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Spectroscopic Microvascular Blood Detection from the Endoscopically Normal Colonic Mucosa: Biomarker for Neoplasia Risk

Hemant K. Roy, MD¹, Andrew Gomes², Vladimir Turzhitsky², Michael J Goldberg, MD¹, Jeremy Rogers, Ph.D², Sarah Ruderman², Young Kim L, Ph.D.³, Alex Kromine², Randall E. Brand, MD⁴, Mohammed Jameel, MD¹, Parmede Vakil², Nahla Hasabou, MD¹, and Vadim Backman, Ph.D.²

¹Dept of Internal Medicine, Evanston-Northwestern Healthcare, Evanston IL

²Biomedical Engineering Department, Northwestern University, Evanston IL

³Dept of Internal Medicine, University of Pittsburgh Medical Center, Pittsburgh PA

⁴Dept of Biomedical Engineering, Purdue University, West Lafayette IN

Abstract

Background & Aims—We have previously utilized a novel biomedical optics technology, four-dimensional elastically-scattered light fingerprinting, to demonstrate that in experimental colon carcinogenesis, the predysplastic epithelial microvascular blood content is markedly elevated. In order to assess the potential clinical translatability of this putative field effect marker, we characterized the early increase in blood supply (EIBS) in humans *in vivo*.

Methods—We developed a novel endoscopically-compatible polarization-gated spectroscopic probe that was capable of measuring oxygenated (Ohb) and deoxygenated (Dhb) hemoglobin specifically in the mucosal microcirculation through polarization-gating. Microvascular blood content was measured in 222 patients from the endoscopically-normal cecum, midtransverse colon and rectum. If polyp was present, readings were taken from the polyp tissue along with the normal mucosa 10 cm and 30 cm proximal and distal to the lesion.

Results—Tissue phantom studies demonstrated that the probe had outstanding accuracy for hemoglobin determination ($r^2=0.99$). Augmentation of microvasculature blood content was most pronounced within the most superficial (~100 μ m) layer and dissipated in deeper layers (i.e. submucosa). EIBS was detectable within 30 cm from lesion and the magnitude mirrored adenoma proximity. This occurred for both OHb and DHb, with the effect size being slightly greater for

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Correspondence Hemant K. Roy MD, Associate Professor, Feinberg School of Medicine at Northwestern University, Section of Gastroenterology, Evanston-Northwestern Healthcare, G208, Evanston Hospital, 2650 Ridge Avenue, Evanston IL 60201, Phone: 847-570-3115, Fax: 847-733-5041, h-roy@northwestern.edu.

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DHb. EIBS correlated with adenoma size and was not engendered by non-neoplastic (hyperplastic) polyps.

Conclusions—We provide, herein, the first demonstration that *in vivo* microvascular blood content can be measured and provides an accurate marker of field carcinogenesis. This technological/biological advance has numerous potential applications in CRC screening such as improved polyp detection and risk-stratification.

INTRODUCTION

The realization that colon carcinogenesis involves both discrete mutational events and more diffuse epigenetic/cellular abnormalities (field carcinogenesis) has potential clinical consequences. For instance, colonoscopic removal of focal lesions (adenomatous polyps) has been shown to dramatically reduce the incidence of future colorectal cancers (CRCs).¹ On the other hand, identification of field effect in the rectosigmoid may predict risk of neoplasia throughout the colon.² Commonly used means of sensing field carcinogenesis include flexible sigmoidoscopy, in which a distal adenoma portends a ~2.5 fold increased risk of neoplasia in the proximal colon.³ Rectal aberrant crypt foci (ACF) number may be a harbinger of adenomas/carcinomas elsewhere in the colon.⁴ However, these field effect identification modalities are generally insensitive, hindering utilization for population screening. Therefore, attention has focused on detecting biomarkers that represent earlier changes of carcinogenesis. Several lines of evidence suggest that there are profound cellular (increased proliferation⁵ and decreased apoptosis⁶), biochemical (e.g. protein kinase C activity⁷), genomic (microarray)⁸ and proteomic⁹ abnormalities in the histologically normal mucosa of patients harboring neoplasia. While this provides powerful corroboration of the field effect, these techniques lack the requisite performance characteristics for clinical use.

