THE UNIVERSITY of ADELAIDE

Towards an Understanding of the Growing Incidence of Colorectal Cancer and Appendiceal Neoplasms in Young Adults

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Abstract

Colorectal cancer (CRC) incidence and mortality rates are rising in young adults aged <50 years old [referred to as young-onset CRC (YOCRC)] in Australia and many other countries while there has been a steady decline in overall rates of this malignancy in individuals aged ≥50 years old. In addition, the incidence and mortality rates of appendiceal neoplasms (ANs) have also been reported to be on the rise in both age groups (<50 years and ≥50) in the United States, Canada and Netherlands. Currently, the causes of these observations remain largely unknown. Identifying the underlying aetiological factors for YOCRC and ANs is a primary public health priority because addressing these contributing factors is a key prevention strategy. The main aim of this thesis is to explore the observation of the increasing incidence of CRC and ANs in young adults.

The aetiology of YOCRC is likely to be heterogeneous, comprising a spectrum of genetic and environmental triggers. To this end, we have investigated the role of type 2 diabetes (T2D) as a marker of increased risk, and explored the exome in YOCRC patients for pathogenic germline variants. Consistent evidence suggests an association between T2D at any age and increased CRC risk. An observational study found that a personal history of T2D was significantly higher in YOCRC patients compared to controls (age- and sex-matched individuals with clear colonoscopies). In addition, analysis of exome sequencing data of YOCRC patients showed that one in six YOCRC patients had clinically actionable germline variants in at least one cancer-predisposing gene, with 35% of these being in genes associated with breast or ovarian cancer. First-degree relatives with CRC were rarely seen in variant carriers and three patients with variants in polyposis associated genes (*MUTYH* (bi-allelic), *RNF43* and *BMPR1A*) showed no polyposis. In addition, two individuals with CRC were identified from a single-family carrying a likely-pathogenic germline variant in *RNF43:c.375+1G>A*. Tumours from both carriers were $BRAF^{V600E}$-mutated and mismatch repair-proficient indicating that the CRCs arose

in sessile serrated lesions. However, the proband did not meet the clinical criteria for serrated polyposis. Both studies taken together suggest that phenotype was a poor predictor of genotype.

Trends in incidence and mortality rates of ANs in Australia were explored by performing a retrospective analysis on national data obtained from the Australian Institute of Health and Welfare from 1982 to 2013. Similar to the observed trend in other countries, this work has demonstrated that the incidence and mortality rates of ANs are alarmingly on the rise in Australia in both age groups (<50 years and ≥50), both genders, and within diverse histological subtypes.

In conclusion, findings from this work suggest that there is an enrichment for personal history of T2D in patients with YOCRC, and that carriers of variants in breast/ovarian cancer-related genes might need to receive surveillance tests for CRC earlier than the general population, and importantly, that multigene panel testing is warranted for all YOCRC patients regardless of family history or phenotype. The findings also lend weight to further consideration for a hereditary role for *RNF43* as a tumour suppressor gene in colorectal tumorigenesis outside the setting of individuals meeting the clinical criteria for serrated polyposis. In addition, an apparent rise in the incidence and mortality rates of ANs in Australia was demonstrated, the causes of which remain unclear. Further research exploring the risk factors for YOCRC and ANs is warranted, to stem the rising trend of both these malignancies.

Declaration by author

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Publication during candidature

Review articles

1. **Mikaeel, R.R.,** Price, T.J., Smith, E., Drew, P.A., Uylaki, W., Horsnell, M., and Young, J.P., 2019. Colorectal cancer in Australian Young Adults. Mathews. J Cancer Sci.; 4(1), pp.1-18, (DOI: 10.30654/MJCS.10018) (A large part of chapter 1).
2. **Mikaeel, R.R.,** Young, J.P., Tapia Rico, G., Hewett, P.J., Hardingham, J.E., Uylaki, W., Horsnell, M. and Price, T.J., 2021. Immunohistochemistry features and molecular pathology of appendiceal neoplasms. *Critical Reviews in Clinical Laboratory Sciences, pp.1-36,* (DOI: 10.1080/10408363.2021.1881756). (Chapter 6).

Research articles

1. **Mikaeel, R.R.,** Symonds, E.L., Kimber, J., Smith, E., Horsnell, M., Uylaki, W., Tapia Rico, G., Hewett, P.J., Yong, J., Tonkin, D. and Jesudason, D., 2021. Young-onset colorectal cancer is associated with a personal history of type 2 diabetes. *Asia-Pacific Journal of Clinical Oncology, 17(1), pp.131-138,* (DOI: 10.1111/ajco.13428). (Chapter 2).
2. **Mikaeel, R.R.,** Young, J.P., Li, Y., Smith, E., Horsnell, M., Uylaki, W., Tapia Rico, G., Poplawski, N.K., Hardingham, J.E., Tomita, Y., and Townsend, A.R., 2021. Survey of Germline Variants in Cancer-Associated Genes in Young Adults with Colorectal *Cancer. Genes, Chromosomes and Cancer, 60(12),* (DOI: 10.1002/gcc.23011). (Chapter 3).
3. **Mikaeel, R.R.,** Young, J.P., Hardingham, J.E., Tapia Rico, G., Hewett, P.J., Symonds, E.L., Edwards, S., Smith, E., Tomita, Y., Uylaki, W. and Horsnell, M., 2021. Appendiceal neoplasm incidence and mortality rates are on the rise in Australia. *Expert Review of Gastroenterology & Hepatology, 15(2), pp.203-210,* (DOI: 10.1080/17474124.2021.1832467). (Chapter 5).

Short report

4. **Mikaeel, R.R.,** Young, J.P., Li, Y., Poplawski, N.K., Smith, E., Horsnell, M., Uylaki, W., Tomita, Y., Townsend, A.R., Feng, J. and Zibat, A., 2021. *RNF43* pathogenic Germline variant in a family with colorectal cancer. *Clinical Genetics*, (DOI: 10.1111/cge.14064). (Chapter 4).

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1. **Mikaeel, R.R.,** Young JP, Li Y, et al. Pathogenic Germline *RNF43* splice-site variant in a proband with colorectal cancer in the absence of serrated polyposis. *2021 Florey Postgraduate Research Conference.* ***The John Barker Bequest Prize.***
2. **Mikaeel RR**, Young JP, Smith E, Price TJ. Tu1018 Incidence and mortality rates of appendiceal neoplasms are on the rise in Australia. *Gastroenterology.* 2020;158(6):S-1008-S-9.
3. Young JP, Horsnell ME, Uylaki W, **Mikaeel RR**, Townsend A, Poplawski N, et al. Tu1218 Relationship between germline mutation, phenotype and family history in young onset colorectal cancer. *Gastroenterology*. 2020; 158(6):S-1023.
4. **Mikaeel R R**, Symonds E, Kimber J, Uylaki W, Horsnell M, Rico G, et al., editors. Personal history of type 2 diabetes is a potential risk marker for colorectal cancer in young people. *Journal of gastroenterology and hepatology; 2019: WILEY 111 RIVER ST, HOBOKEN 07030-5774, NJ USA.*
5. **Mikaeel, R.R.,** Symonds, E.L., Kimber, J., Smith, E., Horsnell, M., Uylaki, W., Tapia Rico, G., Hewett, P.J., Yong, J., Tonkin, D. and Jesudason, D. Distinct Clinical and Molecular Features Presented in Young-Onset Colorectal Cancer. *2019 Florey Postgraduate Research Conference.*

List of abbreviations

Abbreviation	Meaning
AA	Acute appendicitis
ACMG	The American College of Medical Genetics
AGEs	Advanced glycation end-products
AIHW	The Australian Institute of Health and Welfare
AIs/ANs	American Indians/Alaska Natives
ANs	Appendiceal neoplasms
AORs	Adjusted odds ratios
APIs	Asians/Pacific Islanders
BMI	Body mass index
BWA	The Burrows–Wheeler Aligner
CD	Crohn's disease
CGA-IGC	The Collaborative Group of the Americas for Inherited Gastrointestinal Cancer
CIMP	CpG methylator phenotype
CIN	chromosomal instability
CK1	Casein kinase1
CI	Confidence interval
CRC	Colorectal cancer
dMMR	Mismatch repair deficiency
EPCAM	the epithelial cell adhesion molecule
FAP	Familial adenomatous polyposis
FDR	First-degree relative
FIT	Faecal immunochemical test
GATK	The Genome Analysis Tool Kit

HG19	The human reference genome
HR	Hazard ratio
IARC	International Agency for Research on Cancer
IBD	inflammatory bowel disease
IGF-1	insulin-like growth factor-1
IGV	The Integrative Genomic Viewer
IHC	Immunohistochemistry
IRR	Incidence rate ratio
LLS	Lynch-like syndrome
LOCRC	Late-onset CRC
LOH	Loss of heterozygosity
LP	likely pathogenic
LS	Lynch Syndrome
MAF	Minor allele frequency
MAP	MUTYH-associated polyposis
MMR	Mismatch repair
MSI	Microsatellite instability
MSS	Microsatellite stable
NBCSP	The National Bowel Cancer Screening Program
NHBs	Non-Hispanic blacks
NHWs	non-Hispanic whites
NOS	Not otherwise specified
OR	Odd ratio
P	Pathogenic
PCPs	Primary care physicians
RR	Relative risk

SAYO	The South Australian Young Onset Colorectal Cancer
SDR	Second-degree relative
SEER	the Surveillance, Epidemiology and End Results
SIR	Standardized incidence ratio
SPS	Serrated polyposis syndrome
SSLs	Sessile serrated lesions
T2D	Type 2 diabetes
TCF4	T-cell factor 4
UC	Ulcerative colitis
US	United States
VDR	Vitamin D receptor
VUS	Variant of uncertain significance
WES	Whole Exome Sequencing
WHI	The Women's Health Initiative
WHO	The World Health Organisation
YOCRC	Young-onset CRC
25(OH)D	25-hydroxyvitamin D

Chapter One: Introduction

Colorectal cancer (CRC) arises from pre-malignant polyps in the lining of the large bowel. CRC incidence rate is rising in young adults in many developed and developing countries while opposite trends have been observed in adults aged ≥50 years old (1, 2). The drivers of this observed trend are not adequately explained. Young adults with CRC more often present with advanced stages of the disease with increased prevalence of aggressive histopathological features and with recto-sigmoid cancers compared to their older counterparts, and they frequently receive aggressive chemotherapies (1, 3). There is a need to identify young individuals at increased risk for CRC in the general population so that prevention strategies can be instituted.

CRC in young adults is heterogeneous. The majority of CRC cases (80%) in young adults are sporadic and a strong birth cohort effect suggests that changes in CRC incidence are likely to be attributed to behavioural factors that influence cancer risk (1). However, genetic predispositions and a family history of the disease are the strongest risk factors for CRC and a significant proportion of CRC heritability is yet to be explained. Identifying etiological environmental/behavioural and genetic risk factors that could elucidate this increasing trend in CRC in young is a major public health priority.

Similar to CRC, appendiceal neoplasms (ANs) incidence rates have also been rising in the United States, Canada and Netherlands (4, 5). However, the incidence of ANs was reported to be on the rise in both the young and the elderly. Currently, the causes of the rise in the incidence of this malignancy remain unexplained and risk factors for ANs are largely unknown. In addition, it is yet to be established whether the mortality rates of ANs are following a similar

trend to the incidence rates and whether this malignancy is also on the rise in other westernized countries including Australia.

The main aim of this thesis is to explore issues around the rising incidence of both CRC and ANs in young adults.

1. Anatomy and histological considerations of the large bowel and associated structures

The large intestine (bowel), which is the last segment of the gastrointestinal tract and the digestive system, consists of the colon, and more distally the rectum (**Figure 1**) (6). The main functions of the large intestine are the absorption of electrolytes, vitamins, and water from food alongside the elimination of faeces (6). Classical divisions of the colon are the caecum, ascending colon, transverse colon, descending colon, and sigmoid colon (6-8). The rectum, about 10-15 cm, is the last segment of the large intestine which connects the sigmoid colon to the anus and acts as a temporary store for faeces (7, 9). Simple columnar epithelium with long microvilli line the mucosa layer of the large bowel (**Figure 2**).

Colorectal cancer (CRC), also known as bowel cancer, is a common cancer arising within the single cell thick epithelial lining of the large bowel. Tumours originating from the anus are categorized differently from those in the rectum as they arise from different cell types and thereby, have different features. However, cancers within the colon and the rectum also differ in their clinicopathological and molecular characteristics as well as in their associated risk factors (6).

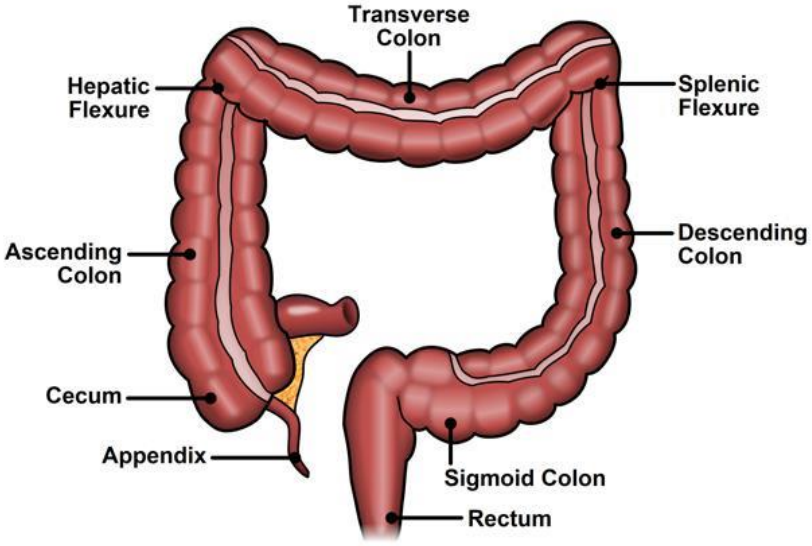

Figure 1. Large intestine and appendix (10).

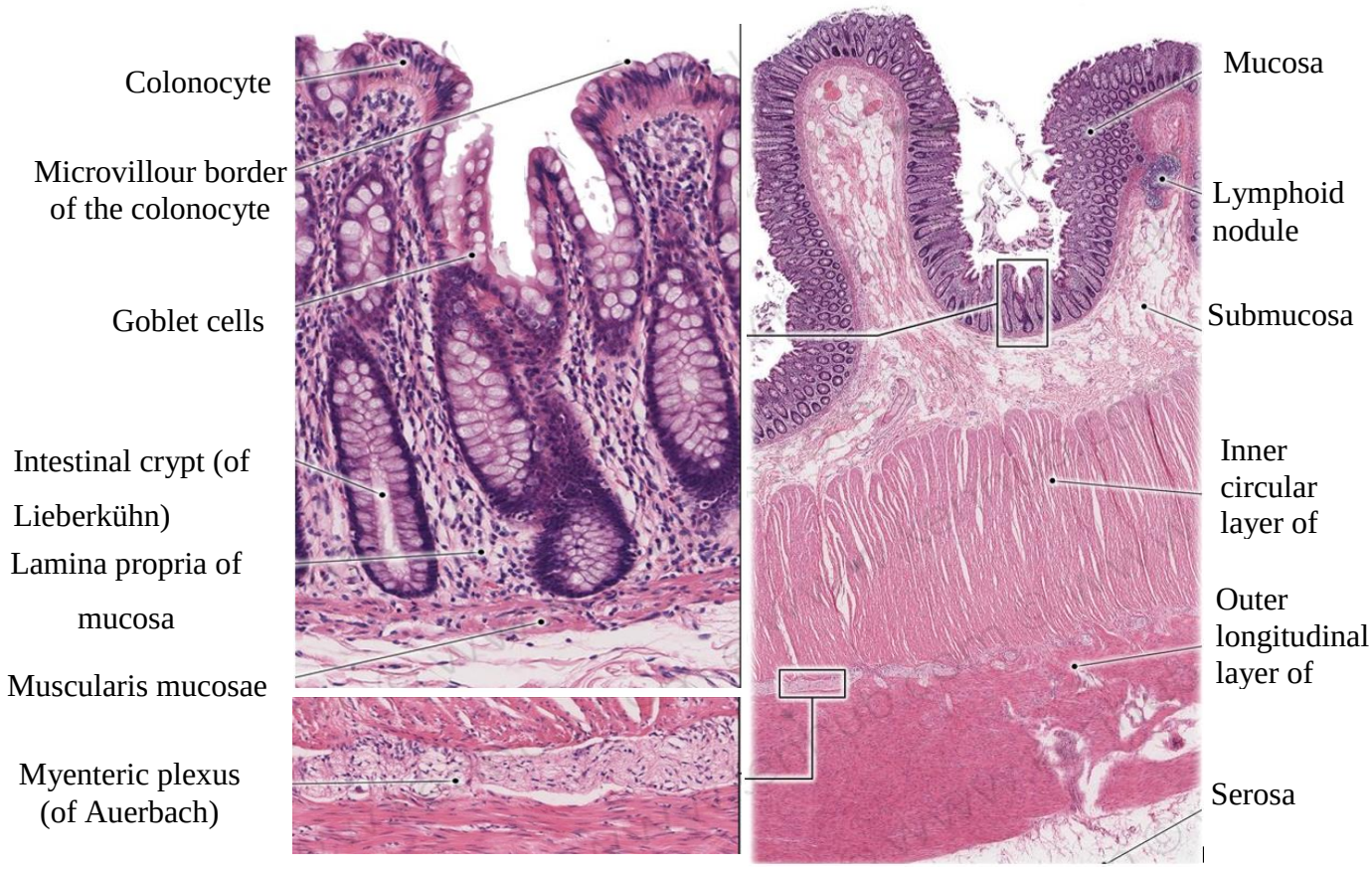

Figure 2. Longitudinal section of the colon. Stain: H&E. Left boxes: High magnification, Right box: Low magnification. (11).

The appendix is a muscular structure attached to the posteromedial end of the cecum. In human, the appendix is 6 mm in diameter and 5 to 35 cm (average 9 cm) in length (12, 13). In the past, the appendix was considered to be a vestigial organ and used to be removed during other abdominal surgeries. However, recent evidence suggests that this organ has an important physiological role in infants and adults. Endocrine cells appear in the human embryo appendix at around the 11th week of development (14). These cells produce various peptide hormones and biogenic amines that have a role in homeostatic mechanisms. The appendix also helps the process of maturation of B-lymphocytes and therefore functions as a lymphoid organ during the early stages of human development. In addition, the appendix can help the growth of beneficial gut bacteria. A study reported that a recurrence of *C. difficile* colitis was 4-fold higher in patients who underwent appendectomy compared to those with an appendix. (15). The appendix is reported to function as a "safe house" for healthy gut bacteria when disease flushes the bacteria from the rest of the bowel (15). The histological structure of the appendix is similar to the colon except that the appendix has masses of lymphoid tissue in the mucosa (11).

2. Colorectal polyps

Colorectal polyps are protrusions arising in the colon or rectum lumen mainly sporadic or as part of other hereditary conditions. Polyps are precursors in the majority of CRC cases, thus allowing for prevention approaches to CRC by the endoscopic removal of these lesions. While most polyps develop in the mucosa layer of the colorectum, some submucosal pathologies can result in mucosal protrusion into the lumen and present as mucosal polyps (6, 16). Polyps are detected in almost 50% of average-risk individuals aged ≥50 years who undergo colonoscopy, with a higher prevalence among males than females (6). However, less than 10% of polyps exhibit advanced neoplastic behaviours and progress to invasive adenocarcinoma in a process that might take 10 to 20 years (6). Colorectal polyps can be classified as adenomatous, serrated lesions, hamartomatous, or inflammatory polyps (16).

Adenomatous polyps are gland-like growths, and are the most commonly diagnosed polyp occurring in 20 to 40% of average-risk individuals aged ≥50 years who undergo colonoscopy (17). Based on histological appearances, adenomatous polyps are sub-classified into three types: tubular adenomas, villous adenomas, and tubulovillous adenomas. Tubular adenomas, which are characterised by having <25% villous component, are the most frequently diagnosed adenomas accounting for 65 to 80% of all polyps removed (16, 18). While the degree of atypia is variable, tubular adenomas usually harbour less atypia compared to villous adenomas and are usually pedunculated. Villous adenomas, which are characterised by having >75% villous features, account for 5 to 10% of neoplastic polyps and more often have severe dysplasia. Tubulovillous adenomas, which are characterised by having 25 to 75% villous features, account for 5 to 15% of adenomas (16). Size of the polyps and their histological types are that factors that have been associated with the risk of malignant features in adenomatous polyps. Advanced adenomatous polyps are defined as those ≥1 cm in size or with villous features or high-grade dysplasia regardless of the size (19). Among young adults and in the general population, ~85% of CRC cases arise through adenomatous polyps (20).

Serrated lesions, which are usually found in the proximal colon, range in morphology from lesions with exaggerated serrated architecture (saw-toothed papillary epithelial infoldings) and overt cytological dysplasia to those with superficial serrations. In addition, these lesions are clinically heterogeneous and can progress to tumours with various clinical features (21). Overall, serrated lesions are classified into four types which are hyperplastic polyps (microvesicular hyperplastic polyps or goblet cell hyperplastic polyps), sessile serrated lesions (SSLs) (with or without dysplasia), traditional serrated adenoma and serrated adenoma unclassified (**Table 1**) (21). Advanced serrated lesions are SSLs ≥ 10 mm, SSLs with dysplasia or any traditional serrated adenomas (19). Among serrated lesions, SSLs with dysplasia are considered to be the most advanced lesions and the most clinically significant subtype. This subtype account for 2 to 5% of all SSLs and less than 0.5% of all colorectal polyps (21). Similar

to other serrated polyps, SSLs are more predominant in females than males, and are frequently found in the caecum and ascending colon. Serrated lesions might progress to *BRAF* mutated and mismatch repair (MMR) deficient-CRC; *BRAF* mutated and MMR proficient-CRC; or *KRAS* mutated and MMR proficient-CRC (22). Up to 20% of CRC cases arise from serrated lesions (20).

Table 1. Morphologic and molecular characteristics of different types of serrated lesions (21).

Histologic features					Molecular features		
Type	**Crypt architecture**	**Proliferation zone**	**Cytologic features**	**Mucin type**	***BRAF* mutation**	***KRAS* mutation**	**CpG island methylation**
Microvesicular hyperplastic polyp	Funnel-shaped crypts with serrations limited to upper two-thirds	Located uniformly in the basal portion of crypts	Small basally located nuclei, no dysplasia	Mixed Micro-vesicular and Goblet cell	70–80%	0%	+
Goblet cell hyperplastic polyp	Elongated crypts that resemble enlarged normal crypts; Little to no serrations	Located uniformly in the basal portion of crypts	Small basally located nuclei, no dysplasia	Goblet cell only	0%	50%	−
Sessile serrated polyp	Horizontal growth along the muscularis mucosae, dilation (often asymmetric) of the crypt base (basal third of the crypt), and/or serrations extending into the crypt base	Proliferation may be abnormally located away from the crypt base, variable from crypt to crypt	Small basally located nuclei with occasional larger nuclei with inconspicuous nucleoli, no dysplasia	Mixed Micro-vesicular and Goblet cell	>90%	0–5%	++
Sessile serrated polyp with dysplasia	As for sessile serrated polyp	As for sessile serrated polyp with more proliferation in dysplastic component	Varied morphologic appearance to dysplastic component	Varied type	>90%	0%	+++
Traditional serrated adenoma	Slit-like serrations, often ectopic crypt foci	Present within ectopic crypt foci and crypt base	Elongated pencillate nuclei with nuclear stratification and cytoplasmic eosinophilia; may develop overt (conventional or serrated) dysplasia	Occasional scattered goblet cells; rare goblet cell variant has been described	20–40%	50–70%	BRAF mutated ++
Serrated adenoma-unclassified	Varied	Varied	Unequivocal dysplasia must be present	Varied	Uncertain	Uncertain	Uncertain

3. Epidemiology of young-onset colorectal cancer

3.1. Incidence and mortality rates of CRC in young adults

In 2020, more than 1.9 million new cases of colorectal cancer (CRC) and more than 935,00 CRC-related deaths were estimated around the world and these numbers are expected to grow to 2.2 million new cases and 1.1 million deaths by 2030 (23, 24). Currently, CRC is the third most commonly diagnosed cancer and the second leading cause of cancer-related death worldwide (25). However, the incidence and mortality of CRC vary substantially across the globe, with more than half of the cases occurring in more developed countries (26). Australia, New Zealand, Europe, and North America have the highest CRC incidence rates (27) while Africa and South-Central Asia have the lowest rates (28). In Australia, an estimated 15,494 new cases of CRC were diagnosed and 5,322 individuals died of this disease in 2020 (29). In 2018, CRC was estimated to be the most frequently diagnosed digestive-tract cancer and was Australia's most frequent digestive-tract cancer killer (30). The geographical variations reflect differences in risk factors including dietary and environmental exposures, uptake of screening and access to appropriate treatment services (26).

The age distribution, however, has altered with a decrease in CRC incidence rates in adults ages ≥50 years old, largely referred to as late-onset CRC (LOCRC), in the United States (US), Australia and many other countries since 1975 and has started to decline at a faster rate since 1998, presumably due to population screening and improvement in treatment (31-33). A population-based study by the Surveillance, Epidemiology, and End Results (SEER) program reported that in the US the CRC incidence rates in adults aged ≥50 years declined from 225.6 per 100,000 population in 1985 to 119.3 in 2013 (34) (**Figure 3**). The incidence rates of CRC among this age group declined by 32% between 2000 and 2013 (34). Siegel et al., reported that CRC incidence rates in adults aged 50 to 64 years old dropped by an average of 1.4% per annum, and by 4% in those aged ≥65 years old (34). From 2011 to 2016, the incidence rates of

CRC decreased by 3.3% per annum among adults aged ≥65 years old (35). In Australia, CRC incidence rates declined in individuals aged 50 to 69 years from the mid-1990s with the decline in the annual percentage change in specific age groups ranging from 0.8%-4.8% per year (36). Similar trends have been reported in Canada, New Zealand, UK, Germany and many other developed and developing countries (1, 37).

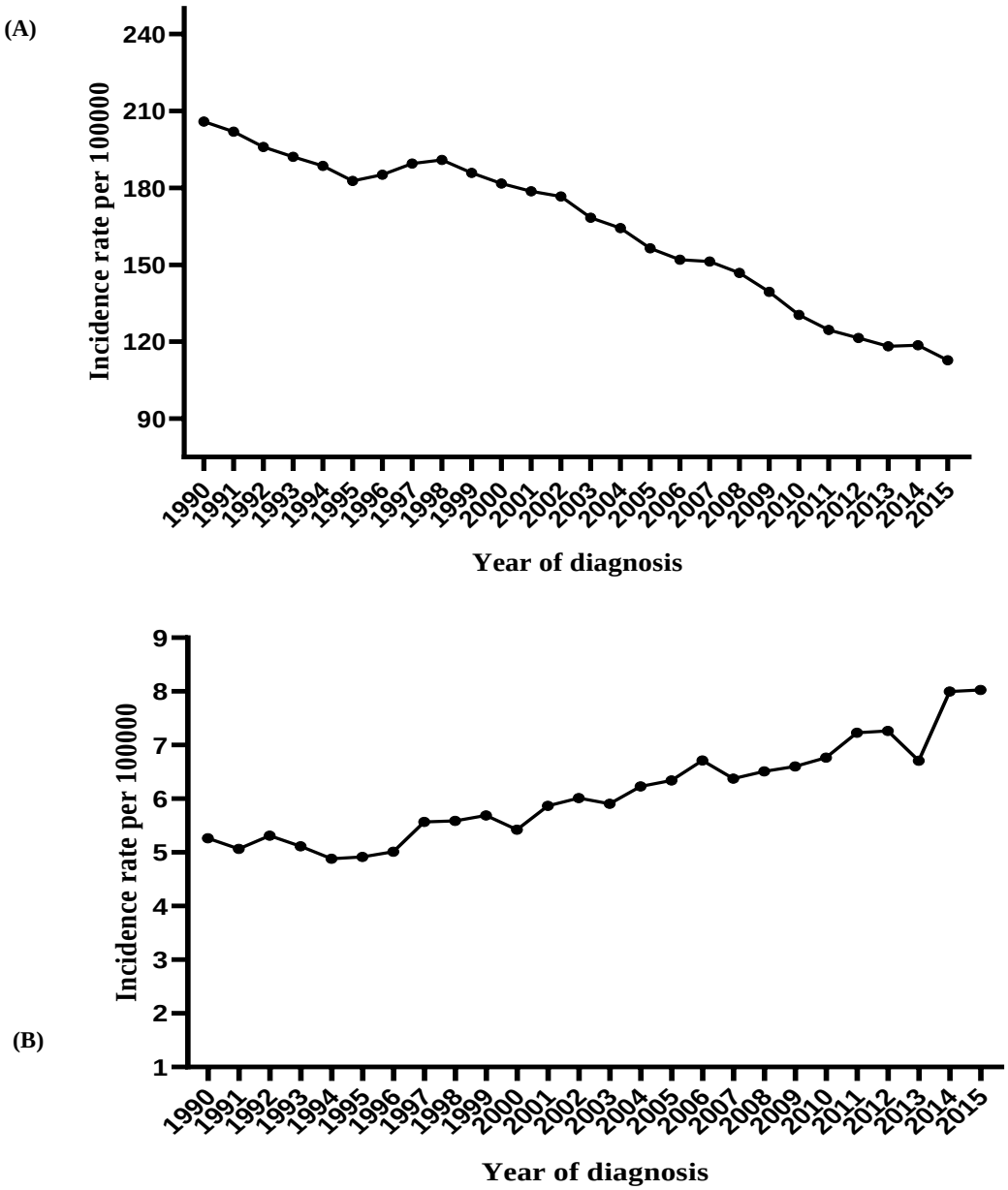

Figure 3. Age-adjusted SEER incidence rates of CRC from 1990 to 2015 among people above and under 50 years of ages in the US. Graph (A) shows the incidence of CRC in people above the age of 50 years. Graph (B) shows the incidence of CRC in people under the age of 50 years. (https://seer.cancer.gov/faststats/selections.php?#Output).

In contrast, a reverse trend of CRC incidence rates has been observed in young adults (1, 35, 37) (**Figure 3**). While there is a difference in the literature regarding the exact definition of young-onset CRC (YOCRC) as different researchers have used various age groups including individuals under 30, 35, 40, 50 or 55 years, it is largely defined as CRC in adults aged <50 years old (38-52). In 2003, O'Connell et al. reported for the first time an increase in the incidence rates of CRC at the population level in adults aged ≤50 years old (53). This observation was confirmed by another study in 2009 (54) and since then YOCRC has gained traction as a public health concern (3). In the US, the overall incidence rates of YOCRC increased from 9.9/100,000 in 1975-1980 to 11.7/100,000 in 2010-2014 (55). Among adults aged 20 to 49 years, the incidence rates of CRC increased from 8.6/100,000 in 1992 to 13.1/100,000 in 2016. In people aged 40 to 49 years, the incidence rate increased from 18.2/100,000 in 1992 to 26.5/100,000 in 2015 (56). In adults aged under 55 years old, the incidence of CRC rose by ~20% per annum from 1994 to 2014 (32). Recently, Zaki et al. reported that the incidence rates of CRC increased from 23.4 to 34.0/100,000 and from 46.6 to 63.8 for adults aged 45-49 years and 50-54 years, respectively, between 1992-1995 and 2016-2018 in the US. In contrast, the incidence rates declined from 81.7 to 63.7 in adults aged 55-59 years (57). In 2020, CRC ranked the fourth most commonly diagnosed cancer in adults aged 30 to 39 years in the US (58).

In Australia, the incidence of CRC has increased by 186% in individuals between the ages of 15 to 24 years in the last three decades (59). Feletto et al. analysed over 375,000 cases of colon and rectal cancers from 1982 to 2014 in Australia. This study confirmed that the incidence rates of colon cancer have increased in young adults under the age of 50 years since the mid-2000s, with the increase in the annual percentage changes ranging from 1.7% to 9.3% per annum depending on specific age group. The incidence rates of rectal cancer have also increased among the same group of ages from the 1990s, with the increase in the annual percentage changes ranging from 0.9% to 7.1% per annum (36). In addition, another study published in

2019 found that CRC incidence has increased by almost 10% in individuals under 50 years of age since 1990 in Australia (60). Between 1990 and 2010, the incidence rate of CRC increased by 85-100% in Australians aged 20-29 years and by 35% in the age group 30-39 years (61). In 2018, as many as 14% of all new CRC cases and 10% of all Australians who died from CRC were <55 years (59). In 2019, CRC was estimated to be the third most commonly diagnosed cancer in Australians aged 25-49 years (62).

Similar trends of increasing YOCRC incidence rates have been observed in various other countries in Europe (63), Asia (64, 65) and the Middle East (1). A recent study found that age-standardised CRC incidence rates in YOCRC were highest in Korea (12.9/100,000) and Australia (11.2/100,000) and lowest in India (3.5/100,000) during 2008 to 2012 among 42 countries (37). The data of the most recent decade showed that YOCRC incidence rates dropped in only three countries (Lithuania, Austria and Italy) and increased in 19 countries including the US, Canada, Australia, and New Zealand (37) (**Figure 4**). By 2030, it has been reported that incidence rates of colon cancer in individuals aged 20-34 years and 35-49 years will grow 90.0% and 27.7% in the US, respectively. However, the incidence rates of a rectal tumour will grow 124.2% and 46.0% in people ages of 20-34 years and 35-49 years, respectively (66). An estimated of 10% and 22% of all colon and rectal tumours, respectively, will be diagnosed in Americans younger than 50 years by 2030 (66).

Similar to incidence rates, mortality rates have also been decreasing in adults ages ≥50 years since the late 1980s in the US, Australia and many other countries (2, 67, 68). Overall mortality rates decreased almost by 50% from 28.1/100,000 in 1975 to 14.5/100,000 in 2013 in the US (67). In Australia, mortality rates decreased by 55% from 32.8/100,000 in 1985 to 18.7/100,000 in 2016 (**Figure 5**) (68). In contrast, mortality rates remained stable in adults with YOCRC from 1998 to 2012 (~ 2.4/100,000) (69) and increased by 1% per annum from 2004 to 2014 (70). The SEER program determined that from 1992 to 2015, CRC mortality rates slightly rose

among adults ages 40 to 49 years from 5.4/100,000 to 6.5/100,000 (56). In adults aged under the age of 55 years, CRC mortality increased by 11% from 2005 to 2015 in the US (32). A recent population-based study in the US found that the mortality rates of CRC in adults aged 30 to 39 years rose ~ 1% per annum from 2008 to 2017. During the same period, the mortality rates declined by 3% per annum in adults aged ≥65 (58). In Australia, CRC is considered to be the most common cancer-related cause of death for those aged 25-29 (43). Young et al. reported that age-specific mortality rates of CRC in Australians aged 20 to 39 years were stable between 1990 and 2010 (61).

In summary, studies have shown that the geographical variation in YOCRC incidence rate mirrors that in older individuals, with a three-fold difference between the highest rates in Korea and lowest in India. However, temporal trends in the age-specific incidence of CRC were variable, with an increase limited to only several countries for LOCRC versus most of the countries for YOCRC (37). The most rapid rise in YOCRC incidence appears to be in countries (such as Korea, Australia, and the US) where the rates are already highest. These data show that YOCRC is a public health concern in many developing and developed countries. (1).

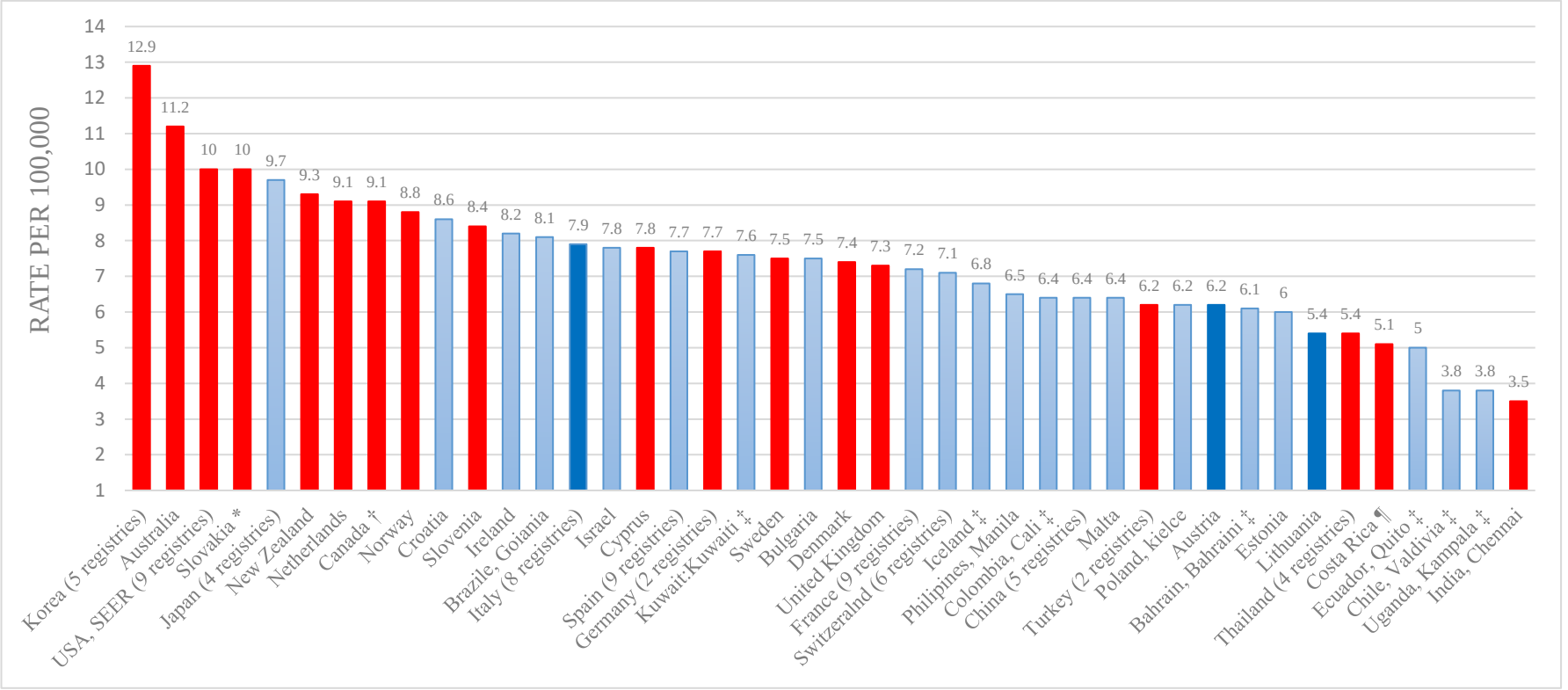

Figure 4. Age-standardized incidence rates for CRC in adults ages 20 to 49 years during 2008 to 2012. Column shading shows trends in incidence rates **bas**ed on ten years average annual percentage. Light blue: stable or insufficient number of cases for trend analysis (‡); Red: statistically significant increase; dark blue: statistically significant decrease. *Rate based on data during 2008 to 2010. †Excludes Nunavut, Quebec, and Yukon. ‡Excluded from trend analysis due to the insufficient number of annual cases. ¶Rate based on data during 2008-2011. Derived from a population-based study published by Siegel et al. (37).

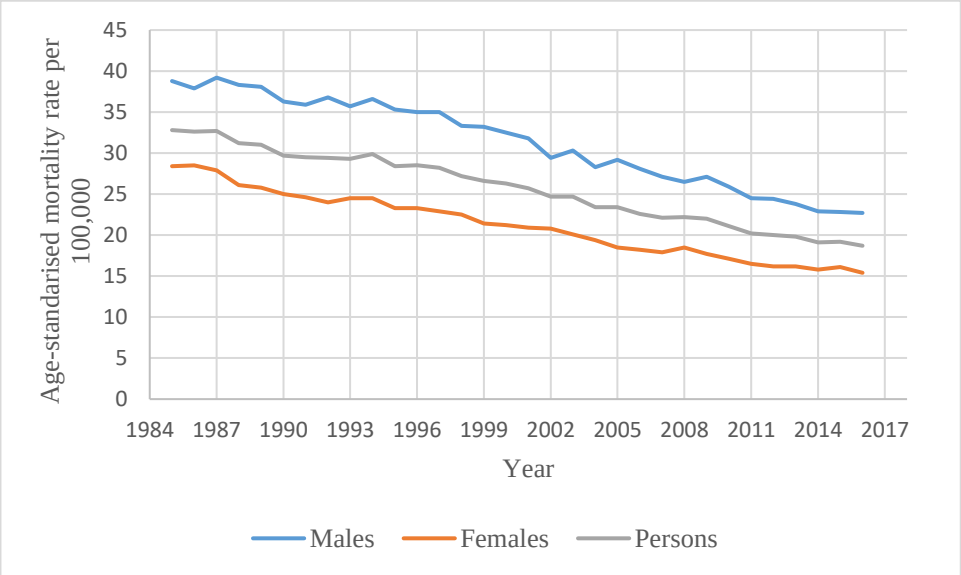

Figure 5. Age-standardised mortality rates in Australia for CRC, 1985 to 2016, by sex (68).

3.2. Ethnicity

The prevalence of YOCRC is not uniform across all racial and ethnic groups, though there is an increase in the prevalence of this disease across the entire population. For example, in the US, African-Americans have higher incidence and mortality rates of YOCRC and LOCRC compared to non-Hispanic whites (NHWs) (71, 72). A study reported that the proportion of YOCRC is approximately two-fold lower among NHWs relative to non-Hispanic blacks (NHBs) (73). According to the SEER database, YOCRC accounts for 16.5% of all CRC cases in American Indians/Alaska Natives (AIs/ANs), 15.4% in Hispanics, 12% in Asians/Pacific Islanders (APIs), 11.9% in African Americans and 6.7% in NHWs (34). The incidence rates of LOCRC for Whites declined by 40% from 1975 to 2013 compared to 26% for African-Americans (67, 72). However, a high-quality population based-study in the US reported that the incidence of CRC in adults aged 20-40 years from 1995 to 2015 increased in NHWs in 40 of 47 states while largely remained stable in Blacks and Hispanics (74). Between 1998 and 2009, YOCRC incidence has grown in White people by 3% each year for stage IV and 1.5% each year for stages I-III, but it was stable in black people (70). Recently, Siegel et al. showed

that in Black people, the average annual incidence rates increased from 11.8/100,000 person-years during 1995-1997 to 13.3/100,000 person-years during 2015-2017, and in White people from 8.3 to 12.4/100,000 person-years (75). The findings showed that Black-White disparities declined from a relative risk (RR) of 1.84 (95%CI, 1.75-1.94) to 1.31 (95%CI, 1.24-1.38). The data showed that over the last two decades, increased YOCRC risk among White people in the US decreased the Black-White disparities from 42% to 6% for incidence rates (75). While the incidence rate of YOCRC is still higher in Blacks than Whites, the increase in the incidence of CRC is mostly confined to Whites (74).

The risk of YOCRC-related death is higher in African Americans [1.35, 95% confidence interval (CI) 1.26-1.45] compared to NHWs, with a five-year survival of 54.9% and 68.1, respectively (73). These findings reflect the higher proportion of advanced stages YOCRC among minorities compared to NHWs. Wu et al. analysed the SEER database from 1973 to 2014 and found that the proportion of metastatic cancer in Blacks was higher than Whites and others (P =0.004) and Blacks had higher mortality rates (P = 0.001) (76). However, a recent study showed that mortality rates slightly declined in Black peoples from 4.6/100,000 during 1995-1997 to 4.1/100,000 during 2016-2018 and increased in White individuals from 2.5 to 3.1/100,000 (75). Black individuals aged 45 to 49 years old had 29% higher mortality rates than White individuals. These findings suggest that while incidence rates of CRC in young White and Black adults are currently comparable, the mortality rates remain considerably higher in Black people (75).

In New Zealand, the death rates of YOCRC were similar among Pacific people, non-Mâori non-Pacific people and Mâori by 1996-99. In contrast, the death rates accelerated up to 10-fold among Pacific people, by 50% among Mâori and decreased 10-20% among non-Mâori non-Pacific people during 1981-1995 (77). In Britain, age-standardized rates for people diagnosed with CRC is significantly higher than for Asian people (78). Weir et al. reported that

Aboriginals are significantly younger than non-aboriginals when diagnosed with CRC (17% compared to 6% of YOCRC). This variation in the proportion of CRC occurring at an earlier age may reflect the expected lifespan of this sub-group in particular countries, as well as genetic background and a greater sensitivity to Western lifestyle risk factors.

3.3. Gender

While the incidence and mortality rates of CRC in both genders are different across countries, incidence and mortality patterns are consistent. Incidence and mortality rates of YOCRC are increasing in men and women while opposite trends have been observed in both genders aged ≥50 years (3). In Australia, overall CRC incidence rates decreased by 18% and 10% in men and women from 2000 to 2015 (79). From 1968 to 2016, overall mortality rates in men and women decreased by 44% and 59%, respectively, in Australia (68). Similar trends have been reported in the US, New Zealand and Europe (33, 80-82). Regarding YOCRC, Vuik et al. reported that the incidence rates of CRC in men aged 20-29 years increased by 7.9% per year between 2005 and 2016 in Europe, and in women by 8.1% between 2003 and 2016. The same trend was observed for men and women aged 30-39 years (82). In the US, YOCRC in men and women increased by 1.5% and 1.6% per year, respectively, from 1992-2005 (54). In contrast, LOCRC in men and women decreased by 2.8% and 2.1%, respectively, since 1998 (54). In 2018, CRC was the second and third leading cause of cancer-related death in men and women aged 20 to 29 years old, respectively, in the US in 2018 (83). YOCRC accounts for 11% and 10% of all CRC cases in men and women, respectively, in the US (34). Among Australians aged 15-24 years from 2010 to 2014, CRC ranked as the fifth and fourth most commonly diagnosed cancer in males and females, respectively (59). In Pakistan, Zahir et al. found that the male to female ratio of YOCRC was 2:1, with a mean age of 33.3 ± 7.9 years (84).

