Comparison of Tissue-Based Molecular Markers in Younger versus Older Patients with Colorectal Neoplasia

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ABSTRACT

Background: Emerging colorectal cancer trends demonstrate increased incidence and mortality in younger populations, prompting consideration of average-risk colorectal cancer screening initiation at age 45 versus 50 years. However, screening test performance characteristics in adults 45–49 years have been minimally described. To inform the biologic rationale for multi-target stool DNA (mt-sDNA) screening in younger patients, we analyzed and compared tissue levels of methylation (*BMP3, NDRG4*) and mutation (*KRAS*) markers included in the FDA-approved, mt-sDNA assay (Cologuard; Exact Sciences Corporation).

Methods: Within 40–44, 45–49, and 50–64 year age groups, archived colorectal tissue specimens were identified for 211 sporadic colorectal cancer cases, 123 advanced precancerous lesions (APLs; adenomas >1 cm, high-grade dysplasia, ≥25% villous morphology, or sessile serrated polyp; 45–49 and 50–64 age groups only), and 204 histologically normal controls. Following DNA extraction, *KRAS, BMP3*, and *NDRG4* were quantified using QuARTS assays, relative to *ACTB* (reference gene).

Results: None of the molecular marker concentrations were significantly associated with age ($P > 0.05$ for all comparisons), with the exception of *NDRG4* concentration in APL samples (higher in older vs. younger cases; $P = 0.008$). However, *NDRG4* levels were also statistically higher in APL case versus normal control samples in both the 45–49 ($P < 0.0001$) and 50–64 ($P < 0.0001$) year age groups.

Conclusions: Overall, these findings support the potential for earlier onset of average-risk colorectal cancer screening with the mt-sDNA assay.

Impact: These novel data address an identified knowledge gap and strengthen the biologic basis for earlier-onset, average-risk screening with the mt-sDNA assay.

Introduction

Average-risk screening for colorectal cancer is recommended by the United States Preventive Services Task Force (USPSTF; ref. 1), Multi-Society Task Force (MSTF; ref. 2), American Cancer Society (ACS; ref. 3), and other national organizations. Structural (colonoscopy, flexible sigmoidoscopy, CT colonography), and stool-based (guaiac-based fecal occult blood test, fecal immunochemical test, multi-target stool DNA) screening strategies are currently endorsed, based on data from randomized trials, epidemiology studies, model simulations, and other sources. Notably, over the past two decades, increased participation in colorectal cancer screening has been associated with accelerated declines in overall colorectal cancer incidence and mortality rates (4–6), providing further support of a favorable public health impact.

In contrast to overall colorectal cancer trends, early onset colorectal cancer (EOCRC; diagnosed prior to age 50 years) incidence and mortality rates are on the rise, with reported increases of 22% and 13%, respectively, during the past two decades (4). Although the absolute rates remain relatively low, EOCRC cases represent 11% of all incident and 7% of all fatal colorectal cancers in the United States (7). Moreover, EOCRC cases account for approximately 22% of the person-years of life lost from colorectal malignancies (3). Birth cohort analyses further demonstrate that colon cancer risk is more than 2-fold higher [IRR = 2.40; 95% confidence interval (CI) = 1.11–5.19] and rectal cancer risk is more than 4-fold higher (IRR = 4.32; 95% CI, = 2.19–8.51) for adults born in 1990 compared with adults born in 1950 (4). In an effort to modify these disturbing trends, ACS recently recommended lowering the age of initiation for colorectal cancer screening from 50–45 years (3). The ACS-endorsed test options remained unchanged, under the assumption that screening performance would be similar in adults 45–49 years compared with older persons. However, the ACS guideline development group acknowledged a paucity of existing data referent to average-risk colorectal cancer screening experiences and outcomes in the sub-50-year-old patient population (3).

To advance current understanding of colorectal cancer screening performance in younger adults, further assessment of technical parameters that might potentially influence test results is needed. Specific to multi-target stool DNA (mt-sDNA) testing, demonstration of age independence in tissue levels for relevant molecular markers (*KRAS, BMP3*, and *NDRG4*; included in the FDA-approved Cologuard assay) would be informative. In this study, we utilized archived, annotated clinical pathology samples to analyze and compare *KRAS, BMP3*, and *NDRG4* by age in malignant, premalignant, and histologically normal colorectal tissues to clarify the biologic potential for leveraging these markers in younger-onset colorectal cancer screening strategies.

