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Interpretation of P16 expression as a marker of HPV in colorectal cancer

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Summary. Background. Colorectal cancer is one of the most prevalent types of tumors worldwide. $P16^{INK4a}$ is a widely used immunohistochemical marker for high-risk HPV infection. The purpose of this study is to explore the relationship between P16 expression as an indicator of HPV infection and colorectal cancer in Egyptian patients, as well as its association with histopathological characteristics.

Material and methods. The study was performed on 59 cases of colorectal carcinoma cases and 30 specimens of normal colonic mucosa.

Results. p16 protein was detected in 22% (13 of 59) of patients with colorectal carcinoma. No evidence of P16 expression in all 30 cases of non-neoplastic colonic mucosa was found. More frequent expression of P16 was seen in distal carcinomas.

Conclusion. our study demonstrated that P16 protein is expressed in a reasonable percent of colorectal carcinoma cases, suggesting a role of HPV in colorectal carcinogenesis. The present study highlights the role of p16 protein expression which is important in the pathogenesis in colorectal carcinoma, especially regarding distal tumors.

Key words: P16 protein, Colorectal carcinoma, Immunohistochemistry, Distal tumor

Introduction

Colorectal cancer (CRC) is the world's third most lethal and fourth most common malignancy. The global incidence of CRC has been continuously increasing, particularly in developing countries that are adopting the "western" way of life (Bray et al., 2018).

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Colorectal cancer is the 7th commonest cancer in Egypt, representing 3.47% of male cancers and 3% of female cancers. The estimated number of colon cancer patients (excluding rectal cancer) in 2015 was slightly more than three thousand (Ibrahim et al., 2014).

Although hereditary forms of colorectal carcinoma have been well established, most cases are sporadic (Lynch and de la Chapelle, 2003). Numerous epidemiological studies have revealed lifestyle and environmental factors that contribute to the occurrence of colorectal carcinoma, such as excessive red meat consumption or tobacco use (Liang et al., 2009; Chan et al., 2011). However, the majority of these risk factors only pose a moderate risk (Brenner et al., 2014).

The p16INK4a (CDKN2A) is a commonly utilised immunohistochemical marker for high-risk HPV infection, particularly in the anogenital region, and it is also a known prognostic marker in oropharyngeal squamous cell carcinoma (Mahajan, 2016).

The p16 tumour suppressor gene (CDKN2A) is situated on chromosome 9p21 and belongs to the INK4 class of cell cycle inhibitors. The p16 protein interacts with cyclin-dependent kinases (CDK) 4 and 6, keeping the retinoblastoma gene product hypo-phosphorylated, which then binds to the E2F transcription factor and stops cell cycle progression (Khleif et al., 1996; Serrano, 1997; Kim and Sharpless, 2006; Iaconis et al., 2007).

In HPV-driven malignancies, virus integration into the host cell genome results in the synthesis of E6 and E7 viral oncoproteins (Khleif et al., 1996; Murphy et al., 2004; Iaconis et al., 2007). E6 disrupts p53 and hence inhibits apoptosis. E7 inactivates pRb function, preventing it from attaching to the E2F transcription factor. As a result of this positive feedback loop, the expression of p16 in both the nucleus and the cytoplasm rises, and can be detected by immunohistochemistry (Khleif et al., 1996; Doorbar, 2006; Iaconis et al., 2007; Wentzensen and von Knebel Doeberitz, 2007).

Human Papillomavirus (HPV) are epitheliotropic,

Abbreviations. CRC, Colorectal carcinoma; CDK, cyclin dependant kinase; HPV, Human papilloma virus.

double-stranded DNA viruses that infect the squamous epithelium of the skin and mucous membranes. Viral multiplication occurs in the nuclei and is closely linked to the differentiation of the cells. There are well over 100 HPV genotypes. Low-risk types include types 6, 11, 40, and 42, which are frequently associated with benign lesions. HPV-16, HPV-18, HPV-31, and HPV-45, on the other hand, are categorised as high-risk types because they have a high carcinogenic potential (Bosch and de Sanjosé, 2007; Giuliano et al., 2008).

The global prevalence of high-risk HPV infection is 10.4 % (de Sanjosé et al., 2007), with some developing nations seeing as high as 36.5 % (Okunade et al., 2017). HPV has been linked as a cause of malignancies of the cervical, vaginal, anal, oral, and penile regions (Schiffman et al., 2007; Crosbie et al., 2013; Larsson et al., 2013; Gami et al., 2014; Diorio and Giuliano, 2016; Tanaka and Alawi, 2018). Important correlations have also been found between HPV and the development of malignancies of the oesophagus, pharynx, and larynx (Gillison et al., 2000; Li et al., 2013, 2014).

