%)	2 (3.5%)	0.76
Rectum	4 (20%)	29 (50.8%)	0.01

FIG. 10C

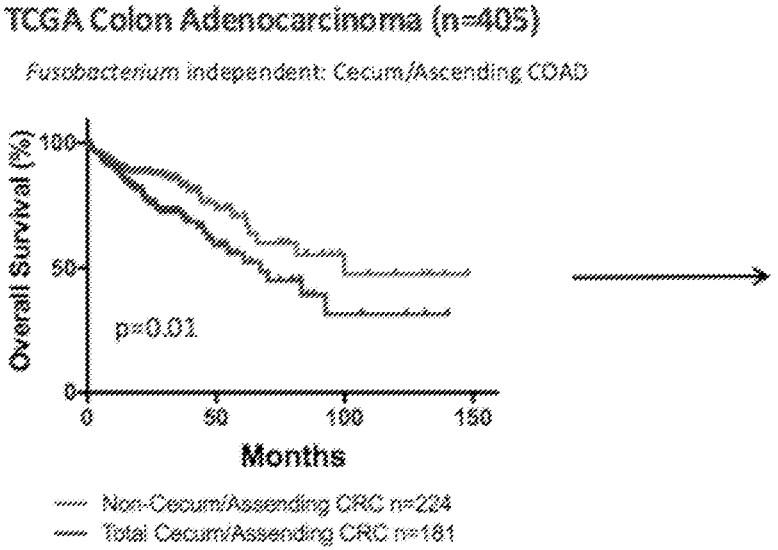
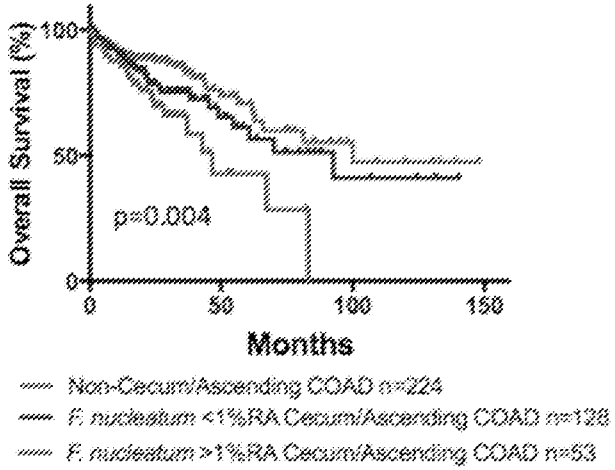


FIG. 10D



Hazard Ratio (Mantel-Haenszel) ; *Fusobacterium* "High" Cecum/Ascending Colon vs. non-Cecum/Ascending Colon: 3.337 (p=0.0007)

