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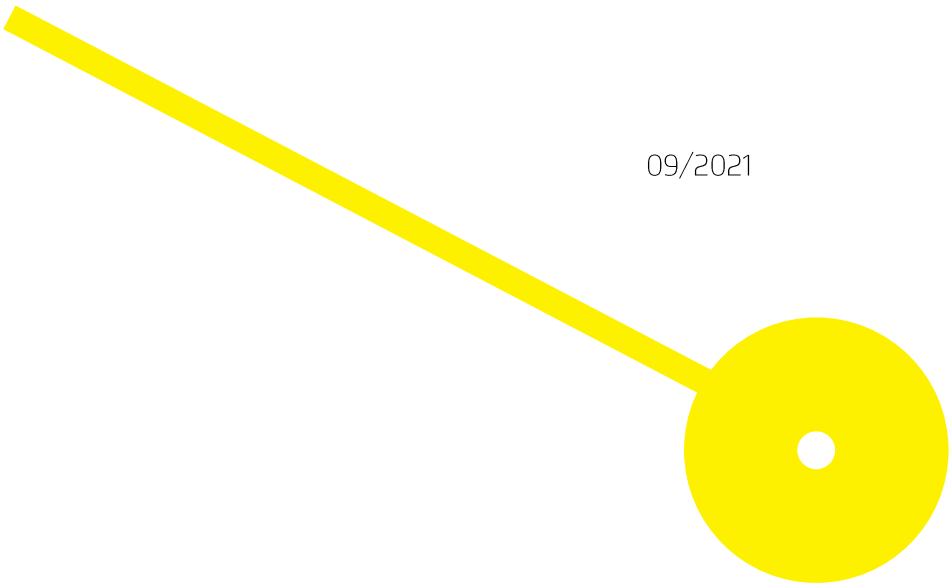
Next generation sequencing of a custom gene panel to improve the diagnosis of patients with inherited predisposition to colorectal polyposis

Miguel Ângelo Sota Porto da Silva



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P.PORTO

ESCOLA SUPERIOR DE SAÚDE

Next generation sequencing of a custom gene panel to improve the diagnosis of patients with inherited predisposition to colorectal polyposis

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Resumo

As síndromes associadas a polipose são um grupo de patologias com predisposição para cancro. Em geral, os mecanismos genéticos estão bem estabelecidos, mas nos últimos anos variantes patogénicas num grupo de genes emergentes foram associadas a este fenótipo.

O objetivo principal deste estudo foi pesquisar variantes patogénicas em genes emergentes através de um painel personalizado de sequenciação de nova geração (NGS) numa série retrospetiva de 189 indivíduos com história pessoal/familiar de polipose previamente negativos para variantes patogénicas nos genes *MUTYH* e/ou *APC*. Foi também completado o estudo do gene *MUTYH* em todos os doentes previamente estudados apenas para as variantes patogénicas fundadoras/recorrentes.

Um total de 18 variantes (15 diferentes) foram encontradas em 17 indivíduos, sete patogénicas (seis diferentes, duas delas no mesmo doente) e 11 (nove diferentes) variantes de significado desconhecido (duas delas não descritas). Nenhum dos 79 casos previamente estudados apenas para as variantes patogénicas recorrentes do *MUTYH* apresentou variantes patogénicas nas restantes regiões codificantes desse gene.

Este estudo demonstra que variantes patogénicas em genes emergentes recentemente associados na literatura a polipose são raras.

Palavras-chave: Síndromes associados a polipose; cancro colorretal hereditário; variantes germinativas; sequenciação de nova geração; sequenciação de genes alvo

Abstract

Polyposis syndromes are a group of diseases predisposing to cancer. In general, the genetic mechanisms are well established, but in recent years several pathogenic variants in emergent genes have been associated to these phenotypes.

The main goal of this study was to search for pathogenic variants in emerging genes through a customized next-generation sequencing (NGS) panel in a retrospective series of 189 individuals with a personal/family history of polyposis previously negative for pathogenic variants in the *MUTYH* and/or *APC* genes. We also aimed to complete the study of the *MUTYH* gene in all patients (79 cases) previously studied only for the recurrent/founder pathogenic variants.

A total of 18 variants (15 different) were found in 17 patients, seven of them deleterious (six different, two of them in the same patient) and 11 (9 different) variants of uncertain significance (two of them novel). None of the 79 cases previously studied for only the recurrent pathogenic *MUTYH* variants presented pathogenic variants in the remaining coding regions of this gene.

This study demonstrates that pathogenic variants in the emerging genes recently associated in the literature with polyposis are rare.

Keywords: Polyposis syndromes; hereditary colorectal cancer; germline variants; next-generation sequencing; targeted sequencing.

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Abbreviature Index

ACMG – American College of Medical Genetics and Genomics

AFAP – Attenuated Familial Adenomatous Polyposis

AMP – Association for Molecular Pathology

BER – Base-excision-repair

BMP – Bone morphogenetic protein

CIMP – CpG island methylator phenotype

CIN – Chromosome instability

CNV – Copy number variations

CRC – Colorectal cancer

DNA – Deoxyribonucleic acid

dsDNA – Double stranded DNA

Dsh – Dishevelled

ED – Exonuclease domain

EDTA – Ethylenediamine tetraacetic acid

ExAC – Exome Aggregation Consortium

FAP – Familial Adenomatous Polyposis

gDNA – Genomic DNA

GLOBOCAN – Global Cancer Observatory: CANCER TODAY

gnomAD – Genome Aggregation Database

HMPS – Hereditary Mixed Polyposis Syndrome

HNPCC – Hereditary nonpolyposis colorectal cancer

HS – High Sensitivity

IPO-Porto – Instituto Português de Oncologia do Porto

LDP – Low density lipoprotein

LoF – Loss-of-function

LRP – Receptor-related protein

MAF – Minor allele frequency

MAP – *MUTYH* Associated Polyposis

MMR – Mismatch repair

MSI – Microsatellite instability

MSS – Microsatellite stability

NCCN – National Comprehensive Cancer Network

NGS – Next-Generation Sequencing

PCR – Polymerase Chain Reaction

SSLs – Sessile serrated lesions

TCF – T-cell factor

UMIs – Unique molecular identifiers

UTR – Untranslated Region

VUS – Variant of uncertain significance

WHO – World Health Organization

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1. Introduction

Cancer is characterized by an uncontrolled proliferation of abnormal cells, which tends to harm other functional cells and organs (Preston-Martin, Pike, Ross, Jones, & Henderson, 1990). A late diagnosis decreases the chance of a successful treatment, because malignant cells can already be spread over the rest of the body, which will result in death (Chambers, Groom, & MacDonald, 2002; Fidler, 1990).

A complex interaction between environmental and genetic factors is the cause of cancer. These environmental factors can be extrinsic (tobacco, drugs, air pollution, infection diseases, food, etc.) and intrinsic (hormonal changes, metabolic products, etc.) (Wogan, Hecht, Felton, Conney, & Loeb, 2004). The interaction between these factors results in DNA and chromosomal alterations that may affect several molecular pathways (Weinstein, 1988).

According to the World Health Organization (WHO), cancer is the leading cause of death in the world. In 2020, cancer was also the first or second cause of premature deaths (<70 years of age) in a great number of countries (Sung et al., 2021).

Epidemiology of cancer is essential for the establishment of preventive measures for cancer development, but also for the implementation of screening and diagnosis programs for early detection, in order to improve life expectancy (Mattiuzzi & Lippi, 2019; Montagnana & Lippi, 2017). It also allowed the development of a great number of studies, trying to find the inherited genetic causes of cancer development, including some uncommon causes that are nowadays emerging.

1.1. Colorectal cancer

1.1.1. Colorectal cancer epidemiology

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide, representing 10% of all new cases of cancer (Figure 1). In 2020, 1,900,000 new cases of CRC were estimated in the global population, being responsible for around 900,000 deaths worldwide, which corresponds to 9.4% of all cancer-related deaths (Sung et al., 2021).

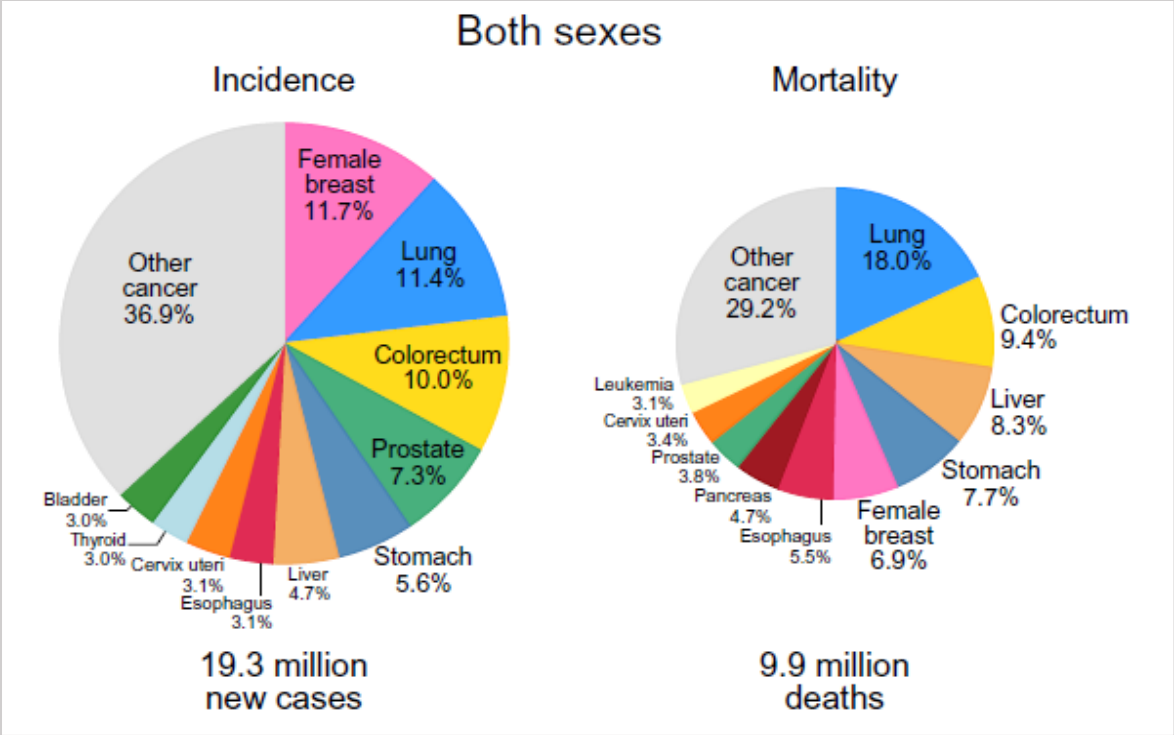

Figure 1 – The percentage of new cases and the mortality for the top 10 most common cancers in 2020 [Adapted from Sung et al 2021]

Colorectal cancer is more prevalent in men than in women. According to GLOBOCAN data, the estimated age-standardized incidence of male cases in 2020 is 1.44-fold increased, in comparison to female cases (Figure 2). The age-standardized incidence in both genders reveals that CRC is the fourth leading cause of new cases of cancer in the world and the third leading cause of cancer-related deaths (Sung et al., 2021).

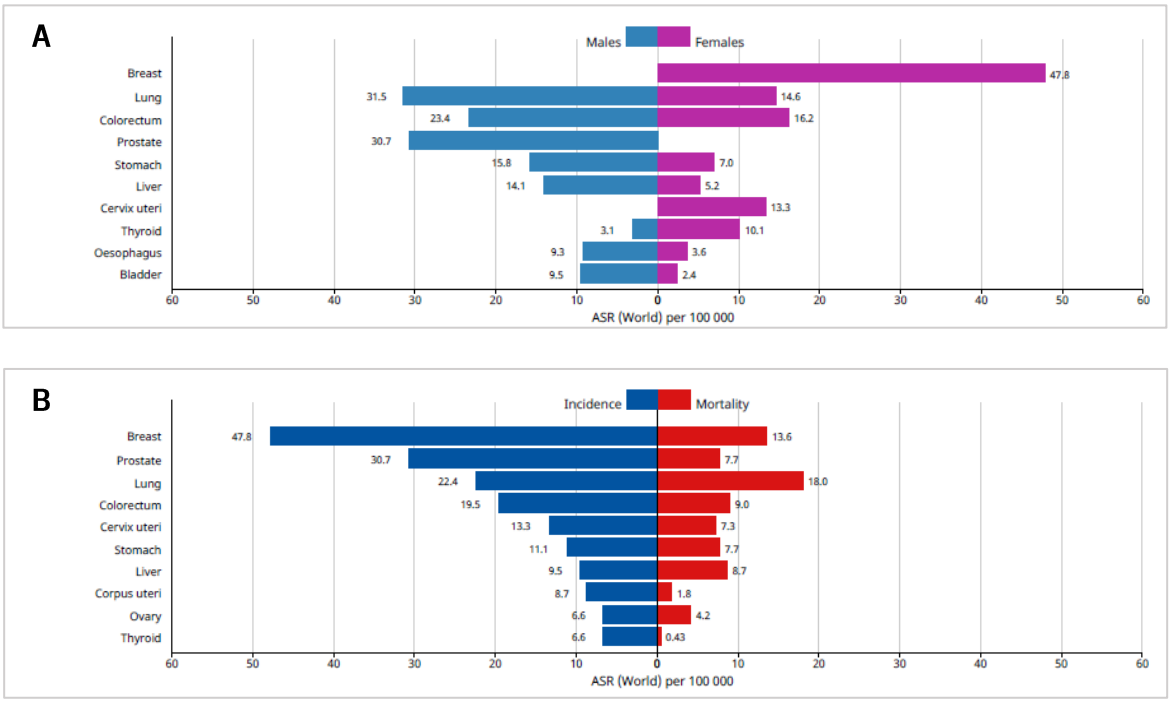

Figure 2 – Age–standardized incidence rates, top 10 cancers, in the world, per 100,000 inhabitants; A – Incidence rates per sex; B – Incidence and mortality rates [Adapted from GLOBOCAN, 2020]

In Portugal, according to GLOBOCAN, CRC represents the second leading cause of death as a consequence of malignant cancers, only behind lung cancer in males and breast cancer in females (GLOBOCAN, 2021; Sung et al., 2021). Although it is the second-leading cause of new cases of cancer in both males and females, when studying both sexes together it becomes the leading cause of new cancer cases (Figure 3).

