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MESTRADO EM ONCOLOGIA
ESPECIALIZAÇÃO EM ONCOLOGIA LABORATORIAL

Molecular diagnosis of *MBD4*-associated neoplasia syndrome

Inês Maria Rosendo da Costa Querido

M

2023

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M. ICBAS. 2023

INSTITUTO DE **CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**

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Molecular diagnosis of *MBD4*-associated neoplasia syndrome

Dissertação de candidatura ao grau de Mestre em Oncologia – especialização em Oncologia Laboratorial – submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Menção de apoio financeiro

Este trabalho foi realizado com o apoio da Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte, através da atribuição de uma das Bolsas de Investigação em Oncologia LPCC-NRN 2023.

Agradecimentos

Em primeiro lugar, quero agradecer ao Professor Manuel Teixeira, meu orientador, pela oportunidade de realizar este trabalho no Serviço de Genética Laboratorial do IPO-Porto. Obrigada por toda a ajuda, ensinamentos, sugestões e por ter partilhado do meu entusiasmo ao longo deste trabalho.

À Carla Pinto, que sempre me apoiou e guiou ao longo deste ano. Obrigada por teres partilhado comigo o teu conhecimento e por arranjares sempre tempo para mim e para qualquer dúvida que me surgisse (sem pressão!). Sei que a sorte esteve do meu lado com o *MBD4*, mas também é uma sorte poder aprender contigo.

À Patrícia Arinto, por toda a ajuda que me deu, especialmente no laboratório. Obrigada por teres partilhado comigo a tua experiência e por estares sempre disponível para me ensinar e ajudar, principalmente a encontrar “aqueles primers que parece que não estão em lado em nenhum”.

Às minhas “lab partners”, Raquel, Elsa e Cátia, pela amizade e pelo companheirismo. Obrigada pelos desabafos, pela paciência, pela motivação e, especialmente, pelos almoços e lanches em que ríamos mais do que falávamos.

A todos os restantes membros do Serviço de Genética Laboratorial do IPO-Porto, obrigada por me terem integrado nesta equipa, pela simpatia constante e por me terem feito sentir tão à vontade desde o início.

A todos os docentes do Mestrado em Oncologia, pela oportunidade de integrar este mestrado e por todos os conhecimentos transmitidos.

Aos bioquímicos que levo para a vida, Kika, Ju, Alex e Pedrelis, por estarem sempre (sempre!) lá, mesmo quando estamos mais distantes. Obrigada por ouvirem os meus disparates e por conseguirem sempre acrescentar algo ainda mais disparatado por cima. Tenho a agradecer-vos por todo o apoio e por partilharem sempre as minhas conquistas. A vida é imensamente mais fácil com amigos como vocês.

Às minhas 3+1 estrelinhas, Alex, Ju, Borer e Cisca, tem sido um prazer crescer convosco. Obrigada pela aventura das nossas vidas, por alinharem em tudo e por nunca me deixarem chorar sozinha (somos um). E ao excelentíssimo Babo, pelos conselhos e por me fazeres rir nos dias maus (ufa). Obrigada por todas as histórias (leia-se dramas) que ficam para contar destes 5 anos que, afinal, passaram mesmo a voar. Levo-vos no coração.

Por último, mas talvez mais importante, quero agradecer à minha família, em especial aos meus pais e aos meus avós. Obrigada por me proporcionarem a oportunidade de estudar, por me incentivarem a seguir os meus sonhos, por terem sempre uma palavra de encorajamento e, acima de tudo, por acreditarem sempre em mim. Já sei que “não percebem nada do que diz aqui”, mas este trabalho também é um bocadinho vosso.

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List of abbreviations

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3-AlkylA	3-Alkyladenine
5mC	5-methylcytosine
8-OxoG	8-Oxoguanine
A	Adenine
ACMG	American College of Medical Genetics and Genomics
AML	Acute myeloid leukemia
AP	Apurinic/apyrimidinic
APC	Adenomatous polyposis coli
APE1	Apurinic endonuclease 1
BCC	Basal cell carcinoma
BER	Base excision repair
BRCA1	BRCA1 DNA repair associated
BRCA2	BRCA2 DNA repair associated
C	Cytosine
CDKN1A/p21	Cyclin dependent kinase inhibitor 1A
COSMIC	Catalogue of Somatic Mutations in Cancer
CpG	5'-C-phosphate-G-3'
CRC	Colorectal cancer
DCIS	Ductal carcinoma *in situ* of the breast
ddNTP	Dideoxynucleotide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT3A	DNA methyltransferase 3 alpha
dNTP	Deoxynucleoside triphosphate
dRP	Deoxyribose phosphate
FADD	Fas-associated death domain protein
FAP	Familial adenomatous polyposis
FDA	Food and Drug Administration

FEN1	Flap endonuclease 1
G	Guanine
GITVA	Gastrointestinal tract tubulovillus adenoma
HBOC	Hereditary breast and ovarian cancer
HDAC	Histone deacetylase
HGVS	Human Genome Variation Society
HR	Homologous recombination
HSCT	Hematopoietic stem cell transplantation
IPO-Porto	Portuguese Oncology Institute of Porto
MANS	*MBD4*-associated neoplasia syndrome
MAP	*MUTYH*-associated polyposis
MBD	Methyl-CpG binding domain
MBD4	Methyl-CpG binding domain protein 4
MeCP2	Methyl-CpG binding protein 2
MLH1	MutL homolog 1
MMR	Mismatch repair
MPG	N-Methylpurine DNA glycosylase
MSH4	MutS homolog 4
MSI	Microsatellite instability
MUTYH	MutY DNA glycosylase
NCCN	National Comprehensive Cancer Network
NEIL	Nei-like DNA glycosylase
NER	Nucleotide excision repair
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NTHL1	Nth-like DNA glycosylase 1
OGG1	8-Oxoguanine glycosylase 1
OvGCT	Ovarian granullosa cell tumor
p16(INK4a)	Cyclin dependent kinase inhibitor 2A
PARP1	Poly(ADP-ribose) polymerase 1

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PM2	Pathogenic moderate 2
Pol	DNA polymerase
PVS1	Pathogenic very strong 1
SBS1	Single base substitution 1
SMUG1	Single strand selective monofunctional uracil DNA glycosylase 1
T	Thymine
TDG	Thymine DNA glycosylase
TP53	Tumor protein p53
U	Uracil
UDG	Uracil DNA glycosylase
UVM	Uveal melanoma
XRCC1	X-ray repair cross complementing 1

Abstract

Abstract

Since the 1990s, the list of genes associated with inherited cancer predisposition syndromes has been progressively increasing. Nonetheless, the cause of hereditary cancer in a considerable proportion of the affected families still remains unknown, underscoring the existence of various cancer predisposing genes that are yet to be uncovered.

The *MBD4* gene encodes a multifunctional protein, which mainly acts as a DNA glycosylase in the base excision repair (BER) pathway and ultimately prevents the formation of C>T mutations, thus contributing to maintain genomic integrity. Moreover, it seems to be able to participate in other key cellular processes, such as apoptosis and epigenetic regulation, although the mechanisms by which MBD4 contributes to these processes have not yet been completely elucidated. There is growing evidence demonstrating that defects in the *MBD4* gene play a role in carcinogenesis by promoting genomic instability, mainly through the accumulation of C>T transitions across the genome. Recently, germline biallelic loss-of-function variants in *MBD4* were shown to be responsible for a novel autosomal recessive multi-tumor predisposition syndrome, provisionally denominated as *MBD4*-associated neoplasia syndrome (MANS) and associated with increased susceptibility to develop adenomatous polyposis, colorectal cancer (CRC) and extracolonic neoplasms, mainly acute myeloid leukemia (AML).

The main aim of this work was to study the *MBD4* gene in five individuals, from four distinct families, affected with adenomatous polyposis and AML, in order to identify germline variants that could explain the phenotype observed in these families. Screening of germline variants in *MBD4* was performed by Sanger sequencing.

All five patients included in this study were shown to present homozygous deleterious germline variants in the *MBD4* gene, allowing the establishment of the molecular diagnosis of the syndrome in these four families. Three individuals presented the *MBD4* variant c.1544-1G>T, which affects splicing, and two individuals presented a previously unreported *MBD4* variant, the c.538del p.(Thr180ProfsTer35), which is presumed to originate a premature stop codon. Both of the detected variants are predicted to result in nonfunctional truncated MBD4 proteins, lacking the glycosylase domain.

This work significantly contributed to expand the knowledge about this emerging phenotype and provided new insights on *MBD4* constitutional deficiency, including broadening of the syndrome tumor spectrum and the identification of a novel *MBD4* deleterious variant. Our findings, along with those described in the literature so far,

strongly suggest that *MBD4* must be included in next generation sequencing (NGS) multi-gene panels used to study patients with personal and/or family history of colorectal polyposis, especially when associated with AML and/or uveal melanoma. Although MANS appears to be a rare hereditary cancer syndrome, it is crucial to perform its molecular diagnosis, since this information will enable personalized medical care for patients and their families, thus potentially contributing to increase their quality of life and overall survival.

Resumo

Resumo

Desde a década de 1990, a lista de genes associados a síndromes hereditárias de predisposição para cancro tem vindo a aumentar progressivamente. No entanto, a causa do cancro hereditário numa proporção considerável das famílias afetadas ainda permanece desconhecida, o que realça a existência de vários genes de predisposição para cancro que ainda estão por ser descobertos.

