# Measurement of the IgM and IgG Autoantibody Immune Responses in Human Serum has High Predictive Value for the Presence of Colorectal Cancer

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## Abstract

**There is an unmet clinical need for a minimally invasive, sensitive, and specific method for detecting the presence of colorectal cancer and pre-malignant lesions. This study describes a novel minimally invasive enzyme-linked immunosorbent assay-based method, capable of identifying patients with colorectal cancer as distinct from both normal and adenoma samples with a cumulative sensitivity and specificity of 70.8% and 86.5%, respectively.**

**Introduction:** Colorectal cancer is a major public health issue, with incidences continuing to rise owing to the growing and aging world population. Current screening strategies for colorectal cancer diagnosis suffer from various limitations, including invasiveness and poor uptake. Consequently, there is an unmet clinical need for a minimally invasive, sensitive, and specific method for detecting the presence of colorectal cancer and pre-malignant lesions. **Patients and Methods:** An indirect enzyme-linked immunosorbent assay was used to measure the primary (IgM) and secondary (IgG) adaptive humoral immune responses to a panel of previously identified cancer antigens in the sera of normal and adenoma samples, and sera from patients with colorectal cancer. **Results:** An optimal panel of 7 biomarkers capable of identifying patients with colorectal cancer as distinct from both normal and adenoma samples is identified. The cumulative sensitivity and specificity of the assay are 70.8% and 86.5%, respectively. The positive and negative predictive values of the cohort are 77.3% and 82.1%. This assay was not able to accurately discriminate between normal and adenoma samples. Patients whose serum was positive for the presence of anti-ICLN IgM autoantibodies had a significantly poorer 5-year survival than patients whose serum was negative ($P = .004$). **Conclusion:** This study describes a novel minimally invasive enzyme-linked immunosorbent assay-based method, capable of identifying patients with colorectal cancer as distinct from both normal and adenoma samples. Patients are likely to be far more amenable to a blood-based test such as the one described herein, rather than a fecal-based test, likely leading to increased patient uptake.

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## Introduction

Colorectal cancer (CRC) is currently the third most commonly diagnosed malignant cancer in the world, with its incidences being indiscriminate and widespread throughout the world.[1] Diagnosing CRC can be a difficult task, as individuals often present to their physician with initial symptoms that are nonspecific for CRC and commonly related to many other conditions.[2,3] Preventative screening for CRC is one course of action used globally as a means to increase early diagnosis and decrease mortality rates. Despite the fact that screening has been proven with certainty to be of substantial net benefit and highly cost-effective,[4-8] many countries have thus far failed to implement population-based CRC screening.

Colonoscopy is currently the gold-standard for CRC diagnosis, yet colonoscopy procedures are expensive, and this often prohibits their use as a primary screening mechanism, particularly in underdeveloped regions. Therefore, several population-based screening programs use a 2-step approach, using colonoscopy only for diagnostic clarification in those with a positive first-line screening test. Fecal (stool)-based screening methods are commonly used as a first-line screening test and have acceptable sensitivity and specificity,[9-11] yet suffer from poor patient uptake, largely owing to the requirement of persons to handle their feces.[12,13] Thus, the evidence surrounding the current screening methods for CRC points to the necessity of a new diagnostic test that is affordable, reliable, and accurate, as well as being accessible to patients.[7,14]

The human body recognizes cancer-associated antigens as foreign or non-self and triggers an immune response that often results in the elimination of cancer cells via the innate immune system.[15-17] However, many malignant cells survive this primary reaction and hence are not eliminated, necessitating a secondary response. Both IgG and IgM autoantibodies are generally produced as part of the secondary adaptive immune response and are secreted into the bloodstream. Serum antibody profiling has been used to identify novel antibodies against a number of tumor antigens in patients with various tumor types[18-22] and is a particularly attractive concept because a single draw of blood could potentially allow for screening for the presence of several different cancers.