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Materials and Methods

Study oversight

Approval for this study was obtained from the Institutional Review Board for Human Research at Mayo Clinic (Rochester, MN).

Subject population

Institutional databases at Mayo Clinic (Rochester, MN) were queried to identify sporadic colorectal cancer cases, advanced precancerous lesion (APL) cases (defined as one or more of the following: adenoma or sessile serrated lesion ≥1 cm in diameter, high-grade dysplasia, or ≥25% villous morphology), and normal controls (colorectal biopsy or resection for nonneoplastic condition), with tissue samples obtained between January 01, 1990 through June 30, 2018. Case and control subjects were selected within 40–44, 45–49, and 50–64 year age groups (based on the date of diagnosis), until targeted sample numbers were achieved or available tissue specimens were exhausted. Because of insufficient numbers of archived APL samples in the 40–44 year age group, APL case subjects were limited to the 45–49 and 50–64 year age groups. Exclusion criteria were defined as: past or current history of colorectal cancer, chemotherapy, radiotherapy, inflammatory bowel disease, primary sclerosing cholangitis, or known heritable predisposition to cancer (Lynch syndrome, familial adenomatous polyposis syndrome, or serrated polyposis syndrome).

Tissue storage, processing, and DNA extraction

Formalin-fixed, paraffin-embedded (FFPE) tissue specimens were archived in a dedicated facility with controlled temperature and humidity, under conditions that facilitate efficient retrieval for IHC and molecular testing (8–10).

Paraffin blocks were serially sectioned in 5-μm increments. One slide was stained with hematoxylin and eosin, with areas of neoplastic or normal tissue identified and confirmed by an experienced gastrointestinal pathologist (to R.P. Graham). Tissue core punches, approximately 200 mm^3 in volume, were shipped to Exact Sciences Laboratories LLC for DNA extraction using Qiagen QIAamp DNA FFPE Tissue Kit, 56404 per manufacturer's protocol. Extracted DNA was then purified and bisulfite converted using ammonium bisulfite chemistry, as described previously (11). All core punches were deidentified prior to shipment and unblinding occurred only after completion of the molecular marker analyses.

Molecular marker analyses

All molecular marker assays were performed at Exact Sciences Laboratories. Quantitative allele–specific real-time target and signal amplification (QuARTS) assays were used to quantify PCR products of mutant *KRAS* relative to *ACTB* (reference gene) and methylated *BMP3* and *NDRG4* relative to bisulfite treated *ACTB* (*BTACT*). Approximately, 10 μL of extracted purified or bisuflite-converted DNA were used for the *KRAS/ACTB* and *BMP3/NDRG4/BTACT* QuARTS reactions, respectively. Plasmid calibrators containing the footprint of the QuARTS reactions were used to quantify the levels of each of the molecular markers, as described (11). Percentage *KRAS* mutation and *BMP3* andf *NDRG4* methylation were computed relative to the reference gene, *ACTB* or *BTACT*, respectively, and used for input to the data analysis.

Demographic factors and tissue characteristics

Electronic medical records were queried to obtain focused, annotated data for evaluable subjects. Demographic factors included age at colorectal tissue acquisition, sex (woman, man), race/ethnicity (Caucasian, African American, other), previous colorectal cancer screening history (yes, no, unknown), personal history of colorectal neoplasia (yes, no, unknown), family history of colorectal neoplasia (yes, no, unknown), and tobacco use (ever, never, unknown). Tissue characteristics included anatomic subsite [proximal colon (cecum, ascending, hepatic flexure, transverse), distal colorectum (splenic flexure, descending, sigmoid, rectum)] for all cases and controls. For colorectal cancer cases, size, stage, grade, and % neoplastic cells per core punch were also recorded.