Our multidisciplinary colon cancer prevention group has been interested in bridging advances in bio-optical technologies into clinical practice. We have recently developed four dimensional elastic light scattering fingerprinting (4D-ELF)¹⁰ which allows heretofore unprecedented quantification of the colonic mucosal microvasculature. Using this approach, we observed that mucosal microvascular blood content was increased in the histologically normal mucosa of two experimental models of colonic neoplasia, the azoxymethane (AOM)-treated rat and the MIN mouse¹¹. These occurred prior to development of adenomas or even ACF. Importantly, this early increase in blood supply (EIBS) was confined to the regions where future neoplasia would develop. This was confirmed using conventional techniques (Western blot analysis for hemoglobin from colonic mucosal scrapings), although latter was markedly less sensitive than 4D-ELF¹¹. While the biological mechanisms remain incompletely elucidated, studies in the AOM-treated rat implicate the role of nitric oxide synthase (iNOS)¹².

Taken together, these findings suggest that EIBS may be a useful marker for colon carcinogenesis. In order to confirm these findings in human and characterize the relationship with concurrent neoplasia we, therefore, performed studies mapping microvascular blood content during colonoscopy. Our results provide the first indication that EIBS is detectable *in vivo* and its magnitude mirrors proximity to neoplasia potentially suggesting future clinical utility for enhancing endoscopic polyp detection.

METHODS

Endoscopically-compatible fiber-optic probe

We developed an endoscopically-compatible fiber-optic probe to assess microvascular blood content *in vivo*. Figure 1(A) illustrates the probe design. This probe provides a simplified

version of 4D-ELF in that it measured wavelength and polarization of scattered light but not scattering angle or azimuthal angle of scattering. One of the three 200 μm -core diameter multimode fibers is used as an illumination channel to deliver broadband light from a xenon-lamp onto tissue surface whereas two fibers serve as collection channels for scattered light from the tissue. Two thin film polarizers are mounted on the proximal tip to polarize the incident light and to enable collections of backscattered light that was polarized parallel (co-polarized signal $I_{\parallel}$) and perpendicular (cross-polarized signal $I_{\perp}$) to the incident light polarization. The graded refractive index (GRIN) lens (outer diameter = 1.8 mm and pitch = 0.25) attached to the fiber tip collimated the light incident onto the tissue with half-angle divergence of 3° and focused backscattered light onto the collection fibers. The design insured that the collection fibers received scattered light from the same tissue site, avoided specular reflectance and improved the signal-to-noise ratio. In the proximal probe end, the linear array of fibers was coupled to an integrated CCD spectrometer that recorded the spectra of $I_{\parallel}$ and $I_{\perp}$ in the spectral range from 280 to 780 nm. The outer diameter of the fiber-optic probe was 2.5 mm (Fig. 1(B) & C), thereby enabling easy passage through the colonoscope biopsy port.

In order to specifically assess the mucosal microvasculature, the probe preferentially sampled backscattered light from superficial tissue depth ($\sim 100 \mu\text{m}$) via polarization gating¹³. The differential polarization signal $\Delta I = I_{\parallel} - I_{\perp}$, co-polarized signal $I_{\parallel}$, and cross-polarized signal $I_{\perp}$ are sensitive to progressively deeper tissue depths. For a given polarization signal, the penetration depth is determined by the angle between incident and collected light beams and the sizes of the illumination and collection spots on tissue surface. These are, in turn, defined by the lens focal length, fiber numeric aperture, and fiber-to-fiber separation. We tested several probe configurations to optimize penetration depth for colonic mucosa.

Spectroscopic determination of blood content

The unique absorption spectra of oxygenated hemoglobin (OHb) and deoxygenated hemoglobin (DHb) in the visible range allowed hemoglobin quantification through a Beer's law inspired algorithm.^{11, 14} Briefly, we assumed minimal path length variability due to differences in optical properties. Therefore, the attenuation due to absorption has an inverse exponential relationship with the absorber concentration:

$$I(\lambda) = I_{\text{scattering}}(\lambda) \cdot e^{-\alpha_{\text{OHb}} \cdot A_{\text{OHb}}(\lambda) - \alpha_{\text{DHb}} \cdot A_{\text{DHb}}(\lambda)} \quad (1)$$