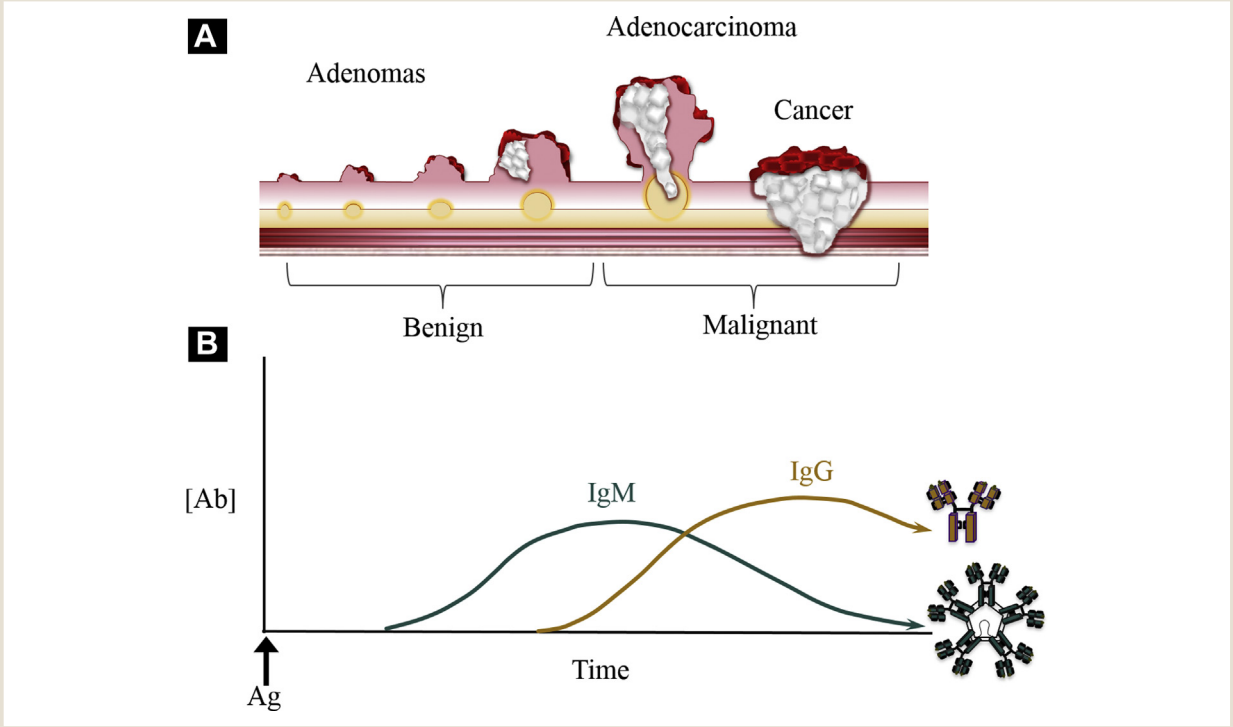
Traditionally, cancer immunology studies have focused mainly on IgG autoantibodies in relation to the diagnosis and treatment of cancer.[23,24] To date, IgM autoantibodies have not been well-studied and may provide an earlier indication of the presence of cancer. This study sets out to measure both the IgM and IgG autoantibody responses to a panel of previously identified cancer-specific antigens[18] in human serum and to determine their ability to differentiate between sera of normal and adenoma samples, and sera from patients with CRC (Figure 1).

## Patients and Methods

### *Patients and Samples*

All patients were symptomatic and therefore referred for colonoscopy. All sample types including normal, adenoma, and cancer were studied, and thus there were no specific inclusion criteria. Patients under 18 years of age were excluded. This study was approved by the Ethics (Medical) Research Committees at Beaumont Hospital,

**Figure 1** Colorectal Cancer Progression and the Related Autoantibody Responses. A, Schematic Representation of Colorectal Cancer Progression From Benign Adenoma to Malignant Cancer; B, Comparison of the IgM and IgG Innate Autoantibody Responses to Cancer



Dublin and Connolly Hospital, Dublin. Written informed consent was obtained from all patients. Subjects were asked to provide a blood sample prior to colonoscopy. Serum was isolated from whole blood by centrifugation and stored at −80°C. Diagnosis of CRC was independently verified by a consultant pathologist.