O gene *MBD4* codifica uma proteína multifuncional, que atua principalmente como DNA glicosilase na via de reparação por excisão de bases (*base excision repair,* BER) e previne a formação de mutações C>T, contribuindo, assim, para manter a integridade genómica. Adicionalmente, parece ter a capacidade de participar noutros processos celulares essenciais, como a apoptose e a regulação epigenética, embora os mecanismos através dos quais a MBD4 participa nestes processos ainda não se encontrem bem estabelecidos. Há evidências crescentes que demonstram que alterações no gene *MBD4* desempenham um papel na carcinogénese, promovendo a instabilidade genómica, principalmente através da acumulação de transições C>T ao longo do genoma. Recentemente, foi demonstrado que variantes germinativas bialélicas de perda de função no *MBD4* são responsáveis por uma nova síndrome autossómica recessiva de predisposição para múltiplos tumores, denominada de Síndrome de neoplasias associadas ao *MBD4* (*MBD4-associated neoplasia syndrome*, MANS) e associada a suscetibilidade aumentada para desenvolver polipose adenomatosa, cancro colorretal (CCR) e outras neoplasias, principalmente leucemia mieloide aguda (LMA).

O principal objetivo deste trabalho consistiu em estudar o gene *MBD4* em cinco indivíduos, pertencentes a quatro famílias distintas, afetados com polipose adenomatosa e LMA, de forma a identificar variantes germinativas que pudessem explicar o fenótipo observado nestas famílias. A pesquisa de variantes germinativas no gene *MBD4* foi realizada através de sequenciação de Sanger.

Todos os pacientes incluídos neste estudo apresentaram variantes homozigóticas deletérias no gene *MBD4*, o que permitiu estabelecer o diagnóstico molecular da síndrome nestas quatro famílias. Três indivíduos apresentaram a variante no *MBD4* c.1544-1G>T, que afeta o *splicing*, e dois indivíduos apresentaram a variante no *MBD4* c.538del p.(Thr180ProfsTer35), ainda não descrita na literatura, que se presume que origine um codão de terminação prematuro. Prevê-se que ambas as variantes detetadas resultem na produção de proteínas MBD4 truncadas não funcionais, com ausência do domínio glicosilase.

Assim, este trabalho contribuiu significativamente para melhorar o conhecimento acerca da deficiência constitucional de *MBD4*, incluindo a expansão do espetro tumoral da síndrome e a identificação de uma nova variante deletéria no *MBD4*. Os nossos resultados, juntamente com os descritos na literatura até à data, sugerem fortemente que o *MBD4* deve ser incluído nos painéis de genes de sequenciação de nova geração (*next generation sequencing*, NGS) utilizados para o estudo de indivíduos com história pessoal e/ou familiar de polipose colorretal, especialmente em associação com LMA ou melanoma uveal. Embora a MANS pareça constituir uma síndrome rara, é crucial realizar o seu diagnóstico molecular, uma vez que esta informação irá permitir oferecer cuidados médicos personalizados para os pacientes e para as suas famílias, potencialmente contribuindo para melhorar a sua qualidade de vida e a sobrevivência global.

I. Introduction

I. Introduction

Cancer consists of a complex group of diseases that arise when normal cells undergo genetic or epigenetic alterations that disrupt regulatory mechanisms, leading to uncontrolled proliferation and dissemination of abnormal cells (Frank, 2001; Wang, 2016). Notably, earlier diagnosis enables timely intervention before the spread of cancer cells, which significantly increases the chance of a successful outcome (Wardle et al., 2015).

Cancer emerges as a consequence of an intricate interaction between extrinsic factors, for instance tobacco use, alcohol intake, infectious organisms, among others, and intrinsic factors, such as hormonal changes, inflammation and hereditary conditions (Wu et al., 2018). The interplay between these factors ultimately results in DNA alterations, which affect several molecular pathways and lead to tumorigenesis. Therefore, cancer constitutes a genetic disease in the truest sense of the word (Frank, 2001).

In the realm of clinical healthcare, cancer currently stands as a global foremost challenge, with approximately 19 to 20 million new cases and 10 million deaths annually worldwide (Chhikara & Parang, 2023). Thus, it becomes essential to unravel the biological pathways and genetic alterations underlying tumor development, which will contribute to improve early detection, treatment and prevention strategies, in order to achieve more favorable outcomes for cancer patients.

1. Hereditary cancer predisposition syndromes

The vast majority of cancers occur sporadically, while approximately 5 to 10% of all cases are attributed to inherited cancer predisposition syndromes, which comprise a heterogeneous group of genetic disorders characterized by a significantly increased risk of developing a certain spectrum of neoplasias (Imyanitov et al., 2023; Rahner & Steinke, 2008; Wang, 2016). These syndromes follow a Mendelian mode of heritance and most of them are transmitted in an autosomal dominant manner. Nevertheless, a few examples of autosomal recessive inheritance of cancer predisposition have been described, although these cases appear to be more difficult to study, especially for common tumor types (Imyanitov et al., 2023).

Hereditary cancer predisposition syndromes occur due to deleterious germline variants in specific key genes, therefore being present in every cell of the human body.

In this context, the full inactivation of the cancer predisposing gene may represent the rate-limiting step for tumor initiation, but the process of neoplastic transformation ultimately requires the gradual accumulation of other cancer-driving events in the same cell clone, particularly to promote or enhance tumor progression (Berger et al., 2011; Rahner & Steinke, 2008). Thus, when an individual carries a cancer-associated germline variant, the first step into carcinogenesis has already been taken in every cell, which explains the increased propensity for tumor development, comparing with the general population. Moreover, in these cases, since tumorigenesis requires less additional somatic events, hereditary cancer is commonly associated with younger age of onset, comparing with sporadic cancer, and with the development of multiple primary malignancies (Imyanitov et al., 2023; Rahner & Steinke, 2008) (Figure 1).

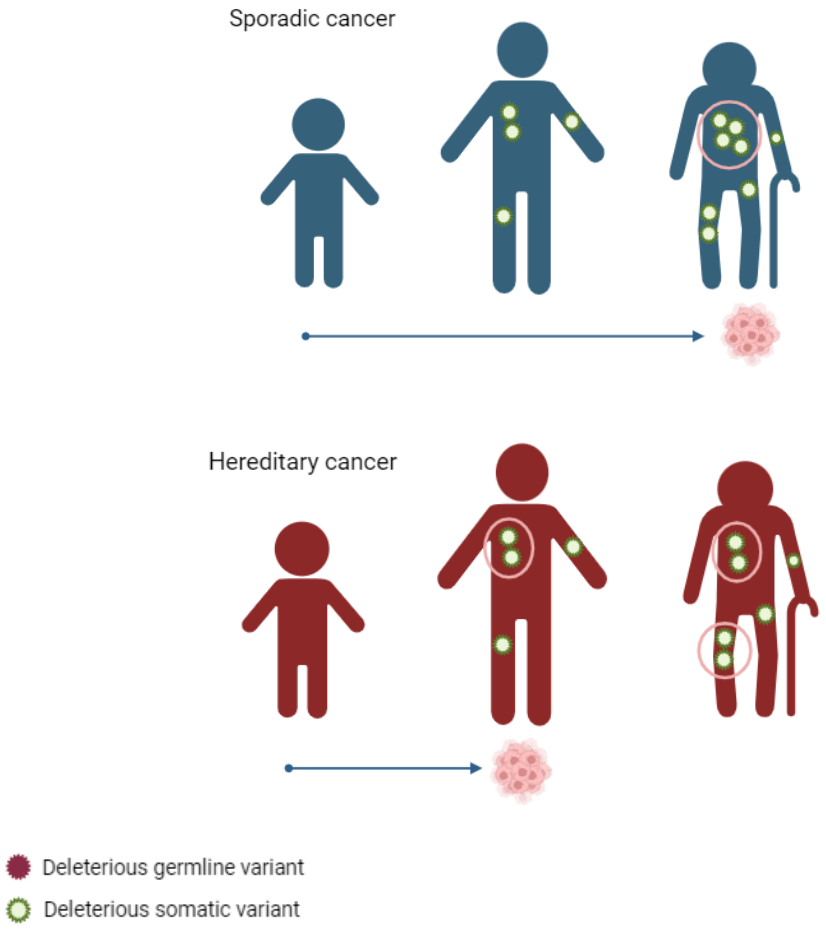

Figure 1. Mechanism of inherited cancer predisposition, comparing with sporadic cancer. In hereditary cancer, the first step into carcinogenesis has already been taken in every cell, meaning that less additional somatic events are necessary for tumor development. Thus, hereditary cancer is characterized by a relatively young age of onset, as well as for the presence of multiple primary malignancies.

Deleterious germline variants that are responsible for hereditary cancer predisposition syndromes occur in genes involved in essential cellular processes, such as cell cycle regulation, gene expression and DNA damage repair (Kentsis, 2020; Rahner & Steinke, 2008). Since the 1990s, the list of established cancer predisposition genes has been progressively increasing. Some examples include the *BRCA1* and *BRCA2* genes responsible for hereditary breast and ovarian cancer (HBOC) syndrome, the *TP53* gene for Li-Fraumeni syndrome, the *APC* gene for familial adenomatous polyposis (FAP), among others (Imyanitov et al., 2023; Wang, 2016). Nonetheless, the cause of hereditary cancer for a substantial proportion of the affected families still remains unknown, meaning that various other cancer predisposition genes are yet to be uncovered (Wang, 2016).

The detection of the causative germline variant in a hereditary cancer patient allows genetic testing for asymptomatic family members, offering the possibility of a reliable predictive diagnosis. Therefore, the at-risk relatives can be screened for the presence of the germline variant, in order to establish whether or not they have inherited the increased cancer susceptibility. With the aim of significantly improving prognosis and outcomes, mutation carriers are subsequently enrolled in specific surveillance programs, potentially complemented by prophylactic measures in certain situations. Moreover, non–mutation carriers may be discharged from unnecessary intensified cancer screening, since their cancer risk resembles that of the general population (Frank, 2001; Rahner & Steinke, 2008; Wang, 2016). Hence, the identification of cancer predisposition genes plays a fundamental role in personalized medical care for patients and their families.

