Name of journal: World Journal of Gastrointestinal Oncology

Manuscript NO: 42891

Title: Persistently elevated plasma levels of 8 proangiogenic proteins after colorectal resection are associated with greatly increased wound fluid levels of the same proteins

Responses for reviewer's comments

Reviewer's code: 03478911

Comment 1: The authors must explain the mechanism of action of several angiogenic factors that listed in the theoretical background.

Author's response:

The introduction was updated and the following paragraph with theoretical background added the reference list updated.

VEGF stimulates multiple early steps in angiogenesis including endothelial cell (EC) proliferation, microtubule formation, invasion and migration. Angiopoetin2 (Ang 2) enhances VEGF's effects by destabilizing the connections between the endothelium and perivascular cells. Ang 2 does this by competitively binding to the Tie-2 receptor with a greater affinity than Ang-1 which, when bound to Tie-2 has anti-angiogenic effects.(1,2) Placenta Growth Factor (PlGF), a member of the Vascular Endothelial Growth Factor (VEGF) family, plays a crucial role in both physiologic and pathologic angiogenesis. PlGF primarily regulates the angiogenic switch under pathologic conditions (3). However, PLGF, by increasing the amount of VEGF available to bind to the key receptor VEGFR2 (and decreasing VEGF's binding to VEGFR1) maximizes VEGF's proangiogeneic effects early in the process of vessel formation. MCP-1 is believed to mediate angiogenesis by recruiting proangiogenic protein producing macrophages and monocytes into wounds and tumors; MCP-1 also promotes EC migration, a critical early step in angiogenesis, by binding to CCR-2 (C-C chemokine Receptor 2) on the surface of endothelial cells (EC's), (4,5). Human chitinase 3-like 1 (CHI3L1), also known as YKL-40, induces IL-8 and MCP-1 secretion through the ERK and JNK signal pathways (6); these chemokines support macrophage recruitment and tumor angiogenesis. Osteopontin (OPN) is an integrin binding phosphorylated acidic glycoprotein that mediates cell-matrix and cell-cell communication.(7,8) OPN has been shown to enhance tumor progression and angiogenesis via the PI3K/AKT and ERK mediated pathways in association with VEGF.(9,10) Matrix Metalloproteinase-2(MMP-2) is an extracellular matrix remodeling enzyme(11, 12) that degrades type IV collagen(13) in the basement membrane which enables EC migration and tumor cell invasion(14,15); it has also been shown to enhance VEGF release.(16) Matrix metalloproteinase-3 (MMP-3) has been

shown to support the process of epithelial-mesenchymal transition (EMT) during which epithelial cells lose adhesion, become invasive, and transition to the mesenchyme which is critical in wound healing, angiogenesis, and the initiation of cancer metastasis (17).

Comment 2: And the purpose of the study that presented in the theoretical background is unclear

Author's response:

Manuscript introduction was updated to reflect below information.

Authors have explained the scope of the study concisely in the introduction. Further explanation follows in response to the reviewer's comment: It has been demonstrated, in previous studies performed by our lab, that all 8 plasma proteins (each with proangiogenic effects) included in the current study are significantly elevated plasma levels for 2-4 weeks after colorectal resection. The etiology of these persistent plasma protein changes is unknown. Because angiogenesis is central to wound healing and because during the first month after surgery the body is tasked with the job of healing both the intra-abdominal and the abdominal wall wounds, the authors hypothesized that the added protein in the bloodstream originates in the healing wounds and then finds its way into the circulation. Of note, previous investigators have noted elevated VEGF levels in wound fluid taken from mastectomy and other surgical patients (15-17). The purpose of this study was to simultaneously measure plasma and wound levels of 8 proteins in patients undergoing colorectal resection for both cancer and benign indications during the first month following surgery. The proteins chosen for study have been previously shown to have persistently elevated plasma levels for 2-4 weeks after MICR for colorectal cancer. The proteins assessed were: vascular endothelial growth factor (VEGF), placental growth factor (PLGF), angiopoetin-2 (ANG2), monocyte chemotactic protein-1 (MCP-1), chitinase 3 like protein-1 (CHI3L1), osteopontin (OPN), matrix metalloproteinase-2 (MMP2) and MMP3. In the previously published studies concerning these proteins, plasma levels of only 1 or 2 proteins were assessed per patient population; in this study, plasma and wound levels of all 8 proteins were determined in all patients at each time point. Further, this study included both patients with cancer and those with benign indications for colorectal resection in order to determine if the indication for surgery influenced the postop levels.