where $I(\lambda)$ is one of the polarization components of the collected light (ΔI , $I_{\parallel}$, or $I_{\perp}$), $I_{\text{scattering}}(\lambda)$ represents the scattering signal if the sample were devoid of absorbers. $A(\lambda)$ represents the absorption spectrum of all of the absorbers present (predominantly OHb and DHb in the colonic mucosa), and α is the coefficient that represents the product of path length and Hb concentration under the constraints of Beer's law. Extinction coefficients α_{OHb} and α_{DHb} . We were used to account for different contributions of oxygenated and deoxygenated hemoglobin. These were obtained from published sources and corrected for hemoglobin packing (originally described by Finlay and Foster)¹⁵. This phenomenon occurs because hemoglobin molecules within the same erythrocyte may shield each other from the incident light. Additionally, the non-erythrocyte sample volume provides many possible light paths that do not sample hemoglobin. This can be corrected through multiplying the standard absorption spectrum in a homogeneous solution by a distortion coefficient described by Foster. In the more realistic scenario of RBCs within a blood vessel, the packaging effect is no longer simply due to the cells themselves and but rather the length scale of the packaging of RBCs inside a blood vessel, R . In our analysis, we used packaging length scale R as a fitting parameter.

Deduction of α_{HbO_2} and α_{Hb} values from Equation 1 required assuming that the form of the scattering spectrum, $I_{\text{scattering}}(\lambda)$ could be represented by a smooth second-order polynomial lacking the characteristic hemoglobin absorption peaks at 542 nm and 576 nm in the case of OHb and 555 nm in the case of DHb). The parameters α_{HbO_2} , α_{Hb} , and R were chosen by a nonlinear optimization MATLAB routine such that the sum of square error between calculated and measured spectra in the range of 480–680 nm is minimized.

The end result of the analysis is six values characterizing blood content in superficial (e.g. mucosal) tissue: concentrations of oxygenated and deoxygenated hemoglobin for each of the three depths of penetration. Although simplistic, this Beer's law-inspired relationship was able to fit our data well, because of the short depth of penetration of the polarization signals (from 100 to 170 μm).

Tissue phantom studies

We used tissue phantom experiments to determine the fiber-optic probe's penetration depth and accuracy of blood content measurement. For penetration depth, aqueous suspensions of microspheres with various scattering properties in the range of colon tissue (anisotropy coefficient $g = 0.73\text{--}0.93$, mean free path length $l_s = 90\text{--}289\ \mu\text{m}$) were used.^{11–14} The polarization signals were collected from tissue phantoms of various thickness ($\sim 50\ \mu\text{m}$ to 4 mm). Then, we estimated each representative penetration depth from ΔI , $I_{\parallel}$, and $I_{\perp}$ as the average of optical thickness (optical thickness $\tau = l_s * L$; $\tau = 1$ represents one scattering event on average) by the rate of the increase of the polarization signals with tissue phantom thickness.

In order to validate the accuracy of spectroscopic measurement of hemoglobin concentration using the fiber-optic probe, we used known concentrations of human Hb (0–15 g/l from Sigma-Aldrich, St. Louis, MO) in the microsphere suspension. Then this true concentration was compared with the one determined using the spectral analysis.

Human Studies

This study was performed under the supervision of the Institutional Review Board of Evanston-Northwestern Healthcare. Patients undergoing screening colonoscopy at Evanston Hospital were recruited for the study. Exclusion criteria included incomplete colonoscopy, poor preparation and colitis. After intubation of the cecum, the polarization-gated spectroscopy probe was inserted and approximately 10 measurements (each requiring 50 milliseconds) were taken from the endoscopically normal mucosa from the cecum, midtransverse colon and rectum. The probe was placed in contact with minimal pressure with the colonic mucosa. If a polyp was seen during the colonoscopy, readings from the polyp, $< 10\ \text{cm}$, and $10\text{--}30\ \text{cm}$ proximal and distal to the lesion were taken prior to polyp removal. All polyps were removed by a technique at the endoscopist's discretion. Polyp size was assessed based on endoscopic estimate (using open biopsy as a comparator). All polyps underwent histological analysis by a board certified pathologist. Based on endoscopy and pathology, patients were classified in one of three groups based on their largest polyp: Advanced adenoma (size $\geq 10\ \text{mm}$, $> 25\%$ villous component or presence of high-grade dysplasia), adenoma (non-advanced) or no dysplasia (no adenoma identified).

Statistical Analysis

To assess possible demographic/lifestyle characteristics that might represent confounding factors, we used multivariable linear regression analyses on EIBS values in the largest subgroup (those without neoplasia). Covariates included gender, age, current status of alcohol intake, current smoking status, personal history of polyps from previous colonoscopy, personal history of CRC, and family history of CRC from first-degree

relatives. All statistical calculations were carried out using STATA 9.0 for Windows (Stata Corp., College Station, TX).