2. DNA damage and repair

The human genome is constantly prone to the action of endogenous and exogenous damaging agents, which induce alterations in DNA and constitute a threat for the integrity of genetic information (Chatterjee & Walker, 2017). Endogenous DNA lesions can result from replication errors, hydrolysis or exposure to reactive metabolites, while exogenous DNA damage arises due to the action of environmental, physical or chemical compounds, such as ultraviolet and ionizing radiation, alkylating agents, chemotherapeutic drugs, among others (Carusillo & Mussolino, 2020; Chatterjee & Walker, 2017). These insults result in a wide variety of DNA lesions, including base

modifications, DNA adducts and dimers, single- and double-strand breaks, insertions or deletions, and crosslinks (Sharma et al., 2020).

In order to combat DNA damage, cells harbor DNA repair systems that collectively act to detect and correct genetic lesions, preventing the accumulation of mutations and contributing to the faithful transmission of genetic information (Chatterjee & Walker, 2017). Notably, it is currently estimated that human cells have to repair more than 10 000 DNA lesions per day (Carusillo & Mussolino, 2020). At least five major DNA repair pathways have been described to be active throughout different cell cycle stages, depending on the specific type of damage – base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR) and non-homologous end joining (NHEJ) (Sharma et al., 2020).

2.1. Base excision repair (BER)

The BER system is a primary mechanism for the correction of non-bulky DNA damage, namely single-base lesions in the form of small chemical modifications, including alkylation, oxidation, methylation and deamination, which do not usually cause distortions to the DNA helix structure (Wallace, 2014). This pathway is presumed to be mainly active throughout the G1 phase of the cell cycle, in order to ensure base damage correction before DNA replication (Chatterjee & Walker, 2017; Dianov & Hübscher, 2013).

The initial step in BER consists in the recognition and excision of a damaged or mismatched base by a specific DNA glycosylase. In humans, at least 11 subtypes of DNA glycosylases are described to participate in the BER pathway, for instance MutY DNA glycosylase (MUTYH), Nth-like DNA glycosylase 1 (NTHL1), thymine DNA glycosylase (TDG), methyl-CpG binding domain protein 4 (MBD4), among others. These enzymes can be mechanistically classified as monofunctional, if they exclusively present glycosylase activity, or as bifunctional, if they exhibit glycosylase and lyase activities, meaning that they are also able to cleave the DNA backbone (Beard et al., 2019; Krokan & Bjoras, 2013). The main characteristics of the 11 described human DNA glycosylases that participate in the BER pathway are summarized in Table 1.

Table 1. List of the described human DNA glycosylases that participate in the BER pathway, with the respective substrates and classification.

Enzyme	Abbreviation	Substrate	Classification
MutY homolog DNA glycosylase	MUTYH	A paired with 8-OxoG	Monofunctional
Thymine DNA glycosylase	TDG	T and U paired with G	
Methyl-CpG binding domain protein 4	MBD4		
Uracil DNA glycosylase	UDG	U	
Single strand selective monofunctional uracil DNA glycosylase 1	SMUG1		
N-Methylpurine DNA glycosylase	MPG	3-AlkylA	
8-Oxoguanine glycosylase 1	OGG1	8-OxoG	Bifunctional
Nth-like DNA glycosylase 1	NTHL1	Oxidized bases	
Nei-like DNA glycosylase 1, 2 and 3	NEIL 1, 2 and 3		

A – Adenine; 8-OxoG – 8-Oxoguanine; T – Thymine; U – Uracil; G – Guanine; 3-AlkylA – 3-Alkyladenine.

This first step generates an apurinic/apyrimidinic (AP) site that is recognized and cleaved by apurinic endonuclease 1 (APE1), leaving a gap flanked by a deoxyribose phosphate (dRP) on the 5' side and a hydroxyl group on the 3' end. Then, the accessory factors poly(ADP-ribose) polymerase 1 (PARP1) and X-ray repair cross complementing 1 (XRCC1) bind at the gap and promote the repair. Thereafter, the process may be conducted following one of two subpathways - short or long-patch BER, depending on the specific lesion type and the complexity of the damage. Regarding short-patch BER, in which a single nucleotide gap is generated, either DNA polymerase β (Pol β) or DNA polymerase λ (Pol λ) uses its phosphodiesterase activity to remove the 5'-dRP, generating the 5'-phosphate end required for ligation. Simultaneously, it conducts gap-filing DNA synthesis by introducing the proper complementary nucleotide. On the other hand, in long-patch BER, in which a gap of two to ten nucleotides is generated, strand-displacement DNA synthesis is conducted either by Pol β or other polymerases, such as DNA polymerase δ (Pol δ) or DNA polymerase ε (Pol ε), which use proliferating cell nuclear antigen (PCNA) as a cofactor. This generates a 5'-flap that is excised by flap endonuclease 1 (FEN1), leaving the nicked DNA necessary for ligation. Finally, either

for short or long patch-BER, these steps are followed by ligation by DNA ligase 1 or 3, thus completing the repair (Beard et al., 2019; Chatterjee & Walker, 2017; Wallace, 2014). This mechanism is briefly represented in Figure 2.

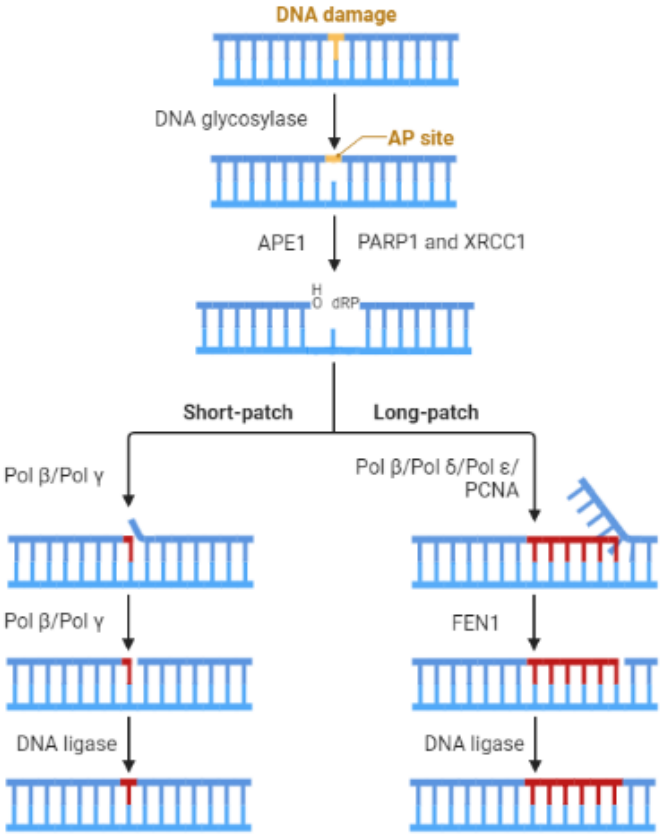

Figure 2. Mechanism of base excision repair (BER), including the short and long-patch subpathways.

2.2. BER defects and cancer

Malfunction or deregulation of DNA repair systems leads to mutagenesis and compromises genomic integrity, thereby promoting the development of DNA damage-induced diseases, particularly cancer (Chatterjee & Walker, 2017; Sharma et al., 2020). In fact, defects in genes encoding key players of the BER pathway, such as DNA glycosylases, have been shown to contribute to tumorigenesis by promoting genomic instability and increasing the susceptibility to develop certain neoplasias, as it is the case of *MUTYH* and *NTHL1*.

MUTYH contributes to the repair of oxidative damage, specifically by recognizing and removing adenine mispaired with 8-oxoguanine, which, if left unrepaired, may lead to G>T mutations upon DNA replication. It is well described that biallelic pathogenic germline variants in the *MUTYH* gene are responsible for an autosomal recessive hereditary cancer predisposition syndrome – *MUTYH*-associated polyposis (MAP), which is mainly characterized by an increased risk of developing adenomatous polyposis and colorectal cancer (CRC) (Al-Tassan et al., 2002; Sieber et al., 2003; Yamaguchi et al., 2014).

The identification of MAP put the BER pathway in the spotlight for a role in hereditary cancer, which led to the detection of new polyposis and CRC predisposing genes, such as *NTHL1*. This DNA glycosylase is also involved in the repair of oxidative lesions, namely oxidized pyrimidines, including 5-hydroxyuracil, 5-hydroxycytosine and thymine glycol (Das et al., 2020). Deleterious biallelic germline variants in *NTHL1* revealed to be the cause of *NTHL1* tumor syndrome, an autosomal recessive disorder mainly associated with increased susceptibility for colorectal polyposis, CRC and extracolonic neoplasias, such as breast cancer (Rebuzzi et al., 2023; Valle et al., 2019; Weren et al., 2015).

More recently, germline biallelic loss-of-function variants in *MBD4*, another gene encoding a DNA glycosylase of the BER system, have also been associated with inherited cancer predisposition.

3. The *MBD4* gene

The *MBD4* gene is located on chromosome 3q21 and encodes a 574 amino acid protein with a tripartite structure, that contains an N-terminal methyl-CpG binding domain (MBD), which allows it to recognize and bind to methylated DNA sequences, a central region currently lacking a recognizable domain, and a C-terminal catalytic DNA glycosylase domain (Bellacosa, 2001) (Figure 3). MBD4 was first characterized by Hendrich and Bird in 1988 (Hendrich & Bird, 1998) and it is notable for its unique structure, remaining the only human protein described to combine an MBD domain and DNA repair activity.

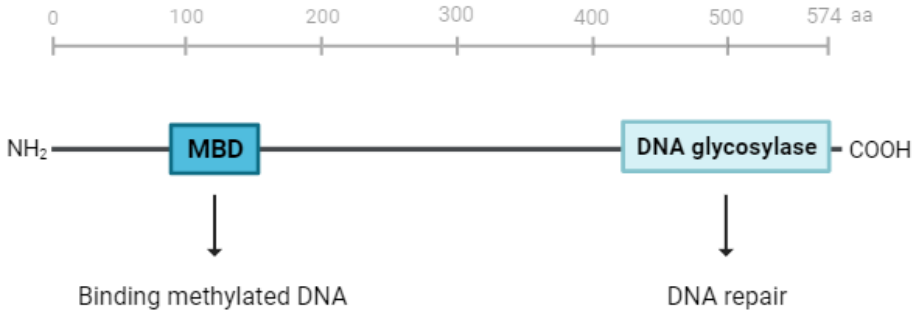

Figure 3. Schematic structure of the MBD4 protein. The N-terminal methyl-CpG-binding domain (MBD) domain allows it to recognize and bind to methylated DNA sequences, while the C-terminal catalytic glycosylase domain allows it to participate in DNA damage repair.

