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LAURA ROHT

Hereditary colorectal cancer syndromes in Estonia

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Paper II: Participation in the study design; consulting patients, collecting, analysing and interpreting the data; and writing the manuscript.

Paper III: Participation in the study design; analysing the data, consulting patients; and writing the manuscript.

Paper IV: writing the manuscript.

ABBREVIATIONS

ACMG	American College of Medical Genetics
AD	Autosomal dominant inheritance
ACPGBI	Association of Coloproctology of Great Britain and Ireland
AFAP	Attenuated familial adenomatous polyposis syndrome
AR	Autosomal recessive inheritance
ASHG	American Society of Human Genetics
BC	Breast cancer
BER	Base excision repair
BSG	British Society of Gastroenterology
CAP	Cancer Prevention Program
CFS	Cancer family syndrome
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal instability
CMMRD	Constitutional mismatch repair deficiency syndrome
CMS	Consensus molecular subtype
CNV	Copy Number Variation
CoNIFER	Copy number inference from exome reads
CP	Cleft palate
CRC	Colorectal cancer
CS	Cowden syndrome
DDR	DNA damage repair (pathways)
DECoN	Detection of Exon Copy Number Variants
DNA	Deoxyribonucleic acid
DSBR	Double-strand break repair
E. coli	Escherichia coli
EC	Endometrial cancer
EHTG	European Hereditary Tumour Group
EPCAM	Epithelial cell adhesion molecule
ERN GENTURIS	European Reference Network for patients with rare genetic tumor risk syndromes
ESCP	European Society of Coloproctology
ESHG	European Society of Human Genetics
ESMO	European Society of Medical Oncology
EstBB	Estonian Biobank
EUS	Endoscopic ultrasound
FAP	Familial adenomatous polyposis syndrome
FH	Familial hypercholesterolemia
FOBT	Faecal occult blood test
FOLFOX/CAPOX	combination of fluorouracil, leucovorin, oxaliplatin/capecitabine plus oxaliplatin
FSP	Frameshift peptide

5-FU	5-fluorouracil
GATK	Genome Analysis Toolkit
GLM	General linear model
GPM Clinic	Genetics and Personalized Medicine Clinic
gnomAD	Genome Aggregation Database
HBOC	Hereditary breast and ovarian cancer syndrome
HGMD	Human Gene Mutation Database
HNPCC	Hereditary non-polyposis colorectal cancer syndrome
ICD-11	International Classification of Diseases 11th Revision
ICI	Immune checkpoint inhibitors
IHC	Immunohistochemistry
IMRC	International Mismatch Repair Consortium
INDELS	Small insertions and/or deletions
JPS	Juvenile polyposis syndrome
LB	Live births
LoF	Loss of function
LP	Likely pathogenic
LS	Lynch syndrome
MAP	*MUTYH*-associated polyposis syndrome
MLH1	MutL homologue 1
MLH3	MutL homologue 3
MLPA	Multiplex Ligation dependent Probe Amplification
MMR	Mismatch repair system
MMR-D	MMR system deficiency
MRI	Magnetic Resonance Imaging
MSH2	MutS homologue 2
MSH6	MutS homologue 6
MSI	Microsatellite instability
MSI-H	Microsatellite instability High
MSI-L	Microsatellite instability Low
MSS	Microsatellite stable
MTS	Muir-Torre syndrome
NAP	*NTHL1*-associated polyposis syndrome
NCCN	National Comprehensive Cancer Network
NGS	Next-Generation Sequencing
OC	Ovarian cancer
OMIM	Online Mendelian Inheritance in Man
OR	Odds ratio
ORPHA	The Orphanet nomenclature of rare diseases
P	Pathogenic gene variant
PARP	Poly ADP-ribose Polymerase
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PJS	Peutz-Jeghers syndrome
PLSD	Prospective Lynch Syndrome Database

PMS1	PMS1 homologue 1
PMS2	PMS1 homologue 2
PREMM	Prediction Model for Gene Mutations
RR	Relative risk
SCNA	Somatic copy number alterations
SPS	Serrated polyposis syndrome
SRS	Silver-Russel syndrome
TCGA	The Cancer Genome Atlas
TSC	Illumina TruSight Cancer panel
TSHC	Illumina TruSight Hereditary Cancer panel
TSO	Illumina TruSight One panel
TSOE	Illumina TruSight One Expanded panel
TUH	Tartu University Hospital
UKCGG	United Kingdom Cancer Genetics Group
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGS	Whole genome sequencing

1. INTRODUCTION

Colorectal cancer (CRC) is defined as a cancer, which starts from the colon or rectum. The two sites are grouped together, because they have common features (*American Cancer Society webpage*). CRC has been the third most frequent cancer in Estonia, as well as worldwide for more than ten years. According to the Estonian National Institute for Health Development, the growth in incidence of CRC has escalated rather quickly: there were 791 primary cases (C18-C21) in 2010, and 963 cases in 2020 (*The National Institute for Health Development webpage*).

