Antibiotic Treatment Decreases Microbial Burden Associated with Pseudomyxoma Peritonei and Affects β-catenin Distribution

Cristina Semino-Mora¹, Traci L. Testerman², Hui Liu¹, Jeannette M. Whitmire¹, Kimberley Studeman³, Yali Jia², Thomas J. McAvoy⁴, Jennifer Francis³, Carol Nieroda³, Armando Sardi³, D. Scott Merrell¹, and Andre Dubois.¹

¹Uniformed Services University and United States Military Cancer Institute, Bethesda, MD, 20814; ²Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA 71030; ³Mercy Medical Center, Baltimore, MD 21202; ⁴University of Maryland, College Park, MD 20742

Correspondence: Thomas McAvoy Ph.D., University of Maryland, College Park, MD 20742
EMAIL: mcavoy@umd.edu

Or D. Scott Merrell, Ph.D., Department of Microbiology, Uniformed Services University of the Health Sciences, Rm. A3046, Bethesda, MD 20814 EMAIL: douglas.merrell@usuhs.edu

The study was approved by the Institutional Review Boards of the Mercy Medical Center and the Uniformed Services University of the Health Sciences, and written informed consent was obtained from all patients before study entry.

Disclosure of Potential Conflicts of Interest: The authors have no potential conflicts of interest to disclose.

Running head: Pseudomyxoma Peritonei, bacteria, and antibiotics
TRANSLATIONAL RELEVANCE

Pseudomyxoma peritonei (PMP) is an under-researched abdominal cancer that originates from an appendiceal neoplasm. Patients frequently relapse and succumb to the disease, necessitating the development of novel therapeutics to treat patients. Recent studies have indicated the presence of bacteria within the tumors and mucin of PMP patients. The study herein indicates that antibiotic treatment significantly reduces bacterial density along with $\beta$-catenin levels in the cytoplasm, the cell nuclei, and mucin-associated cells of PMCA patients. Additionally, the $\beta$-catenin levels within membranes significantly increase in both DPAM and PMCA patients. The re-normalization of $\beta$-catenin distribution combined with the reduction in bacterial density make antibiotic treatment a potential therapeutic for PMP disease.
ABSTRACT

PURPOSE: Pseudomyxoma peritonei (PMP) is an understudied cancer in which an appendiceal neoplasm invades the peritoneum and forms tumor foci on abdominal organs. Previous studies have shown that bacteria reside within PMP tumors and mucin. Thus, we sought to analyze the effect of antibiotics on bacterial density and β-catenin expression within PMP samples.

EXPERIMENTAL DESIGN: The study included 48 patients: 19 with disseminated peritoneal adenomucinosis (DPAM) and 29 with peritoneal mucinous carcinomatosis (PMCA). Fourteen patients were given antibiotics (30mg lansoprazole, 1g amoxicillin and 500mg clarithromycin) BID for 14 days. One week after completion of therapy, surgery was performed and specimens were harvested for pathology, bacterial culture, in situ hybridization (ISH), and immunohistochemistry (IHC).

RESULTS: ISH demonstrated the presence of bacteria in 83% of the patient samples with a higher H. pylori density observed in PMCA vs. DPAM. PMCA patients treated with antibiotics had a significantly lower bacterial density and decreased β-catenin levels in the cytoplasm, the cell nuclei, and mucin-associated cells. Though not significant, similar trends were observed in DPAM patients. Cell membrane β-catenin was significantly increased in both DPAM and PMCA patients receiving antibiotics.

CONCLUSIONS: Bacteria play an important role in PMP. Antibiotic treatment improved the histopathology of tissue, particularly in PMCA patients. In PMCA, antibiotics decreased bacterial density and were associated with a significant β-catenin decrease in the cytoplasm, cell nuclei and mucin along with a small membrane increase. These results suggest that antibiotics offer potential protection against cell detachment, cellular invasion, and metastasis.
INTRODUCTION

Pseudomyxoma peritonei (PMP) is characterized by the presence of multifocal peritoneal and omental implants of mucus-secreting epithelial cells and dissecting gelatinous ascites (1). This complex disease is found in 0.02% of laparotomies, and its incidence is three-fold higher in females than in males (2, 3). PMP usually originates from a perforated low-grade appendiceal mucinous neoplasm or appendiceal adenocarcinoma that protrudes into the peritoneum, and the initial presentation of the disease often includes increased abdominal girth and/or appendicitis-like symptoms (4). PMP can take on a protracted course if the histopathology is that of Disseminated Peritoneal Adenomucinosis (DPAM), or rapidly evolve as a highly malignant tumor with Peritoneal Mucinous Carcinomatosis (PMCA) histopathology. Current treatment combines cytoreductive surgery (resection of the tumor and peritoneal implants) and intraperitoneal hyperthermic chemotherapy (5-8). Despite extensive resections, PMP frequently relapses and 5-year survival is only 53% (9), although survival is significantly better if lymph nodes are negative (5-year survival is 76% for lymph node-negative patients and 11% for lymph node-positive patients; p <0.001) (10). Even in patients with low-grade appendiceal mucinous neoplasms, most patients succumb to the disease due to recurrent mucoid ascites, proliferation of gelatinous mucin, and mechanical compression and obstruction of abdominal organs, heart, and lungs (11).