Statistical analyses

The primary goal of this study was to characterize, and subsequently compare, colorectal tissue levels for methylation (*NDRG4, BMP3*) and mutation (*KRAS*) markers included in the FDA-approved, mt-sDNA assay (Cologuard; Exact Sciences Corporation) in subjects 40–44, 45–49, and 50–64 years of age at the time of colorectal cancer, APL, or normal tissue acquisition. Marker distributions were summarized, by age strata, as a median with corresponding percentiles of 25%, 75%, and 100% and depicted using box-plots. Demographic factors, tissue characteristics, and molecular marker distributions were compared across age groups, overall and by case status, using the Wilcoxon rank sum test/Kruskal–Wallis test for continuous variables or the χ^2 test for categorical variables. This study was powered to detect an average difference in the %-methylation of ± 25% (e.g., 10% vs. 35%) between three age groups. Assuming a SD of 50%, to detect this difference with 80% power using a two-sided significance level of 0.05, an average group size of 54 subjects (total of 162 subjects) was required. When using Bonferroni-corrected significance per age strata for three markers (i.e., 0.05/3 = 0.0167), the average group size required was estimated to be 73 subjects (or 219 subjects in total) to maintain the same level of power.

Results

In total, 788 potentially eligible subjects were identified that met our histologic and age requirements (**Fig. 1**). Of these, 204 did not have enough DNA, 39 were excluded due to assay failure, 4 were excluded for unclear histology findings, and 1 was excluded due to partial removal of the lesion outside of Mayo Clinic, leaving 538 evaluable subjects who met study criteria and had either valid tissue or sufficient DNA recovery samples. By age group and case status, evaluable subjects included: 40–44 year age group (68 total; 32 colorectal cancer cases, 36 normal controls); 45–49 year age group (216 total; 88 colorectal cancer cases, 52 APL cases, 76 normal controls); and 50–64 year age group (254 total; 91 colorectal cancer cases, 71 APL cases, 92 normal controls). Demographic factors including gender, race/ethnicity status, and tobacco use did not differ by age (**Table 1**), while subjects in the older age groups had higher percentages of previous colorectal cancer screening ($P < 0.001$), personal history of colorectal neoplasia ($P = 0.006$), and family history of colorectal cancer ($P = 0.02$).

With respect to tissue characteristics, samples obtained from the proximal and distal colorectum were similarly represented in the 40–44, 45–49, and 50–64 year age groups (**Table 2**). For colorectal cancer cases, histologic grade, and stage at presentation were also proportionally similar for older and younger subjects, while the percentage of neoplastic cells was found to be slightly, but not statistically significantly, higher in the 40–44 year age group compared with the 45–49 year, and 50–64 year age groups ($P = 0.18$).

Overall, none of the tissue-based molecular marker levels were statistically different when compared across the 40–44, 45–49,