In Estonia, a screening programme for CRC started in 2016, which means that males and females aged 60–68 are screened every two years by faecal immunochemical testing, which identifies occult blood, and this is organized and covered by the Estonian Health Insurance Fund (*Estonian Health Insurance Fund webpage*). If testing is positive, patients are sent for colonoscopy to confirm or rule out CRC. Dr Heigo Reima, who defended his thesis on "Colorectal cancer care and outcomes – evaluation and possibilities for improvement in Estonia" in 2022, also noted that due to screening the apparent incidence of CRC in the screening age group had risen and there were more first stage cancer cases. The survival rate had improved, but was still ~10% lower than in Scandinavian countries. Diagnostics and multimodal treatment have improved significantly in time, but there are still problems such as belated diagnosis, high rate of metastatic CRC and need for emergency surgery. He also conducted a randomized study of whether staining operative material with methylene blue helps to improve detection of affected lymph nodes, which in turn helps determine the stage and to decide on the necessity of adjuvant chemotherapy (Reima, 2022).

Regarding hereditary colorectal cancer syndromes, historically Peutz-Jeghers syndrome (PJS) was one of the first described. It was initially documented by an English physician J.T. Connor in 1895, who described twin sisters, who had oral pigmentation: one of them died at the age of 20 years due to intussusception and the other at the age of 52 years because of breast cancer (BC). In 1954 the syndrome was named after Peutz, who described a family with gastrointestinal polyposis and pigmentation of mucous membranes consistent with autosomal-dominant (AD) inheritance, and Jeghers, who defined mucocutaneous pigmentation together with gastrointestinal polyposis as a distinct phenotype (Beggs et al., 2010).

Before 2012, from the perspective of molecular genetics, there were only a few solitary genetic tests available for testing hereditary CRC. In 2012 an article on how to genetically consult and survey patients with or at risk of hereditary colorectal cancer, was published in the journal *Eesti Arst* (Estonian Physician). The authors stated that ~5% of CRC cases are the result of monogenic high penetrance cancer syndromes. At that time, mismatch repair (MMR) genes immunohistochemistry (IHC) and microsatellite instability (MSI) analyses, as well as genetic testing for the most frequent CRC syndromes, were already

available in Estonia. In addition, the authors claimed that effective surveillance improves the quality of life and survival rates (Mikita et al., 2012). In Estonia, unlike other European countries, no systematic review of hereditary colorectal cancer syndromes has been published.

The aims of this study were as follows: to analyse the estimated prevalence of disease-causing gene variants in Estonian CRC patient, to estimate the prevalence of Lynch syndrome in the general adult population and the effectiveness of MMR genes immunohistochemistry tests in CRC patients over 50 years of age. Besides that, the aim was to specify the clinical phenotype and cancer risks of *AXIN2*-related oligodontia-colorectal cancer syndrome carriers.

2. LITERATURE REVIEW

2.1 Colorectal cancer

Cancer is a major cause of death and an important obstacle to higher life expectancy. According to World Cancer Research Fund International, CRC is among the commonest cancers in the world and the second leading cause of cancer-related death. It is the third most common cancer for males and second for females (*World Cancer Research Fund International webpage*). From 1990 to 2012, the incidence of CRC has increased by more than 200,000 new cases per year (Marmol et al., 2017). In 2020, there were more than 1.9 million new cases worldwide and 935,173 people died because of it (*World Cancer Research Fund International webpage*). Regarding geographical differences, the variation in incidence can be up to nine-fold between different regions. The highest rates are seen in some European regions (especially Hungary and Norway), Australia, New Zealand and North America (Sung et al., 2021). Thus, the highest incidence is seen in the most developed countries, although in those highly developed countries CRC incidence seems to be stabilizing and even decreasing (Dekker et al., 2019). If the trend in developing countries continues, however it is predicted that in 2035 there will be 2–5 million new cases worldwide. The increase is most probably the result of changes in lifestyle and diet, which lead to being overweight and physically less active, both of which are independent risk factors for CRC (Sung et al., 2021). Remarkably, an important fact is that the overall incidence of CRC in the age group over 50 years has decreased due to screening, but increased in the younger (<50 years) population (Baidoun et al., 2021).

According to the National Comprehensive Cancer Network (NCCN) Guidelines Version 1.2023, the lifetime risk of CRC in the general United States population is 4.1% (NCCN-guidelines, 2023). Types of personal traits and habits, or other risk factors, can increase the risk of developing CRC. Some of them are modifiable and others are not. The strongest non-modifiable risk factors for pre-cancerous polyps and CRC are age, male sex and family history. As cancer is related to aging, CRC incidence increases after the age of 50 years; in fact, ~90% of global cases and deaths occur in the age group of 50 years and older. Males appear to have a higher tendency to develop CRC for various reasons: they are affected by environmental factors more than females, and in addition, more prone to be exposed to such changeable risk factors as smoking, alcohol consumption, poor dietary choices and visceral fat. Furthermore, males tend to take part in screening programmes less often than females (OR comparing males versus females: 0.84, 95% CI 0.75–0.79), and relative to females, males lack the protective benefit of endogenous oestrogen. Males, compared to females, also have the disadvantage of higher heritability associated with CRC. Regarding family history, approximately 25% of CRC cases are familial, but not related to any distinct hereditary cancer syndrome (Keum & Giovannucci, 2019).