MBD4 consists in a multifunctional protein, which mainly acts as a thymine and uracil glycosylase specific for G:T and G:U mismatches arising from the deamination of 5-methylcytosine (5mC) and cytosine, respectively, preferentially in CpG sites (Bellacosa et al., 1999; Tricarico et al., 2015). Then, the repair process is carried out following the BER steps as aforementioned. These deamination reactions often occur spontaneously, at an estimated rate of 200 to 300 events per cell per day and, if not corrected, may lead to C>T transition mutations upon DNA replication, which represent the most frequent mutations in human cancer (Bellacosa & Drohat, 2015; Sjolund et al., 2013; Tricarico et al., 2015). Therefore, MBD4 avoids mutagenesis and contributes to maintain CpG integrity, being currently considered a candidate tumor suppressor gene (Derrien et al., 2021; Sjolund et al., 2013).

Beyond its primary function in DNA repair through the BER pathway, MBD4 has been described to participate in other crucial cellular processes, such as apoptosis and epigenetic regulation, including transcriptional repression. However, the mechanisms by which MBD4 contributes to these processes remain under investigation and are still poorly established.

3.1. *MBD4* and apoptosis

MBD4 has been identified as a binding partner of MutL homolog 1 (MLH1), which seems to allow it to interact with the MMR pathway (Bellacosa et al., 1999). A study by Cortellino and coworkers using mouse embryo fibroblasts demonstrated that MBD4 is capable of affecting the cell cycle in a similar manner as the MMR system, having the

ability of promoting apoptosis (Cortellino et al., 2003). It was shown that *MBD4*-deficient cells treated with DNA damaging agents fail to undergo apoptosis to the extent observed in *MBD4*-proficient cells. Additionally, Screaton and colleagues identified MBD4 as a binding partner of Fas-associated death domain protein (FADD) in a complex with MLH1, suggesting a possible mechanism by which the apoptotic response is modulated via p53 activation (Screaton et al., 2003) (Figure 4A). Consistently with these evidence, it was observed that *Mbd4*$^{-/-}$ knockout mice displayed reduced apoptosis in the small intestine in response to cytotoxic agents, such as 5-fluorouracil and cisplatin (Sansom et al., 2003).

Therefore, MBD4 seems to be able to mediate the cellular response to DNA damage, which represents a potential link between genome surveillance and apoptosis. Consequently, it also suggests that conventional chemotherapeutic agents may not be the optimal treatment approach for *MBD4*-deficient tumors (Parsons, 2003).

3.2. *MBD4* and epigenetic regulation

Since it contains an MBD domain, MBD4 is considered a member of the MBD protein family, along with MBD1, MBD2, MBD3 and methyl-CpG binding protein 2 (MeCP2). These proteins have the ability to recognize and bind to fully or hemi-methylated DNA sequences, playing a crucial role in epigenetic regulation (Du et al., 2015). Approximately 70 to 80% of all CpGs in the mammalian genome are methylated (in the form of 5mC) and these dinucleotides are most frequently found within promoter regions, being essential for the regulation of gene expression, genomic imprinting and X-chromosome inactivation (Greenberg & Bourc'His, 2019). High levels of DNA methylation at promoter regions are generally associated with gene silencing, while unmethylated promoters correlate with active genes. As such, deregulation of genomic methylation patterns, namely global DNA hypomethylation accompanied by hypermethylation of tumor suppressor genes, is associated with aberrant gene expression, which potentially leads to cancer development and progression (Skvortsova et al., 2019). Given the importance of this epigenetic mark, careful regulation of dynamic DNA methylation and demethylation is required to ensure genomic stability. The establishment and regulation of DNA methylation patterns is carried out by a complex interaction between several proteins, including for instance MBD proteins and DNA methyltransferases (DNMT) (Du et al., 2015).

In particular, DNMT1, also described as “the maintenance DNMT”, is mainly responsible for preserving existing methylated sites (Greenberg & Bourc’His, 2019). In a study using *Xenopus* embryos, it was demonstrated that DNMT1 depletion or inhibition was responsible for developmental defects, which arose due to activation of p53 mediated apoptosis (Stancheva, 2001). These evidence led researchers to further investigate the mechanism by which the apoptotic response was triggered, and a possible hypothesis is through the interaction between DNMT1, MBD4 and MLH1, functioning in a complex (Figure 4B). It was demonstrated that these proteins are colocalized in DNA damage sites, which implies that this complex may participate in a cellular checkpoint that monitors DNA methylation and further decides to either repair the damage or induce apoptosis. Therefore, it was hypothesized that the absence of DNMT1 at this checkpoint triggers cell death, by releasing MBD4 and MLH1 to automatically activate p53 mediated apoptosis (Ruzov et al., 2009). Hence, this process consists in another proposed mechanism by which MBD4 is involved in modulating the apoptotic response, as well as represents a potential functional link between epigenetic regulation, DNA repair and apoptosis.

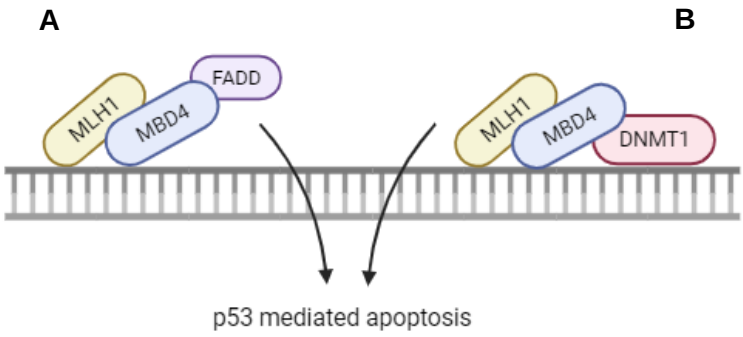

Figure 4. Two proposed mechanisms by which MBD4 seems to modulate the apoptotic response via p53 activation. **(A)** MBD4 has been demonstrated to interact with MLH1 and FADD in a complex that appears to mediate apoptosis in response to DNA damaging agents. **(B)** MBD4 is thought to act in a DNA methylation cellular checkpoint, in a complex with MLH1 and DNMT1, which further decides to either repair methylation damage or activate apoptosis.

Furthermore, it is well established that MBD1, MBD2, MBD3 and MeCP2 can function as transcriptional repressors, by binding methylated DNA in promoter regions and recruiting co-repressors, which leads to the inhibition of the downstream gene (Du et al., 2015). However, the role of MBD4 in repressing transcription has not yet been fully explored. To date, there have only been described four target genes for which MBD4 seems to participate in transcriptional inhibition – *p16(INK4a), MLH1, CDKN1A/p21* and

MSH4 (Kondo et al., 2005; Laget et al., 2014). The mechanisms by which MBD4 represses its target genes are still poorly understood, but evidence collected to date indicates that, regarding *p16(INK4a)* and *MLH1*, the repression mediated by MBD4 may be histone deacetylase (HDAC)-dependent, as it has also been observed for other MBD proteins (Kondo et al., 2005). On the other hand, in the case of *CDKN1A/p21* and *MSH4*, the process appears to be mediated by an interplay between MBD4 and DNMT1 (Laget et al., 2014), possibly comprising an additional context in which these two proteins interact.

3.3. The role of *MBD4* in carcinogenesis

Due to its functions in DNA repair and genetic surveillance, alterations in the *MBD4* gene are expected to lead to an accumulation of DNA damage throughout the genome, which can contribute to tumorigenesis via genomic instability (Sjolund et al., 2013). Moreover, deregulation of apoptosis, DNA methylation patterns and transcriptional repression also comprise different potential mechanisms by which *MBD4* deficiency may contribute to carcinogenesis, although further research is required to confirm these hypotheses.

In fact, it has been observed that *Mbd4*$^{-/-}$ knockout mice are viable and fertile, but exhibit a significant increase in C>T mutations at CpG sites, as well as present accelerated development of intestinal tumors and increased multiplicity in a cancer-predisposing genetic background, such as the presence of a heterozygous mutation in the *APC* gene (Millar et al., 2002; Wong et al., 2002). In this context, it was further noted that the increased tumorigenicity was specifically caused by an increase in inactivating somatic C>T mutations at CpG sites within the coding region of the wildtype *APC* allele. Moreover, a study by Tricarico and colleagues demonstrated that *Mbd4*$^{-/-}$ and *Mlh1*$^{-/-}$ double knockout mice showed a significant reduction in survival and an increased incidence of lymphomas, comparing with *Mlh1*$^{-/-}$ single knockout mice (Tricarico et al., 2015). Additionally, it was also observed that the absence of *MBD4* was capable of modifying the tumor spectrum associated with the *MLH1* defect, leading to the development of more aggressive tumors.

Altogether, these findings corroborate the functional importance of *MBD4* in maintaining DNA integrity and suppressing carcinogenesis, as well as support the notion that *MBD4* defects play a fundamental role in tumor development, by modulating the somatic mutational landscape and influencing cancer progression.