A key aspect of colon cancer and several other cancers is deregulation of the Wnt/β-catenin pathway (12). The transmembrane protein β-catenin is associated with the cytoplasmic region of E-cadherin within adherens junctions. In normal, polarized gut epithelial cells, β-catenin provides a linkage mechanism between cytoskeletal proteins and cell-to-cell junctional proteins (e.g. E-cadherin), allowing cells to tightly bind to each other (13). These interactions are crucial
for maintenance of epithelial cell polarity, regulation of cell growth, and cell-to-cell focal
adhesion. During carcinogenesis, β-catenin relocates into the cytoplasm and nucleus. Loss of
membrane β-catenin leads to cell separation and migration, movement of abnormal neoplastic
cells to the stroma, entry into blood vessels, and metastatic spread to different tissues through the
bloodstream (14). Furthermore, following nuclear translocation, β-catenin interacts with T cell
factors (TCFs) or lymphocyte-enhancer factors that can then act as transcription factors. Thus, β-
catenin nuclear localization triggers expression of various genes, including genes required for
cell proliferation (15).

Several infectious agents have been found to stimulate cell proliferation via the Wnt/β-
catenin pathway. In fact, the potential for certain viruses to cause cancer has been recognized for
some time. Recent studies suggest that Epstein-Barr virus, Kaposi’s sarcoma-associated virus,
and hepatitis C virus promote carcinogenesis by activating the Wnt/β-catenin pathway (16, 17).

Some bacteria also influence the Wnt/β-catenin pathway. Helicobacter pylori expresses
numerous virulence factors that promote carcinogenesis, including the cytotoxin-associated gene
A (CagA). Translocation of CagA into epithelial cells results in changes in expression of β-
catenin (18) as well as causes nuclear accumulation of β-catenin (19), which is associated with
aggressive and invasive tumors (20). Salmonella and Chlamydia trachomatis also stimulate
epithelial proliferation via the Wnt/β-catenin pathway (21, 22).

Although PMP patients have no symptoms of peritonitis, we previously hypothesized that
intestinal bacteria spread to the peritoneum at the time of appendiceal perforation. In keeping
with this idea, we recently demonstrated that H. pylori and other bacteria can be detected in PMP
tissues (23). Given this finding, herein we studied the effect of preoperative antibiotic therapy on
bacterial density, and on the concurrent expression of β-catenin within the neoplastic cancerous
cells of DPAM and PMCA patients. We observed that after antibiotic treatment, PMCA patients showed a significant decrease in bacterial density along with significantly decreased β-catenin expression in the cytoplasm, nuclei and mucin. We found that in neoplastic cancerous cells, β-catenin is redistributed into the cytoplasm and accumulates in different areas of the cytosol and ground substance (connective tissue in the stroma that supports fibers). En masse, our data suggest that antibiotic treatment of PMCA may affect the carcinogen pathway elicited by β-catenin and may serve as a novel treatment of this understudied cancer.

MATERIALS AND METHODS

Patients, histopathology, and treatment. Forty-eight patients with the diagnosis of peritoneal dissemination of appendiceal mucinous neoplasms that had been scheduled to undergo laparotomy for staging, extensive cytoreductive surgery and hyperthermic intraperitoneal chemotherapy were studied. Patients’ age was 53± 2 years and weight 73± 2 kg (Means and SEM).

Observation and analysis of biopsies stained with hematoxylin and eosin (H&E) from cytoreductive surgical specimens by a board certified and experienced pathologist allowed tumor classification as either DPAM or as the more malignant PMCA (24). Tissue from a patient with a non-perforated, non-neoplastic appendix (NNA) was used as a control. Analysis of the grade of inflammation was performed according to conditions previously described (25).

Three weeks before surgery, an open label anti-\textit{H. pylori} triple therapy (Prevpac®, i.e. 30 mg lansoprazole, 1,000 mg amoxicillin and 500 mg clarithromycin given BID for 14 days) was given to a total of 14 of the PMP patients: 6 DPAM and 8 PMCA. A total of 34 patients received no antibiotics and were maintained as untreated controls: 13 DPAM and 21 PMCA. One week
after completion of the antibiotic therapy, patients and controls underwent cytoreductive surgery (resection of the tumor and peritoneal implants) and hyperthermic intraperitoneal chemotherapy to achieve complete or near-complete resection of PMP cancerous tissues (8).

**In situ hybridization (ISH) studies**

**Probes:** Two previously described *16S rDNA* bacterial probes (23) labeled with biotin were as follows: (1) a probe that can detect 19,973 typed and nonculturable bacteria (TNCB) (including *Campylobacter jejuni, Escherichia coli, Salmonella enterica, H. pylori,* and *Enterococcus faecalis*) (5′-AGCAA CAG GAT TAG ATA CCC TGG TAG TCC AC-3′); and (2) a probe specific for *H. pylori* (5′-ATT TCA CAC CTG ACT GAC TAT CCC GCC TAC GCG-3′) (26). In initial experiments, we used both cRNA and cDNA probes concurrently, and confirmed earlier observations that these two probes can detect the same bacteria (27).

Paraffin blocks of formalin-fixed tissue were sectioned (5 μm) and analyzed as previously described (27). Briefly, each unstained section was deparaffinized, prehybridized, hybridized with denatured probe solution, and then incubated for 18 h with a probe labeled with biotin followed by the removal of unbound probe using saline citrate solution. DNA was detected by incubating the slide for 2 h with streptavidin-conjugated alkaline phosphatase (Roche Diagnostic, Indianapolis, IN) (1:500 dilution in blocking immuno-Tris buffer), washing, and applying, the chromogenic substrate BCIP/NBT/ kit (5-bromop-4-chloro-3-indolyl phosphate/nitro-blue-tetrazolium), yielding a blue color reaction (Vector Labs, Burlingame, CA). The slide was then counterstained with Nuclear Fast Red, washed in water, dehydrated, cleared in xylene, air dried, and mounted with permount. TNCB and *H. pylori* *16S rDNA* were detected in serial sections under bright light as follows: 1st section: detection of TNCB *16S rDNA*, 2nd section: detection of
H. pylori 16S rDNA. Parallel dual fluorescence in situ hybridization (FISH) studies (27) were also performed using the 16S rDNA-TNCB probe labeled with biotin and then detected with avidin labeled with fluorescein- FITC (green reaction), and the 16S rDNA-H. pylori probe labeled with digoxigenin and then detected with anti-digoxigenin mouse monoclonal antibody conjugated to biotin (Abcam, Cambridge, MA 02139-1517) and then detected using avidin-Tx red (red reaction). Co-localization of both TNCB and H. pylori was visualized as yellow.