Histopathologically, most CRCs are adenocarcinomas (>90%). Adenocarcinomas develop from glandular epithelial cells. Other types of CRCs, which are rare, include squamous cell carcinoma, adenosquamous carcinoma, spindle cell and undifferentiated carcinoma (Keum & Giovannucci, 2019). Most cancers evolve from a non-cancerous polyp, which is the product of an aberrant crypt. The progression to CRC takes about 10–15 years in sporadic cases. CRC originates from a stem-cell or stem-cell-like cells, as the result of accumulation of genetic alterations that inactivate tumour-suppressor genes and activate oncogenes (Dekker et al., 2019). For nearly 30 years, it has been hypothesized that cancers result from mutations in specific tumour-suppressor genes and oncogenes. This was based on experimental data and proposed models by Armitage and Doll, Nowell, Knudson, and others (Vogelstein & Kinzler, 2015). Besides tumour-suppressor genes and oncogenes, recent analysis of The Cancer Genome Atlas (TCGA) data identified that both colonic and rectal adenocarcinomas, harboured variants in several DNA damage response and repair (DDR) genes (Reilly et al., 2019). DDR pathways play a key role in maintaining the genomic stability. Thus, they modulate cancer risk, progression and therapeutic response (Knijnenburg et al., 2018).

It had been hypothesized that tumorigenesis is a multistep process long before 1990 when Fearon and Vogelstein proposed their genetic model of colorectal carcinogenesis. Their model includes the following outstanding features: coexistence of the activation of oncogenes and inactivation of tumour-suppressor genes, pathogenic variants at least in four to five genes for a malignant tumour, and that the biologic quality of the tumour is rather the result of the accumulation of genetic alterations than their order of occurrence. They also noted that some tumour-suppressor genes might not express a recessive pattern (Fearon & Vogelstein, 1990). Since then many new insights have emerged. For example, genome-wide sequencing has provided new data: there are “driver genes”, which, if mutated, give the tumour cell a growth advantage over the surrounding cells, and “passengers”, which occur coincidentally during tumorigenesis. In adult solid tumours, alterations in as few as three “driver genes” is suffice for a cell to evolve into advanced cancer (Vogelstein & Kinzler, 2015).

Moving from pathogenesis to classification of colorectal tumours, they are differentiated as hypermutated (>12 mutations for 10^6 bases) or non-hypermutated (<12 mutations for 10^6 bases). In 2012, TCGA carried out a description of CRC tumours, including exome sequences, DNA copy numbers and RNA expression levels. Of the cases analysed (276) 16% were classified as hypermutated and exhibited an MSI phenotype. The remaining 84% of the cases were classified as microsatellite stable (MSS) and exhibited a higher frequency of somatic copy number variations (CNVs) (Reilly et al., 2019). To homogenize genetic-based CRC classifications, a new system of four consensus molecular subtypes (CMS) was formed: CMS1 (MSI immune, 14%), CMS2 (canonical, 37%), CMS3 (metabolic, 13%) and CMS4 (mesenchymal, 23%) (Guinney et al., 2015). Details of the characteristics of different subtypes are covered in Table 1. CMS1 is characterized by MSI, high CpG Island Methylator Phenotype

(CIMP) and *BRAF* variants and low prevalence of somatic copy number alterations (SCNA); the cancers are predominantly in the proximal colon. CMS2 hallmarks are high chromosomal instability (CIN), *CIMP* and WNT and MYC pathway regulation alterations; tumours are usually located in the distal colon and rectum. CMS3 does not have a predominance localization-wise; its main features are enrichment in *KRAS* variants, moderate/low MSI, intermedial CIMP, *PIK3CA* variants, over-expression of IGBP3 and absence of *BRAF* pathogenic changes. CMS4 is typical in distal colon and rectal tumours; TGF-β activation, high rate of SCNA and mesenchymal epithelial transition is characteristic. This classification also helps to direct clinical decisions, for example adjuvant chemotherapy etc. (Valenzuela et al., 2021).

Table 1. Consensus molecular subtypes.

	CMS1 MSI immune	**CMS2 Canonical**	**CMS3 Metabolic**	**CMS4 Mesenchymal**
Proportion (%)	14%	37%	13%	23%
Characteristics	Predominantly proximal CRC; MSI, high CIMP, *BRAF* variants, low SCNA	Predominantly distal colon and rectal tumours; high CIN, CIMP, WNT and MYC regulation alterations	No predominant localization; *KRAS* variants, moderate or low MSI, intermedial CIMP, *PIK3CA* variants, over-expression of IGBP3 and absence of *BRAF* alterations	Predominantly distal colon and rectal tumours high rate of SCNA, mesenchymal epithelial transition and TGF- β activation

2.2 Hereditary colorectal cancer syndromes

Most CRC cases are sporadic. As mentioned earlier, many environmental factors are important in developing CRC, but a genetic component also plays an important role. Studies show that in ~10–20% cases there is a positive family history, the proportion depending on the age at CRC diagnosis, and the number and degree (for example first-degree, second-degree etc.) of the relatives affected (Henrikson et al., 2015). Some authors even state that family history accounts for up to 35% of the risk in patients with CRC (Kastrinos et al., 2020). In this publication, which is based on individual studies and systematic reviews, they report an estimated two-fold increase in risk of CRC in individuals with one or more first-degree relatives with CRC, compared to individuals without a family history (Kastrinos et al., 2020). Not only the degree, but also the number of CRC cases in relatives plays an important role: in two large studies an estimated three- to four-fold increase in relative risk (RR) for individuals with two or more CRC cases in their family was seen (Butterworth et al., 2006; Taylor et al., 2010). Family history is, and probably will remain, an important tool in genetic counselling.

