

# World Journal of *Gastrointestinal Oncology*

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## Basic Study

# Plasma and wound fluid levels of eight proangiogenic proteins are elevated after colorectal resection

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## Abstract

### BACKGROUND

Colorectal resection is associated with 3-5 wk long elevations in the plasma levels of at least 11 proangiogenic proteins that may stimulate tumor angiogenesis post-surgery. The increases during the first week after surgery may be related to the acute inflammatory response; the cause(s) of the week 2-5 increases is unknown. The wounds are a possible source because of the important role that angiogenesis plays in the healing process. The main hypothesis of the study is that wound fluid levels of the proteins studied will be elevated well beyond plasma levels which, in turn, are elevated from preoperative baseline levels.

### AIM

To determine plasma and wound fluid levels of 8 proangiogenic proteins after colorectal resection for cancer and benign pathology.

### METHODS

Blood and wound fluid samples were taken simultaneously on postoperative (postop) day 1, 3, and later time points until wound drain removal in 35 colorectal cancer patients and 31 benign disease patients undergoing colorectal resection in whom closed wound drains had been placed in either the pelvis or the subcutaneous space of the abdominal incision. Postop plasma levels were compared to preop plasma and postop wound fluid levels (separate analyses for cancer and benign groups).

### RESULTS

authors have no conflicts of interest or financial ties to disclose.

**Data sharing statement:** No additional data available.

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Sixty-six colorectal disease patients were studied (35 cancer, 31 benign pathology). Most patients underwent minimally invasive surgery (open surgery in 11% of cancer and 6% of benign patients). The majority in the cancer group had rectal resections while in the benign group sigmoid or right colectomy predominated. Plasma levels of all 8 proteins were significantly elevated from baseline ( $P < 0.05$ ) at all post-operative time points in the cancer group and at 90% of time points (29/32) in the benign group. Wound levels of all 8 proteins were 3-106 times higher ( $P < 0.05$ ) than plasma levels at 87-90 percent of postop time points; of note, wound levels were more than 10 times higher at 47-50% of time points.

## CONCLUSION

Plasma protein levels were elevated for 3 weeks after surgery; wound fluid levels were much greater than corresponding blood levels. Healing wounds may be the source of the plasma increases.

**Key words:** Effects of surgery; Colorectal resection; Colorectal cancer; Plasma protein levels; Wound protein levels; Angiogenesis

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**Core tip:** Simultaneous postoperative (postop) measurement of plasma and wound fluid levels of 8 proangiogenic proteins for 3 wk after colorectal resection was carried out in 66 patients. Wound fluid protein levels were 3-106 times greater than postop plasma levels which, in turn, were significantly greater than preoperative plasma levels. Colorectal resection is associated with persistent systemic blood protein changes that might stimulate tumor angiogenesis and, thus, tumor growth in residual tumor deposits during the first month after surgery. It is hypothesized that the healing wounds are a major source of the added protein in the blood stream.

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## INTRODUCTION

In a small percentage of cancer patients surgical excision of the primary tumor is associated with the rapid development of tumor recurrence(s) or the growth of existing metastases<sup>[1-7]</sup>. There is a sizable experimental literature regarding surgery's impact on tumors in the early postoperative (postop) period and numerous hypotheses proposed to account for the phenomenon of accelerated tumor growth in this time period<sup>[8,9]</sup>. Surgery-related immunosuppression and the elimination (*via* resection) of a metastasis suppressing protein generated by the primary tumor are two examples<sup>[10,11]</sup>. Recently, another mechanism has been proposed, namely the stimulation of angiogenesis in residual tumor deposits by persistent blood protein alterations<sup>[12]</sup>.

Over the last decade it has been shown that minimally invasive colorectal resection (MICR) in colorectal cancer (CRC) patients is associated with persistent proangiogenic plasma protein changes that persist for 3 to 5 wk after surgery<sup>[12,13]</sup>. Prior investigators had noted only short lived plasma protein alterations that were attributed to the acute inflammatory and endocrine responses that follow major surgical trauma; these changes lasted hours or, at most, 3 days after MICR or major surgical trauma<sup>[14,15]</sup>. As regards the newly discovered long duration changes, thus far, a total of 11 proteins have been shown to be elevated for much of or all of the first postop month<sup>[12,13,16-21]</sup>. Interestingly, all of these proteins play a role in angiogenesis. It has also been shown that plasma from the second and third weeks after MICR stimulates endothelial cell (EC) proliferation, migration, and invasion in *in vitro* cultures; these results lend support to the hypothesis that the proangiogenic blood protein changes after surgery may promote tumor growth by stimulating tumor angiogenesis<sup>[22]</sup>.



Of note, postop plasma from CRC patients who underwent open resection has been shown to have similar proangiogenic effects on *in vitro* EC cultures, thus both open and minimally invasive methods (MIS) are similar in this regard<sup>[21]</sup>. Finally, similar blood compositional changes and *in vitro* EC culture results have been noted in patients undergoing MICR for benign conditions such as diverticulitis or adenoma, thus, the indication for surgery does not appear to influence or be the source of these surgery related alterations<sup>[13]</sup>.

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 may originate in the healing wounds and then find its way into the circulation. Of note, previous investigators have noted elevated vascular endothelial growth factor (VEGF) levels in wound fluid (WFL) taken from mastectomy and other surgical patients<sup>[23-25]</sup>. The purpose of this study was to assess plasma and wound levels of 8 proteins that have proangiogenic effects in patients undergoing colorectal resection. The chosen proteins, all previously shown to have persistently elevated plasma levels after colorectal resection, are: VEGF, placental growth factor (PLGF), angiopoietin-2 (ANG-2), monocyte chemotactic protein-1 (MCP-1), chitinase 3 like protein-1 (CHI3L1), osteopontin (OPN), matrix metalloproteinase-2 (MMP2) and MMP3. Brief background information regarding the proangiogenic effects of these proteins follows.

VEGF, critical to angiogenesis, stimulates multiple early steps in neovascularization including EC proliferation, microtubule formation, invasion and migration. 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<sup>[26,27]</sup>. PLGF primarily regulates the angiogenic switch under pathologic conditions<sup>[28]</sup>, however, as regards non-pathologic neovascularization, by increasing the amount of VEGF available to bind to the key receptor VEGFR2 it maximizes VEGF's proangiogenic 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 C-C chemokine receptor 2 on the surface of EC's<sup>[29,30]</sup>. Human CHI3L1, also known as YKL-40, induces IL-8 and MCP-1 secretion through the extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase signal pathways<sup>[31]</sup>; these chemokines support macrophage recruitment and tumor angiogenesis. OPN is an integrin binding phosphorylated acidic glycoprotein that mediates cell-matrix and cell-cell communication<sup>[32,33]</sup>. OPN has been shown to enhance tumor progression and angiogenesis *via* the PI3K/AKT and ERK mediated pathways in association with VEGF<sup>[34,35]</sup>. MMP-2 is an extracellular matrix remodeling enzyme<sup>[36,37]</sup> that degrades type IV collagen<sup>[38]</sup> in the basement membrane which enables EC migration and tumor cell invasion<sup>[39,40]</sup>; it has also been shown to enhance VEGF release<sup>[41]</sup>. MMP-3 has been shown to support the process of epithelial-mesenchymal transition (EMT) during which epithelial cells loses adhesion, become invasive, and transition to the mesenchyme which is critical in wound healing, angiogenesis, and the initiation of cancer metastasis<sup>[42]</sup>.

As stated above, the overriding goal of this study was to establish that the wounds are the likely source of the added protein and that plasma levels are persistently increased after surgery. Toward this end, simultaneous postop plasma and wound specimens were collected at multiple time points. Populations of cancer and benign pathology colorectal resection patients were assessed so as to determine if the surgical indication influenced the body's response. If the results support the hypothesis then it will have been can demonstrate that wound healing has potentially important, heretofore unknown, systemic manifestations. This information would provide insight into wound healing and may compel doctors to look for anti-cancer agents that could be given during the early postop period in an effort to negate these potentially tumor stimulatory conditions.