3.4. *MBD4* defects in sporadic tumors

The *MBD4* gene has been identified to be somatically mutated in approximately 26 to 43% of sporadic tumors displaying microsatellite instability (MSI), including CRC as well as gastric, endometrial and pancreatic carcinomas (Bader et al., 1999; Riccio et al., 1999; Yamada et al., 2002). The majority of these inactivating mutations occur via frameshifts within a polyadenine tract of ten consecutive adenine nucleotides (A_{10}) in exon 3, which are predicted to result in the production of truncated nonfunctional MBD4 proteins lacking the glycosylase domain. Given the presence of normal cells within primary tumor samples, researchers found it difficult to define the heterozygous or homozygous nature of these somatic mutations, but Riccio and colleagues provided evidence indicating that at least a small proportion of these tumors exhibits biallelic inactivation of *MBD4* (Riccio et al., 1999). Thus, it remains unclear whether *MBD4* somatic mutations are the cause of these cancers or simply reflect the presence of MSI. Nevertheless, a study using human MSI colon cancer cells demonstrated that these heterozygous *MBD4* mutations exert a dominant negative effect, by competitively inhibiting the glycosylase activity of the remaining wildtype *MBD4* allele and leading to a significant increase in mutation frequency (Bader et al., 2007). Hence, even the *MBD4* mutations that occur as a consequence of MSI seem to be capable of affecting tumor progression.

Furthermore, alterations in the expression levels of *MBD4* have also been observed in hepatocarcinoma, glioma and bladder cancer (Saito, 2001; Schlegel et al., 2002; Wojtczyk-Miaskowska et al., 2017). Beyond that, various studies suggest that some polymorphisms in the *MBD4* gene seem to influence the risk of developing certain neoplasias, namely esophageal, gastric, cervical and lung cancer, although the results remain controversial (Hao et al., 2004; Miao et al., 2008; Shin et al., 2006; Wang et al., 2017; Xiong et al., 2012; Yin et al., 2015).

3.5. *MBD4* heterozygous germline variants

A role for *MBD4* germline mutations in hereditary cancer was hypothesized two decades ago (Hendrich et al., 1999), but only recently concrete evidence has started to emerge regarding this correlation.

In addition to the mouse models experiments, Tricarico and collaborators reported the presence of *MBD4* heterozygous germline variants in CRC patients

(Tricarico et al., 2015). In 2019, Tanakaya and colleagues also identified a *MBD4* deleterious germline variant in a patient with early-onset CRC with oligopolyposis, presenting somatic inactivation of this gene in the cancer tissue (Tanakaya et al., 2019). Furthermore, loss-of-function germline variants in *MBD4* accompanied by somatic loss of heterozygosity were recently identified in patients with uveal melanoma, as well as in a case of glioblastoma (Derrien et al., 2021; Johansson et al., 2019; Rodrigues et al., 2018). Consistently with the observations provided by the previous *in vivo* studies, these reports also found an elevated mutational burden in the *MBD4*-deficient tumors with a predominance of C>T transitions, which corresponds to the Catalogue of Somatic Mutations in Cancer (COSMIC) mutational signature SBS1 (Waszak et al., 2017).

Regarding the uveal melanoma patients harboring deleterious *MBD4* germline variants, it was observed that these individuals presented an unusually good response to immune checkpoint inhibitors therapy. This positive outcome is likely attributed to the hypermutated nature of these tumors, as a higher number of somatic mutations leads to an increased formation of neoantigens, thereby increasing the probability of recognition by immune cells (Johansson et al., 2019; Rodrigues et al., 2018). These findings pave the way for a role of *MBD4* as a potential predictive biomarker of immunotherapy response, as well as open the possibility of conducting clinical trials to evaluate the use of immune checkpoint inhibitors as a treatment option for *MBD4*-deficient cancers.

3.6. *MBD4*-associated neoplasia syndrome (MANS)

More recently, deactivation of *MBD4* due to biallelic loss-of-function germline variants was shown to be associated with hereditary predisposition to adenomatous polyposis, CRC and extracolonic neoplasms, mainly early-onset acute myeloid leukemia (AML) (Blombery et al., 2022; Griffin et al., 2021; Palles et al., 2022; Sanders et al., 2018).

The first evidence that constitutive inactivation of this gene could be the cause of an inherited cancer predisposition syndrome were described in 2018, by Sanders and coworkers, who reported three patients, from two families, affected with early-onset AML, two of which had performed colonoscopy and presented colorectal polyps (Sanders et al., 2018). To our knowledge, these were the first individuals described as carrying biallelic deleterious germline variants in *MBD4*. Since then, other studies have focused on *MBD4* constitutional deficiency and new cases have been reported, allowing a better understanding of this emerging rare syndrome (Blombery et al., 2022; Griffin et al., 2021;

Palles et al., 2022). To date, there have been described a total of eleven individuals, from eight distinct families, among which ten presented colorectal polyps and seven also developed AML. Moreover, biallelic variant carriers seem to frequently present benign lesions, such as schwannoma, reported in three of these patients, and meningioma, which has been reported once. Other described manifestations of this syndrome include uveal melanoma, lymphoma, papillary thyroid cancer, ovarian granulosa cell tumor, breast cancer, liver and renal cysts, and upper gastrointestinal adenomas.

Furthermore, all these reports also noted that the *MBD4*-deficient cancers presented an increased mutation load, heavily dominated by C>T transitions occurring at CpG sites, once again supporting the idea of a mutational spectrum corresponding to the mutational signature SBS1. Notably, five of the described AML cases seem to share a common molecular pathway to malignancy, particularly through the acquisition of pathogenic somatic mutations affecting *DNMT3A* (Blombery et al., 2022; Griffin et al., 2021; Sanders et al., 2018). Mutations in this gene are recognized to alter the self-renewal capacity of hematopoietic stem cells and lead to clonal hematopoiesis, which eventually progresses to AML (Sanders et al., 2018). These findings indicate that *MBD4* inactivation appears to be associated with a conserved path to the development of hematological neoplasms, but additional studies are required to better understand and establish this process.

Thus, *MBD4* constitutional deficiency is responsible for a novel multi-tumor predisposition syndrome, transmitted in an autosomal recessive manner. In 2022, Palles and coworkers (Palles et al., 2022) provisionally denominated this condition as *MBD4*-associated neoplasia syndrome (MANS). Although it appears to be a rare syndrome, the identification of MANS patients is of paramount importance, in order to better characterize the disease tumor spectrum. This knowledge may contribute to a more comprehensive understanding of the mechanisms by which *MBD4* inactivation is involved in carcinogenesis, as well as to the development of personalized diagnostic, surveillance and treatment strategies for biallelic variant carriers.

II. Aims of the study

II. Aims of the study

The main aim of this work was to study the *MBD4* gene in patients affected with adenomatous polyposis and AML, in order to detect germline variants that could explain the phenotype of these families.

Specifically, the objectives of this study were:

- To perform germline variant screening in the complete coding regions and the adjacent exon boundaries of the *MBD4* gene;
- To assess the significance of incorporating *MBD4* into genetic screening protocols for individuals with personal and/or family history of colorectal polyposis, particularly when co-occurring with AML.

III. Materials and Methods

III. Materials and Methods

1. Patients, samples and DNA extraction

This study included all individuals affected with AML and adenomatous polyposis, that had been referred to the Department of Laboratory Genetics of the Portuguese Oncology Institute of Porto (IPO-Porto) between June 2004 and May 2023 for germline variant analysis in genes associated with hereditary predisposition to adenomatous polyposis, namely *APC* and *MUTYH*, in which no deleterious variants were detected.

In order to study the *MBD4* gene by Sanger sequencing, archived DNA samples from the index patients were selected, in which DNA had previously been isolated from peripheral blood using standard procedures. In addition, whenever possible, affected relatives of the index patients were also studied for the presence of the germline variant detected in the family.

2. *MBD4* germline variant analysis

Screening of deleterious germline variants in the *MBD4* gene was performed by Sanger sequencing of the entire coding regions (exons 1-8) and flanking splice junctions. For this purpose, due to the size of the gene in analysis, DNA was amplified using different primer sets, as represented in Table 2.

Table 2. Primers used for the amplification of exons 1-8 (and flanking splice junctions) of the *MBD4* gene.

Exon	Direction	Nucleotide sequence
1	F	5'-TGTAAAACGACGGCCAGTCAACATTCAGACCTCGGTTGC-3'
	R	5'-CAGGAAACAGCTATGACCCTGCCGACCCTCTGTCAAA-3'
2	F	5'-TGTAAAACGACGGCCAGTCATAGCACAAGACTGGTCCAC-3'
	R	5'-CAGGAAACAGCTATGACCATTCCTGCTATGCTCCCACTA-3'
3.1	F	5'-TGTAAAACGGCCAGTAGAAATCTGAAATGTGGTCCA-3'
	R	5'-CAGGAAACAGCTATGACCATAGTCACCTTTCCTTTGGGC-3'
3.2	F	5'-TGTAAAACGACGGCCAGTTGCCGCCAAGTAGTAGTTCAG-3'
	R	5'-CAGGAAACAGCTATGACCTATGGGCACGAATACAAGATG-3'
4-5	F	5'-TGTAAAACGACGGCCAGTTAGAATAGTGCCTGGCATGCT-3'
	R	5'-CAGGAAACAGCTATGACCTTTAAGGGTGAAGGGGGAATG-3'

Table 2. Continued.

Exon	Direction	Nucleotide sequence
6	F	5'-TGTAAAACGACGGCCAGTACTTGAACATGAAATCAGCCC-3'
	R	5'-CAGGAAACAGCTATGACCTGCTTGAGCTGAAAAACCTCC-3'
7	F	5'-TGTAAAACGACGGCCAGTGGCATGTGGGCAGAATTAGG-3'
	R	5'-CAGGAAACAGCTATGACCGGGAGCGCCTACTGATCAAA-3'
8	F	5'-TGTAAAACGACGGCCAGTGCCACCCCACATTCTAAGTC-3'
	R	5'-CAGGAAACAGCTATGACCAAGATCCACCATTGGCACAC-3'

F – Forward; R – Reverse.