Five to ten percent of CRC cases are caused by high or moderate penetrance gene variants associated with different hereditary cancer syndromes (Kastrinos et al., 2020). Hereditary CRC syndromes can be subdivided into non-polyposis [Lynch syndrome (LS) and familial CRC] and polyposis syndromes, of which familial adenomatous polyposis (FAP) is the most common (Dekker et al., 2019). Polyposis syndromes are distinguished mostly by the number and histology of colorectal polyps (Valle, Vilar, et al., 2019). By histology, polyps are classified as adenomatous [different subtypes of FAP, *MUTYH*-associated polyposis (MAP), *NTHL1*-associated polyposis (NAP)] or hamartomatous [PJS, juvenile polyposis syndrome (JPS), and Cowden syndrome (CS)]. Most of these polyposis syndromes are inherited in an AD pattern, except for MAP, and if left untreated, result in CRC, accounting for ~1% of CRCs worldwide (Hryhorowicz et al., 2022). Table 2 shows potential diagnosis in association with the histology and number of gastrointestinal polyps.

Table 2. Histology and number of polyps in association of potential diagnosis (Roht et al., 2020).

No and histology of polyps	Possible diagnosis
>10 colorectal adenomas	FAP–Familial Adenomatous Polyposis AFAP–attenuated FAP MAP–*MUTYH*-associated polyposis
>2 hamartomatous polyps	JPS–Juvenile Polyposis Syndrome PJS–Peutz-Jeghers Syndrome
>5 serrated polyps proximal from sigmoid colon	SPS–Serrated Polyposis Syndrome

2.2.1 Lynch syndrome

2.2.1.1 Historical background of the syndrome

Lynch syndrome (OMIM #120435; ORPHA 144) was one of the first hereditary cancer syndromes recognized as well as one of the most prevalent of them. In order to understand its essence, a little historic perspective is needed. In 1895, Aldred Scott Warthin, a world-renowned pathologist from the University of Michigan, began documenting medical data, pathology findings and cancer history in the family of his seamstress. In one of these pedigrees, Family G (G for Germany), people died of CRC, gastric and endometrial cancer (EC) throughout generations. He published his findings together with data of two other cancer-prone pedigrees documented in the medical school of the University of Michigan in 1913. In the mid-1930s his colleagues published an update on the observations of Family G, which were among the most extensive of familial cancer clustering. In 1962, Dr Henry Lynch consulted with a patient from Nebraska with a comparable family history to that published by Warthin. Dr Lynch noted CRC cases in multiple generations and at first thought of FAP

instead, as this was then the most frequent cause in CRC families. Going through medical and pathology records of the family members (Family N; N for Nebraska), Lynch did not detect the hallmark of FAP – multiple colorectal adenomas, and he began questioning if this might be a new syndrome. In 1966, pedigrees of Family N and another similar family (Family M; M for Michigan) were published. This was the era of environmental factors being the main cause of cancer, but the strong clinical foundation indicating that it might be an inherited condition led to molecular genetic studies of these known familial cases, and it was established that, indeed, LS is an inherited cancer predisposition syndrome. From 1971, LS was referred to as cancer family syndrome (CFS), in 1984 it was renamed LS, but at the same time a term hereditary non-polyposis colorectal cancer (HNPCC) was used. As it became clear that these individuals might have cancers outside the colon as well, and that there can be multiple adenomas, by consensus it is nowadays mostly referred to as LS (Lynch et al., 2015).

2.2.1.2 Clinical spectrum of MMR genes disease-causing variants

Clinically, one of the first features of LS described and reported in 1977 was the high proportion of tumours located in the proximal colon (Lynch et al., 1977). In the 1990s, it was thought that there were two clinical subtypes: LS I, which was thought to be associated only with CRC, and LS II, which in addition was associated with extracolonic cancers, endometrial cancer being the most frequent (Lynch et al., 1991). In 1994, increased risks of cancer of the stomach, small bowel, hepatobiliary system, upper urological tract, and ovary were found and these cancers were added to the LS spectrum (Watson & Lynch, 1994). An update in 2008 linked brain tumours to LS and suggested thinking about preventive measures in some cases and subgroups (Watson et al., 2008). In 2015, specifically glioblastoma, astrocytoma and oligodendroglioma, were associated and added to the spectrum of LS by Danish researchers (Therkildsen et al., 2015). Later on, elevated risk of prostate and pancreatic cancer as well as adrenocortical tumours was shown (Bujanda & Herreros-Villanueva, 2017; Raymond et al., 2013; Ryan et al., 2014). Breast cancer as part of the LS spectrum has been discussed for years. According to the Prospective Lynch Syndrome Database (PLSD), the risk of BC for LS females is ~12–15% by the age of 75 years (*Prospective Lynch Syndrome Database (PLSD) risk calculator*). A systematic review from 2013 stated that this is inconclusive as only one prospective study had reported elevated risk of BC for carriers of disease-causing MMR gene mutations (Win et al., 2013). In 2020, Sheehan et al. suggested a significant link between BC and *PMS2* disease-causing variants (Sheehan et al., 2020). To date, evidence is too inconclusive to support additional screening besides population-based screening for BC (Idos & Valle, 1993; NCCN-guidelines, 2023).