3.4. Anatomic subsite

There is a consensus in the literature that there is an increased prevalence in the distal colon and rectum (sometimes referred to as left-sided CRC) in YOCRC patients (34, 54, 85, 86). By contrast, proximal colon tumour is more predominant within LOCRC patients (51, 86, 87) (**Figure 6**). Analysis from the SEER database has demonstrated substantial growth in distal and rectal cancers among individuals ≤50 (54). The incidence of colon cancer among individuals between 20 and 39 years of age has elevated by 1% to 2.4% each year since 1985. However, the growth in the incidence rates of rectal cancer among young adults increased more significantly, rising by 3.2 annually from 1974 to 2013 (88). After analysing 11,071 cases of CRC in individuals aged 15 to 39 years from 1998 to 2011, Teng et al. concluded that the rectum was the most common site accounting for 25%, and 67% of cases were diagnosed with left-sided CRC (38). In addition, Lu et al. conducted a multicentre study using national data and found that more than 74% of YOCRC patients had left-sided CRC compared to 56% of LOCRC (89). Based on these findings, it has been recommended that flexible sigmoidoscopy screening should be initiated at the age of 40 years in average-risk patients (90). In addition, this variation in incidence by anatomical sites have made scientists suggest to separate risk factors for colon versus rectal cancer. These anatomical subsites are different in terms of embryological origins, level of oxygenation, the concentration of bile salts and the microbial environment (91).

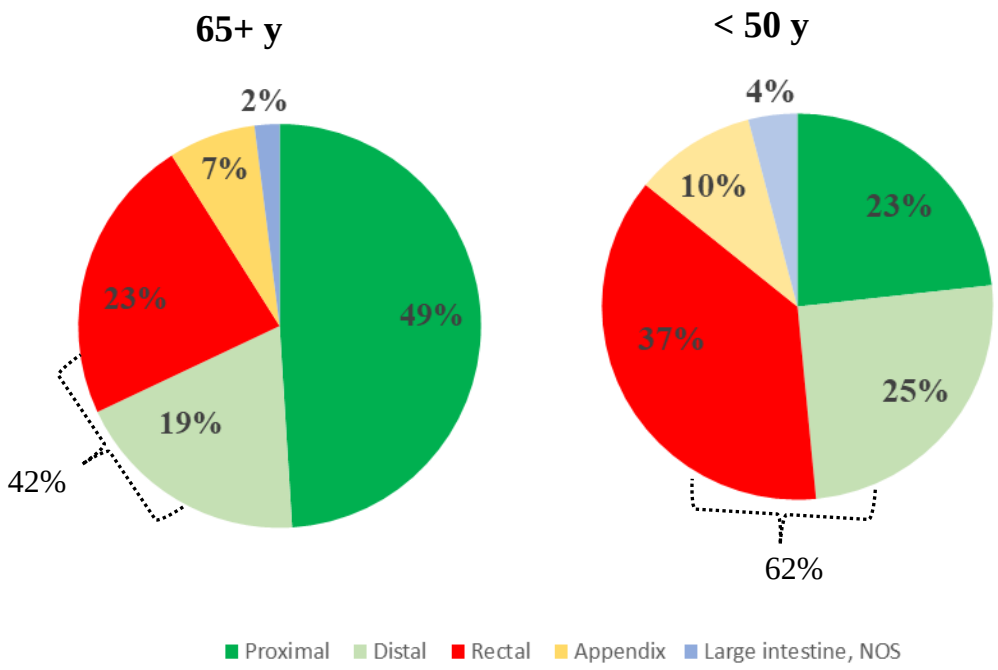

Figure 6. CRC subsite distribution by age (92).

3.5. Survival rate and prognosis

The overall 5-year survival rate of CRC for all stages combined from diagnosis is ~ 69% in Australia (93) and 60% in the US (32, 94). Considering all stages of the disease the 5-year survival rate of CRC has increased by 18% between 1985 to 1989 and 2010 to 2014 in Australia (93). In 2007, Quah et al. reported that the outcomes (recurrence rates and 5-year survival) of CRC stages I, II, III were similar, if not better, between YOCRC and LOCRC patients. In particular, there were no noticeable differences in the local and distant recurrence rates between the groups (17% vs 18%). Moreover, there were no differences in the 5-year recurrence-free survival between YOCRC (80%) and older patients (79%) after four years and eight months of following up (95). Finally, the overall relative survival rate in older patients (73%) was worse than in YOCRC patients (84%) (42, 95). In 2004, O'Connell et al. reported that the 5- year survival rate, when matched for stage, was the same for stage I and II between YOCRC and LOCRC patients, but it was substantially higher in younger individuals for stage II and IV disease (40). A retrospective analysis, including nine phase III trials, reported that progression-free survival- but not response rate or overall survival- was higher among those <40 years of

age compared to LOCRC patients (96). A Population-Based Cohort Study of SEER 9 Registries Data (1988–2011) reported that 5-year CRC-specific survival was the lowest in patients with LOCRC (62.8%), followed by the middle youngest group (41-50 years, 65.1%) and was the highest in patients aged 20-40 years (67.1%) (97). In contrast, reports from different treatment centres showed that YOCRC patients had low survival rate in comparison to LOCRC patients (90-92). For example, Lieu et al. concluded that the older the patients, the higher the prognosis (93). Khan et al. reported that 5-year disease-specific survival in YOCRC patients was 48% compared to 78% in their older counterparts (98). Other studies showed that age does not affect survival rate (99, 100). These findings show that there is inconsistency in the prognostic association of YOCRC. This is likely due to the heterogeneity in study population and design (101).

3.6. Symptoms and diagnosis

Although YOCRC patients frequently present with characteristic symptoms, they are more often diagnosed with advanced stage of the disease compared to patients with LOCRC (3, 101). Approximately 86% of young individuals are found to be symptomatic at the time of CRC diagnosis (54). Symptoms are often not specific such as abdominal pain (55%), fatigue and weight loss (35%); but YOCRC patients also tend to have a predominance of the symptoms in the left side including change in bowel habits (32%) and rectal bleeding (46%) (40). However, it has been reported that the majority of YOCRC cases at the time of diagnosis are already distantly metastatic or regionally advanced (87). According to the data from the National Cancer Database, ~ 63% and 57% of early-onset colon and rectal cancer, respectively, are at stage III/IV in comparison with 49% and 46% of the late-onset colon and rectal cancers (34). Another study showed that 44.5 % of LOCRC cases had metastatic disease at the time of diagnosis in comparison with 61.2% of YOCRC patients (102). Comparable with findings of other studies (38), Chen et al. reported that 71.5% of YOCRC cases were at stages III or IV at the time of diagnosis in comparison with 62.5% of LOCRC cases (102).

Diagnostic delay of YOCRC is probably due to some physician-based factors and/or patient associated factors. One example of this is the fact that many Primary care physicians (PCPs) and patients do not attribute abdominal pain and rectal bleeding to CRC in young adults and consequently do not follow further investigation (103). Physicians commonly attribute rectal bleeding in young patients to haemorrhoids instead of cancer without a proper medical evaluation (103). Physician-related delays in the diagnosis of this disease occur in 15-50% of YOCRC patients. Another explanation for the delay in diagnosis is that young individuals might under-utilize healthcare services probably due to the lack of knowledge about CRC, psychological factors (such as a sense of invincibility) and lack of health insurance (104). On average, patients with symptoms of CRC may wait about six months before seeking medical care (40).

PCPs can increase young patients' awareness of the symptoms of CRC and the importance of seeking medical care earlier by discussing with them the risk factors of this disease, the importance of screening tests and the value of early diagnosis during routine visits (51). Furthermore, PCPs might also reduce the risk of the delay of the CRC diagnosis by bearing in mind that CRC is one of the real potential cancers in young adults who have common CRC symptoms or have a family history with the disease (105). The rate of bowel cancer in both genders with rectal bleeding is about 25 times higher compared to the general population. Anaemia and change of bowel habits are more common symptoms in males than females (106). The risk of developing CRC is doubled in people of all ages combined in the presence of a second symptom (105). Rectal bleeding in combination with anaemia is a common symptom of bowel cancer and should be carefully examined. Symptoms are more common in people with rectal cancer compared to patients with colon cancer (51).

4. Risk Factors for Young-Onset Colorectal Cancer

4.1. Hereditary and family high-risk for CRC syndromes

Hereditary CRC syndromes and family history of the disease are the strongest risk factors for CRC. Compared to individuals without a family history of CRC, people with a first-degree family history of CRC have up to four times the risk of developing this cancer (107). The risk is even higher in patients with a first-degree relative (FDR) of YOCRC and those with multiple affected family members (108). The predisposition genetic syndromes contribute disproportionally to YOCRC (109). The commonly diagnosed CRC syndromes in young adults are Lynch Syndrome (LS), Familial Adenomatous Polyposis (FAP), MUTYH-associated polyposis (MAP), Serrated Polyposis Syndrome (SPS) (103) and Juvenile Polyposis (JP) (110). Other very rare hereditary syndromes that have been contributed to YOCRC include NTHL1-tumour Syndrome (0.1%), Peutz-Jeghers Syndrome (PJS) (<0.05%), PTEN-Hamartoma Tumour (<0.05%), and GREM1-Associated Mixed Polyposis (110).

LS is the most frequently diagnosed CRC inherited syndromes. It is defined as an autosomal dominant cancer predisposition syndrome which is caused by germline variants in one of the four DNA *MMR* genes (*MSH2*, *MLH1*, *MSH6*, and *PMS2*) (**Table 2**). Approximately 35%-45% of LS cases are diagnosed with CRC before the age of 40-45 years, and this syndrome accounts for approximately one-third of YOCRC in people under the age of 30 years (111-114). However, the average age at the CRC diagnosis among people with LS syndrome is 42-45 years (**Table 2**). Germline variants in the *MLH1* and *MSH2* account for ~ 70% to 90% of the LS (115, 116) while variants in *MSH6*, *PMS2* are detected in 10% to 20% of LS cases (117). Activity of the DNA MMR enzyme is lost within LS tumours, which results in the accumulation of multiple frameshift mutations, facilitates cancer growth and metastasis, and is characterized by detection of microsatellite instability (MSI). Furthermore, another alternative silencing cause for *MSH2* and leading to LS is a germline deletion in the epithelial cell adhesion molecule (*EPCAM*) gene

which is located in the upstream of *MSH2* (118). *EPCAM* germline variants are reported to account for 6.3% of all LS cases (119). This germline deletion results in silencing the transcription of *MSH2* by causing its allele-specific methylation. Therefore, the risk of bowel cancer in patients with EPCAM germline variants (75%) is similar to those with *MSH2* variants (77%) by age 70 years (120). The clinicopathological features associated with CRCs in LS include poor tumour differentiation, proximal location, mucinous histology, lymphocytic reactions, and synchronous and metachronous lesions (114, 121). People with LS have a 70% lifetime risk of susceptibility to CRC (122) (**Table 2**). In addition, a recently known Lynch-like syndrome (LLS) is described as an MMR-deficient colorectal tumour without germline variants and/or *MLH1* promoter methylation, and it can be as frequent as 70% of suspected LS patients (123). Lastly, bi-allelic deleterious variants of MMR genes result in constitutional mismatch repair deficiency. This condition predisposes patients to CRC with an average age of 16 years at the time of diagnosis which is much earlier than mono-allelic LS (124-127).

Table 2. Summary of the main hereditary and family high risk of CRC syndromes.

Syndrome	Inheritance	Gene	Average age of CRC diagnosis	Lifetime risk factor	Incidence in CRC population	Clinicopathological features
LS	Dominant	*MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*	40-45	70%	3-4%	Poor differentiation carcinoma, mucinous carcinoma, tumour-infiltrating lymphocytes and more commonly found in the right colon.
FAP	Dominant	*APC*	39	100%	< 1%	Good differentiation carcinoma, no mucinous carcinoma, no lymphocytic reaction and more commonly found in the distal colon.
MAP	Recessive	*MUTYH*	48	43-100%	<1%	Development of 10 to 100 adenomatous polyps in the colon and rectum
SPS	Unknown	Unknown	55-65	<50%	<1%	Multiple and/or large serrated polyps throughout the colon and rectum.
JP	Dominant	*BMPR1A*, *SMAD4*	34-44	20%	0.2%	Presence of juvenile hamartomatous polys in the gastrointestinal tract.

LS: Lynch syndrome, **FAP**; Familial Adenomatous Syndrome, **MAP**: MUTYH-Associated Polyposis, **SPS**: Serrated Polyposis Syndrome., JP: Juvenile polyposis.

FAP is the second most frequently diagnosed autosomal dominant inherited syndrome and clinically, classic FAP is characterized by the development of numerous (hundreds to thousands) adenomatous polyps in the bowel beginning at the age of 10-12 years (**Table 2**). Germline variants in Adenomatous Polyposis Coli (*APC*) gene is an early event in the

progression of FAP and is the main cause of the classic and attenuated FAP syndromes (128) (**Table 2**). Pathogenic germline variants in *APC* have been identified in less than 1% of the overall CRC cases and up to 5% of the YOCRC cases (129). Patients with this syndrome have a 100% lifetime risk of CRC by the age of 40 years if prophylactic colectomy is not performed. However, the median age at CRC diagnosis among people with FAP syndrome is around 39 years (130) (**Table 2**).

MAP is another subtype of adenomatous polyposis which has an autosomal recessive transmission and is related to bi-allelic germline variants in the *MUTYH* gene (131). MAP patients have a lifetime risk of developing CRC from 43%-100% at an average age of 48 years and about 50% of cases present with cancer at the time of diagnosis (132, 133). The phenotypes of attenuated FAP and MAP syndromes are often indistinguishable. There are currently; no exact phenotypes of MAP condition. Some reports show one single CRC and no polyps or less than ten, or cases presenting with mostly hyperplastic/serrated polyps (134) whilst proximal adenomas are commonly seen. Moreover, the cancers related to MAP syndrome are more likely to be found in the proximal side of the colon in comparison to the AFAP related tumours (133). Germline variants in the *MUTYH* gene have also been reported in patients with LLS (**Table 2**). Studies have shown that the MAP accounts for less than 1% of the overall CRC cases as well as YOCRC cases (112, 129).

Further, another syndrome known as SPS is characterized by the presence of many serrated polyps in the colon and rectum (135). Boparai et al. found CRC in 35% of individuals with SPS, with the majority of the cases (94.5%) were diagnosed with CRC at the time of SPS diagnosis. However, 6.5% of patients were under surveillance for SPS while diagnosed with CRC (136). Currently, 40% to 60% of SPS patients demonstrate a family history of bowel cancer rather than of polyposis. Nevertheless, it is worth noting that the exact patterns of

inheritance of this syndrome are still not clear, and autosomal recessive alleles and autosomal dominant alleles are suggested (137, 138) (**Table 2**).

JP is a rare autosomal dominant condition defined by the presence of juvenile hamartomatous polys in the gastrointestinal tract. The clinical diagnosis of this syndrome includes the presence of any number of juvenile polyps in a patient with a family history of the disease, the presence of juvenile polyps along the digestive tract (including the stomach), or the presence of >5 polys in the colon and/or colon (110). Pathogenic germline variants in *BMPRIA* or *SMAD4* are associated with the risk of JP, with the lifetime risk of developing CRC of 20% (139). Germline variants in these genes have been reported in 2% of YOCRC cases (110). The mean age of CRC diagnosis in patients with JP is between 34-43.9 years (140, 141).

CRC has an apparent hereditary component as it is estimated that 40% of CRC risk may be explained by hereditary factors. However, in a large number of cases, no known genetic risk factors or family history can be identified indicating the presence of missing heritability in CRC aetiology (142). This might be explained by using the genome-wide association study (GWAS) approach and identifying common genetic variants linked to the risk of developing CRC. Zhang et al. reviewed the recent newly identified genetic variants associated with the risk of CRC and clinical outcome of CRC patients (143). Currently, common genetic variants associated with CRC risk have been identified across more than 40 chromosome regions. The majority of these identified common variants have a modest impact on the risk of CRC (OR < 1.20) (143). While there has been no study investigating the direct association of genetic variants with the clinical outcome of CRC patients, many others identified a number of genetic variants which are generated from candidate gene or pathway-based studies (143, 144). More recently, Liu et al. investigated CRC risk loci using patients from the Swedish Low-risk CRC study (2663 CRC cases and 1642 controls) and reported that SNP analysis did not generate any significant results (145). It is worth noting that there is a lack of understanding of the mechanism of many single

nucleotide polymorphisms (SNPs) that are associated with the risk of CRC and further studies are needed to evaluate the utility of SNPs as prognostic markers of CRC in clinical sittings.

4.2. Lifestyle and health-related colorectal cancer risk factors

Though YOCRC raises the likelihood of a hereditary predisposition, the majority of cases are sporadic rather than inherited in nature (146). Studies have shown that YOCRC has risen across successive birth cohorts. A recent study analysis found that persons born in 1990 were 4.1 times at higher risk of rectal cancer and 2.6 times at higher risk of distal cancer than persons born in 1950 (147). Another study reported that the incidence rates of CRC among 40 years individuals born in 1950 were lower (18.3/100,000) compared with individuals born in 1970 (24.4/100,000) (148). While the risk by birth cohort differs within each country, rises in YOCRC by birth cohort have occurred globally (36, 149). Some of this increase in the incidence rate of CRC in the recent cohorts may reflect the detection of prevalent subclinical disease due to increasing colonoscopy utilization for screening, diagnostic and surveillance purposes. However, this does not fully explain the trends in YOCRC because inconsistent with a screening effect, the incidence rates have increased at a similar magnitude for early and advanced stage of the disease and the most rapid gains are for adults in their 20s and 30s (the least likely age groups to be screened) (150). The strong birth cohort effect suggests population-level changes in lifestyle-related factors that influence CRC risk (3, 151).

Well established non-hereditary risk factors for CRC are obesity, a high intake of red or processed meat, smoking, lack of physical activity and heavy consumption of alcohol (3, 151). Some factors such as dietary consumption of calcium and folate might decrease the risk of colon tumour but have no link to a rectal tumour, others such as processed meat may increase the risk of colon tumour but have no link to a rectal tumour, and there are factors including obesity and cigarette smoking that might increase the risk of colon tumour as well as rectal tumour (152). However, it is worth noting that these associations are mainly based on CRC occurrence in older cohorts rather than YOCRC. In addition, people with a personal or family health record

of inflammatory bowel disease (IBD) including Crohn's disease (CD) or ulcerative colitis (UC) are more likely to develop chronic inflammatory CRC. Consequently, patients with this chronic disease are at higher risk of developing CRC (153). Moreover, individuals with adenoma (particularly those with multiple polyps), diabetes, solid organ transplantation, appendicitis and with a personal history of other types of cancers such as ovarian cancer, endometrial cancer, and breast cancer are also at higher risk of CRC (154-157).

4.2.1. Obesity and CRC risk

By 2030, more than 2 billion adults are estimated to be overweight, and 1.12 billion to be obese worldwide (158, 159). Obesity has widely been associated with various types of cancers including CRC. Obese patients can have chronic inflammation, insulin resistance, metabolic syndrome as well as modifications of gut microbiota (160). The prevalence of obesity increased among people of all racial groups and ages in the last three decades in the US (54). For example, 35% of Americans aged 20-74 years were obese in 2014 compared to 15% of them in 1979 (161). Therefore, because of the parallel increase in the YOCRC incidence rates and obesity, recognizing the role of obesity and overweight in YOCRC may assist to understand this trend (160).

The risk of developing CRC increased by 13%-18% in each five-unit increase in the body mass index (BMI). This risk was reported to be more significant in males compared to females, and for colon cancer compared to rectal cancer (162). Levi et al. investigated the link between adolescence BMI and CRC risk and found that obese men and women are at higher risk of developing colon cancer (Hazard ratio (HR), 1.54 and 1.51, respectively), and rectal cancer (HR, 1.71 for men and HR, 2.03 for women) (162). The substantial point of this population-based current research was the predominance of YOCRC (162). In addition, another study reported that obese or overweight women had a double higher CRC risk under the age of 50 years compared to healthy body weight women (160). The findings of this study, in which data of 85,256 women under the age of 50 included, showed that higher recent BMI (at the age of

18 years) and obesity since early adulthood are strongly linked to the increased risk of YOCRC in women (160). The link of excess body weight to CRC was shown to be stronger in females relative to males when genes related to obesity were studied according to Mendelian randomization (163, 164). Sanford et al. investigated the association of obesity and YOCRC versus LOCRC by analysing 3,173 CRC cases and found that BMI ≥30.0 kg/m^2 was associated with YOCRC (adjusted odds ratios (AORs) 1.4% CI: 1.00-1.92) but not with LOCRC (AOR 0.93, 95% CI: 0.85-1.03) (165). Another population-based cohort analysis studied around 7000 CRC cases with 8.3% of them being YOCRC patients and reported that several factors were associated with the increased risk of YOCRC versus LOCRC. These factors included having obesity [odd ratio (OR) 1.14, 95%CI: 1.08-1.20, P < 0.001], a family history of any cancer (OR 1.78, 95%CI: 1.67-1.90, P < 0.001) and African-American race (OR 1.18, 95%CI: 1.09-1.27, P < 0.001) (166). In addition, compared to controls, increased YOCRC risk was associated with obesity (OR 2.88, 95%CI: 2.74-3.04, P < 0.001), having a family history of cancer (OR 11.66, 95%CI: 10.97-12.39, P < 0.001), African-American race (OR 1.25, 95%CI: 1.17-1.35, P < 0.001) and male gender (OR 1.34, 95%CI: 1.27-1.41), P < 0.001) (166). A recent systemic literature review and meta-analysis of 20 studies showed that obesity (RR 1.54, 95% CI 1.01-2.35) and hyperlipidemia (RR 1.62, 95% CI 1.22-2.13) were significant risk factors for YOCRC (167).

Nevertheless, while increases in BMI across Europe are almost similar, there are differences in CRC trends. In Germany, BMI rose in men from 24 kg/m^2 in 1975 to 27 kg/m^2 in 2014, where the incidence of YOCRC rose, as well as in Italy, Austria, and Croatia, where the incidence of YOCRC did not rise (3, 168). Low et al. conducted a case-control study and reported that in the adjusted analysis, obesity or overweight and aspirin use were significantly associated with decreased odds of YOCRC. They found a significant association of male sex and increasing age with the increased YOCRC risk (169). Chen et al. reported that the rates of obese (5.5%) and overweight (3.6%) status in young adults with CRC were lower than late-onset CRC

patients (5.6% and 13.4%, respectively) (102). A population-based study analysed changes in YOCRC risk factors and incidence rates in the US and concluded that there was no association between trends in obesity and CRC incidence rates (74). In addition, a retrospective study found no association of obesity with the increased risk of YOCRC (170). Finally, it is worth noting that CRC trends vary by ethnicity, subsite, and state within the US indicating a role for risk factors beyond obesity or overweight (74, 171, 172). To illustrate this, studies have shown that rectal cancer appears to be driving the rise in the incidence rates of YOCRC, although obesity is more significantly associated with colon cancer than rectal cancer (3, 74, 171, 172). The findings of these studies show that obesity does not fully explain the cause of the spike in incidence rates of YOCRC.

4.2.2. Type 2 diabetes and the risk for CRC in young adults

Type 2 diabetes (T2D) is a large and growing global health public concern and has become a serious problem in developing countries. It is estimated that the incidence of T2D will continue to rise in the next two decades, and 70% of cases will be in developing countries (173). The number of T2D patients has doubled in the USA between 2000 (4%) to 2010 (8%) (174, 175). In 2014-2015, an estimated one million Australians had T2D accounting for 85% of all diagnosed diabetes in Australia. The link between T2D and cancer was officially recognized in the American Diabetes Association guidelines (2010) (176). T2D has been associated with some of the other commonly diagnosed cancers that are the leading causes of death such as CRC, liver and breast cancer (177). Tsilidis et al. (2015) investigated the association of T2D with the risk of developing cancer across published meta-analyses or systematic reviews and found that this condition was associated with a 27% increased risk of CRC among older individuals (178). However, the mechanisms causing the initiation and progression of a tumour in T2D are not well known (179) and may involve the interplay of several factors such as insulin signalling, genetic predispositions, adipokines, inflammation, and microbiota.

T2D is often associated with basal hyperinsulinemia, in which there is a high insulin level in the blood. Insulin might stimulate cell proliferation by binding to insulin or insulin-like growth factor-1(IGF-1) receptors and by inhibition of IGF-1 binding proteins which result in the increase of the availability of IGF-1 to the IGF-receptors (180). Therefore, though findings are not consistent across all studies, several studies have associated the high levels of IGF-1 with the risk of bowel cancer and adenoma (180, 181).

There is evidence showing a strong association between genetic basis and T2D (for example, twin and family studies). The concordance rate for T2D in dizygotic twins is significantly lower (20%-30%) relative to monozygotic twins which are about 70% (182). It is reported that people with one T2D parent are ~40% at higher risk of developing this condition compared to the normal population (183). However, when both parents have T2D, the risk of developing T2D in offspring is ~ 70%. The lifetime risk of developing T2D is doubled in the offspring with first-degree family history, and the risk is higher when the mother is affected (184, 185). When a known group of diabetes risk loci were examined in the setting of CRC, evidence for an interaction between T2D-related variants and gender, as well as for T2D status, in modulating CRC risk was demonstrated (186). Sainz et al. found that the *TCF7L2* _rs7903146_T allele was associated with an increased risk of developing CRC in females (P_{trend} = 0.003) but not in males ($P_{interaction}$= 0.06) (186). These studies suggest either that there is overlapping polygenic genetic predisposition between T2D and CRC, or alternatively, the genetic predisposition for T2D creates a physiological milieu in which CRC development becomes more likely in predisposed individuals.

CRC and T2D share similar environmental and lifestyle risk factors such as physical inactivity and obesity. Therefore, this observation suggests that T2D itself might be a risk marker for CRC. Visceral obesity is a state of chronic systemic inflammation, and this might be one of the mechanisms in which intestinal neoplasia develops. The non-steroidal anti-inflammatory drug

group, including aspirin, are reported to be the strongest primary preventative agents for bowel cancer. Therefore, systemic inflammation is suggested to be a driver for CRC development. Non-alcoholic fatty liver disease and other conditions related to obesity are linked to both systemic inflammation and increased risks for adenomas and adenocarcinoma in the large bowel, reviewed in (187), with increasing odds ratios for individuals aged under 40 years for obesity and metabolic syndrome(188). However, even in apparently normal-weight individuals, such a pro-inflammatory state may be present (105, 189).

One of the most important risk factors of CRC is age. Age and methylation of some CpG islands are highly associated, and models using these markers can precisely anticipate the chronological age of a tissue. Changes in DNA methylation due to the age increases the risk of the development of cancers. *De novo* methylation of CpG islands has been well documented in the human colon with age and with inflammation (190), in CRC (191), in mucinous and poorly differentiated CRC (common in YOCRC), and in sessile serrated polyps (SSP) (192). Lifestyle-related factors might considerably change these observations. For example, using insulin and hormone replacement decrease age-related methylation while smoking and obesity increase the DNA methylation (193). Although there is evidence for a genetic predisposition to epigenetic ageing (194), environmental factors such as T2D might be a trigger. Dobson et al. has recently used an animal model and found the impacts of high intake of diets rich in sugar in early life through insulin-like signalling on genes with forkhead box promoters, consequently changing the expression of epigenetic regulators as well as reducing lifespan (195). This mechanism is found to be highly conservative in mammals (**Figure 7**). Epigenetics may also play a role in the transgenerational transmission of risk. The evidence of this is seen in long-term studies of Swedish people who have been exposed to cycles of famine and feast. Overfeeding during developmental windows before the production of sperm or ova resulted in diabetes and cardiovascular disease in subsequent generations (196, 197), with the strong parent of origin effects, suggestive of imprinting. However, although the link between CRC and T2D has been

frequently reported in studies, the association between personal and/or family history of T2D and YOCRC has not been widely investigated. Similar to YOCRC trends, T2D incidence has also been rising in young adults in the US and globally. In addition, T2D in young adults under the age of 45 years appears to be more aggressive. A population-based study concluded that young adults (<45 years) with T2D had 80% higher risk of requiring insulin therapy compared with their older counterparts (198). Therefore, understanding the role of T2D in YOCRC will likely provide a significant insight into the aetiology, prevention and early diagnosis of YOCRC.

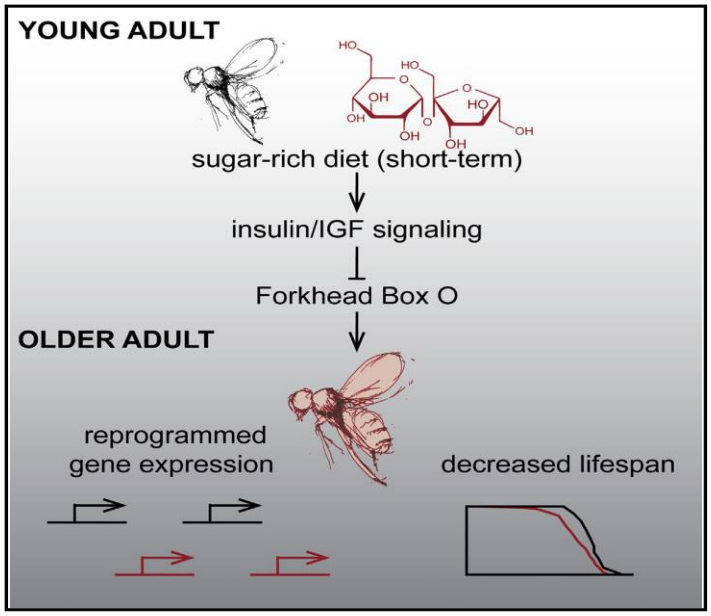

Figure 7. Excess dietary sugar in early life programs Drosophila lifespan through the activation of insulin-like signalling and the regulation of forkhead box O transcription factors (195).

4.2.3. Inflammatory bowel disease

Chronic Inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and Ulcerative disease (UC) are longstanding inflammatory conditions in the colon which are caused by a combination of environmental factors, hereditary predisposition, and disorder in the immune system. The incidence of IBD is more common among young adults in comparison to elderly and pediatric individuals (199). The high mortality rate among people with IBD (UC and CD) is mainly due to CRC. It has been reported that one out of twelve and one out of six mortalities

in people with CD and UC, respectively, is due to the CRC (200). Patients with IBDs have 2-3 fold higher risk of CRC, especially when diagnosed in adolescence age, in comparison to the general population (199). Therefore, IBS ranks as the third most high-risk condition for CRC followed by FAP and LS (201). One study found that around 41% of IBD patients with CRC were ≤ 50 years old at the time of CRC diagnosis (202).

Genetic predisposition factors are found to have a prominent role in paediatrics onset IBDs, but environmental factors have a higher role in the development of elderly onset IBDs than genetic factors (203, 204). In European countries, recent studies show an increase in the number of incidence of a paediatric-onset CD over the past 20-30 years, while the number of UC cases has not changed or slightly declined (205-207). However, the frequency of IBDs is rising globally, and the population aging makes this disease a rising problem in elderly individuals. Moreover, there might be a change in the pattern of elderly onset IBDs because the number of CD is lower than the incidence of UC in the French study (208).

4.2.4. Appendicitis

Acute appendicitis (AA) is one of the most common causes of abdominal emergency operation. The lifetime risk of developing this condition is about 7% and is more common in adolescents and young adults (209). Appendectomy is the standard treatment for AA. In Australia, the appendectomy rate was the second-highest in the Organisation for Economic Co-operation and Development (OECD) in 2013, with 177 per 100,000 population. The rate increased by ~ 22% between 2000 and 2013 in Australia (210). Since the appendix has reported having a defensive role against malignancy, appendectomy has also been associated with some forms of cancer including LOCRC (211, 212). According to Wu et al., individuals undergoing appendectomy are 4.60 times at higher risk of developing any type of malignancy compared to their comparison group. The finding also showed that 1.8% of patients developed some forms of cancer during the first year after an appendectomy. Moreover, the HR for developing CRC was 14.7 (99.9% CI 8.66-25.0) (212). In Taiwan, a retrospective study of 17873 patients with AA

was conducted by Lai et al. in 2006. They found that 0.85% of them had CRC at the time of appendectomy or within three years and 4 months after the operation, and the incidence of CRC in individuals older than 40 years was 1.76% (213). In addition, more recently Mohamed et al. conducted a retrospective study of 1633 patients at the ages of 40 years and older who underwent an appendectomy. The overall incidence of caecal cancer was reported to be 0.7% among the participants and was 2.2% among patients aged ≥ 55 years (214). Cakmak et al. investigated a possible association of a colon tumour with appendix vermiformis length by conducting a retrospective study of abdominal CT scans of 60 healthy individuals and 60 colon tumour patients. The mean length of the appendix vermiformis was found to be significantly shorter ($P < 0.001$) in colon tumour patients [65.178 mm ± 13.46 (SD)] compared to that in the healthy individuals [101.99 mm ± 16.58 (SD)], indicating that this organ might have a critical role in the development of a colon tumour (215).

On the other hand, studies have also shown the association of non-surgical management of AA with increasing the risk of cancer. Shine et al. performed a retrospective study in New Zealand to investigate the prevalence of CRC in 612 patients (aged > 50 years) presenting with AA compared to New Zealand standard rates. The outcomes showed that fifteen patients had a diagnosis of CRC during the period of following-up. The risk of CRC was 6.3-fold (95% CI 3.6-10.2) higher among patients aged 45 and over and was 17-fold (95% CI 8.0- 32.2) higher among individuals aged 45-60 years compared to New Zealand standard rates (216). Enblad et al concluded that 2.5% of 13,595 AA patients with non-operational treatment were diagnosed with small bowel cancer, appendiceal or CRC (SIR 4.1, 95% CI .7-4.6) (157). They also reported that the largest incidence was appendiceal and proximal colon tumour (SIR 35, 95% CI 26-46; SIR 7.5, 95% CI 6.6-8.6, respectively). Moreover, the incidence of CRC was higher among appendicitis patients with abscess and without abscess (SIR 4.6, 95% CI 4.0-5.2; SIR 3.5, 95% CI 2.9-4.1) (157).

4.2.5. Smoking and CRC risk

Smoking is a well-established risk factor for developing CRC. There are meta-analyses showing ~ 20% increase in the risk of developing CRC for former or new smokers than never-smokers (105, 217). In the US, smoking is associated with ~ 20% of all CRC cases (218) and about 12% of overall CRC-related deaths (219, 220). In addition, other studies have found that there was ~ 30% increase in CRC risk in both men and women smokers compared to non-smokers (221-227). This association appears to be different across CRC subtypes, though the results have been discordant. Tobacco smoking has been more strongly linked to the risk of a colon tumour, and particularly with MSI- high (H) CRC (225, 228). Limburg et al. conducted a cohort study of long term postmenopausal women (n = 41836) and concluded that there was a stronger association between women smokers with increased risk of proximal CRC compared to distal CRC (229). In contrast, Diergaarde et al. showed no increase in MSI-H CRC among smokers (230).

Smokers are diagnosed with CRC earlier than non-smokers with at least five years differences in age (231-233). Terry et al. conducted a prospective study and reported that in moderate and heavy smokers, CRC was diagnosed at the age of 48-50 years compared to 56 years of age in non-smokers (234). Likewise, Anderson et al. found that non-smokers were diagnosed with CRC starting at the age of 56.8 years, which was later than smokers (50 years) (235). While the association of smoking with a younger age of CRC diagnosis has been reported in both genders, the risk is higher in females. Studies have also shown that females are more susceptible to the carcinogenic effect of smoking cigarettes than males. The reasons for this observation are still unclear (236).

Additionally, tobacco smokers are more likely to be diagnosed with advanced stages of CRC in comparison with non-smokers. Anderson et al. and Lieberman et al. found that smokers were 2-fold at higher risk of advanced neoplasia than non-smokers, similar or higher than those

patients that have FDR with this disease (235, 237). Zisman et al. found that there was an association between drinking, smoking and smoking plus drinking with the increased risk of YOCRC (adjusted age difference, 5.2, 5.2, and 7.8 years, respectively; P<.001 for all) (233). Therefore, the American College of Gastroenterology recommends physicians to be aware of a higher risk of CRC in tobacco smokers (238). However, it is worth noting that cigarette smoking needs more than 30 years as an induction period to induce tumourigenesis (217). A recent systemic review and meta-analysis showed no statistically significant association between smoking and increased YOCRC risk (RR 1.35, 95% CI 0.81-2.25). A retrospective study found no association of smoking with the increased risk of YOCRC (170). In addition, birth cohort studies have shown that smoking is decreasing in young adults (61), suggesting that smoking is less likely to contribute to the observed increasing trend in the incidence rates of YOCRC.

4.2.6. Alcohol consumption and CRC risk

Although there is an ambiguity in the clinical studies related to the heavy drinking of alcohol, alcohol abuse has been widely associated with an increased risk of CRC. The risk of developing CRC is estimated to be higher by 20% and 40% among individuals who have 2-3 and more than 3 alcoholic drinks per day on average, respectively, in comparison to non-drinkers or light drinkers (239). Findings of a pooled analysis of 8 cohort investigations from EU and North America showed a modestly increased risk of colon tumour and rectal tumour with regular high intake of alcohol (>45 g/day) in both genders combined in relative non-drinkers (240). Another pooled analysis of 5 Japanese cohort studies showed that compared to non-drinkers, the risk of CRC was higher among males and females who drink ≥23 g of ethanol regularly per day (241). However, Pedersen et al. investigated 411 patients with colon tumours and 202 cases of rectal tumours during a mean follow up of 14.7 years. They have reported that alcohol drinkers of >41 drinks per week were at higher risk of developing rectal cancer than non-drinkers (RR= 2.2, 95% CI, 1.0-4.6). The risk of rectal cancer was also higher among drinkers of > 14 drinks of beer and spirits per week, but not wine (RR 3.5, 95% CI, 1.8-6.9) (242). However, the RR

of rectal cancer decreased among those who included 30% of the wine in the alcohol intake. The findings of this study found no significant association of total intake of alcohol, wine, spirits and beer with the RR of colon cancer (242). Fedirko et al. analysed 61 epidemiological studies and reported that the pooled RR was 1.52 for heavy drinking (≥5 drinks per day) and 1.21 for moderate drinking (≥2 drinks per day) (243).

Similar to smoking, alcohol consumption has also been associated with younger age at onset of CRC with adjusted age difference being 5.2 years (P<.001) (233). Rosato et al. reported a 1.6 fold increase in the risk of YOCRC with ≥2 drinks per day (244). A population-based study analysed changes in YOCRC risk factors and incidence rates in the US and concluded that there was no association between trends in alcohol consumption and CRC incidence rates (74). Kim et al. multivariate analysis showed that alcohol intake was an independent risk factor for overall CRC in Koreans aged 30 to 39 years (245). However, the consumption of alcohol among young adults has been decreasing over the last few years (246-248). In addition, alcohol drinkers and non-drinkers develop YOCRC. All of these findings suggest that there are other factors besides alcohol consumption contributing to the increasing incidence of YOCRC.

4.2.7. Physical inactivity

There is ample evidence showing that physical inactivity is widely linked to an increased risk of CRC (15). It has been reported that the least active individuals are by 27% at higher risk of susceptibility to CRC in comparison to those who are physically active (249). Lisa (2007) found the inverse association between CRC and physical activity in both genders and showed that the incidence of this malignancy is 40-50% higher in males and females who are the least physically active than highly physically active individuals (250). The HR of CRC in individuals who did at least one hour of physical activity per day was 0.57 (95% CI, 0.41–0.79) in comparison to those who did ten minutes or less of physical activity each day (251). More recently, Kim et al. conducted a cohort study to analyse the risk factors for CRC in 72,356 asymptomatic participants aged 20 to 39 years who underwent colonoscopies in South Korea from 2004 to

2015. The results of this study revealed that physical activity was a protective factor against the incidence of YOCRC (245). Prolonged watching TV has been associated with an increased risk of YOCRC, especially rectal cancer, independent of obesity and physical activity (252).

Researchers have also seen that the least active individuals are more likely to die after diagnosis with CRC compared to physically active individuals (253). The results of Ratjen et al. showed the inverse relationship between the mortality cause and physical activity in a group of women and men CRC survivors. The mortality rate was also found to be higher among those CRC survivors who were physically inactive and had prolonged watching TV. Thus, it is suggested that survivors of CRC should be advised to stay physically active (254). Some studies reported that physical activity decreases the risk of developing proximal colon cancer but not distal colon or rectal cancers. In contrast, other reports showed that physical activity does not distinguish between proximal and distal cancers (151).

4.2.8. Diet and nutrition

Geographically, the incidence rates of CRC remarkably differ globally. This suggests that an unhealthy lifestyle such as a poor diet has a strong association with the risk of developing CRC (255). Therefore, there have been substantial experimental and epidemiological studies related to the positive association of the high intake of some nutrients and foods with the risk of CRC. The effects of dietary patterns on CRC occurrence might be indirect as through obesity or direct through some of the nutritional components (256, 257). Studies have shown that diet might have an effect on CRC tumorigenesis by genetic/epigenetic changes, inflammation, or alteration of gut microbiota (258, 259). However, there are few reports that investigated the role of diet in YOCRC.

In general, studies have shown that red meat and processed meat increases the risk of CRC, while fibre, whole grain, calcium, and milk decrease the risk of developing this cancer (259). According to the International Agency for Research on Cancer (IARC), red meat is classified

as “probably carcinogenic to humans” and processed meat is categorized as “carcinogenic to humans”, depending on the accumulated scientific studies related to the risk of CRC (260). One study showed that the risk of CRC doubled among individuals who frequently consumed red meat, eggs, refined starches and cheese. On the contrary, a high intake of tomatoes decreased the risk of developing colon cancer by 50% and rectal cancer by 60% (261). A case-control study conducted in the north of Italy showed that a high intake of red meat attributed to 17% of CRC (262). A meta-analysis study reported that there was no significant association between the risk of CRC and overall meat consumption. However, a high intake of red meat and processed meat was linked to ~33% higher risk of CRC occurrence (263). Another meta-analysis study evaluated 8000 CRC cases from nineteenth prospective studies and observed the inverse relationship between the risk of CRC and intake of red meat or processed meat. People in the highest intake category of red meat and processed meat were by 30% and 20%, respectively, at higher risk of CRC in comparison to those in the lowest intake category (251). Similar findings were observed in both genders among Americans and Europeans (103). In terms of YOCRC, case-control and cohort studies have also shown the significant association between YOCRC risks with diets high in processed meat (264). However, it is worth noting that most of the studies that have shown the association of red meat consumption with the risk of CRC were observational studies and residual confounding from other diet and lifestyle factors were difficult to be ruled out. Therefore, chance, bias and confounding could not be excluded with the same degree of confidence for the data on red meat consumption (265).

The intake of fast food increased from 18% of overall calories in 1977-1978 to 32% in 1994-1996 among children and young individuals. Fast food contains a higher saturated fat and total fat; and lower fibre, iron, and calcium on a per-calorie basis compared to homemade foods. These are important potential factors for increasing the risk of CRC occurrence (266). The increase in the incidence rates of YOCRC is the highest in countries such as Korea, where rates are already highest (3). This observation in Korea is yet to be explained but is thought to be due

to the rapid dietary transition that occurred following the Korean War. Production of wheat-derived processed foods increased during the 1970s and shortly followed by increasing fast-food restaurants (3). Notably, a western dietary pattern has specifically been associated with increasing the risk of left-sided CRC which is predominant in YOCRC patients. In contrast, the Mediterranean diet has been associated with decreasing the risk of right-sided and left-sided CRC (151).

The typical cooking style used in westernized countries such as deep-frying appears to be unhealthy as it can produce advanced glycation end-products (AGEs) which are considered to be pro-inflammatory and pro-carcinogenic products (151, 267). The level of AGEs produced depends on the type of foods, cooking style, cooking temperature, cooking time and the presence of moisture. While vegetables, whole grains, fruits and other nutrient-rich foods contain fairly few AGEs, animal-derived foods generally contain high AGEs (151, 267).

Arguing against the association of increasing YOCRC incidence rates with a western diet is considering that YOCRC is rising in countries with heavy consumption of a western diet (such as Australia, Canada and the US) as well as in countries with heavy consumption of Mediterranean diet (such as Egypt) (151). Dietary risk factors also do not elucidate long-standing higher incidence rates of right-sided colon cancer in African Americans aged ≤50 (72). In addition, identifying dietary risk factors is limited to at most two years before the diagnosis. Therefore, it is difficult to determine whether modifications in the quality of diet over the life span can change YOCRC risk (264).

4.2.9. Calcium

Calcium (Ca^{+2}) is not only an essential nutrient for healthy bones and teeth, but is also considered to be anti-neoplastic (268). Ca^{+2} has been reported to affect the risk of developing CRC through various mechanisms. Firstly, ionized Ca^{+2} has the ability to decrease the potentially toxic impact of bile acids as well as free fatty acids in the lumen of the colon by converting these acids into insoluble soaps (268). Secondly, this nutrient has been found to

induce cell apoptosis and differentiation as well as inactivate the proliferation of the cells. Additionally, Ca^{+2} has also been reported to inhibit oxidative DNA damage and "modulate the CRC-related cell signaling pathways" (259). Notably, the involvement of Ca^{+2} in the transcription of the gene is suggested to be through the cAMP response element-binding protein (CREB) (269).

Findings of a prospective cohort study pointed out that the risk of CRC was about 70% lower in people with the highest consumption of Ca^{+2} compared to people with the lowest calcium intake (270). Cho et al. after analysing ten cohort studies concluded that compared to the people with the lowest intake, there was a significant association between the reduction in the incidence of CRC and the highest consumption of dietary Ca+2 (RR = 0.86, 95% CI = 0.78–0.95), milk (RR = 0.85, 95% CI = 0.78–0.94), and total Ca+2 (RR = 0.78, 95% CI = 0.69–0.88) (218). Moreover, some other cohort studies found that there was an increased risk of developing CRC among people who intake lower than 700-1000 mg of Ca^{+2} per day (271-273). Studies have also shown that the association between the risk of developing tumours in the distal colon or rectum with calcium intake is stronger in comparison to the risk of cancers in other anatomic locations (274, 275). However, although this inverse relationship between CRC occurrence and dietary Ca^{+2}, milk and overall Ca^{+2} has been reported (276), findings of epidemiological studies are not consistent regarding the association of Ca^{+2} with the risk of growing adenomas. Some epidemiological studies found no association between the incidence of adenomas and $Ca^{+2,}$ while others showed an unassertive relationship (277).

