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ORIGINAL RESEARCH

Associations of Circulating Inflammatory Biomarkers with Risk Factors for Colorectal Cancer in Colorectal Adenoma Patients

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Abstract: Obesity and central adiposity are associated with colorectal cancer risk and have been linked to inflammation. Inflammation is a complex, interactive response that may most accurately be summarized through multiple, simultaneously measured cytokines. In this cross-sectional analysis, we investigated associations of circulating plasma levels of C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), and a combined inflammation *z* score with risk factors for colorectal cancer in colorectal adenoma patients (n = 92). Multivariable logistic regression was used to investigate associations between cytokine levels and known risk factors for colorectal neoplasms. Mean cytokine levels tended to increase with increasing body mass index (BMI), with statistically significant trends in relation to CRP, IL-6, and the combined inflammation *z* score (*P* for trend < 0.001, 0.02, and <0.001, respectively). The odds ratios for associations of the inflammation *z* score with being overweight (BMI 25–29.9 kg/m^2), obese (BMI $\geq$ 30 kg/m^2), or having a high waist-to-hip ratio were 4.33 (95% CI [confidence interval], 1.04–18.00), 5.54 (95% CI, 1.37–22.42), and 4.09 (95% CI, 1.67–9.98), respectively. Our findings support (1) associations of inflammation with increased general and central adiposity and (2) investigation of a combined inflammation score as a risk factor for colorectal neoplasms.

Keywords: colonic neoplasms, inflammation, biomarkers

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer mortality in the United States with 70% of cases having no family history of the disease, pointing to a large environmental component and, hence, its preventability.[1] Among the potentially modifiable risk factors for CRC, physical activity, non-steroidal anti-inflammatory drug (NSAID) use, and calcium intake have consistently been associated with decreased risk, while adiposity is associated with increased risk.[2–4] Other factors previously examined and fairly well supported to modulate risk for CRC are smoking, serum 25-OH-vitamin D levels, and energy intake.[5–7] Established, non-modifiable risk factors for CRC are old age, family or personal history of CRC, and inflammatory bowel disease.[8]

There is increasing evidence for a link between inflammation and colorectal cancer: NSAID use reduced sporadic colorectal adenoma recurrence in clinical trials, and specific pro-inflammatory markers, such as C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), are elevated in inflammatory bowel disease and colorectal adenoma patients.[3,8–11] CRP, TNF-α, and IL-6 also have been directly associated with adenomas, higher tumor grade, and increased risk of mortality in colorectal cancer patients.[8,12,13] High levels of these inflammatory markers were also found to be directly associated with risk factors for colorectal adenomas, such as age, smoking, and adiposity in a case-control study.[14]

To further investigate potential links between risk factors for CRC and biomarkers of inflammation, individually and in combination, we examined associations between circulating levels of CRP, TNF-α, IL-6, IL-8, and IL-1β as well as a previously reported combined *z* score,[15] with potential colorectal cancer risk factors (sex, age, body mass index [BMI], waist-to-hip ratio [WHR], NSAID use, serum 25-OH-vitamin D, physical activity, and total energy, calcium, and alcohol intakes) in a population of colorectal adenoma patients.

Materials and Methods

This study was approved by the Emory University Institutional Review Board. Written informed consent was obtained from each study participant.

Study population and procedures

This cross-sectional study was conducted using stored blood samples collected at baseline from participants in a randomized controlled chemoprevention trial that administered calcium and/or vitamin D to sporadic colorectal adenoma patients. The detailed protocol of study recruitment and procedures was published previously.[16] Briefly, study participants were recruited from the patient population attending the Digestive Diseases Clinic of Emory University between April 2005 and January 2006. Eligibility criteria included age 30 to 75 years, good general health, capability of giving informed consent, and having at least one pathology-confirmed sporadic colorectal adenoma in the past 36 months. Exclusion criteria included the use of calcium or vitamin D supplements and any medical conditions, habits, or medication usage that would otherwise interfere with the study.[16]

The 92 participants who were randomized into the trial underwent a blood draw and answered questionnaires at baseline that included the Willett Food Frequency questionnaire. Participants were asked to abstain from using aspirin (but not other NSAIDs) for 7 days before the blood draw visit. All visits for a given participant were scheduled at the same time of day to control for possible circadian variability in the outcome measures. Peripheral venous blood samples were taken after subjects had been seated upright with their legs uncrossed for five minutes. Blood was drawn into red-coated, pre-chilled Vacutainer tubes for whole blood, plasma, and serum and then immediately placed on ice and shielded from light. The blood collection tubes for plasma and serum were centrifuged with refrigeration, the blood fractions were aliquotted into amber-colored cryopreservation tubes, the air was displaced with argon gas, and then the aliquots were immediately placed in a −80 °C freezer until analysis.