RESULTS

Probe validation

First, we investigated the depth-selectivity for the three different polarization signals. The phantom studies using optical properties of colonic tissue showed that differential polarization signal ΔI , co-polarized signal $I_{||}$, and cross-polarized signal $I_{\perp}$ selected backscattered light within the optical depth (τ) of 2.0 ± 0.7 , 2.6 ± 0.8 , and 3.4 ± 0.6 , respectively. A $\tau=1$ corresponded to a physical penetration depth of $\sim 50 \mu\text{m}$ given that the mean free path l_s of light has been measured to be $\sim 50 \mu\text{m}$ in colonic mucosa⁹. Therefore, the optical depths correspond to physical penetration depths of $100 \pm 35 \mu\text{m}$, $130 \pm 40 \mu\text{m}$, and $170 \pm 30 \mu\text{m}$, respectively. Thus, the differential polarization signal ΔI corresponded to the epithelial (pericryptal) capillary network.

Second, we determined the accuracy of spectral Hb concentration measurement by utilizing tissue phantoms with known concentrations of Hb. This experiment was repeated for different tissue phantoms scattering properties ($g=0.73-0.9$, $l_s=50 \mu\text{m} - 118 \mu\text{m}$). Figure 2 shows representative measurements of Hb concentrations obtained from one of the tissue phantoms ($l_s = 80 \mu\text{m}$). Calculated Hb concentrations had strong linear correlations with the actual concentrations over the large range of both the physiological Hb concentration and scattering properties in colonic tissue for all three depths ($r^2 > 0.99$ for all depths). The spectral measurement error for the three penetration depths (~ 100 , ~ 130 , and $\sim 170 \mu\text{m}$), were 5%, 12%, and 19%, respectively, over the Hb concentrations (0 g/l – 15 g/l). Thus, the spectroscopic approach allows accurate measurement of Hb concentrations over a wide range of tissue optical properties.

We also wanted to ensure that the probe is capable of providing reliable readings during colonoscopy. To this end, the probe was tested in a series of *in vivo* experiments evaluating mucosal hemoglobin measures under various conditions. 1) Endoscopic illumination had a negligible effect on the signal recorded by the probe ($<1\%$). 2) Endoscope bending and angulation (including 180°) for practically relevant radii of curvatures ($>5 \text{ cm}$) had a minimal effect ($<1\%$ of signal level and $<1\%$ of the difference in blood content values obtained). 3) We investigated the effect on EIBS of the tissue pressure exerted by the probe using 10 patients (106 tissue sites probed) (Fig. 3A). During colonoscopy, measures were taken with low, medium or high pressure against the tissue. Low pressure corresponded to the probe lightly touching the tissue surface, while high pressure was sufficient to visibly indent the tissue surface. There were no significant differences in measured Hb concentration for any of the pressure levels (ANOVA P-value > 0.92 , standard deviation due to the pressure effect 4%). 4) Similarly, the probe tip angle was studied during colonoscopy ($n=10$ patients, 85 tissue sites probed). The angle between the normal axis of the probe tip and the surface normal was varied among $0^\circ-10^\circ$, $30^\circ-50^\circ$, and $60^\circ-90^\circ$. Data indicated that the angle did not have a significant effect on the Hb content measurements (ANOVA P-value > 0.41 , standard deviation 11%, Fig. 3(B)). 5) Finally, we evaluated the effect of colonic distension (10 patients, 148 tissue sites), which was varied from non-distended to a highly distended colon thus spanning the normal operating range during colonoscopy (Fig. 3(C)). The distension did not impact upon Hb measurements (ANOVA P-value > 0.76 , standard deviation 5%). These results indicate that the specifics of probe operation *in vivo* do not have a significant effect on Hb content assessment and the implementation of the endoscopically compatible probe can be feasible in any clinical setting.

Patient characteristics

For the main study assessing EIBS in the colon, we recruited 222 patients undergoing colonoscopy. Of these, 175 subjects had no neoplasia (no adenomas detected), 35 with non-advanced adenomas, and 12 with advanced adenomas. The mean age of the cohort was 56.6 years and 40% were females. There was no significant difference in gender between the control and any adenoma groups (P -value = 0.21 using a test of differences in two proportions). The age of subjects in the no neoplasia and adenoma groups was also comparable (P -value = 0.96). Furthermore, groups did not significantly differ in tobacco or alcohol history.