## MATERIALS AND METHODS

### Methods

This was an Institutional Review Board (IRB) approved prospective study. All colorectal patients undergoing elective MICR, regardless of the indication, who had consented preoperatively to participate in the Mount Sinai West Colorectal service's IRB approved general tissue and data banking protocol, in whom a Jackson Pratt (JP)

drain was placed in the abdominal cavity, pelvis, or subcutaneous space of the main extraction incision were eligible for the study (Institutional Review Board of the Mount Sinai School of Medicine, New York; IRB reference NO: GCO1: 16-2619 and Institutional Review Board of the Mount Sinai School of Medicine, New York; IRB reference NO: GCO1: 16-1863). Independent of this investigation, the authors have been investigating the use of subcutaneous wound drains to lower the incidence of superficial Surgical Site Infections (SSI's). Patients with JP drains were asked to consider participating in the study on postop day (POD) 1 by a Study Registered Nurse and interested patients were consented after they had been given a full explanation of the study and all questions had been answered. Prior consent to the tissue banking program was necessary such that a preoperative (preop) blood sample would be available to determine the baseline, pre-resection plasma protein levels. The tissue banking protocol allows the research dedicated blood samples to be used in IRB approved studies.

Plasma and WFL samples from 35 patents diagnosed with colorectal adenocarcinoma (rectal 21; colon 14; 21 male /14 female, mean age  $63.6 \pm 11.3$  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,  $57.3 \pm 14.1$  years) 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.

### Sample collection

Blood samples and "WFL" samples from the JP suction device were simultaneously 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; in this subgroup later postop samples were 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 WFL specimens on set postop days (for example, POD 7 or 14). Because late samples were obtained on different postop 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 was stored in labeled 500  $\mu$ L cryo storage vials at - 80 °C until the time of analysis. WFL samples, initially placed in sterile plastic containers, were processed promptly *via* centrifugation at 16000 g for 10 min at 6 °C after which the supernatant was divided into 0.5 mL aliquots that were stored in cryo vials at - 80 °C until the analysis was performed. Basic demographic, co-morbidity, operative, pathologic, and clinical data were obtained and recorded.

### Exclusion criteria

Patients undergoing emergent surgery were not eligible. Also, HIV positive patients and those on immunosuppressive medications were not eligible.

### Wound fluid and plasma analyses

WFL and plasma VEGF, PLGF, ANG2, MCP-1, CHI3L1, OPN, MMP2 and MMP3 levels were determined in duplicate *via* highly specific and sensitive commercially available Enzyme-Linked Immunosorbent Assays (ELISA) kits (R and D Systems, Quantikine kit numbers DVE00, DPG00, DANG20, DCP00, DC3L10, DOST00, MMP200 DMP300). The ELISA's used in the study were tested for precision (intra-assay precision and Inter-assay-precision), recovery, sensitivity and linearity by the vendor. Before analysis WFL 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 storing the plasma in 500  $\mu$ L aliquots and by performing several protein ELISA's on the same day such that a given vial of plasma or WFL was fully utilized once thawed. The ELISAs were read using an automated microplate reader (Synergy2; Bio-Tek Instruments, Inc., Winooski, VT, United States). Standard curves were generated on four parameter logistic curve fit and protein concentrations are reported as pg/mL or

ng/mL.

### Statistics

As mentioned, the wound and blood samples after the first week were bundled into 7 days time periods and considered as single time points. Because some JP drains were removed prior to hospital discharge or at the first postop office visit, the “n” for the late bundled time points is notably smaller than for the POD 1 and 3 time points. The cancer and benign indication patient subgroups were assessed separately. Also, the intraperitoneal and subcutaneous WFL samples were considered both separately and together. For the preop *vs* postop plasma protein level comparisons, the data is reported as median and 95% confidence intervals and the Wilcoxon signed-rank match paired test was used. In regards to the postop plasma *vs* WFL comparisons, the results are reported as the median and 95% confidence intervals and the Mann and Whitney test was used. Plasma and WFL protein levels in figures are expressed as median and 75% quartile range. A *P* value < 0.05 was considered statistically significant. All data analysis was performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, United States). As the sample size varies for the POD 7-13 and PODS 14-20 time points, a separate preop results bar is included for each time point in the figures.

## RESULTS

A total of 35 patients with colorectal adenocarcinoma (rectal 21, colon 14) and 31 patients with benign colorectal conditions in whom a JP drain was used in either the intraperitoneal or subcutaneous location were enrolled in this study. **Table 1** provides the demographic and operative data as well as the length of stay for the cancer and benign patient groups. As regards surgical methods, most patients underwent laparoscopic-assisted resections (cancer, 63%; benign, 68%) while the rest underwent either a hand-assisted procedure (cancer, 26%; benign, 26%) or an open resection (cancer, 11%; benign, 6%). There were 2 conversions in the cancer group that underwent MIS (6.5%) and 3 in the benign patient group (9.7%). There were no deaths. The type of resections performed in the cancer group were: Low anterior resection/anterior resection, 12 patients, 34%; abdominoperineal resection, 9 patients, 26%; sigmoid/rectosigmoidectomy, 5, 14%; right colectomy, 4, 11%; transverse colectomy, 3, 9%; and total proctocolectomy with ileal pouch, 2, 6%. The final cancer stage breakdown was Stage 0, 2 rectal cancer patients, 6% (T-0, N-0, pathologic complete response after neoadjuvant RT/chemotherapy), Stage 1, 10, 29%; Stage 2, 11, 31%; Stage 3, 12, 34%, and Stage 4, 0 (**Table 1**).

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 operations performed were: sigmoid/rectosigmoid resection, 15 (48%); right colectomy, 6 (19%); lower anterior resection, 4 (13%); total colectomy/proctocolectomy, 3 (10%); Hartmann takedown with resection, 2 (7%); and transverse colectomy, 1 (**Table 1**). As regards the cancer group, in 23 patients the JP drain was placed in the pelvis whereas in 12 it was placed in the subcutaneous space beneath the main incision; 3 patients had both types of drains. In the benign pathology group the JP drains were placed in the pelvis in 8 patients and in the subcutaneous position in 23; 3 patients had both pelvic and subcutaneous drains. The greater number of pelvic JP drains in the cancer group reflects the fact that over 50 percent of the cancer cases were rectal cancer resections.

### Plasma protein levels

The median plasma level at each postop time point was compared to the median preop level for each of the proteins. The total number of preop *vs* postop protein comparisons for each group (cancer and benign) was 32 (8 proteins × 4 time points). As regards the cancer group, significant elevations from baseline were noted postop at all of the time points while for the benign group significant elevations were noted at 29 of the 32 time points (90%). The 3 non-significant elevations concerned the POD 14-20 time point where the “n” for the benign group was 4 which made statistical analysis difficult. The extent of the increases over baseline varied from protein to protein and from time point to time point (Figures 1-8 and supplementary Tables 1 and 2). The range of the percent change from baseline values for each of the proteins over the 4 postop time points for the cancer group was comparable with the same data for the benign group (**Table 2**).