FIG. 11A

PDX Success	Tumor Grade: High	Tumor Grade: Low
Yes	4	2
No	1	6

Fischer's exact p=0.1

FIG. 11B

Fusobacterium	Tumor Grade: High	Tumor Grade: Low
Culture Pos	4	1
Culture Neg	1	7

Fischer's exact p=0.03

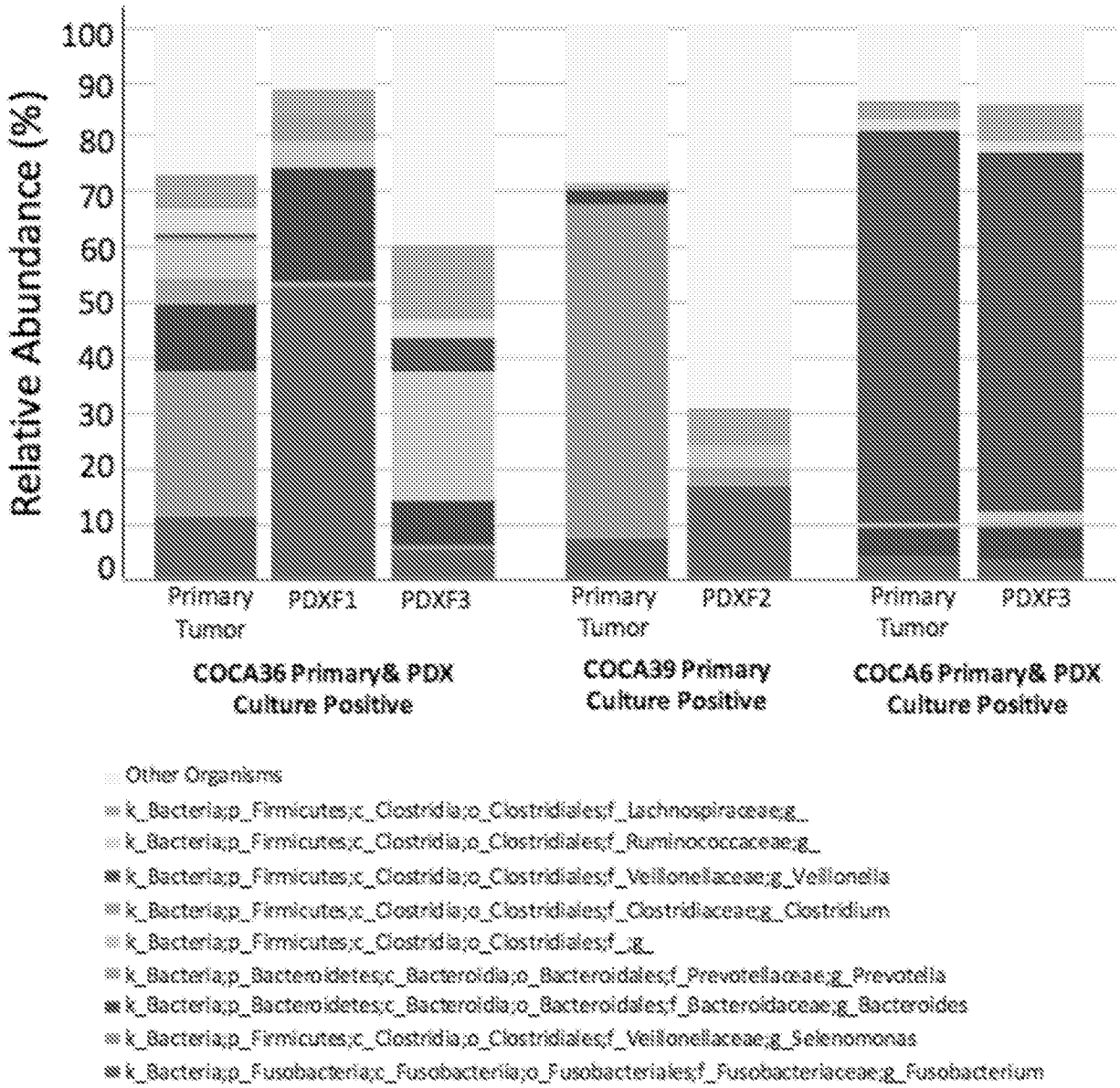

Relative Abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Primary Tumor
PDXF1
PDXF3
Primary Tumor
PDXF2
Primary Tumor
PDXF3
COCA36 Primary& PDX Culture Positive
COCA39 Primary Culture Positive
COCA6 Primary& PDX Culture Positive
Other Organisms
k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_
k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_
k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Veillonella
k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Clostridium
k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_
k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella
k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides
k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Selenomonas
k_Bacteria;p_Fusobacteria;c_Fusobacteria;o_Fusobacteriales;f_Fusobacteriaceae;g_Fusobacterium