Assessment of Diagnostic Depth for EIBS markers

The probe uses polarization-gating to measure OHb and DHb contents at three unique penetration depths. To ascertain the optimal penetration depth for sensing adenomas, the OHb and DHb determinations for each depth from adenoma-free patients were compared to corresponding readings from the uninvolved mucosa in adenoma-harboring patients. The magnitude of difference appeared to be greatest for the ~100 μ m depth for both OHb and DHb (Fig.4). Indeed, the only statistically significant difference for either OHb or DHb readings between controls (no neoplasia) and adenoma-harboring patients was at 100 μ m. This tissue depth corresponded to the mucosal pericryptal capillary network obtained in the MIN mouse and AOM-treated rat using our bench-top instrument¹¹. This supports the concept that only a fraction of the total colonic blood supply (the most superficial) is involved in the phenomena of EIBS thus underscoring the importance of polarization gating to selectively probe this area and avoid being overshadowed by blood from deeper tissues.

EIBS Mirrors Proximity to Neoplastic Lesions

In order to assess the spatial variation in mucosal EIBS (total Hb, OHb and DHb) with respect to distance from adenoma, we focused the tissue depth of ~ 100 μ m. We measured the adenomatous polyp, uninvolved mucosa ~10 cm, and ~ 30 cm from the lesion (both proximally and distally with distance estimated with a straightened colonoscope during withdrawal). The data was normalized to same-site readings from neoplasia-free patients because of the modest variability in basal microvascular readings. For instance, in non-adenoma harboring patients, the absolute values from the rectum, mid-transverse colon and cecum for deoxyhemoglobin was 2.98 g/l, 2.44 g/l and 2.12 g/l respectively (ANOVA p value <0.001) whereas total hemoglobin was 8.62 g/l, 7.65 g/l and 7.63 g/l, respectively (p <0.01). Figures 5 (A) demonstrate that at the adenoma site the total Hb concentration was elevated 75.3% above control levels (comparable region from non-adenoma harboring patient) and persisted in the uninvolved mucosa. Moreover, tissue sites located within 10 and 30 cm away from an adenoma also manifested a highly statistically significant increase in both total Hb, OHb and DHb concentration (P -value<0.001).

Total Hb, OHb and DHb readings gradually diminished with distance from lesion. In general, percent oxygenation was inversely associated with adenoma proximity with a 14.0% reduction as readings taken from ~10 compared to ~30 cm from adenoma leading to a less robust gradient with OHb than DHb or total Hb (data not shown). In Figs. 5 (A) and (B), the spatial gradients for total Hb and DHb are shown individually. Once again, there is a highly statistically significant increase in blood concentration with proximity to the adenoma (ANOVA P -value <0.00001 for both total Hb and DHb). DHb had a slightly stronger spatial gradient than total Hb possibly reflecting increased tissue oxygen extraction in the proximity of the tumor.

With regards to performance characteristics, EIBS measured 30cm away from lesion was reasonably accurate in detecting advanced adenomas (sensitivity =92%, specificity = 78%,

negative predictive value =99.7%, positive predictive value= 9.4%). These estimates should be viewed cautiously as they are based on a relatively modest patient numbers.

Effect of polyp size and histology on EIBS

We noted that the microvascular blood content was elevated in the uninvolved mucosa in both advanced and non-advanced adenomas (Fig. 6a). However, it was apparent that the magnitude of EIBS engendered by larger lesions was greater than smaller lesions. Specifically, in the uninvolved mucosa ~10 cm from a lesion, EIBS (depth of ~ 100 μ m) was higher if the lesion was an advanced adenoma versus a smaller (non-advanced) adenomas (P-value = 0.0150 and 0.000263 for total Hb and DHb, respectively, Figs. 6A). This was even more pronounced if the readings were taken from the adenomatous tissue, as demonstrated in Fig. 6B. Indeed, the differences between Hb concentration measured from advanced adenomatous polyps versus non-advanced adenomas was highly statistically significant (P-value <0.00001 for total Hb and DHb, respectively).

We also wanted to determine whether EIBS is specific for premalignant lesions or may be associated with polyps believed to be innocuous (hyperplastic polyps)? Figures 6C show increased total Hb and DHb readings from the hyperplastic polyp tissue but not from the endoscopically normal tissue surrounding these polyps (ANOVA p-value = 0.437 and 0.392, respectively). These results suggest that EIBS is specific to clinically relevant lesions only and would not be confounded by the presence of hyperplastic polyps.