### Wound fluid results

Study patients had either a pelvic or a subcutaneous JP drain except for 3 in each



**Table 1** Demographic and clinical characteristics of the plasma and wound fluid study population (benign and cancer groups), *n* (%)

	Benign ( <i>n</i> = 31)	Cancer ( <i>n</i> = 35)
Age, yr (mean ± SD)	57.3± 14.1	63.6± 11.3
Sex ( <i>n</i> ):		
Male	11 (35.0)	21 (60)
Female	20 (65.0)	14 (40)
Incision length, cm (mean ± SD)	8.3 ± 5.3	9.8 ± 6.0
Operative time, min (mean ± SD)	339.1 ± 116.1	430.1 ± 121.0
Length of stay, d (mean ± SD)	5.6 ± 2.3	7.7 ± 6.6
Type of resection:		
Right	6 (19.0)	4 (11.0)
Transverse	1 (3.0)	3 (9.0)
Sigmoid/rectosigmoid	15 (48.0)	5 (14.0)
LAR/AR	4 (13.0)	12 (34.0)
APR	0 (0.0)	9 (26.0)
Hartman takedown with resection	2 (7.0)	0(0.0)
Total colectomy/proctocolectomy	3 (10.0)	2 (6.0)
Surgical method:		
Laparoscopic-assisted	21 (68.0)	22 (63.0)
Hand-assisted/hybrid Laparoscopic	8 (26.0)	9 (26.0)
Open	2 (6)	4 (11)

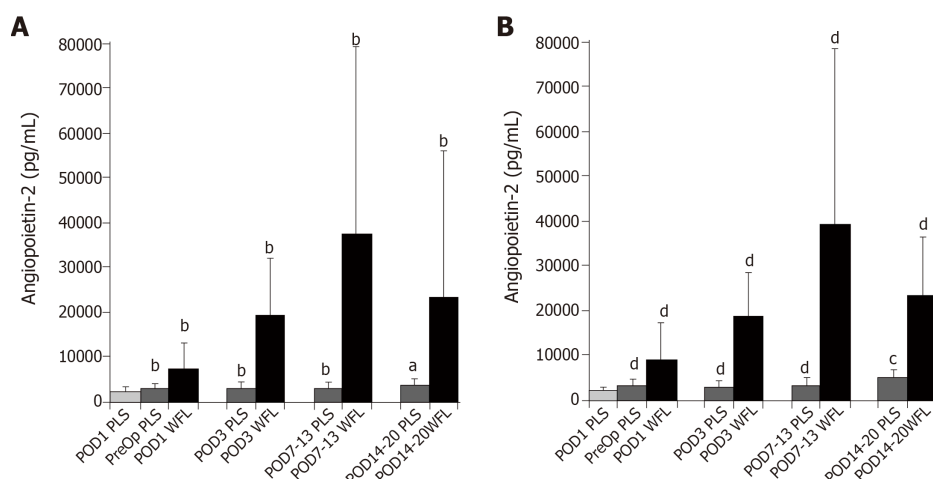
LAR: Lower anterior resection; AR: Anterior resection APR: Abdominoperineal resection.

group that had two drains. Because, many drains were removed prior to hospital discharge and post discharge samples were obtained only once in most patients between POD 7-21, the “*n*”s of the pelvic and subcutaneous fluid subgroups were low for the POD 7-13 (cancer pelvic, 15; cancer subcutaneous, 7; benign pelvic, 6; benign subcutaneous, 10) and the POD 14-21 time points (cancer pelvic, 7; cancer subcutaneous, 2; benign pelvic, 2; benign subcutaneous, 2). The results of the protein assays performed on the WFL samples were first considered as to their origin (the pelvis or subcutaneous space) and the results compared; for all 8 proteins, at the great majority of time points, there was no statistical difference in protein levels between the pelvic and subcutaneous WFL (Supplementary Table 3). Therefore, in order to simplify the analysis and to increase the WFL “*n*” for the later time points, the pelvic and subcutaneous subgroups were combined to form a single larger WFL group the levels of which were compared to the plasma protein concentrations at each time point; the results of that comparison follow.

There were a total of 32 postop data points (8 proteins × 4 postop time points) to consider for both the cancer and benign groups. The median WFL levels at all time points were significantly higher than the corresponding median plasma level for all 8 proteins for both groups. The highest WFL levels were noted at the POD 7-13 or 14-20 time points for 6 of the 8 proteins in both the cancer and benign pathology groups. The magnitude of the difference between wound and plasma levels varied considerably from protein to protein and between the postop time points. What follows is a list of fold changes (multiples of the mean plasma level at each time point) followed by the percent of postop time points (*n* = 32) whose mean WFL value was equal to or greater than the stated fold change for the cancer groups data: ≥ 2 × mean plasma level, 97%; ≥ 3 ×, 91%; ≥ 5 ×, 69%; ≥ 10 ×, 47%; ≥ 30 ×, 29% and ≥ 40 ×, 22%. The comparable results for the benign group are as follows: ≥ 2 ×, 90%; ≥ 3 ×, 87%; ≥ 5 ×, 68%; ≥ 10 ×, 52%; ≥ 30 ×, 29%; and ≥ 40 ×, 26% (Table 3).

When the results for the individual proteins are considered the proteins can be divided into 3 categories. The greatest WFL elevations (*vs* plasma levels) were noted for VEGF, PLGF, and MCP-1 (mean wound value ≥ 30 × plasma levels at 9/12 cancer and 8/12 benign time points). The lowest elevations were noted for MMP-2, MMP-3, Ang-2, and CHI3L1 (cancer and benign groups, < 10 fold change *vs* plasma, 15/16 time points). The OPN results fall between the high and low groups (cancer and benign groups, 10-24 fold elevations at 75% of time points) (Table 3).

### Pelvic vs subcutaneous wound fluid analysis



**Figure 1 Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative angiopoietin 2 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients.** A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 26$ ),  $^bP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^bP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^bP < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ),  $^aP < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^bP < 0.01$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^bP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^bP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 6$ ) vs Postoperative day 14-20 (wound fluid,  $n = 4$ ),  $^bP < 0.01$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as  $^aP < 0.05$ ,  $^bP < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 17$ ),  $^dP < 0.01$ ; postoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^cP < 0.05$ . Plasma vs wound fluid; Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^dP < 0.01$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^dP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 17$ ) vs Postoperative day 7-13 (wound fluid,  $n = 20$ ),  $^dP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^dP < 0.01$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as  $^cP < 0.05$ ,  $^dP < 0.01$ . Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid.

The pelvic and subcutaneous WFL results at each time point were compared for both the cancer and benign pathology groups. Of note, as regards the POD 14-20 data, the  $n$  for subcutaneous WFL was only 2 for the cancer and benign groups and was also 2 for the benign pelvic subgroup; therefore, valid statistical analysis was not possible at that time point. As regards the 24 evaluable data points (8 proteins  $\times$  3 postop time points) no statistically significant differences were found between the subcutaneous and the pelvic WFL protein levels at 19 time points (79%) in the cancer group and at 20 time points (83%) in the benign group (Supplementary Table 3). In the cancer group subcutaneous protein levels were significantly greater than the pelvic results at 4 time points whereas in the benign disease group the pelvic fluid levels were greater than the subcutaneous results at 3 time points. Based on these results, for the comparison of the plasma and WFL protein levels at each time point the subcutaneous and pelvic WFL results were pooled.

Of note, for both the cancer and benign pathology groups, when the pelvic and subcutaneous WFL results were separately compared to the plasma protein results at each of the evaluable time points (POD 1, 3, 7-13), the WFL levels were significantly higher than the corresponding plasma levels at 92 % of time points (Supplementary Tables 4 and 5). Regardless of the source, mean WFL levels were greater than the corresponding mean plasma levels at all time points for all 8 proteins for both groups.