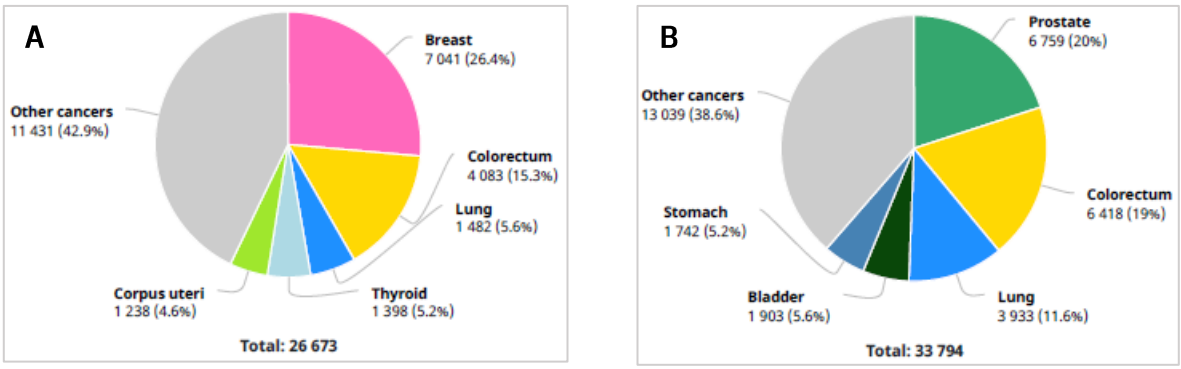

Figure 3 – Number of new cases of cancer in Portugal in 2020; A – females; B – males [Adapted from: Globocan, 2020]

1.1.2. Diagnosis and treatment

Most of CRC are asymptomatic during its early stages, being detected due to its symptoms only in advanced stages. However, some symptoms might indicate the presence of a lesion in the colon or rectum, like changes in bowel habits, rectal bleeding, anemia or abdominal pain (Buccafusca, Proserpio, Tralongo, Rametta Giuliano, & Tralongo, 2019). The clinical presentation of CRC is dependent on the location of the tumor and its stage. Colonic lesions are usually associated with occult bleeding, that can cause anemia and fatigue, while rectal tumors are able to originate more often hematochezia and bleeding (Ford et al., 2008; Labianca et al., 2013).

Screening for colorectal lesions and early stage CRC is essential for a fast and efficient treatment, avoiding fast development of advanced CRC. Since the end of last century, large screening programs have been implemented worldwide, which have been allowing a slow decline not only in mortality, but also in the incidence of CRC (Shaukat et al., 2021; Zauber et al., 2012). Since most CRC develop from adenomas, it is important to remove these precursor lesions before their transformation into malignancy (Cotton, Sharp, & Little, 1996).

Colonoscopy remains as the main strategy for CRC surveillance, diagnosis, and treatment, mainly because it is possible to find the exact location of lesions in the colon or the rectum, and also allows biopsy of the lesions or even their removal (Shaukat et al., 2021). Additionally, colonoscopy is in general considered a safe procedure, with a small number of risks that rarely occur (Feagins, 2019; Reumkens et al., 2016).

Whenever a more advanced CRC appears, surgery or chemotherapy might be required. In this cases, it is extremely important to know the classification of CRCs according to their local invasion depth (T stage), the involvement of lymph nodes (N stage) and the presence of metastases (M stage) (Edge & Compton, 2010). The definition of this TNM stage, combined with the clinical information, will provide the basic information for the therapeutic decisions.

In case of invasive nonmetastatic colonic and rectal cancers, surgical excision is usually the most beneficial treatment to reduce the probability of recurrence and to increase the survival rate (Sehgal & Coffey, 2014; Søndenaa et al., 2014). In high-risk stage II and stage III colonic cancers adjuvant systemic chemotherapy is recommended (Labianca et al., 2013). Usually, neoadjuvant therapy is recommended in rectal cancers (Schrag et al., 2014). In both cancers, it is important for patients to be in a follow-up program to detect early any recurrence that might occur.

1.1.3. Colorectal cancer carcinogenesis

The development of tumors can follow several pathways, depending on the capabilities acquired by the cells and tissues, which represent the several hallmarks of cancer: 1) sustaining proliferative signaling, 2) evading growth suppressors, 3) enabling replicative immortality, 4) activating invasion and metastasis, 5) inducing angiogenesis, 6) resisting to cell death, 7) deregulating cellular energetics, and 8) avoiding immune destruction. Two major characteristics facilitate the acquisition of any of these hallmarks, tumor-promoting inflammation and genome instability and mutation (Hanahan & Weinberg, 2011). While these hallmarks have been accepted in general, recent studies have been proposing a reclassification of these hallmarks (Fouad & Aanei, 2017) or the inclusion of new ones (Pavlova & Thompson, 2016).

During the last few decades, carcinogenesis models of CRC have been changing constantly, due to the uncovering of new genetic and epigenetic changes in several molecular pathways of the colonic epithelium. Initially, Fearon & Vogelstein proposed a model where a multi-step process, involving alterations in oncogenes and tumor suppressor genes, was the main cause for the evolution pathway to CRC (Fearon & Vogelstein, 1990). Since then, the understanding of carcinogenesis and tumorigenesis of CRC has improved and, today, there are at least three main molecular pathways associated to the development of CRC: microsatellite instability (MSI) (Gryfe et al., 2000), chromosome instability (CIN) (Lengauer, Kinzler, & Vogelstein, 1997), and CpG island methylator phenotype (CIMP) (Nazemalhosseini Mojarad, Kuppen, Aghdaei, & Zali, 2013). While this molecular classification seems to be accepted in general, other pathways might be included in the future, as the association between CRC and other genetic events have been described (Hu et al., 2019; Muzny et al., 2012).

In the MSI pathway, short tandem repeat DNA sequences show a general instability. In these cases, it is possible to find deleterious changes in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* or large deletions in the 3´of the *EPCAM* gene (Biller, Syngal, & Yurgelun, 2019), which cause a hypermutated phenotype in the tumor. Most of the inherited cases of CRC are related to this molecular mechanism (Lynch & de la Chapelle, 2003). The MSI phenotype is present in >90% of Lynch syndrome cases, which carry germline variants in the MMR genes, and also in around 15% of sporadic CRC cancers mostly due to *MLH1* silencing by promoter hypermethylation (Pellat et al., 2019; Woerner et al., 2010).

The CIN molecular pathway is mostly related to sporadic cases, and accounts for a large percentage of CRC (Grady & Carethers, 2008). In these tumors, large alterations in chromosome numbers or structure can be found, such as aneuploidy, deletions, insertions, or loss of heterozygosity, largely as consequence of defects in chromosomal segregation (Lengauer et al., 1997). Moreover, CIN tumors also have mutations in some oncogenes and tumor suppressor genes, namely, *APC, TP53, KRAS, BRAF*, or *PIK3CA* (Pino & Chung, 2010). Usually, CIN tumors are associated to microsatellite stability (MSS).

The CIMP pathway is characterized by hypermethylation in a large number of CpG islands in tumor suppressor genes promoters (Toyota & Issa, 1999). Frequently, CIMP tumors often presents hypermethylation of the *MLH1* promotor and *BRAF* mutations (Weisenberger et al., 2006). While CIN and MSI pathways are usually independent to each other, the CIMP pathway sometimes overlaps with the other two.

These pathways organize CRC according to its molecular and genetic characteristics, but it is also important to understand the histological categorization. There are three main groups of histological carcinogenic pathways in CRC: 1) adenoma–carcinoma sequence (Gloor, 1986); 2) serrated pathway (Jass, 2007); and 3) inflammatory pathway (Itzkowitz & Yio, 2004). Although cancer arises from different pathways, they share a common behavior through which CRC naturally evolves through time and it can be divided in four stages (Pitot, 1993): 1) initiation, 2) promotion, 3) progression, and 4) metastasis.

The adenoma–carcinoma sequence is the pathway by which most sporadic cases of CRC arise. In this pathway, cells of the colon epithelium start to develop into a small adenoma, which can progress and become larger, leading eventually to a state of malignancy (Leslie, Carey, Pratt, & Steele, 2002). This type of CRC development is mainly related to the CIN molecular status and can take many years for the progression to be observed.

Around 15% of CRC are formed from the serrated pathway. The progression in this pathway is similar to the observed in the adenoma–carcinoma sequence. In this case, small hyperplastic polyps start to be formed, and may progress into serrated sessile polyps (Fearon & Vogelstein, 1990). Usually these polyps are formed as a consequence of mutations in the oncogene *BRAF,* and also from alterations in methylation that can be associated to the CIMP molecular pathway (De Palma et al., 2019; Leggett & Whitehall, 2010).

A residual percentage (<2%) of sporadic cases emerge through inflammatory pathways. Although a rare event, there is a strong association between patients with inflammatory bowel

disease and progression to CRC. The evolution of this type of CRC begins by an indefinite dysplasia, which eventually can progress into a small dysplasia, larger dysplasia and finally cancer (Itzkowitz & Yio, 2004).

The majority of new CRC cases are sporadic, as a consequence of environmental factors, but 2–8% are associated to germline variants, of which 20% arise in individuals with <50 years of age (AlDubayan et al., 2018; Siegel, Miller, & Jemal, 2019). A large number of the inherited cases of CRC are related to Lynch syndrome, while polyposis syndromes (mainly FAP and MAP) account for a smaller percentage of all inherited CRC cases (Hampel et al., 2008; Jasperson, Tuohy, Neklason, & Burt, 2010).

1.1.4. Polyposis

Polyps are a consequence of a proliferative lesion in the epithelium, presenting an abnormal projection of this layer over the surrounding tissue (Park & Lauwers, 2008). As cells grow and divide through time, some might acquire mutations, resulting in a continuous growth and proliferation, even when new cells are not necessary in the tissue, forming the several types of polyps known today. These lesions are found with higher frequency along the gastrointestinal tract, predominantly in the colon and rectum, due to the interaction of the mucosa with external factors.

Polyposis of the gastrointestinal tract have a high incidence and prevalence worldwide, and can arise both in hereditary or nonhereditary forms (Haggitt & Reid, 1986; Ward & Wolfsen, 2002). Most patients with polyps do not develop any type of problems associated with it, but in a small percentage of cases cells evolve to a malignant state.

Nowadays, the preferred screening tool is endoscopy/colonoscopy, depending on the topography of the polyps. These exams show a direct and easy observation of the gastrointestinal mucosa, allowing a fast examination and, if required, the removal of the polyps with malignant potential (Zauber et al., 2012). The information acquired from an endoscopic approach is vital for follow up decisions, for instance, the number of polyps, the topography, and the histological classification of these lesions. This knowledge, along with the familial and personal history, are essential to study the potential inherited origin of these conditions (Basso, Bianchi, Malesci, & Laghi, 2017).

As previously referred, polyposis can be hereditary or sporadic and in these last few decades a large evolution in molecular knowledge allowed a better characterization of the several pathways involved (Bronner, 2003; I Spier, Hüneburg, & Aretz, 2020). More specifically, hereditary polyposis syndromes have been associated to variants in DNA repair genes, with some specific molecular features (Valle, 2017).

1.1.5. Colorectal polyposis syndromes

There are two main types of colorectal polyps in a histological definition (Figure 4): 1) epithelial polyps, originated from abnormal growth of the surface epithelium, and 2) hamartomatous polyps, developed from the overgrowth of lamina propria in the mucosa (Wills & Burt, 2002).