Lynch syndrome has other clinical subtypes besides classical manifestations. One of them, the Muir-Torre syndrome (MTS, OMIM #158320, ORPHA 587),

was first described by Muir et al. in 1967 and, independently, by Torre et al. one year later, in 1968. It was linked to LS as a rare variant in 1981 (Lynch et al., 1981). MTS is a rare AD genodermatosis characterized by sebaceous neoplasms, which include solitary or multiple sebaceous adenoma and carcinoma, and one or more visceral malignancies, most commonly located in the colon, rectum or endometrium (Bhaijee & Brown, 2014). The coexistence of primary brain tumours with multiple colorectal adenomas is known as the Turcot syndrome (OMIM #276300, ORPHA 252202), which can either be a subtype of FAP or LS, and has AD inheritance. An autosomal recessive (AR) subtype of LS, constitutional mismatch repair deficiency (CMMRD) is a rare childhood cancer predisposition syndrome caused by bi-allelic disease-causing variants in MMR genes. CMMRD often manifests already in childhood with brain tumours, multiple colonic adenomas, CRC or other gastrointestinal cancers; haematological malignancies, such as leukaemias and lymphomas, are also common. Nearly all patients have *café-au-lait* spots (Carethers & Stoffel, 2015). A case report of a female Estonian CMMRD patient has been published. The patient, currently 25 years old, has been diagnosed with two forms of leukaemia at different ages, and at the age of 17 years with extreme colorectal polyposis. Intraepithelial adenocarcinoma was found in the biggest polyp in the sigmoid colon (Soplepmann & Laidre, 2016) and total proctocolectomy was performed. At the age of 21 years she was diagnosed with diffuse astrocytoma of the frontal lobe and at the age of 24 years with acute myeloid leukaemia (personal communication with Dr P. Laidre and Dr J. Soplepmann).

2.2.1.3 Aetiology and genetic background

The central cause of LS is the loss of function of DNA MMR genes. There are four key MMR genes: MutL homologue 1 (*MLH1)*, MutS homologue 2 (*MSH2)*, MutS homologue 6 (*MSH6)*, PMS1 homologue 2 (*PMS2),* and one non-MMR gene, epithelial cell adhesion molecule (*EPCAM)* gene. LS is an AD condition caused by germline heterozygous disease-causing variants in one of the MMR genes. Most of the patients have an affected parent; *de novo* cases are rare, constituting only 2.3% according to the literature (Peltomaki et al., 2023). MMR genes secure the genomic stability by correcting base-base mismatches and small insertions/deletions (indels) resulting from DNA replication and recombination. It is known as the MMR system, which was originally discovered in *Escherichia coli (E. coli),* and is highly conserved from bacteria to humans. The names of MMR genes thus reflect the homology to the *E. coli* system. The discovery and studies of the MMR system in other organisms definitely paved the way to establishing the importance of the MMR system in humans. The knowledge of how the MMR system functions comes primarily from *in vitro* studies (Pecina-Slaus et al., 2020).

The MMR machinery involves four steps: recognition of the lesion, initiation of repair, excision of the lesion and DNA re-synthesis (Pecina-Slaus et al., 2020). In this system, groups of proteins interact as heterodimers. In normal

cells, where MMR functions correctly, *MSH2* and *MSH6* form heterodimers, forming MutSα that is able to repair base-base mismatches and indels, whereas *MSH2-MSH3*, known as MutSβ, recognizes larger indels. Besides recognizing the error, these heterodimers are responsible for initiation of repair. *MLH1* forms heterodimers with *PMS2*, *PMS1*, or *MLH3* to form MutLα, MutLβ, and MutLγ, respectively. Depending on the specificity of the DNA damage, different MMR genes are involved and specific complexes formed (Tamura et al., 2019). If a mismatch occurs, the *MSH2-MSH6* dimer recognizes it and recruits the *MLH1-PMS2* dimer, which then recruits other proteins needed for the process: proliferation-cell-nuclear antigen, replication factor C, exonuclease 1, replication protein A, and DNA ligase (Olave, 2022). Figure 1 illustrates how the functional MMR system works. MMR system enzymes also take part in other DNA repair pathways, for example base excision repair (BER) and interstrand crosslinking (ICL) (Liu et al., 2017).