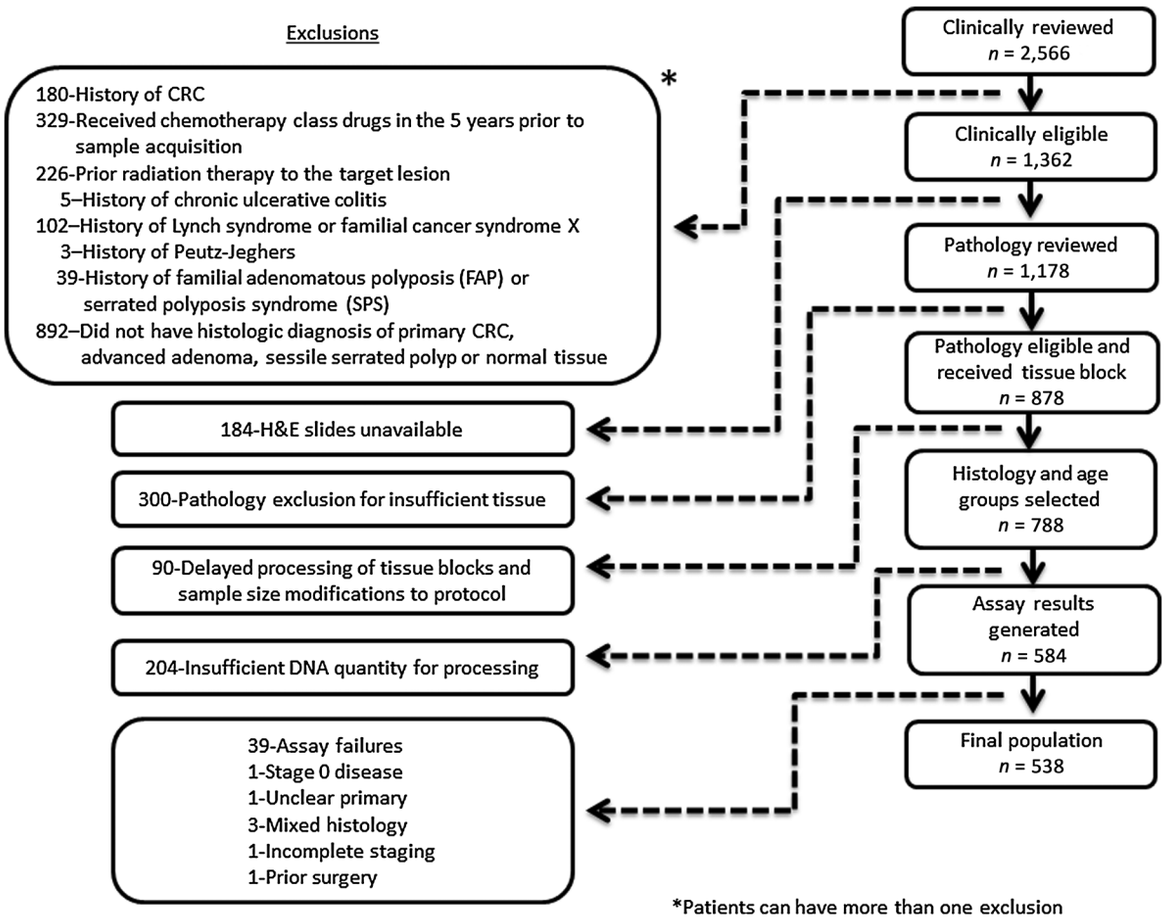

Figure 1.
Flow diagram of study population. Detailed depiction of the process used to identify eligible study subjects. CRC, colorectal cancer.

and 50–64 year age groups (**Table 3** and **Fig. 2**). For colorectal cancer cases and normal control subjects, no age-related patterns in *NDRG4, BMP3*, or *KRAS* were observed. For APL cases, *KRAS* and *BMP3* were statistically similar in the 45–49 and 50–64 year old age groups, while *NDRG4* was higher in the 50–64 year age group compared with the 45–49 year age group ($P = 0.008$). When further analyzed by disease status, *NDRG4* levels in the 45–49 and 50–64 year age groups were higher for colorectal cancer cases ($P < 0.0001$) and APL cases ($P < 0.0001$) than for the corresponding normal controls.

Discussion

In this retrospective, single-center study, tissue levels of mutant *KRAS* and methylated *BMP3* did not differ across age groups, when analyzed overall or by case–control status. For methylated *NDRG4*, colorectal cancer and normal control tissue levels were similar in younger and older subjects, while APL cases exhibited an age-related difference (higher in the older age group). Notably, *NDRG4* tissue levels also remained higher in APL cases than normal controls, regardless of age. In aggregate, these data provide tissue-level support for the "reasonable expectation" (proffered by ACS) that mt-sDNA screening will yield similar performance in adults ages 45–49 years as in persons for whom average-risk colorectal cancer screening is currently recommended (3).

Outside of model simulation studies, data are presently limited with respect to colorectal cancer screening outcomes in the sub-50 year-old population (12–14). Among the guideline-endorsed options, mt-sDNA testing is unique in that the targeted mutation (*KRAS*) and methylation (*BMP3, NDRG4*) markers can be interrogated in malignant, premalignant, and normal colorectal tissue samples from younger patients to inform the biologic rationale for stool-based neoplasia detection. Logically, tissue-based molecular markers that associate with disease state, and are independent of age, should afford the greatest predictive potential. Encouragingly, our findings indicate a high likelihood of comparable colorectal cancer risk stratification with mt-sDNA screening in <50 versus ≥50 year old average-risk screening populations. However, extrapolation of these tissue-based marker concentrations to stool-based assay performance requires additional investigation.

In our study, both *BMP3* and *NDRG4* marker levels were strongly associated with case status (regardless of age), which is consistent with previous reports (15–20). While tissue-based *KRAS* mutation levels were likewise unrelated to age, this marker did not discriminate neoplasia cases from normal controls, most likely reflecting the relatively low overall percentage of colorectal cancer cases that harbor

Table 1. Subject demographics by age group.