As previously described (27), control for nonspecific binding included: a) sense probe instead of antisense probe; b) hybridization buffer instead of the antisense probe; c) unlabeled antisense probe; d) digoxigenin or biotin-labeled probe for a sequence completely unrelated to man and prokaryotes-the scorpion Buthus martensi Karsch neurotoxin sequence [5′-GGC CAC GCG TCG ACT AGT AC-3′] (28); e) RNase A pretreatment (Roche); f) DNase I pretreatment (Roche); and g) RNase + DNase I pretreatment.

For fluorescence, a Nikon Eclipse 80i microscope DS black-white camera with a DS-L2 control unit was used and software from NIS-Elements Advanced Research was used for analysis and reproduction of β-catenin images. For brightfield microscopy, an Eclipse 800 Nikon microscope with digital camera (QCapture Pro, Micropublisher 6.0, Burnaby, BC, Canada) was used for analysis and reproduction of bacterial images (TNCB and H. pylori). Fluorescence immunohistochemistry (FIHC) was used for studies of β-catenin. Two different studies were conducted:

Laser confocal microscopy was used to quantify cell membrane and nuclear localization of β-catenin. Antigen retrieval was first achieved by sequentially treating deparaffinized sections with saline citrate solution in a pressure cooker system for 9 min (27). β-catenin expression was analyzed using a mouse anti-β-catenin monoclonal antibody (Santa Cruz Biotechnology, Inc) at
1:150 dilution followed by biotinylated anti-mouse IgG and avidin, labeled with either fluorescein isothiocyanate (FITC) for β-catenin cell membrane localization or Texas red for β-catenin nuclear localization. For nuclear localization, DNA was also stained using 4’, 6 diamino-2-phenylindole (DAPI) since it specifically binds DNA without overlap with fluorochromes. Importantly, DAPI binds DNA (heterochromatin and euchromatin) of an interphase nucleus. In contrast to usual dual-fluorescence stains, DAPI stain cannot be easily merged with the expression of any fluorochrome (in this case with β-catenin-Texas red) to produce a third color because nuclear DNA is bound to β-catenin (first layer of procedure) and, as a result, DAPI binding to DNA is not as efficient at the same site. However, DAPI can bind to DNA that is not bound to β-catenin.

**β-catenin compartment localization** was determined from standard fluorescence (FIHC) of paraffin sections mounted with Vectashield alone (w/o DAPI) and observed using a Nikon Eclipse 80i microscope as described above. The following tissue types were analyzed: 1) epithelia, 2) lymphocytes, monocytes, and granulocytes, 3) stroma (connective tissue, vessels, fibroblasts and fibers), and 4) mucin pools. In addition, specific analyses of a) lateral cell-cell contact and b) cytoplasm localization were performed.

Fluorescence IHC images were observed and reproduced using the same microscope and digital camera described above for the ISH studies.

For β-catenin nuclear localization, sections were mounted using Vectashield containing DAPI and confocal images were collected on a Zeiss S710 NLO laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) equipped with a 63x oil immersion lens (1.4NA). DAPI stained samples were excited with a 405nm laser diode and emitted light in the 410 - 556nm range was collected. For Texas Red stained samples, a laser line of 561nm was used for
excitation and light between 566 - 690nm was used for imaging (BIC, USUHS). Phosphate buffer solution instead of the first β-catenin antibody was used as a negative control.

**Morphometric Analysis.**

1. **Quantitation of Bacteria and β-catenin.** Determination of bacterial and β-catenin density in cellular compartments was conducted as previously described (27). Briefly, an intraocular grid-based method was used to examine samples by a microscopist who was blinded to the histopathological diagnoses (i.e., DPAM or PMCA) as well as antibiotic treatment category. The number of bacterial clusters expressing 16S rDNA of TNCB and/or *H. pylori* was quantified at 400X magnification in three randomly selected fields of view according to a modification of the point-counting stereological method, and using an intraocular reticle of 27-mm diameter, covering 3578 μm² (i.e., 17,892 μm³ for 5 μm thick sections; Kr409, Klarman Rulings, Inc, Litchfield, NH) (23). Counting of the number of intersections of vertical and horizontal lines that overlapped a bacterium in the area delimited by a projected grid on the tissue was conducted. All data were expressed as means ± standard error of the mean (SEM) number of bacteria per 10⁶ μm³ (representing an imaginary cube with sides of 100 μm or 0.1 mm).

Total β-catenin density in biopsies was determined in a similar fashion and expressed as Vvi (volumetric density or volume occupied for the β-catenin reaction) in 10⁶ μm³ of tissue.

2. **Computerized quantification of β-catenin in the nuclei.** Nuclear localization of β-catenin and analysis were performed using a Zeiss 710 NLO laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) as described above (BIC, USUHS).

Software developed by TM using a MATLAB platform and associated toolboxes (http://www.mathworks.com/) was used to quantify the fraction of the nucleus occupied by β-
catenin in confocal microscopic pictures of IHC sections that had been double-stained for both β-catenin (Texas red) and DNA (DAPI) (Supplementary Methods). In brief, the percent of nuclei volume occupied by β-catenin was calculated as the number of bright red pixels that overlapped the dark blue DAPI stain divided by total nuclei pixels (nuclei in blue) multiplied by 100.