For amplification of all exons (except exon 7), approximately 20 ng of DNA were used in a solution containing 1x *Taq* reaction buffer [Thermo Fisher Scientific, Waltham, MA, USA] (constituted by 75 mM Tris-HCl and 20 mM $(NH_4)_2SO_4$), 1.25 mM of $MgCl_2$ [Thermo Fisher Scientific], 0.5 mM of dNTP mix [Thermo Fisher Scientific], 0.33 mM of each primer (forward and reverse) [Frilabo, Maia, Portugal], 1 U of *Taq* DNA polymerase [Thermo Fisher Scientific] and bidestilled sterile water [B. Braun, Foster City, CA, USA], in a final reaction volume of 25 µL. The PCR reaction was performed in a thermocycler [Gene Amp PCR Systern 9700, Perkin-Elmer, Waltham, MA, USA], according to the conditions presented in Table 3.

Table 3. PCR program for the amplification of exons 1-6 and 8 (including flanking splice junctions) of the *MBD4* gene.

Cycles	Step	Temperature	Time
	Initial denaturation	95ºC	10 min
35x	Denaturation	95ºC	45 sec
	Annealing	58ºC	45 sec
	Extension	72ºC	45 sec
	Final extension	72ºC	10 min
	Pause	4ºC	∞

Regarding exon 7, approximately 20 ng of DNA were amplified in a solution containing 1x PCR Gold buffer [Applied Biosystems, Foster City, CA, USA] (constituted by 150 mM Tris-HCl and 500 mM KCl), 1.5 mM of $MgCl_2$ [Applied Biosystems], 0.75 mM of dNTP mix [Applied Biosystems], 1.25 mM of each primer (forward and reverse) [Frilabo] and 0.5 U of AmpliTaq Gold [Applied Biosystems], in a final reaction volume of 20 µL. The PCR reaction was performed

in a thermocycler [Gene Amp PCR Systern 9700, Perkin-Elmer] according to the conditions represented in Table 4.

Table 4. PCR program for the amplification of exon 7 (including flanking splice junctions) of the *MBD4* gene.

Cycles	Step	Temperature	Time
	Initial denaturation	95ºC	10 min
20x	Denaturation	95ºC	1 min
	Annealing	65ºC	1 min
	Extension	72ºC	1 min
15x	Denaturation	95ºC	1 min
	Annealing	55ºC	1 min
	Extension	72ºC	1 min
	Final extension	72ºC	10 min
	Pause	4ºC	∞

Then, the amplified PCR products were analyzed by high-resolution capillary electrophoresis in a QIAxcel Advanced system [QIAGEN, Hilden, Germany] and the results were evaluated using the QIAxcel ScreenGel software [QIAGEN].

Subsequently, the purification of the PCR products was performed using the ExoSAP-IT method in order to remove excess of primers and dNTPs. The samples were purified adding 2 µL of ExoSAP solution [Exonuclease I [Thermo Fisher Scientific] (20 U/µL) and Fast Thermosensitive Alkaline Phosphatase [Thermo Fisher Scientific] (1 U/µL), in a proportion of 1:2] to 5 µL of the PCR product, followed by incubation at 37ºC for 50 minutes and enzyme inactivation at 80ºC for 15 minutes.

The purification was followed by the sequencing reaction, using the BigDye® Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems]. The reaction consisted of mixing 3.4 µL of sequencing buffer, 0.5 µL of Big Dye® Terminator v3.1 (containing dNTPs, ddNTPs-fluorocromes, $MgCl_2$ and Tris-HCl buffer), 0.3 µL of the adequate primer (forward or reverse), 4.8 µL of bidestilled sterile water [B. Braun] and 1 µL of the previously purified DNA, in a final reaction volume of 10 µL. The sequencing reaction occurred according to the conditions presented in Table 5.

Table 5. PCR program for the sequencing reaction.

Cycles	Step	Temperature	Time
	Initial denaturation	95ºC	4 min
35x	Denaturation	95ºC	10 sec
	Annealing	50ºC	10 sec
	Extension	60ºC	2 sec
	Final extension	60ºC	10 min
	Pause	4ºC	∞

In order to remove excess of dNTPs, labeled ddNTPs and non-incorporated primers, the sequencing products were purified with Illustra Sephadex® G-50 fine [GE Healthcare, Life Sciences, Cleveland, OH, USA], according to standard procedures. After the purification, 12 µL of Hi-DiTM Formamide [Applied Biosystems] were added to the sequencing products to help stabilize the single stranded DNA.

The purified sequencing products were then analyzed in a 3500 Genetic Analyzer [Applied Biosystems] by capillary electrophoresis. The electropherograms of each sample were analyzed with the Sequencing Analysis Software v5.4 [Applied Biosystems]. All of them were analyzed at least twice, reviewed manually and with the Mutation Surveyor® DNA Variant Analysis Software v4.0.8 [Softgenetics, State College, PA, USA].

All variants were described in accordance with the Human Genome Variation Society (HGVS) recommendations, using NM_001276270.2 as the reference sequence

IV. Results

IV. Results

This study included a total of five individuals, from four distinct families, affected with adenomatous polyposis and AML (Figure 5). Screening of deleterious germline variants in *MBD4* by Sanger sequencing revealed that all selected individuals present homozygous germline variants in this gene.

Patient A1 was a woman diagnosed with adenomatous polyposis, CRC and basal cell carcinoma (BCC) at the age of 33 and with AML two years later. Moreover, she presented a schwannoma, for which it was not possible to access information of the exact age of onset. This patient passed away at the age of 35 years old and no other relevant family history or consanguinity was known (Figure 5A). Patient B1 is a woman who was diagnosed with adenomatous polyposis, CRC and AML at 38 years old. She has a deceased brother who was diagnosed with leukemia at 17 years old and passed away two years later. Moreover, she presents family history of several cancers in second- and third-degree relatives (Figure 5B), but no consanguinity was known. Patient C1 was also a woman diagnosed at 36 years old with AML and adenomatous polyposis, as well as with a non-specified benign bone lesion. She eventually passed away at 38 years old. The parents of this patient were consanguineous and she had two deceased maternal uncles, both diagnosed with leukemia at the age of 37 and 39, respectively (Figure 5C). Despite belonging to apparently unrelated families, patients A1, B1 and C1 present the same *MBD4* germline variant in homozygosity, consisting of a substitution of a guanine for a thymine in intron 6 (transversion, c.1544-1G>T), which affects splicing and is predicted to disrupt the glycosylase domain, being currently classified as likely pathogenic in ClinVar (ID 1700251) (Figure 6A).

Finally, patient D1 was a man who was diagnosed with adenomatous polyposis and AML at the age of 55, and eventually passed away at 59 years old. His brother, patient D2, was diagnosed with adenomatous polyposis at 51 years old, with AML one year later and with meningioma at 55 years old. These patients had consanguineous parents, but they are not sure exactly how they were related (Figure 5D). Individual D1 was shown to harbor a previously unreported *MBD4* germline variant in homozygosity, which consists of a deletion of an adenine in exon 3 (c.538del) in the first position of codon 180 (ACC $\rightarrow$ CCC), resulting in a frameshift variant that originates a premature stop codon in the central interdomain region (p.Thr180ProfsTer35) and that is predicted to produce a truncated MBD4 protein lacking the glycosylase domain (Figure 6C).