To date, there is a paucity of data concerning P16 protein expression in colorectal carcinoma. Our study aims to investigate the association of HPV infection in colorectal carcinoma on Egyptian patients through P16 immunohistochemical expression and its correlation with the histopathological parameters.

Materials and methods

Tissue and patient data

Our study included a total number of 59 formalin-fixed and paraffin-embedded colorectal carcinoma and 30 specimens of adjacent normal epithelium, at the pathology department of Ain Shams university hospital Cairo, Egypt, during the period between 2014 and 2018. The patients' clinical data were obtained from their files.

Two specialized expert pathologists examined the specimens independently and confirmed them to be colonic carcinoma. The pathological features were identified as tumor size, grade, pathological stage, lympho-vascular invasion, and presence of tumor budding. The tumor grade was determined based on the WHO criteria (5^{th} edition) (Nagtegaal et al., 2019). The tumor stage was evaluated using the current AJCC/UICC TNM staging (8th edition, 2017) of colorectal carcinoma (Jessup et al., 2017).

Immunohistochemistry

Immunohistochemistry was performed on 59 colorectal cancer cases and 30 normal colonic epithelial specimens. The paraffin-embedded tissue sections were deparaffinized in xylene and subsequently rehydrated in absolute alcohol. After treating the sections in a microwave at 8w for 5-6 minutes, then at 3w for 10 minutes, the antigen retrieval in citrate buffer (pH9 Lab vision cat#AP9003) was conducted; the sections were then allowed to cool for 20 minutes. The peroxidase and protein block were carried out. After that, slides were incubated overnight with the primary antibodies at room temperature using Anti- CDKN2A/$P16^{INK4a}$ antibody (rabbit polyclonal antibody, Chongqing Biospes Co. Ltd., Chongqing, China) at 1:200 dilution.

Immunohistochemical evaluation of P16 was performed. The p16 marker was considered positive if both nuclear and cytoplasmic expressions were present. The following method was used to calculate the immunohistochemical expression score: The staining intensity was recorded as 1 (negative), 2 (weak), 3 (moderate), and 4 (strong). The p16 expression levels were defined as positive (overexpression) and negative (hypo expression), with a cutoff point of 50% of moderate and/or strongly stained cells (Deschoolmeester et al., 2010) The immunostaining was examined by two pathologists in a blinded manner.

Ethical consideration

The Research Ethical Committee at Ain Shams University, Faculty of Medicine, (Cairo, Egypt) reviewed and approved this study protocol with IRB approval number: FMASU R144/2021. This followed the declaration of Helsinki regarding ethical considerations.

Statistical analysis

The interrelationship of P16 expression with clinicopathological parameters was analyzed by the χ^2 test or Fisher's exact test. All statistical procedures were carried out using SPSS version 25 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Results of the clinicopathological data

Among the 59 cases, 22 (37.3%) were males and 37 (62.7%) were females, at a proportion of 1:1.7. The ages ranged from 17 to 80 years, with a mean of 50.69±14.04 years. Half of the patients (50%) were found to be ≤51.5 years. (Table 1)

According to the tumor location, 14 (26.4%) tumors were located in the right colon, 5 (9.4%) tumors were in the left colon, 3 (5.7%) tumors were in the transverse

Table 1. Demographic data.

		Min.	Max.	Median	Mean	SD
Age		17.00	80.00	51.50	50.69	14.04
		N			%	
Gender	Male	22			37.3%	
	Female	37			62.7%	

colon and 31 (58.5%) tumors were in the rectosigmoid region. Our cases included 47 (79.7%) radical colectomy specimens and 12 (20.3%) endoscopic biopsies. As for tumor size, it ranged from 1.5 cm to 12 cm, with a mean of 5.17±2.41 cm. Twenty-one cases (44.7%) measured <5 cm and 26 cases (55.3%) measured ≥5 cm (Table 2).

Regarding the histologic type, 45 (76.3%) cases were adenocarcinoma NOS while 13 (22%) cases were mucinous adenocarcinoma, and 1 (1.7%) case was signet ring carcinoma. Forty-five (76.3%) cases were low grade while 14 (23.7%) cases were high grade. Lympho-vascular invasion was detected in 7 (14.9%) cases and perineural invasion was present in 10 (21.3%) (Table 2).

One (2.1%) case only was evaluated as T1 stage, 9 (19.1%) cases were T2 stage, 25 (53.2%) cases were T3 stage, and 12 (25.5%) cases were T4 stage. Twenty-four (51.1%) cases showed lymph node metastasis, and 23 (48.9%) cases showed free lymph nodes. Tumor budding was detected in 20 (42.6%) cases (Table 2).