Inflammation biomarker analyses

A single enzyme linked immunoassay (ELISA) (R&D systems, Minneapolis, MN) was used to measure CRP, and a high sensitivity multiplex ELISA (R&D systems, Minneapolis, MN) was used to measure TNF-α, IL-6, IL-1β, and IL-8 in duplicate according to the manufacturer's protocol in 2010. The average intra-assay coefficient of variation (CV) for CRP was 6.6%, for TNF-α, 11.5%, for IL-6, 11.7%, for IL-1β,

10.6%, and for IL-8, 7.9%. Cytokine levels below the limits of detection were assigned a value equal to the lower limit of detection for that cytokine.

Statistical analysis

Risk factors for CRC that were evaluated included sex, regular NSAID use (≥once per week), adiposity (indicated by BMI and WHR), age, physical activity (in METs/d), total energy and calcium intakes, and serum 25-OH-vitamin D level. All continuous variables except BMI were dichotomized as low for values below the 50th percentile and high for values at the 50th percentile and above; WHR and alcohol intake were dichotomized at the 50th percentile of the sex-specific distributions. BMI was categorized based on World Health Organization (WHO) definitions (1997 WHO Consultation on Obesity) as normal or underweight (<25 kg/m^2), overweight (25–29.9 kg/m^2), or obese (≥30 kg/m^2). Mean cytokine values were calculated for each risk factor and evaluated by *t* test for dichotomized risk factors and by a general linear model for BMI. Associations between CRC risk factors and high levels of inflammatory cytokines were evaluated using multivariable logistic regression. Inflammatory cytokines and a summary *z* score were classified as high if a subject's level was above the 50th percentile of the population distribution.

Due to the strong associations between BMI and our panel of inflammatory cytokines in our study as well as previous literature, BMI was treated as a confounder in all regression models. Also, physical activity was adjusted for total energy intake and vice versa.

To assess the associations of CRC risk factors with a summary score of the inflammatory cytokines combined, a summary inflammation *z* score was calculated. This score was calculated as follows: first, a normalized *z* score for each individual cytokine value with a mean of zero and standard deviation of 1.0 was calculated as $z = (x - \mu)/\sigma$, where x is a participant's cytokine value and μ and σ are the study population mean and standard deviation, respectively; then, the combined inflammation *z* score for each participant was created by summing the *z* scores of each inflammatory marker.

Results

Study participants

Selected characteristics of the study participants are shown in Table 1. The mean age of participants was 61 years, 70% were men, 70% were white, and 20% had a family history of colorectal cancer in a first-degree relative. Only three participants smoked; therefore, we did not evaluate this risk factor.

Table 1. Selected baseline characteristics of the clinical trial participants (Emory University, 2006).

Characteristics	n = 92
Demographics	
Age (y)	60.7 (8.0)
Men (%)	70.3
White (%)	70.3
College graduate (%)	57.1
Medical history	
History of colorectal cancer in 1° relative (%)	19.8
Take NSAID regularly[a] (%)	17.6
If woman (n = 5), taking estrogens (%)	5.4
Habits	
Current smoker (%)	3.3
Take multivitamin (%)	31.9
Physical activity (METs/d)	18.3 (14.0)
Mean dietary intakes	
Total energy intake (kcal/d)	
Men	1,824 (617)
Women	1,683 (851)
Total fat (g/d)	71.2 (30.9)
Total[b] calcium (mg/d)	761.9 (497.2)
Dietary fiber (g/d)	16.8 (8.9)
Alcohol (g/d)	11.0 (16.9)
Anthropometrics	
Body mass index (kg/m^2)	30.2 (6.1)
Waist-to-hip ratio	0.9 (0.1)
Serum 25-OH-vitamin D (ng/mL)	22.0 (8.5)

Notes: Data are given as means (SD) unless otherwise specified. [a]At least once a week; [b]diet plus supplements.
Abbreviation: NSAID, non-steroidal anti-inflammatory drug.