The Women's Health Initiative (WHI) conducted the largest randomized clinical trials (7 years follow-up) to investigate the potentiality of Ca^{+2} supplements to the risk of CRC. The results revealed that Ca^{+2} supplementations were not associated with the reduction in the incidence of CRC (278). Nevertheless, the reason behind these findings was suggested to be due to the several limitations in the study such as poor patient adherence, high consumption of calcium at

baseline and the unsatisfactory duration of the treatment. Therefore, the data was re-analyzed and showed the decrease in the incidence of CRC with Ca^{+2} supplementations by 17% among participants of WHI who had not already consumed Ca^{+2} at randomization (259, 278). Therefore, the findings of WHI propose that supplementary Ca^{+2} might not decrease the risk of CRC in individuals who already consume high Ca^{+2}. It is also worth noting that there has been a decline in dairy intake among young adults since the 1970s which might have increased the incidence of YOCRC (54). However, the association of Ca^{+2} intake with the risk of YOCRC is not well explored in the research community.

4.2.10. Vitamin D

In 1980, Garland and Garland hypothesized that the mortality rate of CRC was high due to the vitamin D status in a population with insufficient sunlight exposure (279). This suggestion was studied by many researchers utilizing various surrogates for the status of vitamin D (280-285) and some found an inverse relationship between vitamin D and the incidences of adenomas, as well as the incidence and mortality rates of CRC (286, 287). Vitamin D receptor (*VDR*) gene is activated by binding to vitamin D; and *VDR* gene polymorphism, BsmI, has been consistently associated with CRC (288). Vitamin D has been suggested to regulate up to 5% of human genetic materials directly or indirectly and has several anticancer functions such as inducing cell differentiation and apoptosis, suppressing of cell proliferation and angiogenesis and inhibiting metastasis (288, 289). Vitamin D has also been reported to have an anti-inflammation important role and a well-established role in immunity (290). A high vitamin D intake diminished inflammation in mice with ulcerative colitis. This shows that this vitamin might have a significant role in inflammation-related carcinogenesis (291).

Gorham (282)et al. reported that the risk of developing CRC was 50% lower in people with ≥1000 IU/day oral Vitamin D ($p < 0.0001$) or ≥33 ng/ml (82 nmol/l) serum 25-hydroxyvitamin D ($p < 0.01$) (292) compared to those with <100 IU/day Vitamin D or <13 ng/ml serum 25-hydroxyvitamin D (292). McCullough et al. pooled participant-level data from 17 cohorts (5706

CRC patients and 7107 controls) and found that 10 ng/mL increment circulating 25-hydroxyvitamin D (25(OH)D) was associated with a 19% and 7% lower risk of developing CRC in women and men, respectively (P-value for heterogeneity by sex = .008) (293). The findings of Baron et al. clinical trial indicated that the risk of adenoma recurrence was not decreased by daily intake of vitamin D supplementation (1000 IU) (294). A Mendelian randomization study concluded that there was no significant association of genetically determined 25(OH) D levels with the risk of CRC (OR, 0.92; 95% CI, 0.76–1.10) (295). Therefore, it is notable that the findings of studies regarding the role of vitamin D supplementation in the risk of CRC have not been conclusive.

Regarding YOCRC, Kim et al. prospectively investigated the association of vitamin D intake with the risk of YOCRC and colorectal polyps in a cohort of young women (296). The study concluded that a higher total intake of vitamin D was significantly association with decreased risk of developing YOCRC and colorectal polyps (adenoma and serrated polyps) (296). This association was reported to be more significant for vitamin D intake from food sources than supplemental vitamin D (HR per 400 IU/day increase, 0.34; 95% CI, 0.15–0.79, and 0.77; 95% CI, 0.37–1.62, respectively). This study found no association between the risk of CRC in women ≥50 years old with vitamin D level (296). In addition, Harnack et al. found that vitamin D intake from food sources (such as eggs, fish and mushrooms) has declined since the 1980s (297). Vitamin D intake from mile has also decreased in the US (298). Therefore, vitamin D intake is considered to be one of the possible risk factors for increasing the incidence of YOCRC. However, further research is needed to study causality and to determine whether the association of vitamin D with CRC is stronger in young adults compared to their older counterparts (296).

4.3. Microbiota

There is strong scientific evidence associating gut microbial symbiosis with CRC. The microbiota may play a role through impacting on host metabolism, and through the transmission of metabolic and even CRC risk factors in non-Mendelian familial aggregation, as has been shown in co-housed preclinical animal models (299). Obesity and diabetes, and CRC itself have been linked to changes in the gingival and gut microbiota in humans. Though there have been multiple findings suggesting causation in animal models, several confounding factors such as genetic background, and stress may have played a role. It is currently not definitively known whether these observations are readily translated into human settings and whether human studies suggest direct causation or setting-associated colonisation in a predisposed host. For example, findings in humans of insulin sensitivity being improved in obese subjects after faecal transplantation into the small intestine from lean donors (300), indicate that microbiota in the obese does not necessarily cause obesity, but that a certain element of the microbiota from lean individuals lacking in the obese can modify insulin sensitivity. Similarly, obese patients who experienced weight loss had an improved response to periodontal therapy over those who remained obese with persisting gum disease (301, 302), which could be interpreted as setting-associated. The composition of bacteria chronologically alters as people age, as well as depending on location within the bowel (303, 304). Some species of bacteria have been recognized to have a role in instigating bowel cancer pathogenesis such as *Bacteroides fragilis*, *Fusobacterium nucleatum*, *Streptococcus bovis*, and some strains of *Escherichia coli*. Findings of studies in older CRC patients showed a significant role of *F. nucleatum* in the pathogenesis of CRC, particularly in the right-side cancers (304). Other studies found that *F. nucleatum* travels as bowel cancer metastasizes in mice, and murine cancers with this bacterium respond to the metronidazole antibiotic (305). In addition, Pleguezuelos-Manzano et al., (2020) exposed human intestinal organoid genotoxic pks^+ *Escherichia coli* and found a mutational signature that was not observed from organoids injected with isogenic pks-mutant bacteria (306). However, while it can be postulated that microbiota might be involved, there is not yet much

convincing evidence in an area which is difficult to research. For example, human stool and preferably colonic tissue would need to be sampled at multiple times during development and then associated with the CRC risk after decades. Such findings are yet to be reported. It is currently not known whether the microbiota has a role in YOCRC.

4.4. Antibiotics

Antibiotic overexposure is one of the current public health concerns. In the US, over one million unnecessary doses of antibiotics are prescribed each year (151). Early antibiotic exposure has been associated with various adverse health conditions such as obesity which is linked to YOCRC. Antibiotics are prescribed for half of the American infants for more than 5 days and many pregnant women effecting on the microbiota of infants after birth (307). There is inconsistency in the findings of studies regarding the association of antibiotics with the risk of developing CRC. While some epidemiological studies support the notion that antibiotic exposure increases the risk of CRC, other studies showed that antibiotics might have a protective role against CRC (151). This is probably due to the effect of antibiotics in microorganisms such as *Fusobacterium* which can drive CRC (305). Therefore, further studies are needed to determine the role of antibiotic exposure and other medications that target the gastrointestinal tract in developing CRC.

5. Molecular characteristics of YOCRC

Among YOCRC and in the general population, the adenoma-carcinoma pathway contributes to the development of ~ 85% of all CRCs. In contrast, evidence has shown that about 15%-30% of CRCs exhibit the features of an alternative serrated neoplasia pathway (308-310). Three are three underlying molecular mechanisms which have been described in the development of CRC: chromosomal instability (CIN), MSI and CpG methylator phenotype (CIMP) (81). These three mechanisms are not mutually exclusive and may overlap in some subsets of CRC (308, 311).

CIN, which accounts for about 85% of sporadic CRCs (312), is characterized by continuing errors in chromosomal segregation including a high rate of gains or losses of whole chromosomes or large fractions of chromosomes (313). This results in aneuploidy, rearrangement of chromosomes, copy number variations, as well as variants in tumour suppressor genes and oncogenes such as *APC*, *TP53*, *KRAS* and *BRAF* which subsequently contribute to CRC carcinogenesis ((314, 315). In general, the genome of YOCRC patients is more frequently euploid and hypermethylated than LOCRC cases (316, 317). Somatic variants of the *KRAS* gene are found in about 35-45% of CRCs (318) and predict a lack of response to anti-EGFR targeted therapy (109, 319, 320). In YOCRC, the incidence of *KRAS* variants remains questionable, with the incidence of these variants ranging from 4 to 54% in YOCRC (321-327). Similarly, *BRAF* variants in YOCRC are reported to range from 0% to 14.3% (327-333). In addition, loss of the chromosomal regions coding for loci, where *APC*, *SMAD4* and *DCC* genes, is more common in LOCRCs than YOCRC (334-336). In contrast, YOCRC loses the chromosomal regions which code for CRC markers (TJP2) (337-339) and FOX transcriptional factors (340), and gains regions coding for AMP-kinase regulatory subunit and *BMPR1A* (337). Puccini *et al*. reported that variants in genes such as *KDM5C*, *KMT2A*, *KMT2D* and *SETD2* which are involved in the modification of histones are higher in YOCRC than LOCRC.

MSI-H, which represents ~ 21% of the YOCRC, is characterized by the inability of the MMR system to maintain the DNA structure or to correct errors during the process of DNA replication as well as by the accumulation of point variants and changes in the repetitive microsatellite nucleotide sequences (321-323, 325). MSI-H cancers in YOCRC are mostly linked to LS, with some cases having epigenetic inactivation of *MLH1* and wild-type *BRAF* which are categorized as epimutation-type LS. CIMP has been shown to contribute to ~ 40% of all CRCs and is involved in the alternative serrated neoplasia pathway (341, 342). This pathway is categorized by high methylation of CpG islands and early *BRAF* variants. Hypermethylation of the MMR

gene *MLH1* is also frequently reported in this pathway and can result in diploid CRCs that are MSI. Tumours which are CIMP-high are usually found in the right-side colon, have high-MSI, a higher rate of *BRAF* variants and are poorly differentiated CRC with CIMP and *BRAF* that are MSI-H are mostly observed in LOCRC. Tumours which are CIMP-low are also observed in young adults with CRCs (343).

Studies have consistently shown that the prevalence of *KRAS/RAS* and *BRAF* variants, as well as MSI, is higher in right-sided colon tumours than left-side (344-347). This is clinically relevant given survival benefits with selective anti-EGFR inhibitors are higher in patients with left-side *RAS* wild-type colon tumours compared to the individuals with proximal colon tumours. The differing prevalence of primary sites may lead to survival implications based on age. For female YOCRC estrogen may play a role as some studies have suggested that estrogen might be a protective factor for the development of CRC in the proximal colon. As its level decreases with age, this may result in the increasing prevalence of proximal colon tumours in female adults above the age of 50 years (348). Evidence for this hypothesis comes from reports that women with higher estrogen exposure were more protected against high-MSI cancers which are very often found in the proximal colon (349). However, a tumour mutational burden (TMB) is more frequent in young adults with left-sided colon tumours than their older counterparts (9.7% vs 2.8%, $P < .001$). TMB may have relevance to immunotherapy options for this group of patients (350). For example, although RAS WT is higher in left-sided cancer, there is a higher rate of *HER2* amplification and *NF1* mutations in young adults with left-sided colon tumours than older individuals (350) which may have clinical relevance. Therefore, though there is a difference in the proportion of variants between YOCRC and LOCRC, this may reflect, at least in part, the different site distribution of CRC between the two age groups.

6. Screening and prevention of YOCRC

Development of CRC can be prevented by removal of precursor lesions (such as adenomatous polyps and serrated lesions) or if discovered in the early stages, can be treated by surgery alone without the need for chemo-or radiotherapy. This is particularly relevant for YOCRC patients, as they generally present with late-stage disease (4). Due to late and often symptomatic presentations they suffer considerable mortality and morbidity in their most productive time of life, impacting on education, career, family life, and physical and mental health in the survivors (1). Therefore, it is imperative to address contributing factors to the development of YOCRC to predict those most at risk in the population because avoidance of risk factors of the disease is a key primary prevention measure. Additionally, CRC screening with stool-based tests and colonoscopy are potentially beneficial for secondary prevention of YOCRC (5). Screening not only detects cancers but also advanced pre-cancerous polyps, which facilitates both prevention and early detection (6). Therefore, screening could be the main contributor to decreasing the incidence and mortality rates of CRC in people ≥50 years old.

Currently, an individuals' risk of CRC is mainly determined by age, number and histology of colorectal polyps and family history with CRC (148). In Australia and many other countries, in the absence of family history, targeted screening is only carried out for people under 50 years in individuals when there are known predispositions such as inflammatory bowel disease or evidence of pathogenic/likely-germline variants in genes associated with predisposition to hereditary CRC or polyposis. However, less than 10% of CRC cases are clearly inherited in an autosomal-dominant manner, and 3 out of 4 YOCRC patients have no family history of the disease (148). In addition, above 60% of YOCRC patients with FDR with CRC or advanced adenoma receive screening in their 40es as recommended (351) and only less than 40% of them have been asked this information by their physicians (352). Gupta et al. reported that among YOCRC patients in their 40's who met family history–based early screening criteria, nearly all cases (98%) would have been diagnosed earlier or some cases possibly prevented if they had

been screened according to family history–based guidelines (353). In addition, Although patients with a personal history of hereditary syndromes need to start screening in their 20's, most cases are not identified until their CRC diagnosis (92). Following the guidelines and screening young adults with a family history of advanced polyps or CRC would identify those who are at increased risk and decrease the incidence of advanced stages of the disease.

The US Preventive Services Task Force has proposed that CRC screening in average-risk individuals should begin earlier at age 45 years, due to the alarming rise in YOCRC. These new guidelines are consistent with the American Cancer Society recommendations issued in 2018 after studies had shown that starting screening with colonoscopy at 45 years instead of 50 years resulted in greater life-years gained by more than 6% with an additional 810 colonoscopies per 1,000 individuals over a person's lifetime of screening. Screening in high-risk individuals should start before the age of 50 years (354, 355). However, a significant number of YOCRC occurs among people aged <45years old (35) and therefore, lowering the recommended age to initiate screening is only one step in addressing YOCRC. A better understanding of the risk factors related to YOCRC could allow for personalised screening, particularly for those under 50 years deemed to be an elevated risk.

7. Treatment of YOCRC patients

CRC patients are generally treated in a standardized way based on current guidelines (356, 357). Sporadic CRC in individuals younger than 50 years may have a different molecular profile, and treatment may differ based on this (350, 358-360), but ultimately the therapeutic strategy will be guided by the exact profile rather than age. There are subtle but real differences that may reflect age. For example, guidelines allow the choice of first-line systemic chemotherapy schedules, which may vary from single-agent fluoropyrimidine to triplet therapy (FOLFOXIRI), although in general doublet chemotherapy is recommended (361). However, data from registries do suggest that YOCRC patients tend to receive more aggressive

chemotherapies compared to older patients with this disease. This is probably because these patients can tolerate more aggressive regimens and the misconception that YOCRC patients have worse treatment outcomes. YOCRC patients with stage II and III more commonly receive adjuvant therapy more often with multi-agent adjuvant regimens. Despite this trend, there is little evidence this improves outcomes significantly. For example, Kneuertz *et al.* showed no survival gain in their analysis for patients diagnosed with stage II CRC [Relative risk (RR), 0.90; 95% CI, 0.69-1.17]. A minor survival benefit may exist for those diagnosed at stages III-IV (RR, 0.89; 95CI, 0.81-0.97) (359). However, further evidence is required as to which subgroups may benefit most (150). Findings of studies have shown that adding bevacizumab or cetuximab to adjuvant fluorouracil, oxaliplatin and leucovorin regimens do not increase survival, though YOCRC patients have a higher tolerance to multiagent regimens than LOCRC patients (3). YOCRC patients with advanced or early stages of the disease are also more likely to undergo additional surgical treatment than their older counterparts. This difference may be due to both provider and patient age-related biases (3). Resection of primary cancer more commonly performed in young adults with metastatic CRC (mCRC) compared to older mCRC patients (70.8% versus 66.6%; $P < 0.001$) (42, 362-364). Resection of primary may impact on the outcome by preventing future complications (363-365) and may impact on survival (363, 366-368). One study investigated the findings of 9 phase III, fluorouracil-based, single and combination agents and showed that YOCRC patients had similar overall survival or relative risk of death to LOCRC patients but had lower progression-free survival (3). Lieu et al. conducted a systemic review and concluded that CRC patients aged above 65 years and those around the age of 20 years had the lowest progression and overall survival (369).

YOCRC patients are also more likely to undergo radiation therapy in the setting of metastatic rectal cancer than their older counterparts (42). Radiation therapy for the rectal primary in metastatic disease is used to control the local recurrence rate. There are few studies regarding the recurrence rate of rectal cancer in young adults compared to their older counterparts after

radiation therapy. However, You *et al.* reported that the recurrence incidence of the tumour was higher in young adults with rectal cancer, especially distant metastasis than their older counterparts after a similar length of following up (370). In addition, Fossum *et al.* conducted a retrospective review comparing patients with synchronous resectable lung or liver metastasis who did not receive neoadjuvant therapy versus those who received neoadjuvant therapy. It was found that none of the patients who received neoadjuvant therapy had a local recurrence after follow up of 43 months while 26% of patients without neoadjuvant therapy had a local recurrence ($P< 0.001$) (371).

8. Appendiceal neoplasms in Young adults

Appendiceal neoplasms (ANs) are relatively rare and their incidence and mortality rates rarely are reported in epidemiological population studies. In 2020, ANs and other rare cancers of the digestive system accounted for 2.3% of cancers of the digestive system in the US. ANs are often diagnosed as an incidental finding seen on the appendectomy surgical specimen, during the histopathological examination of removed colon specimens for different reasons, or histopathological examination of removed appendix specimens. Singh et al. reported that the overall incidence rate of ANs increased by 292% in Canada and by 232% in the US over the last two decades (4). There was a rise in the incidence rate of ANs across both histological subtypes (appendiceal adenocarcinomas and appendiceal neuroendocrine tumours), both genders, all age groups, and stages of disease (4). Similar findings of increasing the incidence of ANs were reported by two other population studies in the US (5, 372).

Recent analysis from the SEER database showed that ~31% of ANs patients are diagnosed under the age of 50 years. Similar to the YOCRC, ANs in young adults (<50 years) have distinct demographic and histological characteristics (373). For example, the proportion of women with this malignancy was higher in young adults compared to their older counterparts. Young individuals with ANs were 82% more likely to be Hispanic, four times more likely to be

AIs/ANs compared to their older counterparts (373). In addition, young adults with appendiceal neuroendocrine tumours were less likely to be diagnosed with advanced stages of the disease compared to those aged ≥50 years old (373). *KRAS, TP53, GNAS* and *SMAD4* are the most common somatic mutations in ANs (374) (**Figure 8**). Young adults with ANs are less likely to be diagnosed with *GNAS* mutation compared to their older counterparts (374). *GNAS* somatic mutations were reported to be mutually exclusive with *TP53* mutation in both age groups (374). These findings show that young adults with ANs have distinct molecular characteristics.

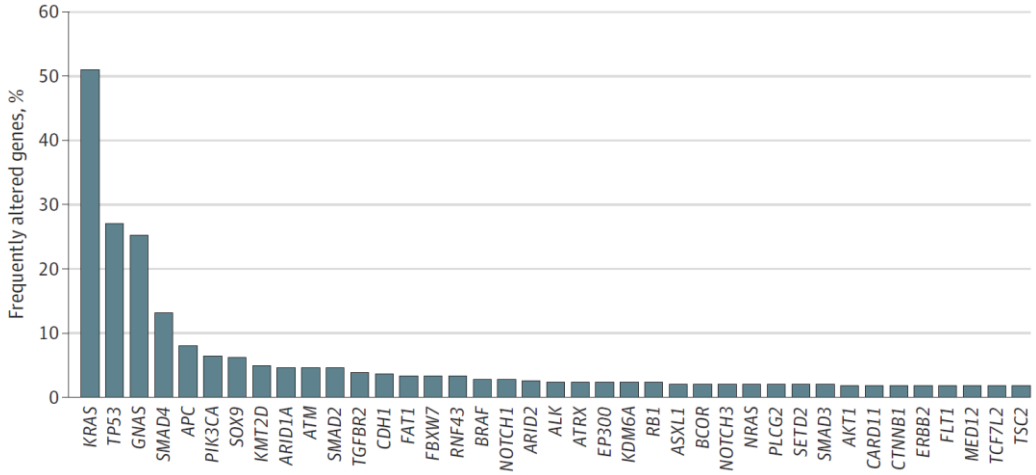

Figure 8. Frequency of somatic mutations in commonly mutated genes in patients with appendiceal neoplasms (374).

ANs are usually aggressive and can have devastating consequences. One study reported that 5-years survival for patients with signet ring cell ANs was 25%, non-mucinous ANs was 46% and mucinous ANs was 54% (375). Another study reported that overall 5-year survival for patients with up to 11 and more than 12 regional lymph nodes were 59% and 74%, respectively (376). Appendiceal cancer survival is significantly worse in NHBs people compared to NHWs. A study reported that 5-years cancer-specific survival for NHBs, NHWs, and Hispanics were 64.5%, 77.0% and 79.2%, respectively (354). Holloway et al. found that men had 55% and 44% higher hazards of deaths in non-mucinous adenocarcinomas and mucinous adenocarcinomas, respectively, compared to women (374). These findings suggest that survival after young-onset

ANs differ significantly by race/ethnicity with NHBs having lower survival rates compared to NHWs.

The risk factors of ANs are not adequately explained and currently, there are no genetic or familial factors known to cause this malignancy. Lu et al. reported that older age and obesity were predictors of ANs diagnosis among patients undergoing appendectomies (377). However, more studies are needed to understand risk factors, etiologies, and prognostic factors of ANs and to utilize this knowledge to reverse the increasing disease burden as well as inform clinical, molecular, and population-level features that contribute to ANs disparities. In addition, it is yet to be reported whether the patterns of increasing the incidence of ANs are consistent in other westernized countries with a lifestyle similar to the USA and whether the mortality rate is following a similar trend of incidence rates of ANs.

9. Rationale and aims

The incidence and mortality rates of CRC in young adults are rising in Australia and worldwide, and the reasons for this observation are currently poorly understood. There is a need to identify at-risk young individuals in primary healthcare settings. Although lifestyle risk-related factors may contribute to the development of YOCRC, the early occurrence of this disease suggests that individual genetic predispositions may also have a strong role. T2D has long been associated with CRC risk in all age groups and has been on the rise among children, young adults and in all racial/ethnic groups. Therefore, T2D could explain patterns of increasing incidence rates of YOCRC. Family history and genetics are the strongest risk factors for YOCRC. Therefore, pinpointing genetic risk factors in these patients is fundamental for the appropriate clinical management of patients and their family members. Currently, the role of genetic susceptibility in a high proportion of YOCRC cases is unknown. Thus, I aimed to investigate the clinicopathological and molecular characteristics of YOCRC, and their association with a personal and family history of T2D. I also aimed to identify hereditary risk

factors for YOCRC. In addition, the incidence rate of ANs has also been rising in the USA and several other countries with unexplained causes. However, further epidemiological studies are needed to determine whether this trend is similar in other westernized countries with similar lifestyles or whether the mortality rate is following a similar trend of incidence rates. Therefore, I aimed to investigate the incidence and mortality rates of ANs in Australia.

10. Objectives of the project

The overall aim of this work was to explore the issues around rising incidence of both CRC and ANs in young adults.

Specific objectives of the study were:

1) To determine the association between a personal history of T2D, and CRC in young adults in South Australia in order to estimate the level of risk. This was achieved by performing a case-control study of T2D and CRC under the age of 55 years.
2) To identify known monogenic CRC predispositions, known common risk loci for T2D and CRC, and novel germline variants and genes associated with cancer-predisposition. For this objective, blood was sampled from YOCRC patients for WES and the data underwent a comprehensive analysis and detailed interpretation and classification of variants in cancer-predisposition/implicated genes.
3) To investigate the role of *RNF43* as a cause of an inherited predisposition to CRC/colorectal polyposis.
4) To investigate the incidence and mortality rates of ANs in Australia by performing a retrospective analysis on national data obtained from the Australian Institute of Health and Welfare (AIHW) from 1982 to 2013.

11. Thesis overview

This thesis is comprised of peer-reviewed publications. Each chapter includes figures, tables, and appendices in the form of supporting/supplementary information, and references relevant

to its content. Chapter 2 focuses on the association of diabetes with the risk of YOCRC. Chapters 3 and 4 address objectives 2 and 3 of the project. Chapter 3 shows the germline variants in cancer-predisposing/implicated genes and chapter 4 demonstrates the hereditary role of *RNF43* in CRC tumorigenesis. Chapter 5 addresses objective 4 of the project. Chapter 6 explains the immunochemistry features and molecular pathology of each histology subtype of ANs. The final chapter of this thesis provides a conclusion of our findings and future studies for the project.

Chapter Two: Young-onset colorectal cancer is associated with a personal history of type 2 diabetes

Asia-Pac J Clin Oncol. 2021;17:131–138.

In this publication, a case-control study was performed to investigate the association of personal history of T2D with the risk of YOCRC. Ninety unrelated YOCRC cases and 240 controls were recruited for this study. Personal and detailed family history of T2D were recorded and whole-exome sequencing was performed for all the cases. Controls were patients with clear colonoscopy and no known CRC predispositions. It was found that younger patients having a personal history of T2D may have an increased risk of developing CRC. These findings could have several implications for policy and practice. For example, this might help GPs to consider screening at an earlier age for young adults with diabetes.

Statement of Authorship

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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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ORIGINAL ARTICLE

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Young-onset colorectal cancer is associated with a personal history of type 2 diabetes

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Abstract

Background: Colorectal cancer (CRC) is rising in incidence in young adults, and this observation is currently unexplained. We investigated whether having a personal history of type 2 diabetes mellitus (T2D) was a potential risk factor for young-onset colorectal cancer (YOCRC).

Methods: The South Australian Young Onset (SAYO) CRC study is a series of young adults with CRC below age 55. Ninety unrelated YOCRC cases were recruited to the study. Personal history and detailed family history of T2D were obtained at face-to-face interview and confirmed from medical records. Whole exome sequencing was conducted on germline DNA from each CRC case. Controls for personal history studies of T2D were 240 patients with proven clear colonoscopies and no known CRC predispositions.

Results: The median age of YOCRC cases was 44 years (18–54) and of controls was 45 years (18–54), and 53% of both cases and controls were females ($P = 0.99$). Left-sided (distal) CRC was seen in 67/89 (75%) of cases. A personal history of T2D was confirmed in 17/90 (19%) YOCRC patients compared with controls (12/240, 5%; $P < 0.001$; odds ratio = 4.4; 95% confidence interval, 2.0–9.7). YOCRC patients frequently reported at least one first-degree relative with T2D (32/85, 38%). Ten of 87 (12%) of YOCRC cases had CRC-related pathogenic germline variants, however, no pathogenic variants in familial diabetes-associated genes were seen.

Conclusions: Though the mechanism remains unclear, our observations suggest that there is enrichment for personal history of T2D in YOCRC patients.

Impact: A diagnosis of T2D could therefore potentially identify a subset of young adults at increased risk for CRC and in whom early screening might be appropriate.

KEYWORDS
colorectal cancer, germline mutations, risk factors, screening, type 2 diabetes, young-onset colorectal cancer

1 | INTRODUCTION

Young-onset colorectal cancer (YOCRC) incidence is rising in Australia,[1,2] and elsewhere in the developed world,[2,3] at a time when the incidence of CRC in older adults is declining.[4] This rise in incidence is currently unexplained. Individuals who develop CRC before the age of 50 years, present at a later stage in their illness,[5-8] and are thus frequently unable to take advantage of the benefits of early detection. Due to patient- and healthcare-associated diagnostic delays, and subsequent late and often symptomatic presentations, young adults suffer considerable morbidity and mortality in their most productive time of life, impacting on education, career, family and social life and physical and mental health in the survivors. In 2013, 1313 Australian young adults developed CRC. In 2017, CRC was responsible for the most cancer deaths in 20- to 29-year-old Australians.[9]

In Australia, population screening is recommended for people aged 50–75 years. This is carried out under the National Bowel Cancer Screening Program (NBCSP) with faecal immunochemical tests (FIT), in those considered to be at average risk, via Medicare-subsidized FIT tests requested by general practitioners (family physicians) and via FIT test kits purchased privately. Screening not only aims to detect cancers, but may also detect advanced precancerous polyps in the ratio of four to five lesions for every one cancer detected,[10] thus facilitating both prevention and early detection. Young adults (<50 years) without a family history of CRC have a lower risk of CRC when compared with their older counterparts and are therefore not included in population screening programs. In this age group, the low yield, potential for harm and anxiety which are associated with screening may outweigh any benefits of early detection. These factors also translate to a lack of cost-effectiveness. Therefore, in the absence of a family history, targeted screening only is carried out for people under 50 years in individuals when there are known predispositions such as inflammatory bowel disease or evidence of an inherited pathogenic or likely pathogenic variant (P/LP variant) in a gene predisposing to CRC.[11]

CRC in young adults is heterogeneous. Approximately 10% arises from inherited DNA mismatch repair deficiency,[12] and a further small proportion from other known high penetrance genetic predispositions. The remainder of CRC in young adults is largely unexplained, apparently sporadic and accounts for >80% of the burden. Importantly, YOCRC examined in retrospect, frequently does not meet the criteria for screening[13] in that there is no significant family history. Though population screening is not justified for those under 50 with no family history,[14] screening of highly targeted young adult subsets with identified risk factors outside the current guidelines has the potential to extend the successes of screening older patients to those aged under 50 with increased risk. Currently, though modern lifestyles are likely to be implicated in the observed rise in incidence of YOCRC, there has been no definitive risk factor identified. Patients diagnosed with T2D at any age have a 20–40% higher risk of CRC than the general population.[15] CRC and T2D are complex diseases resulting from an interaction between acquired as well as genetic factors. Although the link between CRC and T2D has been frequently reported in studies,[16] the association between personal and/or family history of T2D and YOCRC has not been widely investigated.[17] The aim of this study was to investigate whether personal history T2D was associated with YOCRC.

2 | MATERIALS AND METHODS

2.1 | SAYO study

The South Australian Young Onset Colorectal Polyp and Cancer Study (SAYO) is a multidisciplinary state-wide consortium, which seeks to identify the risk factors and warning signs for CRC in young adults. Study activities, including colonoscopy database audits, are carried out under ethics approval HREC/14/TQEHLMH/194 (The Queen Elizabeth Hospital, CALHN Office for Research, Adelaide, South Australia). The study has directly enrolled patients identified with primary adenocarcinoma of the colorectum aged under 55 years from public and private hospitals since 2015 by face-to-face interview. Written informed consent was provided by all study participants. CRC was confirmed from medical records. CRC was divided into right-sided (proximal) cancers (cecum, ascending colon, hepatic flexure and transverse colon) and left-sided (distal) cancers (splenic flexure, sigmoid colon, descending colon, recto-sigmoid and rectum). Though population screening in Australia begins at age 50, younger adults aged up to 55 years with CRC are enrolled in the study due to the low rates of population screening uptake in this overlapping age group (26.4%), the more pronounced risk of CRC in patients under 55 who develop T2D mellitus, and reported increasing mortality in patients under 55 in the United States.[4,9,18,19] Patients enrolled in the study underwent an interview which covered potential risk factors such as personal and family history of any cancers in first- and second-degree relatives, colorectal polyps and T2D mellitus. Blood was sampled for whole-exome sequencing of leucocyte DNA.[20] Recruitment acceptance for SAYO remained high throughout the enrolment period with over 95% of patients approached agreeing to participate.

2.2 | Description of personal history studies

Personal history of T2D was obtained from SAYO CRC cases at face-to-face interview, and confirmed from medical records including notes, blood tests and medication history. Controls for this comparison were age-appropriate patients from a single center with proven clear colonoscopies and no known CRC predisposition (germline P/LP variant, inflammatory bowel disease). Controls ($n = 240$) were drawn from a series of 3130 colonoscopies carried out at a single center (the Queen Elizabeth Hospital) in 2016 using approaches described previously.[21] Patients were deemed eligible to serve as controls if they returned findings of a clear colonoscopy, and had no inflammatory bowel disease, no previous colorectal neoplasms, and no known inherited predispositions to CRC. T2D was confirmed from admission interview and also from medical charts including notes, blood tests and medication history. Family history of CRC was based on interview alone and not confirmed in both cases and controls.

2.3 | Genetic testing

SAYO patients with CRC underwent whole-exome sequencing of their germlines as described previously.[22] Briefly, whole-exome sequencing was performed using the KAPA HyperPrep Kit for library preparation and the Roche SeqCap EZ MedExome Enrichment Kit for sequence capture. The Illumina NextSeq 500 was used to sequence the captured libraries (2×150 bp paired-end reads). The Burrows–Wheeler Aligner (BWA) was used to align sequences to the human reference sequence (hg19). The Genome Analysis ToolKit (GATK) was used for performing variants calling and variants were annotated with ANNOVAR. American College of Medical Genetics (ACMG) guidelines[23] were used to identify likely pathogenic or pathogenic (class 4 or 5, respectively) germline variants in CRC-associated genes and in genes associated with monogenic nonneonatal diabetes,[24] severe insulin-resistant diabetes, mild obesity related diabetes and mild age-related diabetes[25] for deleterious changes (see Table S1). Pathogenicity of putative germline P/LP variants was confirmed using public databases ($n = 8$), explored for functionality using MSI testing ($n = 1$) or lymphoblastoid cell line RNA splicing ($n = 1$). Routine mismatch repair testing of cancer tissue via immunohistochemistry was undertaken to detect potential Lynch syndrome patients as previously described.[26]

2.4 | Statistics

Means in continuous variables were compared using a *t*-test procedure. Prevalence of characteristics in patients was compared between cases and controls using Pearson's chi-squared or Fisher's exact test as

TABLE 1 Summary of features of study participants

Feature	Cases (range or percent)	Controls (range or percent)	Odds ratio	95% CI	*P*-value
	90	240			
Median age	44 (18–54)	45 (18–54)			
Females	48/90 (53%)	127/240 (53%)	1.0	0.6–1.7	0.99
Indications for scope/examination					
Bleeding	44/90 (49%)	90/212 (42%)	1.3	0.8–2.1	0.31
Change of bowel habit	38/90 (42%)	38/212 (18%)	3.3	1.9–5.8	<0.001
Pain	33/90 (37%)	43/212 (20%)	5.0	2.8–8.7	<0.001
*Family history CRC	10/85 (12%)	25/212 (12%)	1.0	0.5–2.2	0.99
Type 2 diabetes					
Personal history	17/90 (19%)	12/240 (5%)	4.4	2.0-9.7	<0.001
Females	9/39 (23%)	8/127 (6%)	3.6	1.32–10.13	0.01
[a]Family history T2D	32/85 (38%)	Unknown			
Females	15/24 (63%)		0.9	0.38–2.26	0.99
Pathology					
[b]Left-sided (distal) cancers	67/89 (75%)				
Left-sided (distal) (females)	30/47 (64%)		0.2	0.07–0.72	0.01
Left-sided (distal) (males)	37/42 (88%)				
MMR deficient CRC	7/83 (8%)				
Confirmed Lynch syndrome	3/87 (3%)				
BRCA2 Mutation	4/87 (5%)				
Bi-allelic *MUTYH* Mutation	2/87 (2%)				

[a]Adoptees $n = 5$ family history unknown.
[b]One site of CRC was unknown.

appropriate. All statistical association tests were performed using SPSS Version 25 for Mac (IBM). Two-tailed statistics were used throughout with a significance level of <0.05.

3 | RESULTS

Summary features of 90 study participants with CRC are shown in Table 1. CRC patients ranged in age from 18 to 54 years (median age 44). Clear colon controls ($n = 240$) ranged in age from 18 to 54 years (median age 45). The main indications for colonoscopy in cases and controls were rectal bleeding, a change in bowel habits or abdominal pain. Forty-four of 90 (49%) YOCRC patients and 90/212 (42%) of controls had bleeding ($P = 0.31$), 38/90 (42%) of CRC patients and 34/212 (18%) of controls had change of bowel habit ($P < 0.001$) and 33/90 (37%) of YOCRC cases and 43/212 (20%) of controls had experienced abdominal pain ($P < 0.001$). The majority of CRC patients were of European ethnicity ($n = 86$), except for four whose ancestors were Filipino ($n = 2$), Iranian ($n = 1$) or Indian ($n = 1$). Forty-eight of 90 (53%) were females. Left-sided (distal) CRC was seen in 67/89 patients (75%), with a distal site of cancer being less common in females (30/47, 64%) compared to males (37/42, 88; $P = 0.01$). First-degree family history of CRC was seen in 10/85 (12%) of YOCRC cases and 25/212 (12%) of controls ($P = 0.99$). Information was not available in five remaining CRC cases due to adoption and an unknown family history. Pathogenicity of putative germline mutations was confirmed using public databases ($n = 8$), explored for functionality using MSI testing ($n = 1$) or lymphoblastoid cell line RNA splicing ($n = 1$). Seven of 83 (8%) patients had a mismatch repair deficient cancer, and three of 87 (3%) YOCRC cases had a mismatch repair deficient cancer and molecularly confirmed Lynch syndrome. The remaining four patients with a mismatch repair deficient CRC did not have a family history meeting the revised Bethesda criteria,[27] germline mutation or methylation in a known mismatch repair gene. Ten patients were found to have deleterious variants in CRC-associated genes, four in *BRCA2*, two in *MSH2*, one in *MSH6*, one in *RNF43* and two patients had biallelic mutations in *MUTYH* (see Table S2). One patient with a deleterious *MSH2* mutation (female aged 29) also carried a mono-allelic deleterious *MUTYH* mutation. It is worth noting that only one of 10 YOCRC patients with a germline mutation had a first-degree family history with CRC (Figure 1), and the details of these findings will be reported in detail in a separate publication.

A personal history of T2D was confirmed in 17/90 (19%) of the series of YOCRC cases, which was significantly higher than the prevalence in the controls (12/240, 5%; $P < 0.001$; odds ratio [OR] = 4.4; 95% confidence interval [CI], 2.0–9.7). This was also true when patients were partitioned for age. Those aged 18 to 44 years at diagnosis (6/50 or 12% *vs* 3/114 or 3%; $P = 0.02$; OR = 5.0; 95% CI, 1.2–21.1) as well

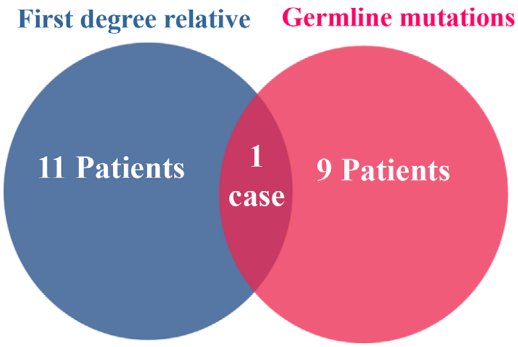

FIGURE 1 First degree relative with CRC and pathogenic germline mutations [Colour figure can be viewed at wileyonlinelibrary.com]

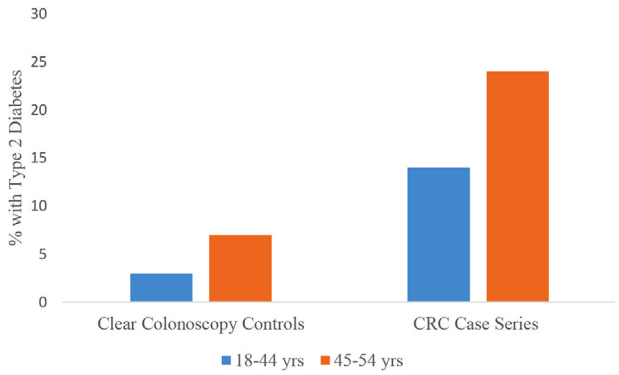

FIGURE 2 Comparison of prevalence of T2Dunder age 55 in (left to right) the clear colonoscopy controls ($n = 240$), and colorectal cancer case series SAYO ($n = 90$) [Colour figure can be viewed at wileyonlinelibrary.com]

as those 45–54 years (11/40 or 28% *vs* 9/126 or 7%; $P = 0.0001$; OR = 4.9; 95% CI, 1.9–13.0) had significantly increased prevalence of T2D (Figure 2). A personal history of T2D remained significantly higher in YOCRC cases (15/90, 17%) after excluding those cases (n = 2) with deleterious variants in CRC-associated genes compared to controls (12/240, 5%; $P < 0.001$; OR = 3.8; 95% CI, 1.7–8.4). The prevalence of T2D in males and females was 24% and 23% in SAYO cases, respectively ($P = 0.99$; Table 1). In all cases where T2D was present, this was identified at ($n = 2$) or before ($n = 15$) the time of diagnosis of CRC.

Patients with CRC frequently reported at least one first-degree relative with T2D (32/85 or 38%). First-degree family history of T2D was seen in one or both parents in 23 cases, siblings only in four cases, and parents and siblings in a further five cases. A first-degree family history of T2D was observed in both males and females (15/39; 38% and 17/46, 37% respectively; $P = 0.99$). Twelve of 16 (75%) patients with personal history of type 2 diabetes, where family history was known, also had first-degree relatives with type 2 diabetes. No previously described diabetes-associated loci were found to harbor deleterious alterations on exome sequencing.

4 | DISCUSSION

Currently, the increased incidence of YOCRC is unexplained. Recent geographical data from the United States have shown that though the prevalence of obesity and heavy alcohol consumption has increased during the time period 1995–2005, there was no correlation between these potential risk factors and increasing incidence rates of YOCRC.[28] In this report, we examined T2D as a risk marker for YOCRC. Diabetes of all types affects 1 in 17 adult Australians (6%), and approximately 5% of the adult population have T2D.[29] The population rate of diabetes in those aged 18–44 is 1.5% increasing to 5% in those aged 45–54 years. This is commensurate with the rate of T2D observed in our series of clear colonoscopy control patients aged under 55 years at 5%. However, our case series of young adults diagnosed with CRC under 55 years of age has a significantly higher personal rate of T2D than is present in clear colonoscopy controls. Our results suggest that young adults with T2D may be at increased risk for developing CRC.

The consistent association between T2D and CRC is postulated to be associated with a proinflammatory milieu involving insulin-dependent growth factors at a molecular level.[30] Lifestyle factors are thought to play a role, and these include lack of physical activity, poor dietary choices and obesity, however, obesity *per se* has not been shown to underlie YOCRC in recent US findings.[28] High levels of insulin signaling in the prediabetic *milieu* are also thought to contribute to the increased incidence of CRC in the immediate post-diagnosis period. A recent report from de Kort et al.[19] has identified a peak incidence of CRC in T2D patients during the 6 months following initial diagnosis (HR = 1.3; 95% CI, 1.2–1.5), and this was significantly more prevalent in the proximal colon (HR = 1.7; 95% CI, 1.4–2.0). The risk was highest in males aged less than 55 years (HR = 2.0; 95% CI, 1.0–3.8). When detection bias is considered by excluding the initial period after diagnosis of type 2 diabetes, the relationship between T2D and CRC continues to be robust albeit with a lower level of risk. Overbeek et al. reported that patients with T2D were 1.3 times at higher risk of developing CRC compared to diabetes free controls, and a higher increased risk of proximal colon cancer was observed among females with T2D (HR = 1.58; 95% CI, 1.13–2.19) than males with this condition compared to controls, and they found males with T2D were at higher increased risk of developing distal colon cancer (HR = 1.42; 95% CI, 1.08–1.88). The authors concluded that more attention should be paid to sex-specific screening and prevention protocols for patients with T2D.[31] Although there was a trend in our results for females to have more proximally located CRC, our numbers are small and therefore cannot be used to support this observation. In addition, the cited report reflects CRC patients of all ages rather than those who are under 55 years. Proximal CRC becomes more common with age in females.[32] Vu et al. found that patients aged 40–49 years with T2D mellitus were at higher risk of developing colorectal adenomas compared to the same age group without this disease (OR = 3.1; 95% CI, 1.5–6.4; $P = 0.002$).[33] Recently, Ali Khan et al. conducted a nationwide cohort study using Swedish family cancer data sets and reported that young adults with diabetes mellitus were at increased risk of developing CRC by 1.9-fold under age of 50 years (95% CI for standardized incidence ratio: 1.6–2.3) and by 1.3-fold at

or after 50 years of age (1.2–1.4). They also found that young patients with diabetes had a similar lifetime risk of developing CRC under the age of 50 years (0.4%; 95% CI, 0.3%–0.4%) to individuals with only a family history of CRC (0.5%; 0.5%–0.5%).[34] These findings are consistent with our Australian cohort results showing the prominent association of diabetes with increased risk of CRC in young adults.

Another factor in the etiology of YOCRC may involve the microbiome. Gut microbiota produce short-chain fatty acids (butyric acid and acetic acid), which protect the intestinal tract by increasing the production of mucus from intestinal goblet cells. The decrease in the production of short-chain fatty acids might suppress the function of goblet cells and results in reducing the function of the intestinal barrier. This results in transferring lipopolysaccharides, mostly produced by protobacteria, from the intestinal side to the lumen where it comes in contact with blood. When the level of lipopolysaccharides increases in the blood, insulin resistance organs such as skeletal muscle and liver become insulin resistant which finally leads to hyperinsulinemia.[35] This might enhance IGF and Wnt signaling systems and result in CRC carcinogenesis.[36] Zhao et al.[37] reported that some dietary fibers manipulated the gut microbiota and enhanced the production of short-chain fatty acids. Overgrowing bacteria which produce these fatty acids directly associated with the reduction in the level of glycated hemoglobin. A systemic review concluded that dietary intervention in patients with T2D was reported to modulate the gut microbiota and improve glycemic control.[38] The risk of CRC associated with T2D has become an issue of concern as the age at which T2D is diagnosed is shifting further towards younger adults,[19] and a diagnosis of T2D in a patient younger than 50 years has the potential to serve as an inclusion criterion for early screening.