	40–44 Years ($N = 68$)	45–49 Years ($N = 216$)	50–64 Years ($N = 254$)
Age, years			
Median (Q1, Q3)	43.59 (42.59, 44.35)	47.75 (46.48, 49.10)	57.69 (53.78, 60.95)
Range	40.42–44.99	45.00–50.00	50.06–64.96
Sex, *n* (%)			
Women	33 (48.5%)	100 (46.3%)	131 (51.6%)
Men	35 (51.5%)	116 (53.7%)	123 (48.4%)
Race/ethnicity, *n* (%)			
African American	0 (0.0%)	3 (1.4%)	2 (0.8%)
Caucasian	64 (94.1%)	194 (89.8%)	238 (93.7%)
Other	4 (5.9%)	19 (8.8%)	14 (5.5%)
Previous CRC screening, *n* (%)			
Yes	1 (1.5%)	14 (6.5%)	110 (43.3%)
No	67 (98.5%)	202 (93.5%)	144 (56.7%)
Personal history of CRN, *n* (%)			
Yes	1 (1.5%)	4 (1.9%)	22 (8.8%)
No	67 (98.5%)	211 (98.1%)	228 (91.2%)
Unknown	0	1	4
Family history of CRC, *n* (%)			
Yes	2 (3.1%)	16 (7.5%)	33 (13.3%)
No	63 (96.9%)	198 (92.5%)	216 (86.7%)
Unknown	3	2	5
Tobacco use, *n* (%)			
Ever	30 (45.5%)	84 (39.4%)	101 (40.1%)
Never	36 (54.5%)	129 (60.6%)	151 (59.9%)
Unknown	2	3	2

Abbreviations: CRC, colorectal cancer; CRN, colorectal neoplasia.

Table 2. Tissue characteristics, by age group.

	40–44 Years ($N = 68$)	45–49 Years ($N = 216$)	50–64 Years ($N = 254$)
Case status			
CRC	32 (47.1%)	88 (40.7%)	91 (35.8%)
Advanced adenoma	0 (0%)	52 (24.1%)	71 (28.0%)
Normal colorectum	36 (52.9%)	76 (35.2%)	92 (36.2%)
Anatomic subsite			
Proximal	50 (73.5%)	162 (75.0%)	183 (72.0%)
Distal	17 (25.0%)	46 (21.3%)	64 (25.2%)
Whole colon	1 (1.5%)	8 (3.7%)	7 (2.8%)
CRC size, cm			
Median (Q1, Q3)	4.75 (3.35, 6.35)	3.00 (1.50, 5.00)	2.20 (1.40, 4.00)
Range	1.20–8.60	0.40–10.00	0.60–20.00
CRC grade, *n* (%)			
2	0 (0.0%)	3 (3.5%)	2 (2.2%)
3	26 (81.2%)	60 (69.8%)	64 (71.9%)
4	6 (18.8%)	23 (26.7%)	23 (25.8%)
CRC stage, *n* (%)			
I	7 (10.3%)	14 (6.5%)	24 (9.4%)
II	6 (8.8%)	15 (6.9%)	20 (7.9%)
III	12 (17.6%)	40 (18.5%)	29 (11.4%)
IV	6 (8.8%)	18 (8.3%)	17 (6.7%)
% Neoplastic cells[a]			
Median (Q1, Q3)	70.00 (60.00, 70.00)	60.00 (60.00, 70.00)	60.00 (60.00, 70.00)
Range	50.00–80.00	50.00–100.00	50.00–90.00

Abbreviation: CRC, colorectal cancer.
[a]Colorectal cancer cases only.

Table 3. Molecular marker concentration by age group.