Although men had statistically significant 44% lower mean CRP levels than did women, the odds ratio for a CRP-sex association was not statistically significant (Table 2). None of the other inflammatory cytokines nor the inflammation *z* score differed by sex. CRP levels in obese participants were significantly higher (138%, *P* for trend < 0.001) than in those with a normal BMI (odds ratio [OR] 5.20; 95% CI [confidence interval], 1.42–19.04). Although there was a trend of higher inflammatory marker levels with increasing BMI, the trends were statistically significant only for CRP, IL-6, and the inflammation *z* score (*P* for trend < 0.001, 0.02, and <0.001, respectively). Mirroring the results for BMI, with a higher WHR CRP, IL-6, and the inflammation *z* score

Table 2. Biomarkers of inflammation according to possible colorectal cancer risk factors in colon adenoma patients.

CRC risk factors	n	Inflammatory biomarkers			
		CRP[a]		TNF-α	
		Mean (SD)	*P*[c]	Mean (SD)	*P*
Sex					
Male	64	2.38 (2.92)		3.96 (1.72)	
Female	27	4.23 (4.72)	0.02	4.77 (7.12)	0.39
OR (95% CI)[d]		1.25 (0.45, 3.46)		1.25 (0.44, 3.55)	
BMI (kg/m^2)					
<25	16	1.80 (3.80)		3.74 (1.76)	
25–29.9	34	2.02 (2.70)		3.82 (1.43)	
≥30	41	4.30 (4.11)	<0.001	4.69 (5.88)	0.24
OR (95% CI) 2 vs.1		1.86 (0.49, 7.00)		1.29 (0.39, 4.25)	
OR (95% CI) 3 vs. 1		5.20 (1.42, 19.04)		1.35 (0.42, 4.32)	
WHR					
Low[e]	42	2.09 (3.14)		4.05 (5.71)	
High	49	3.72 (4.04)	0.04	4.33 (1.90)	0.75
OR (95% CI)		2.91 (1.02, 8.32)		3.59 (1.18, 10.87)	
Age (yrs.)					
≤59	45	3.44 (3.20)		4.74 (7.23)	
>59	46	2.46 (3.52)	0.22	3.43 (1.67)	0.98
OR (95% CI)		0.41 (0.16, 1.04)		1.24 (0.54, 7.83)	
Serum 25-OH-vitamin D (ng/mL)					
≤22.3	47	2.69 (3.07)		4.51 (5.44)	
>22.3	44	3.28 (4.37)	0.47	3.87 (1.84)	0.46
OR (95% CI)		1.09 (0.42, 2.85)		1.58 (0.59, 4.23)	
NSAID[f]					
Non-users	75	2.33 (3.52)		3.62 (1.71)	
Users	16	3.69 (4.67)	0.08	6.88 (8.79)	0.02
OR (95% CI)		1.06 (0.30, 3.68)		2.63 (0.81, 8.51)	
Physical activity (METs/d)					
Low	38	4.64 (4.88)		4.86 (6.03)	
High	39	2.24 (2.53)	0.02	3.78 (1.78)	0.46
OR (95% CI)		0.59 (0.22, 1.68)		1.29 (0.53, 3.13)	
Total energy intake (kcal/d)					
Low	45	3.00 (3.66)		3.47 (1.94)	
High	46	2.92 (3.82)	0.81	4.41 (5.47)	0.37
OR (95% CI)		1.04 (0.42, 2.59)		1.17 (0.51, 2.72)	
Total calcium intake[g] (kg/d)					
Low	46	3.15 (3.62)		4.62 (5.43)	
High	45	2.78 (3.85)	0.99	3.76 (1.97)	0.39
OR (95% CI)		0.95 (0.42, 2.17)		0.80 (0.35, 1.83)	
Alcohol intake (g/day)					
Low	37	3.59 (3.95)		4.64 (5.47)	
High	38	2.33 (3.40)	0.69	3.71 (1.65)	0.47
OR (95% CI)		0.55 (0.22, 1.40)		1.04 (0.46, 2.38)	