Family history of diabetes increases with age in the general population.[27] In the current report, our observations also suggest that an inherited factor which increases the risk of T2D in a family may also increase the risk of YOCRC, and this deserves further exploration as this too has the potential to identify younger adults at risk in the population prior to the onset of CRC. There have been at least two previous reports suggesting a link between family history of T2D and CRC, which lend additional evidence to support our findings. In 2002, Bauer et al.[39] investigated familial aggregation of diabetes and colorectal neoplasia, and found positive associations between familial diabetes and adenomatous polyps or CRC. Ma et al.[17] reported in 2018 that family history of diabetes is associated with risk of CRC in a sex-specific manner, and that the relationship is more pronounced in patients under 60 years, and only significant in males. We found this feature in both sexes with YOCRC, however, the numbers were low and hence it is not possible to confirm this observation. Though there was enrichment for T2D in families, no diabetes-associated variants were noted on exome sequencing.

There are several paradigms which may be drawn upon to explore our findings, however the most plausible is a gene environment interaction associated with modern lifestyles. An enrichment for personal and family history of T2D in the young adult population with CRC may simply reflect shared lifestyle factors, including shared exposure to high calorie load, and at this point, this consideration cannot be excluded. However, the relationship between T2D and CRC has been shown to be independent of obesity in patients under 55 years,[40] and therefore a genetic or epigenetic predisposition may also be a factor in these observations. Metabolically unhealthy phenotypes, including patients with high insulin signaling in the setting of normal weight, indicative of genetic background, increase the risk for CRC.[41] As patients were enrolled at the time of diagnosis in this study, body mass index (BMI), a potential confounder, was not measured due to the possibility that their current BMI did not reflect that when their cancer or its precursor polyp was initiated, which may have been up to a decade earlier.

Transgenerational epigenetic alterations may also play a role in the development of YOCRC. A diabetic parent or grandparent may alter the epigenetics of subsequent generations. Epigenetic effects involving metabolic anomalies were seen in the Överkalix study from Sweden in the 19th Century and the Dutch Hunger Winter of World War II.[42] Mothers who were starved of adequate nutrition in the first trimester of pregnancy produced children who were significantly more likely to develop heart disease, metabolic problems and cancer in their adult life. Gestational diabetes may also be a potential risk factor for CRC in offspring and future studies exploring this concept are warranted.

There are a number of implications of our findings for policy and practice. Among the 35,000 general practitioners in Australia, the number of 1313 people under 50 years diagnosed with CRC means that one general practitioner in 26 had a patient under the age of 50 who was diagnosed with CRC in that year, or that each general practitioner will have only one or two such patients diagnosed in her or his working lifetime. Australians make an average of seven visits annually to general practice, with each visit representing an opportunity for the general practitioners to check the CRC screening status of their patients with diabetes under 50 years. However, it is humanly impossible for general practitioners to remember to monitor this at every visit while attending to their many other tasks. None of the comprehensive clinical record software packages marketed for use in Australian general practice has an automated system to monitor CRC screening status and to remind the patient and the GP when screening or re-screening is due. The vendors of those clinical software packages should add this function. If our findings are confirmed, that automated reminder algorithm should recognize that patients with diabetes should be screened from an earlier age, perhaps 40 years. FIT should be considered for screening these increased risk patients, especially as their cancers tend to be in the distal colon (for which the FIT is more sensitive).

This report confirms findings of previous studies where an a priori relationship between CRC and a personal history of T2D,[19] as well as with having first-degree relatives with type 2 diabetes, has been demonstrated.[17,39] The strength of this report is that it reflects the findings of a contemporary, well-characterized case series of young adults with CRC, including specific data collection regarding family history of T2D at face-to-face interview, and a cohort of well-characterized controls who had undergone a colonoscopy and returned unremarkable findings. Limitations of this report include family history of T2D not being available in controls, and no available data on BMI during a preceding time in which the CRC precursor lesion may have been initiated in cases. BMI is a potential confounder, and

no multivariate analysis was performed to show T2D was an independent predictor of CRC. However, as mentioned previously, the relationship between T2D and CRC has been shown to be independent of obesity in persons under 55 years.[40] Another limitation of this study is unavailability of T2D treatment information. Nevertheless, Peeters et al.[43] reported that there was no association between CRC with T2D treatment stages. Like the explanation for the increase in YOCRC, the exact mechanism to explain our findings remains to be determined, but our report indicates that for some with YOCRC, the excess incidence may relate to T2D. The implication of this being that a young adult with early T2D, particularly when associated with first-degree family history of this condition, may be at increased risk of developing CRC. This warrants further investigation because of the potential to identify young adults in the non-screening population who may benefit from early surveillance.

CONFLICTS OF INTEREST

The authors declare they hold no conflicts of interest with respect to this work.

AUTHORS' CONTRIBUTIONS

R.M., J.Y. study concept and design, collected the data, analyzed results, wrote manuscript. J.Y., E.S., T.P. study concept and design, acquisition of data, analysis and interpretation of data, critical review of manuscript, statistical analysis. P.H., E.L., D.J., N.P., A.R.R., P.A.D., J.H., H.P., S.W., Y.T., D.P., S.V., A.T., G.T., M.H., J.Y., D.T. acquisition of data, interpretation of data, critical revision of manuscript. W.U., M.H., J.K. technical support, acquisition of data, analysis of data, critical revision of manuscript. D.R., G.Y., S.P., I.T., G.W., O.F., D.W, D.L.W., W.J.B. study concept and design, interpretation of data, critical revision of manuscript. The authors declare they hold no conflicts of interest with respect to this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary Table 1: Genes surveyed for genetic variants predisposing to type 2 diabetes

Gene Symbol	Gene Name
TCF7L2	transcription factor 7 like 2
KCNQ1	potassium channel subfamily Q 1
HHEX	haematopoietically expressed homeobox
IGF2BP2	insulin growth factor 2 binding protein 2
CDKN2B	cyclin dependent kinase inhibitor 2B
SLC30A8	solute carrier family 30 member 8
MC4R	melanocortin 4 receptor
TM6SF2	transmembrane 6 superfamily 2
KCNJ11	potassium channel KIR6.2
BLK	B-lymphoid tyrosine kinase
CEL	carboxyl ester lipase
GCK	glucokinase
HNF1A	hepatocyte nuclear factor 1 alpha
HNF1B	hepatocyte nuclear factor 1 beta
HNF4A	hepatocyte nuclear factor 4 alpha
INS	insulin
KLF11	kruppel-like factor 11
NEUROD1	neurogenic differentiation factor 1
PAX4	paired box 4
PDX1	insulin promoter factor 1

Supplementary Table 2: Actionable mutations

Number	*Sex*	*Age*	*Site*	*FDR CRC EC*	*MMR IHC*	*GL Mutation*	*PH T2D*	*FDR T2D*	*Relations T2D*	*Polyposis*
1	*F*	*44*	*D*	*no*	*N*	*BRCA2 (p.Ser3133; c.9398C>G)*	*no*	*yes*	*mother maternal aunt maternal GM maternal GF*	*no*
2	*F*	*29*	*P*	*no*	*MSH2/ MSH6*	*MSH2 (p.Arg680;c.2038C>T)*	*no*	*yes*	*father*	*no*
3	*F*	*43*	*PD*	*no*	*N*	*MUTYH [p.Tyr179cys; exon 7 c.536A>G; p.Gly396Asp;exon 13 c.118G>A]*	*yes*	*yes*	*brother mother maternal GM*	*no*
4	*F*	*50*	*P*	*yes*	*N*	*RNF43 (c.375+1G>A)*	*no*	*no*		*no*
5	*F*	*47*	*P*	*no*	*N*	*MUTYH [p.Trip103; c.309G>A; Gln391;117C>T]*	*no*	*no*		*yes*
6	*F*	*30*	*P*	*yes*	*MSH2/ MSH6*	*MSH2 [p.(Val1265_Gln314del); c.942+3A>T]*	*no*	*no*		*no*
7	*F*	*38*	*D*	*no*	*N*	*BRCA2 (p.Leu1908fs; c.5718_5719CT)*	*no*	*yes*	*mother*	*no*
8	*M*	*38*	*P*	*no*	*N*	*BRCA2 (p.Asn1626fs; 4876_4877delAA)*	*no*	*no*		*yes*
9	*F*	*27*	*P*	*no*	*N*	*BRCA2 (p.Tyr3098; 9294C>G)*	*no*	*no*		*yes*
10	*F*	*45*	*D*	*no*	*Weak MSH2/ MSH6*	*MSH6 (p.Phel323fs; c.3964_3967dupAAT)*	*yes*	*no*		*no*

Abbreviations

FDR T2D = first-degree relatives with type 2 diabetes, FDR CRC EC = first-degree family history of colorectal or endometrial cancer,MMR IHC = mismatch repair immunohistochemistry (N = normal staining)

PH T2D = personal history of type 2 diabetes, P=proximal (right-sided) CRC, PD=proximal (right-sided) and distal (left-sided) CRC were present, D=distal (left-sided) CRC.

Chapter Three: Survey of germline variants in cancer-associated genes in young adults with colorectal cancer

Gene, Chromosome & Cancer. 2021; 60(12).

In this publication, the findings of whole-exome sequencing of 133 unrelated young adults with colorectal cancer (CRC) who underwent a comprehensive analysis and detailed interpretation and classification of variants of 133 cancer-predisposition/implicated genes to determine the prevalence and spectrum of germline variants is presented. It was found that one in six young adults with CRC had clinically actionable germline variant in at least one cancer-predisposing gene, with 35% of these in genes associated with breast or ovarian cancer. The findings add further weight that carriers of variants in breast/ovarian cancer-related genes might need to receive surveillance tests for CRC earlier than the general population. Overall it was observed that phenotype and family history was a poor predictor of genotype.

Statement of Authorship

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Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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RESEARCH ARTICLE

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Survey of germline variants in cancer-associated genes in young adults with colorectal cancer

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Abstract

Colorectal cancer (CRC) incidence in young adults is rising. Identifying genetic risk factors is fundamental for the clinical management of patients and their families. This study aimed to identify clinically significant germline variants among young adults with CRC. Whole-exome sequencing data of blood-derived DNA from 133 unrelated young CRC patients (<55 years of age) underwent a comprehensive analysis of 133 cancer-predisposition/implicated genes. All patient tumors were evaluated for mismatch repair deficiency (dMMR). Among 133 patients (aged 16–54 years), 15% (20/133) had clinically actionable pathogenic or likely pathogenic (P/LP) variants in at least 1 well established cancer-predisposing gene: dMMR genes (6), *MUTYH* [bi-allelic (2), mono-allelic (3)], *RNF43* (1), *BMPR1A* (1), *BRCA2* (4), *ATM* (1), *RAD51C* (1), and *BRIP1* (1). Five patients (4%) had variants in genes implicated in cancer but where the significance of germline variants in CRC risk is uncertain: *GATA2* (1), *ERCC2* (mono-allelic) (1), *ERCC4* (mono-allelic) (1), *CFTR* (2). Fourteen (11%) had dMMR tumors. Eighteen (14%) reported a first-degree relative with CRC, but only three of these carried P/LP variants. Three patients with variants in polyposis-associated genes showed no polyposis (one each in *MUTYH* [bi-allelic], *RNF43*, and *BMPR1A*). Approximately

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one in five young adults in our series carried at least one P/LP variant in a cancer-predisposing/implicated gene; 80% of these variants are currently considered clinically actionable in a familial cancer setting. Family history and phenotype have limitations for genetic risk prediction; therefore multigene panel testing and genetic counseling are warranted for all young adults with CRC regardless of those two factors.

KEYWORDS
BRCA2, mismatch repair, whole-exome sequencing, young-onset CRC

1 | INTRODUCTION

Colorectal cancer (CRC) incidence rates in young adults are rising in high-income countries including Australia, for reasons that remain unknown.[1] Genetic risk factors can predispose to young-onset CRC (YOCRC), largely defined as CRC in individuals <50 years of age, and account for 40% of the variability in the risk of CRC.[2] Individuals with a first-degree relative (FDR) with a history of CRC or advanced adenomas are 2- to 4-fold more likely to develop the disease. More than one-quarter of YOCRC patients have an affected FDR.[3] However, less than 10% of CRC cases are clearly inherited in an autosomal-dominant manner and almost half of these do not have a FDR with the disease[4] suggesting that a large proportion of CRC heritability is yet to be explained.[2,5] Identifying clinically significant germline variants in CRC/cancer susceptibility genes is of utmost importance for the prevention and efficient, cost-effective early detection of the disease as this would facilitate targeted CRC surveillance to young adults at increased risk.

Among the hereditary CRC conditions, Lynch syndrome (LS) is the most frequently diagnosed condition and accounts for half of the cases in YOCRC.[6] This syndrome is caused by germline variants in the mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) or monoallelic deletions of the 3′ end of *EPCAM* that silence the downstream gene, MSH2.[6] Germline variants in polyposis-associated genes including *APC*, *MUTYH*, *SMAD4*, *POLE*, and *POLD1* have also been reported in YOCRC patients.[7] However, up to 30% of CRC patients are considered to be due to poly or oligogenic factors interacting with environmental and other factors (multifactorial CRC).[8] Identification of germline variants in other non-CRC associated genes, such as *BRCA1/2*, *ATM*, and *PALB2*, and/or in genes associated with moderate-penetrance cancer risk may explain part of the missing heritability of CRC. Previous studies have excluded some of these genes (such as *RNF43*, *RSP20*, and *MSH3*) and selected patients from potentially high-risk families.[6,9–12] This study demonstrates the findings of whole-exome sequencing (WES) of 133 unrelated young adults with CRC who underwent a comprehensive analysis and detailed interpretation and classification of variants of 133 cancer-predisposition/implicated genes to determine the prevalence and spectrum of germline variants.

2 | PATIENTS AND METHODS

2.1 | Patients

The South Australian Young Onset Colorectal Cancer (SAYO) study is a unique and well-characterized case series of young adults diagnosed with CRC. Between 2014 and 2020, we recruited 133 patients for this study. All patients underwent a face-to-face interview and their detailed family history was recorded as previously described.[13] The study was conducted under ethics approval HREC/14/TQEHLMH/194 (The Queen Elizabeth Hospital, CALHN Office for Research, Adelaide, South Australia) and all patients provided written informed consent before inclusion in the study and providing blood samples. While individuals aged ≥50–74 years are eligible for National Bowel Cancer Screening Program in Australia and YOCRC is widely defined as individuals diagnosed with CRC under 50 years of age, patients ≤55 years have been enrolled in this study because of the low rates of population screening uptake in this overlapping age group (26.4%) and reported increased incidence and mortality in patients under 55 in the United States.[1,13–15]

2.2 | Mismatch repair deficiency testing and somatic variants

Medical records were audited for tumor-based testing results. Mismatch repair deficiency (dMMR) was determined by immunohistochemical (IHC) analysis for the 4 MMR proteins (MLH1, PMS2, MSH2, and MSH6) for 116 patients, and by microsatellite instability (MSI) and IHC analysis for 17 patients.[16] MSI analysis involved the comparison of the allelic profiles of normal and tumor samples at five microsatellite loci: Bat25, Bat26, NR-21 and NR-24, and Mono-27 (Promega MSI Analysis System). An abnormality in two or more loci was defined as MSI-high.[17] Somatic *MLH1* promoter methylation analysis was performed for tumors exhibiting a loss of MLH1 or PMS2 staining. Targeted variant testing was performed on DNA isolated from paraffin-embedded tumor tissue for previously reported clinically significant variants in the *BRAF*, *EGFR*, *KIT*, *KRAS*, and *NRAS* genes using a commercial panel (OncoFOCUS™) for 78 patients.[18]

2.3 | Whole-exome sequencing on leucocyte DNA and multigene panel testing

WES was performed on DNA isolated from lymphocytes for all participants as previously reported.[13] The multigene panel included 26 genes which have recently been recommended by the Collaborative Group of the Americas for Inherited Gastrointestinal Cancer (CGA-IGC) to be used for evaluation of hereditary CRC[19] and 107 more genes which are present in the commercial multigene cancer panels[20,21] and/or have been reported in the literature to be associated with hereditary CRC/cancers[6,7,9,22] (Table S1).

2.4 | WES data filtration and interpretation

The WES data filtration and interpretation were carried out using the Varvis and the Varfish exome analysis pipelines. The data were filtered for high-quality (coverage of >6 reads, a minimum quality score of 10, the alternative allele frequency of ≥25%), and rare (minor allele frequency [MAF] <0.001) variants. MAFs were taken from public (GnomAD, dbSNP) and an in-house database.[23] The American College of Medical Genetics (ACMG) guidelines[24] were used to evaluate the sequence variants as pathogenic (P), likely-pathogenic (LP), or variant of uncertain significance (VUS). Pathogenicity of putative germline variants was confirmed using public databases (ClinGen, ClinVar, InSiGHT, PubMed, OMIM, and HGMD) as previously reported.[23] Five in silico prediction tools (SIFT and Provean [http://provean.jcvi.org/index.php], CADD [https://cadd.gs.wash-ington.edu/], PolyPhene-2 [http://genetics.bwh.harvard.edu/pph2/], and Mutation Taster [http://mutationtaster.org/]) were used for predicting the sequence variants for their protein deleteriousness potential. Pfam server (http://pfam.xfam.org/) was used to analyze protein sequences and identify protein domains. NetGene 2 (http://www.cbs.dtu.dk/services/NetGene2/), Alternative Splice Site Predictor (http://wangcomputing.com/assp/index.html), varSEAK (https://varseak.bio/index.php) and Fruit Fly Splice Predictor (https://www.fruitfly.org/seq_tools/splice.html), servers were used to predict the potential splice site alterations. The Integrative Genomic Viewer tool (IGV2.8.10) was utilized for the visual exploration of sequence variants and facilitating the detection of real variants. Mutalyzer software (https://mutalyzer.nl/) was used to check the mutation nomenclature.

2.5 | Statistics

Prevalence of characteristics was compared between patients with proximal colon cancer and patients with distal colon cancer using Pearson's chi-squared or Fisher's exact test as appropriate. All statistical association tests were performed using SPSS for Windows, Version 26.0, IBM Corp, Armonk, NY. Two-tailed statistics were used throughout with a significance level of $p < 0.05$.

3 | RESULTS

Summary features of 133 study participants with CRC, diagnosed between 2014 and 2020, are shown in Table 1. The median age of these patients was 44 years (range, 16–54); 68 (51%) were female.

Eighteen (14%) had at least one FDR with CRC, and 17 (13%) had at least one FDR with breast cancer. Six (5%) had at least one FDR with CRC and one FDR with breast cancer. Ninety-three of 127 (73%) had cancer in the distal colon, 71/104 (68%) of patients had advanced CRC (stage III or IV), 19/112 (17%) had poorly differentiated cancer, 46/91 (51%) had lymphovascular invasion, and 12/125 (10%) had mucinous adenocarcinoma.

Five (4%) patients met the World Health Organisation (WHO) clinical criteria (2019) for serrated polyposis syndrome (SPS). *BRAF* and *KRAS* somatic variants were detected in 9/78 (12%) and 31/78 (40%) colorectal tumors, respectively (Table 1). There was a significant association between male gender and distal colon cancer ($p < 0.002$), and proximal colon cancer and FDR with breast cancer ($p < 0.03$) (Table S2).

3.1 | Germline variants

Pathogenic or likely pathogenic (P/LP) germline variants were identified in 25 of 133 (19%) YOCRC patients (Table 2, Table S3). Five patients had two P/LP variants, whilst 20 patients had one.

Of all YOCRC patients, 15% (20/133) had clinically actionable P/LP variants in at least one gene clearly associated with cancer predisposition. Thirteen (10%) patients had P/LP variants in genes associated with CRC. Six patients had P variants in one of the MMR genes with *MLH1*, *MSH2*, and *MSH6* variants identified in two patients each. One patient with a *MSH6* variant also had an additional mono-allelic variant in *MUTYH*.

Seven patients had a variant in a polyposis gene only. Two had bi-allelic variants in *MUTYH*, and three had a mono-allelic variant in this gene. Two of the three patients with mono-allelic variants in *MUTYH* had P somatic variants in *KRAS* (one with *KRAS*: c.437C > T [Ala146Val] and the other one with *KRAS*: c.34G > T [Gly12Cys]). One patient had a LP variant in *RNF43*, and one had a LP variant in *BMPR1A*. Three of the seven patients with variants in the polyposis genes had no polyps (one each *MUTYH* [bi-allelic], *RNF43*, and *BMPR1A*). Seven (5%) patients had P/LP variants in the breast or ovarian cancer-predisposing genes (4 *BRCA2*, and 1 each in *ATM*, *RAD51C*, and *BRIP1*) (Table 2).

In addition, 2 of 133 YOCRC patients had a mono-allelic variant in the DNA repair genes (*ERCC2*/4) associated with the recessive cancer predisposition syndrome xeroderma pigmentosum. Three patients had P/LP variants in genes [*GATA2* (1) and *CFTR* (2)] implicated in cancer but where the significance of germline variants in CRC risk is uncertain (Table 2).

Fourteen of 133 (11%) had MMR deficient tumors (Table 1), 2 of these were analyzed by both IHC and MSI which showed an abnormal pattern of staining and MSI-high. Seven patients were diagnosed with

TABLE 1 Characteristics of 133 young adults with CRC

Characteristics	Frequency	Percentage
Median age	44 years	
16–40 years	58/133	44
40–54 years	75/133	56
Female	68/133	51
FDR with CRC	18/133	14
FDR with breast cancer	17/133	13
FDR with other cancers	33/133	25
Personal history of T2D	14/133	11
FDR with T2D	44/133	33
Smoking		
Previous smoker	65/130	50
Current smoker	18/130	14
Left-sided (distal) cancers	93/127	73
Splenic flexure	5/127	3.9
Descending colon	2/127	1.6
Sigmoid colon	38/127	29.9
Recto-sigmoid	13/127	10.2
Rectum	35/127	27.6
Right-sided (proximal) cancers	34/127	27
Caecum	11/127	8.7
Ascending colon	8/127	6.3
Hepatic flexure	6/127	4.7
Transverse colon	9/127	7.1
Stage		
I	13/104	13
II	20/104	19
III	59/104	57
IV	12/104	12
Well-differentiated	2/112	2
Moderately differentiated	91/112	81
Poorly differentiated	19/112	17
Lymphovascular invasion	46/91	51
Neural invasion	29/91	32
Synchronous tumors	7/131	5
Metachronous tumors	2/131	2
Mucinous adenocarcinoma	12/125	10
Signet-ring cell adenocarcinoma	2/125	2
Somatic variants		
BRAF	9/78	12
KRAS	31/78	40
NRAS	0/78	0
EGFR	0/78	0
KIT	0/78	0
MMR deficiency[a]	14/133	11
Lynch syndrome	7/133	5
Lynch-like syndrome[b]	6/133	5
Unclassified[c]	1/133	1
Serrated polyposis syndrome	5/133	4

Abbreviations: CRC, colorectal cancer; FDR, first-degree relative.
[a]Twelve of 14 patients were determined by IHC only and 2 were determined by IHC and MSI analysis.
[b]Patients with MMR deficient tumors but without pathogenic/likely pathogenic germline variants or MLH1-promoter hypermethylation.
[c]Patient with MMR deficient tumor (loss of expression in MLH1 and PMS2) but without pathogenic/likely pathogenic germline variants and not tested for MLH1-promoter hypermethylation.

LS (six with germline P variant in an MMR gene and one patient had a constitutional epimutation of MLH1). Six patients had MMR deficient tumors [loss of expression in MSH2 and MSH6 (2), MSH6 (1), MLH1 and PMS2 (1), and PMS2 and MSH6 (1)] but without P/LP germline variants or *MLH1* promoter hypermethylation (Lynch-like syndrome [LLS]). One patient with MMR deficient tumor (loss of expression in MLH1 and PMS2) was unclassified because there were no known P/LP germline MMR gene variants and the tumor was not tested for somatic *MLH1*-promoter hypermethylation.

It is worth noting that of all the YOCRC patients with variants in cancer-associated/implicated genes, only three patients had a FDR, and one had a second-degree relative (SDR), diagnosed with CRC. Three out of four patients with *BRCA2* variants had FDR with breast cancer; two of whom had also a FDR with polyps (Table 2).

In addition to the P/LP variants, 91 VUS were identified across 46 genes (Table S4). VUS in MMR genes and *POLE* were the most frequently identified in this category. Notably, two patients had frameshift variants in *POLE* (p.Ser233Hisfs*2 and p.Phe1900Serfs*4). There was no significant association between somatic variants in *KRAS* or *BRAF* with the identification of germline variants in YOCRC patients ($p = 0.9$) (Table 2).

4 | DISCUSSION

This study shows that 19% of YOCRC patients had P/LP variants in at least one cancer-predisposing/implicated gene detected through WES, and 80% of these variants were clinically actionable in a familial cancer setting. Among YOCRC cases, 1 in 10 had P/LP germline variants in genes with well-established CRC risk, and 1 in 20 had P/LP variants in breast/ovarian cancer-related genes. P germline variants in MMR-associated genes, *MUTYH* and *BRCA2* were the most frequently encountered variants in this cohort. Three patients with P germline variants in polyposis-associated genes (*MUTYH*, *BMPRIA* and *RNF43*) showed no polyposis. The vast majority of the patients with germline variants did not have FDR or SDR with CRC. This is the first study to our knowledge to determine the prevalence and spectrum of germline variants in large number of cancer-predisposition/implicated genes in unrelated young adults with CRC. These findings show that

TABLE 2 Pathogenic/likely-pathogenic germline variants in cancer-associated genes in young adults with CRC

Patient	Gender	Age	CRC	dMMR	Gene	cDNA	AA change	Classification	FDR CRC	SDR CRC	FDR BC	FDR other cancers	*KRAS/BRAF* variants
1	M	33	Distal	Yes	*MLH1*	NM_000249.3:c.350C > T	p.(Thr117Met)	Pathogenic	Yes	No	No	No	No
2	M	52	Proximal	Yes	*MLH1*	NM_000249.3c.350C > T	p.(Thr117Met)	Pathogenic	Yes	No	Yes	No	No
3	F	30	Proximal	Yes	*MSH2*	NM_000251.2:c.942 + 3A > T	Proximal	Pathogenic	No	Yes	No	No	No
4	F	29	Distal	Yes	*MSH2*	NM_000251.2:c.2038C > T	p.(Arg680*)	Pathogenic	No	No	No	No	*BRAF*
					MUTYH	NM_001048171.1:c.898C > T	p.(Gln300*)	Pathogenic					
5	F	46	Distal	Yes	*MSH6*	NM_000179.2:c.3964_3967dup	p.(Phe1323*)	Pathogenic	No	No	No	Yes	No
6	M	54	Distal	Yes	*MSH6*	NM_000179.2:c.3312del	p.(Phe1104Leufs*11)	Pathogenic	No	No	No	No	No
					MUTYH	NM_001048171.1:c.1145G > A	p.(Gly382Asp)	Pathogenic					
7	F	47	Proximal	No	*MUTYH*	NM_001048171.1:c.1129C > T	p.(Gln377*)	Pathogenic	No	No	No	No	No
						NM_001048171.1:c.267G > A	p.(Trp89*)	Likely-pathogenic					
8	F	42	Distal	No	*MUTYH*	NM_001048171.1:c.1145G > A	p.(Gly382Asp)	Pathogenic	No	No	No	No	No
						NM_001048171.1:c.494A > G	p.(Tyr165Cys)	Pathogenic					
9	M	46	Distal	No	*MUTYH*	NM_001048171.1:c.1145G > A	p.(Gly382Asp)	Pathogenic	No	No	No	No	*KRAS*
10	M	36	Distal	No	*MUTYH*	NM_001048171.1:c.494A > G	p.(Tyr165Cys)	Pathogenic	No	No	No	No	*KRAS*
11	M	44	Distal	No	*MUTYH*	NM_001048171.1:c.494A > G	p.(Tyr165Cys)	Pathogenic	No	No	No	Yes	No
					FANCC	NM_000136.2:c.67del	p.(Asp23Ilefs*23)	Pathogenic					
12	F	40	Proximal	No	*BMPR1A*	NM_004329.2:c.531-1G > T	p.?	Likely-pathogenic	No	No	No	No	*KRAS*
13	F	50	Distal	No	*RNF43*	NM_017763.5:c.375 + 1G > A	p.?	Likely-pathogenic	Yes	No	No	No	*KRAS*
14	F	38	Distal	No	*BRCA2*	NM_000059.3:c.5722_5723del	p.(Leu1908Argfs*2)	Pathogenic	No	No	Yes	No	*KRAS*
15	M	38	Proximal	No	*BRCA2*	NM_000059.3:c.4876_4877del	p.(Asn1626Serfs*12)	Pathogenic	No	No	Yes	No	*KRAS*
16	F	44	Distal	No	*BRCA2*	NM_000059.3:c.9398C > G	p.(Ser3133*)	Likely-pathogenic	No	No	No	No	No
17	F	27	Proximal	No	*BRCA2*	NM_000059.3:c.9294C > G	p.(Tyr3098*)	Pathogenic	No	No	Yes	No	No
18	M	42	Distal	No	*ATM*	NM_000051:c.5979_5983del	p.(Ser1993Argfs*23)	Pathogenic	No	No	No	Yes	No
19	F	46	Proximal	Yes	*RAD51C*	NM_058216.2:c.656 T > C	p.(Leu219Ser)	Pathogenic	No	No	No	No	No
20	F	50	Proximal	No	*BRIP1*	NM_032043.2:c.2392C > T	p.(Arg798*)	Pathogenic	No	No	No	No	No
21	M	28	Distal	No	*ERCC2*	NM_000400.3:c.2068C > T	p.(Arg690Trp)	Pathogenic	No	No	No	No	No
22	M	49	Distal	No	*GATA2*	NM_001145661.1: c.526A > C	p.(Thr176Pro)	Likely-pathogenic	No	No	No	No	No
23	M	54	Distal	No	*GJB2*	NM_004004.6: c.101 T > C	p.(Met34Thr)	Pathogenic	No	No	No	No	No
					ERCC4	NM_000179.2: c.1765C > T	p.(Arg589Trp)	Pathogenic					
24	M	37	Distal	No	*CFTR*	NM_000492.3:c.1000C > T	p.(Arg334Trp)	Pathogenic	No	No	No	No	No
25	F	33	Proximal	No	*CFTR*	NM_000492.3:c.178G > T	p.(E60*)	Pathogenic	No	No	No	No	No

Abbreviations: BC, breast cancer; CRC, colorectal cancer; dMMR, mismatch repair-deficient; F, female; FDR, first-degree relative; M, male; SDR, second-degree relative.

phenotype/family history does not tightly predict genotype and that current CRC screening clinical practice guidelines[25] of offering genetic screening to only those with significant family history, polyposis, or dMMR tumors will miss a significant fraction of patients who might be at higher risk of developing CRC. Therefore, YOCRC patients should be considered for genetic counseling and testing with a broad multigene panel beyond the current clinical genetic testing. This would assist to identify individuals with germline variants in genes that are not traditionally associated with CRC and who might be at higher risk of developing hereditary CRC. However, further accumulation of large international datasets and extensive testing are needed to determine the magnitude of the CRC risk and complete cancer spectrum for variants in genes that are currently excluded from routine hereditary CRC panel testing.[26] In addition, consistent with previously published studies,[27–39] our findings showed that the majority of YOCRC patients at the time of diagnosis were already distantly metastatic or regionally advanced and there was an increased prevalence of distal colon and rectum tumors.[28,35,40–42] YOCRC tend to present with aggressive pathological features with almost one in two cases having lymphovascular invasion and 1 in 10 patients having mucinous adenocarcinoma. We found a male predominance of cancer in the distal colon, and there was a significant association between proximal colon cancer and FDR with breast cancer. A population-based study in Sweden reported that breast cancer women were at higher risk of developing CRC than the general population (standardized incidence ratio [SIR], 1.59; 95% confidence interval [CI], 1.53–1.65) and the risk was higher for proximal colon cancer than distal colon cancer (SIR, 1.72; 95% CI, 1.61–1.82 vs. SIR, 1.46; 95% CI, 1.34–1.58). These results suggest that sex hormones, especially estrogen, might have a role in the initiation and progression of CRC as previously reported.[43–45]

Using multigene panel testing, genetic predispositions have been associated with 14%–25% of CRC in young adults aged <50 years old,[6,10,46] and this proportion was higher (34%) in younger patients (≤35 years).[46] Pearlman et al. identified 16% of YOCRC patients with at least one P variant in high or moderate penetrance genes associated with CRC with around half of them having P variants in MMR genes associated with LS.[6] Stoffel et al. using a research-based next-generation sequencing multigene panel found that 18% of YOCRC patients had pathogenic germline variants associated with cancer predisposition genes with 71% of them having pathogenic variants in MMR genes associated with LS.[47] Recently, Uson et al. conducted a prospective multisite study of germline sequencing using >80-gene next-generation sequencing platform and identified approximately 22% of YOCRC patients, comparable to our findings, with P germline variants.[12] In our study, 24% of patients with germline variants had LS, with all tumors being MMR-deficient on MMR immunohistochemistry. It is possible that other studies may have recruited more YOCRC cases from familial clusters. For example, 18% of our cohort had FDR with CRC while this rate was higher (26%) in the study conducted by Stoffel et al.[47] Additionally, six more patients had an abnormal expression of DNA MMR proteins but without a known P germline in MMR genes suggesting LLS. However, it is worth noting that failure in detecting germline variants in LLS patients does not mean that they do not have genetic cancer predispositions. Rodriguez-Soler et al. found that the incidence of CRC in families of individuals with LLS is higher than families of patients with sporadic CRC (SIR for LLS, 2.12; 95% CI, 1.16–3.56; SIR for sporadic CRC, 0.48; 95% CI, 0.27–0.79; $p < 0.001$) but it is lower in those with LS (SIR for LS, 6.04; 95% CI, $p < 0.001$).[48] There are multiple and redundant DNA repair pathways within the cells and many components, which are organized in multimeric structures, coordinate DNA repair. MMR proteins may cooperate with other components involved in other DNA repair pathways and thus, MMR deficiency in patients with LLS is likely due to the aberrations in other genes involved in DNA repair.[49] For example, in one patient with LLS, a P variant in *RAD51C* was detected. This gene is involved in homologous recombination-mediated DNA double-strand break repair and functions in concert with *BRCA1/2* to ensure genomic stability.[50] However, bi-allelic somatic variants in MMR genes are the major cause of abnormal expression of MMR proteins in LLS patients and have been found in 27%–82% of LLS patients.[51–56]

Other genetic predisposition syndromes including familial adenomatous polyposis (FAP) and *MUTYH*-associated polyposis account for 2%–3% of YOCRC.[6] In our study, none of the YOCRC patients was diagnosed with FAP, but two patients had bi-allelic variants in *MUTYH*, and another three had mono-allelic variants in this gene. A large meta-analysis study has shown that bi-allelic variants in *MUTYH* increase the risk of developing CRC by 28-fold (95% CI: 6.95–115). When considering the 2 most common *MUTYH* variants, CRC risk varied with genotype; G396D/G396D OR = 23.09 (95% CI: 3.15–169.15); Y179C/G396D OR = 21.6 (95% CI: 2.94–159); and Y179C/Y179C OR = 4.10 (95% CI: 0.91–18.48). However, no significant effect was observed in mono-allelic *MUTYH* variant carriers (OR = 1.12; 95% CI: 0.90–1.40).[57] In contrast, Win et al. studied 2332 mono-allelic MUTYH variant carriers and concluded that CRC risk for these carriers with a FDR with CRC is high enough to warrant more intensive screening compared to the general population.[58] Several case–control studies[57,59–61] and family-based studies[62,63] have also reported the effect of mono-allelic variants in *MUTYH* on CRC.

P germline variants in *BMPR1A* have also been associated with YOCRC.[4] This gene is a serine–threonine kinase receptor involving the TGF-β signaling pathway which is a vital regulator of different cellular processes (such as cell proliferation, cell differentiation, and migration). Germline variants in *BMPR1A* are associated with juvenile polyposis syndrome, MMR-proficient YOCRC, and unexplained adenomatous polyposis.[64] We identified one patient with a LP variant in *BMPR1A* and with an MMR-proficient tumor.

Another novel finding of this study is identifying the *RNF43* germline variant in one patient with sigmoid colon cancer without polyposis. *RNF43* is suggested to function as a tumor suppressor gene inhibiting Wnt signaling, and knocking out this gene in mice leads to an intestinal polyposis phenotype.[65] Germline variants in *RNF43* have been previously reported in SPS.[7,66–70] Quintana et al. investigated *RNF43* mutation in 96 SPS patients and found the p.Arg132* variant in a woman diagnosed with CRC and more than 50 polyps.[71] To the best of our knowledge, this is the first study to find a LP variant in *RNF43* in non-polyposis CRC.

CRC has been reported in families carrying a P/LP germline variant in non-CRC associated genes, though the evidence of the CRC causality of these genes has been conflicting.[6,7,9] We identified seven patients with P/LP germline variants in breast/ovarian cancer-related genes, four of whom had variants in *BRCA2*. Another study, consistent with our findings, reported four YOCRC patients with germline P variants in *BRCA2* and two patients with *BRCA1* variants.[6] Similar findings were reported by Yurgelun et al.[10] *BRCA1* and *BRCA2* are tumor suppressor genes involved in homologous recombination which is a major pathway for the error-free repair DNA double-strand breaks.[7,72,73] Garre et al. reported the evidence of the association between germline P variants in *BRCA2* and CRC risk after screening the 27 coding exons and exon-intron boundaries of *BRCA2* in 48 probands from families with a dominant inheritance pattern of CRC.[74] Our findings further support the hypothesis that germline variants in *BRCA1/2* predispose to a wider diversity of cancers than traditionally reported,[75] potentially including YOCRC. However, there is still an ongoing debate about the association of pathogenic *BRCA* variants with the risk of CRC, and there is no consensus whether carriers of these variants need to receive screening tests for CRC or whether they may benefit from poly (ADP-ribose) polymerase (PARP) inhibitors or platinum-based chemotherapy that are used in *BRCA*-associated breast and ovarian cancers.

Screening of somatic variants in tumor tissues has proven to be fundamental in the detection of novel cancer-predisposing genes. Currently, molecular tumor testing can aid in the diagnosis of inherited CRC that arises through germline DNA repair defects, as seen in LS and MUTYH-associated polyposis, as well as define LLS and inform the choice of targeted drug therapy. Somatic *KRAS* and *BRAF* variants in late-onset CRCs have been widely explored in the research community, but they have not been well investigated in YOCRC and the variant rates in these genes in this group of patients have been inconclusive. Characterization of *KRAS* and *NRAS* somatic variants in CRC is useful in predicting the effectiveness of targeted drug therapy and determining polyp precursor and risk factors. Somatic variants in *KRAS* are found in about 35%–45% of CRCs.[76] Studies have reported somatic variants in this gene in 4%–54% of YOCRC patients.[1] We detected *KRAS* variants in 40% of our YOCRC patients. Similar findings were reported by other studies.[77–79] Regarding *BRAF* variants, 7%–10% of CRC patients have been reported to harbor these variants and are significantly associated with elderly female, CpG island methylator phenotype, MSI, right-side tumor, a higher grade, and poor outcomes especially when the patients are metastatic.[80,81] In YOCRC, these variants have been reported in 0%–14% of cases.[1] In this report, 12% of YOCRC patients were diagnosed with $BRAF^{V600E}$ which is comparable to its rates in their older counterparts. The findings of our study show that there is no difference between the rates of *KRAS* and *BRAF* variants in YOCRC patients and their older counterparts. In addition, there was no significant association between somatic variants in *KRAS* or *BRAF* with the identification of germline variants in YPCRC patients.

In conclusion, approximately one in five young adults with CRC in our series carried at least one P/LP variant in a cancer-associated or cancer-implicated gene, and 80% of these variants were clearly clinically actionable in a familial cancer setting. Half of the variants were in genes currently excluded from routine hereditary CRC panel testing. FDR with CRC was rarely seen in variant carriers and three patients with variants in polyposis-associated genes (*MUTYH* [bi-allelic], *RNF43*, and *BMPR1A*) showed no polyposis. Family history and phenotype are not strongly predictive of germline variants in cancer-predisposition genes, and therefore, broad multigene panel testing and genetic counseling are warranted for all YOCRC patients regardless of those two factors. Given the high proportion of unexplained YOCRC patients, further research is needed to identify and evaluate hereditary risk factors in patients with YOCRC. In addition, more research is needed to determine whether carriers of variants in breast/ovarian cancer-related genes need to receive surveillance tests for CRC or whether they may benefit from therapies used in *BRCA*-associated breast and ovarian cancers.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Reger R. Mikaeel: study concept and design, collecting the data, bioinformatics analysis, identification and interpretation of germline variants, funding acquisition, writing – original draft. Joanne P. Young, Yun Li, Timothy J. Price, Bernd Wollnik: supervision, study concept and design, validation, writing – review and editing. Mehgan Horsnell, Wendy Uylaki: collecting the data, software, writing – review and editing. Eric Smith, Gonzalo Tapia Rico, Nicola K. Poplawski, Jennifer E. Hardingham, Yoko Tomita, Amanda R. Townsend, Gökhan Yigit, Silke Kaulfuß, Christian Müller: study concept and design, validation, writing – review and editing. Jinghua Feng, Arne Zibat: software and data curation, writing – review and editing. Each author has approved the submitted version of the manuscript.