Immunohistochemical results

$P16^{INK4a}$ staining pattern was nuclear and cytoplasmic in the tumor cells. All 30 cases of non-neoplastic colonic mucosa showed no evidence of p16 protein expression (Fig. 1a,e). $P16^{INK4a}$ expression was divided into positive in 13 (22%) cases and negative in 46 (78%) cases (Fig. 1).

The relationship between colorectal carcinoma clinicopathological variables and $p16^{INK4a}$ overexpression was investigated as shown in Table 3. There was no significant correlation present.

Discussion

Colorectal carcinoma is a common and fatal disease, as advanced colorectal carcinoma has a high mortality rate, consistently ranking in the top three causes of death related to cancer worldwide. Both inherited and environmental influences contribute to the risk of progression (Hull et al., 2020).

Recently, HPV has been shown to have a role in colorectal carcinogenesis. The source of HPV in the colon is not clear. The ascending or retrograde infection from the perineum, sexual intercourse, inoculation with fomites, or preventive colonoscopy could be the sources of HPV infection in the colon (Deschoolmeester et al., 2010; Pérez et al., 2010; Bucchi et al., 2016).

The frequency of $p16^{ink4a}$ immunohistochemical expression is variable in different studies and ranges from 17% to 80% (Carneiro et al., 2006; Lam et al., 2008; Al-Ahwal et al., 2016; Dalla Libera et al., 2020). Our study reported that $p16^{ink4a}$ expression in colorectal carcinoma is 22%.

In our work, the expression of $p16^{ink4a}$ was nucleocytoplasmic and the cytoplasmic only expression was considered negative. Lam et al. (2008) reported that p16 is a nucleoprotein; the nucleocytoplasmic pattern of staining suggests overexpression of the P16 gene. The change in the subcellular position of this overexpressed nucleoprotein may justify the pathogenesis of colorectal carcinoma. It is obvious that the overexpression of p16 instead of loss of its protein contributes to the pathogenetic mechanism of colorectal carcinoma.

The normal non-malignant epithelium is negative for $p16^{ink4a}$ expression. Many studies stated the same result (Carneiro et al., 2006; Lam et al., 2008; Al-Ahwal et al., 2016; Dalla Libera et al., 2020). This could be suggestive of the role of HPV in the pathogenesis of colorectal carcinoma.

The immunohistochemical overexpression of $p16^{INK4a}$ is considered a sensitive marker for the existence of transcriptionally active infection by high-risk HPV genotypes, mainly in cervical and oropharyngeal tumors related to the virus (Darragh et al., 2013; Hellman et al., 2014; Westra, 2015; Mahajan, 2016). On the other hand, other studies reported that the relation between $p16^{INK4a}$ protein expression and presence of HPV genome is debatable in colorectal carcinoma (Deschoolmeester et al., 2010; Dalla Libera et al., 2020).

Lorenzon et al. (2011) stated that it is unclear whether HPV plays a role in the pathogenesis of colorectal carcinoma. The virus's presence in tumour cells could be coincidental; otherwise, it may have a role

Table 2. Histopathological parameters of colorectal carcinoma.

		Min.	Max.	Median	Mean	SD
Size (cm)		1.50	12.00	5.00	5.17	2.41
		N		%		
Size	<5 cm	21		44.7%		
	≥5 cm	26		55.3%		
Site	Rt colon	14		26.4%		
	Lt colon	5		9.4%		
	Transverse	3		5.7%		
	Rectal/Sigmoid/ Rectosigmoid	31		58.5%		
Type of specimen	Radical specimen	47		79.7%		
	Biopsy	12		20.3%		
Histologic type	adenocarcinoma	45		76.3%		
	mucinous adenocarcinoma	13		22.0%		
	Signet ring carcinoma	1		1.7%		
Tumor grade	Low grade	45		76.3%		
	High grade	14		23.7%		
Lympho-vascular invasion	No	40		85.1%		
	Yes	7		14.9%		
Perineural invasion	No	37		78.7%		
	Yes	10		21.3%		
T stage	T1	1		2.1%		
	T2	9		19.1%		
	T3	25		53.2%		
	T4	12		25.5%		
Lymph node metastasis	No	23		48.9%		
	Yes	24		51.1%		
Tumor budding	Present	20		42.6%		
	Absent	27		57.4%		