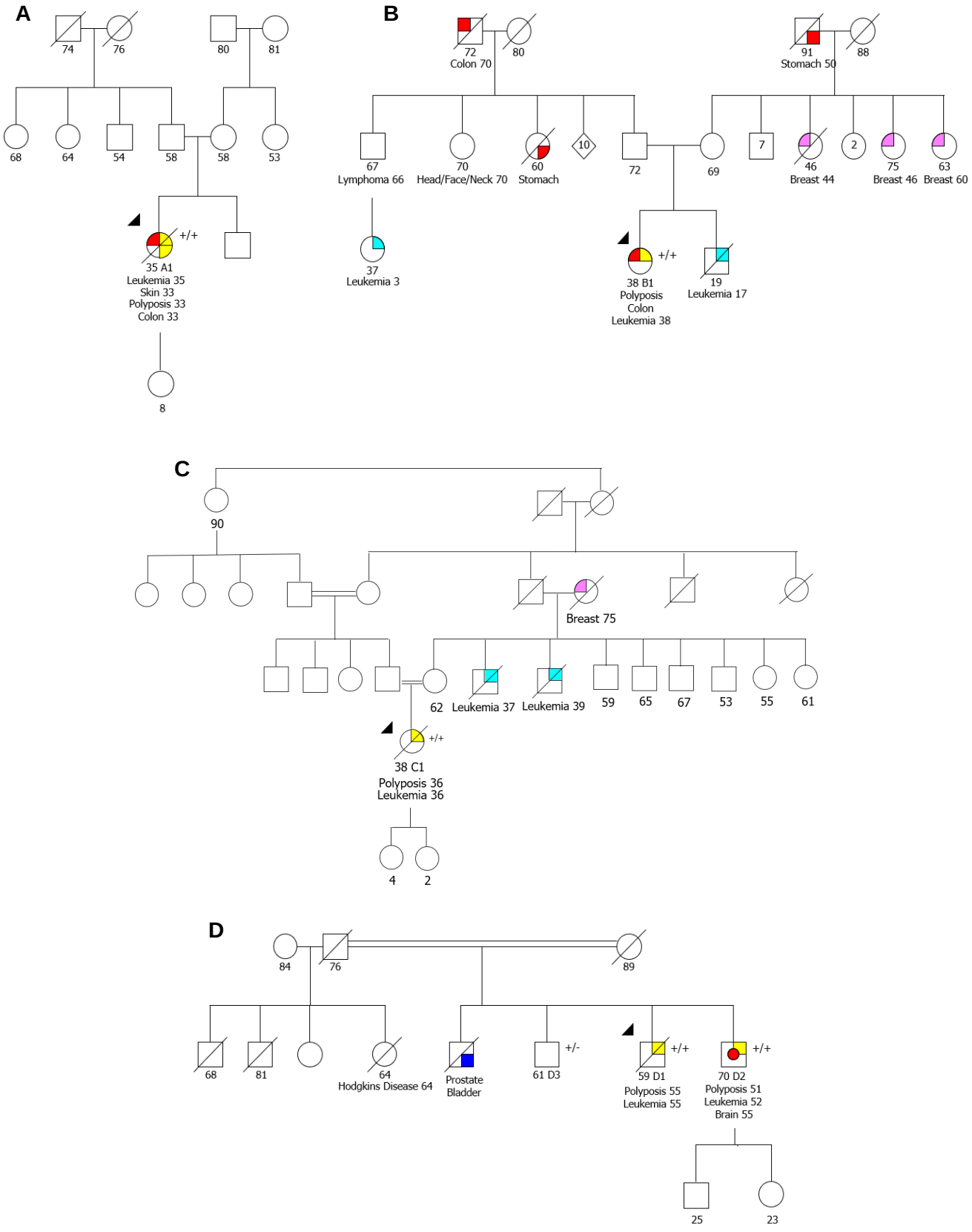

Figure 5. Pedigrees of the four families with biallelic deleterious variants in the *MBD4* gene. **(A), (B)** and **(C)** Families with the splicing variant NM_001276270.2:c.[1544-1G>T];[1544-1G>T]. **(D)** Family with the novel variant NM_001276270.2:c.[538del];[538del] p.[(Thr180ProfsTer35)];[(Thr180ProfsTer35)]. +/+ represents biallelic variant carrier; +/- represents heterozygous variant carrier.

Since we had an archived DNA sample isolated from peripheral blood from patient D2 (the affected sibling of patient D1), we searched for the presence of the *MBD4* germline variant previously detected in his brother, and this sample presented the aforementioned variant in heterozygosity. Upon reviewing the patient's medical records, we noticed that he had undergone allogeneic hematopoietic stem cell transplantation (HSCT), whose donor was a healthy sibling (relative D3). Consequently, relative D3 is presumed to be a heterozygous variant carrier. Then, in order to properly analyze the presence of the *MBD4* germline variant in patient D2, we used DNA isolated from a buccal swab sample, which revealed that this patient also harbors the *MBD4* variant in homozygosity (Figure 5D).

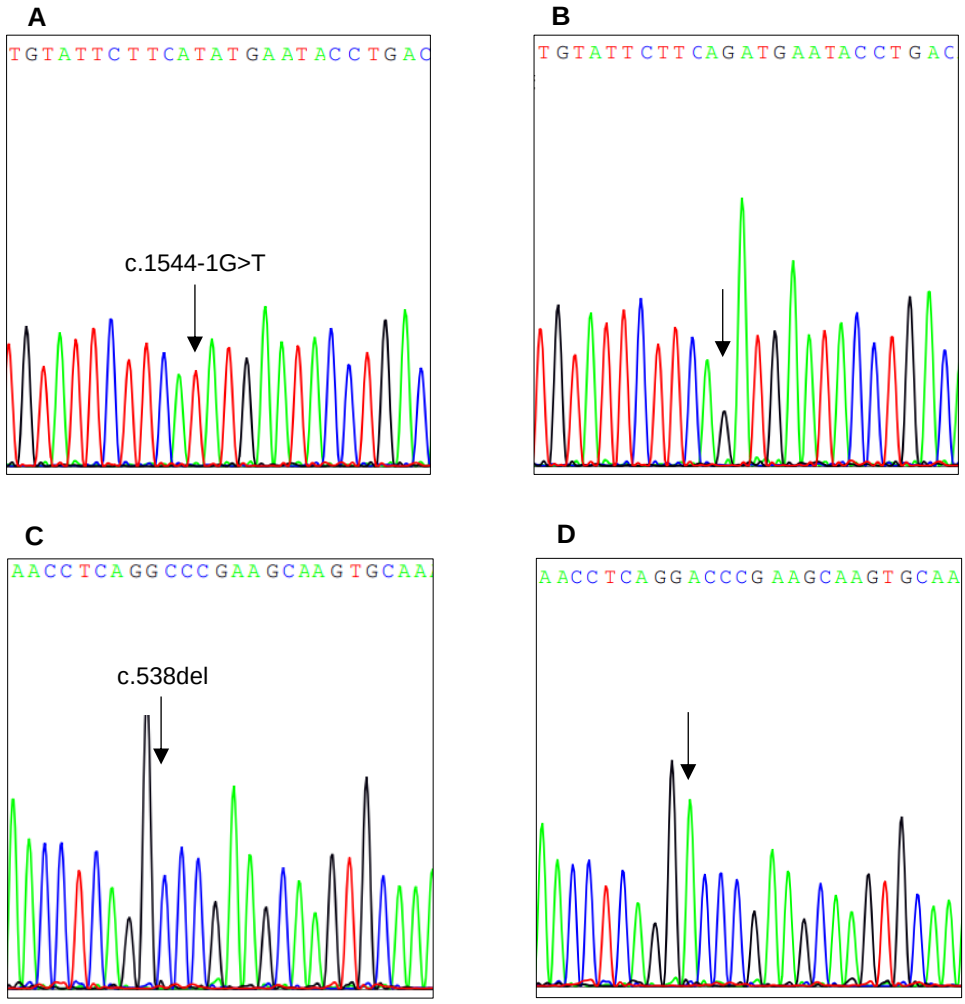

Figure 6. DNA sequence electropherograms obtained from the germline analysis of the *MBD4* gene, corresponding to **(A)** patient A1, presenting the splicing variant NM_001276270.1:c.[1544-1G>T];[1544-1G>T], and **(C)** patient D1, with the variant NM_001276270.2:c.[538del];[538del] p.[Thr180ProfsTer35];[Thr180ProfsTer35]. **(B)** and **(D)** correspond to reference sequences, in which the arrows indicate the normal DNA base.

V. Discussion

V. Discussion

The MBD4 protein has a multifaceted nature, primarily functioning as a DNA glycosylase in the BER pathway and acting to safeguard genetic integrity. Beyond that, it appears to be able to participate in various other critical cellular processes, such as modulation of the apoptotic response and epigenetic regulation, although the mechanisms by which MBD4 contributes to these processes remain under investigation. Nonetheless, there is growing evidence demonstrating that alterations in the *MBD4* gene contribute to carcinogenesis by promoting genomic instability, most likely due to loss of DNA repair activity, leading to the accumulation of C>T transitions along the genome, which represent the most frequent mutations in human cancer.

Similarly to other DNA glycosylases of the BER system, namely *MUTYH* and *NTHL1*, constitutive loss of *MBD4* function has recently been associated with inherited cancer predisposition. Deleterious biallelic germline variants in this gene were shown to be responsible for an increased susceptibility to develop adenomatous polyposis, CRC and other neoplasms, mainly early-onset AML. Thus, constitutional deficiency of *MBD4* is the cause of a novel autosomal recessive hereditary cancer syndrome, which has been provisionally denominated as MANS and has so far only been reported in eight families (Blombery et al., 2022; Griffin et al., 2021; Palles et al., 2022; Sanders et al., 2018).

In the present work, we describe a set of four new families presenting five individuals affected with AML and adenomatous polyposis, which are homozygous for deleterious germline variants in the *MBD4* gene. Hence, the families identified in this study constitute one third of the total of families with MANS currently described (Table 6).

The phenotype of the patients included in our study is consistent with that described in the literature, although there is limited available information (Blombery et al., 2022; Griffin et al., 2021; Palles et al., 2022; Sanders et al., 2018). Nevertheless, this evidence indicates that the main clinical manifestations of MANS appear to be predisposition to adenomatous polyposis, CRC and AML. In addition, it is possible to observe that the affected individuals, as it has been previously documented (Blombery et al., 2022; Palles et al., 2022), frequently present benign lesions, namely schwannoma and meningioma. However, this study is the first to describe a BCC and a benign bone lesion in the context of MANS, thus contributing to expand the tumor spectrum of the syndrome, if these findings are further validated by other studies.

Table 6. Clinicopathological characteristics of the patients presenting biallelic *MBD4* deleterious germline variants.

Family	Patient	Gender	Malignancies (age of onset*)	Benign lesions (age of onset*)	*MBD4* germline variants		Publication
					DNA (NM_001276270.2)	Protein effect prediction	
1	EMC-AML-1	M	Polyposis; AML (33)	-	c.[1681_1683del]; [1681_1683del]	p.[(His561del)]; [(His561del)]	(Sanders et al., 2018)
2	WEHI-AML-1	F	AML (31)	-	c.[939dup]; [1544-1G>T]	p.[(Glu314ArgfsTer13)]; [?]	
	WEHI-AML-2	F	AML (34); Polyposis; CRC (40)	-			
3	-	M	Polyposis; CRC (24); Lymphoma (28); AML (30)	-	c.[1273C>T]; [1670T>A]	p.[(Arg425Ter)]; [(Leu557Ter)]	(Griffin et al., 2021)
	-	F	Polyposis (33); AML (35); Papillary thyroid cancer (44)	Schwannoma (44)			
4	D:II-1	M	Polyposis (36); AML (49)	-	c.[612_615del]; [612_615del]	p.[(Ser205ThrfsTer9)]; [(Ser205ThrfsTer9)]	(Palles et al., 2022)
5	CRDFF-292-1:II-3	M	Polyposis, UVM (53)	Liver and small renal cysts (53)	c.[939dup];[939dup]	p.[(Glu314ArgfsTer13)]; [(Glu314ArgfsTer13)]	
6	CRDFF-336-1:II-1	F	OvGCT (12); Polyposis (39)	-			
	CRDFF-336-2:II-2	M	Polyposis (39)	-			

Table 6. Continued.