### *Protein Expression and Purification*

*E. coli* expression clones encoding recombinant human CADM1, HMGB1, ICLN, p53, SEC16, zinc finger protein 700 (ZNF700), and zinc finger protein 768 (ZNF768) were obtained from ImaGenes GmbH (Berlin, Germany) and were verified by sequencing (Source Bioscience Sequencing, Dublin, Ireland). Each recombinant human protein was overexpressed in *E. coli*, as described previously.[25] The *E. coli* cells were lysed using lysis buffer, releasing the recombinant human protein. The proteins were purified using immobilized-metal affinity (Nickel-NTA resin, Qiagen) chromatography under denaturing conditions. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting were used to confirm the successful expression and isolation of each protein of interest. Protein concentrations were determined using absorbance determination with a Nanodrop spectrophotometer (ND- 1000, Thermo Scientific) at 280 nm.

### *Autoantibody Detection*

An indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect the presence/absence of autoantibodies in human serum.[25] In brief, microtiter plates were coated with the recombinant human proteins CADM1, HMGB1, ICLN, p53, SEC16, ZNF700, and ZNF768 (overnight incubation (o/n) at 4°C; 100 µL/well; 5 µg/mL). Plates were washed in 3× washes of 1 × phosphate-buffered saline-Tween followed by 1 × phosphate-buffered saline and blocked with 5% (w/v) bovine serum albumin (3-4 hours, 37°C). The plates were then incubated with human sera from the patient cohorts diluted in assay diluent (15 µL/mL; 100 µL/well; o/n at room temperature). Following subsequent washing, plates were incubated with either (1) mouse anti-human IgG (1 hour at 37°C; 100 µL/well; 1 in 10,000 dilution) and followed by peroxidase-labelled rabbit anti-mouse IgG (1 hour at 37°C; 100 µL/well; 1 in 5000 dilution) or (2) mouse anti-human IgM antibody (1 hour at 37°C; 100 µL/well; 1 in 1000 dilution) followed by peroxidase-labeled goat anti-mouse IgM antibody (1 hour at 37°C; 100 µL/well; 1 in 1000 dilution). Tetramethylbenzidine was used as the substrate for the peroxidase reaction (RT; 100 µL/well), and the enzymatic reaction was stopped after 10 minutes with 1 N HCl (room temperature; 50 µL/well). The optical density (OD) was read at 450 nm using an ELISA plate reader (Safire2, Tecan). Human IgG and IgM (50 µg/mL) was used in duplicate as a positive control in respective microtiter plates and used to determine relative OD ELISA values.

### *Statistical Analysis*

ELISA cutoffs were calculated as the average of the normal relative OD values + 2 × standard deviation. Serum samples with ELISA values above the 'cutoff' were identified as positive for autoantibodies against the proteins of interest. Specificities and sensitivities for individual and grouped antigens were calculated according to Altman and Bland.[21] Briefly, specificity was calculated as the proportion of true negatives correctly identified as normal by the assay from the total number of normal patients. Sensitivity was calculated as the proportion of true positives correctly identified as such by the assay (presence of at least 1 autoantibody response). Cumulative specificity and sensitivity were calculated by merging individual specificity and sensitivity values, whereby samples with more than 1 of the 5 autoantibodies contributed only once to the calculation. The positive and negative predictive values (PPV and NPV, respectively) refer to the proportions of positive and negative results in diagnostic tests that are truly positive and negative results, respectively. Five-year survival analysis was plotted according to the Kaplan-Meier method. Patients with follow-up information over 5 years were censored at year 5 post-diagnosis. All tests were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA), and the findings were considered statistically significant at $P < .05$.