## DISCUSSION

This study accomplishes 2 goals. First, it confirms that plasma levels of these 8 proangiogenic proteins are elevated from baseline for at least 3 wk after colorectal resection. Secondly, it demonstrates that the levels of these proteins in WFL samples taken simultaneously from the pelvic and abdominal wall wounds of CRC patients are significantly higher than the corresponding plasma levels at all time points. Notably, wound levels were 3 times higher than plasma levels at 87%-91% of time points and 10 times higher at 47%-52% of time points. Similar results were noted for both the cancer and benign pathology patient subgroups which suggests that it is the tissue trauma and/or subsequent healing rather than the indication for surgery that is the source of these elevations. Although the acute inflammatory response may contribute to the protein elevations during the first 4-7 d, in the authors' opinion, the most likely source of the added protein in the bloodstream for most of the postop

**Table 2** Range of mean % of increase of plasma proteins during post-operative period from pre-operative mean value

	Benign	Malignant
ANG-2	49-101	38-70
VEGF	64-169	104-202
MMP-2	19-39	16-37
PLGF	36-55	29-53
MMP-3	60-162	43-143
OPN	85-187	108-146
MCP-1	28-344	44-63
CHI3L1	45-946	196-1006

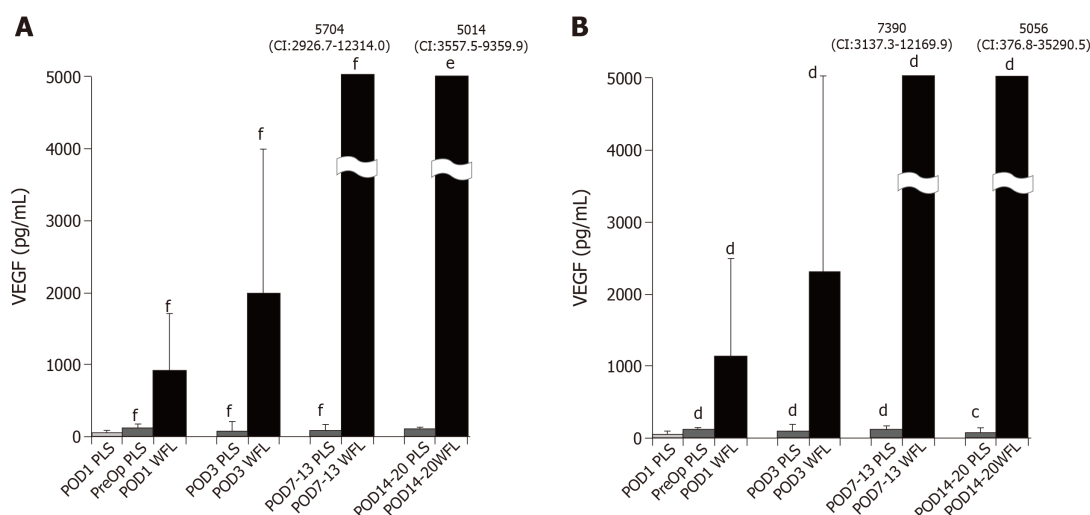
ANG2: Angiopoetin-2; VEGF: Vascular endothelial growth factor; MMP-2: Matrix metalloproteinase-2; PLGF: Placental growth factor; MMP3: Matrix metalloproteinase-3; OPN: Osteopontin; MCP-1: Monocyte chemotactic protein-1; CHI3L1: Chitinase 3 like protein-1.

period are the healing surgical wounds. Since angiogenesis is a critical component of wound healing it is not surprising that the levels of these proteins would be increased in the wound where considerable neovascularization is occurring. Numerous previous investigators have documented the proangiogenic properties of WFL<sup>[43-47]</sup>. It is speculated that the proteins follow the concentration gradient from the wounds (3 to 106 × higher) to the bloodstream.

Also, the persistent and concomitant elevation of both wound and plasma protein levels for 2 wk or more suggests an association between the two sites. Further, blood levels have been shown to be increased for a month or longer<sup>[48-51]</sup>, which is the time frame within which the lion's share of wound healing occurs. If the added proteins were generated elsewhere in the body after surgery and then were transported, *via* the bloodstream, to the wound, they would be doing so against the concentration gradient which seems highly unlikely. Also, since the primary tumor has been resected it cannot be the source of the protein increases noted post-surgery.

As mentioned, it has previously been shown that postop plasma stimulates proangiogenic EC behavior *in vitro*<sup>[12,13,22]</sup>. 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. Support for this hypothesis can be found in a clinical study that demonstrated that colorectal resection was associated with an increase in the size and intra-tumoral and peri-tumoral vascular density of pre-existing liver metastases 6-12 wk after resection of the primary colorectal tumor<sup>[52]</sup>.

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<sup>[28]</sup>. 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<sup>[53,54]</sup>. 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<sup>[55]</sup>. Also, elevated blood levels of Chi3l1 have been noted in a large variety of cancer patients<sup>[56-61]</sup> and are associated with a poor prognosis in many<sup>[56,59,60]</sup>. OPN has been shown in some studies to enhance tumor progression and angiogenesis in association with VEGF<sup>[34,35]</sup>. Overexpression of OPN has been noted in breast, lung, liver and CRC patients and is associated with worse prognosis and early recurrence in patients with hepatocellular cancer<sup>[62]</sup>. 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<sup>[63]</sup>, breast<sup>[64]</sup>, prostate<sup>[65]</sup> and CRC<sup>[66]</sup>. As mentioned, MMP-3 has been shown to play a role in the process of 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



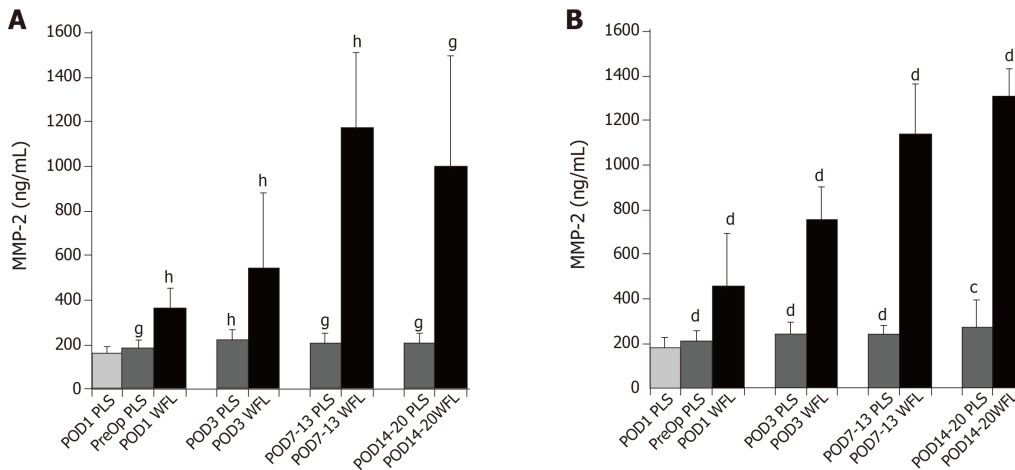
**Figure 2** Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative vascular endothelial growth factor levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs. Postoperative day 1 ( $n = 26$ ),  $^{\dagger}P < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^{\dagger}P < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^{\dagger}P < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ), ns. Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^{\dagger}P < 0.01$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^{\dagger}P < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^{\dagger}P < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 6$ ) vs Postoperative day 14-20 (wound fluid,  $n = 4$ ),  $^{\circ}P < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as  $^{\circ}P < 0.05$ ,  $^{\dagger}P < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^{\dagger}P < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^{\dagger}P < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 17$ ),  $^{\dagger}P < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^{\circ}P < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^{\dagger}P < 0.01$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^{\dagger}P < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 17$ ) vs Postoperative day 7-13 (wound fluid,  $n = 20$ ),  $^{\dagger}P < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^{\dagger}P < 0.01$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. (Statistical significance is expressed as  $^{\circ}P < 0.05$ ,  $^{\dagger}P < 0.01$  and  $^{\circ}P < 0.05$ ). Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; VEGF: Vascular endothelial growth factor.

and/or metastatic transformation of cancers including breast cancer and hepatocellular carcinoma<sup>[67-74]</sup> and is overexpressed in some gastric and liver cancers.

Unfortunately, this study was quite small and, thus, it is not reasonable to draw any firm conclusions. It must be acknowledged, therefore, that there is no definitive evidence directly linking these plasma compositional changes to early recurrence or accelerated tumor growth after surgery. As mentioned, there is substantial clinical data supporting the concept that major surgery is associated with rapid cancer growth<sup>[3-5,75]</sup>. In 4 studies regarding patients with synchronous colon and liver lesions, rapid growth (within 2-3 mo) of the pre-existing liver metastases was noted after resection of the primary CRC as measured by serial computed tomography<sup>[4,5]</sup> and positron emission tomography scans<sup>[6,7]</sup>. Clearly, further studies are needed to determine the clinical ramifications of the persistent proangiogenic plasma compositional changes that have been noted.