**Statistical Analysis**

Statistical significance was determined using t tests to compare results in patients receiving antibiotics versus no antibiotics in each cell of the tables. Values were expressed as means ± SEM.

**Results**

**Pathological evaluation of Disseminated Peritoneal Adenomucinosis (DPAM) and Peritoneal Mucinous Carcinomatosis (PMCA):**

H&E-stained sections were examined to determine the histopathology of the pseudomyxoma peritonei samples. Sections from DPAM patients (Fig. 1A) demonstrated the presence of abundant extracellular mucin (pale pink areas with blue streaks) surrounded by dark pink collagen bands. Mucin pools were surrounded by connective tissue septa supporting the mucin deposits. Strips of mucinous epithelial lining and scattered chronic inflammatory cells, including lymphocytes and macrophages, were also characteristics of DPAM (Fig. 1A, left panel). Low-grade adenomatous dysplasia was seen without evidence of mitosis, which is consistent with the more indolent nature of DPAM. Mesothelial hyperplasia, vascular congestion (engorged blood
vessels) and chronic inflammation were present in the peritoneal lining adjacent to extracellular mucin in DPAM sections (Fig. 1A, right panel).

In PMCA patient sections (Fig. 1B-D), glands consisting of angulated gland-forming mucinous epithelium were frequent, often with destructive stromal invasion (Fig. 1B). High-grade cytologic atypia, mitotic activity (Fig. 1C), and inflammatory cells within a desmoplastic stroma with fibrotic adhesions differentiate PMCA from DPAM (Fig. 1D); desmoplasia is a fibrotic reaction to malignant cells invading normal tissue. No significant differences in inflammatory cell densities were observed across the two disease types in H&E slides (scores of 0-3) (29).

**Presence of Bacteria in DPAM and PMCA as Determined by ISH**

Our work has previously shown that *H. pylori* and other bacteria can be found in PMP samples (23). We therefore sought to confirm these results using these independently obtained PMP samples and ISH with probes that detect TNCB or *H. pylori*. Our initial qualitative analysis of samples from all 48 patients revealed that clusters of TNCB (Fig. 2A-C, left panels) and *H. pylori* (Fig. 2A-C, right panels) were present within 83% of the specimens: 76.9% of the untreated DPAM patients, 83.3% of the antibiotic treated DPAM patients, 90.5% of the untreated PMCA patients, and 75% of the antibiotic treated PMCA patients (Table 1). Both TNCB and *H. pylori* were present in mucinous epithelia and were associated with mucin-secreting and goblet cells, as well as pools of mucin. Figure 2B (left panel) shows TNCB bacteria within mucin deposits and in areas of abnormal neoplastic epithelia. *H. pylori* was similarly located in the mucin surrounded by abnormal neoplastic epithelia and goblet cells and attached to the cell membrane of neoplastic cells (Fig. 2B, right panel, and inset). TNCB and *H. pylori* were also
observed in the connective tissue of the lamina propria (stroma) (Fig. 2C, left and right panels, respectively), touching inflammatory cells located in the stroma, and surrounding epithelial cells in the intestinal glands.

To determine whether there was any effect of antibiotic treatment on the quantity of bacteria found per sample, individual bacteria were counted manually in 3 fields of view. As with the qualitative analysis, *H. pylori* and TNCB were detected in 83% of the patients. The 8 patients negative for both probes were as follows: 1 DPAM-A, 3 DPAM-noA; 2 PMCA-A, and 2 PMCA-noA, where “A” indicates antibiotic treatment and “noA” indicates no antibiotic treatment. On average, and as previously reported (23), *H. pylori* and TNCB densities were significantly higher in PMCA than in DPAM patients (*H. pylori*: 32.4 ± 5.2 vs. 13.0 ± 3.2/10^6 μm^3, p<0.008; TNCB: 63.3 ± 10.4 vs. 26.8 ± 5.4/10^6 μm^3, p<0.01). In addition, *H. pylori* and TNCB densities were significantly lower in PMCA patients who received preoperative antibiotics (*H. pylori* 12 ± 6 vs. 40 ± 6/10^6 μm^3, p <0.01 and TNCB 18.6 ± 7.4 vs. 80.4 ± 12.2/10^6 μm^3, p <0.006, respectively). However, there was no significant difference in bacterial density in DPAM patients after antibiotic treatment. Examples of the bacterial density with and without antibiotic treatment are illustrated in an area of pooled mucin using FISH staining (Fig. 3 A-C; A: TNCB, B: *H. pylori*, C: merge). The TNCB probe binds to *H. pylori* as well as other species. Therefore, *H. pylori* overlaps TNCB and appears yellow when the images are merged, while non-*H. pylori* bacteria remain green in the merged image.

The above results were obtained using probes that recognize 16S rDNA, which detects both live and dead bacteria. Since it was possible that some dead bacteria could still be detected, we evaluated *H. pylori* viability in a subset of PMCA patients using a 16S rRNA probe combined with polyclonal anti-*H. pylori* antiserum. As shown in Supplementary Fig. 1, virtually
all of the *H. pylori* were alive in untreated PMCA patients, whereas very few remained viable in antibiotic-treated patients. These data suggest that the determined densities of bacteria are likely artificially high in patients that received antibiotic treatment.