**Table 1** Clinical and Pathologic Details of Patient Cohort

| | Normal (n = 37) | Adenoma (n = 53) | Cancer (n = 24) |
|---|---|---|---|
| Gender | | | |
| Female | 17 | 18 | 12 |
| Male | 20 | 35 | 12 |
| Age, y | | | |
| Mean | 67 | 67 | 67 |
| Adenomas | | | |
| 1 | – | 26 | – |
| 2 | – | 14 | – |
| 3 | – | 6 | – |
| 3+ | – | 7 | – |
| Adenoma type | | | |
| Tubular | – | 34 | – |
| Tubulovillous | – | 25 | – |
| Serrated | – | 7 | – |
| Tumor site | | | |
| Colon | – | – | 13 |
| Rectum | – | – | 10 |
| Not stated | – | – | 1 |
| Tumor stage | | | |
| pT1 | – | – | 2 |
| pT2 | – | – | 5 |
| pT3 | – | – | 12 |
| pT4 | – | – | 5 |
| Nodes | | | |
| N0 | – | – | 16 |
| N1 | – | – | 5 |
| N2 | – | – | 3 |
| Metastasis | | | |
| Mx | – | – | 19 |
| M1 | – | – | 5 |
| Vascular invasion | | | |
| Yes | – | – | 5 |
| No | – | – | 19 |
| Follow-up, mo | | | |
| Mean | – | – | 56 |

**Table 2** Sensitivity and Specificity of IgG and IgM Autoantibody Panel at Detecting the Presence of Colorectal Cancer

| | Individual Sensitivity, % | Individual Specificity, % | Positive Predictive Value, % | Negative Predictive Value, % | Cumulative Sensitivity, % | 95% Confidence Intervals | Cumulative Specificity, % | 95% Confidence Intervals |
|---|---|---|---|---|---|---|---|---|
| SEC 16 IgM | 25.0 | 97.3 | 85.7 | 66.7 | 25.0 | 9.78-46.7 | 97.3 | 85.8-99.9 |
| ZNF 768 IgM | 33.3 | 94.6 | 83.3 | 71.4 | 41.7 | 22.1-63.4 | 94.6 | 81.8-99.3 |
| ZNF 700 IgG | 25.0 | 91.9 | 76.5 | 75.0 | 54.2 | 32.8-74.5 | 89.2 | 74.6-97.0 |
| p53 IgG | 12.5 | 97.3 | 77.8 | 76.7 | 58.3 | 36.6-77.9 | 89.2 | 74.6-97.0 |
| HMGB 1 IgG | 12.5 | 94.6 | 75.0 | 78.1 | 62.5 | 40.6-81.2 | 86.5 | 71.2-95.5 |
| CADM 1 IgM | 16.7 | 94.6 | 76.2 | 80.0 | 66.7 | 44.7-84.4 | 86.5 | 71.2-95.5 |
| ICLN IgM | 16.7 | 94.6 | 77.3 | 82.1 | 70.8 | 48.9-87.4 | 86.5 | 71.2-95.5 |

## Results

### *Patient Characteristics*

In total, 37 individuals with normal colonoscopies, 53 patients diagnosed with adenomas present, and 24 patients with newly diagnosed CRC were included in the study. In total, 67 men and 47 women were included. The average age of diagnosis in each cohort was 67 years. In the adenoma cohort, tubular adenomas (n = 34) were the most common type of adenoma diagnosed, followed by tubulovillous (n = 25) and serrated (n = 7) adenomas. Twenty-six patients had only 1 adenoma lesion present, whereas 27 patients had more than 1 adenoma lesion present. In the colorectal cancer cohort, 13 patients were diagnosed as having a tumor in their colon, 10 patients had rectal tumors, and in one case, the exact location of the tumor was not reported. One-half (12/24) of the CRC cases were diagnosed as having stage 3 CRC. The majority of patients were found to be negative for the presence of cancer cells in surrounding lymph nodes (16/24) and in secondary organs (19/24). The clinical and pathologic characteristics of the cohort are shown in Table 1.