Comment 3: The authors need to describe the scientific and clinical implications to be gained by investigating the increase in angiogenic factors.

Author's response:

A little background is needed to provide the context for the study under consideration. The motivation for the initial perioperative plasma protein analyses (mostly immune system and

stress response related factors such as IL-6, CRP, TNF, IL-2, etc) done by our group over a decade ago was to determine the systemic response to laparoscopic colorectal resection (vs open resection). We also looked at cell mediated immune function (via serial DTH testing) and noted that MIS surgery was associated with significantly less immunosuppression. Next, we looked at perioperative tumor growth after laparotomy vs pneumoperitoneum in murine models and noted that tumor growth and rates of tumor establishment were significantly greater after laparotomy. The lab then began to explore the question of how surgical trauma may impact the growth of residual tumor in cancer patients who underwent resection of the primary tumor. Clinically, in a small percentage of patients tumor recurrences develop within 1-2 months after surgery; similarly, in a proportion of patients with unresectable metastases who undergo major surgery rapid progression is noted. We began looking for the mechanism of this rapid growth after surgery. Since we were interested in a systemic effect, it was decided to assess blood protein composition. Also, because we were looking for concentration changes that had the potential to have a significant clinical impact we sought to determine plasma levels at multiple points during the first month after surgery (rather than the standard preop, postop day 1, pod 3 or 4 time points). The lab studied a wide range of proteins including FGF, TGF, HGF, IGF, and VEGF. The only protein whose levels remained elevated for more than 2 weeks was VEGF. This led to perioperative investigations of least 13 proteins with proangiogenic effects. To our surprise it has been shown that the levels of at least 11 such proteins are elevated for 3 to 5 weeks after surgery. The paper you are reviewing presents data to suggest that source of the added protein in the blood are the surgical wounds.

The potential clinical impact of this work is that it might lead to a close examination of the effects of surgical trauma and an awareness that, following major surgery, cancer patients that have small residual tumor deposits are at risk for accelerated tumor growth during the first month after surgery. The data contained in this paper presents sound data (in our opinion) that supports the hypothesis that wound healing results are the source of the high levels of 8 proangiogenic proteins which might stimulate tumor angiogenesis postoperatively. **This awareness will, hopefully, lead to the development of perioperative anti-cancer treatments the goal of which would be to limit or prevent the tumor stimulatory effects of surgery.** The administration of anti-cancer therapy during the first month after surgery would represent a basic change in the way adjuvant treatment is given postoperatively since, presently, standard adjuvant chemotherapy is started 4 to 8 weeks after surgery. Please note: we do not think that anti-angiogenesis drugs should be given during the periop period since they would interfere with wound healing, instead, other anti-tumor strategies would be pursued (immunomodulation, tumor vaccines, and, perhaps, checkpoint inhibition are examples). Awareness of the potential for rapid tumor growth after surgery is also likely to motivate clinicians to find ways to avoid surgery, where possible, in cancer patients in whom an R-0

tumor resection is not possible.

There are several potential scientific benefits that may arise as a result of this study. First, this avenue of research is the first to prove that surgery significantly alters the plasma protein composition for up to 5 weeks after colorectal resection. The literature that existed prior to these studies suggested that surgery's impact on blood protein levels lasted, at most, for 1 week. This new awareness will alter our thinking about the potential impact of surgery. Also, it may be that it is the wound healing rather than the surgical trauma itself which poses the most problems. Further, this research also strongly suggests that the wound healing process is a lengthy process marked by active angiogenesis for up to 5 weeks. This work may also lead to other studies regarding the first month after surgery.

Independent of the above comments a new lengthy paragraph has been added to the discussion of the revised paper that addresses this question.

Comment 4: In the methodology, the factors such as the disease stage, sex, and age were not clearly distinguished.

Author's response:

The following demographic, clinical and pathologic data is included in the paper for both the benign and cancer patients who participated in the study can be found in the methods section and table 1 and 4. Please note that another review requested ethnicity/race information which has been added as well.