If a clear link between surgery and accelerated tumor growth early postoperatively can be established then it would be logical to look for anti-cancer treatments that could safely be used during the first month after surgery which may be a particularly dangerous period for cancer patients with residual lesions. This is a time period that presently, with few exceptions, is not utilized for anti-cancer treatment; standard adjuvant chemotherapy is usually started 4 to 8 wk after surgery. Immunomodulating agents, tumor vaccines, anti-oxidants, and perhaps select monoclonal antibodies may be candidates for use in the early postop period. It is critical that any anti-cancer agent used in this time period not interfere with the healing process since that would likely lead to increased rates of anastomotic leaks and wound complications. As an example, anti-angiogenic therapy with agents such as bevacizumab is not a viable option because it would likely strongly interfere with wound healing.

This study assessed WFL from the pelvis and subcutaneous space within the main abdominal incision. Since prior evaluations of WFL's from different sources had not been performed, it was not known if the makeup of the 2 types of WFL would be similar. The results suggest that there are no significant differences in the levels of the 8 proteins in the 2 types of fluid at the great majority of time points, however, the study is underpowered in this regard and was not designed to answer this question. The dissimilar numbers of pelvic and subcutaneous samples in each group also makes comparison difficult. To definitively determine if the WFL source impacts WFL



**Figure 3** Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative matrix metalloproteinase-2 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 26$ ),  $^{\#}P < 0.05$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^{\text{h}}P < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^{\#}P < 0.05$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ),  $^{\#}P < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^{\text{h}}P < 0.01$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^{\text{h}}P < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^{\text{h}}P < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 6$ ) vs Postoperative day 14-20 (wound fluid,  $n = 4$ ),  $^{\#}P < 0.05$ ; Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as  $^{\#}P < 0.05$ ,  $^{\text{h}}P < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^{\text{d}}P < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^{\text{d}}P < 0.01$ ; Preoperative vs. Postoperative day 7-13 ( $n = 17$ ),  $^{\text{d}}P < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^{\text{c}}P < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^{\text{d}}P < 0.01$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^{\text{d}}P < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 17$ ) vs Postoperative day 7-13 (wound fluid,  $n = 20$ ),  $^{\text{d}}P < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^{\text{d}}P < 0.01$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as  $^{\text{c}}P < 0.05$ ,  $^{\text{d}}P < 0.01$ . Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; MMP-2: Matrix metalloproteinase-2.

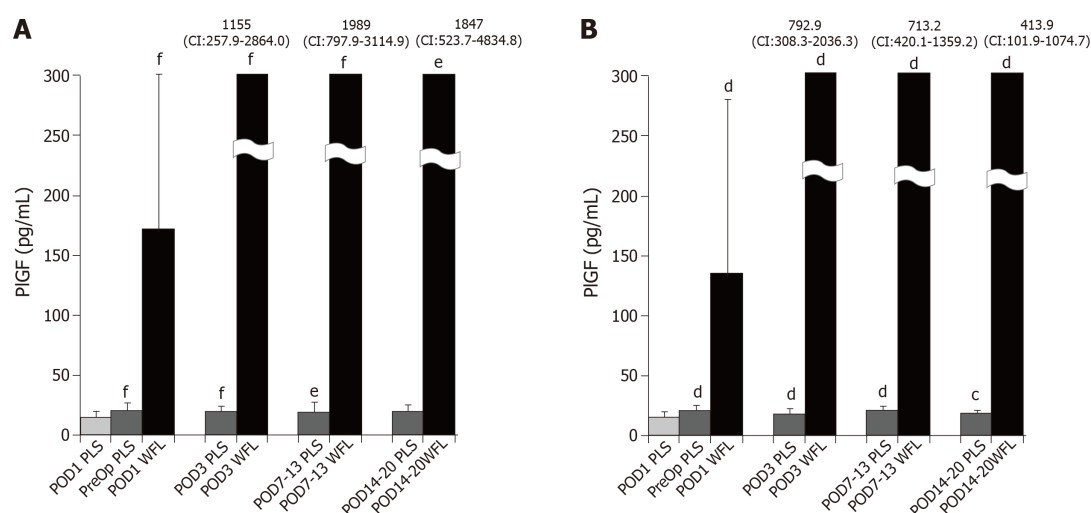
protein levels a substantially larger study would be needed. Despite these limitations it can be confidently stated that wound levels of these proteins are notably higher than corresponding plasma levels.

As mentioned, when the plasma protein results were considered alone, postop plasma protein levels were shown to be significantly elevated from their preop baseline levels for the 8 proteins at all time points in the cancer group and at 93% of time points in the benign pathology group. Of note, this is the first study to determine the late postop plasma levels of these 8 proteins simultaneously in a given population of patients. As mentioned, prior studies that looked at 1-3 proteins per study noted similar persistent late elevations in the plasma levels of the 8 proteins assessed in this study plus an additional 3 proangiogenic proteins (IL-8, progranulin, and keratinocyte growth factor) after MICK<sup>[12,16-21]</sup>. Of note, the current study did not assess plasma after the 3<sup>rd</sup> week after surgery since all wound drains had been removed by that point, however, prior studies have shown that the plasma elevations persist for 4-5 wk for some proteins.

Clear drawbacks to this study are the small “n”s for the post discharge time points (especially POD13-20) and the fact that the postop day on which the late sample(s) were obtained varied widely. As mentioned, once patients were discharged it was not possible to coordinate office visits so as to get study samples on a specific postop day. Therefore, late samples, by necessity, were “bundled”. Also, because many of the drains had been removed in hospital, it was not possible to obtain late specimens from a good proportion of the patients. In addition, as mentioned, there were dissimilar numbers of pelvic and subcutaneous drains used in each group because the drains were not uniformly utilized (placed at the discretion of the surgeon) and because of the high proportion of rectal resections in the cancer group (more pelvic drains). A larger study would increase the “n”s but would likely still require bundling.

A comment must be made regarding the inclusion of open surgery patients in this study. Short lived increases in the extent and degree of the acute inflammatory response as judged by blood levels have been demonstrated in past studies for some cytokines in open (*vs* MIS) colorectal resection patients<sup>[76]</sup>. Further, no late postop plasma protein data was available for open CRC patients prior to this study. However, based on fact that the open colorectal resection *in vitro* EC culture results for the second and third postop weeks were similar to the MIS patients results, as mentioned earlier, the authors speculated that the proangiogenic cytokine response in





**Figure 4** Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative placental growth factor levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 26$ ),  $^iP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^iP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^eP < 0.05$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ), ns. Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^iP < 0.01$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^iP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^iP < 0.01$ ; POD14-20 (plasma,  $n = 6$ ) vs POD14-20 (wound fluid,  $n = 4$ ),  $^eP < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as  $^eP < 0.05$ ,  $^iP < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 17$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^eP < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^dP < 0.01$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^dP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 17$ ) vs Postoperative day 7-13 (wound fluid,  $n = 20$ ),  $^dP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^dP < 0.01$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as  $^eP < 0.05$ ,  $^dP < 0.01$ ). Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; PLGF: Placental growth factor.

the wounds and plasma would be similar. Of note, the small number of open patients in the present study (cancer, 4; benign, 2) precludes meaningful comparison between the open and MIS patients at most time points, however, clearly, wound levels are substantially increased for both methods. Also, when the wound and plasma results of the MIS patients (laparoscopic-assisted and hand-assisted laparoscopic) are assessed alone, significant differences persist for both the wound *vs* plasma and the preop *vs* postop plasma at all time points (data not shown).