IL-6		IL-8		IL-1β		Inflammation Z-score[b]	
Mean (SD)	*P*	Mean (SD)	*P*	Mean (SD)	*P*	Mean (SD)	*P*
2.48 (3.56)		6.47 (4.17)		0.34 (0.61)		–0.13 (2.60)	
2.69 (3.77)	0.78	5.66 (3.27)	0.37	0.40 (0.66)	0.66	–0.03 (3.43)	0.88
1.79 (0.66, 4.83)		0.77 (0.28, 2.11)		1.77 (0.67, 4.63)		1.23 (0.45, 3.33)	
1.33 (1.91)		4.72 (2.01)		0.15 (0.21)		–1.96 (2.85)	
2.79 (4.44)		6.85 (3.94)		0.32 (0.63)		–0.09 (2.63)	
2.78 (3.30)	0.02	6.31 (4.36)	0.37	0.46 (0.71)	0.19	0.71 (2.78)	<0.001
2.10 (0.56, 7.88)		3.55 (1.01, 12.57)		1.96 (0.56, 6.85)		4.33 (1.04, 18.00)	
4.69 (1.29, 17.10)		1.90 (0.56, 6.45)		3.11 (0.91, 10.58)		5.54 (1.37, 22.42)	
1.65 (2.02)		5.70 (4.35)		0.41 (0.71)		–1.23 (2.67)	
3.28 (4.43)	0.03	6.69 (3.49)	0.23	0.31 (0.52)	0.44	0.90 (2.68)	<0.001
1.88 (0.70, 5.00)		2.69 (1.03, 7.04)		1.24 (0.50, 3.11)		4.09 (1.67, 9.98)	
3.22 (4.31)		5.60 (3.29)		0.31 (0.53)		–0.22 (3.20)	
1.85 (2.63)	0.07	6.85 (4.41)	0.13	0.40 (0.70	0.47	0.04 (2.51)	0.68
0.81 (0.33, 1.97)		0.23 (0.53, 2.84)		1.50 (0.60, 3.74)		1.37 (0.56, 3.34)	
2.31 (3.13)		5.91 (3.64)		0.42 (0.70)		–0.26 (3.16)	
2.76 (4.08)	0.55	6.58 (4.22)	0.42	0.29 (0.52)	0.36	0.05 (2.62)	0.63
1.23 (0.48, 3.16)		1.22 (0.50, 3.00)		0.71 (0.28, 1.78)		1.20 (0.56, 3.05)	
2.59 (3.82)		6.33 (4.14)		0.36 (0.66)		–0.23 (2.85)	
2.25 (2.46)	0.73	5.78 (2.74)	0.61	0.35 (0.44)	0.95	0.53 (2.96)	0.36
1.44 (0.44, 4.70)		0.75 (0.22, 2.59)		1.65 (0.53, 5.13)		1.24 (0.38, 4.07)	
2.18 (2.02)		6.65 (4.79)		0.39 (0.59)		0.56 (2.76)	
3.59 (4.89)	0.07	6.18 (3.59)	0.76	0.34 (0.58)	0.74	0.11 (3.03)	0.78
1.14 (0.45, 2.87)		0.45 (0.18, 1.11)		1.19 (0.48, 3.00)		1.17 (0.46, 2.97)	
2.40 (2.96)		6.80 (3.80)		0.34 (0.58)		0.43 (2.46)	
2.64 (4.17)	0.62	5.68 (4.00)	0.25	0.37 (0.66)	0.90	–0.67 (3.19)	0.14
1.01 (0.42, 2.46)		2.24 (0.93, 5.41)		0.99 (0.41, 2.40)		1.38 (0.59, 3.43)	
2.58 (3.57)		6.17 (4.13)		0.49 (0.79)		0.39 (2.91)	
2.47 (3.68)	0.88	6.29 (3.73)	0.80	0.21 (0.48)	0.09	–0.56 (2.78)	0.28
0.47 (0.20, 1.08)		0.96 (0.42, 2.18)		0.46 (0.16, 1.34)		0.67 (0.29, 1.53)	
2.31 (2.49)		7.16 (4.89)		0.47 (0.69)		0.90 (2.60)	
2.74 (4.53)	0.13	5.24 (2.16)	0.03	0.24 (0.53)	0.17	–1.19 (2.80)	0.04
0.63 (0.26, 1.57)		0.50 (0.21, 1.21)		0.52 (0.19, 1.47)		0.51 (0.20, 1.26)	