"Plasma and wound fluid samples from 35 patents diagnosed with colorectal adenocarcinoma (rectal 21; colon 14; 21 male /14 female, mean age 63.6± 11.3 years) were collected and included into the study. The CRC stage distribution was: Stage 1, 10,(29%); Stage 2, 11, (31%); Stage 3, 12, (34%), and Stage 4, 0(0%). The ethnic/race breakdown of the patients was as follows: Caucasian (40%) , Hispanic (29%), African American (28%) and Asian (3%).

In addition, a total of 31 patients with benign pathology who met the entry criteria (11 male/ 20 female, mean age, 57.3± 14.1years) consented to participate in this study. The indications for surgery in the benign disease group were diverticulitis, 18 patients, 58%; benign neoplasm, 10, 32%; ulcerative colitis, 2, 7%; constipation, 1 (3.2%). The ethnicity/race breakdown was as follows: Caucasian (78%), Hispanic (12%), African American (7%) and Asian (3%) patients."

Comment 5: The various factors that presented by this reviewer are related to the outcome of treatment. Therefore, it can be an important parameter.

Author's response:

The reviewer suggests, we believe, that the long term outcome of the cancer patients is germaine to this study given our hypothesis that the surgery may put patients at risk for tumor recurrence. We fully agree with this opinion, however, to prove our hypothesis (that residual tumor deposits may be stimulated to grow during the first postoperative month because of the wound healing related proangiogenic plasma protein elevations) we would need to have a control group of patients with cancers who did not undergo surgery whose rate of tumor growth would be determined. Without a "no surgery" control group we have nothing to compare the recurrence rates of the study patients who underwent cancer resection to. Furthermore, since only a fraction of the patients undergoing "curative" colorectal cancer resection will harbor residual cancer deposits after surgery (unknown to the surgeon or patient) and would be at risk for rapid growth, a much larger number of patients would need to be studied in order for meaningful data to be obtained. Our study of 35 cancer patients is far too small for outcomes analysis.

Comment 6: The process of extracting proteins from tissues or blood is missing, therefore it needs further explanation

Author's response:

As regards the analyses of the blood samples that were performed, the proteins studied were not "extracted" from the blood but rather the concentration of the proteins in the plasma samples were determined using Enzyme-Linked Immunosorbent Assay (ELISA) which is a plate-based assay technique designed for detecting and quantifying soluble plasma proteins. Each protein has its own ELISA, thus, 8 different ELISA kits were used to determine the plasma protein levels. After collection, blood samples (collected in heparin containing tubes) were centrifuged and the plasma fraction placed in 500 ul storage vials that were frozen until used.

As regards the wounds, what was studied was the drainage fluid from Jackson Pratt suction devices that were connected to tube drains that had been left in pelvis and/or abdominal wall wounds at the time of surgery. The drainage fluid from the wound was collected in sterile fashion and the centrifuged for 10 minutes after which the supernatant was collected and placed in 500 ul vials that were frozen until used. Identical to the plasma analyses, different ELISA's were used to determine the concentration of the 8 proteins in the wound fluid supernatant. The proteins were not extracted.

The following R&D Systems Quantikine ELISA kits were used for this study; DVE00, DPG00, DANG20, DCP00, DC3L10, DOST00, MMP200 and DMP300. Each kit and assay had been tested

for precision (intra-assay precision and Inter-assay-precision), recovery, and sensitivity and for linearity by the vendor. Protein levels were determined in duplicate for both the plasma and wound fluid samples. Before analysis wound fluid samples were diluted 10-20 times and plasma samples diluted as per the manufacturer's recommendations for each protein. The plasma and WFL samples from each patient were analyzed on the same ELISA plate in duplicate for each protein and standards were included in each ELISA assay. As regards the frozen specimens, freeze thaw cycles were avoided in the utilization of the samples by utilizing 500 ul cryovials and coordination the individual protein ELISA's such that a given vial of plasma or wound fluid was fully utilized on the same day it was thawed. The ELISAs were read using an automated microplate reader (Synergy2; Bio-Tek Instruments, Inc., Winooski, VT, USA). Standard curves were generated on four parameter logistic curve fit and protein concentrations are reported as pg/ml or ng/ml.

The blood and wound fluid processing methods, reviewed above, as well as the ELISA kit information are in the revised manuscript.

Comment 7: The method for calculating the error bar is missing.