	40–44 Years	45–49 Years	50–64 Years
NDRG4, %			
Overall ($n = 538$)	68	216	254
Median (Q1, Q3)	0.00 (0.00, 17.58)	0.26 (0.00, 17.97)	0.47 (0.00, 24.50)
Range	0.00–116.47	0.00–102.90	0.00–77.35
CRC ($n = 211$)	32	88	91
Median (Q1, Q3)	19.78 (3.53, 45.46)	9.74 (0.11, 27.98)	17.03 (0.00, 30.87)
Range	0.00–116.47	0.00–102.90	0.00–63.62
APLs ($n = 123$)	N/A	52	71
Median (Q1, Q3)	N/A	8.75 (0.49, 21.79)	21.12 (2.26, 34.69)
Range	N/A	0.00–54.57	0.00–77.35
Normal colorectal tissue ($n = 204$)	36	76	92
Median (Q1, Q3)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)
Range	0.00–1.75	0.00–0.75	0.00–2.88
BMP3, %			
Overall ($n = 538$)	68	216	254
Median (Q1, Q3)	0.00 (0.00, 1.73)	0.05 (0.00, 5.14)	0.09 (0.00, 7.75)
Range	0.00–92.72	0.00–113.21	0.00–74.97
CRC ($n = 211$)	32	88	91
Median (Q1, Q3)	1.98 (0.00, 10.10)	0.17 (0.00, 10.02)	0.18 (0.00, 12.50)
Range	0.00–92.72	0.00–113.21	0.00–68.45
APLs ($n = 123$)	N/A	52	71
Median (Q1, Q3)	N/A	4.47 (0.00, 18.62)	9.25 (0.12, 29.27)
Range	N/A	0.00–76.15	0.00–74.97
Normal colorectal tissue ($n = 204$)	36	76	92
Median (Q1, Q3)	0.00 (0.00, 0.00)	0.00 (0.00, 0.01)	0.00 (0.00, 0.09)
Range	0.00–3.25	0.00–0.58	0.00–0.84
KRAS, %			
Overall ($n = 546$)	68	216	254
Median (Q1, Q3)	0.24 (0.10, 1.29)	0.37 (0.14, 0.96)	0.35 (0.16, 0.88)
Range	0.01–178.65	0.00–520.95	0.02–718.09
CRC ($n = 219$)	32	88	91
Median (Q1, Q3)	0.29 (0.07, 12.90)	0.27 (0.10, 7.76)	0.27 (0.12, 25.83)
Range	0.01–178.65	0.00–520.95	0.02–243.87
APLs ($n = 123$)	N/A	52	71
Median (Q1, Q3)	N/A	0.31 (0.15, 0.95)	0.37 (0.22, 0.63)
Range	N/A	0.02–63.35	0.03–718.09
Normal colorectal tissue ($n = 204$)	36	76	92
Median (Q1, Q3)	0.23 (0.13, 0.54)	0.44 (0.23, 0.74)	0.37 (0.24, 0.70)
Range	0.03–2.44	0.02–6.43	0.03–30.91

Abbreviation: CRC, colorectal cancer.

a *KRAS* mutation (31%; https://cancer.sanger.ac.uk/cosmic; accessed August 30, 2019). Ongoing assessment of novel molecular marker combinations (without *KRAS*) appears promising (21) and may serve to increase the attainable accuracy of noninvasive colorectal cancer and APL detection with mt-sDNA screening.

To our knowledge, we report the first comparison of screen-relevant molecular markers in younger versus older colorectal cancer, APL, and normal control subjects. These findings add to the paucity of empirical data related to the molecular biology of EOCRCs, and bolster a critical underpinning of the recent ACS guideline recommendation (3). However, recognized limitations of our study should also be considered. First, the molecular analyses were based on archived, FFPE tissue specimens from which DNA could be adequately extracted, potentially biasing our results toward larger, more advanced-stage lesions. Second, while sufficient for the described molecular marker associations with age group and disease state, the statistical power provided by our relatively small case and control sample set did not permit more detailed analyses of additional demographic factors or tumor characteristics. Third, an insufficient number of archived APL tissue samples were available for the 40–44 year age group, which prevented us from conducting molecular marker analyses on precancerous neoplasia in this subject subset. Fourth, our subjects were predominately Caucasian, which may influence the generalizability of our findings to more racially/ethnically diverse populations.

In summary, we found that tissue levels of mt-sDNA markers *KRAS, BMP3*, and *NDRG4* were statistically similar and/or associated with neoplastic lesions when analyzed across 40–44, 45–49, and 50–64 year age groups. These data address an identified knowledge gap and strengthen the biologic basis for earlier-onset, average-risk screening with the mt-sDNA assay. Forthcoming results from a prospective, multicenter study of mt-sDNA screening in average-risk 45–49 year olds (ClinicalTrials.gov Identifier NCT03728348) will augment the current findings and provide further clarity regarding the clinical application of these results.