### *Multiplexing the IgM and IgG Responses Predicts the Presence of CRC in Human Serum*

We measured the IgG and IgM autoantibody responses of a previously identified multi-marker panel and identified an optimal panel of 7 biomarkers. The antigens included were Cell Adhesion Molecule 1 (CADM1), High mobility group protein B1 (HMGB1), Methylosome subunit pICln (ICLN), Cellular tumor antigen p53 (p53), SEC16 Homolog A (SEC 16), Zinc finger protein 700 (ZNF 700), and Zinc finger protein 768 (ZNF768). The panel was comprised of 4 IgM responses (CADM1, ICLN, SEC 16, and ZNF 768) and 3 IgG responses (HMGB1, p53, ZNF 700). Each biomarker had an individual sensitivity of between 12.5% and 33.3%, and each had an individual specificity of greater than 91.9% (Table 2). The cumulative sensitivity of the panel is 70.8%, and the cumulative specificity is 86.5%. The PPV of the cohort is 77.3% and the NPV is 82.1% (Table 2).

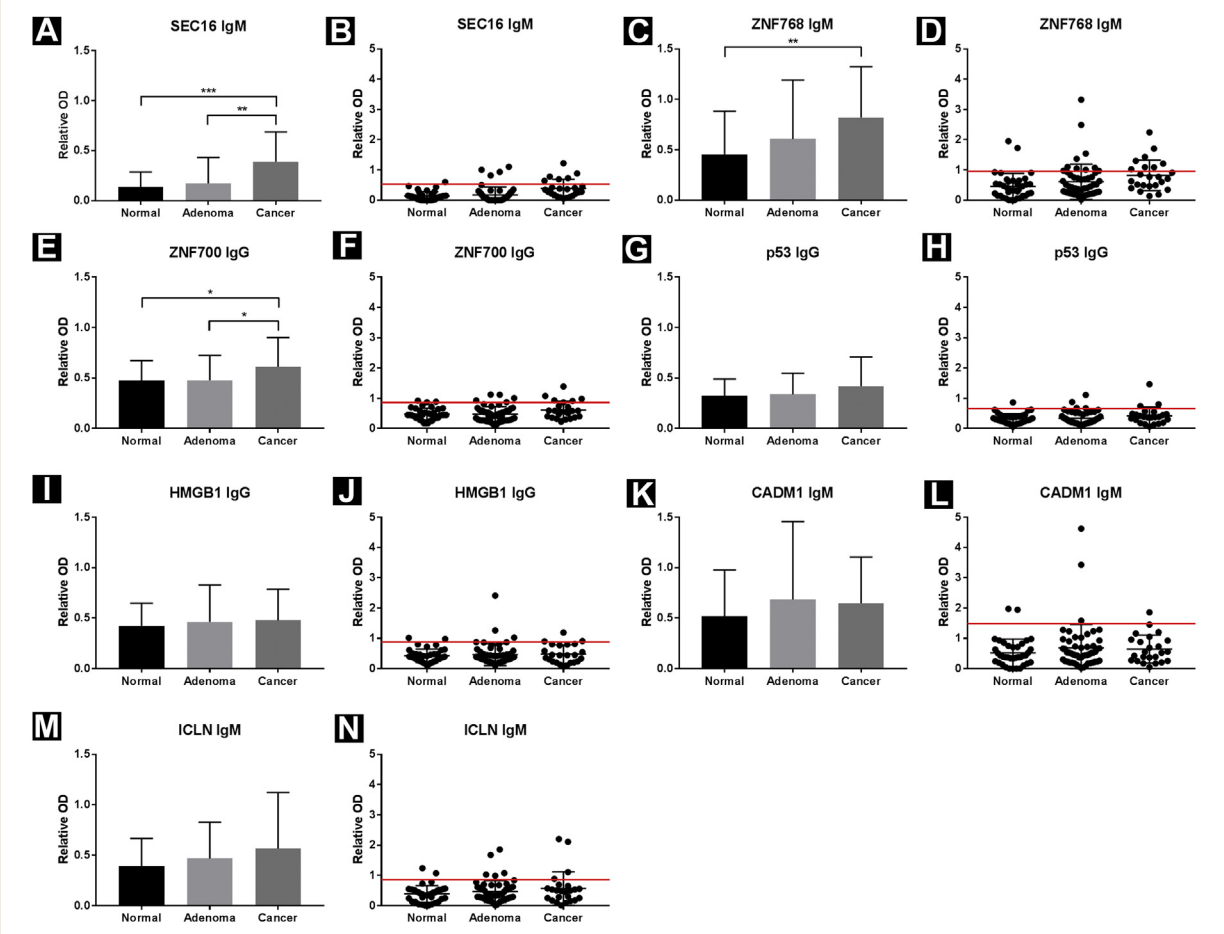
### *The IgM and IgG Autoantibody Responses Are Increased in CRC but Do Not Differentiate Between Adenoma and Normal*