FIG. 12

FIG. 13A

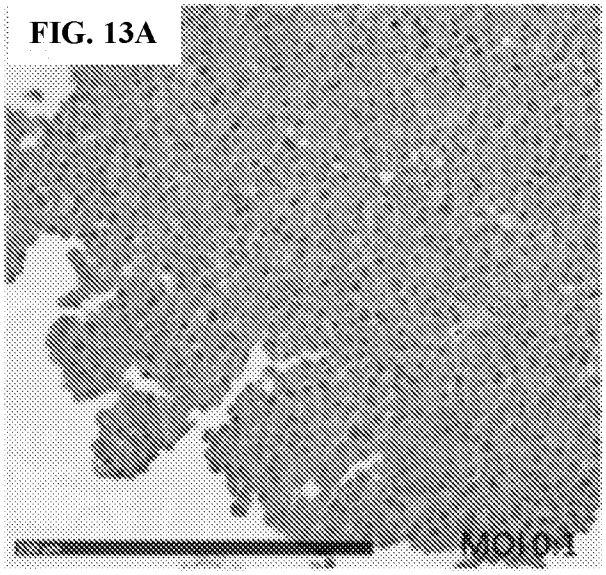


FIG. 13B

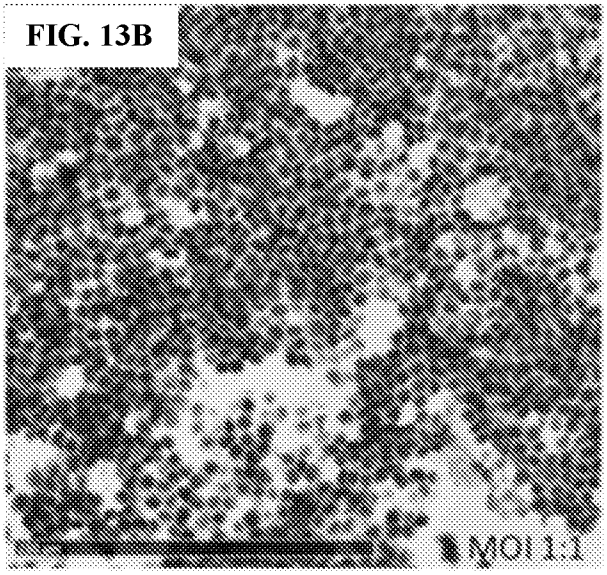


FIG. 13C

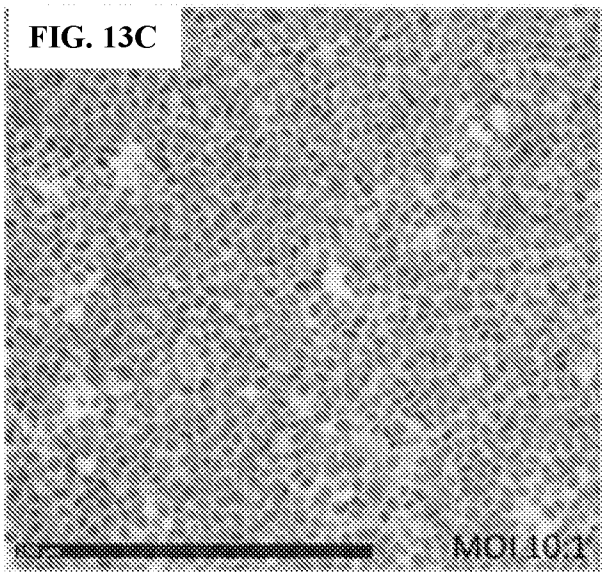
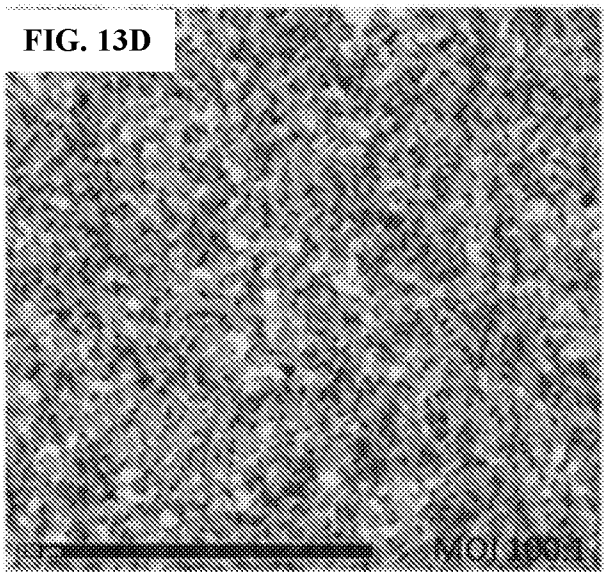