Author's response:
Method and figure legends were updated as per the reviewer's comment.

Plasma and wound fluid protein levels are expressed as median and 75% quartile range in each figure.

Comment 8: In the POD1 results in Figures 1a, 1b, 2a, 2b, and 4b it seems that there are no statistical significance considering the error bars. Therefore, reanalyze is required.

Author's response:
The authors have performed the analyses as per the reviewer's comment. The statistical significance results reported in the paper and figures remains the same. The inclusion of the results for multiple proteins in each of the papers figures may have made it difficult to comprehend. In the revised manuscript, individual figures and data tables for each protein are provided which, hopefully, will be easier for the reader to understand.

Comment 9: Discussion: It is better to describe the clinical implications, point by point, that can be gleaned from the author's findings, rather than theoretical explanations.

Author's response:

The clinical implications were described in response to reviewer's comment 3 (below) and the discussion was updated as per reviewers comment.

The overall clinical implication of the finding that the plasma, in general, is proangiogenic, is that tumor angiogenesis may be stimulated in metastases that are present after surgery. We have in no way proven this (which would be very hard to demonstrate, as explained in the above response to question 5) nor can we in this paper. It is also possible to provide the reader with summaries of what is known about the actions of each protein as regards angiogenesis. We have done this in the revised introduction of the paper (this can be found in response to question 1) and in a paragraph added to the discussion. Again, we can build the case that the protein changes noted may promote tumor angiogenesis but cannot provide actual proof of this. The addition to the discussion section follows.

"As mentioned in the introduction, each of the 8 proteins included in this study have been noted to have proangiogenic effects. It is important to also note that practically all of these proteins are overexpressed in a large variety of cancers and that, for some of the proteins, elevated serum or plasma levels have also been noted. Further, in many cases increased tumor expression or elevated blood levels have been associated with worse cancer outcomes. VEGF, the best studied and well known of the group, is absolutely critical to the process of neovascularization and is overexpressed by many cancers. PLGF may facilitate metastasis by increasing the motility and invasion of malignant cells; also, tumor overexpression of PlGF and VEGF together is associated with increased tumor angiogenesis and cancer growth (3). MCP-1, in addition to promoting EC migration, has been shown to be overexpressed in multiple human cancers and is associated with tumor grade in ovarian cancer patients.(18,19) In regards to Chi3l1, in the murine setting, Chi3l1-overexpressing cancer cell lines exhibited 4.0-8.0 fold greater tumor growth and 1.8-2.0 fold greater vasculature density than controls(20). Also, elevated blood levels of Chi3l1 have been noted in a large variety of cancer patients (21-26) and are associated with a poor prognosis in many.(21,24,25) OPN has been shown in some studies to enhance tumor progression and angiogenesis in association with VEGF. (9,10). Overexpression of OPN has been noted in breast, lung, liver and colorectal cancer patients and is associated with worse prognosis and early recurrence in patients with hepatocellular cancer (27). MMP2 plays a unique role in tissue remodeling as regards angiogenesis and is associated with tumor progression and metastasis. Elevated MMP-2 activity has been linked to a poor prognosis in lung (28), breast (29), prostate (30) and colorectal cancer (31). As mentioned, MMP-3 has been shown to play a role in the process of epithelial-mesenchymal transition (EMT) which is an important component of wound healing and angiogenesis. MMP-3 has also been shown to play an important role in the growth and/or metastatic transformation of cancers

including breast cancer and hepatocellular carcinoma (32-37) and is overexpressed in some gastric and liver cancers."

References:

1. Tait CR, Jones PF. Angiopoetins in tumors: The angiogenic switch. J Pathol 2004;204:1-10.
2. Liekens S, DeClercq E, Neyts J. Angiogenesis: regulators and clinical applications (commentary) Biochemical Pharmacology 2001;61:253-270.
3. Carmeliet P, Moons L, Luttun A, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001; 7(5):575-83
4. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ ,"Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression" Blood 2000;96(1) 34-40.
5. Weber KS, Nelson PJ, Gröne HJ, Weber C, "Expression of CCR2 by endothelial cells: implications for MCP-1 mediated wound injury repair and in vivo inflammatory activation of endothelium" Arteriosclerosis, Thrombosis, and Vascular Biology 1999, 19(9): 2085-2093.
6. Kawada M, Seno H, Kanda K, Nakanishi Y, Akitake R, Komekado H, Kawada K, Sakai Y, Mizoguchi E, Chiba T.Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer Oncogene. 2012; 31(26): 3111–3123
7. Giachelli CM, Steitz S. Osteopontin: a versatile regulator of inflammation and biomineralization. Matrix Biol.2000; (19):615-622.
8. Mazzali M, Kipari T, Ophascharoensuk V, Wesson JA, Johnson R, Hughes J. Osteopontin—a molecule for all seasons (2002) QJM. 95:3-12
9. Dai J, Peng L, Fan K, Wang H, Wei R, Ji G, Cai J, Lu B, Li B, Zhang D, Kang Y, Tan M, Qian W, Guo Y. Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. Oncogene 2009; 28 (38):3412-22.
10. Chakraborty G, Jain S, Kundu GC. Osteopontin Promotes Vascular Endothelial Growth Factor–Dependent Breast Tumor Growth and Angiogenesis via Autocrine and Paracrine Mechanisms. Cancer Res.2008; 68(1):152-161.
11. Baek Moo-Jun Prognostic Role of MMPs in Colorectal Cancer.J of Korean Soc.Coloproctol. 2011;27(3):105-106
12. Page-McCaw A, Ewald AJ, Werb Z.Matrix metalloproteinases and the regulation of tissue remodellingNat Rev Mol Cell Biol.. 2007; 8(3):221-233
13. Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He CS, Bauer EA,Goldberg GI H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem. 1988 May 15;263(14):6579-87
14. Giannelli, G., Falk-Marzillier, J., Schiraldi, O., StetlerStevenson, W. G. & Quaranta, V Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science 1977; (277), 225–228.

15. Xu J, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo J Cell Biol. 2001;154(5):1069-1079.
16. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumorsJ Cell Biol. 2005 May 23;169(4):681-691.
17. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646-674.
18. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, Matsushima K.(2000)Significance of MacrophageChemoattractant Protein-1 in Macrophage Recruitment, Angiogenesis, and Survival in Human Breast Cancer. Clin Cancer Res 6:3282-3289
19. Hefler L, Tempfer C, Heinze G, Mayerhofer K, Breitenecker G, Leodolter S, Reinthaller A, Kainz C. Monocyte chemoattractant protein-1 serum levels in ovarian cancer patients. Br J Cancer 1999; 81(5):855-859.
20. Shao R, Hamel K, Petersen L, Cao QJ, Arenas RB, Bigelow C, Bentley B, Yan W. YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. Oncogene. 2009;28(50):4456-4468.
21. Cintin C, Johansen JS, Christensen IJ, Price PA, Sørensen S, Nielsen HJ. Serum YKL-40 and colorectal cancer. Br J Cancer. 1999;79(9-10):1494-1499
22. Jensen BV, Johansen JS, Price PA. High levels of serum HER-2/neu and YKL-40 independently reflect aggressiveness of metastatic breast cancer. Clin Cancer Res. 2003;9(12):4423-4434.
23. Kucur M, Isman FK, Balci C, Onal B, Hacibekiroglu M, Ozkan F, Ozkan A. Serum YKL-40 levels and chitotriosidase activity as potential biomarkers in primary prostate cancer and benign prostatic hyperplasia. Urol Oncol. 2008;26(1):47-52.
24. Johansen JS, Drivsholm L, Price PA, Christensen IJ. High serum YKL-40 level in patients with small cell lung cancer is related to early death. Lung Cancer. 2004 ;46(3):333-334.
25. Pan JJ, Ge YS, Xu GL, Jia WD, Liu WF, Li JS, Liu WB. The expression of chitinase 3-like 1: a novel prognostic predictor for hepatocellular carcinoma. J Cancer Res Clin Oncol. 2013 ;139(6):1043-1054.
26. Dupont J, Tanwar MK, Thaler HT, Fleisher M, Kauff N, Hensley ML, Sabbatini P, Anderson S, Aghajanian C, Holland EC, Spriggs DR. Early detection and prognosis of ovarian cancer using serum YKL-40. J Clin Oncol. 2004;22(16):3330-3339.
27. Pan HW, Ou YH, Peng SY, Liu SH, Lai PL, Lee PH, Sheu JC, Chen CL, Hsu HC. Overexpression of osteopontin is associated with intrahepatic metastasis, early recurrence, and poorer prognosis of surgically resected hepatocellular carcinoma. 2003; Cancer (98):119-127.
28. Rollin J, Régina S, Vourc'h P, Iochmann S, Bléchet C, Reverdiau P, Gruel Y.Lung Cancer.2007;56(2):273-280
29. Nakopoulou L, Tsirmpa I, Alexandrou P, Louvrou A, Ampela C, Markaki S, Davaris PS. MMP-2 protein in invasive breast cancer and the impact of MMP-2/TIMP-2 phenotype on overall survival. Breast Cancer Res Treat. 2003 Jan;77(2):145-155.
30. Brehmer B, Biesterfeld S, Jakse G Prostate Cancer Expression of matrix metalloproteinases (MMP-2 and -9) and their inhibitors (TIMP-1 and -2) in prostate cancer tissue Prostatic Dis. 2003;6(3):217-222.