Notes: [a]Categories of biomarkers for odds ratio calculations were set at the median for each cytokine: CRP, 1.40 ug/mL; TNF-α, 3.59 pg/mL; IL-6, 1.23 pg/mL; IL-8, 5.11 pg/mL; IL-1β, 0.20 pg/mL; combined inflammation z-score, –0.31; [b]inflammation z-score (CRP, TNF-α, IL-6, IL-8, and IL-1β) was calculated by (1) subtracting the mean and dividing by the standard deviation (thus creating a mean of zero and standard deviation of 1.0) for each participant's individual biomarker value, and then (2) summing the biomarker z-score values for each participant; [c]*P* value based on *t* test for comparing two means, except for BMI, which is a *P* for trend from a linear model; [d]Odds ratios adjusted for BMI, except WHR, which is adjusted for sex. Physical activity is adjusted for total energy intake, and vice versa; [e]throughout the table "Low," below the 50th percentile distribution, and "High," above the 50th percentile distribution; cutoff points for WHR are sex specific (for men, 0.97, women, 0.84); Physical activity, 16 METs/d; Total energy intake, 1,704 kcal/d; Calcium intake, 629.6 mg/d; Alcohol intake, 8.66 g/day in men and 2.35 g/day in women; [f]≥ once per week, not including aspirin; [g]supplements plus dietary intake.
Abbreviations: CRP, C-reactive protein; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1beta; BMI, body mass index; WHR, waist-hip ratio; NSAID, non-steroidal anti-inflammatory drug.

were statistically significantly higher by 77%, 98%, and 173%, respectively. The inflammation *z* score was strongly associated with being overweight or obese (ORs 4.33 [95% CI, 1.04–18.00] and 5.54 [95% CI, 1.37–22.42], respectively) and having a higher WHR (OR 4.09 [95% CI, 1.67–9.98]). High TNF-α and IL-8 were associated with a high WHR, although their mean levels were not statistically significantly different.

Inflammatory cytokine levels were consistently lower among those who consumed a moderate amount of alcohol, with statistically significantly lower mean IL-8 ($P = 0.03$) levels and inflammation *z* scores ($P = 0.04$) found in those with moderate daily alcohol consumption; however, the OR for these associations were not statistically significant. Although mean TNF-α levels were statistically significantly higher by 90% among those who took NSAIDs regularly, the OR for the association included 1.0. Similarly, higher CRP levels were found in participants who were less physically active (107%); however, the OR for the association also included 1.0. There were no statistically significant findings in relation to age, serum 25-OH-vitamin D levels, or total energy or calcium intakes.

Discussion

The results of this study support a direct association of inflammatory cytokines with adiposity-related risk factors for colorectal cancer. Our findings indicate that associations of risk factors for colorectal neoplasms with inflammation may be more strongly reflected by the use of a combined inflammation *z* score than by any single cytokine that reflects only a small aspect of inflammation/immunomodulation.

Previous studies suggested that there is a direct association between colorectal cancer risk and the inflammatory cytokines assessed in this study. Several CRC case-control studies found blood levels of CRP, TNF-α, IL-6, and IL-8 to be higher in cases.[8,11,17] Expression-enhancing polymorphisms in the genes for IL-6, TNF-α, IL-1β, and IL-8 were associated with increased adenoma risk in two case-control studies.[18,19] IL-1β is involved in COX-2 activation and activates the Wnt cell cycle pathway, the primary pathway of colon cell proliferation.[20] While these inflammatory cytokines are increasingly supported as risk factors for CRC, more research is needed to examine their usefulness as biomarkers of risk for colorectal adenomas.