FIG. 13D



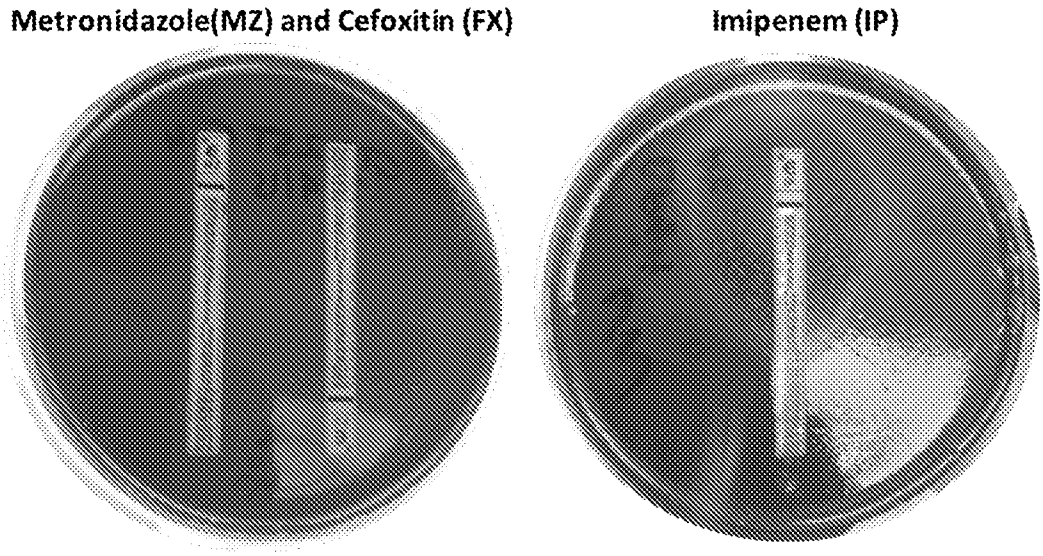


FIG. 14

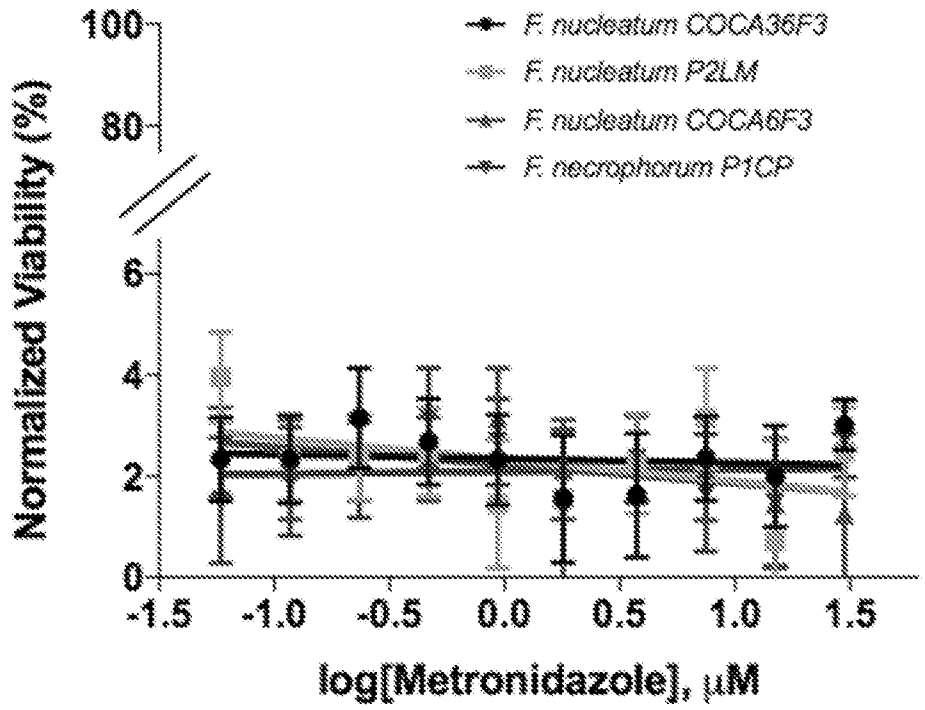

F. nucleatum CCCA36F3
F. nucleatum P2LM
F. nucleatum CCCA6F3
F. necrophorum P1CP
Normalized Viability (%)
100
80
6
4
2
0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
log[Metronidazole], μM