**β-catenin in PMP**

Given that the Wnt pathway and β-catenin have been shown to be important in numerous forms of cancer, we investigated whether β-catenin quantities and localization were affected in PMP and across PMP types. We assessed this by initially quantifying total β-catenin in five random fields per sample from a NNA control and in the DPAM and PMCA sections. When all DPAM and PMCA cases were compared regardless of antibiotic treatment status, we observed that total β-catenin expression tended to be lower in DPAM than in PMCA (1,721±297 vs 3,083±542 Vvi/10^6 μm^3, p<0.062; NS). When the antibiotic treatment state was taken into consideration across the disease states, antibiotics tended to decrease total β-catenin expression in PMCA patients (p <0.036). However, expression was not significantly different in DPAM patients (p<0.36; NS). Given that localization of β-catenin is crucial to its function inside the cell, we next examined localization within different cellular compartments qualitatively as well as quantitatively. Analysis of β-catenin staining within the NNA control indicated the presence of β-catenin in cell membranes and the lateral junctional complex (Fig. 4A and inset). In contrast, biopsies obtained from PMCA patients that did not receive antibiotics showed virtually no β-catenin staining at the intercellular boundary (Fig. 4B and inset); staining appears primarily cytoplasmic. However, as was noted with the total β-catenin assay, antibiotic treatment appeared to have some effect on β-catenin localization since a moderate to intense reaction was observed in the junctional complexes between some of the PMCA neoplastic cells after antibiotic
However, these values did not reach statistical significance when the junctional staining was quantitated for PMCA-A (451±67 Vvi/10^6 μm^3) and compared to PMCA-noA (751±207 Vvi/10^6 μm^3) (p<0.36). Antibiotics decreased β-catenin expression in the cytoplasm of PMCA patients who received treatment as compared to no antibiotics (739.9±63.1 vs. 216.6±41.5 Vvi/10^6 μm^3; p<0.0001). When considered as a whole, β-catenin was increased in the stromal compartment of PMCA (A + noA) (1046.5±193.4 Vvi/10^6 μm^3) as compared to DPAM (A + noA) (516.8±70.1 Vvi/10^6 μm^3) (p=0.0369). This was especially true in the desmoplastic reactions of untreated PMCA patients (Fig. 4D). Finally, β-catenin levels found within the mucin, presumably due to the presence of infiltrating inflammatory cells, decreased in PMCA patients after antibiotic treatment (464.58±139.418 Vvi/10^6 μm^3) as compared to no antibiotics (2,179.70±303.38 Vvi/10^6 μm^3) (p<0.0021). Conversely, DPAM did not show a significant difference in the mucin due to antibiotic treatment. As a result, there was a significant difference (p<0.005) in the overall β-catenin staining of PMCA (A + NoA) mucin (1,706.56±264.45 Vvi/10^6 μm^3) as compared to DPAM (A + NoA) mucin (433.14±104.55 Vvi/10^6 μm^3). Finally, though some change in β-catenin distribution was seen in the stromal region of PMCA patients, these changes were not significant.

Given that β-catenin is known to enter the nucleus and function as a transcription factor, and because our initial staining did not allow us to discern which portion of the cytoplasmic β-catenin was actually in the nucleus, we utilized dual staining with DAPI and β-catenin along with a computerized quantification method to determine the fraction of the nucleus occupied by β-catenin. Control NNA tissue exhibited intense bright DAPI staining, but was negative for nuclear β-catenin (data not shown). Conversely, nuclear β-catenin was common in both PMCA and DPAM samples, as exemplified by the fluorescent staining of the nuclei shown in Fig. 5. In the
high magnification insets, bright red color signifies β-catenin expression (A, arrow), while dark blue DAPI staining is seen when nuclear β-catenin is absent (B, arrowhead). When the images are merged (C), the intense red shows up without being affected by the blue DAPI stain. Of note, faint perinuclear β-catenin staining is seen around most DAPI-stained nuclei, indicating that β-catenin was present elsewhere in those cells. The percent of DAPI-stained nuclei occupied by β-catenin was not significantly different when all PMCA samples (A + NoA) were compared to DPAM samples (A + NoA) (20±2.9 vs. 16.5±3.5 %, p<0.451; NS). However, administration of antibiotics resulted in a significant decrease in the percent of nuclear β-catenin present in PMCA (from 23.8±3.4% to 11±4.4%, p=0.048). Despite the fact that there was a strong trend toward decreased nuclear β-catenin, this significant difference was not seen with DPAM (from 18.9±4.8% to 11.3±3%, p<0.152; NS) (Fig. 6A). As nuclear β-catenin dropped as a result of antibiotic treatment, the percentage of β-catenin located in the membrane as compared to the cytoplasm increased (Fig. 6B). This rise in membrane β-catenin was significant for both PMCA and DPAM patients.

Discussion

It has been proposed that a prolonged inflammatory response to foreign microorganisms promotes cancer, whereas certain commensal organisms reduce inflammation and prevent cancer development (30). H. pylori is a well-established cause of gastric adenocarcinoma and MALT lymphoma, but the hypothesis that H. pylori and/or other bacterial species contribute to a range of cancers is only beginning to gain traction. For example, Helicobacter hepaticus, which colonizes the intestine, has been shown to synergize with aflatoxin or hepatitis B virus to cause liver cancer in mice. In that model, β-catenin nuclear translocation was observed in tumors from
animals exposed to both aflatoxin and *H. hepaticus*, but not in those treated with aflatoxin alone, thereby suggesting that both stimuli are involved in the cancer process (31). Other bacteria are also likely to influence cancer risk. *Salmonella typhi* is a known risk factor for gallbladder cancer, and several species, including *Propionibacterium acnes*, have been proposed as contributors to prostate cancer (32, 33). *Klebsiella pneumoniae* and *Proteus mirabilis* cause colon cancer in *Tbet*<sup>−/−</sup> and *Rag2*<sup>−/−</sup> ulcerative colitis (TRUC) mice through undetermined mechanisms (30). Furthermore, it is worth noting that MALT lymphoma and diffuse large B-cell lymphoma can often be treated solely by eradicating *H. pylori*, indicating that ongoing interactions with *H. pylori* are crucial for the survival of these tumors (34, 35).