DATA AVAILABILITY STATEMENT

Whole-exome sequencing data will be available on request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Supplementary Table 1. Multigene Panel Gene List						
Gene Symbol	**Gene Name**	**Cancer syndrome(s)**	**Major associated tumour types**	**Mechanism of action of CPG mutations**	**Mode of inheritance**	**Reference (PubMed ID)**
ABCB11	ATP-binding cassette, sub-family B (MDR/TAP), member 11	Progressive familial intrahepatic cholestasis	Hepatocellular carcinoma Cholangiocarcinoma	loss-of-function	autosomal recessive	9806540
AIP	aryl hydrocarbon receptor-interacting protein	Pituitary adenoma	Pituitary adenoma 1, multiple types, Pituitary adenoma predisposition	loss-of-function	autosomal recessive	16728643 17244780
ALK	anaplastic lymphoma receptor tyrosine kinase	Neuroblastoma	Neuroblastoma	gain-of-function	autosomal dominant	18724359
ANKRD26	ankyrin repeat domain 26		Myeloid malignancies		autosomal dominant	24030261 24628296 28600339
APC	adenomatous polyposis coli	Familial adenomatous polyposis (FAP)	Colorectal cancer Hepatoblastoma Desmoid tumour	loss-of-function	autosomal dominant	1651174 1651562 1651563 1678319
ATM	ataxia-telangiectasia mutated	Ataxia-Telangiectasia (biallelic mutations)	Biallelic mutations: Lymphoid haematological malignancy (leukaemia, lymphoma) Monoallelic mutations: Breast cancer	loss-of-function	autosomal recessive autosomal dominant	7792600
AXIN2	axin 2	oligodentia-colorectal cancer syndrome	Colorectal cancer	loss-of-function	autosomal dominant	15042511 16110024 27696107
BAP1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)	Tumour predisposition syndrome	Melanoma (cutaneous, uveal) Mesothelioma Meningioma Lung cancer (adenocarcinoma)	loss-of-function	autosomal dominant	21874003
BARD1	BRCA1 associated RING domain 1	Breast cancer	Breast cancer, Ovarian cancer, Pancreatic cancer and endometrial cancer, colon cancer	loss-of-function	autosomal dominant	15342711 18481171 20077502 23334666 26010302 26483394 26720728
BLM	Bloom syndrome, RecQ helicase-like	Bloom syndrome	Lymphoma and ALL haematological malignancy Myeloid haematological malignancy Squamous cell carcinoma, SCC gastric, colorectal cancers	loss-of-function	autosomal recessive	7585968
BMPR1A	bone morphogenetic protein receptor, type IA	Juvenile polyposis syndrome	Colorectal cancer, gastric cancer, hamartoma	loss-of-function	autosomal dominant	11381269
BRCA1	breast cancer 1, early onset	Hereditary breast-ovarian cancer	Breast cancer Ovarian cancer	loss-of-function	autosomal dominant	7545954
BRCA2	breast cancer 2, early onset	Hereditary breast-ovarian cancer Fanconi anaemia (D1) (biallelic mutations)	Biallelic mutations: Myeloid haematological malignancy (Medulloblastoma Wilms tumour Monoallelic mutations: Breast cancer Ovarian cancer Prostate cancer Pancreas cancer	loss-of-function	autosomal recessive autosomal dominant	8524414

Gene Symbol	Gene Name	Cancer syndrome(s)	Major associated tumour types	Mechanism of action of CPG mutations	Mode of inheritance	Reference (PubMed ID)
BRIP1	BRCA1 interacting protein C-terminal helicase 1	Fanconi anaemia (J) (biallelic mutations)	Biallelic mutations: Myeloid haematological malignancy Squamous cell carcinoma (head and neck, oesophagus, genital tract) Monoallelic mutations: Breast cancer Ovarian cancer	loss-of-function	autosomal recessive autosomal dominant	16153896 16116424 16116423
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta (yeast)	Mosaic variegated aneuploidy Syndrome	Wilms Tumor Rhabdomyosarcoma Myeloid haematological malignancy	loss-of-function	autosomal recessive	15475955
CASR	calcium-sensing receptor		Colorectal cancer, breast cancer and prostate cancer	loss-of-function, a gain of function	autosomal dominant	26929638 23555732
CBL	Cbl proto-oncogene, E3 ubiquitin-protein ligase	Noonan syndrome	JMML	loss-of-function	autosomal dominant	20694012
CDC73	cell division cycle 73, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	Hyperparathyroidism-jaw tumour syndrome	Parathyroid cancer Ossifying fibroma (bone)	loss-of-function	autosomal dominant	12434154
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	Hereditary diffuse gastric cancer	Breast cancer (lobular) Gastric cancer (diffuse)	loss-of-function	autosomal dominant	9537325
CDK4	cyclin-dependent kinase 4	Melanoma	Melanoma	gain-of-function	autosomal dominant	8528263
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	Multiple endocrine neoplasias, type IV	Thyroid cancer, Pituitary adenoma	loss-of-function	autosomal recessive autosomal dominant	17030811
CDKN1C	cyclin-dependent kinase inhibitor 1C		Wilms tumour and hepatoblastoma	loss-of-function	autosomal dominant	27419809
CDKN2A	cyclin-dependent kinase inhibitor 2A	Melanoma and neural system tumour syndrome Melanoma-pancreatic cancer syndrome	Melanoma [p16 and p14ARF] Pancreas cancer [p16] Astrocytoma [p14ARF]	loss-of-function	autosomal dominant	7987387 7987388
CEBPA	CCAAT/enhancer-binding protein (C/EBP), alpha	Leukaemia, acute myeloid	Myeloid haematological malignancy	loss-of-function	autosomal dominant	15575056
CFTR	CF transmembrane conductance regulator		Colorectal cancer, pancreatic cancer,	loss-of-function	autosomal dominant	14576497 26751771
CHEK2	checkpoint kinase 2	Breast cancer, Prostate cancer	Breast cancer, Prostate cancer and colorectal cancer	loss-of-function	autosomal dominant	11967536 12094328
COL7A1	collagen, type VII, alpha 1	Epidermolysis bullosa	Squamous cell carcinoma (skin)	loss-of-function	autosomal recessive autosomal dominant	8513326
CTRC	chymotrypsin C		Pancreatic cancer	loss-of-function	autosomal dominant	24600409 30134356
CYLD	cylindromatosis (turban tumour syndrome)	Brooke-Spiegler syndrome	Cylindroma spiroadenocarcinoma Basal cell carcinoma	loss-of-function	autosomal dominant	10835629
DDB2	damage-specific DNA binding protein 2, 48kDa	Xeroderma Pigmentosum (E)	Basal cell carcinoma Squamous cell carcinoma Melanoma	loss-of-function	autosomal dominant	8798680
DICER1	dicer 1, ribonuclease type III	DICER1 syndrome	Pleuropulmonary blastoma Cystic nephroma Ovarian sex cord tumour	loss-of-function	autosomal dominant	19556464
DIS3L2	DIS3 mitotic control homolog (S. cerevisiae)-like 2	Perlman syndrome	Wilms tumour	loss-of-function	autosomal recessive	22306653
DKC1	dyskeratosis congenita 1, dyskerin	Dyskeratosis congenita	acute myeloid leukaemia Squamous cell carcinoma (head + neck, anorectal)	loss-of-function	X-linked recessive	9590285

Gene Symbol	Gene Name	Cancer syndrome(s)	Major associated tumour types	Mechanism of action of CPG mutations	Mode of inheritance	Reference (PubMed ID)
DOCK8	dedicator of cytokinesis 8	HyperIgE syndrome	Squamous cell carcinoma Lymphoma	loss-of-function	autosomal recessive	19776401
EGFR	epidermal growth factor receptor		Non-small cell lung cancer	gain-of-function	autosomal dominant	16258541
ELANE	elastase, neutrophil expressed	Severe congenital neutropenia	Leukaemia	loss-of-function	autosomal dominant	11001877
EPCAM	epithelial cell adhesion molecule	Colorectal cancer, hereditary nonpolyposis, Lynch syndrome	Colorectal cancer, Endometrial cancer, Prostate cancer, Gastric cancer, and Ovarian cancer	loss-of-function	autosomal dominant	
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2	Xeroderma pigmentosum (D)	Basal cell carcinoma Squamous cell carcinoma Melanoma	loss-of-function	autosomal recessive	7849702
ERCC3	excision repair cross-complementing rodent repair deficiency, complementation group 3	Xeroderma pigmentosum (B)	Basal cell carcinoma Squamous cell carcinoma Melanoma	loss-of-function	autosomal recessive	2167179
ERCC4	excision repair cross-complementing rodent repair deficiency, complementation group 4	Xeroderma pigmentosum (F) Fanconi anaemia (Q)	Basal cell carcinoma Squamous cell carcinoma Melanoma	loss-of-function	autosomal recessive	8797827
ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5	Xeroderma pigmentosum (G)	Basal cell carcinoma Squamous cell carcinoma Melanoma	loss-of-function	autosomal recessive	7951246
EXT1	exostosin 1	Chondrosarcoma	Chondrosarcoma	loss-of-function	autosomal dominant	7550340
EXT2	exostosin 2		Chondrosarcoma	loss-of-function	autosomal dominant	8782816
FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	Tyrosinemia	Hepatocellular carcinoma	loss-of-function	autosomal recessive	8318997
FANCA	Fanconi anemia, complementation group A	Fanconi anaemia (A)	Myeloid haematological malignancy Squamous cell carcinoma (head and neck, oesophagus, genital tract)	loss-of-function	autosomal recessive	8896564 8896563
FANCC	Fanconi anemia, complementation group C	Fanconi anaemia (C)	Myeloid haematological malignancy Squamous cell carcinoma (head and neck, oesophagus, genital tract)	loss-of-function	autosomal recessive	1574115
FANCG	Fanconi anemia, complementation group G	Fanconi anaemia (G)	Myeloid haematological malignancy Squamous cell carcinoma (head and neck, oesophagus, genital tract)	loss-of-function	autosomal recessive	9806548
FH	fumarate hydratase	Hereditary leiomyomatosis and renal cell cancer (HLRCC)	Renal cell cancer Leiomyosarcoma (uterus)	loss-of-function	autosomal recessive autosomal dominant	11865300
FLCN	folliculin	Birt-Hogg-Dube syndrome	Renal cell cancer Oncocytoma	loss-of-function	autosomal dominant	12204536
GALNT12	polypeptide N-acetylgalactosaminyltransferase 12	Colorectal cancer	Colorectal cancer	loss-of-function	autosomal dominant	19617566
GATA2	GATA binding protein 2	Emberger MonoMAC syndrome	Myeloid haematological malignancy	loss-of-function	autosomal dominant	21892158 21892162 21765025 21670465
GBA	glucosidase, beta, acid	Gauchers type 1	Myeloma Lymphoma Hepatocellular carcinoma	loss-of-function	autosomal recessive	2880291
GJB2	gap junction protein, beta 2, 26kDa	Keratosis-ichthyosis-deafness syndrome (KID)	Squamous cell carcinoma	loss-of-function	autosomal dominant	11912510
GPC3	glypican 3	Simpson-Golabi-Behmel syndrome	Wilms tumour Hepatoblastoma, hepatocellular carcinoma Neuroblastoma Gonadoblastoma	loss-of-function	X-linked recessive	8589713

Gene Symbol	Gene Name	Cancer syndrome(s)	Major associated tumour types	Mechanism of action of CPG mutations	Mode of inheritance	Reference (PubMed ID)
GREM1	gremlin 1, DAN family BMP antagonist	Hereditary mixed polyposis syndrome (AD)	Hamartomatous polyps, Adenomatous polyps Colorectal cancer	gain-of-function	autosomal dominant	22561515 26493165
HFE	hemochromatosis	Haemochromatosis	Hepatocellular carcinoma Cholangiocarcinoma	loss-of-function	autosomal recessive	8696333
HMBS	hydroxymethylbilane synthase	Porphyria (AI)	hepatocellular carcinoma	loss-of-function	autosomal dominant	2563167
HOXB13	homeobox B13	Prostate cancer	Prostate cancer	loss-of-function gain-of-function	autosomal dominant	22236224
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	Costello syndrome	Rhabdomyosarcoma Neuroblastoma Transitional cell carcinoma (bladder)	gain-of-function	autosomal dominant	16170316
ITK	IL2-inducible T-cell kinase	Lymphoproliferative syndrome 1	Hodgkins lymphoma	loss-of-function	autosomal recessive	19425169
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	Gastrointestinal stromal tumour, familial	Gastro-Intestinal Stromal Tumor	gain-of-function	autosomal dominant	9697690
MAX	MYC associated factor X	Familial paraganglioma-pheochromocytoma syndrome	Paraganglioma Pheochromocytoma	loss-of-function	autosomal dominant	21685915
MEN1	multiple endocrine neoplasia I	Multiple endocrine neoplasia Type 1	Parathyroid, pituitary adenoma Neuroendocrine tumour Carcinoid tumour Adrenocortical carcinoma	loss-of-function	autosomal dominant	9103196
MET	met proto-oncogene (hepatocyte growth factor receptor)	Renal cell carcinoma, papillary, 1, familial and somatic	Renal cell cancer (papillary carcinoma) Osteofibrous dysplasia	gain-of-function	autosomal dominant	9140397
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	MMR deficiency syndrome (biallelic mutations) Lynch syndrome / Hereditary Non-Polyposis Colon Cancer (monoallelic mutations)	Biallelic mutations: Brain tumours Haematological malignancy Embryonal tumours Monoallelic mutations: Colorectal cancer Endometrial cancer Ovarian cancer	loss-of-function	autosomal recessive autosomal dominant	8128251 8145827
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	MMR deficiency syndrome (biallelic mutations) Lynch syndrome / Hereditary Non-Polyposis Colon Cancer (monoallelic mutations)	Biallelic mutations: Brain tumours Haematological malignancy Embryonal tumours Monoallelic mutations: Colorectal cancer Endometrial cancer Ovarian cancer Sebaceous adenoma, carcinoma, epithelioma	loss-of-function	autosomal recessive autosomal dominant	8252616 8261515
MSH3	mutS homolog 3	Familial adenomatous polyposis	Colorectal cancer	loss-of-function	autosomal recessive	27476653
MSH6	mutS homolog 6 (E. coli)	MMR deficiency syndrome (biallelic mutations) Lynch syndrome / Hereditary Non-Polyposis Colon Cancer (monoallelic mutations)	Biallelic mutations: Brain tumours Haematological malignancy Embryonal tumours Monoallelic mutations: Colorectal cancer Endometrial cancer Ovarian cancer	loss-of-function	autosomal recessive autosomal dominant	9354786
MTAP	methylthioadenosine phosphorylase	Diaphyseal medullary stenosis with malignant fibrous histiocytoma (DMS-MFH)	malignant fibrous histiocytoma (sarcoma)	loss-of-function	autosomal dominant	22464254

Gene Symbol	Gene Name	Cancer syndrome(s)	Major associated tumour types	Mechanism of action of CPG mutations	Mode of inheritance	Reference (PubMed ID)
MUTYH	mutY homolog (E. coli)	Adenomas, multiple colorectal	Colorectal cancer	loss-of-function	autosomal recessive	11818965
NBN	nibrin	Nijmegen breakage syndrome	Lymphoma Medulloblastoma Glioma Rhabdomyosarcoma	loss-of-function	autosomal recessive	9590180 9620777
NF1	neurofibromin 1	Neurofibromatosis type 1	Glioma Malignant peripheral nerve sheath tumour	loss-of-function	autosomal dominant	2134734 1694727
NF2	neurofibromin 2 (merlin)	Neurofibromatosis type 2	Vestibular schwannoma Meningioma Ependymoma	loss-of-function	autosomal dominant	8453669 8379998
NTHL1	nth like DNA glycosylase 1	Familial adenomatous polyposis 3	Colorectal cancer Endometrial cancer	loss-of-function	autosomal recessive	25938944 27720914 27713038 30248171
PALB2	partner and localizer of BRCA2	Fanconi anaemia (N) (biallelic mutations)	Biallelic mutations: Myeloid haematological malignancy Medulloblastoma Neuroblastoma Wilms tumour Monoallelic mutations: Breast cancer Pancreas cancer	loss-of-function	autosomal recessive autosomal dominant	17200671 17200672 17200668 17287723
PALLD	palladin, cytoskeletal associated protein	Pancreatic cancer	Pancreatic cancer	gain-of-function	autosomal dominant	17194196 17415588 19336541
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide		Gastro-Intestinal Stromal Tumor	gain-of-function	autosomal dominant	14699510
PHOX2B	paired-like homeobox 2b		Neuroblastoma	loss-of-function	autosomal dominant	12640453
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)	MMR deficiency syndrome (biallelic mutations) Lynch syndrome / Hereditary Non-Polyposis Colon Cancer (monoallelic mutations)	Biallelic mutations: Brain tumours Haematological malignancy Supratentorial primitive neuroectodermal tumors Monoallelic mutations: Colorectal cancer Endometrial cancer Ovarian cancer	loss-of-function	autosomal recessive autosomal dominant	8072530
POLD1	polymerase (DNA directed), delta 1, catalytic subunit	PPAP (polymerase proofreading associated polyposis)	Colorectal cancer Endometrial cancer	loss-of-function	autosomal dominant	23263490
POLE	polymerase (DNA directed), epsilon, catalytic subunit	PPAP (polymerase proofreading associated polyposis)	Colorectal cancer	loss-of-function	autosomal recessive autosomal dominant	23263490
POLH	polymerase (DNA directed), eta	Xeroderma pigmentosa V	Squamous cell cancer (skin)	loss-of-function	autosomal recessive	10385124
POT1	protection of telomeres 1	Glioma, Melanoma	Cutaneous malignant melanoma thyroid cancer Li-Fraumeni-like syndrome	loss-of-function	autosomal recessive	28389767 24686846 24686849 25482530
PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha	Carney complex	Myxoma (cardiac/cutaneous/breast) Thyroid cancer Sex cord-stromal tumor	loss-of-function	autosomal dominant	10973256
PRSS1	protease, serine, 1 (trypsin 1)		Pancreatic cancer	loss-of-function	autosomal dominant	8841182

Gene Symbol	Gene Name	Cancer syndrome(s)	Major associated tumour types	Mechanism of action of CPG mutations	Mode of inheritance	Reference (PubMed ID)
PTCH1	patched 1	Nevoid basal cell carcinoma syndrome Gorlin Syndrome	Basal cell carcinoma Medulloblastoma	loss-of-function	autosomal dominant	8658145 8681379
PTEN	phosphatase and tensin homolog	Cowden Syndrome PTEN hamartoma tumor syndrome	Breast cancer Thyroid cancer Endometrial cancer	loss-of-function	autosomal dominant	9140396
PTPN11	protein tyrosine phosphatase, non-receptor type 11	Noonan syndrome	JMML neuroblastoma	gain-of-function	autosomal dominant	11704759 12717436
RAD51C	RAD51 homolog C (S. cerevisiae)	Fanconi anaemia (O) (biallelic mutations)	Monoallelic mutations: Ovarian cancer	loss-of-function	autosomal recessive autosomal dominant	20400964
RAD51D	RAD51 homolog D (S. cerevisiae)		Ovarian cancer	loss-of-function	autosomal dominant	21822267
RB1	retinoblastoma 1	retinoblastoma	Retinoblastoma Pinealoma Sarcoma Melanoma	loss-of-function	autosomal dominant	2885916
RECQL4	RecQ protein-like 4	Rothmund-Thompson syndrome	Osteosarcoma Basal cell carcinoma Squamous cell carcinoma	loss-of-function	autosomal recessive	10319867
RET	ret proto-oncogene	Multiple endocrine neoplasia 2A/2B Familial medullary thyroid carcinoma	Medullary thyroid cancer Pheochromocytoma	gain-of-function	autosomal dominant	8099202
RHBDF2	rhomboid 5 homolog 2 (Drosophila)		Oesophagal cancer	gain-of-function	autosomal dominant	22265016
RMRP	The RNA component of mitochondrial RNA processing endoribonuclease	Cartilage-hair hypoplasia syndrome	Non-Hodgkin lymphoma Squamous carcinoma (bcc) Leukaemia	loss-of-function	autosomal recessive	11207361
RNF43	ring finger protein 43	Sessile serrated polyposis cancer syndrome	Colorectal cancer	loss-of-function	autosomal dominant	24512911 22895187 27081527
RPS20	ribosomal protein S20		hereditary nonpolyposis CRC	loss-of-function	autosomal dominant	24941021 27713038
RUNX1	runt-related transcription factor 1		Myeloid haematological malignancy (leukaemia)	loss-of-function	autosomal dominant	10508512
SBDS	Shwachman-Bodian-Diamond syndrome	Schwachman-Diamond syndrome	Myeloid haematological malignancy	loss-of-function	autosomal recessive	12496757
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Carney-Stratakis syndrome	Paraganglioma Pheochromocytoma A gastrointestinal stromal tumour (GIST)	loss-of-function	autosomal recessive autosomal dominant	20484225
SDHAF2	succinate dehydrogenase complex assembly factor 2	Familial paraganglioma-pheochromocytoma syndrome	Paraganglioma Pheochromocytoma	loss-of-function	autosomal dominant	19628817
SDHB	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	Familial paraganglioma-pheochromocytoma syndrome	Paraganglioma Pheochromocytoma Renal cell cancer	loss-of-function	autosomal dominant	11404820
SDHC	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	Familial paraganglioma-pheochromocytoma syndrome	Paraganglioma Pheochromocytoma Gastrointestinal stromal tumour (GIST)	loss-of-function	autosomal dominant	11062460
SDHD	succinate dehydrogenase complex, subunit D, integral membrane protein	Familial paraganglioma-pheochromocytoma syndrome	Paraganglioma Pheochromocytoma Gastrointestinal stromal tumour (GIST)	loss-of-function	autosomal dominant	10657297

Gene Symbol	Gene Name	Cancer syndrome(s)	Major associated tumour types	Mechanism of action of CPG mutations	Mode of inheritance	Reference (PubMed ID)
SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Alpha1 antitrypsin deficiency	Hepatocellular carcinoma	loss-of-function	autosomal recessive	3485248 7045697
SH2D1A	SH2 domain-containing 1A	Lymphoproliferative disease	Lymphoma	loss-of-function	X-linked recessive	9771704
SLC25A13	solute carrier family 25 (aspartate/glutamate carrier), member 13	Citrullinaemia	Hepatocellular carcinoma	loss-of-function	autosomal recessive	10369257
SMAD4	SMAD family member 4	Juvenile polyposis syndrome	Colorectal cancer	loss-of-function	autosomal dominant	9582123
SMARCB1	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily b, member 1	Rhabdoid predisposition syndrome	Rhabdoid tumour (renal, extra-renal) Central primitive neuroectodermal tumour	loss-of-function	autosomal dominant	10521299
SMARCE1	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily e, member 1		Meningioma	loss-of-function	autosomal dominant	23377182
SOS1	son of sevenless homolog 1 (Drosophila)	Noonan syndrome	Rhabdomyosarcoma	gain-of-function	autosomal dominant	17143285
SPINK1	serine peptidase inhibitor Kazal type 1		Pancreatic cancer Colorectal cancer Breast cancer	gain-of-function	autosomal recessive autosomal dominant	26656134
SRY	sex-determining region Y		Gonadoblastoma	loss-of-function	Y-linked	2247149 2247151
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	Hyper-immunoglobulin E syndrome	Lymphoma	loss-of-function	autosomal dominant	17676033
STK11	serine/threonine kinase 11	Peutz-Jeghers syndrome	Colorectal cancer Gastric cancer Breast cancer Sex cord-stromal tumor	loss-of-function	autosomal dominant	9425897 9428765
SUFU	suppressor of fused homolog (Drosophila)		Medulloblastoma, meningioma	loss-of-function	autosomal dominant	12068298
TERT	telomerase reverse transcriptase	Dyskeratosis congenita	acute myeloid leukaemia Squamous cell carcinoma (head + neck, anorectal) Melanoma	loss-of-function	autosomal recessive autosomal dominant	16247010
TGFBR1	transforming growth factor, beta receptor 1	Multiple self-healing squamous epithelioma (MSSE) Ferguson-Smith syndrome	Squamous cell carcinoma (skin)	loss-of-function	autosomal dominant	21358634
TMEM127	transmembrane protein 127		Pheochromocytoma	loss-of-function	autosomal dominant	20154675
TNFRSF6 (FAS)	transforming growth factor, beta receptor 1	Autoimmune lymphoproliferative syndrome	Lymphoma	loss-of-function	autosomal dominant	7540117
TP53	tumour protein p53	Li-Fraumeni syndrome	Breast cancer Sarcoma Adrenocortical carcinoma Astrocytoma	loss-of-function	autosomal dominant	1978757
TRIM37	tripartite motif containing 37	Mulibrey-nanism	Wilms tumour	loss-of-function	autosomal recessive	10888877
TSC2	tuberous sclerosis 2	Tuberous sclerosis 2	Renal cell cancer, angiomyolipoma Subependymal giant cell astrocytoma Rhabdomyoma (cardiac)	loss-of-function	autosomal dominant	8269512
UROD	uroporphyrinogen decarboxylase	Porphyria (cutanea tarda)	hepatocellular carcinoma	loss-of-function	autosomal recessive autosomal dominant	3775362
VHL	von Hippel-Lindau tumour suppressor, E3 ubiquitin protein ligase	Von Hippel-Lindau syndrome	Renal cell cancer Pheochromocytoma Neuroendocrine tumour (pancreas) Hemangioblastoma (central nervous system, retina)	loss-of-function	autosomal dominant	8493574

Gene Symbol	Gene Name	Cancer syndrome(s)	Major associated tumour types	Mechanism of action of CPG mutations	Mode of inheritance	Reference (PubMed ID)
WAS	Wiskott-Aldrich syndrome	Wiskott-Aldrich syndrome WAS-related syndrome	Lymphoma	loss-of-function	X-linked recessive	8069912
WRN	Werner syndrome, RecQ helicase-like	Werner syndrome	Sarcoma Melanoma Thyroid cancer	loss-of-function	autosomal recessive	8602509
WT1	Wilms tumour 1	WAGR syndrome Denys-Drash syndrome Frasier syndrome	Wilms tumour Gonadoblastoma	loss-of-function	autosomal dominant	1673293
XPA	xeroderma pigmentosum, complementation group A	Xeroderma pigmentosum (A)	Basal cell carcinoma Squamous cell carcinoma Melanoma	loss-of-function	autosomal recessive	2234061
XPC	xeroderma pigmentosum, complementation group C	Xeroderma pigmentosum (C)	Basal cell carcinoma Squamous cell carcinoma Melanoma	loss-of-function	autosomal recessive	8298653

Supplementary Table 2. Association of characteristics with patients with proximal colon cancer vs distal colon cancer.			
Features (No. of the patient)	**% Proximal colon cancer**	**% Distal colon cancer**	**P-Value**
Male (62)	14.5	85.5	**0.003**
Female (65)	38.5	61.5	
FDR with CRC (18)	38.9	61.1	0.3
NO FDR with CRC (109)	24.8	75.2	
FDR with BC (16)	50	50	**0.03**
NO FDR with BC (111)	23.4	76.6	
FDR with T2D (43)	20.9	79.1	0.4
No FDR with T2D (84)	29.8	70.2	
With *KRAS* variants (30)	36.7	63.3	0.3
Without *KRAS* variants (46)	21.7	78.3	
With *BRAF* variants (9)	44.4	55.6	0.4
Without *BRAF* variants (68)	26.5	73.5	
Ever smoked (64)	18.8	81.2	0.1
Never smoked (60)	35	65	
Current smoker (18)	5.6	94.4	0.09
Non-smoker (106)	30.2	69.8	

Supplementary Table 3. Germline variants in young adults with CRC with evidence of pathogenicity according to the ACMG guidelines.

Chr	Start	Ref	Alt	Gene	cDNA	AA Change	ACMG criteria	Classification
1	45797348	G	A	MUTYH	NM_001048171.1:c.1129C>T	p.(Gln377*)	PVS1, PS1, PS3, PM2	Pathogenic
1	45799124	C	T	MUTYH	NM_001048171.1:c.267G>A	p.(Trp89*)	PVS1, PM2	Likely-pathogenic
1	45797228	C	T	MUTYH	NM_001048171.1:c.1145G>A	p.(Gly382Asp)	PS1, PS3, PP3	Pathogenic
1	45798475	T	C	MUTYH	NM_001048171.1:c.494A>G	p.(Tyr165Cys)	PS1, PS3, PP1, PP3	Pathogenic
2	48030691	CT	C	MSH6	NM_000179.2:c.3312del	p.(Phe1104Leufs*11)	PVS1, PS1, PM2, PP4	Pathogenic
1	45797752	G	A	MUTYH	NM_001048171.1:c.898C>T	p.(Gln300*)	PVS1, PS1, PM2	Pathogenic
2	47703538	C	T	MSH2	NM_000251.2:c.2038C>T	p.(Arg680*)	PVS1, PS1, PM2, PP4	Pathogenic
2	48033752	A	AGAAT	MSH6	c.3964_3967dup	p.(Phe1323*)	PVS1, PS1, PM2, PP4	Pathogenic
9	98011506	TC	T	FANCC	NM_000136.2:c.67del	p.(Asp23Ilefs*23)	PVS1, PS1, PM2	Pathogenic
3	37045935	C	T	MLH1	NM_000249.3:c.350C>T	p.(Thr117Met)	PS1, PS3, PM2, PP3, PP4	Pathogenic
2	47641560	A	T	MSH2	NM_000251.2:c.942+3A>T	p.?	PVS1, PS1, PS3, PM2, PP4	Pathogenic
10	88671996	G	T	BMPR1A	NM_004329.2:c.531-1G>T	p.?	PVS1, PM2	Likely-pathogenic
17	56448271	C	T	RNF43	NM_017763.5:c.375+1G>A	p.?	PVS1_M, PM2, PS3, PP4	Likely-pathogenic
13	32914209	ACT	A	BRCA2	NM_000059.3:c.5722_5723del	p.(Leu1908Argfs*2)	PVS1, PS1, PS3, PM2	Pathogenic
13	32913365	GAA	G	BRCA2	NM_000059.3:c.4876_4877del	p.(Asn1626Serfs*12)	PVS1, PS1, PS3, PM2	Pathogenic
13	32968967	C	G	BRCA2	NM_000059.3:c.9398C>G	p.(Ser3133*)	PVS1, PM2	Likely-pathogenic
13	32968863	C	G	BRCA2	NM_000059.3:c.9294C>G	p.(Tyr3098*)	PVS1, PS1, PS3, PM2	Pathogenic
11	108183193	AAAAGT	A	ATM	NM_000051:c.5979_5983del	p.(Ser1993Argfs*23)	PVS1, PS1, PS4, PM2	Pathogenic
17	59793412	G	A	BRIP1	NM_032043.2:c.2392C>T	p.(Arg798*)	PVS1, PS1, PS3, PM2	Pathogenic
17	56780641	T	C	RAD51C	NM_058216.2:c.656T>C	p.(Leu219Ser)	PS1, PS3, PS4, PM2, PP3	Pathogenic
19	45855589	G	A	ERCC2	NM_000400.3:c.2068C>T	p.(Arg690Trp)	PS1, PM2, PS3, PP3	Pathogenic
3	128204915	T	G	GATA2	NM_001145661.1.c.526A>C	p.(Thr176Pro)	PP1-S, PM2, PM6, PP3	Likely-pathogenic
13	20763620	A	G	GJB2	c.101T>C	p.(Met34Thr)	PS1, PS3, PP1-S, PP3	Pathogenic
16	14029554	C	T	ERCC4	c.1765C>T	p.(Arg589Trp)	PSI, PM2, PS3, PP3	Pathogenic
7	117180284	C	T	CFTR	NM_000492.3:c.1000C>T	p.(Arg334Trp)	PS1, PS3, PP1, PM2, PP3	Pathogenic
7	17149101	G	T	CFTR	NM_000492.3:c.178G>T	p.(E60*)	PVS1, PS1, PS3, PM2	Pathogenic

Supplementary Table 4. Variants of unknown significance in cancer-associated genes detected in young adults with CRC.

Patient	Chr	Start	Ref	Alt	*Gene*	Transcript	cDNA	AA Change
SAYO 018100	9	420563	G	T	*DOCK8*	NM_001190458.1	c.3703G>T	p.(Val1235Leu)
SAYO_015028	7	6038762	C	T	*PMS2*	NM_000535.6	c.682G>A	p.(Gly228Ser)
SAYO 014001	8	90993086	CAAG	C	*NBN*	NM_002485.4	c.353_355del	p.(Ser118del)
	19	45856019	C	G	*ERCC2*	NM_000400.3	c.1887G>C	p.(Gln629His)
SAYO 015002	12	1.33E+08	AG	A	*POLE*	NM_006231.3	c.5697del	p.(Phe1900Serfs*4)
SAYO 015007	4	41747904	C	T	*PHOX2B*	NM_003924.3	c.865G>A	p.(Gly289Ser)
SAYO 015014	2	47707945	A	G	*MSH2*	NM_000251.2	c.2569A>G	p.(Ile857Val)
	19	1207006	A	G	*STK11*	NM_000455.4	c.94A>G	p.(Thr32Ala)
SAYO 015017	16	89805934	CGGA	C	*FANCA*	NM_000135.2	c.3959_3961del	p.(Leu1320del)
SAYO 016043	19	11097598	A	C	*SMARCA4*	NM_001128844.1	c.778A>C	p.(Met260Leu)
SAYO 017056	8	30977876	G	GGTC	*WRN*	NM_000553.4	c.2569_2571dup	p.(Arg857dup)
SAYO 018059	1	17349129	T	C	*SDHB*	NM_003000.2	c.739A>G	p.(Met247Val)
SAYO 018060	17	59926584	A	G	*BRIP1*	NM_032043.2	c.413T>C	p.(Leu138Ser)
SAYO 018063	8	1.46E+08	C	T	*RECQL4*	NM_004260.3	c.3185G>A	p.(Arg1062Gln)
	19	33792754	GGGCGGCGGC	G	*CEBPA*	NM_004364.4	c.558_566del	p.(Pro187_Pro189del)
SAYO 018065	8	31000191	C	T	*WRN*	NM_000553.4	c.3283C>T	p.(Pro1095Ser)
SAYO 018070	3	14187542	G	A	*XPC*	NM_001145769.1	c.2611C>T	p.(Arg871*)
SAYO 018072	2	48033640	C	T	*MSH6*	NM_000179.2	c.3851C>T	p.(Thr1284Met)
	9	1.36E+08	G	A	*TSC1*	NM_000368.4	c.1922C>T	p.(Pro641Leu)
	14	95571450	C	T	*DICER1*	NM_001195573.1	c.3227G>A	p.(Ser1076Asn)
SAYO 018074	22	29095881	C	T	*CHEK2*	NM_001005735.1	c.1082G>A	p.(Arg361His)
SAYO 018077	17	33443879	G	A	*RAD51D*	NM_001142571.1	c.322C>T	p.(Arg108Cys)
SAYO 018083	9	420563	G	T	*DOCK8*	NM_001190458.1	c.3703G>T	p.(Val1235Leu)
	9	98011565	T	A	*FANCC*	NM_000136.2	c.9A>T	p.(Gln3His)
	17	41246198	T	A	*BRCA1*	NM_007294.3	c.1350A>T	p.(Lys450Asn)
SAYO 018086	9	420563	G	T	*DOCK8*	NM_001190458.1	c.3703G>T	p.(Val1235Leu)
SAYO018087	15	80450427	T	C	*FAH*	NM_000137.2	c.107T>C	p.(Ile36Thr)
SAYO 018088	14	65543267	C	T	*MAX*	NM_001320415.1	c.221G>A	p.(Gly74Asp)
	17	59760943	C	T	*BRIP1*	NM_032043.2	c.3464G>A	p.(Gly1155Glu)
SAYO 018094	2	48023078	C	G	*MSH6*	NM_000179.2	c.503C>G	p.(Ala168Gly)
	2	48026159	C	T	*MSH6*	NM_000179.2	c.1037C>T	p.(Ser346Phe)
SAYO 018096	13	32929030	C	A	*BRCA2*	NM_000059.3	c.7040C>A	p.(Pro2347Gln)
SAYO 019106	2	47630477	C	G	*MSH2*	NM_000251.2	c.147C>G	p.(Asp49Glu)
	16	2097827	C	G	*NTHL1*	NM_002528.6	c.22G>C	p.(Gly8Arg)
SAYO 019109	15	91347479	T	C	*BLM*	NM_000057.3	c.3641T>C	p.(Met1214Thr)
	12	1.33E+08	C	T	*POLE*	NM_006231.3	c.2706+5G>A	p.?

SAYO 019112	19	11170550	C	G	*SMARCA4*	NM_001128844.1	c.4757C>G	p.(Ser1586Cys)
SAYO019192	12	11254185	GGA	G	*POLE*	NM_006231.3	c.5697del	p.(Phe1900Serfs*4)
SAYO 019116	9	98011431	A	G	*FANCC*	NM_000136.2	c.143T>C	p.(Met48Thr)
	16	89836642	C	T	*FANCA*	NM_000135.2	c.2248G>A	p.(Val750Met)
SAYO 019118	4	55575650	C	G	*KIT*	NM_000222.2	c.1176C>G	p.(Phe392Leu)
	15	40492521	C	T	*BUB1B*	NM_001211.5	c.1478C>T	p.(Thr493Ile)
SAYO019122	11	1.08E+08	A	G	*ATM*	NM_000051.3	c.3080A>G	p.(His1027Arg)
	17	59760676	A	T	*BRIP1*	NM_032043.2	c.3731T>A	p.(Met1244Lys)
SAYO 019125	1	45797860	C	T	*MUTYH*	NM_001048171.1	c.869G>A	p.(Ser290Asn)
SAYO 019133	5	236628	C	T	*SDHA*	NM_004168.3	c.1346C>T	p.(Ala449Val)
	9	97864059	A	G	*FANCC*	NM_000136.2	c.1607T>C	p.(Leu536Pro)
	12	58145346	C	T	*CDK4*	NM_000075.3	c.155G>A	p.(Ser52Asn)
SAYO 019135	3	14200115	C	T	*XPC*	NM_001145769.1	c.1157G>A	p.(Arg386Gln)
	8	1.46E+08	C	T	*RECQL4*	NM_004260.3	c.2087G>A	p.(Arg696His)
SAYO 019136	1	45796909	C	T	*MUTYH*	NM_001048171.1	c.1379G>A	p.(Arg460His)
	12	1.33E+08	C	G	*POLE*	NM_006231.3	c.3140G>C	p.(Gly1047Ala)
SAYO 019137	11	1.08E+08	A	G	*ATM*	NM_000051.3	c.185+3A>G	p.?
	19	50905076	G	C	*POLD1*	NM_001256849.1	c.358G>C	p.(Gly120Arg)
SAYO 019141	12	1.33E+08	C	T	*POLE*	NM_006231.3	c.3245G>A	p.(Arg1082His)
	16	68844167	T	C	*CDH1*	NM_004360.4	c.755T>C	p.(Val252Ala)
SAYO 019142	19	11141459	G	A	*SMARCA4*	NM_001128844.1	c.3436G>A	p.(Gly1146Ser)
SAYO 019143	22	29091782	G	A	*CHEK2*	NM_001005735.1	c.1304C>T	p.(Ala435Val)
SAYO 019144	11	1.08E+08	A	G	*ATM*	NM_000051.3	c.8734A>G	p.(Arg2912Gly)
	15	91295064	A	G	*BLM*	NM_000057.3	c.847A>G	p.(Thr283Ala)
SAYO 019149	4	55161348	T	A	*PDGFRA*	NM_006206.5	c.3179T>A	p.(Ile1060Asn)
SAYO 019152	10	43622132	G	A	*RET*	NM_020630.4	c.3149G>A	p.(Arg1050Gln)
SAYO 019153	7	55227923	T	A	*EGFR*	NM_005228.4	c.1390T>A	p.(Ser464Thr)
	8	30948047	T	G	*WRN*	NM_000553.4	c.1719T>G	p.(Thr573=)
SAYO 019159	2	48028048	C	T	*MSH6*	NM_000179.2	c.2926C>T	p.(Arg976Cys)
	12	1.33E+08	G	A	*POLE*	NM_006231.3	c.3229C>T	p.(Arg1077Cys)
SAYO 19160	2	47630335	C	T	*MSH2*	NM_000251.2	c.5C>T	p.(Ala2Val)
SAYO 019162	19	50910593	G	A	*POLD1*	NM_001256849.1	c.1696G>A	p.(Glu566Lys)
SAYO 019163	19	1226569	C	T	*STK11*	NM_000455.4	c.1225C>T	p.(Arg409Trp)
SAYO 019164	2	29917860	A	G	*ALK*	NM_004304.4	c.808T>C	p.(Phe270Leu)
SAYO 019167	2	47630350	A	G	*MSH2*	NM_000251.2	c.20A>G	p.(Glu7Gly)
	2	1.28E+08	C	A	*ERCC3*	NM_000122.1	c.2207G>T	p.(Arg736Ile)
SAYO 019170	2	1.28E+08	C	T	*ERCC3*	NM_000122.1	c.2228G>A	p.(Arg743His)
SAYO 020209	3	128200785	C	T	GATA2	NM_032638.5	c.1020G>A	p.=
SAYO 020215	16	89849480	C	T	FANCA	NM_000135.2	c.1501G>A	p.(Gly501Ser)
SAYO 020204	2	48023171	C	T	MSH6	NM_000179.2	c.596C>T	p.(Pro199Leu)
	8	145736926	T	C	RECQL4	NM_004260.3	c.3515A>G	p.(Tyr1172Cys)

SAYO 20218	2	47657058	A	G	MSH2	NM_000251.2	c.1254A>G	p.(Ile418Met)
	2	232995393	A	C	DIS3L2	NM_152383.5	c.666A>C	p.(Arg222Ser)
	9	98270607	G	C	PTCH1	NM_000264.5	c.37C>G	p.(Arg13Gly)
SAYO 020202	8	145738720	C	T	RECQL4	NM_004260.3	c.2344G>A	p.(Asp782Asn)
SAYO 019180	15	91304000	G	A	BLM	NM_000057.3	c.1397G>A	p.(Gly466Glu)
	19	1207006	A	G	STK11	NM_000455.4	c.94A>G	p.(Thr32Ala)
	19	33792731	GGCGGGT	G	CEBPA	NM_004364.4	c.584_589del	p.(His195_Pro196del)
SAYO 019197	11	10818319 3	AAAAGT	A	ATM	NM_000051.3	c.5979_5983del	p.(Ser1993Argfs*23)
	16	2130208	C	T	TSC2	NM_000548.5	c.3440C>T	p.(Ser1147Phe)
	16	23646911	G	A	PALB2	NM_024675.4	c.956C>T	p.(Ser319phe)
	16	89865593	G	C	FANCA	NM_000135.2	c.874C>G	p.(His292Asp)
SAYO 019196	3	142200 32	C	G	XPC	NM_001145769.1	c.37G>C	p.(Gly13Arg)
SAYO 019193	1	45798269	T	C	MUTYH	NM_001048171.1	c.625A>G	p.(Ile209Val)
SAYO 019187	8	145738508	C	T	RECQL4	NM_004260.3	c.2477G>A	p.(Arg826Gln)

Chapter Four: *RNF43* pathogenic germline variant in a family with colorectal cancer

Clinical Genetics. 2021; 1–5.

In this report, the findings from two individuals with CRC from a single family both carrying a likely-pathogenic inherited germline splice variant in *RNF43:c.375+1G>A* are presented. This report is supported by functional evidence of the deleterious nature of this variant. These observations add further evidence to the hereditary role of *RNF43* as a tumour suppressor gene in colorectal tumorigenesis and support the inclusion of *RNF43* as gene of interest in panels for the investigation of CRC predispositions with or without polyposis.

Statement of Authorship

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Contribution to the Paper	First author and corresponding Author Conceptualization, investigation, formal analysis, funding acquisition, methodology, bioinformatics analysis, visualisation, and writing original draft		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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SHORT REPORT

RNF43 pathogenic Germline variant in a family with colorectal cancer

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Abstract

The role of *RNF43* as a cause of an inherited predisposition to colorectal cancer (CRC) is yet to be fully explored. This report presents our findings of two individuals with CRC from a single family carrying a likely-pathogenic inherited germline variant in *RNF43*. The proband (III:1) and the proband's mother (II:2) were diagnosed with mismatch repair proficient CRCs at the age of 50 years and 65 years, respectively. Both patients had *BRAF*^{V600E} mutated colon tumours, indicating that the CRCs arose in sessile serrated lesions. The germline variant *RNF43*:*c.375+1G>A* was identified in both patients. RNA studies showed that this variant resulted in an aberrantly spliced transcript, which was predicted to encode *RNF43*:p.Ala126Ilefs*50 resulting in premature termination of protein synthesis and was classified as a likely-pathogenic variant. Our report adds further evidence to the hereditary role of *RNF43* as a tumour suppressor gene in colorectal tumorigenesis and supports the inclusion of *RNF43* as a gene of interest in the investigation of CRC predispositions outside the setting of serrated polyposis.

Andrew Dubowsky and Timothy J. Price are joint senior authors

KEYWORDS
Colorectal cancer, Germline variant, RNA splicing, *RNF43*, Serrated polyposis

1 | INTRODUCTION

The serrated neoplasia pathway accounts for >25% of colorectal cancer (CRC) cases and has been associated with the activation of the Wnt-signalling pathway.[1] *RNF43* (RING-type E3 ubiquitin ligase) inhibits the Wnt pathway.[2] Somatic *RNF43* pathogenic variants are identified in ~18% of sporadic CRC[3] and have also been reported in colorectal polyps, including both adenomatous and serrated polyp subtypes.[4] Serrated polyps that have acquired *RNF43* variants independent of a CpG island methylator phenotype may progress primarily to microsatellite stable (MSS) CRC.[5] In addition, in familial cases, germline *RNF43* variants have been reported in mismatch repair (MMR) proficient-CRC; mainly in *BRAF* mutant/microsatellite stable CRC, where second-hit *RNF43* inactivation may precede the *BRAF* mutational event.[5] Although *RNF43* is a candidate gene for serrated polyposis syndrome (SPS), germline variants have only rarely been reported.[4–7] Experts have concluded that additional supporting segregation evidence is required to confirm the role of *RNF43* in a dominantly inherited form of SPS, and recommend exercising caution when interpreting *RNF43* results.[6,7] We present our findings of two related individuals with CRC carrying a likely-pathogenic germline variant in *RNF43* from one family recruited to the South Australian Young Onset (SAYO) CRC Registry.

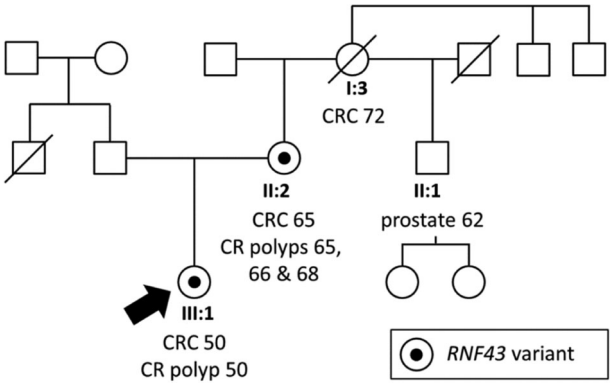

FIGURE 1 Pedigree of the family with colorectal cancer

2 | METHODS

All of the methods in this report are described in detail in the Data supplement.

3 | RESULTS

An extended results section is available in the data supplement.

The family pedigree is shown in Figure 1. The proband (III:1) and her mother II:2 were diagnosed with CRC at the age of 50 years and 65 years, respectively (data supplement).

3.1 | *RNF43:c.375+1G>A* Germline variant

The whole exome sequencing data of III:1 was analysed and a canonical splice site variant *RNF43:c.375+1G>A* was identified in the proband. This variant was subsequently confirmed in II:2 (Figure 2). Sequencing of cDNA identified a single transcript, not observed in

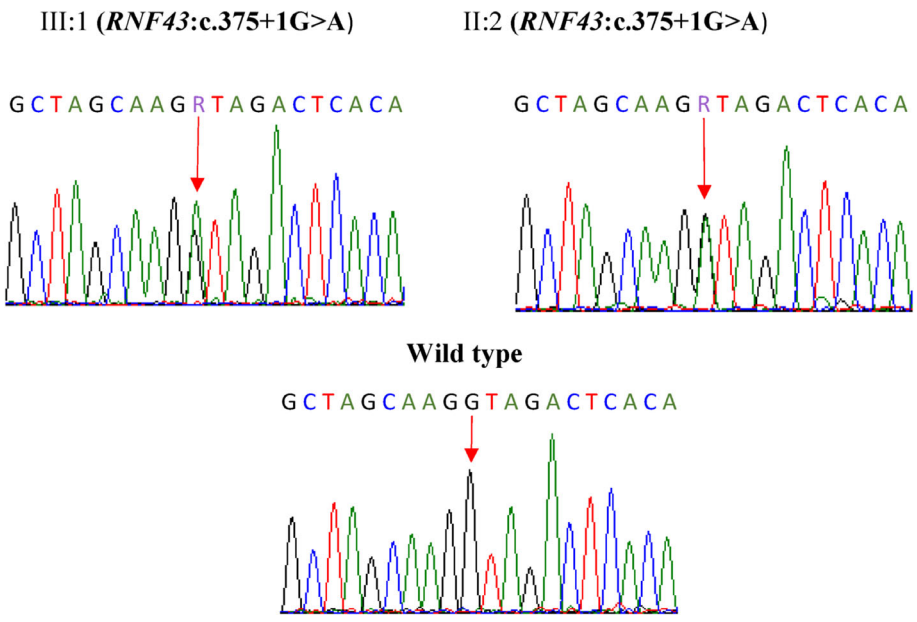

FIGURE 2 Electropherogram of the identified *RNF43:c.375+1G>A variant* [Colour figure can be viewed at wileyonlinelibrary.com]

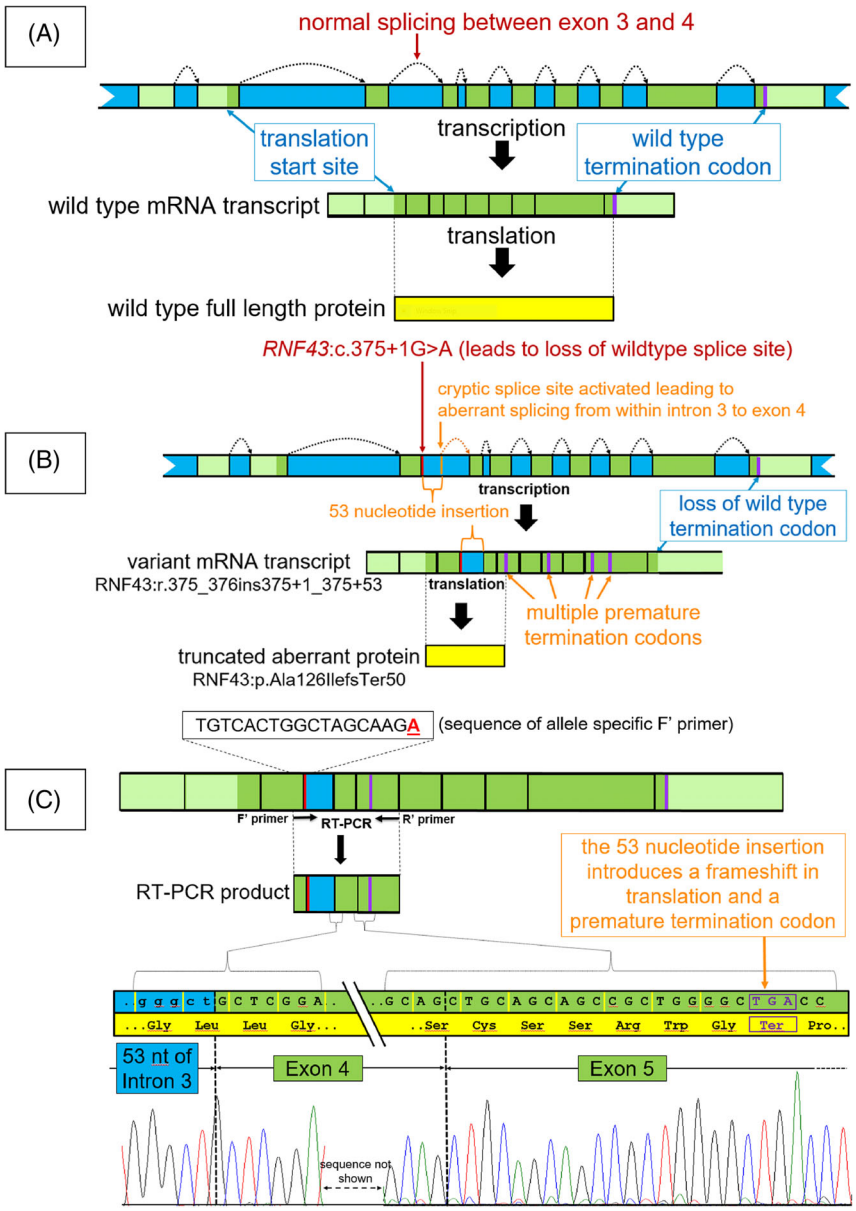

FIGURE 3 Splicing study of *RNF43: c.375+1G>A*. (A) Normal splicing of the wild type *RNF43* allele. (B) Abnormal splicing of the *RNF43:c.375+1G>A* variant allele. The new splice site results in the insertion of 53 nucleotides of intron sequence encoding 17 amino acids and altered downstream reading frame encoding multiple premature termination codons. (C) *RNF43* variant allele-specific RT-PCR amplification and sequencing. Dashed black arrow = normal splicing; dashed orange arrow = aberrant splicing; blue box = intron; green box = exon (non-coding 5′ & 3′ UTR light green); yellow box = translated protein [Colour figure can be viewed at wileyonlinelibrary.com]

control samples, containing an insertion of 53 nucleotides from intron 3, spliced to the first nucleotide of exon 4. This product results from preferential activation of a cryptic splice donor site beginning at position *RNF43:c.375+54* effected by the *RNF43:c.375+1G>A* sequence change. The aberrant spliced transcript (*RNF43*: NC_000017.10[NM_017763.5]: r.375_376ins375+1_375+53) is predicted to encode *RNF43*:p.Ala126Ilefs*50 and result in premature termination of protein synthesis (Figure 3, Figures S1–S3). *RNF43*:c.375+1G>A was therefore classified as a likely-pathogenic variant.