In general, the measured autoantibody responses are higher in the CRC cancer cohort than in the adenoma and normal cohorts (Figure 2). There was a significantly higher autoantibody response to antigens SEC 16 (IgM), ZNF 768 (IgM), and ZNF 700 (IgG) in the CRC cohort than in the normal cohort ($P \leq .05$). The CRC cohort also had significantly higher autoantibody response to SEC 16 (IgM) and ZNF 700 (IgG) than the adenoma cohort ($P \leq .05$). No significant differences in the measured IgG and IgM autoantibody responses were found between the adenoma and normal cohorts. This is true of all of the antigens in the cohort (Figure 2).

### *The Presence of Anti-ICLN IgM Autoantibodies May Predict Prognosis in Patients With CRC*

Kaplan-Meier analysis was performed to assess the association between patient survival and autoantibody response (Figure 3). The 5-year overall survival (OS) analysis showed that patients with CRC

**Figure 2** Relative OD Autoantibody Signals Detected in Normal, Adenoma, and Patients With Colorectal Cancer by Indirect ELISA. SEC16 IgM, ZNF768 IgM, ZNF700 IgG, p53 IgG, HMGB1 IgG, CADM1 IgM, and ICLN IgM Were Used as Capture Antigens. Mean Relative OD Responses (A, C, E, G, I, K, M) and Individual OD Values (B, D, F, H, J, L, N) in Each Cohort Are Represented. The Middle Black Line Represents the Mean of Relative ODs Within Each Patient Cohort. The Red Line Represents ELISA Cut-offs Calculated as the Average of the Normal Relative OD Values + 2 × Standard Deviation. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$




Abbreviations: ELISA = enzyme-linked immunosorbent assay; OD = optical density.

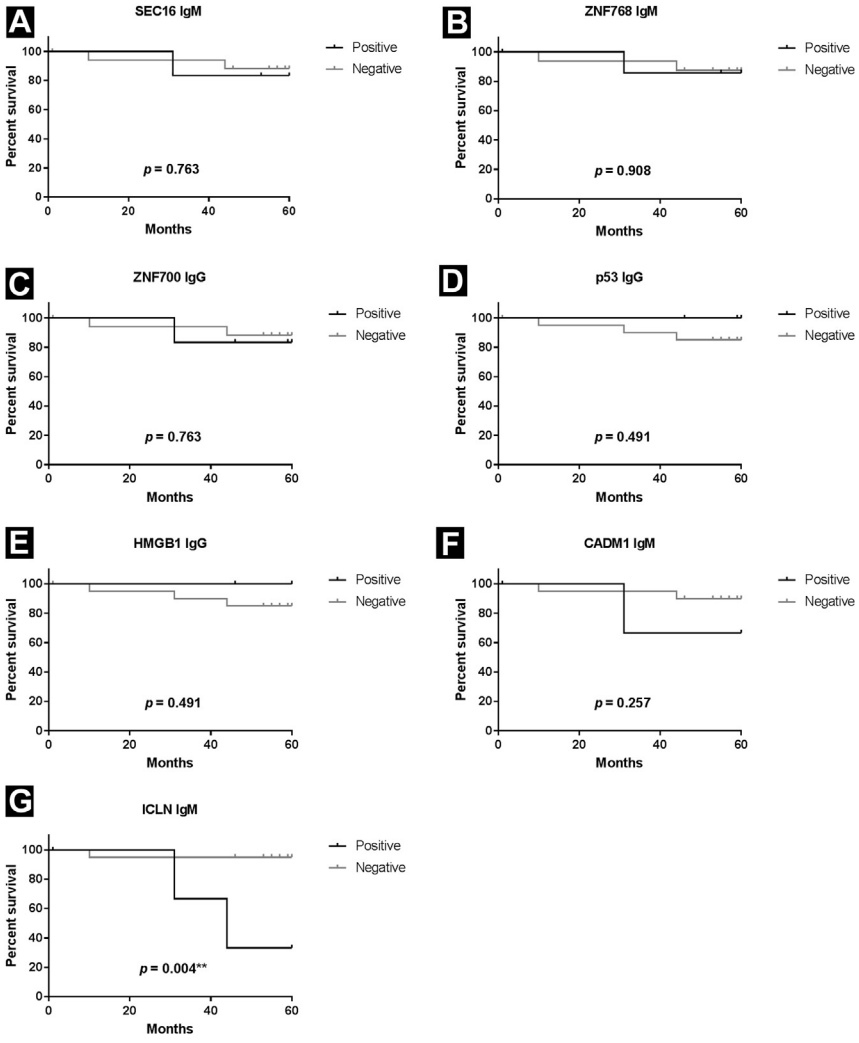
whose serum was positive for the presence of anti-ICLN IgM autoantibodies had a significantly poorer 5-year survival than patients whose serum was negative ($P = .004$). This suggests that testing for the presence of anti-ICLN IgM autoantibodies in patients with CRC patients may help to predict prognosis in addition to its usefulness in disease diagnosis.