FIG. 15

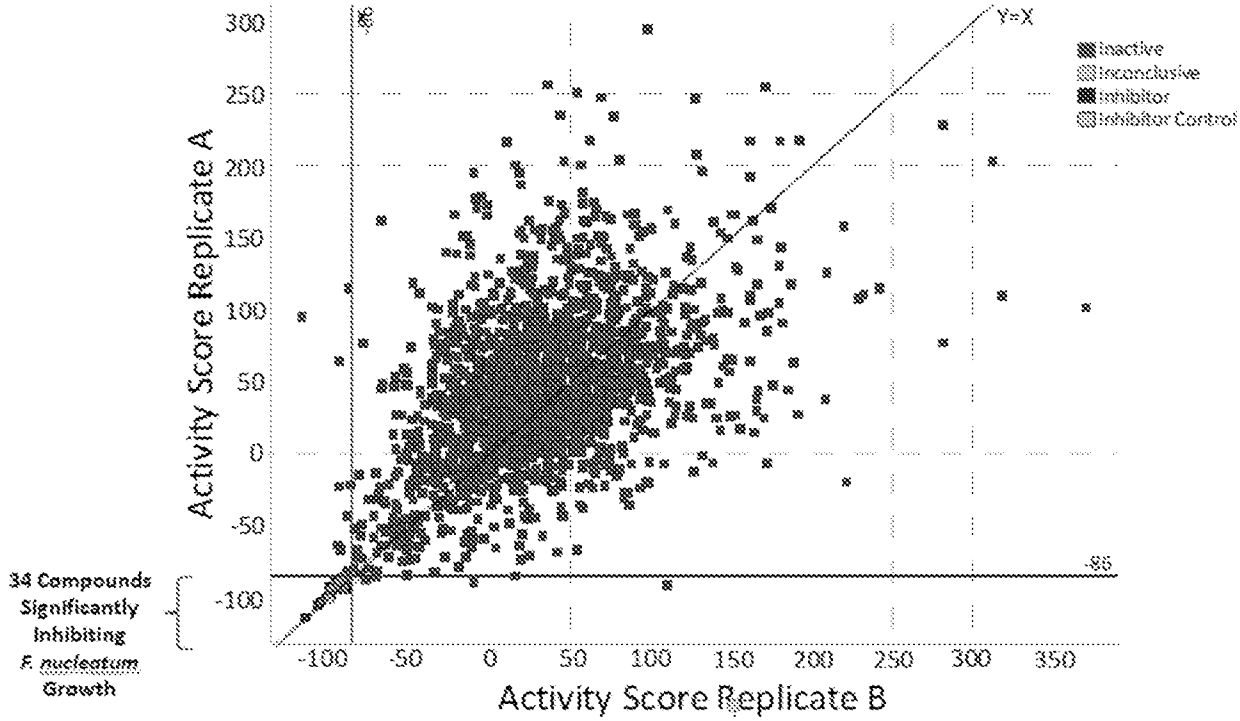

Activity Score Replicate A
Activity Score Replicate B
Y=X
-85
Inactive
Inconclusive
Inhibitor
Inhibitor Control
34 Compounds Significantly Inhibiting F. nucleatum Growth
300
250
200
150
100
50
0
-50
-100
-100
-50
0
50
100
150
200
250
300
350