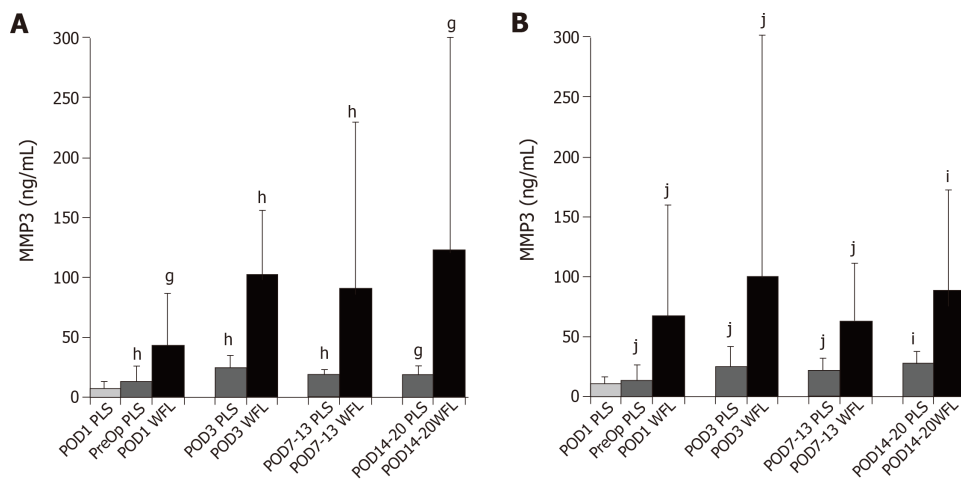
The fact that these 8 proteins, shown to be increased late after surgery, all influence neovascularization suggests that there is considerable angiogenic activity in the wound late in the first postop month well after initial wound healing has occurred. Given these results, one would think that the other cytokines that play prominent roles in the wound healing process would also be persistently elevated after surgery. Interestingly, similar studies that measured postop plasma levels of FGF, TGF, HGF and EGF<sup>[77,78]</sup>, however, have not demonstrated late elevations. However, further studies of other growth factors in this time window are warranted.

In summary, this study has demonstrated that plasma levels of the 8 proangiogenic proteins in question are significantly elevated over preop levels for 3 wk 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.

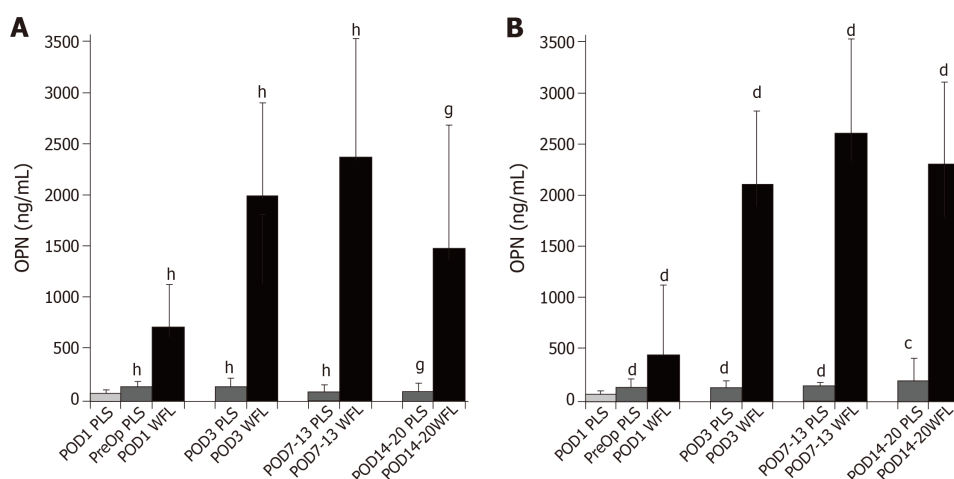
**Table 3** The fall increased in wound fluid per corresponding mean plasma levels

Analyzed protein	The fall increased in wound fluid per corresponding mean plasma levels								Increased range	
	benign	cancer	benign	cancer	benign	cancer	benign	cancer	benign	cancer
	POD1	POD1	POD3	POD3	POD7-13	POD7-13	POD14-20	POD14-20		
ANG-2	3.3	3.0	5.5	6.0	12.8	12.0	8.0	3.5	3-13	3-12
VEGF	15.5	19.0	22.9	36.0	59.0	61.0	60.5	106.0	16-61	19-106
MMP-2	0.96	2.0	1.8	2.0	4.5	4.0	4.5	3.3	1-5	2-4
PLGF	40.8	16.0	88.2	61.0	100.3	46.0	106.1	27.2	41-106	16-61
MMP-3	2.6	5.2	4.6	5.0	6.5	3.2	8.8	5.4	3-9	3.2-5.3
OPN	4.9	3.1	14.0	12.0	24.2	14.0	15.5	9.3	5-23	3.1-14
MCP-1	39.7	40.0	74.7	37.0	85.2	59.0	16.0	57.1	16-86	37-59
CHI3L1	0.6	0.7	6.9	3.0	5.7	9.0	3.6	5.0	1-7	3-9

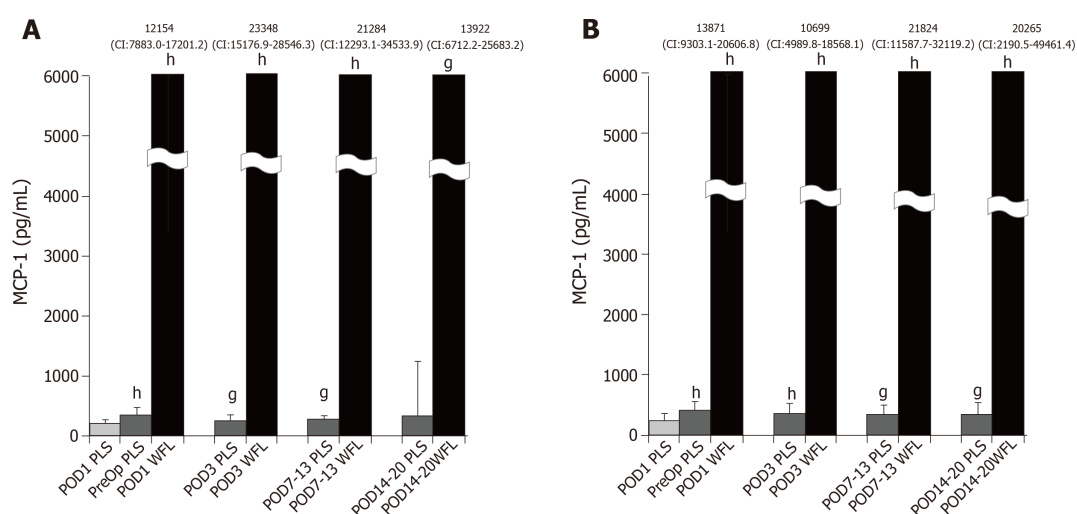
ANG2: Angiopoetin-2; VEGF: Vascular endothelial growth factor; MMP2: Matrix metalloproteinase-2; PLGF: Placental growth factor; MMP3: Matrix metalloproteinase-3; OPN: Osteopontin; MCP-1: Monocyte chemotactic protein-1; CHI3L1: Chitinase 3 like protein-1; POD: Post-operative day.



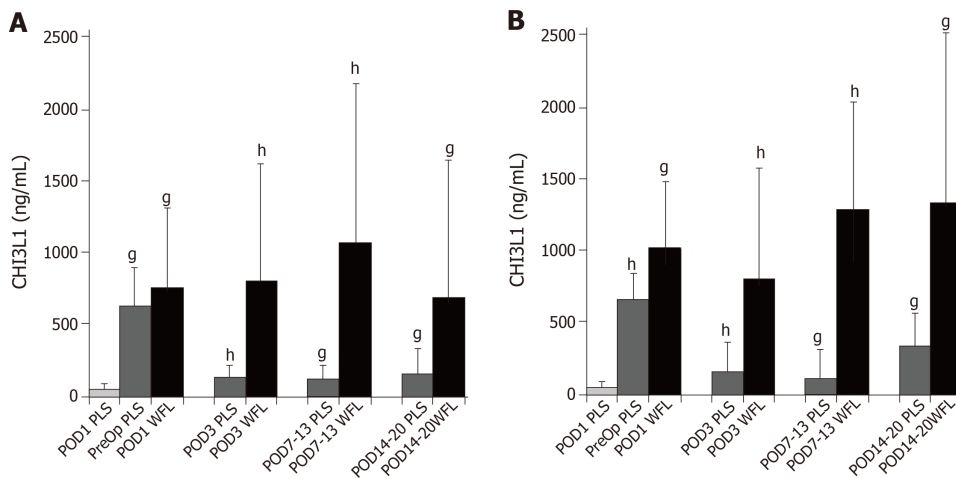
**Figure 5** Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative matrix metalloproteinase-3 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 26$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ),  $^sP < 0.05$ ; Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^sP < 0.05$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^hP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^hP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 6$ ) vs Postoperative day 14-20 (wound fluid,  $n = 4$ ),  $^sP < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. (Statistical significance is expressed as  $^sP < 0.05$ ,  $^hP < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^iP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^iP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 17$ ),  $^iP < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^iP < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^iP < 0.01$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^iP < 0.01$ ; POD7-13 (plasma,  $n = 17$ ) vs POD7-13 (wound fluid,  $n = 20$ ),  $^iP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^iP < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as  $^iP < 0.05$ ,  $^iP < 0.01$ . Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; MMP3: Matrix metalloproteinase-3.