3.2 | Tumour analysis

The tumour samples were observed to be heterogeneous, with an estimate of tumour cells to be 50% and 60% for III:1 and II:2, respectively. The whole *RNF43* gene sequencing detected the *RNF43:c.375+1G>A* variant in both samples. In the tumour of II:2, a pathogenic somatic heterozygous variant c.433C>T; p. (Arg145*) in *RNF43* was detected at the allele frequency corresponding to the heterogeneous nature of the tumour sample (data supplement) and was absent from germline DNA (data not shown). Both variant carriers had $BRAF^{V600E}$ mutated colon tumours that were mismatch repair proficient (Table S1).

4 | DISCUSSION

A probable role for *RNF43* in human gastrointestinal malignancy has been reported and several studies have associated pathogenic germline variants in this gene with SPS.[5–7] However, *RNF43* is yet to be consistently included in multigene panel testing for evaluation of hereditary CRC, polyposis, or other hereditary cancer syndromes.[8] In this study, the likely-pathogenic germline splice site variant (*RNF43:*

c.375+1G>A) was identified in a mother and daughter, both with CRC. Importantly, the proband was aged 50 years at diagnosis (not defined as young-onset CRC) and did not demonstrate polyposis. The absence of both of these features would not have triggered genetic testing, under normal clinical circumstances. In addition to the *RNF43*:*c.375+1G>A* germline variant, a somatic pathogenic variant *RNF43*:*c.433C>T* was detected in the mother's tumour, supporting *RNF43* as the primary driver. We could not determine the phase of the somatic and germline variants. Should they be *in trans*, this could cause a biallelic null effect and no functional protein. We did not identify a second hit in *RNF43* in the proband, but speculate loss of heterozygosity (LOH) involving the wild type *RNF43* allele not detectable by the testing available to us. Both carriers had *BRAF*-mutated colorectal tumours indicating origin in a serrated polyp, and both tumours were MMR-proficient. This study adds to the reported number of patients with serrated polyps and a germline variant in *RNF43*, now totalling 15 patients from eight families. Our findings add further weight to the potential hereditary role of *RNF43* in colorectal tumorigenesis.

Membrane E3 ligase RNF43 and its functional homologue, ZNRF3, under normal circumstances inhibit the Wnt pathway by ubiquitination and lysosomal degradation of Wnt receptors of the Frizzled family. Another alternative mechanism by which *RNF43* negatively regulates the Wnt signalling pathway is by interacting with T cell factor 4 (TCF4) and silencing TCF4-mediated transcription.[9] Recently, Spit et al. identified a class of *RNF43* truncating variants that promote β-catenin-mediated transcription, despite exhibiting normal Wnt receptor downregulation. These variants were found to bind to Casein kinase 1 (CK1) and prevent β-catenin turnover, and consequently induce the transcriptional activation of Wnt target genes.[10] The Wnt pathway is a key component of the intestinal stem cell niche and its activation by *RNF43* mutations might be enough to induce hyperproliferation of intestinal crypts and polyp formation.[11] Deletion of *RNF43* and *ZNRF3*, which are highly expressed in murine intestinal stem cells, has been found to induce intestinal epithelial hyperproliferation resulting in intestinal adenoma.[12]

The findings of studies have shown that germline variants in *RNF43* are rare in patients with SPS.[5–7] Yan et al. identified six individuals from one family carrying a likely-pathogenic germline variant in *RNF43*, five of whom met the WHO diagnostic criteria for SPS and one was diagnosed with young-onset CRC with no polyposis.[5,6] They also showed a second-hit inactivation by somatic *RNF43* variants or LOH in all germline variant carriers.[5] Buchanan et al. screened 74 individuals with SPS and detected two rare germline missense variants [(c.443C>G (p.Ala148Gly) and c.640C>G (p.Leu214Val)], which have been reported to diminish the inhibitory effect of *RNF3* on Wnt signalling.[6] Another study screened 96 unrelated patients with SPS and reported the germline variant c.394C > T (p.Arg132*) in one patient with CRC and SPS.[7] In addition, van Herwaarden et al. recently reported no germline variant in *RNF43* in 26 SPS patients. Of the 15 germline variant carriers reported so far, 14 developed serrated polyps and/or CRC (mean age at diagnosis: 44 years; range: 18–65 years). In addition, 24 out of 25 colonic tumours analysed showed *RNF43* somatic loss or mutation supporting the potential hereditary role of this gene in colorectal tumorigenesis.[7] The most common forms of second hit inactivation are allele loss and somatic variant of a coding sequence. A second hit in the tumour of III:1 may have not been detected due to the failure to amplify the tumour DNA which was extracted from a tissue sample containing normal and malignant tissues or the somatic variant might be located in a critical non-coding region that was not assessed by exome sequencing. It is also possible that haploinsufficiency alone may have determined the phenotype. Overall, the findings suggest the need for identifying additional hereditary risk factors for patients with SPS because *RNF43* germline variants have only rarely been reported. Pathogenic somatic variants of *RNF43* in CRC have been widely associated with *BRAF* mutations in MSI-high tumours, signet-ring cell carcinoma and are mutually exclusive to *APC* pathogenic variants.[5,13–15] Giannakis et al. and others[16] suggested that *RNF43* is less likely to be a driver of carcinogenesis, when frameshift mutations occur at high frequency due to a defective MMR mechanism.[3] However, pathogenic somatic *RNF43* variants have been reported in MMR proficient-CRC; mainly in *BRAF* mutant /microsatellite stable CRC and consistent with our findings, all the germline *RNF43* variant carriers reported by other studies were MSS.[5,7,13,17]

The lack of a genetic aetiology renders SPS one of the most poorly understood and under-recognised of the colorectal polyposes,[18,19] despite being the most common. Taken together with other reports,[5,7,17,20] our findings show that pathogenic truncating variants in *RNF43* may underlie a serrated polyp phenotype in families.[17,21] A limitation of the SPS clinical criteria is the phenotypic variability contained within them.[22] Implicit in the criteria as currently proposed, is a spectrum of disease which can range from a patient with five serrated polyps proximal to the rectum through to patients with hundreds of serrated polyps of any size throughout the colorectum. Cases of oligo-polyposis which do not fit into any known syndromic group are relatively common, particularly involving the co-occurrence of serrated and adenomatous polyps. Conventional adenomas which co-occur with sessile serrated polyps have been found to have specific features which may render them more likely to undergo malignant transformation.[23] The presence of adenomas has been associated with an increased risk of CRC in SPS,[24] and there is evidence that at least half of the CRC in this condition arises from these pre-malignant lesions rather than from sessile serrated lesions.[21] One patient described elsewhere[5] had a CRC arising in an adenoma. Hence, the phenotype of individuals carrying germline variants in *RNF43* may be wider than that described in the current arbitrary clinical criteria for recognition of SPS.

In summary, we have identified a likely-pathogenic germline splice site variant in *RNF43* in two patients from a single family with CRC, the proband showed no polyposis indicating that colonic phenotype does not tightly predict genotype. Tumours from both carriers were $BRAF^{V600E}$-mutated and MMR-proficient. Our report lends weight to further consideration for a hereditary role for *RNF43* as a tumour suppressor gene in colorectal tumorigenesis.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Reger R. Mikaeel Conceptualization, Investigation, Formal analysis, Funding acquisition, Methodology, Bioinformatics analysis, Visualisation, and Writing original draft. Timothy J. Price, Joanne P. Young, Eric Smith Supervision, Conceptualization, Funding acquisition, Writing-review and editing. Andrew Dubowsky Conceptualization, Bioinformatics analysis, Supervision, Methodology, Validation and Writing-review and editing. Yun Li, Nicola K. Poplawski, Yoko Tomita, Amanda R. Townsend, Silke Kaulfuß, Gökhan Yigit, Bernd Wollnik Validation, and Writing-review and editing. Jinghua Feng, Arne Zibat Bioinformatics analysis, Writing-review and editing. Mehgan Horsnell, Wendy Uylaki Software, Writing-review and editing. Christian Müller Methodology, Writing-review and editing. Hamish Scott, Lesley Rawlings, Denae Henry, Cassandra Vakulin Validation, tumour testing, Writing-review and editing.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/cge.14064.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request and subject to ethical approval from the ethics committee that aproved the original study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Data Supplementary (S)

Methods

Family Data

The proband (III:1) was recruited to the SAYO CRC Registry, as previously reported[1] and gave informed consent in writing. Further investigations were conducted clinically on the proband's mother (II:2). The study was conducted under ethics approval HREC/14/TQEHLMH/194 (The Queen Elizabeth Hospital, CALHN Office for Research, Adelaide, South Australia). Blood was sampled from both patients and whole exome sequencing (WES) of leucocyte DNA was performed for the proband as previously reported.[1] Subsequent sanger sequencing was performed on II:2. Medical records were audited for tumour-based testing results (MMR deficiency (dMMR) and somatic mutation testing). Next generation sequencing of *RNF43* and allelic loss studies in tumour DNA from both patients were performed to identify second hit mutations.

Whole Exome Sequencing Data Filtration and Interpretation

The WES data of III:1 underwent a comprehensive analysis of 133 genes that have been associated with cancer/CRC predisposition.[2-4] Data filtration and interpretation were carried out using the "Varvis" exome analysis pipeline. The data were filtered for high-quality (coverage of >6 reads, a minimum quality score of 10, the alternative allele frequency of ≥25%), and rare variants (minor allele frequency (MAF) <1%) variants. The MAFs were taken from public databases (GnomAD, dbSNP) and an in-house database [5]. The American College of Medical Genetics and Genomics guidelines (ACMG) [6] were used to evaluate sequence variants. Pathogenicity of putative germline variants were evaluated using classifications from public databases (ClinGen, ClinVar, InSiGHT, PubMed, OMIM, and HGMD). In silico prediction tools [SIFT and Provean (http://provean.jcvi.org/genome_submit_2.php?species=human), CADD (https://cadd.gs.wash-ington.edu/), PolyPhene-2 (http://genetics.bwh.harvard.edu/pph2/), and Mutation Taster (http://mutationtaster.org/)] were used for predicting the sequence variants for their protein deleteriousness potential. Pfam server (http://pfam.xfam.org/) was used to analyse protein sequences and identify protein domains. NetGene 2 (http://www.cbs.dtu.dk/services/NetGene2/), Alternative Splice Site Predictor (http://wangcomputing.com/assp/index.html), and Fruit Fly Splice Predictor (https://www.fruitfly.org/seq_tools/splice.html) servers were used for predicting the potential

splice site alterations. The Integrative Genomic Viewer (IGV) tool (IGV2.8.10) was utilized for the visualisation of sequence data and variants calls and Sanger sequencing was performed to confirm potentially damaging variants. Mutalyzer software (https://mutalyzer.nl/) was used to check the variant nomenclature.

Splicing Study of *RNF43*:c.375+1G>A

For cDNA analysis of the *RNF43* transcript, RNA was extracted from blood and mRNA was transcribed into cDNA by performing reverse transcriptase PCR and using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Exon and intron specific PCR primers were employed to investigate the *RNF43*:c.375+1G>A variant by PCR amplification of the relevant *RNF43* transcript region with the purpose to generate a product which a) span the wild-type normal splice boundaries of the *RNF43* exons which include exon 2 ending at coding nucleotide c.252, exon 3 spanning coding nucleotides c.253 to c.375, exon 4 spanning coding nucleotides c.376 to c.450, and exon 5 beginning at coding nucleotide c.451 and b) identify abnormal splicing which may occur within the amplified region including the use of allele-specific PCR primers targeted to *RNF43*:c.375+1G>A, and intronic primers downstream of exon 3. Primer sequences are available on request. PCR products were analyzed using bi-directional Sanger sequencing.

Mismatch Repair Deficiency Testing and Somatic Variants

Medical records were audited for tumour-based testing results. Mismatch repair deficiency (dMMR) was determined by immunohistochemical analysis [7]. MLH1 methylation analysis was performed for tumours exhibiting a loss of MLH1 or PMS2 staining. Somatic mutation testing was performed on DNA isolated from paraffin-embedded tumour tissue. Previously reported clinically significant variants in the *BRAF*, *EGFR*, *KIT*, *KRAS*, and *NRAS* genes were screened using a commercial panel (OncoFOCUS™) [8]. Next-generation sequencing of RNF43 was performed using a custom IDT design (GMPfocus v2) sequenced on the Illumina NextSeq Sequencing System. Sequences were aligned to the human reference genome (hg19) using Burrows-Wheeler Aligner (BWA-mem). Variant calling was performed using the Genome Analysis Toolkit (GATK). GATK and Freebayes were used to analyse tumour samples. Variant annotation was performed using VariantGrid v2 and Alamut v2.11.

Extended results

Clinical Data

The proband (III:1) was diagnosed with a splenic flexure CRC, tubular adenoma (7mm; low-grade dysplasia), and ovarian mass at the age of 50 years. Histology confirmed a mucinous adenocarcinoma of the colon (120 x 85 x 30 mm) and a benign ovarian serous cystadeno-fibroma. II:2 was subsequently diagnosed with high grade, mucinous adenocarcinoma of the colon at the age of 65 years; and 7 polyps [2 hyperplastic (7 mm in sigmoid colon; 11 mm in ascending colon), 3 sessile serrated polyps (10 mm in sigmoid colon; 13 mm in ascending colon; unknown size in sigmoid colon); 2 tubular adenomas (30 mm with high-grade dysplasia in ascending colon; 15 mm with low-grade dysplasia in descending colon)]. The proband's maternal grandmother (I:3) was diagnosed with papillary invasive rectal adenocarcinoma of the colon at 72 years of age, but the polyp status was unknown. The proband's maternal half-uncle (II:1) was diagnosed with prostate cancer at the age of 62 years.

RNF43:c.375+1G>A Germline Variant

The WES data of III:1 was analyzed for 26 genes [9] which have recently been recommended by the Collaborative Group of the Americas for Inherited Gastrointestinal Cancer to be used for evaluation of hereditary CRC [2]. After applying a variant prioritization strategy to the WES data, no pathogenic/likely-pathogenic variant was identified. The data were screened using a further 107 genes present in commercial multigene panels [10,11] and/or have been reported in the literature to be associated with hereditary CRC or cancer [3,4,9,12]. Variant *RNF43*:c.375+1G>A was identified in the proband. This variant consists of a G>A nucleotide substitution at the +1 position of intron 3 of *RNF43*. In silico splice analysis tools predicted this variant to disrupt mRNA splicing and expected to result in an absent or disrupted protein product. The CADD score for this variant is 34 predicting its high potential for deleterious effects on the resulting protein. Exon 3 was present in the biologically relevant transcripts. ClinVar has no entries for this variant. This variant is neither found in population databases (GnomAD) nor in the Leiden Open Variation Database. This variant was confirmed in III:1 and II:2 by Sanger Sequencing (**Figure 2**).

Agarose gel electrophoresis demonstrated a PCR product from both wild-type control, III:1 and II:2 patient samples consistent in size with a transcript resulting from normal splicing. Additionally, in the III:1 and II:2 patient samples, a weakly amplified larger product was also observed.

DNA sequencing of the amplified products from the patient sample confirmed the presence of wild-type transcript (*RNF43*:r=) produced by normal splicing from the wild-type allele (*RNF43*:c.=). Notable, however, a weakly amplified product of the same size, but just above the limit of detection (<10% total mRNA), was also observed corresponding to the allele carrying *RNF43*:c.375+1G>A (**Figure S1**).

Next, we investigated if the weakly amplified *RNF43*:c.375+1G>A product was the consequence of allele-specific nonsense-mediated decay, and/or due to limitations of the assay to detect longer transcripts resulting from PCR bias in favour of shorter products. Allele-specific-PCR identified a single transcript, not observed in control samples, containing an insertion of 53 nucleotides from intron 3, spliced to the first nucleotide of exon 4. This product almost certainly results from preferential activation of a cryptic splice donor site beginning at position *RNF43*:c.375+54 effected by the *RNF43*:c.375+1G>A sequence change; the transcript is therefore described as NC_000017.10(NM_017763.5):r.375_376ins375+1_375+53, and predicted to encode RNF43:p.Ala126Ilefs*50 (**Figure 3, Figure S2**).

Bias amplification by allele-specific PCR of this shorter product precluded the detection of longer transcripts. However, downstream stepwise positioning of PCR primers within intron 3 enabled detection of an additional separate transcript containing an intronic insertion of greater than 226 nucleotides. While limitations of our assay prevent the precise characterisation of the cryptic splice donor site expected for this longer transcript, the resulting transcript may be described using position interval uncertainty, as NC_000017.10(NM_017763.5):r.375_376ins375+1_(375+226_376-1). This transcript is predicted to encode an aberrant protein (RNF43:p.Ala126Ilefs*50). Thus, independent of the exact position of the cryptic donor site activated downstream of position 375+226, a premature termination codon is encoded within the characterised region of the retained intron 3 sequence (226 nucleotides) (**Figure 3, Figure S3**).

These results demonstrate *RNF43*:c.375+1G>A effects almost complete loss of the normal wild type transcript from this allele in this family. It must be noted that the longer aberrant transcript (NC_000017.10(NM_017763.5):r.375_376ins375+1_(375+226_376-1) is also observed in the wild type allele (and normal control) (**Figure 3, Figure S3**). However, our findings indicate that this product is weakly expressed, comprising less than 5% of the total *RNF43* transcript. Therefore, *RNF43*:c.375+1G>A is responsible for abrogating normal splicing while concomitantly favouring at least one downstream cryptic splice donor site effecting abrogation

of normal splicing on the allele in which it resides. That normal splicing was absent and the resulting transcripts effect a frameshift insertion, identified *RNF43*:c.375+1G>A as equivalent to a constitutional germline variant in which a premature termination codon is encoded (**Figure 3**). According to the ACMG guidelines[6], *RNF43:c.375+1G>A* was therefore classified as a likely-pathogenic variant (PVS1_Moderate, PM2, PS3, PP4).

Figure S

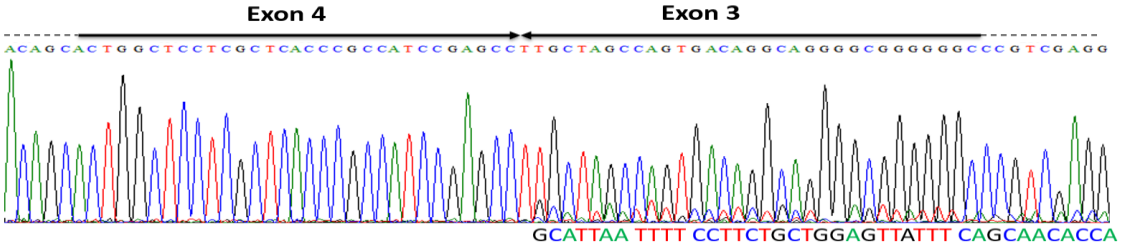

Figure S1. DNA sequence analysis of the amplified products from the patient sample confirmed the presence of wild-type transcript from the wild-type allele (*RNF43*:c.=) and a weakly amplified product from allele carrying *RNF43*:c.375+1G>A.

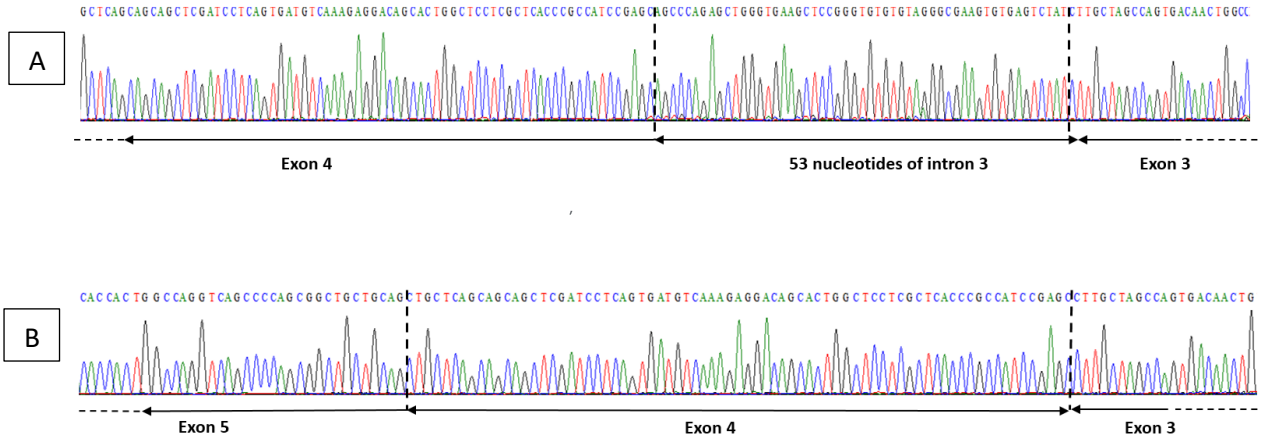

Figure S2. *RNF43* variant (A) and wild-type (B) allele-specific RT-PCR amplification and sequencing.

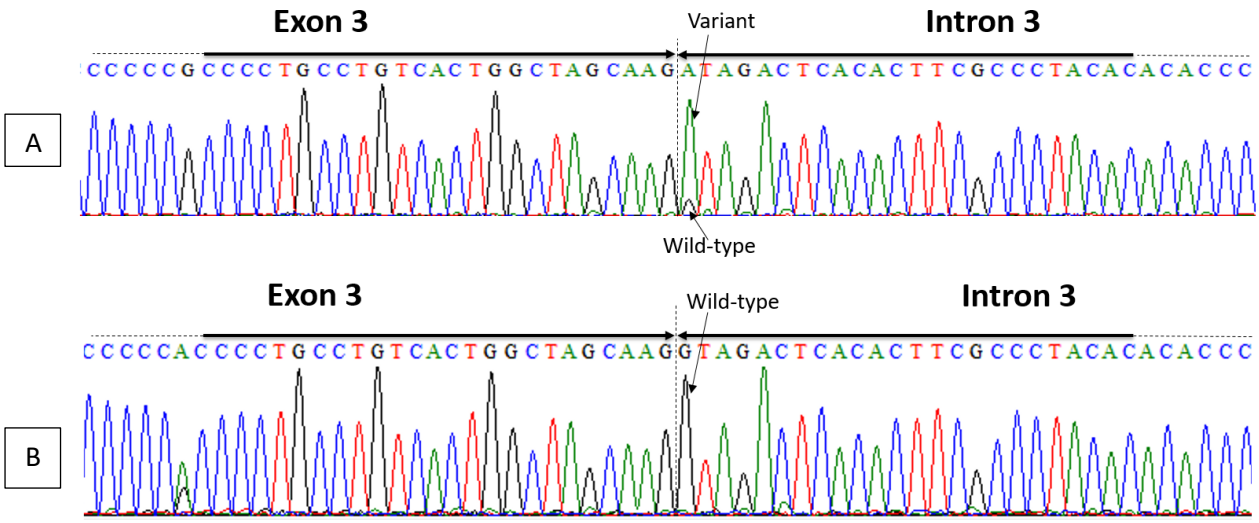

Figure S3. Downstream stepwise positioning of PCR primers within intron 3 in the patient (A) and the control (B) showing detection of an additional separate transcript containing an intronic insertion of greater than 226 nucleotides.

Table S1. Mismatch Repair Deficiency Testing and Identified Variants in Tumour.

Patient	Tissue	*BRAF* ^{V600E}	IHC	*RNF43**	*Allele fraction*	*Tumour Purity*
III:1	CRC	mutated	MMR proficient	c.375+1G>A	61%	50%
II:2	CRC	mutated	MMR proficient	c.375+1G>A c.433C>T; p.(Arg145*)	50% 25%	60%

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Chapter Five: Appendiceal neoplasm incidence and mortality rates are on the rise in Australia

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Expert Review of Gastroenterology & Hepatology. 2021, VOL. 15, NO. 2, 203-210

This publication shows a retrospective analysis performed on national data obtained from the Australian Institute of Health and Welfare from 1982 to 2013 to examine the incidence and mortality rates of appendiceal neoplasms in Australia. The novel aspect of this manuscript is that it shows there is a significant rise in the incidence and mortality rates of appendiceal neoplasms across both genders and age groups (<50 years and ≥50 years) in Australia. It also demonstrates that there are significant differences in incidence rates between gender and age groups, and in mortality rates between age groups. This apparent rise in the incidence of appendiceal neoplasms in Australia might in part be due to the increasing use of CT scanning, improvements in pathological assessment of the appendix, and the growing aging population.

Statement of Authorship

Title of Paper	Appendiceal neoplasm incidence and mortality rates are on the rise in Australia.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
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Contribution to the Paper	First author and corresponding Author Study concept and design, data collection and interpretation, statistical analysis, wrote the manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	16/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	20/10/2021

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Signature		Date	18/10/2021

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ORIGINAL RESEARCH

Appendiceal neoplasm incidence and mortality rates are on the rise in Australia

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ABSTRACT

Objectives: The study aimed to examine the incidence and mortality rates of appendiceal neoplasms (ANs) in Australia.

Methods: A retrospective analysis was performed on national data obtained from the Australian Institute of Health and Welfare (AIHW) from 1982 to 2013. Changes to the incidence, and the cancer-specific mortality following the diagnosis of ANs were analyzed over this time period, with stratification performed for histological subtype, gender, and age groups (<50y and ≥50y).

Results: Incidence and mortality rates of ANs increased significantly across both genders and age groups. Incidence rates increased by 415%, from 0.40/100 000 population in 1982 to 2.06/100 000 in 2013. Overall mortality rates increased by 130%, from 0.057/100 000 during 1982–1985 to 0.131/100 000 during 2010–2013. Controlling for age group and gender, the incidence rates increased by 20% every four years (Incidence rate ratio (IRR) = 1.20, 95% confidence interval (CI): 1.17, 1.23, global P value<0.0001), and controlling for age, the mortality rates increased by 8% every four years (IRR = 1.08, 95% CI: 1.00, 1.17, global P-value = 0.0401).

Conclusion: The increasing use of CT scanning, improvements in pathological assessment of the appendix, and the growing aging population may have contributed in part to the apparent rise in the incidence of ANs.

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Appendiceal neoplasms; incidence rates; mortality rates; risk factors

1. Background

Primary appendiceal neoplasms (ANs) are a rare group of tumors consisting of multiple types. They are usually aggressive and are associated with significant mortality due to late diagnosis and a lack of standard systemic treatment options [1]. ANs are often an incidental finding seen on the appendectomy surgical specimen which is mainly performed following a clinical diagnosis of appendicitis. The fact that most diagnoses are incidental may relate to the low incidence of these neoplasms and the poor understanding of their risk factors.

There is accumulating evidence that the incidence of ANs is increasing. A population-based study conducted by the Surveillance, Epidemiology and End Results (SEER) program determined that over the period from 1973 to 1998, the incidence of ANs was 0.12 cases per 100 000 population per year in the United States of America (USA), accounting for less than 1% of all tumors [2]. Subsequently, Gustafsson et al. reported that the incidence of the appendiceal adenocarcinoma subtype increased 2.6-fold from 1973 to 2004 in the USA, while the incidence of appendiceal neuroendocrine tumors remained stable [3]. In 2015, a retrospective cohort analysis by SEER reported a significant increase in the overall incidence of ANs by 54%, from 2000 to 2009 [4]. Siegel et al. reported that the incidence rates of ANs in the USA rose by 9–10% per year in people aged ≥50 years and increased by 24% per year in people <50 years of age, from 2012 to 2016 [5]. A population-based study in Netherlands showed an increasing trend in the incidence of appendiceal mucinous adenocarcinoma in males and females. The age-standardized rate increased from 0.4/100 000 during 1982–1989 to 1.0/100 000 during 2000–2010 in males and rose from 0.6 to1.9/100 000 in females [6]. The causes of these reported increases in incidence are currently unknown, and it has not yet been established whether the mortality rate of ANs is following a similar trend of incidence rate. In addition, there are limited studies on the incidence rate changes with time in other westernized countries with similar lifestyle to the USA and the Netherlands.

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Supplemental data for this article can be accessed here.

To the best of our knowledge, there has been no report describing trends in incidence and mortality rates of ANs in Australia. This report uses national data from the Australian Institute of Health and Welfare (AIHW), which covers the entire population of Australia, to examine the incidence and mortality rates of ANs from 1982 to 2013.

2. Patients and methods

The AIHW is an independent statutory agency that receives data of all cancers (except non-melanotic skin cancers) from all Australian states and territories and produces authoritative and accessible national reports on cancers. Incidence and mortality of appendiceal cancer from 1982 to 2013 by sex and 5 years age groups were obtained from the AIHW. ANs were identified in the database by ICD-10 code C18.1 and ICD-9 code 153.5 (for mortality data from 1982 to 1996). Appendiceal neoplasm cases were classified according to codes from the fifth edition of the WHO classification system (2019) (Table 1) as: appendiceal neuroendocrine neoplasms [WHO (2019) codes: 8240/3, 8249/3, 8152/3, 8241/3, 8246/3, 8013/3, 8041/3, 8154/3], appendiceal adenocarcinoma [WHO (2019) codes: 8140/3, 8480/3, 8490/3, 8020/3], goblet cell adenocarcinoma [WHO (2019) code: 8243/3] and other rare types such as (haemangiosarcoma and leiomyosarcoma) [7]. Age at diagnosis was categorized into two groups, less than 50 years of age (<50y) and 50 years of age or older (≥50y) to allow the data set to be relevant against colorectal cancer screening ages that start at 50y in Australia.

Table 1. Classification of epithelial appendiceal neoplasms by WHO (2019) [7].

Type	Definition	Subtype
Appendiceal serrated lesions and polyps	Mucosal epithelial polyps characterized by a serrated (sawtooth or stellate) architecture of the crypt lumen).	None
Appendiceal mucinous neoplasms	Mucinous neoplasms characterized by mucinous epithelial proliferation with extracellular mucin and pushing tumor margins.	None
Appendiceal adenocarcinoma	Malignant glandular neoplasms characterized by invasion.	A-signet-ring cell adenocarcinoma, B-mucinous adenocarcinoma, C-carcinoma, undifferentiated, not otherwise specified (NOS).
Appendiceal goblet cell adenocarcinoma	These are amphicrine tumors composed of goblet-like mucinous cells, as well as variable numbers of endocrine cells and paneth-like cells, typically arranged as tubules resembling intestinal crypts.	None
Appendiceal neuroendocrine neoplasms	Neoplasms with neuroendocrine differentiation	A- neuroendocrine tumors (NETs), B-neuroendocrine carcinomas (NECs)

Overall incidence rates of ANs were calculated per 100 000 population for each year of our study period. Incidence rates of ANs by age group, gender, and histological subtype were calculated per 100 000 people for each four-year interval periods, starting in 1982 and until 2013. Overall mortality rates of ANs as well as mortality rates by age group, gender, and histological subtypes were calculated per 100 000 individuals for each four years of the study period. Data on Australia's population as well as its stratification by gender and age during the audited study period was obtained from the Australia Bureau of Statistics [8].

Negative binomial models were performed for AN incidence, with the outcome being number of cases, the predictors being one year or 4-year periods, age group and gender, and the offset being the natural logarithm of the population. Similar models were performed for number of adenocarcinoma cases, number of neuroendocrine neoplasm cases, number of goblet cell adenocarcinoma cases, and number of AN deaths (AN mortality rate). In this analysis, interaction models were performed between 4-year periods and gender, and then 4-year periods and age groups, for the outcomes rate of ANs and rate of ANs mortality. The purpose of these interaction models was to investigate whether, for example the association between rate of ANs and 4-year periods was significantly different across genders (or across age groups). If the interaction was not significant then the interaction term was removed and a main effects model was reported. All statistical tests were conducted using the statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Incidence rates of ANs in Australia (1982–2013)

From 1982 to 2013, there were 5 471 ANs reported in Australia; of those, 3 262 (60%) were females and 2 924 (53%) were <50 y of age. Appendiceal neuroendocrine neoplasms were the most frequently diagnosed tumors accounting for 56.5% (3 090/5 471) of the cases. Appendiceal adenocarcinoma and goblet cell adenocarcinoma accounted for 36.0% (1 969/5 471) and 7.0% (382/5 471) of the cases, respectively. Other rarer cases including haemangiosarcoma, leiomyosarcoma, and not otherwise specified (NOS) only accounted for 0.1% (5/5 471) of the cases, and 0.4% (25/5 471) were classified as neoplasm/malignant by the AIHW. Appendiceal serrated lesions and appendiceal mucinous neoplasms were not reported among the appendiceal neoplasm cases from the AIHW because these neoplasms were considered as benign tumors during the audited period.

Incidence rates of ANs increased by 415% from 0.40/100 000 population in 1982 to 2.06/100 000 in 2013 (Figure 1(a)). When controlling for age group and gender, for every increase in one year, the rate of ANs increased by 20% (IRR = 1.20, 95% confidence interval (CI): 1.17, 1.23, global P value<0.0001). Controlling for year and gender, the ≥50y age group had a rate of ANs 2.12 times that of the <50y age group (IRR = 2.12, 95% CI: 1.89, 2.39, global P value<0.0001) (Table 2). The incidence rate increased during the time period by 286% in those <50y from 0.35 to 1.35/100 000 and rose by

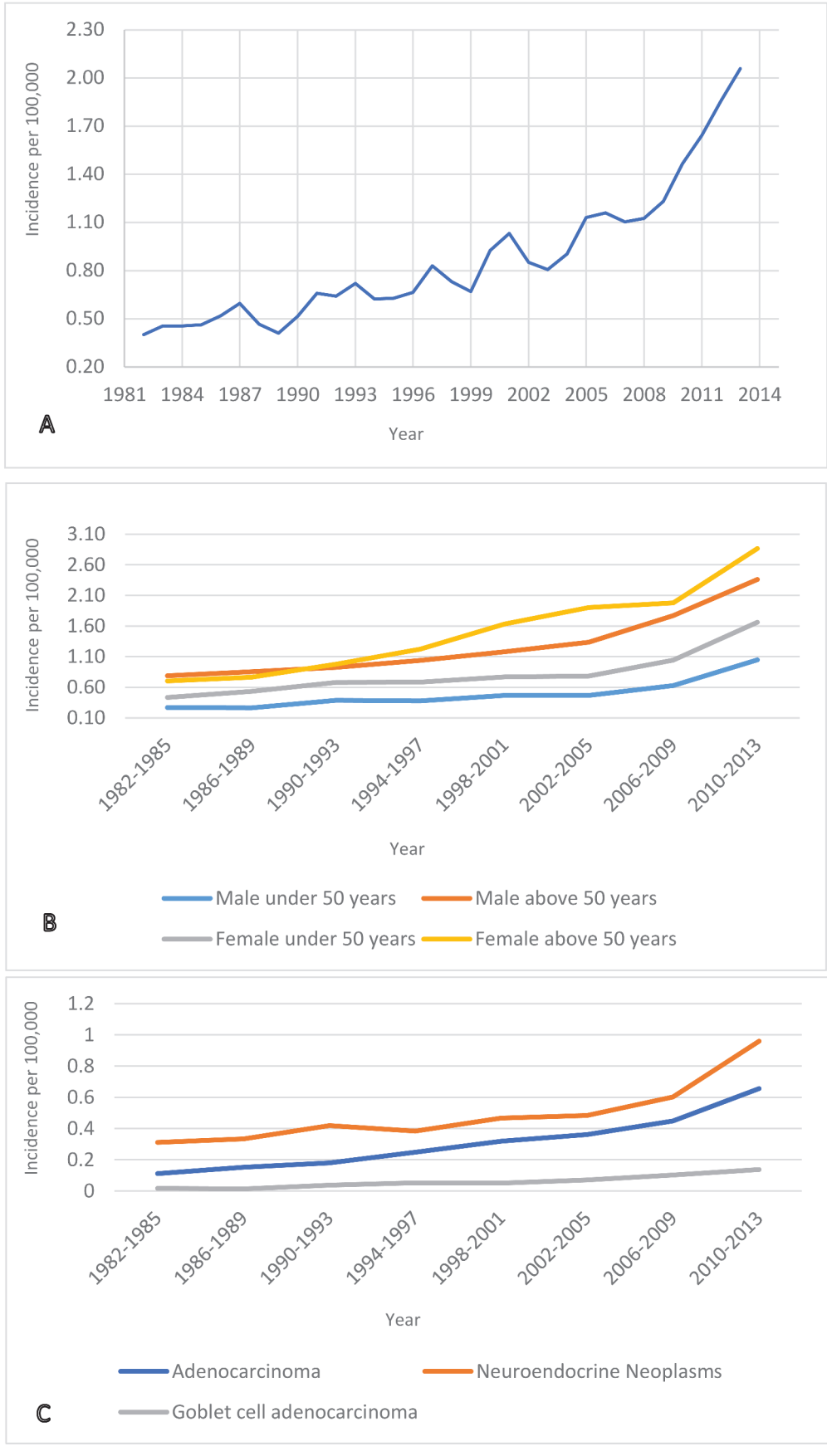

Figure 1. Incidence rates of appendiceal neoplasms (ANs) per 100,000 population in Australia from 1982 to 2013. (a) Incidence rates of ANs from 1982 to 2013. (b) Incidence rates of ANs from 1982 to 2013 in males and females under and above 50 years of age. (c) Incidence rates of three histological subtypes of ANs from 1982 to 2013.

Table 2. Negative binomial regression results.

Outcome	Predictor/Interaction	Comparison	IRR (95% CI)*	*P*-value
Number of AN cases	Age	≥50 vs <50	2.12 (1.89, 2.39)	<0.0001
	Year periods		1.20 (1.17, 1.23)	<0.0001
	Gender	F vs M	1.43 (1.27, 1.60)	<0.0001
Number of AN cases	Year periods*Age groups			0.7811
Number of AN cases	Year periods*Gender			0.7758
Number of AN cases	Age groups*Gender			0.2101
Number of Adenocarcinoma cases	Year period		1.27 (1.21, 1.34)	<0.0001
Number of Neuroendocrine Neoplasm cases	Year period		1.16 (1.11, 1.21)	<0.0001
Number of Goblet cell adenocarcinoma cases	Year period		1.35 (1.21, 1.49)	<0.0001
Number of AN deaths	Age	≥50 vs <50	10.38 (6.81, 15.82)	<0.0001
	Year period		1.08 (1.00, 1.17)	0.0401
Number of AN deaths	Gender	F vs M	1.35 (0.96, 1.89)	0.0808
	Year period		1.12 (1.04, 1.21)	0.0035
Number of AN deaths	Year period*Age groups			0.1479
Number of AN deaths	Year period*Gender			0.8397

*Incidence Rate Ratio (95% Confidence Interval). Offset is natural logarithm of population. AN, appendiceal neoplasm; F, female; M, male; IRR, incidence rate ratio.

255% in those ≥50y from 0.74 to 2.63/100 000 (Supplementary Figure 1(a)). However, there was not a statistically significant interaction between 4-year periods and age group (interaction P value = 0.7811) (Table 2).

Controlling for year and age group, females had a rate of ANs 43% more than males (IRR = 1.43, 95% CI: 1.27, 1.60, global P value<0.0001) (Table 2). The incidence of ANs in men increased by 282% from 0.38/100 000 to 1.45/100 000; and in women, the incidence increased by 312% from 0.50 to 2.06/100 000 (Supplementary Figure 1(b)). There was not a statistically significant interaction between 4-year periods and gender (interaction P value = 0.7758) (Supplementary Table 1, Table 2).

The incidence rates of ANs in males <50y went up by 289%, from 0.27 to 1.05/100 000. Similarly, the incidence rates of ANs in males ≥50y also increased by 199%, from 0.79 to 2.36/100 000. In females under 50y, the incidence rates increased by 286%, from 0.43 to 1.66/100 000. In females ≥50y, the incidence rates increased by 310%, from 0.70 to 2.87/100 000 (Figure 1(b)). However, there was not a statistically significant interaction between age group and gender (interaction P value = 0.2101) (Table 2). Over the period from 1982 to 2013, the incidence rates rose across all three major AN histological subtypes. For every 4-year, the incidence rate increased by 27% for adenocarcinomas, by 16% for neuroendocrine neoplasms, and by 35% for goblet cell adenocarcinomas (all global P value<0.0001) (Figure 1(c), Table 2, and Supplementary Table 1).

3.2. Mortality rate of ANs in Australia (1982–2013)

Overall mortality rates of ANs rose by 130% from 0.057 to 0.131/100 000 (Figure 2(a)). A negative binomial model demonstrated that, controlling for age group, for every 4-year increase in year, the AN mortality rate increased by 8% (IRR = 1.08, 95% CI: 1.00, 1.17, global P-value = 0.0401). Controlling for year, the ≥50y age group had a mortality rate 10.38 times that of the <50y group (IRR = 10.38, 95% CI: 6.81, 15.82, global P value<0.0001) (Table 2). Regarding the increase in the mortality rates, in patients <50y of age, the mortality rate increased by 950% from 0.004 to 0.042/100 000; and rose by 44% in patients ≥50y from 0.221 to 0.318/100 000 (Supplementary Figure 1(c)). However, there was not statistically significant interaction between age group and 4-year periods for mortality rates (interaction P value = 0.1479) (Table 2 and Supplementary Table 1).

Controlling for year, females had an AN mortality rate of 35% more than males (IRR = 1.35, 95% CI: 0.96, 1.89). However, this association was not statistically significant (global P-value = 0.0808). The mortality rates increased in males by 91% from 0.055 to 0.105/100 000 and rose by 171% in females from 0.058 to 0.157/100 000 (Supplementary Figure 1(d)). However, there was not a statistically significant interaction between gender and 4-year periods for mortality rate (interaction P value = 0.8397) (Table 2). The mortality rates of ANs in males <50y went up by 550%, from 0.004 to 0.026/100 000; and in males ≥50y, the mortality rates increased by 23%, from 0.229 to 0.281/100 000. In females <50y, the mortality rates increased by 1375%, from 0.004 to 0.059/100 000. In females ≥50y, the mortality rates rose by 66%, from 0.213 to 0.353/100 000 (Figure 2(b), and Supplementary Table 1).

Interestingly, the linear regression model showed that the slope of incidence rates was significantly higher (0.15 units greater) than the slope of mortality rates over four year periods (interaction P value<0.0001, mean estimate = 0.15, 95% confidence interval (CI): 0.10, 0.20) (Figure 3).

4. Discussion

Primary ANs are rare entities accounting for less than 2% of all appendectomies. However, we found a significant rise in incidence and mortality rates of ANs in Australia from 1982 to 2013. To our knowledge, this is the first report analyzing the incidence and mortality rates of these neoplasms in Australia. In the USA, Marmor *et al*. analyzed data from the SEER database and concluded that the overall incidence rate of ANs increased by 54% from 2000 (0.63/100 000) to 2009 (0.97/100 000) [4]. It was reported that the incidence rates of ANs increased in all three histological subtypes, in both genders and age groups, which is similar to what we observed in the

Figure 2. Appendiceal neoplasm-specific mortality rates per 100,000 population in Australia from 1982 to 2013. (a). Overall mortality rates between the periods 1982 to 2013. (b) Mortality rates of ANs from 1982 to 2013 in males and females under and above 50 years of age.