FIG. 16

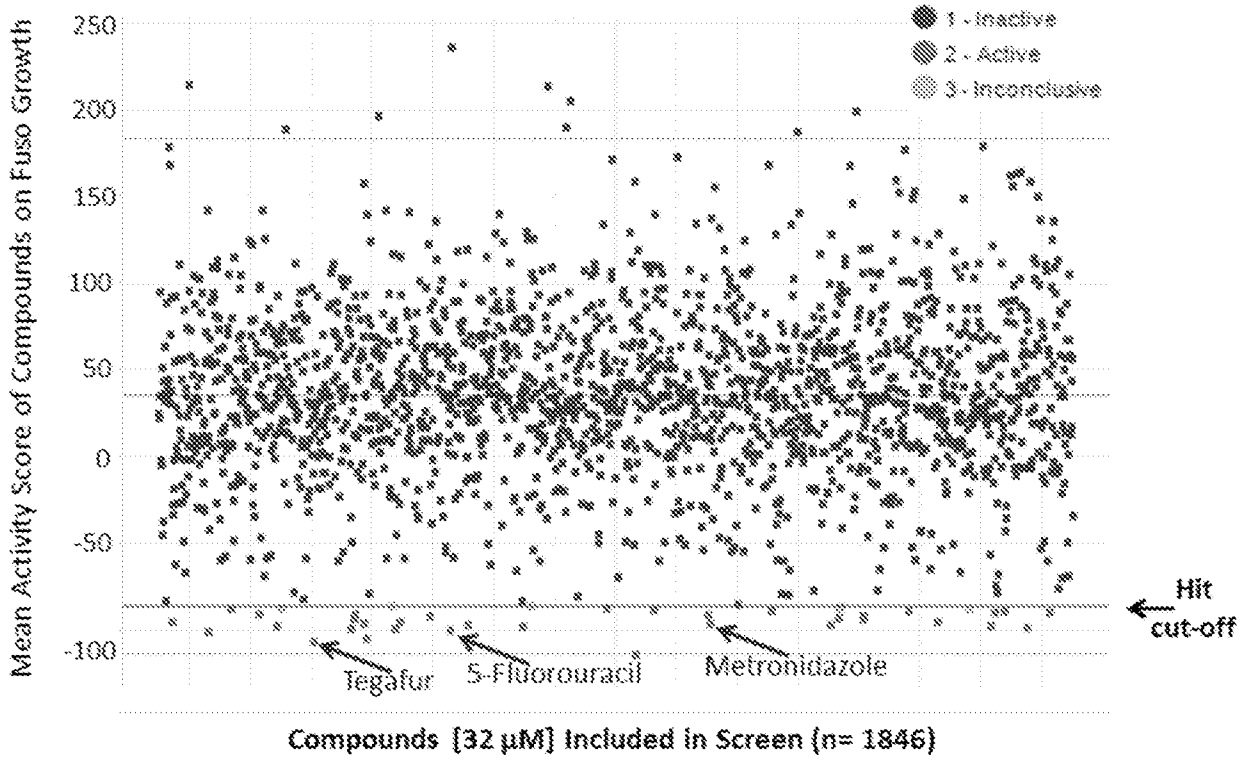
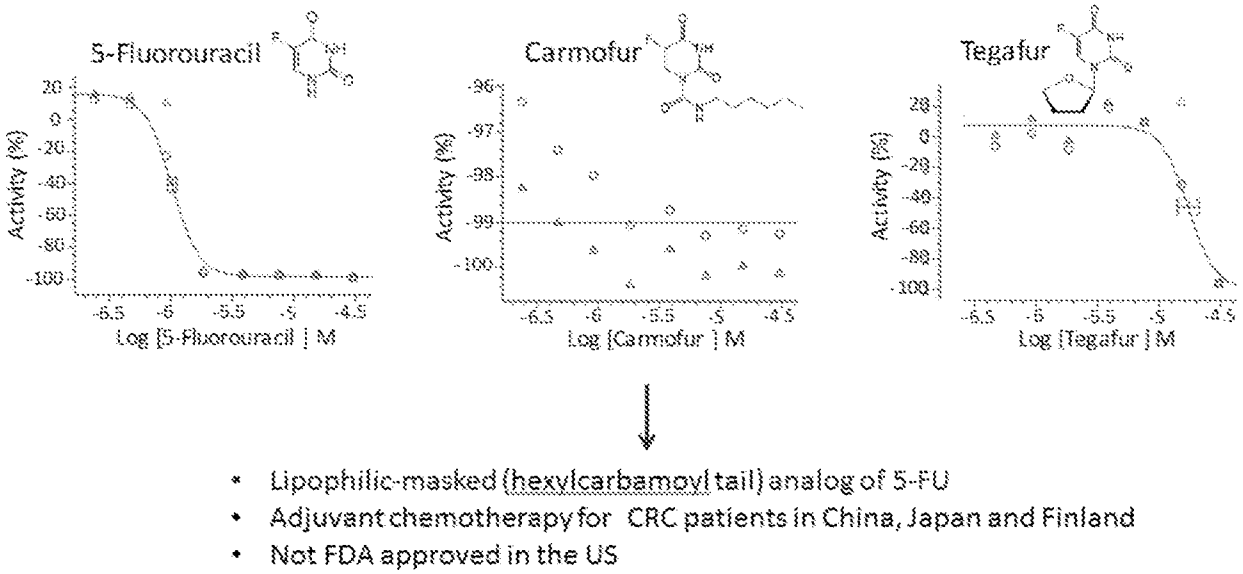