31. Tutton MG, George ML, Eccles SA, Burton S, Swift RI, Abulafi AM Use of plasma MMP-2 and MMP-9 levels as a surrogate for tumour expression in colorectal cancer patients. Int J Cancer. 2003;107(4):541-550
32. Mendes O, Kim HT, Stoica G Expression of MMP2, MMP9 and MMP-3 in breast cancer brain metastasis in a rat model. Clin Exp Metastasis, 2005;22:237-246.
33. Radisky ES, Radisky DC Matrix metalloproteinase induced epithelial-mesenchymal transition in breast cancer. J Mammary Gland Biol Neoplasia .2010; 15:201-212.
34. Lee EJ, Kim SY, Hyun JW, Min SW, Kim DH, Kim HS Glycitein inhibits glioma cell invasion through down-regulation of MMP-3 and MMP-9 gene expression. Chem Biol Interact 2010; 185:18-24.
35. Jung K, Nowak L, Lein M, Priem F, Schnorr D, Loening SA Matrix metalloproteinases 1 and 3, tissue inhibitor of metalloproteinases1 and the complex of metalloproteinases-1/tissue inhibitor in plasma of patients with prostate cancer. Int J Cancer 1997;74:220-223.
36. Tang CH, Yamamoto A, Lin YT, Fong YC, Tan TW Involvement of matrix metalloproteinases-3 in CCL5/CCR5 pathway of chondrosarcoma metastasis. Biochem Pharmacol 2010; 79:209-217.
37. Ishii Y, Nakasato Y, Kobayashi S, Yamazaki Y, Aoki T A study on angiogenesis-related matrix metalloproteinase networks in primary hepatocellular carcinoma. J Exp Clin Cancer Res 2003;22:461-470.

Reviewer's code: 03721686

Comment 1: Besides the small number of patients included, the study has many limitations. Follow up of those patients would be of interest, especially the group with malignant pathology, to draw conclusions that are speculated in the text (e.g. Conclusion section- „These proangiogenic plasma changes may stimulate tumor angiogenesis during the first month after surgery in patients with residual tumor deposits post resection of the primary lesion").Therefore, the conclusion may be revised to be concise. A table with histopathological details about tumors could be added (TNM staging, location of cancer, grading etc), other causes, such as comorbidities, that could influence the results.

Author's response:
The TNM stages and the cancer locations in the cancer group are included in the results section. The reviewer is correct in stating that knowing the long term outcome of the cancer patients is germaine to this study given our hypothesis that the surgery may put patients at risk for tumor recurrence. We fully agree with this opinion, however, to prove our hypothesis (that residual tumor deposits may be stimulated to grow during the first postoperative month because of the wound healing related proangiogenic plasma protein elevations) we would need to have a control group of patients with cancers who did not undergo surgery whose rate of tumor growth would be determined. Without a "no surgery" control group we have nothing to compare the recurrence rates of the study patients who underwent cancer resection to. Furthermore, since only a fraction of the patients undergoing "curative" colorectal cancer

resection will harbor residual cancer deposits after surgery (unknown to the surgeon or patient) and would be at risk for rapid growth, a much larger number of patients would need to be studied in order for meaningful data to be obtained. Our study of 35 cancer patients is far too small for outcomes analysis. Therefore, we acknowledge that long term outcome data is lacking, however, even if added it would not prove or disprove our hypothesis given the small number of patients studied and the lack of a control group of cancer patients who did not undergo surgery. Similarly, in regards to comorbidities and their potential impact on the systemic response to surgery, the study size will not allow any meaningful conclusions to be drawn. A far larger study is needed that would have reasonably sized subgroups with comorbidities such as diabetes, cardiac, pulmonary, or renal disease that could be compared as regards postop plasma protein levels. Regardless, the co-morbidities of the patients are provided below.