Potential Confounders of EIBS: Patient factors

There are numerous factors that are known to affect colonic blood flow. Most of these factors, however, to date, have only been assessed at the larger blood vessels (large arterioles and arteries) and not the microvasculature. We, therefore, evaluated the influence of non-neoplastic factors on EIBS. We performed multivariable linear regression analysis on OHb and DHb (depth ~100 μ m) using no neoplasia subjects (our largest category). The coefficient β represents EIBS markers change expressed as a percent of mean values of EIBS markers in the control group. As demonstrated in Table I, gender was the only demographic factor that had significant effect on OHb with males values averaging 15.0% greater than females. This was consistent with the DHb data (19.1% increase in males). DHb had significant associations with personal and family history of colonic neoplasia (46.4 and 29.1% lower, respectively) but it needs to be emphasized the sample size was very small (4 patients) highlighting the need for caution in interpreting this data. We also looked at the differences in colonoscopic preparations (oral saline phosphate solution versus polyethylene glycol) and noted no alterations in either total or deoxygenated Hb, $p=0.83$ and 0.87 , respectively (data not shown).

DISCUSSION

We report, herein, that *in vivo* microvascular blood contents were elevated in the endoscopically normal mucosa of patients who harbored colonic neoplasia. This data provides human validation for our previous *ex vivo* report that EIBS is an early event in field carcinogenesis in both the AOM-treated rat and the MIN mouse. Importantly, EIBS increased in magnitude with proximity to the lesion. While there did appear to be “dose-dependence” (i.e. the magnitude of EIBS was greater in advanced adenomas than diminutive), it should be noted that even smaller adenomatous polyps manifested EIBS. However, hyperplastic polyps (non-neoplastic lesions) did not engendered EIBS in the uninvolved mucosa. This suggests that EIBS may have both biological and clinical significance.

Previous studies had demonstrated that neo-vascularization could occur as early as the small adenoma¹⁶ or even aberrant crypt foci stage¹⁷. The only report to date on histologically normal mucosa is a vascular casting study that showed that the “transitional mucosa” (normal epithelium adjacent to CRCs) had abnormal microcirculation.¹⁸ Our detection of EIBS is largely due to the unique ability of 4D-ELF to accurately assess the superficial microvasculature (peri-cryptal capillary plexus). While representing only a small amount of the colonic blood supply, it is the compartment that would be anticipated to be most responsive to the hyperproliferative and hence hypermetabolic needs of field carcinogenesis. Consistent with this was our finding that when we gated the blood content analysis to 170 μm (submucosa), the difference in hemoglobin content between adenoma-harboring and no neoplasia patients was markedly diminished. In this regard, it is interesting to note that the spatial trend in DHb was more pronounced than OHb leading us to postulate that the hypermetabolic epithelium adjacent to a lesion derives sustenance by both increasing the micro-circulatory volume and also improving oxygen extraction efficiency.

The other major technological innovation that permitted *in vivo* EIBS detection was simplification and miniaturization of the equipment to allow the development of a light scattering probe. This enabled more accurate Hb content determination without the potential artifacts associated with *ex vivo* analysis. Moreover, measuring *in vivo* enabled assessment oxygenation status of the microvasculature given the distinctive light absorption signature of oxygenated versus deoxygenated blood. Thus, the polarization-gated spectroscopy probe enables a more accurate probing of the microvasculature.

The increased microvascular blood content is a promising marker of field carcinogenesis. The phenomena of EIBS appeared to have two distinct facets: a low-amplitude diffuse increase throughout the colonic mucosa and more marked augmentation in the region of a tumor. Other markers of the field effect have been reported to also have diffuse and/or localized characteristics. For instance, the ubiquitous nature of field carcinogenesis is underscored by the ability of the rectal mucosa to predict proximal neoplasia through analysis of cellular markers (decreased apoptosis⁶ and increased proliferation⁵) and morphological markers (ACF⁴ or adenomas³). Furthermore, genomic (cyclooxygenase 2, osteopontin)⁸ and immunohistochemical (e.g. cytochrome C oxidase subunit I)¹⁹ markers showed no clear diminution with distance. On the other hand, markers such as carcinoembryonic antigen, while found in the peri-tumor histologically normal mucosa rapidly dissipated with distance²⁰. Thus, there is biological precedence that EIBS, being a biomarker for field carcinogenesis, would manifest both diffuse and localized elements.