**Figure 6** Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative osteopontin levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 26$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ),  $^sP < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^hP < 0.01$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^hP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^hP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 6$ ) vs Postoperative day 14-20 (wound fluid,  $n = 4$ ),  $^sP < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as  $^hP < 0.05$ ,  $^sP < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 17$ ),  $^dP < 0.01$ ; Preoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^cP < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^dP < 0.01$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^dP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 17$ ) vs Postoperative day 7-13 (wound fluid,  $n = 20$ ),  $^dP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^dP < 0.01$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as  $^cP < 0.05$ ,  $^dP < 0.01$ . Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; OPN: Osteopontin.



**Figure 7** Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative monocyte chemotactic protein-1 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 26$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^sP < 0.05$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^sP < 0.05$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ), ns. Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^hP < 0.01$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^hP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^hP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 6$ ) vs Postoperative day 14-20 (wound fluid,  $n = 4$ ),  $^sP < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as  $^sP < 0.05$ ,  $^hP < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^hP < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 17$ ),  $^sP < 0.05$ ; Preoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^sP < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^hP < 0.01$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^hP < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 17$ ) vs Postoperative day 7-13 (wound fluid,  $n = 20$ ),  $^hP < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^hP < 0.01$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as  $^sP < 0.05$ ,  $^hP < 0.01$ . Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; MCP-1: Monocyte chemotactic protein-1.



**Figure 8** Enzyme-Linked Immuno-Sorbent Assays determined preoperative and postoperative chitinase 3 like protein-1 levels of plasma and wound fluid levels in colorectal surgery benign patients and malignant patients. A: Colorectal surgery benign patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 26$ ),  $^{\#}P < 0.05$ ; Preoperative vs Postoperative day 3 ( $n = 23$ ),  $^{\text{h}}P < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 16$ ),  $^{\#}P < 0.05$ ; Preoperative vs Postoperative day 14-20 ( $n = 6$ ),  $^{\#}P < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 26$ ) vs Postoperative day 1 (wound fluid,  $n = 30$ ),  $^{\#}P < 0.05$ ; Postoperative day 3 (plasma,  $n = 23$ ) vs Postoperative day 3 (wound fluid,  $n = 28$ ),  $^{\text{h}}P < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 16$ ) vs Postoperative day 7-13 (wound fluid,  $n = 14$ ),  $^{\text{h}}P < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 6$ ) vs POD14-20 (wound fluid,  $n = 4$ ),  $^{\#}P < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range (Statistical significance is expressed as  $^{\#}P < 0.05$ ,  $^{\text{h}}P < 0.01$ ); B: Colorectal surgery malignant patients. Preoperative vs Postoperative plasma: Preoperative vs Postoperative day 1 ( $n = 35$ ),  $^{\text{h}}P < 0.01$ ; Preoperative vs Postoperative day 3 ( $n = 28$ ),  $^{\text{h}}P < 0.01$ ; Preoperative vs Postoperative day 7-13 ( $n = 17$ ),  $^{\#}P < 0.05$ ; Preoperative vs Postoperative day 14-20 ( $n = 7$ ),  $^{\#}P < 0.05$ . Plasma vs wound fluid: Postoperative day 1 (plasma,  $n = 35$ ) vs Postoperative day 1 (wound fluid,  $n = 33$ ),  $^{\#}P < 0.05$ ; Postoperative day 3 (plasma,  $n = 28$ ) vs Postoperative day 3 (wound fluid,  $n = 29$ ),  $^{\text{h}}P < 0.01$ ; Postoperative day 7-13 (plasma,  $n = 17$ ) vs Postoperative day 7-13 (wound fluid,  $n = 20$ ),  $^{\text{h}}P < 0.01$ ; Postoperative day 14-20 (plasma,  $n = 7$ ) vs Postoperative day 14-20 (wound fluid,  $n = 8$ ),  $^{\#}P < 0.05$ . Plasma and wound fluid protein levels are expressed as median and 75% quartile range. Statistical significance is expressed as  $^{\#}P < 0.05$ ,  $^{\text{h}}P < 0.01$ . Preop: Preoperative; POD: Postoperative day; PLS: Plasma; WFL: Wound fluid; CHI3L1: Chitinase 3 like protein-1.

## ARTICLE HIGHLIGHTS

### Research background

Colorectal resection (CRR) has been previously shown to be associated with elevations in plasma levels of 11 proteins with proangiogenic effects [including vascular endothelial growth factor (VEGF)] that persist for 3 to 5 wk. Further, plasma from the second and third postop weeks has also been shown to promote endothelial cell proliferation, migration, and invasion which are critical to neovascularization. The noted persistent proangiogenic blood protein alterations might stimulate the growth of residual metastases that remain after resection of the primary tumor. The etiology of these elevations is unknown. The time course of the healing process and the fact that angiogenesis plays a critical role in wound healing makes the surgical wounds a possible source of the added protein in the blood. This study was done to simultaneously measure the levels of 8 proteins (VEGF, placental growth factor, angiopoietin-2, monocyte chemotactic protein-1, chitinase 3 like 1 protein, osteopontin, matrix metallo-proteinase-2 and matrix metallo-proteinase-3), all previously shown to be persistently elevated after CRR, in both the blood and fluid from the surgical wounds at multiple postop time points. The significance of this study is that it might demonstrate potentially important, heretofore unknown, systemic manifestations of wound healing.

### Research motivation

The main topics of this study were: (1) Determination of the impact of CRR on blood levels of 8 proteins during the first 2 to 3 wks; and (2) To measure, at the same time points, the levels of the same 8 proteins in fluid from either pelvic or abdominal wall wounds. This is the first study to determine the perioperative levels of 8 proteins in the same population of patients and also the first to assess a population of benign pathology (cancer free) patients in addition to a group of cancer patients. If similar blood protein elevations were noted in the benign and cancer groups then it would be clear that the noted blood compositional changes were not related to the cancer diagnosis. A key motivation for this study was to determine the wound fluid (WFL) levels of 8 proangiogenic proteins as this would provide insight into wound healing, in general, and also might reveal a source of the plasma protein increases. Another motivation for this study was the desire to determine if the makeup of WFL from the pelvis in patients who had rectal resections would be similar to that obtained from the abdominal wall wounds. Therefore, this data should provide insight into wound healing in 2 different locations. Determining that wound levels of the proteins in question were notably higher than blood levels (which are also elevated from their baseline) at the same time points would establish that the healing wounds transiently but significantly alter the blood composition. This information would make clear the importance of minimizing the overall surgical trauma incurred in cancer patients. Also, this knowledge, by confirming the proangiogenic nature of the blood for 1 mo post-surgery, may compel doctors to

look for anti-cancer agents that could be given during the early postop period in an effort to negate these potentially tumor stimulatory conditions.