Obesity was previously linked to inflammation and is proposed to be an inflammatory condition.[21] CRP was associated with BMI in obese or overweight young adults in a large cross-sectional study (NHANES III).[22] IL-6, which stimulates CRP release from the liver, is positively correlated with CRP levels in the adipose tissue of obese individuals.[21] Obesity, insulin resistance, and atherosclerosis are associated with higher levels of TNF-α, a strong mediator of inflammation and reactive oxygen/nitrogen species.[21] We found statistically significantly higher levels of CRP, IL-6, and the inflammation *z* score in our obese participants, as well as in those with a higher WHR, further supporting that higher levels of inflammatory cytokines are associated with general and central adiposity in patients at risk for colorectal cancer.

We found a consistent pattern of lower inflammatory cytokine levels with moderate alcohol consumption, with statistically significant lower mean IL-8 levels and inflammation *z* scores. This is consistent with the finding of Pai et al who observed that in those who consume a moderate amount of alcohol, CRP, IL-6, and TNF-α receptor levels were significantly lower than in non-drinkers.[23] In our study, the average alcohol consumption was quite moderate: in women, it was less than one drink per day, and in men, it was around 1.5 drinks per day. In addition, the ORs for the associations of alcohol consumption with the inflammatory markers were not statistically significant after adjusting for BMI.

We found no statistically significant associations of sex, age, serum 25-OH-vitamin D levels, NSAID use, or total energy or calcium intakes with inflammatory cytokines or the inflammation *z* score. This may have been due to our small sample size and the relatively homogenous population. We found that CRP was statistically significantly higher in women and TNF-α was statistically significantly higher in NSAID users; however, the ORs for the associations were not statistically significant, possibly due to the small number of women and NSAID users. Larger studies are needed to more adequately assess these possible associations.

CRP stimulates the release of IL-1β, IL-6, IL-8, and TNF-α from mononuclear phagocytes, which supports a collective analysis of these cytokines, such

as by using a combined *z* score.[15,24] Previously, we reported the effects of vitamin D_3 supplementation on a combined inflammation *z* score (including CRP, IL-6, TNF-α, IL-1β, and IL-8) in a randomized placebo-controlled clinical trial of colorectal adenoma patients. We found that, although vitamin D_3 did not significantly reduce each individual cytokine, it statistically significantly reduced the inflammation *z* score in this study population by 77% ($P = 0.003$) relative to placebo. In our current study, we found that the combined inflammation score was more strongly associated with measures of adiposity than were any of the individual cytokines. In light of these data, we propose further investigation of an inflammation *z* score as a biomarker of risk for colorectal adenomas or cancer.

Our small cross-sectional study has several limitations. First, the small sample size, especially the small sample size of important subpopulations (ie, smokers and NSAID users), limited our analyses and conclusions. Also, the cross-sectional study design does not address temporality, and our results may not be generalizable beyond a limited colorectal adenoma patient population. Finally, about half of the study population was obese, with greater representation of overweight individuals than normal weight individuals. While this limits the comparison of obese to normal weight individuals, the high number of obese and overweight individuals allowed for an important comparison between these groups.

Strengths of this study included that it used standardized methods of collecting blood and patient information from questionnaires as well as standardized methods of assessing vitamin D and cytokine levels. All of the participants had a colorectal adenoma removed in the previous 36 months and, therefore, were unlikely to have current colorectal cancer, which could alter cytokine levels. Other strengths of this study included the array of cytokines investigated, which are well supported and linked to colorectal cancer, and the use of a summary *z* score to analyze the cytokines collectively.

In summary, our findings in this small cross-sectional study support direct associations of CRP, IL-6, and a combined *z* score of inflammatory cytokines with adiposity in colorectal adenoma patients. This study shows that the use of a combined inflammation *z* score may have promise in revealing stronger associations of inflammation with colorectal cancer risk than can be discerned by measuring and analyzing only individual aspects of inflammation, a large, complex system. Larger case-control and prospective studies are needed, however, to further assess the validity and usefulness of these cytokines and the combined inflammation *z* score in relation to risk for colorectal neoplasms or other chronic diseases.

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Author Contributions

Conceived and designed the experiments: RMB. Analysed the data: MHH, WDF, RMB. Wrote the first draft of the manuscript: MHH. Contributed to the writing of the manuscript: RMB, WDF. Agree with manuscript results and conclusions: MHH, RMB, WDF. Jointly developed the structure and arguments for the paper: MHH, RMB. Made critical revisions and approved final version: MHH, RMB, WDF. All authors reviewed and approved of the final manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have

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