One would anticipate that the mechanisms involved in EIBS would also reflect this two component model. While the biological underpinnings of EIBS remain incompletely understood, several lines of evidence implicate nitric oxide, a potent vasodilator and pro-angiogenic factor in colon carcinogenesis. For instance, in the premalignant AOM-treated rat colonic mucosa, iNOS expression was increased diffusely in the uninvolved mucosa and paralleled the magnitude of colonic mucosal microvascular blood content¹². Importantly, short-term treatment with an iNOS inhibitor completely abrogated EIBS arguing for causality¹². With regards to the localized effects, we postulate that it is related to factors elaborated by the tumor (resulting in vasodilation and/or angiogenesis). This may be also nitric oxide related since numerous reports show that iNOS is overexpressed in human colonic adenomas and carcinomas²¹. Other putative factors would include vascular endothelial growth factor (VEGF), prostaglandins etc. Further studies are needed to elucidate the role of iNOS and other vasodilatory/pro-angiogenic factors as mediators for the two components of EIBS.

The critical role of augmented blood supply (angiogenesis) in established CRC leads us to postulate that EIBS may be biologically important in early neoplastic transformation of the colon. From a teleological perspective, increased microvascular blood supply would be essential for supporting the hyperproliferative epithelium that is a hallmark of early colon carcinogenesis. This is further supported by the observation that germline mutations in the vascular-regulatory endoglin 1 is associated with an increased CRC risk²². Indeed, as of late, there has been interest in using anti-vascular strategies to prevent neoplasia (angioprevention)²³. Intriguingly, many agents that target putative EIBS mediators have been also demonstrated outstanding chemopreventive activities. For example, there are number of studies indicating iNOS inhibitors prevent neoplasia in experimental models of colonic neoplasia²⁴. Cyclooxygenase (COX)2 is a potent regulator of angiogenesis in colon carcinogenesis and is overexpressed in both the uninvolved mucosa and dysplastic tissues²⁵. COX-2 inhibitors, such as celecoxib, have been shown to be both anti-angiogenic and potent in suppressing colon neoplasia in clinical trials^{25, 26}. Moreover, in the MIN mouse, the observation that targeting the VEGF receptor-2 decreased tumor multiplicity is consistent with our data on the role of EIBS in the initiation of colon carcinogenesis²⁷. Thus, our finding of EIBS may provide important biological insights of early colon carcinogenesis.

The endoscopic detection of EIBS has several potential applications, especially given the two-component model discussed. For instance, the EIBS gradient may potentially be able to serve as a tool to improve colonoscopic detection of adenomas. The clinical imperative is underscored by the observation that 5.1% of patients with newly diagnosed CRCs had a negative colonoscopy within the past 5 years²⁸. Moreover, tandem colonoscopy studies report 22% of all adenomas are missed²⁹, while CT colography indicates that colonoscopy fails to detect 12% of advanced adenomas³⁰. In clinical practice, the miss rate is manifested by the large inter-endoscopist variability in the detection rate for all adenomas and advanced adenomas (3 to 6-fold respectively)³¹. One could envision microvascular blood content being sampled in each third of the colon (each measurement taking ~50 milliseconds). If, EIBS is elevated, this could serve as a “red flag” for the endoscopist. If the lesion is not visualized, then mapping the EIBS gradient could potentially allow more precise localization. This approach could be of particular value in patients with inadequate preparation or if cecal intubation was not achieved. Potential applications of the diffuse component of EIBS would be for risk stratification through interrogation of the rectal mucosa (data not shown). Finally, this approach may be useful as the “optical biopsy” in distinguishing hyperplastic polyps from adenomas through probing the surrounding mucosa. However, at this juncture, all these potential applications of EIBS detection are purely theoretical.

There are a number of limitations to our studies. Firstly, the colonoscopy miss rate means that we probably underestimated the magnitude of the increased microvascular blood content related to carcinogenesis (some controls actually harbored adenomas). In addition, the colon distance measures are inexact secondary to colonoscope looping. Also, polyp size estimated by endoscopy may be somewhat inaccurate³². While hyperplastic polyps are generally considered innocuous, right-sided lesions may be a precursor of the sessile serrated adenoma which are associated with microsatellite unstable cancer³³. However, the vast majority of hyperplastic polyps in our study were located in the distal colon. Colitis was an exclusion criteria since mucosal blood content can be altered by inflammation. However, confounding is unlikely given colitis is generally readily endoscopically recognizable. Elucidating the mechanisms for EIBS (vasodilation and/or neo-angiogenesis) is of importance application development. Finally, while our data does support the concept of EIBS measurements to improve colonoscopic polyp detection, risk-stratification and optical biopsy, this has not been proven. Future studies are planned to address all these issues.