### Research objectives

The main objectives of this study were the determination of plasma and WFL levels of the 8 proteins in question, simultaneously, at multiple postop time points. The hypothesis was that WFL levels of these proteins would be greater than blood levels because of the angiogenesis occurring in the healing wounds. Another objective was to confirm that after CRR the blood levels of the 8 proteins were persistently elevated for the first 3 wk. Yet other objectives were to determine if similar postop plasma increases were noted in cancer and benign colon disease patients and to ascertain if the protein concentrations in fluid from pelvic and abdominal wounds were similar or different. As mentioned, demonstrating that blood levels of these proangiogenic proteins remain elevated for 3 wk after surgery would confirm that surgery has long lasting systemic manifestations that have the potential to impact growth in residual cancer postop. If true, these results may motivate researchers to look for new anti-cancer agents that could be used early after surgery. Establishing that wound levels are higher than the corresponding blood levels would show that there is a concentration gradient between the healing wounds and the circulation; this would also suggest that the wounds may be a source of the additional protein in the blood. Determination of the similarity or difference between pelvic and abdominal wall WFL will provide insight into wound healing and will also guide future studies.

### Research methods

This study concerned patients who underwent CRR for cancer or for benign colorectal pathology. This study was carried out under the auspices of two separate IRB protocols, one that called for obtaining multiple perioperative blood samples and clinical data for research purposes and the second that concerns harvesting of WFL samples from patients in whom Jackson Pratt drains were placed in either the pelvis or the main abdominal incision (consent obtained post-surgery). Preop blood samples were obtained before surgery from all patients. Blood and WFL samples were simultaneously obtained by research personnel on postop day (POD) 1, 3, and at least 1 late post-discharge time point provided the wound drain remained in place. The late samples, by necessity, were bundled into 2 "time points" (POD 7-13, POD 14-20). Post discharge late samples were obtained in only a fraction of the overall populations due to drain removal and the timing of the first office visit. WFL and blood samples were processed and aliquots of plasma and WFL frozen in a timely fashion. This is one of a small number of studies to collect fluid samples from both abdominal wall and pelvic wounds. WFL and plasma protein levels were determined in duplicate *via* highly specific commercially available Enzyme-Linked ImmunoSorbent Assays. This is the first study to assess perioperative blood levels of 8 proteins at multiple postop time points and the first, to our knowledge, to assess WFL levels for this number of proteins. Demographic, clinical, perioperative, and pathology data were obtained prospectively and entered into the IRB approved above mentioned data bank. The Wilcoxon signed-rank match paired test was used for the pre *vs* postop plasma comparison while the Mann-Whitney test was utilized for the plasma *vs* WFL comparisons.

### Research results

A total of 35 cancer and 31 benign disease patients were studied. The vast majority underwent minimally invasive procedures; 11% of the cancer group and 6% of the benign disease group had open procedures. The majority of the cancer cases were rectal resections (60%) whereas the majority of the benign patients had either a sigmoid or right colectomy (67%). As regards the location of the Jackson Pratt drains, in the cancer group there were 23 pelvic and 12 subcutaneous abdominal wound drains whereas in the benign group there were 8 pelvic and 23 subcutaneous drains. As regards the preop *vs* postop plasma comparisons, there were a total of 32 points of comparison (8 proteins  $\times$  4 postop sampling points). The postop median plasma levels were significantly elevated from preop baseline at all 32 cancer time points and at 29 of 32 of the benign group time points. Of note, the range of the percent change from baseline values for the cancer and benign pathology groups were similar. This assessment of 8 proteins in the two populations verifies and substantiates the results of previous studies that each concerned 1 or, at most, 2 proteins. The results demonstrate that CRR is associated with plasma elevations that persist for at least 3 wk post-surgery. Further, these results prove that the elevations are related to the surgical procedure itself and not the indication for surgery (cancer *vs* benign pathology). These results also make clear the need to determine the oncologic consequences of the 3 to 5 wk long period when the blood is decidedly proangiogenic. New anti-cancer treatments that can be given during the first post month should be considered. Of note, when the pelvic and subcutaneous WFL results were compared, for all 8 proteins, at the great majority of time points, there was no statistical difference in protein levels between the 2 locations, thus, for the following analysis the WFL results from the 2 drain locations were combined. As regards the WFL *vs* plasma level comparisons for the 8 proteins, the median WFL levels were significantly greater than the corresponding plasma level at all 32 time points in both groups. The WFL median level was at least 3  $\times$  higher than plasma levels in 90%-91%, 5  $\times$  higher (or greater) in 68%-69%, and 30  $\times$  greater in 29% of patients in both groups. Of note, the highest WFL levels were noted at the POD 7-13 or 14-20 time points for 6 of the 8 proteins in both groups. These results prove that median wound levels of these proangiogenic proteins are notably greater than the corresponding plasma levels and that there is a substantial gradient between the wounds and circulation. Also, these results strongly support the hypothesis that the healing wounds are the source of the added protein in the blood. Similar studies that assessed different groups of proteins or different operations (gastrectomy, hepatectomy, pneumonectomy, *etc.*) would



increase our understanding of surgery's systemic impact and perhaps lead to attempts to block, in some way, the deleterious systemic manifestations of major surgical trauma. Larger studies of this type would also allow a more detailed comparison between WFL from the pelvic and subcutaneous locations.

### Research conclusions

There are 4 new findings of this study. The first is that WFL levels of the 8 proteins assessed are notably higher than the corresponding plasma levels which, in turn, are elevated from their preop baselines. These results support the hypothesis that the wounds are a major source of the added protein in the blood. These results also suggest that angiogenesis plays a prominent role in wound healing during the first month after surgery. The second new finding is the demonstration in this population of CRR patients that the plasma levels of all 8 proteins were significantly elevated for at least 3 wk after surgery (prior studies considered only 1-2 proteins per population). The third new finding is that plasma protein elevations similar to those found in cancer populations are found following surgery for benign pathology; thus the changes are related to the surgery and not the indication. The fourth new finding is that the make-up of WFL from 2 different locations are similar, as regards the levels of the proteins in question. This aspect needs further study and verification since the numbers of samples from each location limited the ability to detect differences at the later time points. The plasma results, by proving that long duration proangiogenic protein increases are present, raises the fear that these changes may promote tumor growth postoperatively in patients with residual disease. This realization should logically prompt studies to verify this hypothesis as well as to search for ways to limit these deleterious oncologic effects. The plasma and WFL results regarding 8 proteins in both cancer and benign pathology patients makes clear the fact that major surgery results in systemic blood compositional changes that last far longer than previously imagined; further, there is the potential that these changes may negatively impact cancer patients with residual disease. The new methods and study approaches put forth in this study are the simultaneous obtaining of blood and WFL samples at multiple time points during the first 3 wk after surgery and the assessment of 8 different proteins in a single population. As stated above, these results support the main hypothesis that the surgical wounds are the source of the added protein in the blood which significantly elevates plasma levels for weeks after surgery. The results also verify that long lasting plasma protein changes occur after surgery done for benign indications (as is the case for cancer populations).

### Research perspectives

The results of this study add further evidence and support for the concept that CRR (and likely major surgery, in general) results in significant changes in the plasma levels of a substantial number of proteins that persist for at least 3 wk after surgery. Prior studies regarding the 8 proteins assessed in the present study have demonstrated that the full duration of the significant elevations is 3 to 5 wks. Documenting that all 8 proteins are persistently increased after surgery in patients with cancer or benign problems proves that these effects are related to the surgical procedure and not the presence of a cancer. The finding of much higher levels of these proteins in WFL than in the blood makes clear that wound healing is an involved and lengthy process in which that angiogenesis plays a central role. It also strongly suggests that the wounds are the source of the added protein. The fact that major surgery (tissue trauma) and the process of healing that follows alters the blood composition so that it is decidedly proangiogenic for a month's time has important implications. These systemic changes may accelerate the growth of residual tumor metastases by stimulating tumor angiogenesis. Future studies are needed to determine the clinical ramifications of the demonstrated plasma changes. Also, perioperative levels of other proteins that may influence tumor growth and establishment are indicated to better define surgery's effects. A better understanding of surgery's effects may lead to modifications in technique that will ameliorate the deleterious transient effects. Finally, the authors believe that anti-cancer drugs that can be used early after surgery should be sought so as to provide protection against accelerated tumor growth during the early postop period.

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