As suggested, the conclusion has been edited (see below)

"Conclusion:

In summary, this study has demonstrated that plasma levels of the 8 proangiogenic proteins in question are significantly elevated over preoperative levels for 3 weeks after colorectal resection AND that protein levels in WFL samples taken at the same time points are many fold higher than the comparable plasma levels. Although not proven, the healing wounds appear to be a source of the added protein that raises plasma levels postoperatively, especially during weeks 2 and 3 after surgery. The indication for surgery (benign vs malignant) does not appear to impact these surgery-related changes (Supplementary Table 6). These proangiogenic plasma changes may stimulate tumor angiogenesis during the first month after surgery in patients with residual tumor deposits post resection of the primary lesion. Further study is needed to determine if the persistent proangiogenic plasma compositional changes are clinically relevant in cancer patients and, if so, then anti-cancer therapies that can be safely used in the perioperative time window need to be developed."

Requested Supplementary Table 6: TNM staging, location of cancer, grading of the adenocarcinoma group (n=35) . [Note: The 2 rectal cancer patients with Stage 0 lesions (Stage 0;T-0, N-0) had complete pathologic responses following neoadjuvant RT/chemotherapy].

TNM stages	Malignant group (n)	Pathological grading			Location of Cancer	
		Low	Intermediate	High	Colon	Rectal
Stage 0	2 (6%)	0	0	0	0	2
Stage 1	10 (29%)	7	3	0	4	6
Stage 2	11 (31%)	5	5	1	5	6
Stage 3	12 (34%)	5	5	2	5	7

[Comorbidities and past medical history of the malignant group follows: hyperlipidemia, 6(17%); gastroesophageal reflux disease, 5 (14%); asthma,4(11%); sleep apnea ,4(11%); Diabetes mellitus,3(8%); Pulmonary embolism ,1 (3%);Hypertension ,7(20%);Benign prostatic hyperplasia,2 (6%);;Coronary artery disease,1(3%); myocardial infarction,1(3%); depression ,2 (6%), anxiety,1(3%); Heart Murmur ,1(3%); Chronic obstructive pulmonary disease ,1(3%);Hypothyroidism,1(3%);osteoporosis,1(3%) and arrhythmia ,1(3%)].

Comment 2: Page 5, citation must be added- Recently, another mechanism has been proposed, namely the stimulation of angiogenesis in residual tumor deposits by persistent blood protein alterations.

Author's response:
Reference was included as suggested by the reviewer.

Comment 3: Page 6- the last phrase before Methods is unfinished-„ Of note, unlike previous plasma studies in which only 1 or 2 proteins were assessed on a given patient population, in this study, in all patients, plasma and wound levels of all 8 proteins were determined at each time point.

Author's response:
The sentence is updated as follows: "The purpose of the present study was to simultaneously determine the levels of 8 proteins in both plasma and wound fluid at multiple points during the first month after surgery in patients undergoing colorectal resection.

Comment 4: In this study" Appropriate description should be added either in brackets for the first use of the abbreviation, or in legend – e.g. page 6-7- „This was an IRB approved prospective study."; „Independent of this investigation, the authors have been investigating the use of subcutaneous wound drains to lower the incidence of superficial SSI's" etc.

Author's response:
The manuscript has been updated as per this reviewer's comment and suggestion.

Reviewer's code: 00183086

Comment 1: The Introduction section is too long. The number of paragraphs should be reduced and the aim of the study should be placed at the end of the section.

Author's response:

Please note that one of the other reviewers (Reviewer's code: 03478911) requested more information regarding the justification for the selection of the 8 proangiogenic proteins (mechanism of action for the proteins). Therefore, we actually added a sizable paragraph to the introduction to provide the requested information and background. In the rest of the introduction we have tried to be as concise as possible. We apologize for the length of this section. We were not sure how to handle the 2 opposing requests (yours to shorten and the other reviewers request for more background data).