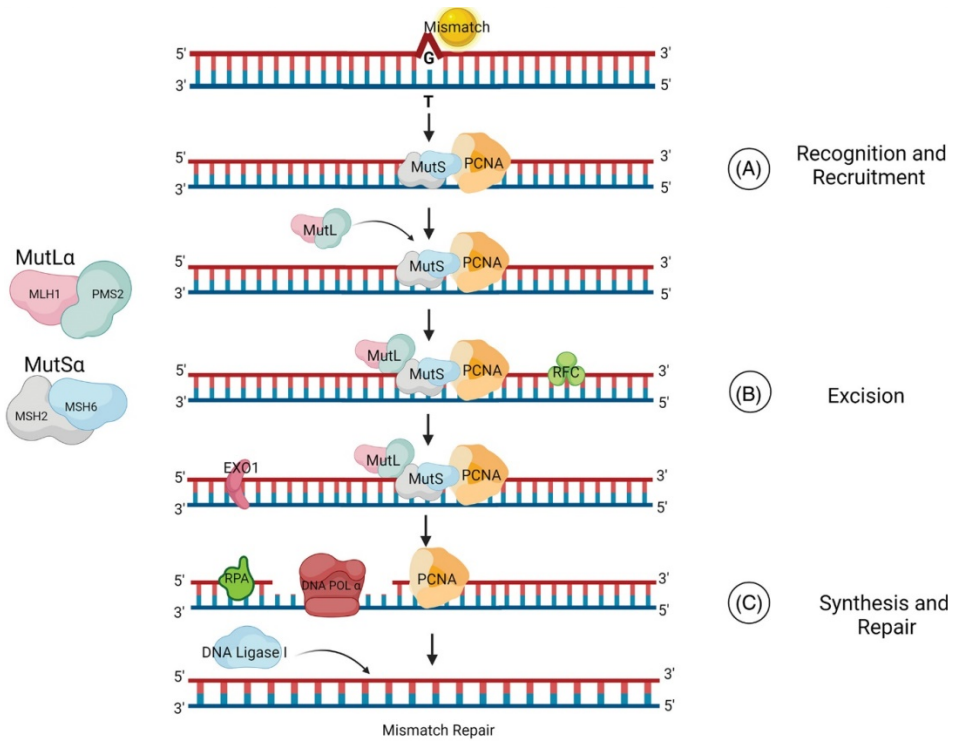


Figure 1. How the functional MMR system works [(Olave, 2022) *permission to use granted]. **Step A:** Recognition of the mismatch by MutS complex, followed by recruitment of proliferating cell nuclear antigen and replication factor C; **Step B:** Excision of the mismatched base by MutL; **Step C:** synthesis of the strand and final repair.

When the MMR system is deficient, it fails to correct the errors, increasing the mutational rate in cells.

Microsatellites are short, tandemly repeated (usually 10–60 times) sequences of mononucleotide, dinucleotide or higher order nucleotide repeats (unit length ranging from 1 to 6 bases), that are scattered throughout the human genome (Baretti & Le, 2018). An increased level of mismatches in microsatellites is called microsatellite instability, and it is associated with the hypermutated phenotype (Capasso et al., 2023). It is one type of genomic instability characteristic of cancer cells. MSI secondary to germline mutations in MMR genes is the molecular fingerprint of LS, while epigenetic inactivation of these genes is more characteristic of sporadic MSI tumours (Baretti & Le, 2018). Deficiency of the MMR system increases the risk of CRC, EC, ovarian and gastric cancer (Liu et al., 2017).

2.2.1.4 Prevalence of Lynch syndrome

Over the years, numerous studies have shown that LS is the most common hereditary colorectal cancer syndrome with an estimated prevalence of 1–3% (Peltomaki, 2016) or nearly 5% (Menahem et al., 2019) of all CRC cases. CRC is the most common cancer in the spectrum of LS, therefore most studies are connected to its prevalence. In 2022 a systematic review and meta-analysis was published by Abu-Ghazaleh et al. where they report that the overall pooled prevalence is 2.2%, which is similar to that suggested earlier, and is remarkably similar across different ethnic, geographical and clinical groups (Abu-Ghazaleh et al., 2022). The second most common cancer related to LS is EC: its prevalence among LS patients is estimated at 2% (Pellat et al., 2019).

There is rather scant information on the prevalence of LS in the general population, but there are data from Iceland, and Win's article considered individuals from the United States, Canada, Australia, and China (Haraldsdottir et al., 2017; Zhang et al., 2022; Win et al., 2017). An Icelandic study from 2017 reported a prevalence of 1/226 (Haraldsdottir et al., 2017). Using population-based family data from the Colon Cancer Family Registry (CCFR), in 2017 Win et al. estimated the prevalence of LS in the general population to be actually as high as 0.35% or 1:279, (95% CI 1:192 to 1:403) (Win et al., 2017). It was estimated to be 1:100–1:180 in 2019 by the International Agency for Research on Cancer (Frankel, 2019). The latest work of Zhang et al. (Zhang et al., 2022) estimated the prevalence of disease-causing MMR gene variants in the Chinese general population to be 0.18%. There are no recent data to be found in the literature for European populations, most probably because not many countries have biobanks. In Estonia, a population-based biobank has been established in 2001 that includes over 200,000 participants' genetic and health data, representing more than 20% of the Estonian adult population (Jurgens et al., 2022). In one of our first studies on LS, we estimated the prevalence of LS-associated MMR gene variants to be 1:485 (Poisson 95% CI: 1:263–1:1009) in the general population as based on Estonian Biobank data (EstBB) (Roht, 2020).