Epithelial polyps are divided in two main groups, adenomas, and serrated polyps. Although etiologically they arise from the same type of cells, they differ in their histological and molecular characteristics that may lead to cancer.

Adenomas, or adenomatous polyps, are the most common histological type of polyps found in the colon and rectum and are the main precursor of CRC (Markowitz & Bertagnolli, 2009). Depending on their evolution, adenomatous polyps can present three main histological forms, tubular, villous, or tubulovillous. A great set of adenomas arise sporadically, but there are two major inherited syndromes characterized by the presence of multiple adenomas in the colon mucosa, Familial Adenomatous Polyposis (FAP) and *MUTYH* Associated Polyposis (MAP). FAP is the most common inherited polyposis syndrome, and it is transmitted in an autosomal-dominant manner due to germline pathogenic variants within the coding region of the *APC* gene, and usually is associated with the development of more than 100 adenomatous polyps in the colon and several extra-colonic manifestations (Galiatsatos & Foulkes, 2006). On the other hand, MAP is an autosomal-recessive syndrome caused by biallelic germline pathogenic variants in the *MUTYH* gene, which tends to resemble the FAP phenotype but usually presents a lower number

of histologically variable (adenomatous and serrated/hyperplastic) polyps (Nielsen, Morreau, Vasen, & Hes, 2011).

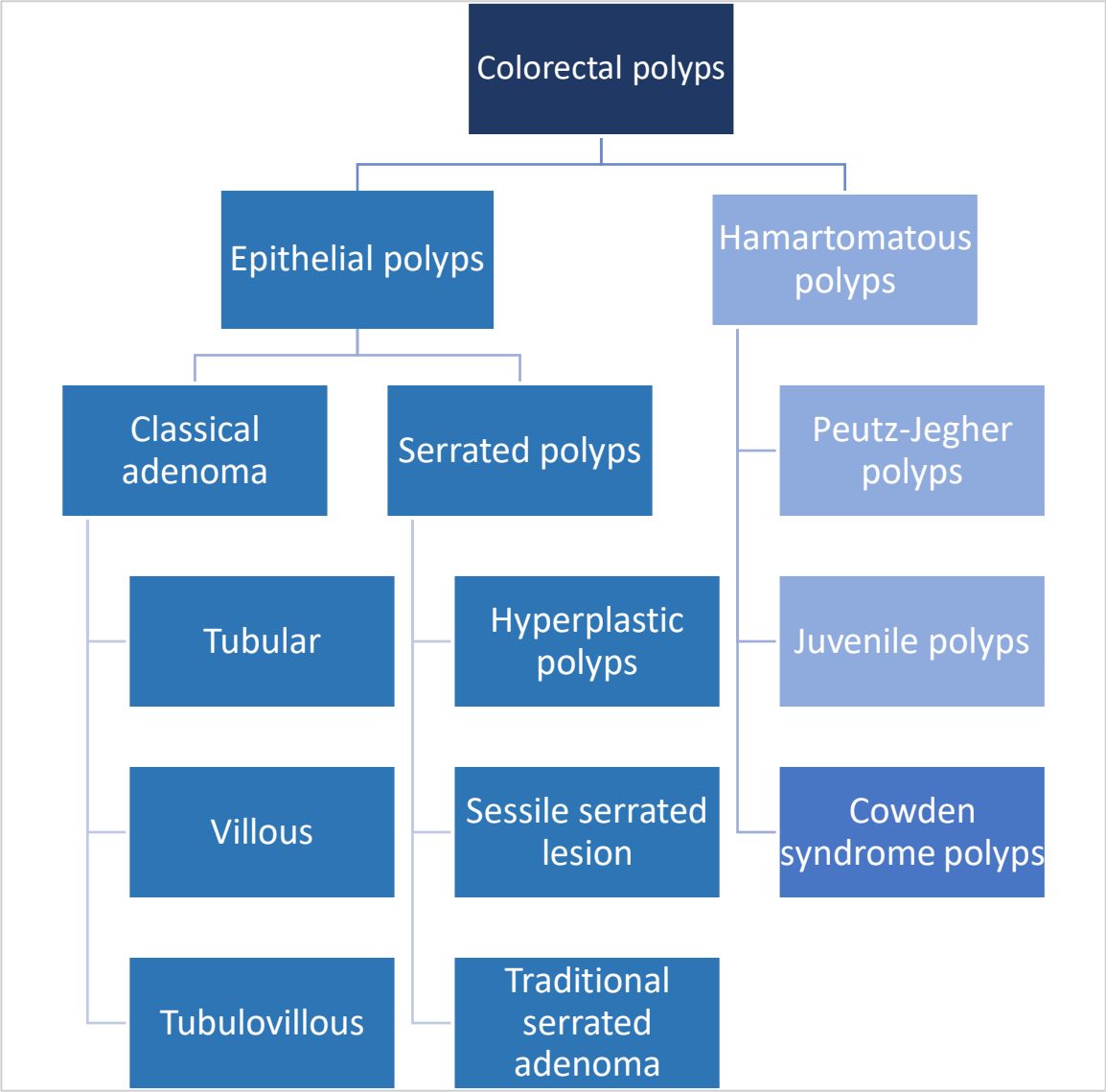

Figure 4 – Histological classification and subdivision of colorectal polyps

Serrated precursor lesions have been increasingly associated with CRC. While initially serrated polyps were just categorized as hyperplastic polyps, nowadays, there are three distinct types identified: hyperplastic polyps, sessile serrated lesions (SSLs), and traditional serrated adenomas (Crockett & Nagtegaal, 2019).

Hamartomatous polyps are considered a non-neoplastic tumor-like growth, which means that they are composed of normal mature cells in a normal tissue, but in an abnormal number and distribution (Jelsig, 2016). These polyps can appear anywhere in the body, consisting of different cell types. In the gastrointestinal tract, there are three types of hamartomatous polyps, Peutz-Jeghers polyps, juvenile polyps, and Cowden syndrome polyps. Although rare

events, it is possible for these polyps to increase the risk of CRC (Cone, 2016). In the colorectum, there are three main types of hamartomatous polyposis syndromes: Peutz–Jeghers syndrome (caused by germline mutations in *STK11*), juvenile polyposis syndrome (caused by germline mutations in *SMAD4* or *BMPR1A*), and PTEN–hamartoma tumor syndrome, also known as Cowden syndrome (caused by germline mutations in *PTEN*). Depending on the clinical, histological, and molecular characteristics of polyps, they can be divided into several distinct polyposis syndromes (Figure 5). For effects of risk assessment of the several polyposis syndromes, serrated polyps are considered apart from the rest of the epithelial polyps, due to their histological characteristics and etiology (NCCN, 2020).

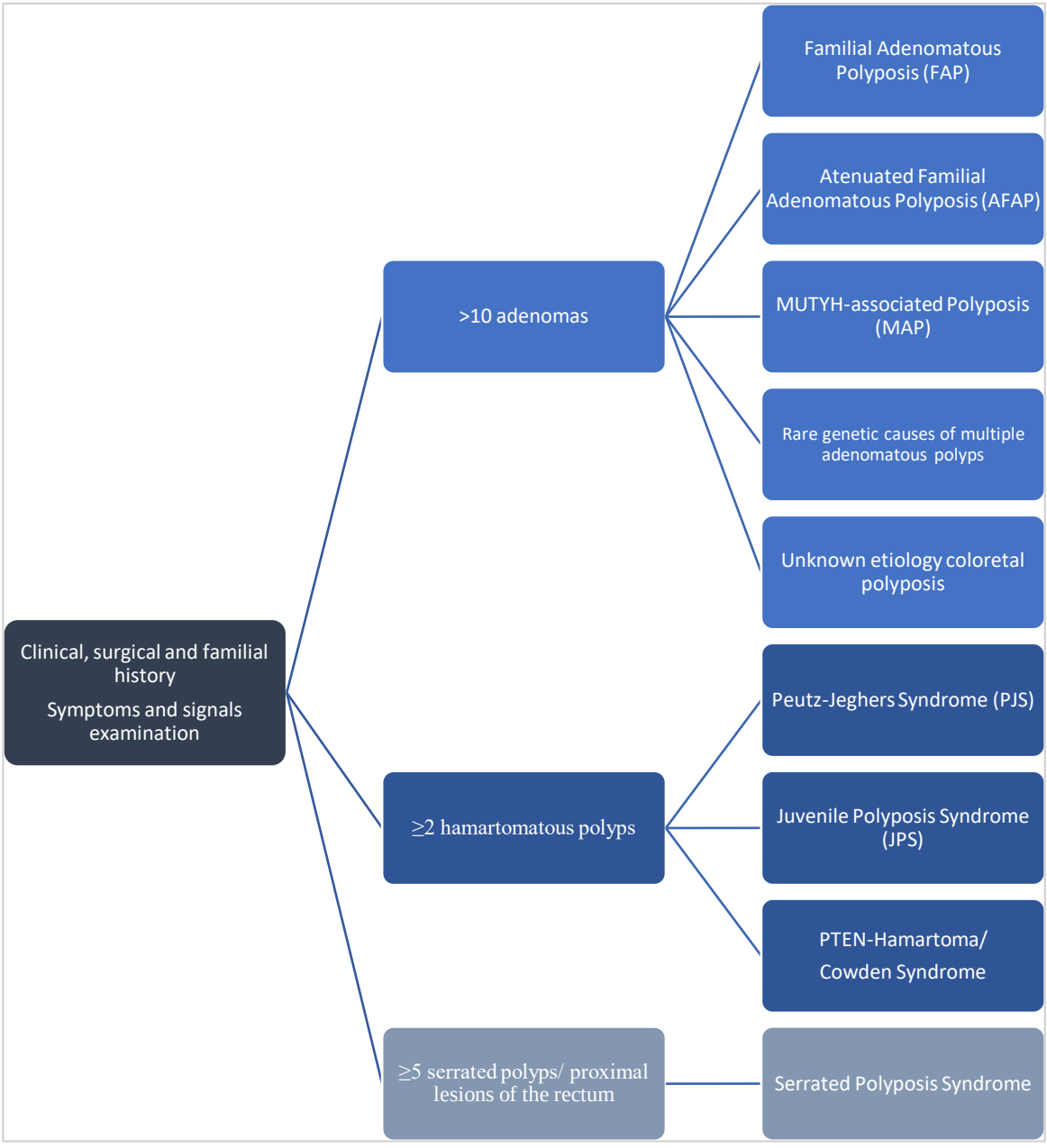

Figure 5 – Risk assessment and genetic evaluation for possible polyposis syndromes [Adapted from NCCN, 2020]

1.2. Emerging genes associated with polyposis

In the National Comprehensive Cancer Network (NCCN) guidelines, beyond the most common genes included in the multi-gene panels, it is recommended the evaluation of a set of emerging genes, where several deleterious variants have been associated with polyposis and CRC (Burke & Dallas, 2021). In this group several genes are included: *RNF43, POLE, POLD1, GREM1, MSH3, NTHL1,* and the promoter 1B of *APC*.

The study of these set of emerging genes is important to characterize cases of CRC associated to polyposis syndromes, in order to determine the genetic origin of the inherited predisposition in patients where no variants were found in the well-established inherited predisposition genes *APC* and *MUTYH*.

1.2.1. *RNF43*

RNF43 encodes a transmembrane E3 ubiquitin ligase, which acts as powerful inhibitor of the Wnt pathway (Figure 6). The encoded protein acts as a negative regulator of the Wnt pathway, promoting the degradation of its receptors by ubiquitination (Koo et al., 2012).