Comment 2: Comparative evaluation of study's results should be referred in the Discussion.

Response:
We have done our best to comply with this request, however, the results of the comparisons between the pelvic and subcutaneous wound fluid results and the results of the combined wound fluid analysis have been left in the Results section. Also, in response to this comment and the third comment of reviewer 03478911 we have updated the discussions as well as the references. Updated sections in the manuscript are highlighted in yellow color.

Comment 3 : In the Methods specific details of fluid collection could be omitted.

Author's response:
The methods section was edited and modified so as to better explain the blood and wound fluid sample collection and processing procedures. The revised text follows:

Sample collection:

Blood samples and "wound fluid" (WFL) samples from the Jackson Pratt (JP) suction device were taken from patients on POD 1 and 3 as well as at the time of post discharge office appointments (provided the JP drain remained). Patients with high drain output were sent home with the JP drain(s) in place; this allowed for later postop samples to be obtained at the time of office visits. The initial office follow up appointment was usually between POD 7-13; however, some patients were seen between POD 14 and 21 as well. After hospital discharge it was not possible to collect the blood and wound fluid specimens on set postoperative days (for example, POD 7,14, or 21). Because late samples were obtained on different postoperative days the samples for each 7 day period were "bundled" together and considered as a single time point (POD 7-13,14-20, etc). Blood samples, collected in heparin coated vacutainers, were collected at the same time the WFL samples were obtained and then promptly processed via centrifugation at 450 G after which the plasma fraction were stored in labeled 500 ul cryo storage vials at -80ºC until the time of analysis. WFL samples, initially placed in sterile plastic containers, were processed promptly via centrifugation at 16,000 G for 10 min at 6^0C after which the supernatant was divided into 0.5 ml aliquots that were stored in cryo vials at -80^0C until the analysis was performed. Basic demographic, co-morbidity, operative, pathologic, and clinical data were obtained and recorded.

Comment 4: On the other hand epidemiological data are missing.

Author's response:

Most of demographic and clinical data of both benign and malignant and are given in the table 1 and further clinical and pathological data are described in the beginning of the results section. Additionally, as per above comment, specific population data regarding ethnicity has now been included;

The benign group comprises Caucasian (78%) , Hispanic (12%), African American (7%) and Asian (3%) and the malignant group consisted Caucasian (40%) , Hispanic (29%), African American (28%) and Asian (3%) patients.

Comment 5 : the Results information included in Tables should not be repeated in the text.

Author's response:

Some of the results interpreted in tables were presented in the manuscript to support the reader to follow the study's outcome parameters. Most of the additional data have been submitted as supplementary tables. Authors have taken great effort to critically discuss the

results in the manuscript. However authors have taken efforts to avoid giving repeat results in the manuscript.

Comment 6: the Discussion comparative analysis with other surveys is missing

Author's response:
As per this reviewer's comment and also in response to comment 9 of reviewer 03478911 and comment 1 of reviewer 03721686, the discussion and the conclusion of the manuscript were updated.

Reviewer's code: 03004570

Comment 1 : Abstract contains two paragraphs of Results; this should be a repetition and must be corrected. The last sentence just before Methods must be corrected grammatically.

Author's response:
Abstract has been corrected as per reviewers comment.

Comment 2 : When we look at the Results section in the manuscript, the number of APR is 10 (26%) in the cancer group, but it looks 9 (26%) in the Table 1. This must be corrected.

Author's response:
Abstract is corrected as per reviewers comment.

Comment 3: Similarly, the number of Hartmann takedown with resection for benign group is 2 (6.5%) in the article, but 2 (7%) in the Table 1.

Author's response:
Abstract is corrected as per reviewers comment.

Comment 4: The number of total colectomy/proctocolectomy is 3 (9.7) in the article, but 3 (10%) in the Table 1.

Author's response:
The manuscript has been corrected as per the reviewers comment

Comment 5: On the other hand, the figures are not visible clearly, it is not understood whether they are removed or not.

Author's response:

We had included four (4) figures on each page in the original original submission. This made review of the figures more confusing and less legible. We have now resubmitted all figures as single figures (1 per page) which we think makes it easier to look and understand the data.

Comment 6: I highly recommend those figures in the article.

Author's response:

All figures are submitted as single figures (including figure legends) together with revised manuscript.