2.2.1.5 Diagnostics of LS

Traditionally, the diagnosis of LS has consisted of two stages: firstly, evaluation of the fulfilment of clinical criteria, and secondly, genetic testing. The first set of clinical criteria, the Amsterdam criteria, arose from the meeting of the international collaborative group on hereditary non-polyposis colon cancer held in Amsterdam in 1990. The Amsterdam I criteria included only CRC and family history, and all six elements had to be positive to fulfil the criteria (Table 3). The Amsterdam II criteria, published in 1999, covered other LS-associated cancers (endometrial, small bowel, etc.). The Amsterdam criteria were not specific and sensitive enough (sensitivity 60% and specificity 70% for Amsterdam I criteria and 78% and 61%, respectively, for Amsterdam II criteria) to decide which of the families should be genetically tested. In 1997, the Bethesda Guidelines were published to detect patients whose tumours should be tested for MSI. In 2004, an update, the revised Bethesda Guidelines, were published. All of the features of the four clinical criteria guidelines (Amsterdam I and II criteria and the Bethesda Guideline and its update) are shown in Table 3. Besides an expanded cancer spectrum and family history, the Bethesda Guidelines also encompass microsatellite instability high (MSI-H) histology (lymphocytes infiltrating tumour, Crohn's-like lymphocytic reaction, mucinous or signet ring differentiation, or medullary growth pattern) and only one positive criterion was enough to fulfil the criteria and suggest MSI testing (Schneider et al., 2012). The sensitivity of the Bethesda Guidelines for identifying families with pathogenic variants has been estimated to be 94% (Syngal et al., 2000). In 2009, Slovenian scientists showed that using the revised Bethesda Guidelines, only 43% of all LS patients would have been discovered (Berginc et al., 2009). Therefore, using clinical guidelines only, many LS individuals might be missed. In 2018, Schwark et al. published a paper on MSI as a predictive factor of LS across different tumour types, including classic LS-associated cancers like CRC or EC, but also cancers classically not linked to LS, such as soft tissue sarcoma, melanoma, etc. They concluded that the LS tumour spectrum is probably more heterogeneous than suggested. In addition, Schwark et al. showed that regardless of cancer type and family history, identifying MSI-H/MMR-D (mismatch repair deficiency) tumours should lead to germline genetic testing due to the fact that ~40% of LS patients with LS-associated cancers that are not classical LS spectrum cancers, would have been missed due to not meeting the clinical criteria (Schwark et al., 2018).

Table 3. Criteria of Amsterdam I, Amsterdam II, Bethesda guidelines and revised Bethesda guidelines (Lipton et al., 2004; Lynch et al., 2015; Umar et al., 2004).

Amsterdam I	Amsterdam II	Bethesda guidelines	Revised Bethesda guidelines
Three relatives with CRC and the following criteria should be fulfilled	Three relatives with HNPCC-related cancers and the following criteria should be fulfilled	Tumours with any of the following should be tested for MSI	Tumours should be tested for MSI in the following situations
One patient is a first-degree relative of the other two	One patient is a first-degree relative of the other two	Individuals with cancer in families that meet Amsterdam Criteria	CRC in a patient < 50 years
CRC affects more than one generation	HNPCC-related cancers affect more than one generation	Individuals with two HNPCC-associated cancers, including synchronous and metachronous CRC or associated extracolonic cancers	Presence of synchronous, metachronous colorectal, or other LS-related tumours, regardless of age
At least one CRC case diagnosed <50 years	At least one HNPCC-related cancer diagnosed <50 years	Individuals with CRC and a first-degree relative with CRC and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma diagnosed at age < 40 years	CRC with MSI-H histology in a patient <60 years
FAP has been ruled out	FAP has been ruled out	Individuals with CRC or endometrial cancer diagnosed at age < 45 years	CRC in one or more first-degree relatives with LS-related cancer, with one of the cancers diagnosed <50 years
Tumours verified by pathologic examination	Tumours verified by pathologic examination	Individuals with right-sided CRC with an undifferentiated pattern (solid or cribriform) on histopathology diagnosed at age < 45 years Individuals with signet-ring–cell-type CRC diagnosed at age < 45 years Individuals with adenomas diagnosed at age < 40 years	CRC diagnosed in two or more first-degree or second-degree relatives with LS-related cancer, regardless of age

CRC-colorectal cancer; HNPCC-hereditary nonpolyposis colorectal cancer; FAP-familial adenomatous polyposis; LS-related cancer sites included the colon, rectum, endometrium, gastric, ovary (including fallopian tube), sebaceous gland adenomas/carcinoma, small bowel, ureteric/renal pelvis, or central nervous system gliomas (including glioblastoma and astrocytoma); MSI-H- microsatellite instability-high; MSI-H histology: presence of tumour infiltrating lymphocytes, Crohn's like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.