1 - Inactive
2 - Active
3 - Inconclusive
Mean Activity Score of Compounds on Fuso Growth
250
200
150
100
50
0
-50
-100
Hit
cut-off
Tegafur
5-Fluorouracil
Metronidazole
Compounds [32 µM] Included in Screen (n= 1846)

FIG. 17



- Lipophilic-masked (hexylcarbamoyl tail) analog of 5-FU
- Adjuvant chemotherapy for CRC patients in China, Japan and Finland
- Not FDA approved in the US

FIG. 18

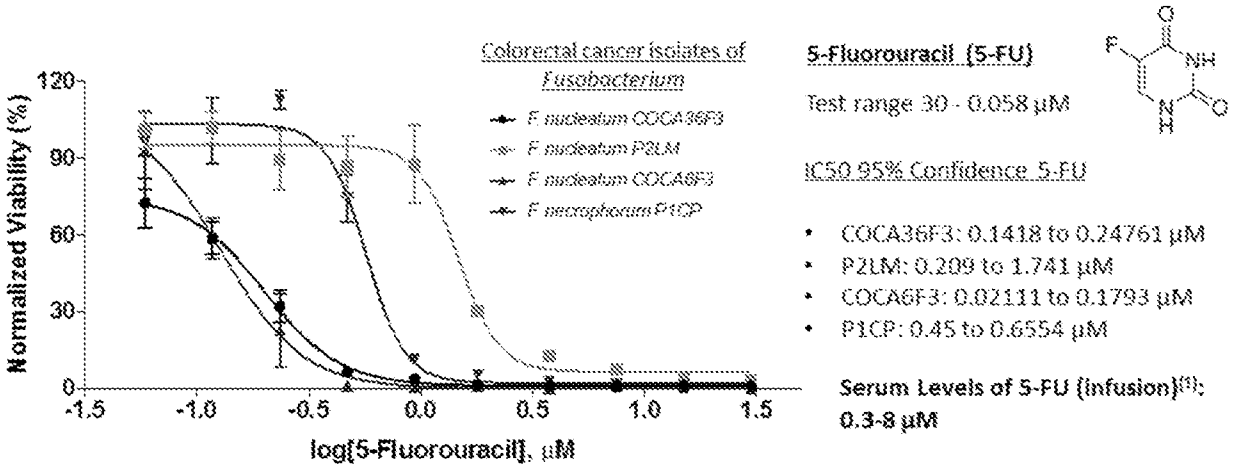

Colorectal cancer isolates of Fusobacterium
F nucleatum COCA36F3
F nucleatum P2LM
F nucleatum COCA6F3
F necrophorum P1CP
Normalized Viability (%)
120
90
60
30
0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
log[5-Fluorouracil], µM
5-Fluorouracil (5-FU)
Test range 30 - 0.058 µM
IC50 95% Confidence 5-FU
COCA36F3: 0.1418 to 0.24761 µM
P2LM: 0.209 to 1.741 µM
COCA6F3: 0.02111 to 0.1793 µM
P1CP: 0.45 to 0.6554 µM
Serum Levels of 5-FU (infusion)[5]:
0.3-8 µM

FIG. 19

Tumors treated with 5FU or a 5-FU prodrug (n=43)

Fusobacterium	Recurrence: Yes	Recurrence: No
High*	6	26
Low/Neg	6	5

Fischer's exact p=0.047
Z- score two population proportions p=0.02

Tumors with no treatment information (n=48)

Fusobacterium	Recurrence: Yes	Recurrence: No
High*	17	19
Low/Neg	6	6

Fischer's exact p>0.999

FIG. 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/42966

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/16, A61K 39/395, A61K 45/06 (2018.01)
CPC - A61K 38/164, A61K 39/39558, A61K 45/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/0107092 A1 (Dana-Farber Cancer Institute, Inc.) 17 April 2014 (17.04.2014); entire document, especially [0008], [0011], [0035], [0067]	1-7, 14-15
X	US 2016/0220619 A1 (John Wayne Cancer Institute) 04 August 2016 (04.08.2016); entire document, especially [0034], [0100], [0102]	1, 16
A	Microbewiki, "Fusobacterium nucleatum", 18 August 2010 (18.08.2010), retrieved on 12 September 2018 from https://microbewiki.kenyon.edu/index.php?title=Fusobacterium_nucleatum&oldid=54788 ; entire document, especially pg 3 para 1	1-7, 14-16
A	US 2010/0040614 A1 (Ahmed et al.) 18 February 2010 (18.02.2010); entire document	1-7, 14-16
A	US 3,326,761 A (Kent) 20 June 1964 (20.06.1964); entire document	1-7, 14-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2018	15 NOV 2018