Given this burgeoning role of bacteria in carcinogenesis and two previous reports that suggested that PMP patients showed positive outcomes following antibiotic treatment (36, 37), we have investigated the molecular mechanisms by which bacteria may influence PMP development. Importantly, our studies provide the first mechanistic data on the role of bacterial infection and β-catenin in PMP. The data presented herein confirm our previous study that demonstrated that bacterial density is higher in PMCA patients than in DPAM patients (23). If inflammation-inducing bacteria contribute to carcinogenesis, then one might posit that tumors with higher bacterial densities would be more malignant than those with fewer bacteria. Thus, our results are consistent with this hypothesis, since we found that the more aggressive PMCA tumors harbor more bacteria than the less aggressive DPAM tumors. Using a specific ISH method to identify bacteria, TNCB and *H. pylori* densities decreased significantly after antibiotic treatment in PMCA patients and to a lesser extent in DPAM patients. It is worth noting that a small number of bacteria remained alive following antibiotic treatment; thus, a different antimicrobial regimen, an extended treatment period, or the alteration of the timing or route of
administration may be necessary to prevent regrowth of bacteria and possible stimulation of
tumor growth. However, the antibiotic treatment utilized in this study clearly reduced the
bacterial density within the tumors.

In contrast to the recent identification of the influence of bacterial species on cancer
development, aberrant β-catenin localization and signaling is a well-established hallmark of
carcinogenesis and metastasis. β-catenin is a transmembrane protein that aids in cell-to-cell
junctions, but it can also influence gene transcription within the nucleus. Translocation of β-
catenin to the nucleus through its association with the Wnt signaling pathway is very important
in embryogenesis and stem cell maintenance; however, it also contributes to abnormal
proliferation during carcinogenesis. Indeed, the heterogeneous distribution of β-catenin within
tumors suggests crosstalk between tumor cells and the tumor microenvironment, including
epithelial-mesenchymal interaction and the vasculature (38). Moreover, in cancer, the Wnt-β
catenin-T cell factor (TCF) signaling plays an important role in nuclear β-catenin accumulation
(39), but the mechanisms governing this translocation are poorly understood and controversial
(40).

Given the established role of β-catenin in carcinogenesis, it was pertinent to investigate
the effect of antibiotic treatment and reduction in bacterial density on β-catenin expression and
localization. Careful analysis of β-catenin expression and cellular distribution after antibiotic
treatment targeted against *H. pylori* infection revealed important reductions in nuclear and total
β-catenin levels in antibiotic-treated PMCA patients. The effects of antibiotic treatment reached
statistical significance primarily in PMCA samples and not in DPAM samples, which could be
the result of the small sample size (n=6) of antibiotic treated DPAM patients, making it more
difficult to reach statistical significance; similar trends were seen in both groups of antibiotic
treated patients. The levels and localization of β-catenin were also altered throughout the various cell types within the analyzed peritoneal tissue, where antibiotic treatment significantly decreased β-catenin expression in the cytoplasm of epithelial and inflammatory cells, in the nuclei, and in the mucin of PMCA patients. Though not statistically significant, stromal β-catenin localization was elevated in PMCA patients not treated with antibiotics. Increased levels of β-catenin within the stroma is associated with desmoplastic reaction in malignancy (Fig. 4D), where β-catenin is attached to the fibrous elements of connective tissue of the stromal compartment (41). The stromal compartment plays an important role during neoplastic cell invasion, detaching from neoplastic intestinal glands, invading the stromal blood vessels and lymphatics, and initiating metastasis (42). β-catenin within mucin was higher in PMCA patients as compared to DPAM patients. Unlike the stroma, antibiotic treatment had a significant effect on decreasing β-catenin in the mucin. Since mucin is the most important histopathology hallmark of PMP, decreased levels of β-catenin within mucin after antibiotic treatment suggests that antimicrobials might be useful in the treatment of this disease. The effectiveness of antibiotic treatment is further evidenced by the significant elevation of β-catenin levels within the membrane in both PMCA and DPAM patients accompanied by a reduction in nuclear β-catenin levels following administration of antibiotics. In normal cells, the β-catenin complex is present within the adherens junctions, as confirmed by immunogold and transmission electron microscopy (43, 44). β-catenin within the membrane promotes cell-to-cell communication and is likely to reduce neoplastic cell migration towards the stroma and metastasis; thus, increased levels of β-catenin within the membrane could be helpful in improving the prognosis of PMP disease. Moreover, the re-normalization of β-catenin distribution in PMCA patients after antibiotic treatment confirms the pivotal contribution of bacteria to the PMP disease process.
Targeting nuclear β-catenin is a potential strategy for cancer therapy. Other reports have discussed the benefit of using antimicrobial agents to treat cancer; however, most attribute normalization of β-catenin with specific effects of antimicrobial agents on the Wnt/β-catenin pathway unrelated to the killing of microorganisms. For example, patients with ovarian endometric adenocarcinoma receiving rapamycin had a demonstrated inhibition of the Wnt/β-catenin pathway along with a decrease in tumor burden (45). Rapamycin blocks Wnt-mediated cell growth by interacting with mTOR, which is a central regulator of cell growth (46).