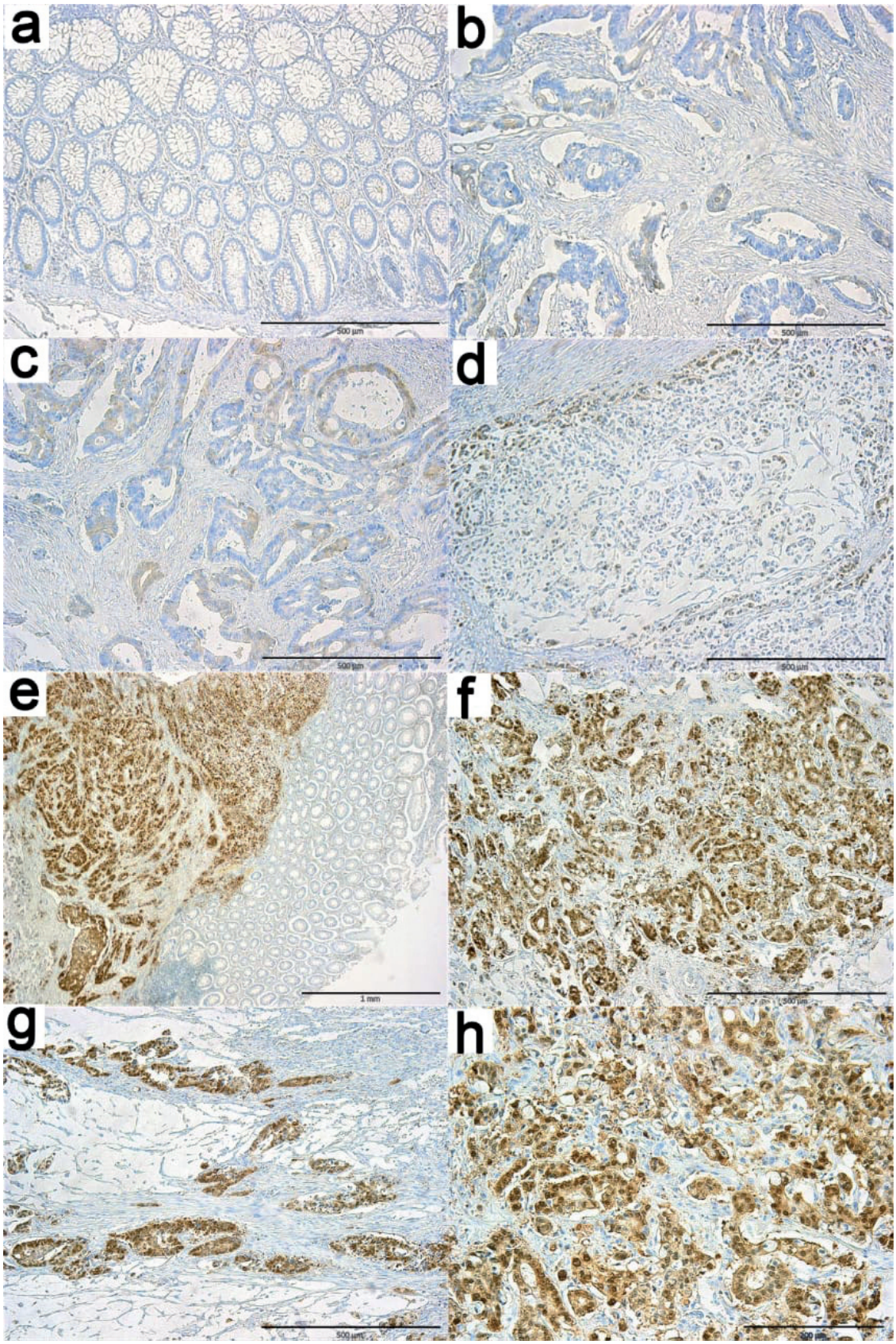

Fig. 1. P16 expression in adenocarcinomas. **a.** Negative P16 expression in normal epithelium. **b, c.** Negative P16 expression (<50%) in adenocarcinoma. **d.** Negative P16 expression (<50%) in signet ring carcinoma. **e.** Positive P16 expression in adenocarcinoma with negative staining of the adjacent normal epithelium. **f, g.** Positive staining of P16 of adenocarcinoma and mucinous adenocarcinoma respectively. **h.** Positive staining of P16 of adenocarcinoma. a-d, f, g, x 100; e, x 40; h, x 200.

in chromosomal instability, oncogene activation, p53 inactivation, or tumour suppressor inactivation via insertional mutagenesis caused by virus integration into the host genome.

The abnormal function of p16 was found to be one of the earliest events happening in cancer progression (Tanaka, 2009; Shi and Washington, 2012; Colussi et al., 2013). In cervical biopsies and Barrett's esophagus, current clinical work reported a consistent link between p16 and premalignant lesions, and p16 was successfully utilized to predict progression to high-grade dysplasia or carcinoma (Lesnikova et al., 2009; Wang et al., 2009).