## Discussion

Globally, there are currently 2main categories of population-based screening tests in use in CRC detection; bowel visualization techniques and fecal-based tests.[26-28] Though the sensitivity and specificity of these screening modalities have increased in recent years, these tests still have many limitations. Colonoscopies are expensive and can expose asymptomatic individuals to unnecessary risk such as cardiovascular events as a result of the need for sedation and the risk of colonic perforation as a result of the procedure itself.[13,29] Fecal-based screening tests suffer from poor patient uptake due to the unpleasant nature of these tests.[12,13] Therefore, there remains an unmet clinical need for a noninvasive, sensitive, and specific method for detecting the presence of CRC and premalignant lesions. This could lead to significantly increased uptake of CRC screening and hence decreased mortality rates in future. The indirect ELISA-based screening method described in this study offers simple solutions to many of the issues of the current screening methods. Sample collection for serum assays is simpler, requiring only a minimally invasive blood draw versus a single or multiple stool samples for fecal test kits and invasive bowel visualization techniques. ELISA testing also has the advantage of being far more cost-effective than endoscopic-radiologic procedures and is also likely to be far better tolerated by patients than the current unpleasant fecal-based tests.

Our previous work used high-density protein arrays to identify a unique antibody profile capable of discriminating between symptomatic patients with and without CRC.[18] Several of the antigens

**Figure 3** Presence of Autoantibodies and Colorectal Cancer Prognosis. Kaplan-Meier Analysis Revealed That Presence of IgM Autoantibodies Specific to the ICLN Protein (G) Predicts 5-year Survival in Colorectal Cancer (**$P \leq .01$). A, SEC16 IgM; B, ZNF768 IgM; C, ZNF700 IgG; D, p53 IgG; E, HMGB1 IgG; and F, CADM1 IgM Do Not Predict 5-year Survival in Colorectal Cancer




corresponding to the autoantibodies identified previously have since been implicated in the progression of CRC,[30,31] which further validates the rationale for measuring the autoantibody response to disease as a tool for diagnosis. Multiplexing the measurement of the IgM autoantibody response with the IgG response has the advantage of potentially identifying malignant lesions at an earlier stage than with the IgG response alone, as IgM antibodies have been shown to play an important role in immunosurveillance mechanisms against epithelial tumors in humans.[32]