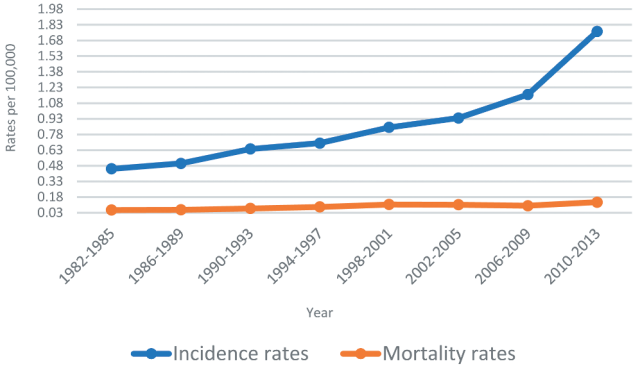

Figure 3. Trends in the incidence and mortality rates of ANs in Australia between the periods 1982 to 2013.

Australian cohort, but over a longer time period [4]. The rise in incidence and mortality rates of ANs is concerning and the exact cause of this observation remains unexplained.

Incidence rates of ANs were found to be significantly higher in women than men across both age cohorts. In women, ANs can be easily confused with primary ovarian cancer because ANs tend to metastasize to ovaries. Therefore, the WHO highlighted the importance of distinguishing between ANs and ovarian cancer in 1999 [9]. However, similar to the findings of Marmor *et al*., our data showed that incidence rates of ANs increased across both genders.

Similar to colorectal cancer, the incidence rates of ANs were significantly higher in people ≥50 years of age compared to those under 50 years [10]. Regardless of other different mechanisms that may lead aging to predispose to cancer (such as changes in internal homeostasis and tissue

accumulation of cells in advanced stages of carcinogenesis), the presentation of acute appendicitis (AA) is atypical in the elderly population with up to 70% of them having perforation at the time of diagnosis and is associated with poor outcome from surgery [10–13]. Although colonoscopy may allow for increased diagnosis of cancers of appendix origin through identifying the appendiceal orifice, the rise in the incidence of ANs was found in individuals under and above 50 years of age as well as across all three histological subtypes. These findings suggest that reclassification of ovarian cancers to appendiceal cancers and increasing use of colonoscopy were less likely to be the cause of the increase in the incidence of ANs, at least for the younger (<50y) age group.

The risk factors for developing ANs are still poorly understood. One study has reported that certain clinical conditions associated with appendiceal adenocarcinomas (e.g. Pseudomyxoma peritonei) are more likely in patients infected with intestinal bacterium *Helicobacter pylori*, with *H. pylori* incidence increasing with age [14,15]. However, studies have shown that the prevalence of *H.pylori* infection is decreasing in Australia and therefore this association observed in other studies is unlikely to explain the increased incidence of ANs among the older population in Australia [16].

Another possible cause of the rise in the incidence rates of ANs is the change in treatment of AA. Managing AA with antibiotics instead of appendectomy is emerging [15–17]. This new approach theoretically increases the risk of developing cancer in patients by creating chronic inflammation and changing appendix microbiome and host health. In addition, this non-operative therapy might also lead to a delay in diagnosis and treatment of ANs [18]. Studies have shown that there is a higher risk of developing ANs in patients with complicated AA (such as those with a peri-appendiceal abscess) compared to uncomplicated appendicitis [19–23]. One meta-analysis studied the risk of complications of non-surgery management in comparison with standard surgical treatment. The findings showed that among 2771 patients with non-operational treatment of appendiceal phlegmon or abscess, 1.2% of them were subsequently diagnosed with cancer [24]. In addition, studies have shown the risk of non-surgical management of AA in patients who underwent interval appendectomy. Wright et al. identified ANs in 12% (11/89) of patients after interval appendectomy, and two smaller studies identified higher proportions of patients (28% and 29%) with these neoplasms [21, 22, and 25].

Active smokers and children exposed to passive smoke have been reported to be at higher risk of appendectomy, with one study showing a 65% increase in the risk of appendectomy among current smokers compared to never-smokers after adjustment for gender, year of birth, and age with a stronger effect in females [26,27]. However, Greenhalgh *et al*. showed that there has been a statistically significant decline in the prevalence of smoking for Australian men and women [28]. These data suggest that smoking does not appear to have a role in the increasing incidence of appendectomies or ANs in Australia, although population studies can mask granularity in datasets.

From 1998 to 2013, Australia increased its availability of CT scans from 24.2 per million population to 53.7 per million [29]. Thus, better detection through increasing the use of CT scanning may have contributed in part to the apparent rise in the incidence of ANs. Age, as in many other types of cancers, has been reported to be a risk factor for developing ANs [19,21,22,25]. Another hypothesis for this rise in the incidence and mortality rates of ANs may be the growing aging Australian population. For example, in 1982, 24% of Australian people were above 50 years of age, but the percentage increased to 33% in 2013. In addition, the mortality rates for both genders considerably decreased in Australia. For people aged 50–59 years, the mortality rates decreased by 57% in 2004 (448/100,000) compared to 1981 (1033/100,000). The mortality rates for Australian women aged 50–59 years declined by 45% in 2004 (272/100,000) compared to 1981 (496/100,000) [30]. Moreover, in 2013, 34% of Australian women were above 50 years of age, but 26% of them were in this age group in 1982. This shows that the female aging population is increasing in Australia and this trend might have caused this rise of ANs [8].

Regarding the rise in the mortality rates, it was reported by the AIHW that although it was possible to isolate ANs (C18.1) from other colon cancers (C18), the precision of the code can only be as good as the precision of the text on the death certificate. It is possible that some appendiceal-cancer-related deaths were simply documented as 'colon cancer' on the death certificate. However, AIHW was unable to quantify the undercount. This suggests that improvements in reporting of ANs during the study period may have resulted in this observed increase in mortality rates.

Similar to incidence, mortality rates of ANs were significantly higher in people ≥50 years of age compared to those under 50 years. This may in part reflect age differences in incidence trend, delay to diagnosis, low survival rates, and barriers to appropriate appendiceal cancer care among elderly patients such as not offering a surgery to elderly patients concerning for a high risk of surgical morbidity and mortality [4]. Studies have reported that appendiceal adenocarcinoma which has poor prognosis is more frequent in elderly patients than in young [31, 32, and 33]. The higher relative odds of distant metastatic disease at diagnosis has also been reported to be significantly associated with elderly patients [4]. However, the increase in the mortality rates was not statistically significant between the two aging groups during the study period.

Notably, while there was a rise in the overall numbers of deaths from ANs in the population, the rate of incidence over time periods was significantly higher than the mortality rates. These findings can partly be reflected to improving the survival rates over the years, early detection of these tumors, as well as adoption and implementation of the best clinical practices in management and treatment of ANs.

There were some data-related limitations in this study; firstly, the lack of staging data at diagnosis and the ability to monitor tumor progression over the years, and secondly, the lack of mortality rate data for different ANs subtypes. Our analysis stops at 2013 because of the new ANs classification system. Finally, our study also had no data to investigate whether the overall survival or 5 years survival of ANs changed over the years and to assess the association between survival rates and different histological subtypes.

5. Conclusion

While the current incidence of ANs is still low, the overall incidence and mortality rates of these tumors rose significantly in Australia from 1982 to 2013. Increases in the incidence of ANs were observed across all histological subtypes, in both genders and in both age groups (<50 and ≥50 years of age). The increasing use of CT scanning as well as improvements in pathological assessment of appendicitis may have contributed in part to the apparent rise in the incidence rates of ANs in Australia. However, further studies are needed to determine the risk factors of ANs and the exact causes of the rising trend of these tumors.

Expert opinion

Incidence and mortality rates of ANs are on the rise in Australia across both genders and within older and young patient groups. This is an emerging health concern because ANs are usually aggressive and are associated with significant mortality. Although the increasing use of CT scanning, improvements in pathological assessment of the appendix, and the growing aging population may have contributed in part to the apparent rise in the incidence of ANs, further studies are needed to identify genetic and environmental risk factors of ANs and the exact causes of the current trends.

Authors' contributions

R.M. study concept and design, data collection and interpretation, statistical analysis, wrote the manuscript. J.Y., T.P. study concept and design, interpretation of data, critical review of the manuscript. J.H., G.R., P.H., E.L. S., E.S., Y.T., W.U., M.H., interpretation of data, critical revision of the manuscript. S.E. statistical analysis, interpretation of data, critical revision of the manuscript. All authors have approved of the final version of the manuscript to be published.

Ethical approval

Approvals for releasing the data were obtained from the data custodian of the Australian Cancer Database (who is also the Head of the CDMU), the Head of the Health Group, and the CEO of AIHW.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. The authors declare that they have no conflict of interest.

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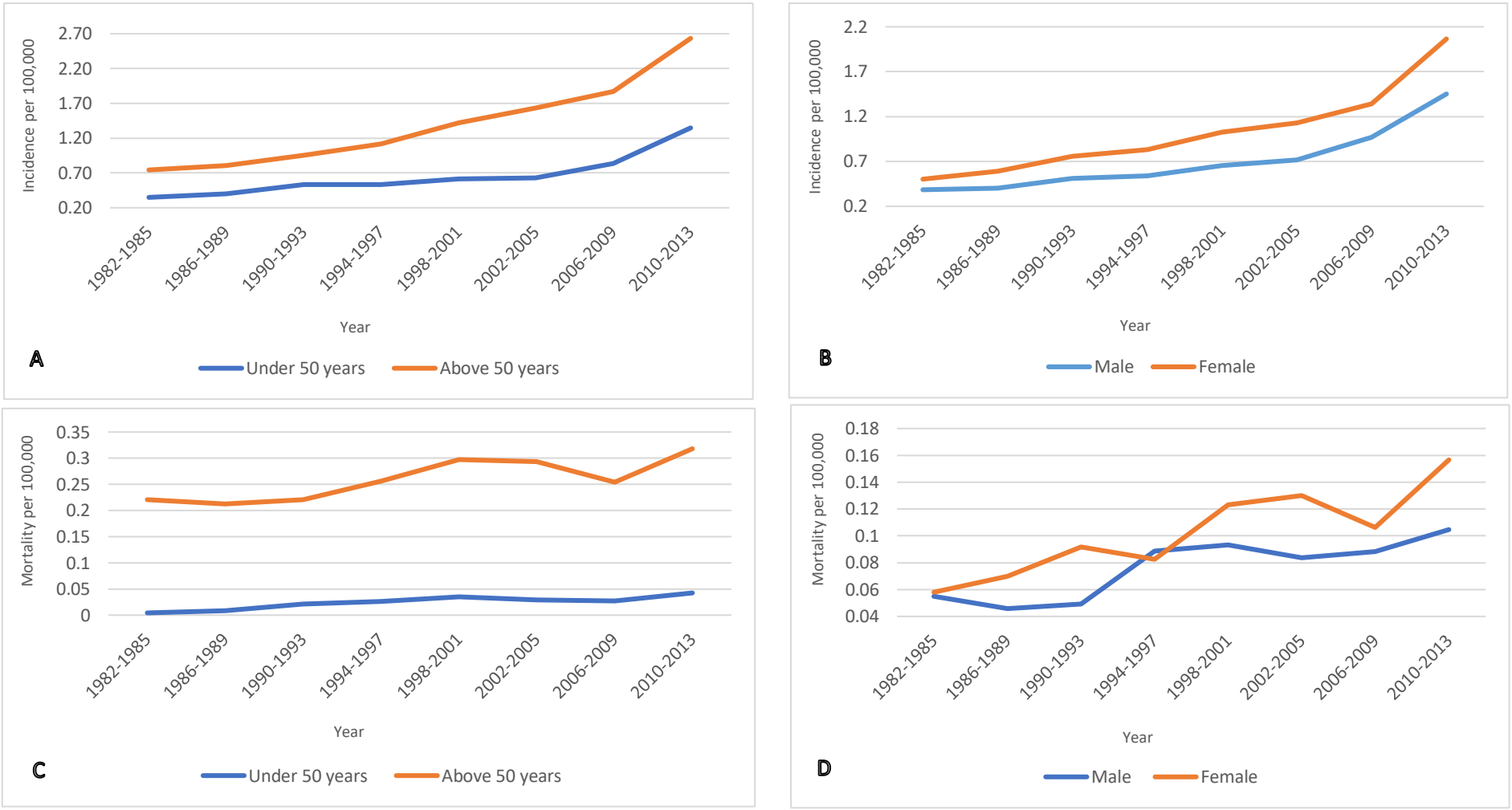

Supplementary Figure 1 Incidence and mortality rates of ANs in Australia from 1982 to 2013. **A.** Incidence rates of ANs from 1982 to 2013 in people under and above 50 years of age. **B.** Incidence rates of ANs from 1982 to 2013 in males and females. **C.** Mortality rates among individuals under and above 50 years of age diagnosed with ANs from 1982 to 2013. **D.** Mortality rates of males and females diagnosed with ANs from 1982 to 2013.

Supplementary Table 1 Incidence and mortality rates per 100 000 by patient characteristics and year (1982-2013) in Australia.

Incidence rates								
Year period	1982-1985	1986-1989	1990-1993	1994-1997	1998-2001	2002-2005	2006-2009	2010-2013
Overall	0.45	0.50	0.64	0.70	0.85	0.94	1.16	1.77
Males	0.38	0.40	0.51	0.54	0.66	0.72	0.97	1.45
Females	0.50	0.59	0.76	0.83	1.03	1.13	1.34	2.06
Under 50 years	0.35	0.40	0.53	0.54	0.62	0.63	0.83	1.35
Above 50 years	0.74	0.81	0.95	1.12	1.42	1.63	1.87	2.63
Male under 50 years	0.27	0.27	0.38	0.38	0.47	0.47	0.63	1.05
Male above 50 years	0.79	0.85	0.93	1.04	1.18	1.33	1.77	2.36
Female under 50 years	0.43	0.53	0.68	0.69	0.77	0.78	1.04	1.66
Female above 50 years	0.70	0.76	0.98	1.22	1.63	1.90	1.98	2.87
Adenocarcinoma	0.11	0.15	0.18	0.25	0.32	0.36	0.45	0.66
Neuroendocrine Neoplasms	0.31	0.33	0.42	0.38	0.47	0.48	0.60	0.96
Goblet cell adenocarcinoma	0.02	0.01	0.04	0.05	0.05	0.07	0.10	0.14
Mortality rates								
Overall	0.057	0.058	0.071	0.086	0.108	0.107	0.097	0.131
Male	0.055	0.046	0.049	0.089	0.093	0.084	0.088	0.105
Female	0.058	0.070	0.092	0.082	0.123	0.130	0.106	0.157
Under 50 years	0.004	0.008	0.021	0.026	0.035	0.029	0.027	0.042
Above 50 years	0.221	0.213	0.221	0.256	0.298	0.293	0.254	0.318
Male under 50 years	0.004	0.012	0.015	0.033	0.033	0.014	0.014	0.026
Male above 50 years	0.229	0.173	0.148	0.259	0.238	0.258	0.265	0.281
Female under 50 years	0.004	0.008	0.027	0.019	0.037	0.043	0.042	0.059
Female above 50 years	0.213	0.235	0.297	0.253	0.361	0.325	0.280	0.353

Chapter Six: Immunohistochemistry features and molecular pathology of appendiceal neoplasms

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While investigating the trends in incidence and mortality rates of ANs in Australia, it was observed that there were controversies regarding the pathology and classification of these neoplasms. In addition, immunohistochemistry features of ANs have not been explained in the fifth edition of the World Health Organization (WHO) classification of tumours (2019) and there was inconsistency in the findings of studies regarding the molecular pathology of these neoplasms. In this publication, the immunohistochemistry features and molecular pathology of each pathological subtypes of epithelial appendiceal neoplasms based on the current classification system are presented. Identifying biomarkers appropriate for each subtype would be useful to better in treatment selection and improve clinical applicability.

Statement of Authorship

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Contribution to the Paper	First author and corresponding author Contributed to the study concept and design, data collection and interpretation, and wrote the manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	16/10/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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REVIEW ARTICLE

Immunohistochemistry features and molecular pathology of appendiceal neoplasms

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ABSTRACT

Primary appendiceal neoplasms (ANs) comprise a heterogeneous group of tumors. The pathology and classification of ANs have been controversial, and thus, a new classification of these neoplasms was published in the World Health Organization (WHO) classification of tumors (5th edition, 2019). However, immunohistochemistry (IHC) features of epithelial ANs are not explained in this edition and the limited data on the molecular pathology of these tumors shows inconsistent findings in various studies. It would be useful to identify biomarkers appropriate for each subtype to better aid in treatment selection. Therefore, we reviewed the literature to investigate what is known of the molecular pathology and IHC features of the most frequently diagnosed pathological subtypes of epithelial ANs based on the recent classification. The inconsistencies in research findings regarding the IHC features and molecular pathology of ANs could be due to differences in the number of samples and their collection and preparation as well as to the lack of a universally accepted classification system for these neoplasms. However, the literature shows that epithelial ANs typically stain positive for MUC2, CK20, and CDX2 and that the expression of SATB2 protein could be used as a biomarker for appendix tumor origin. Low-grade appendiceal mucinous neoplasms tend to have mutations in *KRAS* and *GNAS* but are usually wild-type for *BRAF*, *APC*, and *P53*. Conversely, appendiceal adenocarcinomas are frequently found with mutations in *KRAS*, *GNAS*, *P53*, *PIK3CA*, and *APC*, and have significant nuclear expression of β-catenin, loss of nuclear or nuclear and cytoplasmic expression of SMAD4, and loss of cytoplasmic membranous expression of E-cadherin. Goblet cell carcinomas (GCCs) typically stain positive for keratin and mucin markers and are frequently mutated in *P53* and chromatin-modifier genes, but they tend to be wild-type for *KRAS*, *GNAS*, *APC*, and *PIK3CA*. The expression of CK7 and SATB2 proteins is usually negative in appendiceal neuroendocrine neoplasms and they lack the mutations in common cancer-associated genes including *APC*, *BRAF*, *SMAD4*, and *PIK3C*. The available data suggest that GCCs have distinct molecular and immunohistochemical features and that they have characteristics more in common with adenocarcinoma than classical neuroendocrine tumors. In addition, MSI does not seem to have a role in the pathogenesis of epithelial ANs because they are rarely detected in these tumors. Finally, hereditary predisposition may have a role in the development of ANs because heterozygous *CTNNβ1*, *NOTCH1,* and *NOTCH4* germline mutations have recently been identified in low and high grades ANs.

Abbreviations: AJCC: American Joint Committee on Cancer; AMNs: appendiceal mucinous neoplasms; ANs: appendiceal neoplasms; CgA: chromogranin A; CI: confidence interval; CRC: colorectal cancer; EGFR: epidermal growth factor receptor; GCCs: goblet cell carcinomas; HAMNs: high-grade appendiceal mucinous neoplasms; IHC: immunohistochemistry; LAMNs: low-grade appendiceal mucinous neoplasms; MACs: mucinous adenocarcinomas; MiN-ENs: mixed neuroendocrine-non-neuroendocrine neoplasms; MSI: microsatellite instability; NECs: neuroendocrine carcinomas; NENs: neuroendocrine neoplasms; NETs: neuroendocrine tumors; PCR: polymerase chain reaction; PMP: pseudomyxoma peritonei; PSOGI: Peritoneal Surface Oncology Group International; SATB2: special AT-rich sequence-binding protein 2; WHO: World Health Organization

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Supplemental data for this article can be accessed here.

Introduction

Primary appendiceal neoplasms (ANs) are rare, accounting for less than 1% of all tumors and less than 2% of all appendectomies; the age-adjusted incidence is 0.12 cases per 100,000 individuals per year [1–5]. Analysis of the Surveillance, Epidemiology, and End Results (SEER) database revealed that the overall incidence rate of ANs increased by 54% from 2000 to 2009 in the USA [6]. A recent study based on a retrospective analysis of national data from the Australian Institute of Health and Welfare (AIHW) found that the incidence rates of ANs rose by 415%, from 0.40/100 000 population in 1982 to 2.06/100 000 in 2013, and overall mortality rates rose by 130%, from 0.057/100 000 during 1982–1985 to 0.131/100 000 during 2010–2013 [7]. These increases were not likely due to the increasing use of colonoscopy or reclassification of ovarian cancers to ANs because the incidence went up consistently across gender, histology, age, and ethnic/racial groups. While these observations are currently largely unexplained, better detection through CT scanning may have contributed to them [6,7]. The risk factors for developing ANs are still poorly understood.

While rare, ANs are frequently an aggressive group of neoplasms that are associated with significant mortality thought to be due to late diagnosis. Epithelial ANs are the most frequently diagnosed ANs. However, there is tremendous heterogeneity within the histology and biology of these broad types. The pathology and classification of ANs have been controversial with different proposed classifications [8], and a new classification of epithelial appendiceal tumors was recently published in the World Health Organization (WHO) classification of tumors (5th edition, 2019) [9]. Having a universally accepted classification system for ANs will assist clinicians and pathologists to better diagnose, prognosticate and manage ANs. Diagnosis is based largely on the histological types and stages of the disease. Despite recent advances, the molecular pathogenesis of ANs has yet to be described, and at present, little is known regarding the mutational spectrum of each subtype of epithelial ANs. In addition, immunohistochemistry (IHC) features of different histopathological subtypes of ANs and their association with clinical phenotypes and improvement in accuracy of diagnosis are not discussed in the WHO classification of tumors (2019).

There is ongoing debate about which is the best chemotherapy or targeted agent to use for each subtype of ANs. Currently, treatment is guided by the pathological subgroups but there are no standardized treatment protocols for these neoplasms because of lack of data regarding optimal chemotherapy treatment and poor treatment responses observed with cytotoxics, mainly fluorouracil-based combinations. Treatments used in colon cancer are often used for the management of appendiceal adenocarcinoma. The role and optimal treatment schedule in the management of ANs have not been established in prospective studies. Thus, there is a great need to identify biomarkers and to develop improved treatment regimens [10]. In this review, the molecular pathology and IHC features of each pathological subtype of epithelial ANs, based on the current classification system, will be presented, and the rates of molecular and IHC biomarkers for ANs in various studies, and their differences, will be discussed. Finally, the association of genetic alterations and IHC biomarkers of each pathological subtype, with their heterogeneity, mechanism of progression, clinical phenotypes, and potential actionable targets for therapy, will be provided.

Classification of appendiceal neoplasms

The appendix is a small organ that gives rise to remarkably diverse histologic cancer types. The differential diagnosis of ANs includes epithelial ANs, lymphomas, and mesenchymal tumors [11]. Epithelial ANs are the most frequently diagnosed tumors. However, there is tremendous heterogeneity within the histology and biology of this type (Table 1). Therefore, the

Table 1. Classification of epithelial appendiceal neoplasms by PSOGI, excluding goblet cell tumors [13].

Histological type	Features
Low-grade appendiceal mucinous neoplasms (LAMNs)	Mucinous neoplasm without infiltrative invasion but with any of the following: loss of muscularis mucosae, fibrosis of submucosa "Pushing invasion" (expansile or diverticulum-like growth), dissection of acellular mucin in the wall, undulating or flattened epithelial growth, rupture of appendix, mucin and/or cells outside appendix
High-grade appendiceal mucinous neoplasms (HAMN)	
Serrated lesions	Tumor with serrated features confined to the mucosa, muscularis mucosae intact
Adenoma	Adenoma resembling usual colorectal type, confined to mucosa, muscularis mucosae intact
Mucinous adenocarcinoma	Mucinous neoplasm with infiltrative invasion (40% of all appendiceal adenocarcinomas).
Poorly differentiated (mucinous) adenocarcinoma with signet ring cells	Signet ring cells present $< 50\%$ of the cells in adenocarcinoma
Mucinous signet ring cell carcinoma	Signet ring cells present $> 50\%$ of the cells in adenocarcinoma
Non-mucinous adenocarcinoma	Non-mucinous adenocarcinoma resembling usual colorectal type

Table 2. Classification of epithelial appendiceal neoplasms according to the WHO classification system (2019) [9].

Type	Definition	Subtype
Appendiceal serrated lesions and polyps	Appendiceal serrated lesions and polyps are mucosal epithelial polyps characterized by a serrated (sawtooth or stellate) architecture of the crypt lumen).	None
Appendiceal mucinous neoplasms	Mucinous neoplasms are characterized by mucinous epithelial proliferation with extracellular mucin and pushing tumor margins	None
Appendiceal adenocarcinoma	Malignant glandular neoplasms characterized by invasion	A- signet-ring cell adenocarcinoma, B- mucinous adenocarcinoma, C- carcinoma, undifferentiated, not otherwise specified
Appendiceal goblet cell adenocarcinoma	These are an amphicrine tumor composed of goblet-like mucinous cells, as well as variable numbers of endocrine cells and paneth-like cells, typically arranged as tubules resembling intestinal crypts	None
Appendiceal neuroendocrine neoplasms	Neoplasms with neuroendocrine differentiation	A-neuroendocrine tumors, B-neuroendocrine carcinomas

pathological classification of ANs, particularly appendiceal mucinous neoplasms (AMNs), has been confusing. The majority of pseudomyxoma peritonei (PMP) cases arise from AMNs that exhibit a wide spectrum of clinical and biological behaviors, ranging from significantly aggressive tumors with high risk for recurrence to slow-growing tumors with a decreased likelihood of recurrence. Clinically, PMP is characterized by a diffuse collection of mucinous tumor nodules and mucinous ascites throughout the abdominal cavity. PMP represents local spread within the abdominal peritoneal cavity and most frequently arises from ANs [1]. The prognosis of ANs has been associated mainly with the neoplastic cells within the mucinous tumors of PMP.

The Peritoneal Surface Oncology Group International (PSOGI) recognized that the lack of universally accepted terminology and classification system for AMNs posed problems for clinicians and pathologists. This matter was discussed at the 2012 PSOGI World Congress in Berlin [12]. A consensus regarding the terminology for AMNs was reached by 34 international pathologists, 37 medical oncologists, and surgeons in 2016 [13]. The PSOGI classifies AMNs into non-infiltrative invasive neoplasms [low-grade appendiceal mucinous neoplasm (LAMN), high-grade mucinous neoplasms (HAMN), serrated lesions, and adenoma], and infiltrative invasive neoplasms [mucinous adenocarcinoma, poorly differentiated adenocarcinoma with signet ring cells, and signet ring cell carcinoma] [14] (Table 1). The American Joint Committee on Cancer (AJCC) 8th staging system and the PSOGI use similar diagnostic terminology for AMNs. However, the AJCC system also uses descriptive terminology and a three-tiered grading approach: G1, well-differentiated; G2, low-grade tumors, moderately differentiated; and G3, high-grade tumors, poorly differentiated). The WHO classification system (2019) classifies epithelial ANs into: A – appendiceal serrated lesions and polyps, B – appendiceal mucinous neoplasms, C – appendiceal adenocarcinoma, D – appendiceal goblet cell adenocarcinoma, and E – appendiceal neuroendocrine neoplasms [9] (Table 2). In addition, like the AJCC system, the WHO classification system uses descriptive terminology and a three-tiered grading approach (Grade 1: low-grade appendiceal mucinous neoplasms (LAMNs); Grade 2: high-grade appendiceal mucinous neoplasms (HAMNs) and invasive adenocarcinoma without a signet-ring cell component; and Grade 3: signet-ring cell adenocarcinoma with numerous signet-ring cells in mucin pools or infiltrating tissue [9].

Appendiceal mucinous neoplasms

AMNs are extremely rare malignancies that account for 0.4–1% of all tumors and 0.2–0.3% of appendectomies, with a slight female predominance (50–55%) [1,15]. AMNs are usually indolent tumors and often do not metastasize beyond the abdominal cavity. Moreover, although the age range in AMN patients is broad, the tumors usually occur in the fifth and sixth decade of life [16–18]. AMN patients with early-stage disease typically present with acute appendicitis. However, patients with disseminated disease may present with abdominal or pelvic masses or with features of PMP. Other symptoms include weight loss, abdominal pain, and new hernias [1]. The classifications of primary AMNs is challenging, especially when there are no obvious malignant features but they are associated with PMP. In the early classification systems, AMNs were considered as a benign disease with various diagnostic terminologies such as cystadenoma, appendiceal mucocele, and cystadenocarcinoma. However, these terminologies are no

longer used, and new criteria and diagnosed terminologies are recommended by the recent classification systems [9,13].

Immunohistochemistry features and molecular pathology of low-grade appendiceal mucinous neoplasms and high-grade appendiceal mucinous neoplasms

According to the PSOGI and the WHO classification system (2019), LAMNs are non-infiltrative invasive mucinous neoplasms with low-grade cytological atypia and any of the following characteristics: loss of the muscularis mucosae and lamina propria, fibrosis of submucosa, different forms of "pushing" invasions (expansile or diverticulum-like growth), dissection of acellular mucin in the wall, different patterns of epithelial growth (undulating or flattened epithelial growth), rupture of the appendix, and mucin and/or cells outside the appendix [9,14] (Table 1 and Figure 1). In addition, the neoplastic epithelium in LAMNs usually exhibits circumferential involvement of the mucosa, and microscopically, neoplastic cells display characteristic small, uniform, darkly stained and basally orientated nuclei that preserve nuclear polarity with large cytoplasmic mucin. Macroscopically, the appendix often appears dilated because of the abundant accumulation of mucin within the lumen [12–14].

HAMNs show similar histological features to LAMNs, but the neoplastic epithelium has unequivocal high-grade features that may include vesicular enlarged nuclei with full-thickness stratification, loss of nuclear polarity, numerous mitotic figures, and prominent nucleoli [14,19] (Table 1 and Figure 1). HAMNs are very rare neoplasms and the appendix must be submitted entirely for evaluation for associated infiltrative adenocarcinoma invasion. Neoplastic epithelial cells in the extra-appendiceal mucin were less likely to be observed in patients with LAMNs than HAMNs [20–23], and up to 2/3 of patients with HAMNs developed recurrent adenocarcinoma in the peritoneum [16,22].

Distinguishing secondary LAMNs that have metastasized from primary mucinous ovarian tumors to the ovary is diagnostically challenging because LAMNs

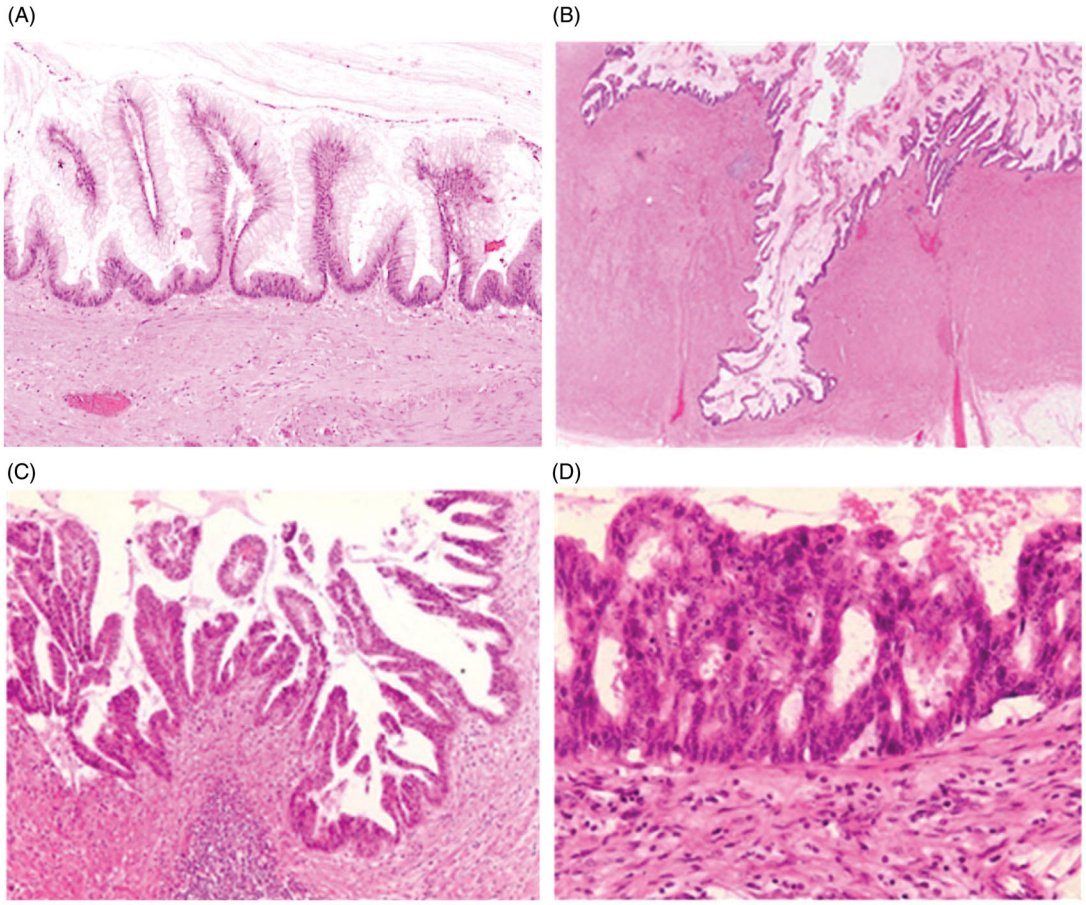

Figure 1. Appendiceal mucinous neoplasms. (A) Low-grade appendiceal mucinous neoplasms showing villiform pattern of growth with low-grade cytological atypia. (B) Low-grade appendiceal mucinous neoplasms showing pushing invasion of appendiceal wall but without infiltrative invasion. (C) High-grade appendiceal mucinous neoplasms showing high-grade cytological atypia but without infiltrative invasion. (D) High-grade appendiceal mucinous neoplasms showing a cribriform pattern [14].

frequently mimic mucinous ovarian carcinoma by appearing as cystic tumors. Because patient with primary ovarian adenocarcinomas are usually treated with paclitaxel and a platinum agent, and those with ANs are usually treated with 5-fluorouracil, distinguishing these two entities is vital for treatment [24]. Pathologists use IHC with variable frequency, and staining results are not definitive or conclusive in many cases. LAMNs typically stain positive for CK20 (90–100%), math1 (100%), MUC2 (92%), PGP (83%), MUC5AC (72%), and CDX2 (92–100%), stain patchy positive for CK7 (14–36%), and stain negative for P53, PAX8, MUC1, ER, cKIT, and SMAD4 (Supplementary Table A). However, the expression of some of these markers overlap, making accurate diagnosis of these tumors difficult. For example, though PAX8 positivity shows a primary ovarian origin, this protein is negative in 50-60% cases of this malignancy. In addition, CDX2 may be helpful to determine intestinal tumor origin [25,26], but it is also expressed in primary ovarian mucinous carcinoma [27]. Vang et al. evaluated CK20/CK7/CDX2 markers to differentiate primary ovarian origin from gastrointestinal tumors and noted that no single marker was adequate as a diagnostic marker. They concluded that CDX2 should be evaluated in the context of coordinate expression with CK7 [27]. More recently, expression of special AT-rich sequence-binding protein 2 (SATB2) was reported in almost all LAMN patients (96–100%), while it was negative or expressed in a minority of patients with primary ovarian carcinoma [25,26,28]. Therefore, the expression of this protein could be used as a marker for appendix tumor origin.

One study that investigated the marker, β-catenin, in 25 LAMN cases found that none had nuclear expression of β-catenin [24]. In contrast, Hara et al. (2015) reported the nuclear expression of β-catenin in 73% of LAMN cases, but the number of samples was small (11 cases), and only two patients had mutations in *CTNNB1* (29) (Supplementary Table A).

Data on the molecular pathology of LAMNs compiled from multiple studies shows that overall, these tumors tend to harbor *KRAS* (83%, 95% confidence interval [CI] 70.6–92.6) and *GNAS* (55%, 95% CI 47.3–62.3) mutations. However, mutation rates of these genes vary significantly across different studies (Supplementary Table B). For example, Hara et al. found only 27% (3/11) of LAMNs with *KRAS* mutations using polymerase chain reaction (PCR). The specificity and sensitivity of the detection technique and the ratio of tumor cells compared to wild type cells of the samples may generate this degree of variation in findings [29]. Nonetheless, the data suggests that activation of the RAS-MAPK pathway plays a significant role in LAMNs and that therapies targeting this pathway should be considered to manage LAMNs. Moreover, *GNAS* mutations, rare in colon cancer, also appear to have a role in the development of LAMNs (Supplementary Table B). This gene has been reported to regulate MUC2 and MUC5AC expressions in colon tumors, and its expression has not been found to be associated with cell proliferation *in vivo* and *in vitro*. Therefore, *GNAS* mutations may have implications for the exaggerated mucin production seen in PMP [30,31]. *GNAS* mutations have consistently been reported in fibrous dysplasia [32], colonic villous adenoma [33], pituitary adenoma [34], and pancreatic intraductal papillary mucinous neoplasms [35].

In addition, four studies reported that *P53* mutations, which are key pathogenic mutations in colorectal cancer (CRC), were not detected in any LAMN cases [30]. On the other hand, Hara et al. detected *P53* mutations in 27% (3/11) of patients with LAMNs, a finding that suggested a potential role of P53 in the progression of LAMNs to high-grade tumors [29]; however, because the expression of P53 was not detected by IHC, the authors concluded that the detected *P53* mutations did not induce conformational changes that inhibited protein degradation, and that this led to false-negative expression by IHC [29] (Supplementary Table B). Zhu et al. found *P53* mutations in 10% (2/21) of cases of LAMNs, but a relatively higher proportion in high-grade tumors [36]. These studies suggest that *P53* deregulation is more likely to be associated with high-grade tumors. Zauber et al. investigated *APC* mutations in 31 LAMN patients and found none; similar findings were observed by others, indicating that *APC* mutations do not seem to have a role in the developing of LAMNs [37–39] (Supplementary Table B). However, in CRC, *APC* has long been considered as the central "gatekeeper" gene in the majority of cases and has been reported to be an early event in CRC carcinogenesis and in the initiation of the adenoma–carcinoma pathway [40–42]. Though *APC* mutations have been found in 33% of LAMN cases [10], it is not known whether these cases had similar histopathological features to patients studied by other investigators. More importantly, when high-resolution sequencing that targeted 409 genes was performed on five primary LAMNs with their corresponding low-grade metastasis tissues, all cases had heterozygous germline mutations in the *CTNNβ1*, *NOTCH1,* and *NOTCH4;* these findings suggested a potential role of hereditary predisposition in developing AMNs [43].

There are limited data in the literature on the IHC features and molecular pathology of HAMNs. However,

a study of five HAMN cases showed that all of them had *KRAS* mutations and one tumor had a *BRAF* mutation. Additionally, a missense mutation in *P53* and non-sense mutations in *APC* and *RNF43* were each demonstrated in one HAMN [39]. RNF43 negatively regulates the Wnt/β-catenin pathway by inhibiting the downstream signaling of mutated β-catenin. Consequently, *RNF43* has been identified as a tumor suppressor gene and its mutations have been commonly detected in malignancies that include CRC, gastric cancer [44] and ovarian cancer [45,46]. Therefore, *RNF43* mutations may have a role in the progression of LAMNs to HAMN through the activation of the Wnt/β-catenin pathway [39]. Additionally, Liao et al. reported *KRAS* and *GNAS* mutations in 9/9 (100%) and 5/9 (56%) of HAMN cases, respectively, compared to 8/8 (100%) and 5/8 (63%) of LAMN cases [47]. However, while 4/9 (44%) and 2/9 (22%) of HAMN cases had mutations in *P53* and *ATM,* no LAMN cases had mutations in these genes. The authors suggested that mutations in *P53* and perhaps *ATM* may cause LAMN to progress to HAMN. Conversely, the comparable high rate of *GNAS* mutations in both LAMN and HAMN suggest that this gene is not a driving factor for transforming LAMNs to HAMNs [47]. Finally, LAMNs tend to be *PIK3CA*, *PTEN,* and *BRAF* wild type, and the microsatellite instability (MSI) pathway does not seem to have a role in the development of these tumors because it has not been reported in any studies [38,48,49] (Supplementary Table B). For example, Zauber et al. analyzed 31 LAMN cases and found all to be microsatellite stable [38].

Serrated lesions and adenoma

Serrated lesions and adenoma are precursors for ANs with serrated features and tumors resembling villous, tubular, or tubulovillous adenoma of the colo-rectum, respectively. Serrated lesions and adenoma confined to the mucosa and muscularis mucosa of the appendix do not have the potential to cause PMP [14]. ANs with serrated features are frequently diagnosed, with the majority lacking cytological dysplasia. However, appendiceal adenomas are rare, and when they occur, they are more frequently of the villous type. In contrast, colonic adenomas are more commonly of the tubular type. Appendiceal serrated lesions may be dysplastic polyps and take the form of traditional serrated adenoma-like dysplasia, serrated-type dysplasia or conventional adenoma-like dysplasia, or they can be non-dysplastic polyps [12,14,48]. Notably, though appendiceal serrated lesions may show comparable histological features to those observed in the colo-rectum, molecular data has shown that they have different frequencies and genetic abnormalities. Serrated lesions in the appendix with or without dysplasia tend to be *KRAS* but not *BRAF* mutated in contrast to the lesions of the colo-rectum that are typically *BRAF* but not *KRAS* mutated. Pai et al. studied appendiceal serrated lesions with and without dysplasia and found that only 5 of 126 cases (4%) had *BRAF* mutations while approximately 50% had *KRAS* mutations [50]. However, *BRAF* mutations account for 90% of the serrated neoplastic pathway in the colo-rectum [51]. These molecular differences suggest that the appendix has a distinct serrated neoplastic pathway and that *BRAF* may be less biologically important than *KRAS* [9]. Therefore, for appendiceal serrated lesions, the PSOGI expert panel and the WHO classification system (2019) have used the categories, serrated polyps with or without dysplasia, instead of sessile serrated adenoma or sessile serrated lesions [12–14]. The WHO classification system (2019) categorized appendiceal serrated lesions and polyps into three different types based on histological and molecular features. Hyperplastic polyps are often present with *KRAS* mutations but rarely with *BRAF* mutations. In contrast, serrated lesions with and without dysplasia typically present with *KRAS* mutations and rarely with *BRAF* mutations (Table 3). It is worth noting that Tsai et al.

Table 3. Appendiceal serrated lesions and polyps based on molecular and histopathological features according to the WHO classification system (2019) [9].

	Histological features			Molecular features	
Polyp type	Crypt	Cytological dysplasia	Architecture	*KRAS* mutation	*BRAF* mutation
Hyperplastic polyp	Straight crypt with serration limited to luminal aspect of the crypt	Absent	Discrete polyp or circumferential mucosal involvement, villous uncommon	Often present	Rarely present
Serrated lesion without dysplasia	Distorted crypt with serration and crypt dilation extending to crypt bases	Absent	Often with circumferential mucosal involvement, villous uncommon	Typically present	Rarely present
Serrated lesion with dysplasia	Distorted crypts with serration and crypt dilation extending to crypt bases	Present	Often with circumferential mucosal involvement, villous variable	Typically present	Rarely present

found *BRAF* mutations in approximately 78% (7/9) of serrated polyps, while *KRAS* mutations occurred in only 22% (2/9) of the cases. They suggested that this discrepancy could be due to variation in the histological criteria for diagnosis of appendiceal serrated polyps. For example, Tsai et al. defined neoplasms with mucinous cells arranged with villous structure with prominent serration and fibrovascular cores as LAMN with serrated architecture, and all the cases had *KRAS* mutations (5/5) and no *BRAF* mutations. In contrast, tumors with these features and epithelial serrations were defined as serrated lesions by Pai et al. and Yantiss et al. In addition, the race and ethnicity of the study cohort could have caused the discrepancy in the genetic alterations [16,23,39].

Immunohistochemistry features and molecular pathology of adenocarcinoma of the appendix

The PSOGI expert panel recommended that the term, adenocarcinoma, be reserved for tumors with infiltrative invasion, which refers to destructive stromal invasion into the appendiceal wall. In addition, in contrast to "pushing" invasion of LAMNs and HAMNs that is associated with poorly cellular, dense and often hyalinized fibrosis, features of infiltrative invasion include desmoplastic reaction, small angulated irregular glans or tumor budding [14] (Figure 2). Accordingly, the PSOGI system classifies AMNs with infiltrative invasion into three types: mucinous adenocarcinomas (MACs), poorly differentiated adenocarcinoma with signet ring cells, and signet ring cell carcinoma [14]. The AJCC system classifies MACs as either moderately differentiated (G2) neoplasms that exhibit high-grade cytology with infiltrative invasion but without signet ring cells, or poorly differentiated (G3) neoplasms that exhibit high-grade cytology with signet ring cells and typically infiltrative invasion [12]. However, the WHO classification system (2019) uses a three-tiered grading system for grading LAMNs, HAMNs and mucinous adenocarcinoma, and a two-tiered grading system for grading non-mucinous adenocarcinoma that is similar to the system for grading CRC [9].

MACs refer to tumors in which histologically extracellular mucin comprises >50% of the cross-sectional area. According to the PSOGI, primary appendiceal mucinous adenocarcinoma can be classified into well-differentiated, moderately differentiated, and poorly differentiated MACs. Well-differentiated MACs often consist of neoplastic epithelium with minimal nuclear atypia lining the cystic mucin pools, while poorly differentiated MACs show no or little gland formation [14]. However, the diagnostic criteria for these three descriptive terms are not provided by the PSOGI. Poorly differentiated (mucinous) adenocarcinoma with signet ring cells are defined as neoplasms in which signet ring cells are present in <50% of the cells. PMP patients with signet ring cells tend to be associated with poor prognosis, and therefore, this group of patients are classified separately [52–54]. Signet ring cell carcinoma refers to neoplasms in which signet ring cells are present in >50% of the cells in adenocarcinoma. In addition, primary appendiceal adenocarcinoma, which can be non-mucinous, resembles colorectal adenocarcinoma radiologically and histologically and is further classified into well-differentiated, moderately differentiated, and poorly differentiated non-mucinous adenocarcinoma. Most appendiceal adenocarcinomas are of the AMN subtype and frequently arise from LAMNs. However, these carcinomas have also been reported to arise from adenomatous polyps or/and serrated adenomas [1,55,56].