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/42966

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-13, 17, 35, 40-42
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. Ill Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1-7 and 14-16, directed to a method of treating a neoplasm in a subject.

Group II: Claims 18-26, directed to a method of identifying a cellular component or pathway linked to sensitivity to growth induction by a Fusobacterium and a method of identifying a compound or composition that inhibits induction of a cell by Fusobacterium.

Group III: Claims 27-34 and 36-39, directed to a method of identifying a subpopulation of a population of patients having a neoplasm, a method of testing a tumor for the presence of a bacterial infection, and a method of identifying a treatment regimen for a patient having a neoplasm.

"'See supplemental box" *

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7, 14-16

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/42966

Continuation of Box No. III

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I requires a method of treating a neoplasm in a subject comprising administering to the subject an agent, compound, or composition that inhibits bacterial growth in the subject, not required by group II-III.

Group II requires a method of identifying a cellular component or pathway linked to sensitivity to growth induction by a Fusobacterium, which comprises contacting a test cell which comprises a mutation or phenotype linked to the cellular component or pathway with a Fusobacterium, contacting a control cell with the Fusobacterium, and identifying the cellular component or pathway as linked to sensitivity to induction by Fusobacterium if the test cell and control cell display a Fusobacterium-dependent phenotypic change; and method of identifying a compound or composition that inhibits induction of a cell by Fusobacterium, which comprises culturing a mammalian cell with a Fusobacterium in the presence of a test compound, culturing the mammalian cell with a Fusobacterium in the absence of the test compound, and identifying the compound as an induction inhibitor if the test compound inhibits growth of the test culture, not required by group I and III.

Group III requires a method of identifying a subpopulation of a population of patients having a neoplasm comprising diagnosing and identifying therefrom patients in the population having a bacterial infection in or associated with the neoplasm, whereby those patients having the bacterial infection in or associated with the neoplasm are the subpopulation; method of identifying a treatment regimen for a patient having a neoplasm comprising diagnosing whether the patient has a bacterial infection in or associated with the neoplasm; and method for removal of a tumor in a subject comprising systemically and/or locally to the tumor testing for the presence of a bacterial infection, optionally wherein the testing comprises a rapid diagnostic that identifies an RNA and/or DNA signature of a bacterial, and further optionally wherein the testing includes measuring bacterial load, not required by group I-II.

Common Technical Features:

Groups I-II share the technical feature of inhibiting bacterial growth. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being anticipated by US 2014/0107092 A1 to Dana-Farber Cancer Institute Inc (hereinafter "Dana"). Dana teaches inhibiting bacterial growth (para [0011], "The methods include administering to the subject a therapeutically effective amount of an antibiotic effective against Fusobacteria, e.g., F. nucleatum").

Groups I and III share the technical feature of bacterial infections associated with neoplasm. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being anticipated by Dana. Dana teaches bacterial infections associated with neoplasm (para [0006], "At least in part, the present invention is based on the discovery that Fusobacterium can be a proximal cause of colorectal cancer").

Groups II-III share the technical feature of diagnosing bacterial infections. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being anticipated by Dana. Dana teaches diagnosing bacterial infections (para [0008], "The methods include obtaining a stool sample from the subject; detecting a level, e.g. a normalized level, of Fusobacteria, e.g., F. nucleatum, in the sample").

As the shared technical features were known in the art at the time of the invention, they cannot be considered common technical features that would otherwise unify the groups. Therefore, Groups I-III lack unity under PCT Rule 13.

Note:
Claims 8-13, 17, 35, and 40-42 are held unsearchable because they are not drafted in accordance with the second and third sentences of Rule 6.4(a).