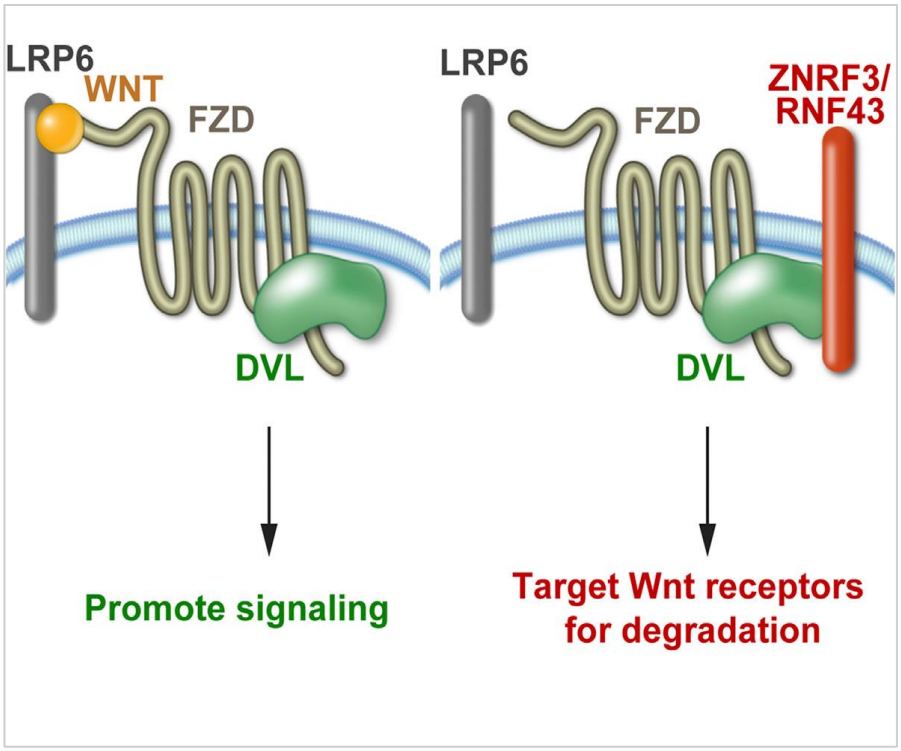

Figure 6 – RNF43 role in Wnt pathway inhibition through the targeting of Wnt receptors for degradation, functioning as a tumor suppressor [Adapted from Jiang et al., 2015]

Wnt proteins act on the surface of target cells, by binding to a complex of receptor proteins, resulting in the direct stimulation of several internal transduction pathways, divided in two main groups, β-catenin dependent and β-catenin independent pathways. Wnt proteins act by binding to the Frizzled/low density lipoprotein (LDP) receptor-related protein (LRP) complex, presented on cell surface (Figure 7) (Logan & Nusse, 2004). The glycoproteins of the Wnt pathway are responsible for the control of several cellular characteristics, such as: autonomous specification, motility, polarity, primary axis formation during embryogenesis and the renewal of stem cells (Habas & Dawid, 2005; Logan & Nusse, 2004; Wodarz & Nusse, 1998). The dysregulation of cellular pathways, responsible for critical steps during cell/organ development, is related to some serious pathologies like neural tube defects, or the development of tumors in several parts of the human organism (Bugter, Fenderico, & Maurice, 2021; Patapoutian & Reichardt, 2000; Ryland et al., 2013).

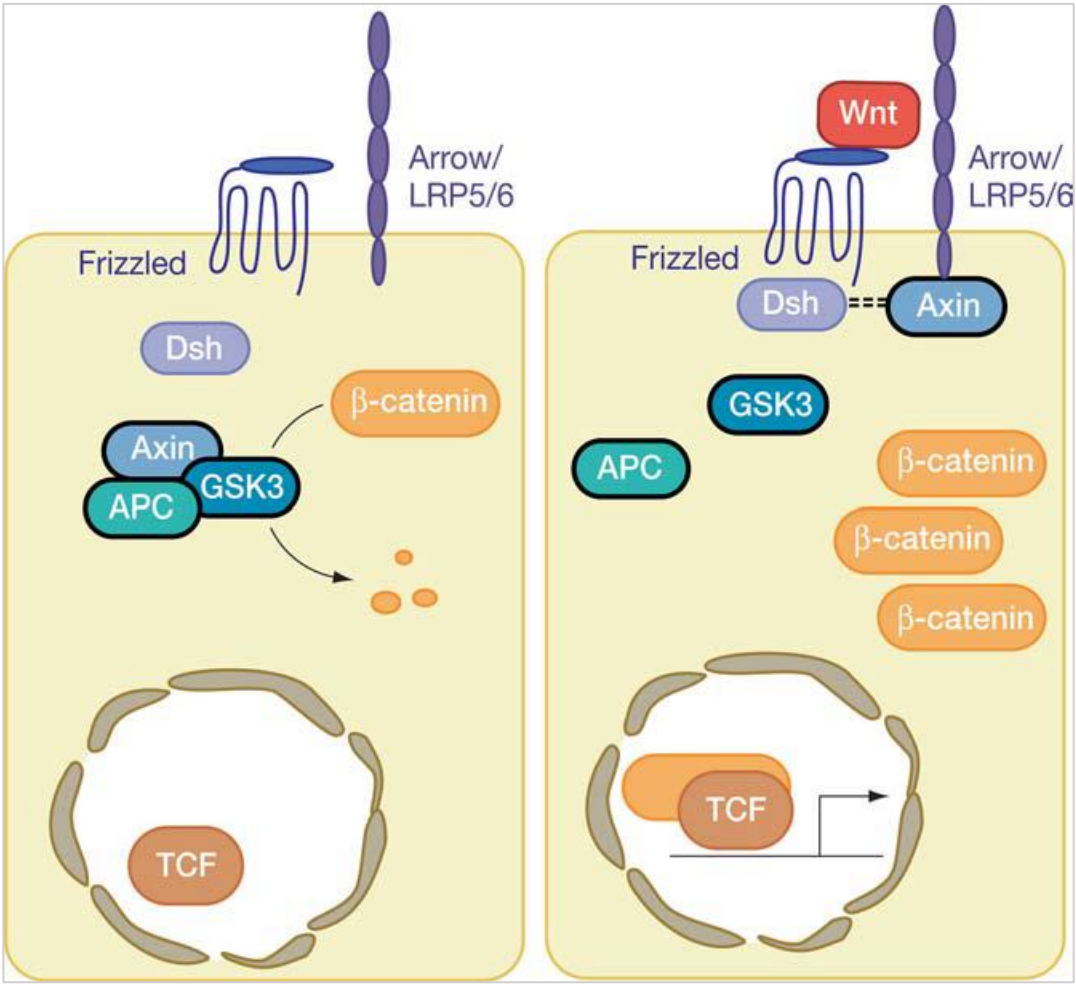

Figure 7 – The canonical Wnt signaling pathway. The Wnt signal enables the interaction between Dishevelled (Dsh) and Axin, inhibiting the degradation of β-catenin, which accumulates in the cytoplasm and nucleus. β-catenin then is able to interact with TCF, which will control transcription in the nucleus [Adapted from: Catriona, et al., 2004]

The *RNF43* gene inactivation can lead to an uncontrolled cellular growth, contributing to the development and progression of several tumors, including CRC (Koo et al., 2012; Spit et al., 2019).

Gala et al. first described that patients with variants in the *RNF43* gene have a high-risk of developing serrated polyposis syndrome (Gala et al., 2014). More recently, Yan et al. has confirmed that deleterious variants in this gene can lead to tumor formation as a consequence of serrated polyposis in both sporadic and familial polyposis cases, oftentimes in association with mutations in *BRAF* (Yan et al., 2017).

1.2.2. *POLE / POLD1*

Both *POLE* and *POLD1* genes encode the exonuclease domain of DNA polymerases Polε and Polδ, respectively (Preston, Albertson, & Herr, 2010). The several subunits encoded by these genes are responsible for the catalyzation and proofreading of the enzymatic complexes of the DNA polymerases (Figure 8).

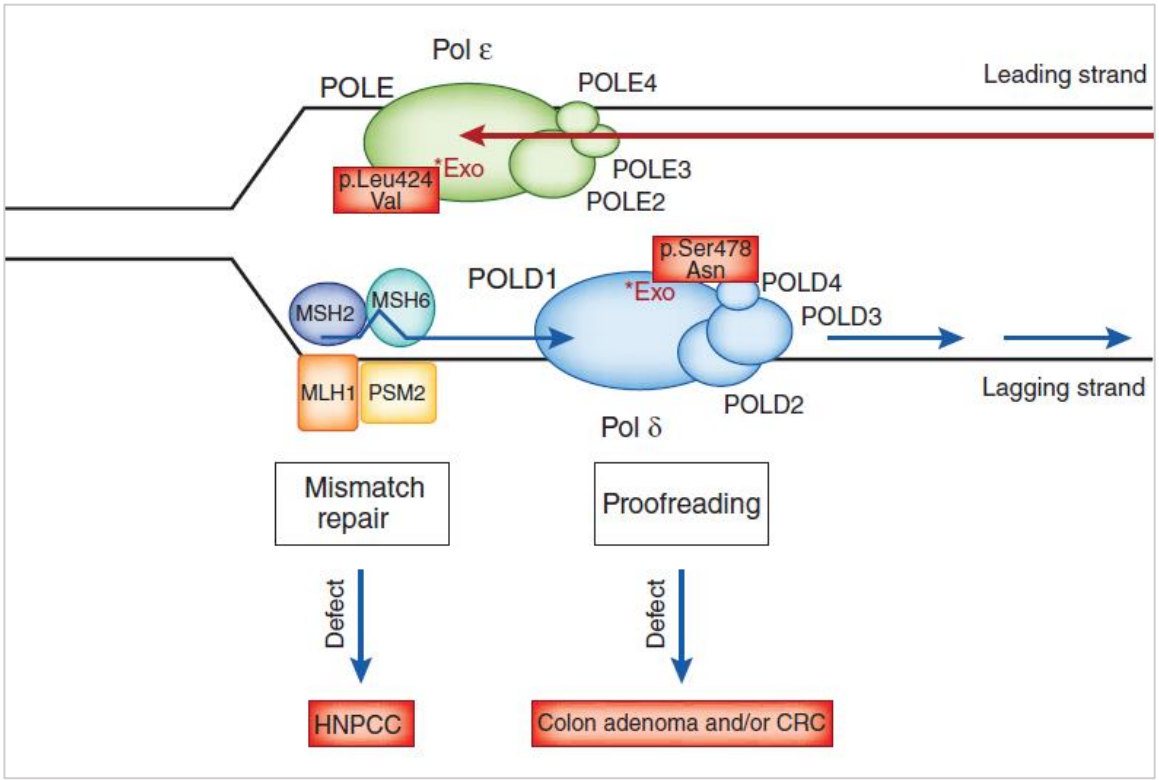

Figure 8 – Deleterious variants in *POLE* and *POLD1* causes defective DNA polymerase subunits resulting in a malfunctioning proofreading, which leads to an increased risk for the development of adenomas and CRC [Adapted from: Seshagiri, 2013].

Palles et al. first described a deleterious variant in the *POLD1* gene (NM_002691: c.1433G>A, p.Ser478Asn), which increases predisposition to polyposis and CRC, but also cancer in other parts of the human organism, including the endometrium and the brain (Palles et al., 2013). The same author also described, in the same study, another deleterious variant (NM_006231: c.1270C>G, p.Leu424Val) in the exonuclease domain of the *POLE* gene, being also associated with a high probability of polyposis and CRC development. Spier et al. later confirmed the deleterious potential of this last variant (Isabel Spier et al., 2015), and has further identified other nine deleterious variants in the exonuclease domains of these genes in a study involving the analysis of over 200 individuals. Thereafter, another study by Bellido et al. described four new deleterious variants in the *POLD1* gene in patients with polymerase proofreading-associated polyposis, and CRC (Bellido et al., 2016).

1.2.3. *GREM1*

This gene is responsible for encoding an antagonist protein of the bone morphogenetic protein (BMP) pathway. Initially, it was identified as a group of inducting proteins of the ectopic bone formation (Urist & Strates, 1971), but later it was shown to be present in other metabolic processes, namely, morphogenesis, tissue and cell differentiation, proliferation, and apoptosis in several cell types of the organism (Reddi & Reddi, 2009).

In the colon, BMP has a fundamental role in tissue differentiation, in particular away from the crypts base (Scoville, Sato, He, & Li, 2008). In the mesenchyme of the crypt's base, there is an increased expression of the *GREM1* gene, suppressing the activity of the BMP pathway and promoting Wnt as a consequence, which stimulates an increased stem cell concentration in this region with a critical role in tissue cell regeneration (Figure 9) (Davis et al., 2015; Voorneveld et al., 2015).

Jaeger et al. showed that a 40kb upstream duplication in the promoter of the *GREM1* gene (Figure 10), results in the transcription of an antagonist protein of the BMP pathway and as a consequence there is a change in the phenotype characteristics of the crypt's cells to stem cells (Jaeger et al., 2012). A considerable part of this duplication is located within the *SCG5* gene, upstream of *GREM1*, but no impact was detected in its expression. This variant was found in a family of Ashkenazi Jews with Hereditary Mixed Polyposis Syndrome (HMPS).