Our study reported no significant correlation between $p16^{ink4a}$ expression and clinicopathological parameters. This is in concordance with Al-Ahwal et al. (2016) and Dalla Libera et al. (2020) who stated the same result.

Concerning tumor location, researchers reported that right-sided and left-sided colorectal cancers can be distinguished by clinical and molecular criteria because right-sided carcinomas are more common in older people, women, and benefit more from 5-fluorouracil-based chemotherapy (Birkenkamp-Demtroder et al., 2005; Lam et al., 2006). Nuclear b-catenin and p53 overexpression were likewise much lower in right-sided carcinomas than in left-sided carcinomas (Iacopetta, 2002; Birkenkamp-Demtroder et al., 2005).

Regarding P16 expression, Lam et al. (2006) reported that p16 protein expression was more frequently seen in mucinous carcinoma of the distal colorectum. Other studies reported that p16 protein was more frequently expressed in adenocarcinoma of the distal colon and rectum (Lam et al., 2008; Deschoolmeester et al., 2010). These results are close to ours as we found more frequent expression of P16 seen in distal carcinomas.

For p16 protein expression concerning tumor grade, it is reported to be higher in low-grade adenocarcinomas and decreased in mucinous and high-grade adenocarcinomas. Furthermore, the p16 expression was reported to be seen in well-differentiated glandular structures within the tumor rather than isolated tumor cells. This indicated that P16 is a marker of differentiation in colorectal carcinoma. (Palmqvist et al., 2000; Tada et al., 2003; Lam et al., 2008). However, our work showed no difference in both tumor grades.

Zhou and Gu (2018) study demonstrated that overexpression of p16 protein was correlated with the Dukes stage, lymph node metastasis, and TNM stage (only in Caucasians) of colorectal carcinoma, indicating that p16 protein overexpression may have a crucial role in the development of colorectal cancer. On the contrary, our study showed no significant correlation with stage or lymph node metastasis. This might be attributed to sample size or lack of standardization of immunohistochemical techniques between different studies.

Conclusion

Our study demonstrated that P16 protein is expressed in a reasonable percent of colorectal carcinoma cases. P16 is a sensitive marker for HPV. However, the HPV-related carcinogenesis should be confirmed by the detection of HPV DNA in host cells. The present study highlighted the role of p16 protein

Table 3. Correlation between P16 IHC expression and histopathological parameters of colorectal carcinoma.

		P16 IHC				X2*	P value
		<50%		≥50%			
		N	%	N	%		
Size	<5 cm	15	71.4%	6	28.6%	1.21	0.27 NS
	≥5 cm	22	84.6%	4	15.4%		
Site	Rt colon	13	92.9%	1	7.1%	3.56 FE	0.30 NS
	Lt colon	3	60.0%	2	40.0%		
	Transverse	2	66.7%	1	33.3%		
	Rectal/Sigmoid/ Rectosigmoid	24	77.4%	7	22.6%		
Histologic type	adenocarcinoma	34	75.6%	11	24.4%	0.82 FE	0.78 NS
	mucinous adenocarcinoma	11	84.6%	2	15.4%		
	Signet ring carcinoma	1	100.0%	0	0.0%		
Tumor grade	Low grade	36	80.0%	9	20.0%	0.46	0.50 NS
	High grade	10	71.4%	4	28.6%		
Lympho-vascular invasion	No	31	77.5%	9	22.5%	0.24	0.62 NS
	Yes	6	85.7%	1	14.3%		
Perineural invasion	No	31	83.8%	6	16.2%	2.66	0.10 NS
	Yes	6	60.0%	4	40.0%		
T stage	T1/T2	7	70.0%	3	30.0%	0.58	0.45 NS
	T3/T4	30	81.1%	7	18.9%		
Lymph node metastasis	No	19	82.6%	4	17.4%	0.41	0.52 NS
	Yes	18	75.0%	6	25.0%		
Tumor budding	Present	17	85.0%	3	15.0%	0.82	0.37 NS
	Absent	20	74.1%	7	25.9%		

expression, which is important in the pathogenesis of colorectal carcinoma, especially regarding distal tumors, which have become much more common in recent years and need more intensive research on larger sample sizes and clinical correlation.

Conflict of Interest. None.

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Author contributions. Conceptualization: MA; Methodology: MMS, FSS, GRM, MA; Supervision: MMS; Writing – original draft: FSH; Writing – review and editing: MMS, FSH, GRM; Approval of final manuscript: all authors.

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