Similarly, the antibiotic Streptonigrin was reported to block the complex formation of β-catenin/TCF (T cell factor signaling) with DNA in human cell lines (47). In our study, the reduction of nuclear β-catenin following treatment with antimicrobial agents is likely due to bacterial killing, since, to our knowledge, no evidence indicates that amoxicillin or clarithromycin influence the Wnt/β-catenin signaling pathway. Of note, companion studies conducted by our group (48) have defined the microbiome of PMP tumors from 11 patients and shown the ability to culture bacteria directly from PMP tumor tissue. That study further indicated that antibiotic administration improves the survival of PMCA patients without PMP observed in the lymph nodes when compared with untreated patients and historical data. Antibiotic treatment did not improve the survival of patients with lymph nodes positive for PMP. Thus, treatment with antibiotics may be a beneficial therapy for PMP patients with non-metastatic disease.

We have not determined which mechanism is responsible for the reduction of nuclear β-catenin following antibiotic treatment or whether bacteria other than *H. pylori* contribute to abnormal β-catenin localization. It is plausible that the diminished β-catenin levels could be due to the loss of direct contact between tumor cells and bacteria or caused by a reduced inflammatory response to bacteria. In fact, DeNardo, *et al.* propose that chronic inflammation
could lead to Wnt/β-catenin activation without specific interactions between bacteria and epithelial cells (49). In this model, TNFα produced by activated macrophages binds to TNF receptors on nearby epithelial cells, leading to AKT phosphorylation and the subsequent stabilization of β-catenin.

Recent studies have revealed the influence of several bacterial species on β-catenin expression and localization. *H. pylori* is present in the intercellular spaces, where the bacteria disrupt apical tight junctions as well as perturb molecular expression of β-catenin and other proteins of the lateral cell-to-cell membrane (50, 51). *Salmonella* activates the Wnt/β-catenin pathway via the secreted effector protein AvrA (21). *Campylobacter rodentium, Bacteroides fragilis* and *Chlamydia trachomatis* also influence Wnt/β-catenin signaling (15, 22). Given the effect of bacteria on β-catenin signaling, which is known to contribute to the carcinogenic process, it is interesting to note that many common cancers occur in anatomical regions frequently exposed to bacteria, including cancers of the throat, lung, gastrointestinal tract, reproductive tract, and skin. Other locations, such as the breast, may seem to exist as sterile sites; however, bacteria can often gain entry, raising the possibility that individual bacterial species or a combination of different species could contribute to the carcinogenic process in multiple cancers. Thus, determining which bacterial species are involved in these processes could lead to targeted antimicrobial therapy and help to improve current methods for preventing and treating cancer.

**Acknowledgements:**

During the writing of this manuscript, Dr. Andre Dubois passed away unexpectedly. Though ill for a length of time, Dr. Dubois did not want others to be concerned and was silent about this
fact. Throughout his illness, he worked diligently and passionately on the PMP research. His wisdom, generosity and expertise will be sorely missed by his colleagues and the research community. Additionally, Dr. Cristina Semino-Mora passed away after the submission of the original manuscript. Despite a lengthy illness, she continued contributing her abilities to several research projects. Her extensive skills and expertise were a tremendous asset that cannot be replaced. The authors thank Dr. Dennis McDaniel for his assistance with laser confocal imaging along with Michelle Sittig and Megan Putman for their contributions to this project. This work was supported by R0832L, which was funded by Uniformed Services University, as well as funding from the US Military Cancer Institute.
References


16. Hayward SD, Liu J, Fujimuro M. Notch and Wnt signaling: mimicry and manipulation by 

upregulation of miR-155 promotes hepatocarcinogenesis by activating Wnt signaling. 

effects of CagA during interaction between Helicobacter pylori and T84 polarized monolayers. J 
Infect Dis 2004;190: 1516-23.

beta-catenin by carcinogenic Helicobacter pylori. Proc Natl Acad Sci U S A 2005;102: 10646-
51.

of phospho-beta-catenin subcellular distribution in invasive breast carcinomas in relation to their 

21. Liu X, Lu R, Wu S, Sun J. Salmonella regulation of intestinal stem cells through the 

trachomatis disturbs epithelial tissue homeostasis in fallopian tubes via paracrine Wnt signaling. 
The Am J Pathol 2012;180: 186-98.

peritonei: is disease progression related to microbial agents? A study of bacteria, MUC2 AND 
MUC5AC expression in disseminated peritoneal adenomucinosis and peritoneal mucinous 

24. Ronnett BM, Zahn CM, Kurman RJ, Kass ME, Sugarbaker PH, Shmookler BM. 
Disseminated peritoneal adenomucinosis and peritoneal mucinous carcinomatosis. A 
clinicopathologic analysis of 109 cases with emphasis on distinguishing pathologic features, site 
of origin, prognosis, and relationship to "pseudomyxoma peritonei". Am J Surg Pathol 1995;19: 
1390-408.

synergistically affects Helicobacter pylori-induced gastric carcinogenesis in nonhuman primates. 

26. Liu H, Rahman A, Semino-Mora C, Doi SQ, Dubois A. Specific and sensitive detection of H. 
pylori in biological specimens by real-time RT-PCR and in situ hybridization. PLoS One 2008;3: 
e2689.

27. Semino-Mora C, Doi SQ, Marty A, Simko V, Carlstedt I, Dubois A. Intracellular and 
interstitial expression of Helicobacter pylori virulence genes in gastric precancerous intestinal 


47. Park S, Chun S. Streptonigrin inhibits beta-Catenin/Tcf signaling and shows cytotoxicity in beta-catenin-activated cells. Biochim Biophys Acta 2011;1810: 1340-5.