IHC studies have shown that MACs, like LAMNs, are positive for CK20 (96–100%), MUC2 (96–100%), SATB2 (83–100%) [25,28,57], CDX2 (93–96%), and MUC5AC (40–86%), suggesting that these markers cannot be used for discriminating low-grade tumors from high-grade tumors (Supplementary Table C). Furthermore,

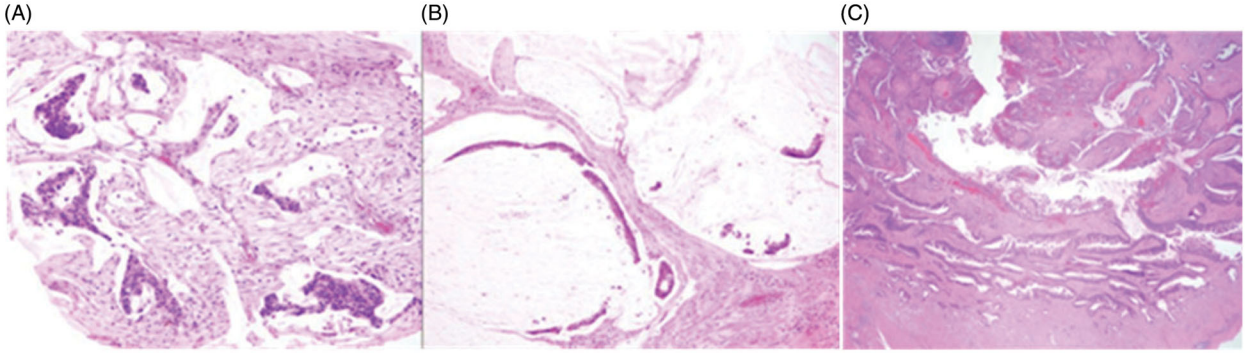

Figure 2. Appendiceal adenocarcinoma. (A and B) Appendiceal mucinous neoplasms showing high-grade cytological atypia with infiltrative invasion and irregular epithelial-lined mucin pools. (C) Non-mucinous appendiceal adenocarcinoma [1].

MACs are also positive but have a lower frequency for CK7 (28–50%), epidermal growth factor receptor (EGFR) (68%) [37], TOPOI, cyclin D1, cKit, and PGP. However, appendiceal adenocarcinoma is often negative for PAX8 (0%), ER (0%) [25,58], cKIT (18%) [37], and CK14 (0%) [59]. Notably, a review of the literature showed that the overall expression of CK7 was higher, but not significantly higher, in appendiceal adenocarcinoma (35%, 95% CI 26.7–44.4) compared to LAMNs (31%, 95% CI 20.3–42.8, $p = 0.6$). The combined expression of CK20 and CK7 is the most commonly used IHC marker for distinguishing primary ovarian mucinous adenocarcinoma from metastatic colorectal adenocarcinoma because colorectal adenocarcinoma is typically CK20 positive and CK7 negative, and ovarian carcinoma is usually CK20 negative and CK7 positive [24]. However, a small fraction of primary ovarian mucinous adenocarcinoma is CK7 negative, and colonic mucinous adenocarcinoma and appendiceal adenocarcinoma are often CK7 positive [60–63] (Supplementary Table C). Thus, these markers are not as valuable for distinguishing primary mucinous adenocarcinoma from metastasized appendiceal adenocarcinoma.

MUC1 protein has been found to be significantly overexpressed (up to 17%) in appendiceal adenocarcinoma compared to LAMNs (Supplementary Tables A and C). Overexpression of this protein is reported in most human tumors and it has been suggested to have a potential role in carcinogenesis, invasiveness and metastasis of cancers, although several studies have reported contrary effects of MUC1 in tumor cells [64]. A systemic review by Zeng et al. concluded that overexpression of MUC1 was significantly associated with CRC metastasis [64].

SMAD4 protein was also found to be significantly expressed in high-grade tumors but not in low-grade tumors. Davison et al. described the loss of nuclear or nuclear and cytoplasmic expression of SAMD4 in 19% of appendiceal adenocarcinoma but in none of the LAMN cases, suggesting that SMAD4 IHC may be useful to confirm the diagnosis of adenocarcinoma (Supplementary Tables A and C) [65]. Loss of expression of SMAD4 protein has also been reported to be higher in aggressive pancreatic and colonic adenocarcinoma compared to noninvasive precursor lesions [65–68]. In addition, there is high expression of TOPO1 in LAMNs and appendiceal adenocarcinoma, suggesting that irinotecan could be combined with fluorouracil for the treatment of these malignancies. However, appendiceal adenocarcinoma is often negative for PAX8, ER [25,58], cKIT (18%) [37], and CK14 [59]. For biomarkers that show specific mutations or indicate tumor pathogenetic pathways, aberrant expression of E-cadherin and β-catenin is found in 29–47% and 12–60% of appendiceal adenocarcinoma, respectively [29,69–71] (Supplementary Table C).

Molecular pathology data show that *KRAS* mutations are frequently detected in patients with MACs (60%, 95% CI 55.5–64.3) and non-mucinous appendiceal adenocarcinoma (54%, 95% CI 48.2–59.7) (Supplementary Tables D and E). Borazanci et al. studied 442 appendiceal adenocarcinoma cases, and *KRAS* mutations were found in 65%, 47%, and 7% cases of AMC, non-mucinous appendiceal adenocarcinoma and signet ring cell carcinoma, respectively [37,48]. Liu et al. studied 19 cases of appendiceal adenocarcinoma and reported that *KRAS* mutations were demonstrated in 6/8 (75%) and 0/11(0%) cases of well-differentiated MAC and signet ring cell carcinoma, respectively [10]. These studies show that the molecular profiles of different appendiceal adenocarcinoma subtypes vary. *KRAS* mutations, which result in constant stimulation of the MAPK pathway, subsequently transform basal cells into malignant cells. Selective inhibitors of this pathway, including cetuximab and panitumumab, have been used in clinical practice since 2004. In CRC, *KRAS* mutations, which occur in 30–50% of cases [72–75], not only predict lack of response to therapy but are also associated with an increased risk of reoccurrence and death [76,77]. However, in ANs, while most studies have shown lack of a significant association of *KRAS/GNAS* mutations with survival [78], Pietrantonio et al. showed that *KRAS* mutations were independently associated with worse progression free survival ($p = 0.012$) [79]. Studies have shown significant differences in the mutation frequency of *GNAS* mutations across different histological subtypes: 24% (95% CI 18.9–29.5) of non-mucinous appendiceal adenocarcinoma, 37% (95% CI 22.6–52.3) of MACs and 55% (95% CI 47.3–62.3) of LAMN cases (Supplementary Tables B, D, and E). The data suggest that *GNAS* mutations may not have a role in the progression of appendiceal tumors.

In addition, in contrast to LAMNs, *P53* mutations are frequently detected in appendiceal adenocarcinoma, with differences in the frequency observed across studies. The overall mutation rate of *P53* in MACs and non-mucinous appendiceal adenocarcinoma from the literature was 37% (95% CI 25.8–49.3) and 46% (95% CI 32.5–59.7), respectively. One study reported *P53* mutations in 24% cases of MAC, 32% of non-mucinous adenocarcinomas, and 15% of signet ring cell carcinomas [37]. Yoon et al. and Hara et al. found a high expression of P53 (40%) in MACs by IHC staining, suggesting that this protein may be a useful biomarker for appendiceal adenocarcinoma (Supplementary Tables D and E) [29,69]. P53 inactivation is involved in malignant

transformation; thus, aggressive properties of appendiceal adenocarcinoma may be due to the inactivated P53 pathway. LaFramboise et al. found that appendiceal adenocarcinoma as LAMNs demonstrated damaging heterozygous germline mutations in *CTNNβ1*, *NOTCH1,* and *NOTCH4,* but damaging variants were significantly higher in appendiceal adenocarcinoma in comparison to LAMNs [43]. The findings showed that somatically acquired mutations suppressing the tumor suppressor gene, *TP53,* and enhancing oncogenes *DAXX* and *MYC* were consistent with manifestation of appendiceal adenocarcinoma. Some studies have shown a significant association of overexpression of P53 with lower overall survival and of *GNAS* inactivation with decreased progression free survival. Thus, systemic therapy in addition to surgery should be considered for patients with *P53* mutations [78].

SMAD4 has a critical role in the TGFβ signal transduction pathway and its mutations that cause disruption of TGFβ signaling have been reported frequently in CRC and pancreatic adenocarcinoma [80]. *SMAD4* mutations have been detected in up to 20% of MACs, a finding that is comparable to its mutation rate in LAMNs (14–20%). In non-mucinous appendiceal adenocarcinomas, Ang et al. detected *SMAD4* mutations in 18% of 208 patients, which is similar to the 21% reported by Borazanic et al. [37,81]. However, two other studies did not detect these mutations in any non-mucinous appendiceal adenocarcinoma cases (the number of samples was very small) (Supplementary Tables D and E). SMAD4 function may be lost by different mechanisms such as point mutation and homozygous deletion with loss of the wild-type allele (genomic deletion). IHC is a reliable surrogate for SMAD4 mutational analysis because loss of expression correlates with genetic mutations in more than 90% of cases, and it detects loss of expression caused by different mechanisms [82,83].

APC mutations are frequently diagnosed in non-mucinous appendiceal adenocarcinoma (20%, 95% CI 12.2–29.8), and at a lower frequency in MAC (9%%, 95% 4.8–13.1) (Supplementary Tables D and E). The tumor suppressor gene, *APC,* encodes APC protein that is involved in the Wnt pathway and has critical functions in many cellular processes. This protein has become a target in clinical trials [37]. In contrast to LAMNs where *BRAF* mutations have not been reported [36,37,84], these mutations have been found in up to 9% of MACs and in up to 14% of non-mucinous appendiceal carcinomas [37,77,85]. *BRAF* is a member of the serine/threonine protein kinase family and is an integrated member of the MAPK pathway that has an active role in other cellular processes including apoptosis, cell migration and survival [86]. *BRAF* ^{V600E} mutations have been found in approximately 8% of all human cancers, with cutaneous melanoma having the highest rate followed by papillary thyroid cancer and serous ovarian cancer (66%, 53%, and 30%, respectively) [87,88]. *BRAF* ^{V600E} is found in approximately 15–20% of sporadic CRCs, and is significantly associated with the elderly female, CpG island methylator phenotype, MSI, RAS tumors, and a higher grade and poor outcomes, especially in advanced stages of the disease [89]. Patients with *BRAF* mutations have been reported to be resistant to anti-EGFR therapies (such as cetuximab or panitumumab) [90,91]. Graf *et al.* recently showed a negative prognostic impact of *BRAF* mutations in patients with colorectal or appendiceal peritoneal metastases scheduled for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, with no patient surviving for more than 2 years [92]. These findings suggest that different therapeutic approaches such as *BRAF* inhibitors plus other targeted drugs should be considered for patients with *BRAF* mutations [92,93]. Tokunaga et al. found *PIK3CA* and *RNF43* mutations in 6% and 7% of MACs cases, respectively [85]. Another study with small sample size found *PIK3CA* mutations in 20% (2/10) of MAC cases [47]. In addition, *PIK3CA* mutations have been reported in non-mucinous appendiceal adenocarcinoma (21%) [37,94] and in 10–20% of CRC [95]. *PIK3CA* mutations have been associated with proximal colon and MSI tumors. Gain of function mutations in *PIK3CA* induce cell proliferation by activating AKT signaling [95]. Wang et al. evaluated the correlation of *PIK3CA* mutations and first-line chemotherapy in 440 CRC patients, and concluded that CRC patients with *PIK3CA* mutations had a worse response to chemotherapy compared to those with wild type *PIK3CA* [96]. Finally, Taggart et al. analyzed 108 cases, utilizing IHC, of appendiceal adenocarcinomas and found three of them (3%) to be MSI-high, but none had a pathogenic germline mutation in any of the mismatch repair genes [97]. Raghav et al. evaluated the loss of the mismatch repair proteins in 35 cases of appendiceal adenocarcinomas and found 94% (33/35) to be microsatellite stable [77]. Another smaller study using PCR investigated MSI in 30 cases of appendiceal adenocarcinoma and found all of them to be microsatellite stable [56]. Findings of studies show, similar to LAMNs, that the MSI pathway appears to have a less important role in the carcinogenesis of appendiceal adenocarcinomas than in CRC.

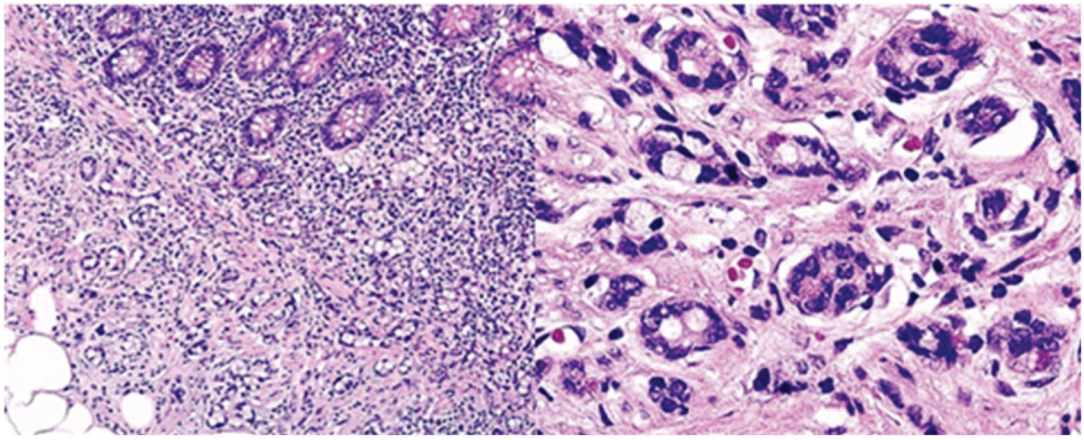

Figure 3. Appendiceal goblet cell carcinoma showing (A) the typical pattern of cells nests separated by stroma and (B) goblet cells and granules-containing cells reminiscent of paneth cells and endocrine cells [60].

Immunohistochemistry features and molecular pathology of goblet cell carcinomas

GCCs are rare but distinct subtypes of epithelial ANs that have both exocrine and endocrine features. These tumors consist of goblet like mucinous cells with variable numbers of endocrine cells and paneth-like cells that are usually arranged as tubules identical to intestinal crypts [9] (Figure 3). The WHO classification system (2010) identified GCCs as carcinoid tumors because they display some features of neuroendocrine tumors (NETs) and are relatively indolent neoplasms. However, the term, goblet cell carcinoid, is no longer recommended by pathologists because appendiceal goblet cell tumors behave more like adenocarcinoma than NETs [98]. Therefore, the WHO classification system (2019) recommended the terms appendiceal goblet cell adenocarcinoma or goblet cell carcinoma instead of goblet cell carcinoid, crypt cell carcinoma or microglandular carcinoma or adenocarcinoma [9]. GCCs appear to be more specific for the appendix in contrast to intestinal, tubular and signet-ring cell carcinomas that can be found throughout the intestinal tract [98]. GGCs are characterized histologically by the presence of small, rounded clusters that usually contain cells with mucin filled cytoplasm and small peripheral nuclei in contrast to the cells in NETs in which the cytoplasm is composed of lipid [60,99,100]. Moreover, a few cells within cell clusters in GCCs can have features of neuroendocrine cells or paneth cells (Figure 3). Thus, it has been suggested that GCCs may arise from pluripotent stem cells. GCCs can be classified into different groups/grades with various clinical behaviors and prognosis [60,101,102] (Supplementary Table F).

IHC markers are used to characterize GCCs and to compare these tumors to well-differentiated NETs and colonic adenocarcinoma. Like colon and appendiceal adenocarcinomas, GGCs are usually positive for CK20 (81–100%), CK19 (42–100%), MUC2 (88%), MUC1 (80%), CDX2 (100%), and CEA (100%) (Supplementary Table G). Moreover, while carcinoids are negative for CK7, the expression of this protein has been frequently reported in GCCs. Though the frequency rates vary, the overall frequency of CK7 expression in appendiceal GCCs was 56% (95% CI 43.5–68.3), which appears to be significantly higher than appendiceal adenocarcinoma (35%, 95% CI 26.7–44.4, $p < 0.005$) and LAMNS (31%, 95% CI 20.3–42.8, $p < 0.003$) (Supplementary Table G). In addition, the majority of well-differentiated NETs of the appendix are negative for CK20 protein, although some NETs, particularly tubular carcinoids, are positive for this protein [60,63,103].

Some authors found that the majority of GCCs were negative for neuroendocrine markers [60,104], while others have shown various rates; for example, chromogranin A (CgA) was reported to be expressed in 12–91% of GCCs cases, and synaptophysin, in 18–86% (Supplementary Table G). However, expression of these markers was limited to cells with eosinophilic cytoplasm that are morphologically similar to neuroendocrine cells [99,105]. In addition, the pattern of positivity for neuroendocrine markers in GCCs is patchy and focal, in contrast to carcinoid tumors where it is diffuse and strong [106]. Thus, the expression of neuroendocrine markers in appendiceal GCCs has been shown to be more similar to their expression in colonic adenocarcinoma than to well-differentiated NETs of the appendix.

Gui et al. investigated the morphological characteristics of different types of ANs and concluded that the protein expression of GCCs was more similar to adenocarcinoma than to classical NETs. Like MACs, overexpression of MUC1 has been observed frequently in GCCs, and has been associated with more aggressive

tumor biology [106]. IHC markers show that up to 31% of GCC cases have mutations of the P53 protein. However, in contrast to appendiceal adenocarcinoma, studies consistently show normal cytoplasmic membranous expression of E-cadherin and nuclear expression of β-catenin proteins in GCCs, suggesting that the metastatic capacity of these neoplasms is lower than adenocarcinoma (Supplementary Table G).

Data on molecular alterations in GCCs shows that mutations in *KRAS* (0–13%), GNAS (6%), *BRAF* (4–11%), *APC* (0–2%), *PIK3CA* (0–2%), RB1 (0–4%), and *EGFR* are either absent or detected in a minority of the cases (Supplementary Table H). The data indicates that carcinogenesis in these tumors follows a different pathway from that involving *RAS* oncogenes. This suggests that selective inhibitors such anti-EGFR agents should be considered for GCC patients with wild-type *KRAS* and *GNAS*. However, *P53* mutations have been reported in up to 31% of GCCs, suggesting a potential role of this gene in the pathogenesis of these carcinomas (Supplementary Table H). In addition, while Ang et al. found *SMAD4* mutations in 19% of GCC cases, Wen et al. and Stancu et al. reported that these mutations were not detected in any cases (Supplementary Table H) [81,107,108]. Therefore, the role of *SMAD4* in the pathogenesis of GCCs remains unclear. Jesinghaus et al. found mutations in *USP9X*, *NOTCH1*, *CTNNB1*, and *TRRAP,* which are Wnt signaling-associated genes, in three GCCs. Moreover, the chromatin-modifier gene, *ARIDIA,* has been observed in up to 23% of GCC cases (Supplementary Table H) [94]. Finally, as with other epithelial appendiceal cancers, MSI does not appear to have a role in the development of GCCs, as defective mismatch repair genes are rarely found in these tumors [94,97]. Overall, there is limited data regarding the molecular pathology of GCCs and the number of samples investigated in studies is small. However, the available data suggest that GCCs have distinct molecular and immunohistochemical features and that they have more characteristics in common with adenocarcinoma than classical NETs (Supplementary Table H).

Immunohistochemistry features and molecular pathology of appendiceal neuroendocrine neoplasms

Appendiceal neuroendocrine neoplasms (NENs) are diverse epithelial tumors that include poorly differentiated neuroendocrine carcinoma (NECs) and well-differentiated NETs with neuroendocrine differentiation. Mixed neuroendocrine-non-neuroendocrine neoplasms (MiN-ENs) are neoplasms that contain ≥30% each of epithelial and neuroendocrine cell types, and each cell type is immunohistochemically and morphologically recognizable as a discrete component [9]. The WHO classification system (2019) considers mixed adenoneuroendocrine carcinoma as a comparable term to MiN-ENs [9]. True appendiceal MiN-ENs are rare and comparable to MiN-ENs of the colon, and are currently considered as an adenocarcinoma subtype, not a type of NEN of the appendix [109]. Brathwaite et al. reported that, like appendiceal adenocarcinoma, MiN-ENs are typically positive for CK20, CDX2, SATB2, and CEA markers. They also found that CK7 protein was expressed in 35% of MiN-ENs cases [110]. In the genetic profile of MiN-ENs, Jesinghaus et al. found *P53*, *BRAF*, and *KRAS* mutations in 47%, 37%, and 21% of cases, respectively. Almost all cases with *BRAF* mutations were wild-type for *KRAS*. These findings show that *BRAF* mutations are more common in MiN-ENs than in other ANs and in conventional colorectal adenocarcinoma [111].

While NECs can be found throughout the appendix, NETs typically occur in its tip. In addition, like appendiceal adenocarcinomas, while patients with NECs often present with advanced disease and have an aggressive clinical course, 80% of NETs are diagnosed incidentally during surgery for appendicitis [9]. IHC studies have shown that in contrast to other ANs, NENs tend to be less positive for CK20 (0–32%) [60,61,63] and are often negative for CK7 (0–11%) [38,75,77], SATB2 (4%) [112] and β-catenin (0%) [60]. These findings clearly show the differences between IHC features of NENs and GCCs. However, appendiceal NENs are typically positive for CgA (100%) [60,63,113], CDX2 (86–100%) [114–116], synaptophysin (79%) [60], CK19 (80%) [61], and CD99 (70%) [117]. The molecular pathology of NENs is largely unknown and there is limited data regarding the molecular profile of these neoplasms. Nevertheless, NENs seem to lack the mutational changes common in other colorectal neoplasms or ANs. Borazanic et al. detected *KRAS*, *GNAS*, *SMAD4,* and *P53* mutations in 9%, 3%, 13%, and 11% of NET cases, respectively. However, three other studies reported no *KRAS* in any NET cases [38,107,113], while Ramnani et al. detected *P53* mutations in 44% of cases. In addition, Wen et al. reported no *APC*, *BRAF*, *SMAD4,* and *PIK3CA* mutation in any NET cases [107,113].

It is important to note that a systemic review has showed that out of nineteen studies related to ANs, three used laser microdissection, thirteen studies used manual microdissection and the other three reaming studies used macrodissection [78]. The review also showed that PCR, Sanger sequencing and next-

generation sequencing were used by 28%, 27%, and 9% of the studies, respectively, for identifying somatic mutations. The differences in specimen retrieval and sequencing methods used in these studies have resulted in inconsistency in research findings regarding the proportion of somatic mutations in ANs [78].

Conclusion

ANs are a heterogeneous group of tumors with a rising incidence. There is ongoing debate regarding a universally accepted classification system for these neoplasms. The findings of several studies on the IHC features and molecular pathology of ANs are inconsistent. This is probably because of differences in the number of samples and their collection and preparation as well as the lack of a universally accepted classification system for ANs. However, the literature shows that epithelial ANs are typically positive for the expression of MUC2, CK20, and CDX2 and that the expression of SATB2 protein could be used as a biomarker for appendix tumor origin. Though the molecular pathogenesis of ANs has yet to be classified, LAMNs tend to be *KRAS* and *GNAS* mutated, but are usually wild- type for *BRAF*, *APC*, and *P53*. Conversely, appendiceal adenocarcinomas are usually found with mutations in *KRAS*, *GNAS*, *P53*, *PIK3CA,* and *APC* as well as significant nuclear expression of β-catenin, loss of nuclear or nuclear and cytoplasmic expression of SMAD4, and loss of cytoplasmic membranous expression of E-cadherin. Keratin and mucin markers are commonly expressed in GCCs, which tend to be wild-type for *KRAS*, *GNAS*, *APC*, and *PIK3CA* but frequently are mutated in *P53* and chromatin-modifier genes. In addition, NENs are typically negative for CK7 and SATB2 as well as seeming to lack the mutations in common cancer-associated genes including *APC*, *BRAF*, *SMAD4,* and *PIK3C*. Moreover, MSIs are rarely detected in epithelial ANs and do not seem to have a role in the pathogenesis of these tumors. Finally, hereditary preposition may have a role in the development of ANs because heterozygous *CTNNβ1*, *NOTCH1,* and *NOTCH4* germline mutations have recently been identified in low and high grades ANs.

Author contributions

RM contributed to the study concept and design, data collection and interpretation, and wrote the manuscript. JY, TP contributed to the study concept and design and interpretation of data, and critical review of the manuscript. JH, GTR, PH, WU, MH contributed to the interpretation of data and critical revision of the manuscript. All authors read and approved the final manuscript.

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Supplementary Table A: Immunohistochemistry features of low-grade mucinous appendiceal neoplasms (LAMNs)

Proteins	No. of samples	% Positive	Author
CK7	25	36	(25)
	16	31	(118)
	12	25	(119)
	7	14	(26)
CK20	7	100	(26)
	16	94	(118)
	25	92	(25)
	10	90	(60)
CDX2	7	100	(26)
	25	92	(25)
ER	27	0	(37)
	25	0	(25)
	7	0	(26)
PAX8	25	0	(25)
	24	0	(28)
	7	0	(26)
SATB2	4	100	(28)
	25	96	(25)
	7	100	(26)
MUC2	25	92	(25)
MUC1	25	0	(25)
MUC5AC	25	72	(25)
P53	10	0	(30)
	11	0	(29)
Math1	10	100	(60)
SMAD4	42	0	(65)
β-catenin	11	73	(29)
	25	0	(25)
cKIT	5	0	(37)
PGP	6	83	(37)
TOPO1	7	57	(37)

Supplementary Table B: Molecular pathology of low-grade mucinous appendiceal neoplasms (LAMNs)

Gene	No. of samples	% Positive	Author
KRAS	32	94	(31)
	15	53	(10)
	11	27	(29)
	31	100	(38)
	13	92	(39)
	10	80	(30)
	18	83	(37)
	9	100	(49)
	69	61	(52)
	29	90	(47)
	8	100	(84)
	21	90	(36)
GNAS	23	35	(120)
	32	50	(31)
	15	53	(10)
	13	62	(39)
	10	50	(30)
	7	57	(37)
	29	69	(47)
	8	63	(84)
	21	62	(36)
APC	15	33	(10)
	31	0	(38)
	7	0	(37)
	13	0	(39)
	8	0	(84)
P53	11	27	(29)
	23	9	(10)
	10	0	(30)
	7	0	(37)
	9	0	(49)
	8	0	(84)
	21	10	(36)
SMAD 4	10	20	(30)
	7	14	(37)
	8	0	(84)
	21	10	(36)
PTEN	21	0	(36)
	8	0	(84)
	7	0	(37)
CTNNB1	11	18	(29)
BRAF	21	0	(36)
	8	0	(84)
	13	0	(37)
PIK3CA	21	0	(36)
	8	0	(84)

Supplementary Table C: Immunohistochemistry features of mucinous appendiceal adenocarcinoma

Proteins	No. of samples	% Positive	Author
CK7	27	37	(71)
	26	31	(58)
	18	28	(59)
	14	29	(121)
	14	50	(70)
	5	40	(119)
CK20	40	98	(57)
	27	96	(71)
	26	96	(58)
	18	100	(59)
	14	100	(70)
	14	100	(121)
CK8	18	100	(59)
CK19	18	100	(59)
MUC2	26	96	(58)
	26	100	(71)
	18	100	(59)
	14	100	(70)
	7	100	(25)
	15	67	(69)
	108	71	(122)
MUC1	18	17	(59)
	14	14	(70)
	7	0	(25)
	108	47	(122)
MUC5AC	18	67	(59)
	15	40	(69)
	14	64	(70)
	7	86	(121)
	7	43	(25)
	108	50	(122)
SATB2	40	83	(57)
	7	86	(25)
CDX2	40	93	(58)
	26	93	(57)
EGFR	19	68	(37)
PGP	253	61	(37)
Villin	40	95	(57)
cKIT	160	18	(37)
P53	15	40	(69)
	5	40	(29)
PAX8	26	0	(58)
	7	0	(25)
ER	291	0	(37)
	7	0	(25)

β-catenin	27	12	(71)
	5	40	(29)
	15	60	(69)
TOPO1	279	61	(37)
	44	70	(85)
COX2	49	61	(77)
	5	100	(123)
MUC6	18	0	(59)
	14	14	(70)
CK14	18	0	(59)
E-Cadherin	15	47	(69)
	14	29	(70)
Cyclin D1	15	87	(69)
SMAD4	67	19	(52)
PTEN	44	88	(85)

Supplementary Table D: Molecular pathology of mucinous appendiceal adenocarcinoma

Gene	No. of samples	% Positive	Author
KRAS	263	65	(37)
	320	56	(81)
	111	61	(124)
	16	50	(56)
	19	84	(125)
	5	60	(29)
	108	55	(77)
	44	64	(85)
	81	54	(52)
	10	70	(47)
BARF	239	2	(37)
	19	0	(125)
	50	4	(77)
	44	9	(85)
	10	0	(47)
APC	88	7	(37)
	320	6	(81)
	44	16	(85)
	10	10	(47)
GNAS	80	34	(37)
	320	52	(81)
	19	37	(120)
	10	10	(47)
P53	82	24	(37)
	320	33	(81)
	5	20	(29)
	44	57	(85)
	10	50	(47)
SMAD4	86	15	(37)
	320	23	(81)
	44	20	(85)
	14	21	(126)
	10	10	(47)
ARID1A	44	15	(85)
	320	8	(81)
PTEN	83	1	(37)
PIK3CA	114	6	(37)
	10	20	(47)
RB1	320	2	(81)
RNF43	44	7	(85)
RB1	320	2	(81)

Supplementary Table E: Molecular pathology of non-mucinous appendiceal adenocarcinoma

Gene	Number of samples	% Positive	Author
KRAS	68	47	-37
	208	56	-81
	4	75	-56
	7	43	-94
BRAF	66	8	-37
	7	14	-94
APC	22	32	-37
	208	17	-81
	7	14	-94
ATM	23	0	-37
	7	14	-94
GNAS	23	17	-37
	208	25	-81
	13	15	-120
PIK3CA	29	17	-37
	7	29	-94
P53	22	32	-37
	208	47	-81
	7	71	-94
SMAD4	23	21	-37
	208	18	-81
	7	0	-94
	8	0	-126
PTEN	22	4.5	-37
	7	14	-94
CTNNB1	7	14	-94
	10	0	-126
ARID1A	208	11	-81
BRACA2	8	38	-37

Supplementary Table F: Classification of goblet cell carcinomas (GCCs)

Group (No. of patients)	Pathological Features	Prognosis	Author
A- Goblet cell carcinoids (22), B- Mixed carcinoid-adenocarcinomas (32)	**A-** Confined to the appendix and mesoappendix, circumferentially surrounded the appendiceal lumen and were often not suspected grossly; histologically, they were often mixed with small crypt-like glands and were serotonin positive. **B-** Spread into the cecum or adjacent viscera at the time of diagnosis and had a large carcinomatous pattern with areas of mucinous, signet-ring, or single-file structure, in addition to goblet cell or insular carcinoid.	**A-** All 22 with follow-up (mean, 19 months) were without metastasis whether or not right hemicolectomy was performed. **B-** All patients had right hemicolectomies, and all but two with follow-up died of the disease (mean, 16 months).	(99)
A- Typical GCC (14), **B**- Adenocarcinoma ex GCC, signet ring cell type (11), **C**- Adenocarcinoma ex GCC, poorly differentiated adenocarcinoma type (6)	**A-** Well-defined goblet cells arranged in clusters or cohesive linear pattern, minimal cytologic atypia, minimal to no desmoplasia, minimal architectural distortion of the appendiceal wall, degenerative change with extracellular mucin is acceptable. **B-** Goblet cells or signet ring cells arranged in irregular large clusters, but lack of confluent sheets of cells, discohesive single file or single-cell infiltrating pattern, significant cytologic atypia; desmoplasia and associated destruction of the appendiceal wall. **C-** At least focal evidence of, goblet cell morphology, A component (>1 low power field or 1 mm2) not otherwise distinguishable from a poorly differentiated, adenocarcinoma, which may appear as either (a) gland forming, (b) confluent sheets of signet ring cells, or (c), undifferentiated carcinoma	**A-** 5- years disease-specific survival (100%), metastasis at the time of presentation (33%), **B-** 5- years disease-specific survival (36%), metastasis at the time of presentation (88%), **C-** 5- years disease-specific survival (0%), metastasis at the time of presentation (100%)	(106)
A- Low-grade GCC (55)	A histologic scoring system	**A-** good prognosis	(127)

B- high-grade GCC (23)	was created whereby 1 point was given for the presence of each of cytologic atypia, peritumoral stromal desmoplasia, and solid growth pattern (score ranges from 0 to 3); patients were divided into 2 groups: **A-** low grade with histologic score 0 or 1, **B-** high grade with histologic score 2 or 3.	with median and 10-year overall survival of 51.0 months and 80.5%, respectively, **B-** poor prognosis with median and 10-year overall survival of 16.5 months (P = .006) and 0% (P < .001), respectively.	
A- Tumors with less than 25% of adenocarcinoma components (23), **B**- Tumors with 25% to 50% adenocarcinoma components (27), **C**- Tumors with more than 50% adenocarcinoma components (24).	Adenocarcinoma was considered to be present when: there were individual dyshesive cells, solid sheets of cells, infiltrative cords of cells or a complex glandular architecture. The presence of destructive invasion or desmoplasia. All of the available sections of the appendiceal tumor were evaluated for the presence of adenocarcinomatous and GCC components, and the percentage of an adenocarcinomatous component was estimated from the mean percentage of all sections.	The mean (standard deviation) overall survival for patients in groups **A, B,** and **C** was 83.8 (34.6) months, 60.6 (30.3) months, and 45.6 (39.7), respectively.	(97)
A- Low-grade goblet cell adenocarcinoma (47), **B**- Intermediate-grade goblet cell adenocarcinoma (22), **C**- High-grade Goblet Cell Adenocarcinoma (57)	**A-** Low-grade goblet cell adenocarcinoma was characterized by >75% of the tumor having clustered or tubular growth and with up to 25% high-grade component. **B** and **C**- Tumors with 50% to 75% tubular growth were classified as intermediate-grade goblet cell adenocarcinoma, and tumors with <50% tubular growth were classified as high-grade goblet cell adenocarcinoma.	Median overall survival was 204, 86, and 29 months for low-grade, intermediate-grade, and high-grade tumors, respectively	(128) (9)

Supplementary Table G: Immunohistochemistry features of goblet cell carcinomas (GCCs)

Proteins	No. of samples	% positive	Author
CK20	19	100	(129)
	18	100	(60)
	17	100	(117)
	16	81	(130)
CK7	19	42	(129)
	18	44	(63)
	17	71	(117)
	16	56	(60)
	9	78	(62)
CK19	19	42	(129)
	18	100	(61)
	9	100	(62)
MUC2	83	88	(131)
MUC1	83	80	(131)
SATB2	19	100	(129)
CDX2	26	100	(129)
	19	100	(129)
	7	100	(94)
Chromogranin A	83	86	(131)
	19	63	(129)
	9	33	(62)
	7	57	(132)
	11	55	(105)
	16	38	(104)
	18	89	(133)
	22	91	(113)
	16	44	(60)
Synaptophysin	83	83	(131)
	19	84	(134)
	9	56	(62)
	7	86	(132)
	15	40	(104)
	18	56	(133)
	16	75	(60)
P53	83	21	(131)
	49	8	(106)
	22	0	(113)
	16	0	(108)
	7	11	(62)
	7	29	(132)
	16	31	(60)
NSE	18	72	(63)
	11	91	(105)
	18	72	(133)
p63	18	0	(63)
β-catenin	18	0	(133)

	11	0	(135)
	49	0	(106)
E-cadherin	18	0	(133)
	6	0	(62)
	11	0	(135)
	49	0	(106)
CD99	18	78	(117)
Somatostatin	11	18	(105)
CD56	18	44	(133)
CEA	18	100	(133)
	16	100	(60)

Supplementary Table H: Molecular pathology of goblet cell carcinomas (GCCs)

Gene	No. of samples	% Mutation	Author
KRAS	84	13	(81)
	16	0	(108)
	18	0	(136)
	18	0	(94)
	14	0	(137)
	22	0	(113)
	13	0	(107)
	16	6	(60)
	53	8	(138)
BRAF	16	6	(107)
	18	11	(94)
	13	8	(107)
	53	4	(138)
P53	84	33	(81)
	16	0	(108)
	16	25	(113)
	18	6	(94)
	13	8	(107)
	50	24	(138)
APC	84	2	(81)
	13	0	(107)
	18	0	(94)
	53	2	(138)
SMAD4	84	19	(81)
	18	6	(94)
	13	0	(107)
	16	0	(108)
	53	9	(138)
ARID1A	84	15	(81)
	11	0	(94)
	13	23	(107)
	13	15	(138)
EGFR	14	0	(137)
	18	0	(94)
GNAs	84	6	(81)
	53	4	(138)
PTEN	18	0	(94)
RB1	84	4	(81)
	18	0	(94)
PIK3CA	18	0	(94)
	13	0	(107)
	53	2	(138)
CTNNB1	18	0	(94)
	16	0	(108)

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See original article for earlier references.

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Chapter Seven: Conclusion and future direction

There has been an alarming rise in the incidence and mortality rates of YOCRC and the drivers of this upward trend are currently unexplained. A better understanding of the risk factors related to YOCRC could allow for personalised screening, particularly for those under 50 years deemed to be an elevated risk.

In this project, an observational study was performed to investigate the association of personal history of T2D with the risk of developing YOCRC. Personal rate of T2D in CRC patients aged < 55 years old was significantly higher compared to clear colonoscopy controls. In accordance with several previous studies (98, 378, 379), these findings show that patients with T2D might be at a higher risk of developing YOCRC. In contrast, other studies found no significant link between these two diseases (15, 17, 25, 40-42). Rosato et al. conducted a case-control study and found no significant association between diabetes and YOCRC risk (OR= 0.94; 95% CI: 0.2–4.47) (244). Recently, Archambault et al. using data pooled from 13 population-based studies found no significant association between diabetes in patients <50 years old and the risk of YOCRC (OR = 1.25; 95% CI: 0.93-1.68; P-value = 0.14) (380). Therefore, there is conflicting evidence in the current literature as to whether T2D in young adults increases the risk of colorectal polyps or YOCRC. Since the genetic background contributes to developing both diseases, the association of T2D predisposing genes with the risk of developing YOCRC was investigated, and no pathogenic germline variants were identified in genes related to *T2D*. Studies have reported a T2D_independent association of TCF7L2_rs7903146 with the risk of developing colon cancer (186, 381). This increase in CRC risk was also independent of a BMI (186, 381). Another multiethnic study found that *THADA*_ rs7578597, *JAZF1*_ rs864745, *KCNJ11*_ rs5219 and *TSPAN8*_ rs7961581 were associated with CRC risk (382). Currently, the underline molecular mechanisms of these variants in CRC carcinogenesis are largely

unknown and more clinical and experimental studies are required to examine the implication of common risk loci for T2D in pathogenic of CRC.

Some studies have also shown a higher prevalence of colorectal polyps, precursors to CRC, in T2D patients compared to those without this disease. Elwing et al. (383) conducted a case-control study and found that there was an independent association between T2D and the presence of advanced adenomas and adenomas. A colonoscopy study of patients aged 40-49 years with and without diabetes in the USA found a three-fold increase in risk for adenomatous polyps in those with diabetes (384). Furthermore, Joh et al. conducted a large prospective cohort study in the US and reported a significant association between sugar-sweetened beverages and sugar intake during adolescence with the increased risk of total and advanced adenomatous polyps (385). Another report compared women with and without T2D and showed that diabetes was a significant independent predictor of significant adenomas and any adenomas (P-value = 0.05) (34). More recently, Ottaviano et al. reported a significant association between adenoma detection rate and T2D in the multivariable analysis (OR = 1.49; 95% CI: 1.13–1.97; P-value = 0.0047), and this link was higher in those who were not on diabetes medications (OR = 2.38; 95% CI: 1.09–5.2; P-value = 0.03) (35). However, both of these studies did not investigate the association of T2D with the risk of colorectal polyps in patients <50 years old (30). A number of other reports showed no significant association between T2D and colorectal polyps (9, 29-32). Recently, Hsu et al. conducted a nationwide population-based study and showed that in the multivariate analysis, there was no significant difference in the risk of developing colorectal polyps in patients with and without T2D (HR = 1.04; 95% CI: 0.98-1.10; P-value = 0.159). Reasons for the discordant findings regarding the risk of CRC and colorectal polyps in young adults could be related to the differences in the study cohort size, type of diabetes, duration of diabetes and follow up time, medications (such as metformin, insulin therapy, and anti-inflammatory drugs), the difference in the primary colonoscopy outcome (CRC or colorectal polyps) or most importantly study participants and control of potential

confounders. Overall, the findings of studies suggest that further research is needed to investigate the role of T2D in developing CRC and colorectal polyps in young adults.

Currently, half of the YOCRC heritability is unknown (129). This missing heritability of YOCRC might be explained by determining the prevalence and spectrum of germline variants in both CRC and non-CRC-associated predisposing genes as well as in genes associated with moderate-penetrance cancer risk. Identifying genetic risk factors is vital for the prevention and early detection of the disease. WES for the patients in this study was performed and analysed by using innovative bioinformatics pipelines. Approximately one in five cases had a P/LP variant in at least one cancer-predisposing gene. One in 20 YOCRC cases had P/LP variants in BC/OC related genes. Among germline variant carriers, only ~ 16% had FDR with CRC, and three patients with germline variants in polyposis associated genes showed no polyposis. The findings suggest that patients with germline variants in BC/OC related genes might be at higher risk of developing other types of cancers including YOCRC and these patients and their relatives might need to receive screening earlier than the general population. Supporting the results of other studies (111, 123, 129), it was shown that family history and phenotypes often do not predict genotypes. Using family history to identify patients at higher risk for hereditary CRC syndromes mainly depends on physicians to be familiar with the guidelines and diagnostic criteria of these syndromes and to have time to create a 3-generation pedigree. A strategy that still has limitations even when it is perfectly accurate (3). In addition, studies have shown that around one in third of patients with classic FAP do not have a family history with the disease and the polyps arise due to novel germline variants in APC. Hampel et al. reported that one in four patients with LS is missed by limiting tumour analysis to those who fulfill Bethesda criteria (4). Therefore, given the limitations of identifying hereditary CRC risk factors by family history and phenotype, multigene panel testing is warranted for all YOCRC patients.

Germline variants in *RNF43*, a tumour suppressor gene, have been associated with SPS. However, these variants have been rarely reported in families with SPS and the hereditary role of *RNF43* in CRC tumourigenesis has yet to be explained. In this project, two CRC patients within a single-family were identified with a likely-pathogenic germline splice variant in *RNF43:c.375+1G>A.* A splicing study was performed to confirm the pathogenicity of this identified variant. Tumours from both carriers were *BRAF*^{V600E}-mutated and MMR-proficient, indicating that the CRCs arose in sessile serrated lesions. These findings add further weight to the potential hereditary role of *RNF43* in colorectal tumorigenesis.

In addition, in this project, a retrospective analysis on national data to investigate the incidence and mortality rates of ANs in Australia was carried out. Similar to the trends observed in other countries (5), the data showed that the incidence and mortality rates of ANs are alarmingly rising in Australia. Opposite to CRC, the increase in incidence and mortality rates of ANs is not limited to young adults but also in elderly individuals. While the drivers of this observation remain largely unknown, increasing the use of CT scanning and improvements in ANs reporting may have contributed in part to the apparent rise in the incidence and mortality rates of these tumours in Australia.

Overall, the findings show that further research is needed to identify the contributing factors of YOCRC and ANs. The majority of YOCRC patients do not have any known pathogenic or likely pathogenic germline variants associated with CRC (111, 123, 129) and experts have recommended exercising cautions when interpreting these results because this could be due to the various possibilities including A) these individuals do not inherit the familial germline variants; B) they might carry variants that are not detectable by current technology; or C) they might carry germline variants in other genes not traditionally associated with the hereditary CRC risk but still increase the risk of developing cancer (3). Therefore, there is a need to identify whether patients with germline variants in non-CRC-associated genes including

BC/OC-associated genes might need to receive screening earlier than the general population. In addition, the majority of YOCRC patients with FDR of the disease also do not have any germline variants in any known cancer-predisposing genes. Therefore, the impact of family history on YOCRC needs further explanation to allow for identifying patients with a higher risk of developing this malignancy. It is also worth noting that WES has several disadvantages including not covering all of the exome, low sensitivity for structural variations, and not sequencing of non-coding intron regions. A study reported that approximately 3% of coding variants were detected by whole-genome sequencing but not by WES (386). Some patients could have pathogenic germline variants in cancer predisposing genes but not detected due to these limitations of WES. Another major factor affecting the clinical utility of genetic tests for cancer predisposition is the ability to provide an accurate actionable classification. However, a large number of variants detected in cancer predisposing genes are classified as a variant of unknown significance (VUS) and therefore, cannot be used for clinical purposes. Many patients in this project had more than one VUS in different cancer predisposing genes, particularly in MMR genes and *POLE*. Further functional studies are needed to evaluate the role of VUS in cancer predisposition. Comprehensive understanding is currently limited regarding the prevalence of germline variants in minority populations and thus, further investigations are also required in this area. Finally, a number of common genetic risk loci for CRC have recently been identified using GWAS approach (143). Confirming the association of these variants with the CRC risk would help to improve risk prediction models within the average risk population and, ultimately, offer more intensive personalized surveillance to those at highest risk (144). However, the vast majority of currently identified SNPs lack known functional significance. Therefore, whether they are causal variants or just surrogates that are in linkage disequilibrium with the functional loci remains largely unknown. Thus, more functional studies are needed to evaluate the causality of these common variants in CRC (143, 144).

Beyond hereditary risk factors, a strong birth cohort effect (150) indicates that lifestyle and environmental-related risk factors, mainly including exposures during early life, such as caesarean birth, gestational diabetes, lack of breastfeeding and childhood antibiotics, may play a role in developing YOCRC (101). These exposures can result in genetic and epigenetic changes in epithelial cells of the colon and rectum, as well as influence the gut microbiota. Therefore, life-course epidemiological studies are needed, and notably, these investigations should be combined with the prospective collections of appropriate bio-specimens, advanced "omics" technologies and bioinformatics, and comprehensive analysis of the gut microbiome (101, 264). Finally, the genetic and environmental/lifestyle-associated risk factors for developing ANs remain largely unknown highlighting that further work is also required to identify those who are at higher risk of developing ANs and explain the drivers of this upward trend in increasing the incidence and mortality rates of this malignancy in an organ contiguous with the large bowel.

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Appendix

Statement of Authorship

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Contribution to the Paper	First author and corresponding Author Study concept and design, collection and interpretation of data, and wrote the manuscript.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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