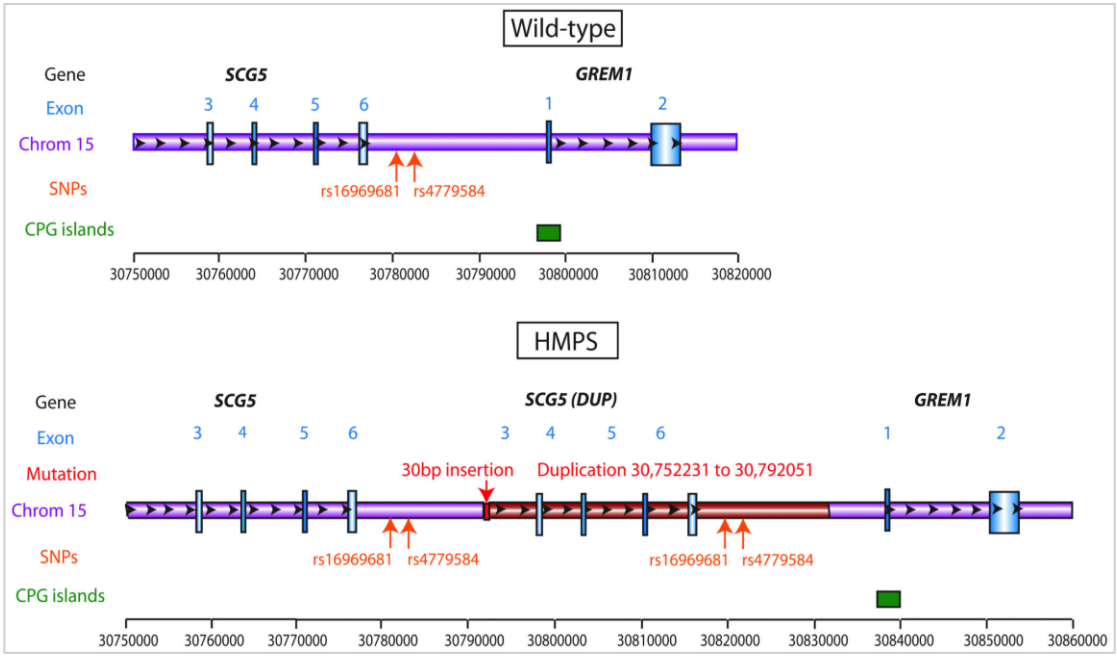

Figure 10 – Schematic representation of the upstream duplication in the promoter of the *GREM1* gene [Adapted from Jaeger et al., 2012]

In recent years, other studies have shown the presence of other smaller upstream duplications of the *GREM1* gene. Rohlin et al. identified a 16kb duplication in the same region, with same enhancer effect in the expression of *GREM1*, resulting in a similar phenotype, but this time in a family with no Ashkenazi ancestry (Anna Rohlin et al., 2016). More recently, McKenna et al. found a third duplication in this same region, a 24kb duplication, also not associated with Ashkenazi ancestry, and with a phenotype of hyperplastic polyps and tubular adenomas. (McKenna et al., 2019).

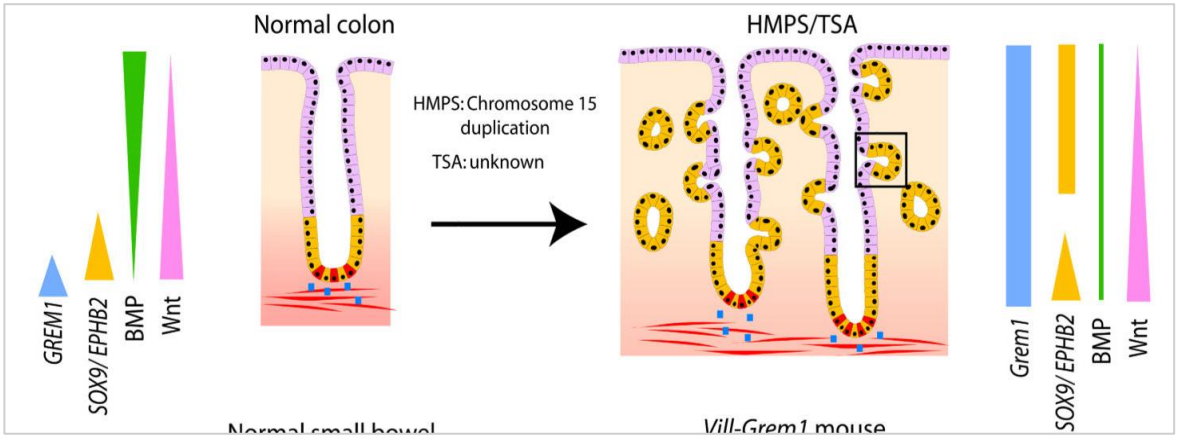

Figure 9 – Model summarizing the proposed mechanistic consequences of disrupted GREM1 morphogen gradients. In a normal colon, GREM1 suppresses BMP activity in the crypts base and promotes stem cells proliferation due to the Wnt pathway activity. A duplication in GREM1 promoter disrupts the activity of this gene and consequently the stem cell activity regulation, leading to the formation of several types of polyps [Adapted from Davis et al., 2015]

1.2.4. *MSH3*

MSH3 is a gene involved in DNA mismatch repair (MMR), responsible to encode a protein that, together with *MSH2*, forms an heterodimer that detects and eliminates mispaired nucleotides, thus reducing the mutational rate and maintaining genomic stability (Gupta, Gellert, & Yang, 2012). This heterodimer is largely responsible for the recognition of large insertions and/or deletions (Figure 11), even though it can recognize a small number of base mispairs (Graham V, Putnam, & Kolodner, 2019).

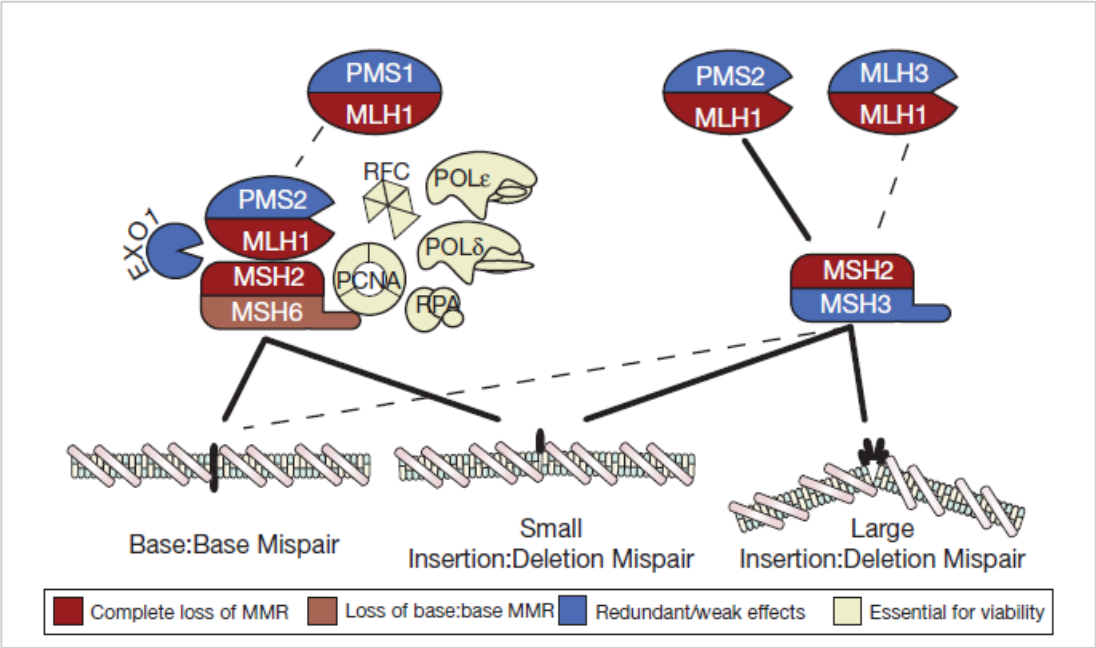

Figure 11 – The different specificity of the several MMR heterodimers. While MSH2-MSH6 heterodimer is responsible for most mispairs recognition, the MSH2-MSH3 heterodimer has an important role mainly in recognizing large insertions and deletions [Adapted form: Graham et al., 2018]

The MMR proteins have particular importance in the correction of errors on microsatellites, sequences of short tandem DNA repeats, in both coding and non-coding regions of human DNA. The repetitive structure of microsatellites makes them prone to the occurrence of errors that MMR, usually, resolve.

As previously referred, individuals with MMR deficiency are associated with Lynch syndrome when having germline heterozygous variants in *MLH1*, *MSH2*, *MSH6* or *PMS2* (Kolodner, 1995). On the other hand, it was recently shown that biallelic *MSH3* deleterious variants are associated with a phenotype of Attenuated Familial Adenomatous Polyposis (AFAP). In 2016, Adam et al., described two different deleterious germline variants in *MSH3* in each of two unrelated individuals with adenomatous polyposis (Adam et al., 2016). The

compound-heterozygous loss-of-function (LoF) variants were NM_002439.4: c.1148del, p.Lys383ArgfsTer32; c.2319-1G>A, p.Thr774_Glu812del; c.2760del, p.Tyr921MetfsTer36; and c.3001-2A>C, p.Val1001ArgfsTer16. Interestingly, both index cases presented one frameshift and one splice-site variant (Figure 12) in different alleles and they also had instability in dinucleotide and tetranucleotide markers in the adenoma-derived DNA. No other mutation was found in genes implicated in polyposis syndromes or in MMR.

Despite the NCCN's inclusion of *MSH3* as an emerging gene associated to colorectal

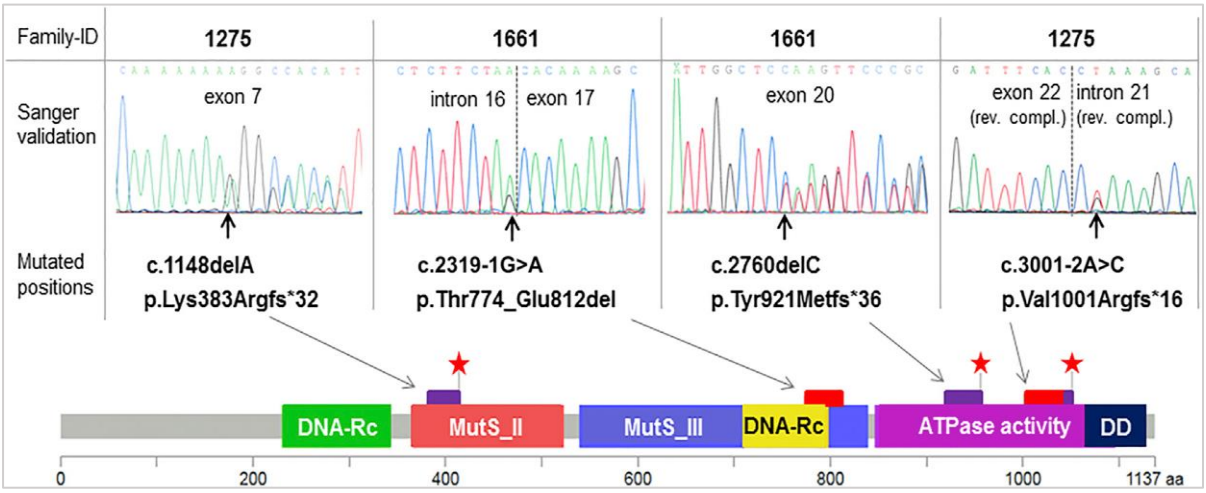

Figure 12 – Germline variants in *MSH3* – Sanger confirmation of all four variants within *MSH3* gene and the affected regions are shown under it. These variants cause premature stop codons, and alterations or loss of some amino acids, affecting the MSH3 role in MMR [Adapted from Adam et al., 2016]

adenomatous polyposis and CRC, this is the only study that reported a possible correlation between deleterious variants in this gene and CRC. Several other authors have tried to prove the same association in other set of cases but were unsuccessful, confirming the infrequency of *MSH3* in polyposis. Other study, from Huang et al., has associated defects in *MSH3* gene to *PTEN* mutations and hamartomatous polyps, but more evidence is still necessary to confirm this association (Huang et al., 2011). Lastly, Terradas et al., tried to study the association of *MSH3* and polyposis, but they were only able to confirm the infrequency of it (Terradas et al., 2019).

1.2.5. *NTHL1*

The *NTHL1* gene encodes a DNA glycosylase of the Base-excision-repair (BER) mechanism, responsible to remove endogenous damaged nucleotides (Figure 13) (Robertson, Klungland, Rognes, & Leiros, 2009). BER has a particular important role in colon epithelium, as this tissue is a target of large number of environmental factors, with particular incidence of oxygen radicals, formed by the natural flora of the intestine, and by carcinogenic molecules directly from the diet (Aceto, Catalano, & Curia, 2020; Huycke & Gaskins, 2004). The endonuclease III-like 1, encoded by *NTHL1,* acts on oxidized pyrimidine residues, initiating the BER mechanism and preventing an incorrect pairing of DNA nitrogenous bases (David & Williams, 1998).