In conclusion, we provide the first demonstration that the microvascular blood content increased diffusely in the microscopically normal colonic mucosa of patients harboring adenomas. Moreover, the magnitude mirrored the proximity to the neoplasia. This observation was detectable because of the technological breakthrough in biomedical optics, polarization-gated spectroscopy. EIBS may provide potentially important insights into early events in colon carcinogenesis. Future studies will further investigate both the biological mechanisms and potential clinical applications.

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Abbreviations used

CRC	colorectal cancer
EIBS	early increase in blood supply
iNOS	inducible nitric oxide synthase
Hb	hemoglobin
OHb	oxyhemoglobin
DHb	deoxyhemoglobin

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Figure 1. (A) Schematic diagram (frontal view) of the endoscopically-compatible polarization-gating spectroscopy fiber-optic probe. (B & C) Photographs of probe.

Figure 2.

Polarization-gating spectroscopy probe accurately measured hemoglobin (Hb) concentrations over a large range of physiological values (tissue phantom study). The solid lines represent linear approximations. Measured Hb concentrations showed excellent correlations with actual Hb contents with r^2 -value = 0.99 for all depths. The root mean squared (RMS) errors from the probing depths of ~100 μm (Fig 2(A)), ~130 μm (Fig 2(B)), and ~170 μm (Fig 2(C)) were 0.33 g/l, 0.36 g/l, and 1.20 g/l, respectively.

Figure 3.
 Probe operation characteristics have a negligible effect on the blood content measurement using the fiber-optic probe. (A): Probe pressure (B): Probe angle (C): Colon distention.

Figure 4.

Determination of interrogation depth for hemoglobin in the endoscopically-normal mucosa that allowed optimal discrimination of patients harboring neoplasia (~30 cm away from adenoma) from those who were neoplasia-free. Panels A, B and C compare OHb and DHb contents were assessed within three different depths of ~100 μ m, ~130 μ m, and ~170 μ m, respectively. The ~100 μ m depth had provided the greatest distinction with progressively lower effect sizes at 130 μ m, and ~170 μ m respectively.

Figure 5. Microvascular blood content (depth ~100 μ m) varies with distance from adenomas. Total hemoglobin (A) and deoxygenated hemoglobin (B) mirrored distance from lesion.

(i) Total Hb (depth ~ 100 μm) (ii) DHb (depth ~ 100 μm)

6 (a)

(i) Total Hb (depth ~ 100 μm) (ii) DHb (depth ~ 100 μm)

6 (b)

6 (c)

Figure 6.

Polyp size and histology impact upon microvascular blood content (measured at ~100 μm depth).

- a. EIBS in the uninvolved mucosa. (~10cm away from lesion) was greater in larger (advanced) adenomas versus non-advanced adenomas.
- b. **i.** Total hemoglobin and **ii.** deoxygenated hemoglobin from polyp tissue paralleled uninvolved mucosal data.
- c. Hyperplastic polyps did not engender EIBS in uninvolved mucosa but was associated with increased blood content in polyp tissue **i.** Total hemoglobin **ii.** Deoxygenate hemoglobin.

Multivariable linear regression coefficients of cross-sectional associations of EIBS markers with patient characteristics (from the neoplasia-free group).

Table 1

	OHb			DHb		
	β (%)	SE	P-value	β (%)	SE	P-value
Gender: Male = 1 or Female = 0	15.0	4.11	3.04×10^{-4}	19.1	6.88	0.00168
Age: <55 yrs = 0 or 55 yrs = 1	6.43	4.03	0.116	5.45	6.01	0.366
Race: White = 0 or Nonwhite = 1	-8.76	7.40	0.237	-9.79	10.9	0.369
Current smoking status: No = 0 or Yes = 1	10.7	5.95	0.073	9.24	8.76	0.292
Current alcohol intake: No = 0 or Yes = 1	3.76	4.30	0.382	-7.12	6.33	0.261
Personal history of polyp: No = 0 or Yes = 1	5.76	8.50	0.498	-18.0	16.0	0.151
Personal history of CRC: No = 0 or Yes = 1	-3.92	14.8	0.791	-46.4	21.8	0.0330
Family history of CRC: No = 0 or Yes = 1	7.47	6.58	0.258	29.1	9.69	0.00284