In this study, we identify an optimal panel of 7 biomarkers from a previously identified panel of 18 biomarkers,[18] capable of identifying patients with CRC as distinct from both normal and adenoma samples. The optimized panel of antigens included 4 IgM responses (CADM1 IgM, ICLN IgM, SEC 16 IgM, and ZNF 768 IgM) and 3 IgG responses (HMGB1 IgG, p53 IgG, and ZNF 700 IgG). The individual sensitivity of each biomarker was between 12.5% and 33.3%, and each had an individual specificity of greater than 91.9% (Table 2). However, combining the antigens in a multiplexed fashion results in a significant increase in both sensitivity and specificity, as well as the PPV and NPV of the assay. The cumulative sensitivity of the panel is 70.8%, the cumulative specificity is 86.5%, and the PPV and NPV of the cohort are 77.3% and 82.1%, respectively. These results are comparable with currently used noninvasive screening tests.[29,33,34]

It is well-established that most adenomas are benign, with fewer than 10% of adenomas going on to become malignant.[35] This assay was not able to accurately discriminate between normal and adenoma samples (Figure 2), which advocates that adenomas more closely resemble normal tissue than cancer. These findings also suggest that the immune response is only activated after an adenoma undergoes a malignant transformation, as depicted in Figure 1. Taken together, these results indicate that this novel screening test

can accurately differentiate between benign and malignant lesions, thus making it more effective as a cancer-specific screening method.

Kaplan-Meier analysis revealed that patients with CRC who were found to have autoantibodies against the methylosome subunit pICln (ICLN) protein had significantly poorer 5-year survival than patients whose serum was negative ($P = .004$). This suggests that testing for the presence of anti-ICLN IgM autoantibodies in the serum of patients with CRC, in addition to its usefulness in diagnosis, may also be a prognostic biomarker. Prognostic biomarkers can help clinicians to identify patients who may potentially benefit from further treatment.[36]

A limitation of this study is that the sensitivity and specificity of the current assay is not greater than 90% and thus is not accurate enough to be used as a stand-alone diagnostic test for the detection of CRC. The assay would require a confirmatory colonoscopy to be carried out as with the currently used fecal-based screening tests. One potential application for this current assay is as a triage tool for colonoscopy in cases where other tests or presenting symptoms were inconclusive, or the colonoscopy procedure bears an increased risk to the patient. The sensitivity and specificity of the assay could potentially be further improved by adding biomarker proteins to the panel that are known to be altered in cancer such as KRAS, matrix metalloproteinases, and carcinoembryonic antigen, thereby improving the overall performance of the test.

This study describes a minimally invasive, cost-effective ELISA-based method that combines both the IgM and IgG innate immune responses in measuring the humoral autoantibody response to previously identified biomarkers of CRC,[18] using only a small volume of patient serum. An optimum panel of biomarkers capable of identifying patients with CRC as distinct from both normal and adenoma samples is identified, and the results demonstrate that the assay has comparable sensitivity and specificity and PPV and NPV to the current fecal exam kits.[26,33,34] However, patients are likely to be far more amenable to a blood-based test such as the one described herein, rather than a fecal-based test, and therefore, this novel assay could provide significant clinical utility if used as a first-line screening method in population-based screening methods for CRC.

### *Clinical Practice Points*

- CRC is currently the third most commonly diagnosed malignant cancer in the world.
- Cancer screening has been proven with certainty to be of substantial net benefit and highly cost-effective, yet many countries have thus far failed to implement population-based CRC screening.
- Current screening strategies for CRC diagnosis suffer from various limitations, including invasiveness and poor patient uptake.
- IgG and IgM autoantibodies are generally produced as part of the secondary adaptive immune response and are secreted into the bloodstream.
- This study sets out to measure both the IgM and IgG autoantibody responses to a panel of previously identified cancer-specific antigens in human serum and to determine their ability to differentiate between normal tissue, adenoma, and patients with CRC.
- The assay can detect cancer samples as distinct from normal and adenoma samples.
- The cumulative sensitivity of the panel is 70.8%, the cumulative specificity is 86.5%, and the PPV and NPV of the cohort are 77.3% and 82.1%, respectively.
- These results are comparable with currently used noninvasive screening tests.
- Evidence suggests patients are likely to be more amenable to a blood-based test such as the one described herein, rather than a fecal-based test, leading to increased patient uptake.

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## Disclosure

The authors have stated that they have no conflicts of interest.

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