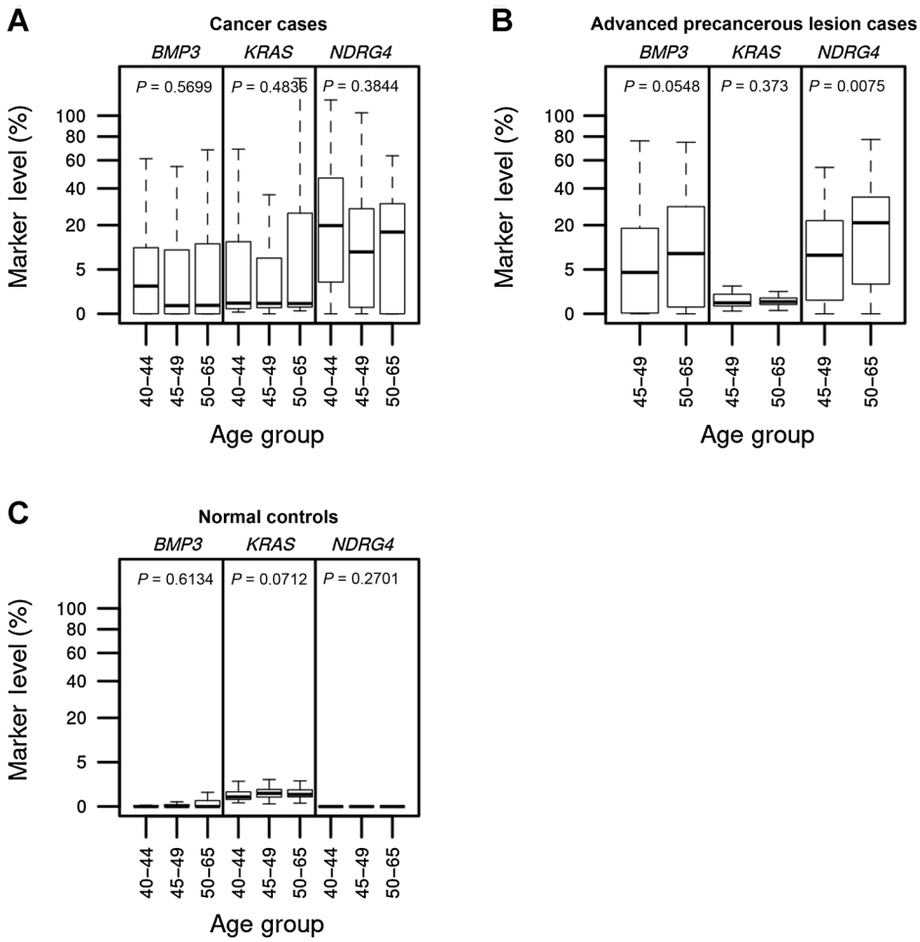

Figure 2.

Molecular marker levels, by age group and case status. Box-and-whisker plots demonstrating *BMP3, KRAS*, and *NDRG4* tissue levels by age group, for colorectal cancer (CRC) cases (**A**), advanced precancerous lesion (APL) cases (**B**), and normal controls (**C**).

Disclosure of Potential Conflicts of Interest

P.J. Limburg is a chief medical officer for screening at and has ownership interest (stock) in Exact Sciences. D.W. Mahoney has ownership interest (including patents) in Exact Sciences. D.A. Ahlquist is a consultant for and has ownership interest (including patents) in Exact Sciences. H.T. Allawi is Vice Present, Research at Exact Sciences. S.C. Johnson is Senior Vice President, R&D Systems at Exact Sciences. M. Kaiser is a principal scientist at and has ownership interest in Exact Sciences Development Company, LLC. V.E. Katerov is a senior research scientist at Exact Sciences. S. Statz is Senior Vice President, Clinical & Regulatory Affairs at and has ownership interest (including patents) in Exact Sciences. G.P. Lidgard reports receiving other remuneration from Exact Sciences. J.B. Kisiel reports receiving a commercial research grant from and has ownership interest (including patents) in Exact Sciences. No potential conflicts of interest were disclosed by the other authors.

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