Table 1. Bacterial distribution in PMP samples

<table>
<thead>
<tr>
<th>Group</th>
<th>% of samples containing bacteria</th>
<th>Relative number of <em>H. pylori</em></th>
<th>Relative number of TNCB</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPAM</td>
<td>76.9</td>
<td>14.46</td>
<td>29.54</td>
</tr>
<tr>
<td>DPAM, antibiotic treated</td>
<td>83.3</td>
<td>9.90</td>
<td>21.0</td>
</tr>
<tr>
<td>PMCA</td>
<td>90.5</td>
<td>40.37</td>
<td>80.36</td>
</tr>
<tr>
<td>PMCA, antibiotic treated</td>
<td>75</td>
<td>11.54</td>
<td>18.54</td>
</tr>
</tbody>
</table>
Figure 1. Pathological Examination of DPAM and PMCA Tissue Samples. Cytoreductive surgical specimens were collected, sectioned, stained with H&E, and evaluated to determine tumor classification, histopathology, and inflammation grade. DPAM samples (A) displayed extracellular mucin (pale pink areas with blue streaks) bordered by dark pink collagen bands supporting the mucin deposits. DPAM samples were further characterized by a mucinous epithelial lining, chronic inflammatory cells scattered throughout the tissue, and low-grade adenomatous dysplasia without mitosis (A, left panel). The peritoneal lining adjacent to the extracellular matrix in DPAM sections exhibited mesothelial hyperplasia, vascular congestion, and chronic inflammation (A, right panel). PMCA samples (B-D) presented with glands comprised of angulated gland-forming mucinous epithelium with destructive stromal invasion (B). Additionally, the PMCA samples are distinctively identified from the DPAM sections based on the presence of high-grade cytologic atypia (C), mitotic activity (C), and inflammatory cells within desmoplastic stroma with fibrotic adhesions (D).

Figure 2. Identification of Bacteria through *In situ* Hybridization (ISH). Formalin-fixed tissue specimens from PMP patients were serially sectioned and analyzed with (ISH) probes to detect all TNCB present (A, B, C, left panels) or specifically *H. pylori* (A, B, C, right panels). Clusters of TNCB (A, left panel) and *H. pylori* (A, right panel) were found in 83% of the analyzed tissue samples. TNCB (B, left panel) and *H. pylori* (B, right panel) were observed within the mucin encompassed by neoplastic epithelia and goblet cells. Both TNCB (C, left panel) and *H. pylori* (C, right panel) were also present in the connective tissue of the lamina propria (stroma), contacting inflammatory cells and epithelial cells in the intestinal glands.
Figure 3. FISH Analysis of Bacterial Density in Mucin. Formalin-fixed tissue specimens from PMP patients were serially sectioned and analyzed with FISH to identify all TNCB present (green; A) or specifically *H. pylori* (red; B) within a region of pooled mucin. In the merged images (C), the *H. pylori* staining converges with the TNCB staining to reveal a yellow color for *H. pylori* while all non-*H. pylori* bacteria appear green. A clear reduction in bacterial density is observed in a PMP patient receiving antibiotic treatment (A-C, bottom panels) compared to an untreated PMP patient (A-C, top panels).

Figure 4. Qualitative Assessment of β-catenin localization in PMP Samples. Formalin-fixed tissue samples from PMP patients and an NNA control were sectioned and stained with an anti-β-catenin antibody. The NNA control (A) displayed strong staining at the boundaries between adjacent cells, showing the localization of β-catenin to cell membranes and the lateral junctional complex. Conversely, PMCA sections (B) exhibited primarily cytoplasmic staining of β-catenin and virtually no staining at the intercellular boundaries. Some staining was observed between PMCA neoplastic cells following antibiotic treatment (C). β-catenin was very visible in the desmoplastic reactions within the stromal compartment of untreated PMCA patients (D).

Figure 5. Immunofluorescence Analysis of Nuclear β-catenin. Formalin-fixed tissue samples from PMP patients were sectioned and dual stained with an anti-β-catenin antibody and DAPI to observe the localization of nuclear β-catenin. As shown in panel A, bright red staining (arrow)
indicates the presence of β-catenin, whereas panel B reveals the dark blue DAPI staining (arrowhead) appearing when β-catenin staining is absent. The merged image in panel C exhibits the intense red β-catenin staining unaffected by blue DAPI staining.

**Figure 6. Quantitative Evaluation of β-catenin Localization.** Formalin-fixed tissue samples from PMP patients were sectioned and either stained solely with an anti-β-catenin antibody to determine membrane localization or stained with both an anti-β-catenin antibody and DAPI to determine the proportion of the nucleus occupied by β-catenin. Antibiotic treatment significantly reduced (p=0.048) the proportion of the nucleus occupied by β-catenin (A) in PMCA patients. Though a reduction in nuclear β-catenin was observed in DPAM patients following antibiotic treatment as well, this change was not significant (A). The reduction in nuclear β-catenin was accompanied by a significant elevation in β-catenin within the membrane (B) for both PMCA (p<0.05) and DPAM (p<0.05) patients receiving antibiotic treatment. (Error bars represent the SEM; * represents a significant difference.)
Figure 3

A: TNCB
B: H. pylori
C: Merge

10um
Figure 5

A

B

C

10um

10um
Figure 6

A

Proportion of nucleus occupied by β-catenin (%)

PMCA | PMCA+A | DPAM | DPAM+A

B

Proportion of β-catenin within the Membrane (%)

PMCA | PMCA+A | DPAM | DPAM+A
Antibiotic Treatment Decreases Microbial Burden Associated with Pseudomyxoma Peritonei and Affects $\beta$-catenin Distribution

Cristina Semino-Mora, Traci L Testerman, Hui Liu, et al.

Clin Cancer Res  Published OnlineFirst June 6, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-0616

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/06/05/1078-0432.CCR-13-0616.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.