Family	Patient	Gender	Malignancies (age of onset*)	Benign lesions (age of onset*)	*MBD4* germline variants		Publication
					DNA (NM_001276270.2)	Protein effect prediction	
7	DB1-70:II-3	F	Polyposis (35); UVM (38, 45); Upper GITVA (49)	Meningioma (41); DCIS, Schwannoma (50)	c.[939dup];[1670T>A]	p.[(Glu314ArgfsTer13)]; [(Leu557Ter)]	(Palles et al., 2022)
8	-	M	Polyposis (32); AML (37)	Schwannoma (34)	c.[1517G>A]; [1517G>A]	p.[(Arg506Gln)]; [(Arg506Gln)]	(Blombery et al., 2022)
9	A1	F	Polyposis, CRC, BCC (33); AML (35)	Schwannoma	c.[1544-1G>T]; [1544-1G>T]	p.[?];[?]	This study
10	B1	F	Polyposis, CRC, AML (38)	-			
11	C1	F	Polyposis, AML (36)	Bone lesion (36)			
12	D1	M	Polyposis, AML (55)	-	c.[538del];[538del]	p.[(Thr180ProfsTer35)]; [(Thr180ProfsTer35)]	
	D2	M	Polyposis (51); AML (52)	Meningioma (55)			

F – Female; M – Male; AML - Acute myeloid leukemia; CRC – Colorectal cancer; BCC – Basal cell carcinoma; UVM – Uveal melanoma; OvGCT – Ovarian granulosa cell tumor, GITVA – Gastrointestinal tract tubulovillus adenoma; DCIS – Ductal carcinoma *in situ* of the breast; *Whenever available.

The *MBD4* variant c.1544-1G>T has been previously described in heterozygosity associated with predisposition to uveal melanoma (Derrien et al., 2021; Rodrigues et al., 2018). Furthermore, this variant has only been previously reported in biallelic inactivation once, by Sanders and colleagues in 2018, in two siblings presenting AML, one of which was also affected with colorectal polyps and CRC (Sanders et al., 2018). Notably, these individuals carried the variant in compound heterozygosity, hence the three families we here present are the first to be reported with this variant in homozygosity.

On the other hand, to our knowledge, the *MBD4* variant c.538del p.(Thr180ProfsTer35) has not been previously described in the literature (Figure 7). Following the American College of Medical Genetics and Genomics (ACMG) guidelines, we classify it as likely pathogenic, since it consists in a frameshift mutation that most likely originates a premature stop codon in the central interdomain region, which is predicted to result in a truncated nonfunctional MBD4 protein lacking the glycosylase domain (PVS1) and presents an extremely low population frequency in population databases (PM2).

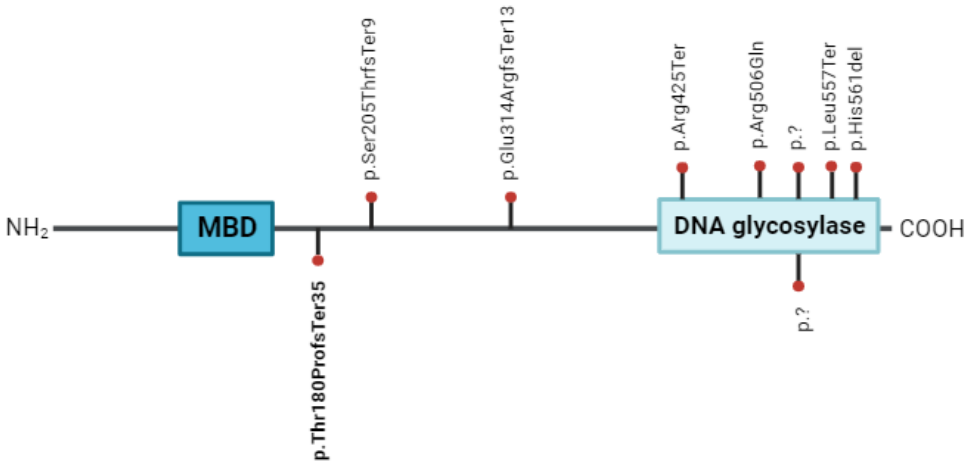

Figure 7. Schematic structure of the MBD4 protein and the spectrum of deleterious germline variants reported in the context of *MBD4*-associated neoplasia syndrome (MANS). The variants previously described in the literature are indicated on the top, while the variants detected in our study are represented on the bottom. The novel variant identified in this study is highlighted in bold. The variant represented by p.? corresponds to c.1544-1G>T, which affects splicing. MBD - Methyl-CpG binding domain.

Importantly, two of the four families identified in our study exhibit consanguinity (families C and D). Given that MANS constitutes an autosomal recessive hereditary syndrome, consanguinity represents a significant risk factor for the disease, since it increases the probability of inheriting two copies of the cancer-associated germline

variant. However, consanguinity is not a mandatory condition for the appearance of autosomal recessive diseases, especially when there are recurrent variants that are relatively frequent in the general population in heterozygosity, as described for MAP and *NTHL1* tumor syndrome (Cleary et al., 2009; Grolleman et al., 2019; Nielsen et al., 2009; Weren et al., 2015). Of note, as aforementioned, the variant c.1544-1G>T in the *MBD4* gene has been previously reported in one MANS family in compound heterozygosity (Sanders et al., 2018) and, in our study, it was detected in homozygosity in three of the four families here identified (families A, B and C), with consanguinity reported for only one of them. These findings may indicate that this variant may be a recurrent change in this gene, but we cannot exclude the possibility, at least in these three families, of the existence of a common ancestor.

The detection of deleterious germline variants in these families enables genetic testing for patients' relatives, which allows pre-symptomatic diagnosis and may eventually contribute to the development of surveillance programs and potential prophylactic measures for biallelic mutation carriers, with the aim of facilitating early detection and proactive management of their cancer risk. Additionally, it allows non-variant carriers to be exempted from unnecessary intensified cancer screening, as their cancer risk resembles that of the general population. Based on the described phenotypes and in accordance with the National Comprehensive Cancer Network (NCCN) guidelines, clinical surveillance concerning MANS should primarily focus on the high risk for colorectal polyposis and early-onset AML for biallelic variant carriers, which may include regular colonoscopies starting at 18-20 years old and complete blood counts (NCCN Guidelines, 2023). Moreover, considering the recurrence of other malignancies and benign lesions, regular clinical monitoring should also be recommended for these individuals. Of note, the scarce available data indicate that heterozygous variant carriers do not present an increased susceptibility for the most common MANS manifestations, including adenomatous polyposis and AML. However, some reports suggest that these individuals may harbor an increased risk for uveal melanoma (Derrien et al., 2021; Johansson et al., 2019; Rodrigues et al., 2018). Although the five MANS patients we here describe do not present uveal melanoma, two of the eleven cases previously described presented that phenotype. Hence, regular ophthalmologic exams are also recommended both for heterozygous variant carriers and for biallelic variant carriers.

Finally, the identification of the molecular diagnosis of the syndrome and the causative germline variants may have an influence in therapeutics. For example, regarding AML patients, the selection of a sibling as bone marrow donor for allogenic HSCT should ideally be guided by prior genetic testing. Moreover, it is worth noting that

MANS patients present a high mortality rate, which highlights the importance of exploring alternative treatment approaches in order to eventually improve their outcomes. Some studies reported that patients with *MBD4*-deficient uveal melanoma, which harbor a heterozygous *MBD4* deleterious germline variant accompanied by somatic loss of the second normal allele, exhibit a good response to immune checkpoint inhibitors (Johansson et al., 2019; Rodrigues et al., 2018), suggesting that *MBD4* may potentially constitute a predictive biomarker of immunotherapy response. Immunotherapy has been approved by the US Food and Drug Administration (FDA) for the treatment of patients with metastatic CRC displaying MSI or high tumor mutational burden (Casak et al., 2021; Marcus et al., 2021) and, although patients harboring BER deficiency are not formally eligible for this type of treatment, there are some case reports, at least in MAP patients, demonstrating a good clinical response (Mathias-Machado et al., 2023; Volkov et al., 2020). Therefore, we speculate that MANS patients may possibly represent good candidates for immune checkpoint inhibitors therapy, since these tumors have also been described to harbor a hypermutated nature, thus probably being more immunogenic and responsive to immunotherapy. However, further investigation is required to evaluate this possibility.

VI. Conclusions

VI. Conclusions

After the completion of this study, we are able to conclude that *MBD4* represents a novel hereditary cancer predisposing gene, that seems to explain most cases of association of colorectal polyposis and AML. Constitutional biallelic inactivation of *MBD4* leads to a defect in the BER pathway, which compromises DNA repair and is responsible for MANS. By describing five new individuals, from four families, presenting homozygous *MBD4* deleterious germline variants, this work significantly contributes to expand the knowledge about this emerging syndrome, as well as provides new insights about MANS, including broadening of the syndrome tumor spectrum and the identification of a novel *MBD4* deleterious variant.

The findings provided by this work, along with those described in the literature so far, strongly suggest that the *MBD4* gene must be included in the next generation sequencing (NGS) multi-gene panels used to study patients with personal and/or family history of adenomatous polyposis, especially when associated with AML and/or uveal melanoma. Although MANS appears to be a rare hereditary cancer syndrome, it is fundamental to establish its molecular diagnosis, since this information will enable specific and personalized clinical healthcare for patients and their families, with the ultimate goal of increasing their life expectancy, quality of life and overall survival.

VII. Future perspectives

VII. Future perspectives

This study may benefit from further analyses in order to support our conclusions and enable a more specific evaluation of the germline variants detected in the *MBD4* gene. Therefore, we intend:

- To study the *MBD4* gene in first-degree relatives of the index patients, with the purpose of confirming the homozygous nature of the detected germline variants;
- To characterize the somatic mutational profile of the MANS tumors, in order to assess the frequency of C>T transitions in CpG sites caused by *MBD4* deactivation;
- To perform haplotype studies in the three families that present the *MBD4* germline variant c.1544-1G>T, which will allow us to understand if these families had a common ancestor.

VII. Bibliography

VII. Bibliography

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