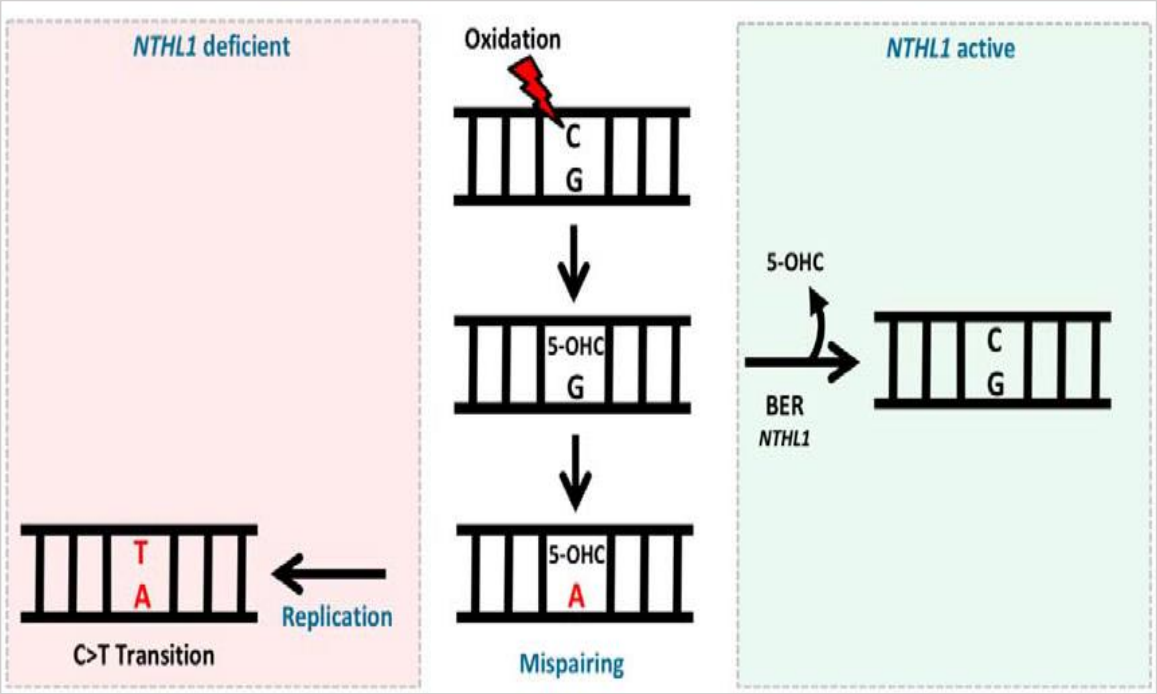

Figure 13 – Representation of NTHL1 glycosylase deficiency in tumor development. NTHL1 is responsible for the recognition and removal of oxidized pyrimidines, which explains why defects in this gene usually results in C>T transitions accumulation [Adapted from: Weren et al., 2018]

The *NTHL1* gene acts as a tumor suppressor, and the dysregulation of its function has a great impact in the development of polyposis and CRC. Weren et al. described a deleterious nonsense variant (NM_002528.6: c.268C>T, p.Gln90Ter) in seven individuals of three different families in homozygosity (Weren et al., 2015). Most individuals in the study had several adenomas in the colon and a familial history of polyposis and CRC. Rivera et al. also described this same variant in another individual with similar clinical criteria (Rivera, Castellsagué, Bah, van Kempen,

& Foulkes, 2015b). In these last few years, several authors identified this variant in several individuals, all of them presenting a similar clinical and familial history (Altaraihi, Gerdes, & Wadt, 2019; Belhadj et al., 2017; Fostira et al., 2018; Grolleman et al., 2019; Groves, Gleeson, & Spigelman, 2019; Ryland et al., 2013).

This same deleterious variant had been described previously in several studies, where the association between genetic damage in the genes of the BER mechanism and the development of polyposis and CRC, but it was only found in heterozygosity, with harmless potential (Dallosso et al., 2008; Smith et al., 2013).

Patients with *NTHL1* tumor syndrome, as a consequence of *NTHL1* loss of function, often present lesions with a mutation signature with high prevalence of C>T transitions in several parts of the genome, which may be responsible for the development of these pathologies (Weren et al., 2015).

1.2.6. *APC* promoter *1B*

Mutations in the *APC* gene are generally associated to FAP and its screening is usually performed nowadays in most cases of polyposis (Mao et al., 2021). In the gastrointestinal tract, the *APC* gene is able to encode two different proteins. While in the colon *APC* generates the most common protein (1A isoform), in the stomach the activation of a second promoter (promoter 1B) encodes an protein that has slight differences in the N terminus due to the inclusion of exon 1B (Santoro & Groden, 1997)

Promoter 1B transcription is 15-fold higher in gastric mucosa, in comparison with the colon. This is a consequence of an almost complete methylation of the promotor 1A in the gastric mucosa, suggesting the importance of the 1B isoform transcription in this region (Hosoya et al., 2009).

Some studies have identified individuals with deletions in the *APC* promotor 1B (Figure 14), but these deletions only affect the regulatory region upstream of the 1A isoform, which have been associated with the FAP phenotype (Kadiyska et al., 2014; Lin et al., 2015; Pavicic et al., 2014; A Rohlin et al., 2011; Snow et al., 2015). This also might suggest that a germline deletion in the promotor 1B will only affect the 1B isoform, since only one promotor is available to regulate the transcription of the *APC* gene.

While large deletions in *APC* gene (including in the promoter 1B region) are found in families with colonic polyps in FAP, point mutation in the promotor 1B were only shown to be associated with fundic gastric polyps (FGPs) (J. Li et al., 2016). Three different point mutations were found (NM_001127511: c.-191T>C, c.-192A>G, and c.-195A>C), as shown in Figure 14, confirming that point mutations and large deletions are affecting the same region of the *APC* promoter 1B, but are displayed in two different phenotypes, FAP and gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS).

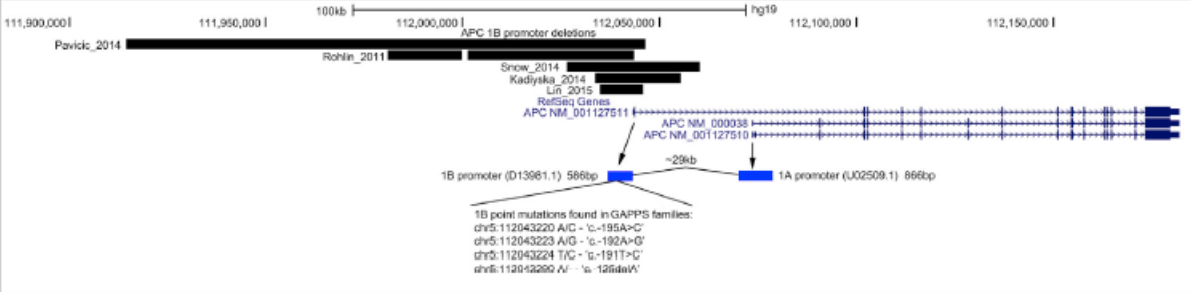

Figure 14 – Schematic representation of the APC 1A and 1B promoters showing the location of deletions described in FAP and point mutations associated to gastric polyposis [Adapted from Li et al., 2016]

2. Aims

The overall goal of this project was to identify novel deleterious germline variants that could explain the inherited predisposition to colorectal polyposis in patients with no pathogenic germline variants detected in the well-established genes associated with polyposis.

Specifically, the objectives of this project were:

1. To screen for pathogenic variants in the emerging genes described in the literature associated to polyposis, namely, *RNF43, POLE, POLD1, GREM1, MSH3*, *NTHL1,* and the promoter 1B of *APC*, in a series of patients selected retrospectively with colorectal polyposis that were negative for *APC* and/or *MUTYH* pathogenic variants.

2. To complete the *MUTYH* variant screening in all patients with an attenuated polyposis phenotype that had previously been studied only for the recurrent/founder pathogenic variants in this gene.

3. To correlate the variants found in the emerging genes with the histological classification of the polyps by the Anatomical Pathology Service.

3. Materials and Methods

3.1. Patients and sample collection

The study included a total of 189 DNA samples obtained from unrelated patients previously diagnosed with personal/family history of colorectal polyposis (serrated polyps, or >10 polyps). All patients had been referred to the Genetics Department of IPO-Porto between 1999 and 2021, and samples were collected after genetic counselling and informed consent. This department proceeded for the identification of germline variants related to their polyposis phenotype, namely the analysis of *APC* and/or *MUTYH* variants. Patients with deleterious variants identified in these genes, or with other variants with a strong connection to their polyposis phenotype, were excluded from the study. Of the 189 patients, 79 presented at the time of diagnosis less than 20 polyps and, according to the routine criteria, only frequent *MUTYH* variants in our population, namely, c.536A>G, c.1187G>A, and c.1227_1228dup (NM_001128425.1), were previously screened and reported.

The DNA was obtained after extraction from peripheral blood collected in EDTA tubes, following standard procedures. After extraction, DNA samples were stored at -80°C in ultra-cold freezers.

3.2. Custom gene-panel analysis by next generation sequencing

Germline variants were screened using a next generation sequencing (NGS) QIAseq Targeted DNA custom panel [Qiagen, Hilden, Germany] designed to cover all regions of interest in the emerging genes associated to polyposis, namely, all coding regions of *RNF43, MSH3* and *NTHL1,* the exonuclease domain of *POLE* and *POLD,* the promotor 1B of *APC*, and three regions of the promoter and exon 1 and 2 of *GREM1.* Additionally, this panel also covers all coding regions of the *MUTYH* gene (Table 1).

Table 1 – List of genes and the region of interest included in the customized NGS panel

Gene	Region of interest	Reference Sequence
RNF43	all coding sequence	NM_017763.6
POLE	Exons 9 – 14 (ED)	NM_006231.2
POLD1	Exons 8 -12 (ED)	NM_002691.4
NTHL1	all coding sequence	NM_002528.6
MSH3	all coding sequence	NM_002439.4
APC	promoter 1B	NM_001127511.2
GREM1	Exons 1 – 2 3 regions within the promotor: - chr15:32988695-32989205 - chr15:33000845-33001855 - chr15:33003695-33004205	NM_013372.7
MUTYH	all coding sequence	NM_001128425.1

ED, exonuclease domain.

Library preparation was performed according to the manufacturer's instructions. In more detail, approximately 10 to 40ng of input genomic DNA (gDNA) was used for fragmentation, end-repair and A-addition, in a single multi-enzyme reaction followed by an incubation in a thermocycler [Verity TM Thermal Cycler, Applied Biosystems, Foster City, CA, USA] according to the conditions of Table 2.

Table 2 – Incubation conditions in the fragmentation, end-repair and A-addition

Step	Temperature	Incubation Time
1	4°C	1 min
2	32°C	24 min
3	72°C	30 min
4	4°C	∞

Adapter ligation was then performed, allowing the attachment of the unique molecular indices (UMIs) and the sample index to the fragmented gDNA. This reaction consisted on mixing the fragmented DNA with a ligation buffer, a DNA ligase and a ligation solution, as well as single-index adapters, followed by an incubation in a thermocycler at 20ºC for 15 minutes.

The target enrichment was performed in the adapter-ligated DNA after a purification reaction using the QIAseq beads [Qiagen]. For enrichment, the purified adapter-ligated DNA was amplified in a solution containing the QIAseq targeted DNA panel and a universal primer complementary to the adapter. The amplification conditions for the target enrichemnt are described in Table 3.

Table 3 – Amplification conditions of Target Enrichment

Step	Time	Temperature
Initial denaturation	13 min 2 min	95°C 98°C
8 cycles	15 s 10 min	98°C 68°C
1 cycle	5 min	72°C
Hold	5 min	4°C
Hold	∞	4°C

The target enrichment reaction was purified using the QIAseq Beads [Qiagen] and a Universal PCR was performed to amplify the library and add the platform specific adapter sequences and additional sample indices. The thermal cycler conditions for the Universal PCR are described in Table 4.

Table 4 – Thermal cycler condition of Universal PCR

Step	Time	Temperature
Initial denaturation	13 min 2 min	95°C 98°C
Amplification cycles (x22)	15 s 2 min	98°C 60°C
1 cycle	5 min	72°C
Hold	5 min	4°C
Hold	∞	4°C

After the cleanup of PCR products using the QIAseq Beads [Qiagen] the libraries were quantified using the dsDNA HS Assay in a Qubit 2.0 Fluorometer [Invitrogen, Waltham, MA, USA], and the quality was checked on a 4200 TapeStation equipment [Agilent Technologies, Santa Clara, CA, USA]. The samples

were diluted to 4 nM and final pooled library (12pM) was loaded and sequenced on the MiSeq™ platform [Illumina, San Diego, USA] according to the manufacturer's instructions.

Sequencing alignment and variant calling were performed using GeneGlobe Data Analysis Center [Qiagen] (Xu, 2018), and the resulting .vcf files were imported to GeneticistAssistant™ software [SoftGenetics, LLC, State College, PA, USA].

The filters used in the GeneticistAssistant™ software (Table 5) included the frequency within the panel, a minimum coverage, and the minor allele frequency (MAF) of the variants in two different databases, namely the 1000 Genomes Project (Auton et al., 2015; Clarke et al., 2017) and the Genome Aggregation Database (gnomAD) (Koch, 2020). To gather information about clinical and phenotype significance of the variants, the ClinVar database was consulted, since it addresses several categories of rare germline variants (Landrum et al., 2020).

Table 5 – Filters applied for the analysis in the GeneticistAssistant™ software

Criteria	Score
Panel Frequency	≤5%
Coverage	≥20
The 1000 Genomes Project*	≤1%
gnomAD*	≤1%

*Not applicable for *MUTYH*

Copy number variations (CNV) analysis were performed using the CNV tool and Batch CNV tool of the NextGENe software [SoftGenetics].

3.3. Variant interpretation and classification

The sequence variants were classified according to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines, using the five degree system that classified all variants as pathogenic, likely pathogenic, uncertain significance (VUS), likely benign, or benign (Richards et al., 2015). According to these recommendations, variants classified with a general consensus as benign, and likely benign were excluded. Nonsense, frameshift, indels and canonical splice-site variants, as well as variants classified as pathogenic and/or likely pathogenic in the literature were considered deleterious.

4. Results

4.1. Germline variant screening

A total of 189 DNA samples from patients with a personal/familial history of polyposis were analyzed using a custom NGS panel. In this series, a total of 18 variants (15 different) were found (Table 6 and 7) in 17 different patients. Six of these variants were previously reported as pathogenic or likely pathogenic, seven as VUS, and the remaining two variants were not described in the literature.

None of the 79 patients previously studied only for the *MUTYH* recurrent variants presented pathogenic variants in the remaining coding region of this gene.

The CNVs assays showed no alterations in the regions of interest of the emerging genes in all the 189 samples analyzed.

4.2. Deleterious variants

Seven (six different) deleterious variants, detected in six samples (two different variants in one of them), were classified as pathogenic or likely pathogenic according to the ACMG recommendations (four in *MUTYH* and three in *NTHL1*) (Table 6). These variants are well described in the literature and have strong evidence about their deleterious effect in polyposis syndromes and CRC when found in homozigosity or in compound heterozigozity due to the recessive form of transmission of the phenotypes associated with these two genes.

The variants within *MUTYH* coding region, namelly, the c.536A>G, p.(Tyr179Cys), the c.1187G>A, p.(Gly396Asp), and the c.1437_1439del, p.(Glu480del), have been reported in individuals with MAP, in a homozygous state, or in compound heterozigosity with other deleterious variants in the same gene (Abduljaleel, Athar, Al-Allaf, Al-Dehlawi, & Vazquez, 2019; Al-Tassan et al., 2002; Gismondi et al., 2004; Halford et al., 2003; Nielsen et al., 2009; Peterlongo et al., 2006; Tsaousis et al., 2019).

Table 6 – Deleterious germline variants found in our series.

Sample	Gene	Exon	cDNA variant	Protein alteration	Effect	ClinVar Clinical Significance (*)
#7 #159	***MUTYH***	**7**	**c.536A>G**	**p.(Tyr179Cys)**	**Missense**	**Pathogenic/Likely pathogenic (34)**
#7	***MUTYH***	**13**	**c.1187G>A**	**p.(Gly396Asp)**	**Missense**	**Pathogenic/Likely pathogenic (42)**
#27	*MUTYH*	14	c.1437_1439del	p.(Glu480del)	In frame Deletion	Pathogenic (18)
#188	*NTHL1*	intron 1	c.139+1G>A	–	Splice-site variant	Likely pathogenic (3)
#176	*NTHL1*	2	c.268C>T	p.(Gln90Ter)	Missense	Pathogenic (6), VUS (1)
#138	*NTHL1*	intron 3	c.550-1G>A	–	Splice-site variant	Pathogenic/Likely pathogenic (2)

*, number of citations in ClinVar; VUS, variant of unknown significance. The patient that has two pathogenic *MUTYH* variants is higlighted in bold.

The *NTHL1* pathogenic and likely pathogenic variants found in this study are reported in ClinVar with strong evidence for their deleterious effect when in a homozygous state, or in compound heterozigosity with other deleterious variants in the same gene. The *NTHL1* variant c.268C>T, p.(Gln90Ter), results in the creation of a premature translational stop signal. This variant has been described in homozigosity in several families presenting a phenotype of base excision repair-associated adenomatous polyposis and CRC (Weren et al., 2015), or in compound heterozigosity with other variants (Belhadj et al., 2019; Chubb et al., 2016; Rivera, Castellsagué, Bah, van Kempen, & Foulkes, 2015a; Staninova-Stojovska et al., 2019). There is also a report that found the presence of this variant in homozygosity in individuals that do not present polyposis (Lorca et al., 2019). The remaining variants found in this gene were splice-site variants. The variant c.139+1G>A that affects the splice-site of exon 1 is expected to result in aberrant splicing, and consequently in a abnormal protein, thus being classified as likely pathogenic (Boulouard et al., 2021). The variant c.550-1G>A that affects the splice-site of exon 3 has been described one time in compound heterozigosity with the variant c.268C>T in an individual diagnosed with polyposis and CRC, and also with a meningioma (Belhadj et al., 2019).

Only one of the six samples showing deleterious variants (sample #7) had two In the same gene, namely, two deleterious variants within the coding region of *MUTYH* in compound heterozigozity. This patient presents the *MUTYH* variants c.536A>G, p.(Tyr179Cys), and the

c.1187G>A, p.(Gly396Asp), widelly described in the literature as recurrent pathogenic variants (Al-Tassan et al., 2002; Aretz et al., 2014; Nielsen et al., 2009; Pin et al., 2013).

4.2.1. Clinicopathological characteristics of *MUTYH* c.536A>G and c.1187G>A carrier

The patient corresponding to sample #7 carrying the two *MUTYH* deleterious variants (c.536A>G and c.1187G>A) is a woman that presented 35 polyps in the colon mucosa and CRC diagnosed at 40 years of age, and who later was also diagnosed with breast cancer at 45 years of age. This patient showed a family history of polyposis affecting two consecutive generations (her son presented 5 polyps at age 29) and early-onset gastric cancer in a third generation (her mother died of gastric cancer at age 49; Figure 15). In 2005 no germline pathogenic variants in were detected in the *APC* gene, the only known at the time to be associated with a seamingly autossomal dominant form of polyposis.

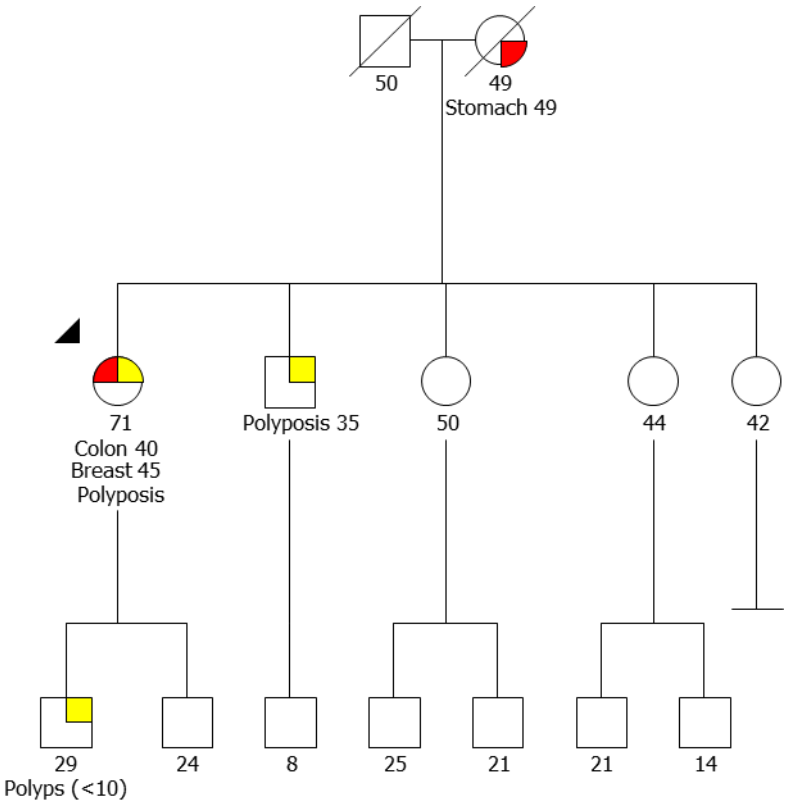

Figure 15 – Pedigree of the patient corresponding to sample #7

4.3. Variants of uncertain significance

In this study we identified 11 VUS (9 different) in 11 patients, two of them not described in the ClinVar database (Table 7).

Table 7 – Variants of uncertain significance identified in our series.

Sample	Gene	Exon	cDNA variant	Protein alteration	Effect	ClinVar Clinical Significance (*)
#150	*GREM1*	1	c.-111G>A	-	5' UTR Variant	N/A
#42	*MSH3*	4	c.739G>A	p.(Val247Ile)	Missense	N/A
#12	*MSH3*	9	c.1394A>G	p.(Tyr465Cys)	Missense	VUS (2)
#3	*MSH3*	13	c.1777C>T	p.(Arg593Trp)	Missense	VUS (2)
#40 #173	*MSH3*	20	c.2732T>G	p.(Leu911Trp)	Missense	VUS (3), Benign (1)
#5	*MUTYH*	10	c.925C>T	p.(Arg309Cys)	Missense	Benign (1), Likely benign (1), VUS (10)
#145	*POLE*	9	c.861T>A	p.(Asp287Glu)	Missense	Benign (1), Likely benign (3), VUS (8)
#4 #84	*RNF43*	2	c.172A>G	p.(Thr58Ala)	Missense	VUS (1)
#36	*RNF43*	9	c.1114C>T	p.(Pro372Ser)	Missense	VUS (1)

*, number of citations in ClinVar; VUS, variant of uncertain significance; N/A, not available.

The *MSH3* variant c.1394A>G results in a conservative amino acid substitution of a Tyrosine for a Cysteine, p.(Tyr465Cys). This variant has been described once in heterozigosity in a patient presenting a phenotype of polyposis (Terradas et al., 2019) and has been submitted two times to ClinVar. The *MSH3* variant c.1777C>T translates into a conservative amino acid substitution of a Arginine for a Tryptophan, p.(Arg593Trp), has only been submitted two times to ClinVar. The *MSH3* variant c.2732T>G results in a conservative amino acid substitution of a Leucine for a Tryptophan, p.(Leu911Trp), and has been described several times in patients with personal or familial history of CRC (Anna Rohlin et al., 2017), most of which in heterozigosity but in some cases also with the presence of a second hit mutation in the same gene (Dámaso et al., 2020; DeRycke et al., 2017; Duraturo et al., 2011), or in *MSH6* (Morak et al., 2017). The same variant was also described in heterozigosity in two unrelated patients with polyposis (Terradas

et al., 2019). Beyond its association with CRC, this variant was also identified in two unrelated individuals with luminal breast cancer (Cortes-Urrea et al., 2020).

The *MUTYH* variant c.925C>T results into a non-conservative amino acid substitution of Arginine to Cysteine, p.(Arg309Cys). This variant has been described multiple times in the literature, usually related to cases of personal history of polyposis or CRC (Goto et al., 2010; Halford et al., 2003; Komine et al., 2015; Nielsen et al., 2009; Anna Rohlin et al., 2017; Sieber et al., 2003; Vogt et al., 2009), but it was also found with a higher frequency in heterozigosity in breast cancer patients negative for *BRCA1* and *BRCA2* mutations in a case-control study (Out et al., 2012)

The *POLE* variant c.861T>A results in a conservative amino acid substituition of an Aspartic Acid for a Glutamic Acid p.(Asp287Glu). This *POLE* variant has been previously described in nine patients with cutaneous melanoma (Potjer et al., 2019), In three unrelated patients with cutaneous melanoma and other tumors in the family including squamous cell carcinoma, non-Hogkin lymphoma and breast cancer (Aoude et al., 2015), in patients with Lynch Syndrome associated tumours, including in the colorectum and endometrium (Billingsley et al., 2015; Jansen et al., 2016), in patients with a high-risk of hereditary CRC (Buchanan et al., 2018), and in two patients with breast cancer, one of whom also diagnosed with CRC (Mur et al., 2020).

The *RNF43* variants c.172A>G and c.1114C>T result into moderately conservative substitutions, p.(Thr58Ala) and p.(Pro372Ser), respectively. These variants were submitted only once to ClinVar.

The *MSH3* variant, c.739G>A results in a conservative substitution of a Valine for a Isoleucine p.(Val247Ile). This variant has not been reported to ClinVar, and there is no literature describing it.

The *GREM1* variant c.-111G>A is located in the 5' UTR of this gene, but no reports were submitted to ClinVar, until this date, and no literature was found about it.

5. Discussion

Polyposis syndromes affect a large number of individuals around the world, often without a known genetic cause associated to it. Although the majority of these polyps do not develop beyond the benign state, in a considerable number of cases an early detection and removal can prevent an evolution into malignancy. The well-known FAP and MAP syndromes remain as the most common inherited causes of polyposis. However, there is a great number of family aggregations of individuals with polyposis that may include inherited polyposis syndromes with genetic cause yet to uncover. In recent years, some deleterious variants in emerging genes have been associated to the phenotype of polyposis syndromes and CRC, so it is important to screen retrospective samples taking into consideration these new findings.

This study aimed for the detection of deleterious variants in emerging genes associated with polyposis syndromes and CRC, in a total of 189 samples negative for *APC* and/or *MUTYH* pathogenic variants. A total of 18 variants (15 different) were found in 17 patients, seven of them deleterious (two of them in the same patient) and 11 VUS (9 different, two of them novel).

5.1. Genetic diagnosis of a patient with MAP and its implications

The patient corresponding to from sample #7 presents two deleterious variants within the coding region of *MUTYH*. Both variants, c.536A>G, p.(Tyr179Cys), and c.1187G>A, p.(Gly396Asp), are recurrent variants in *MUTYH* associated to MAP in several European populations, including the Portuguese population, representing 80% of *MUTYH*-associated disease observed in Caucasian individuals (Kantor, Sobrado, Patel, Eiseler, & Ochner, 2017; Landon et al., 2015). This patient is a female who presented 35 polyps in the colon and was diagnosed with CRC at the age of 40 and with breast cancer five years later. This index case had a brother with multiple polyps and a 29 year-old son who also had the presence of five polyps in the colon in addition to her mother having died of gastric cancer at age 49. According to the pedigree of this patient, an autosomal recessive disease was not expected, namely due to two consecutive generations with early-onset polyposis. This family had been studied in 2005 for germline variants in *APC* and no deleterious variants were found. This finding highlights the importance of the implementation of NGS panels covering a set of genes associated to polyposis syndromes and CRC in the routine analyses, since the family information can be scarce or (as it

happened in this case) the family phenotype can resemble other hereditary syndromes and may mislead the choice of the genetic test. These data are supported by recent literature (Sutcliffe et al., 2019) that confirms the importance of the implementation of a multi-gene NGS panel due to MAP phenotypic variability and to the possible presence of less frequent deleterious variants within the coding region of *MUTYH*.

Since MAP is an autosomal recessive Mendelian disease, the siblings of an index patient have 25% likelihood of inheriting biallelic mutation and these at-risk family members should be offered site-specific testing for the familial pathogenic variants. Full sequencing of *MUTYH* should only be considered in an unaffected parent if the other parent was diagnosed with MAP. No further studies in the children are necessary if no *MUTYH* pathogenic variant is found in the unaffected parent. When the unaffected parent was not tested for *MUTYH* pathogenic variants, the children should be indicated for *MUTYH* testing in the adult age (Burke & Dallas, 2021). The genetic diagnosis of MAP in this family will therefore help guide genetic counseling and surveillance colonoscopy recommendations for the various family members.

According to the NCCN guidelines for genetic/familial high-risk assessment for colorectal cancer, in all biallelic *MUTYH* pathogenic variants carriers surveillance colonoscopy should begin between 25 and 30 years old (earlier if family history justifies it) and should be repeated every 1 to 2 years if negative (Burke & Dallas, 2021).

5.2. The significance of heterozygous *MUTYH* variant carriers

Two patients presented a *MUTYH* deleterious variant in heterozygosity. One patient presented the recurrent/founder pathogenic variant c.536A>G, p.(Tyr179Cys), and the other the variant c.1437_1439del, p.(Glu480del). Despite MAP being an autosomal recessive disease caused by homozygous or compound heterozygous germline variants, there are reports of increased CRC predisposition in individuals with heterozygous *MUTYH* pathogenic variants. Carriers of monoallelic deleterious variants within the coding region of *MUTYH* were shown to have a 1.15-fold increased predisposition for CRC development (Win, Hopper, & Jenkins, 2011) and a 2.5-fold increased risk if family history for CRC is present (Win et al., 2014). This increased risk, although not comparable to that of a Mendelian disease with high penetrance, is well-established for both *MUTYH* founding mutations, but is yet to be established for less frequent variants (Theodoratou et al., 2010).

Recommendations for surveillance in heterozygous carriers of *MUTYH* pathogenic variants depends on the family history (Burke & Dallas, 2021). If a first-degree relative has been diagnosed with CRC, colonoscopy is recommended to be performed every 10 years, starting at 40 years old, or 10 years prior to the age of the first-degree relative with CRC at the time of the diagnosis. In cases where no relative has been diagnosed with CRC, there is no clear data about the screening and surveillance programs for heterozygous carriers to be included (Katona et al., 2018).

5.3. The role of testing of common *MUTYH* variants in Portugal

At the Department of Genetics of IPO Porto, whenever there is a clinical suspicion of MAP, we search for variants in the entire *MUTYH* gene only when the patient presents more than 20 polyps or family history of polyposis with a recessive inheritance pattern. In patients with 10–20 polyps, especially in those older than 60 years of age and without personal or familial history of colorectal cancer or polyposis, we start by testing specifically the recurrent pathogenic *MUTYH* variants in Portugal (c.536A>G, p.(Tyr179Cys); c.1187G>A, p.(Gly396Asp); and c.1227_1228dup, p.(Glu410GlyfsTer43)) and only sequence the entire gene if one of these variants is found in heterozygosity. Applying these criteria, we were able to identify a total of 41 families with MAP. Of these families, 26 (63.4%) presented only recurrent pathogenic variants, nine families (21.9%) presented one recurrent variant in concomitance with other less frequent variants, and only six families (14.6%) presented pathogenic variants that did not include any of the three most common (and which typically have more than 20 polyps).

In this study, all 79 patients with less than 20 polyps, over 60 years old, and without a personal/family history for polyposis or CRC, previously studied only for *MUTYH* recurrent pathogenic variants, were re-evaluated and none of them presented any pathogenic variant in the remaining coding and flanking regions of this gene. These data supports our strategy and reinforces the idea that MAP genetic diagnosis in patients with these characteristics are quite rare.

5.4. The significance of heterozygous *NTHL1* variant carriers

In this study we also found the presence of three variants classified as pathogenic or likely pathogenic within the coding region of *NTHL1*. These three variants were all in heterozygosity.

The *NTHL1* tumor syndrome is a multi-tumor spectrum disease that includes polyposis caused by biallelic inactivating variants in the *NTHL1* gene (Grolleman et al., 2019). Studies indicate that a heterozygous state of variants in this gene are not associated with this syndrome (Kumpula et al., 2020) However, a recent study from Li et al. described the possibility of a low to moderate increased risk for breast cancer (N. Li et al., 2021), but further studies are necessary to confirm this report. Individuals carrying *NTHL1* deleterious heterozygous variants or VUS should participate in screening studies for a better evaluation of their risk, if any, for polyposis and/or CRC.

Since there is scarce literature about the impact of *NTHL1* pathogenic variants in heterozygosity, there are presently no recommendations in clinical guidelines concerning surveillance in individuals who present this genotype.

5.5. The role of testing of the common *NTHL1* variant in Portugal

The *NTHL1* gene only recently has been associated to a multi-tumor that includes polyposis. At the Department of Genetics of IPO Porto, *NTHL1* biallelic pathogenic variants were found in three different families. Two of these families carry the recurrent variant c.268C>T, p.(Gln90Ter), in homozygosity, and the other family also carries this recurrent variant in concomitance with an in-frame likely pathogenic variant within the coding region of this gene. In addition, three other unrelated individuals with a *NTHL1* pathogenic variant in heterozygosity have been Identified, one of them carrying the recurrent variant, while the others carried two distinct less common pathogenic variants.

In this study three different pathogenic variants were found in three individuals, the recurrent variant c.268C>T, p.(Gln90Ter), and two splice-site variants: c.139+1G>A and c.550-1G>A. Although the data is still scarce, our findings suggest that in Portugal screening of the entire coding region of *NTHL1* in all individuals with polyposis may be the best approach, at least for those that have the same clinical criteria for full *MUTYH* testing (but also having in mind the clinical differences between the two syndromes). This somehow contrasts with the initial idea at our department based on literature data and the initial finding that all three biallelic pathogenic *NTHL1* variant families presented the recurrent variant in at least one allele. However, there might be a role for testing of the most common *NTHL1* variant together with the three most common

MUTYH variants in patients with 10–20 polyps older than 60 years of age and without personal or familial history of colorectal cancer or polyposis.

5.6. Variants of uncertain significance

One individual presented a VUS within exon 9 of *POLE*, the c.861T>A, p.(Asp287Glu), variant. This variant has been described several times in individuals with a variety of different phenotypes, including, cutaneous melanoma (Potjer et al., 2019), squamous cell carcinoma, non-Hodgkin lymphoma (Aoude et al., 2015), breast cancer (Mur et al., 2020), Lynch Syndrome associated tumors (Billingsley et al., 2015; Jansen et al., 2016), and high-risk hereditary CRC (Buchanan et al., 2018; Mur et al., 2020). Reports on ClinVar claim that this variant may have a damaging effect on the protein function when using some *in-silico* tools. Since this variant affects the exonuclease domain of Pol ε and there is evidence in the literature about the deleterious effects of several variants in this domain (Ahn et al., 2016; Palles et al., 2013), a pathogenic potential was initially expected. However, some other evidence supports a possible benign classification for this variant, namely, the frequency of this variant being higher in comparison with the average of other pathogenic variants (expected frequency in the Non-Finnish European population is 0.20% in 1000 Genomes, 0.14% in ExAC, and 0.18% in GnomAD) (Kobayashi et al., 2017) and the study from Aoude et al. showing that this variant did not segregate along with the disease phenotype (Aoude et al., 2015). Recently, Mur et al. classified this variant as likely benign since they demonstrate that it lacks the ED-associated mutational signature (Mur et al., 2020). Considering the disparate nature of current evidence around this variant, further studies should be held to determine its disease-causing or benign nature.

Four VUS were found in the coding region of *MSH3*, one of them not reported in the literature. These variants have been reported mostly in cases with predisposition for hereditary cancer, but the literature is scarce about the phenotype consequences of these alterations in *MSH3*, except for the c.2732T>G, p.(Leu911Trp), which was only reported in heterozygosity in individuals with several pathological features, including polyposis and CRC (Dámaso et al., 2020; DeRycke et al., 2017; Duraturo et al., 2011; Morak et al., 2017; Anna Rohlin et al., 2017; Terradas et al., 2019). Taking this evidence into consideration, the role of *MSH3* in predisposition to polyposis and CRC is only confirmed when biallelic mutation are present (Adam et al., 2016), which was not observed in any of the cases in this study.

In this study, two VUS within *RNF43* coding region were found. Deleterious variants in this gene have been associated to a specific form of CRC, arising from serrated polyps (Bond et al., 2016; Gala et al., 2014; Yan et al., 2017). Since then, several deleterious variants within the coding region of *RNF43* have been strongly associated to CRC, and other tumors, including the endometrium (Giannakis et al., 2014; Lai et al., 2019; S. Li et al., 2020; Matsumoto et al., 2020; Sackstein, Buechner, & Weinberg, 2021). The *RNF43* gene has only recently been associated to a tumorigenesis phenotype in the colon, and its related to the occurrence of serrated polyposis, a less frequent polyposis syndrome in the colon and rectum (IJspeert, Vermeulen, Meijer, & Dekker, 2015). However, the frequency of deleterious variants in *RNF43* is quite low in serrated polyposis patients and other yet unknown genes may explain most cases of this type of polyposis.

The variant c.-111G>A within the *GREM1* promotor was also Identified in this study. No literature is available for this variant, and there are no reports on ClinVar. Although duplications of the *GREM1* upstream region have been associated with HMPS (Arnau-Collell et al., 2020; Jaeger et al., 2012; Lieberman et al., 2017), further studies are necessary to understand the pathologic potential of other types of variants in that region of the genome.

Finally, although the *MUTYH* missense variant c.925C>T has been described in the literature In patients with polyposis or CRC (Goto et al., 2010; Halford et al., 2003; Komine et al., 2015; Nielsen et al., 2009; Anna Rohlin et al., 2017; Sieber et al., 2003; Vogt et al., 2009), the data is still not enough to conclusively determine its pathological or benign nature.

Although the *GREM1*, *MSH3*, *POLE*, *POLD1*, and *RNF43* genes have all been associated to some form of polyposis and high risk of CRC, this work shows that they may explain only a minority of patients with polyposis and that more studies are required to explain the eventual genetic predisposition of many patients with polyposis besides those having FAP or MAP.

6. Conclusion

After the evaluation of the results of this study, we are able to conclude that deleterious variants in emerging genes are a rare event in polyposis patients. We were only able to identify carriers of deleterious variants in *NTHL1* (only in heterozygosity), while in the other emerging genes no clearly pathogenic variants were detected. However, considering the literature data about these genes, we still believe that these genes should be included in large gene panels for the study of individuals with any form of hereditary colorectal polyposis and CRC.

We also confirm that the current strategy routinely applied in cases with less than 20 polyps in older patients without a family history is cost-effective, since none of the 79 cases with these criteria previously studied for only the recurrent pathogenic *MUTYH* variants presented pathogenic variants in the remaining coding regions of this gene. On the other hand, this study reinforces the importance of large NGS panels for the molecular diagnosis of hereditary polyposis since the clinical criteria do not have full sensitivity to decide which genes to test due to the phenotype overlap of several of these syndromes.

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