Thus, currently two general diagnostic approaches are used. First, molecular screening of CRC and EC patients for MMR deficiency or MSI status to identify individuals with MSI-H cancers who need germline testing of MMR genes (Yurgelun & Hampel, 2018). MSI and MMR IHC are two methods used for LS screening. Evaluating MMR status serves three major purposes: it screens individuals for potential LS, predicts response to programmed cell death protein 1 (PD-1) inhibition, and gives prognostic value as a biomarker (Olave, 2022). MSI can be differentiated into three types: high microsatellite instability (MSI-H), low microsatellite instability (MSI-L) and microsatellite stability (MSS). At present, clinical research tends to classify MSI-L and MSS as one (Li et al., 2020). In clinical practice, MSI is referred to as high if the threshold of mismatches, which is dependent on the panel used, is exceeded. MSI status can be detected by different methods: conventional polymerase chain reaction (PCR), closed real-time PCR systems or Next Generation Sequencing (NGS). Tumour MMR status can also be tested by IHC (Olave, 2022). In 1996, Leach et al. developed monoclonal antibodies that detected MSH2 protein in DNA mismatch-proficient cell lines (Leach et al., 1996). Given the IHC test's simplicity, availability, reproducibility and equally informative features to that of PCR based MSI detection, there are data suggesting that IHC should be the preferred screening method. In 2017, Hoogerbrugge et al. presented their thesis to the European Society of Human Genetics Conference, that raising the age limit from 50 to 70 years of age for MMR IHC testing concerning colorectal cancer patients, would improve the efficacy of LS diagnosis by 40% (Hoogerbrugge, 2017). Slovenian scientists had shown similar results already in 2009 (Berginc et al., 2009).

When interpreting MMR IHC, loss of protein expression refers to complete absence of nuclear staining in tumour cells (McCarthy et al., 2019). The concordance of MMR-D assessment by IHC and MSI analysis is reported to be 94% (Carnevali et al., 2022). The pros and cons of PCR-based MSI tests and MMR IHC are shown in Table 4. While universal tumour screening is an important instrument in discovering LS, it works efficiently on individuals with tumour(s) with an available tumour sample (Biller et al., 2019). The latest joint guideline from the British Society of Gastroenterology (BSG), Association of Coloproctology of Great Britain and Ireland (ACPGBI) and United Kingdom Cancer Genetics Group (UKCGG) suggests that all people with CRC should be screened for LS either by MMR IHC or MSI, and if MMR-D is identified in the tumour, these individuals should be further tested for germline disease-causing variants of MMR and *EPCAM* genes (Monahan et al., 2020). The NCCN Guidelines suggest MMR status testing in all CRC and EC patients independent of their age when possible. In low-resource situations, Amsterdam II and revised Bethesda guidelines may still be helpful in deciding when the MMR status of a tumour should be tested (NCCN-guidelines, 2023).

Table 4. Comparison between MMR IHC and PCR based MSI method in CRC (adapted from Saeed et al., [(Saeed et al., 2021) *permission to use granted].

Methodology	Sensitivity	Specificity	Pros	Cons
MMR IHC	~90%	~99%	Widely available; Rapid; May identify the target gene; Cost-effective	Subjective interpretation; Staining of non-functional proteins; Certain MMR variants, that result in an abnormal/ dysfunctional protein, can still retain IHC expression and cause trouble in inter-preting; Misses MMR intact MSI-H tumours;
MSI (PCR)	~95%	~99%	More objective (threshold-wise); Few confounders	Not so widely available; Longer turnaround time than MMR IHC; Requires normal tissue for control; Misses MMR-D tumours that are not MSI-H

Another diagnostic approach is direct germline testing if the patient's personal and/or family history is suggestive of LS. Instead of testing every individual with a personal and/or family history of LS, Biller et al. suggest using clinical prediction models, for example the Prediction Model for Gene Mutations or PREMM (*PREMM5 model*), for screening (Biller et al., 2019). Currently, $PREMM_5$ is used, which launched in 2017, and provides the estimated probability of carrying a disease-causing variant in any of the four MMR genes or the *EPCAM* gene. $PREMM_5$ takes into account patient's sex, age, tumour type and age at diagnosis, and cancer history among first- and second-degree relatives. If the individual exceeds a 2.5% threshold, germline testing is recommended (Kastrinos et al., 2017). While tumour screening and clinical prediction models for LS are of great help, nothing replaces molecular genetics or germline testing for discovering variants in MMR and *EPCAM* genes. Development of Sanger sequencing in 1977, laid the foundations of the molecular genetics we know today. It still has its place in research and clinical settings, but NGS panel testing is currently most used due to its utility and cost-effectiveness. It is the same with LS testing, which is supported by multiple studies carried out on different cohorts (unselected or high risk populations). NGS panel testing is very appealing, but like any other method, it also comprises potential risks: misinterpretation, discovering variants that have no clear management, variants of unknown significance (VUS) etc. (Biller et al., 2019). Furthermore, NGS panel testing assists in finding alternative diagnosis for colorectal and other

cancers, which can alter the treatment or surveillance of these patients and their family members, for example in case of FAP and other hereditary colorectal cancer syndromes. Routinely, to detect CNVs in MMR genes, the Multiplex Ligation-dependent Probe Amplification (MLPA) technique has been used for further diagnostics (Schouten et al., 2002). Fowler et al. developed a tool for Detection of Exon Copy Number Variants (DECoN), the purpose of which was to detect exon CNVs simultaneously with NGS panel testing in clinical settings (Fowler et al., 2016). This improvement, when used, has made LS genetic testing faster and more cost-effective. An example of the algorithm for Lynch syndrome diagnosis is presented in Figure 2.

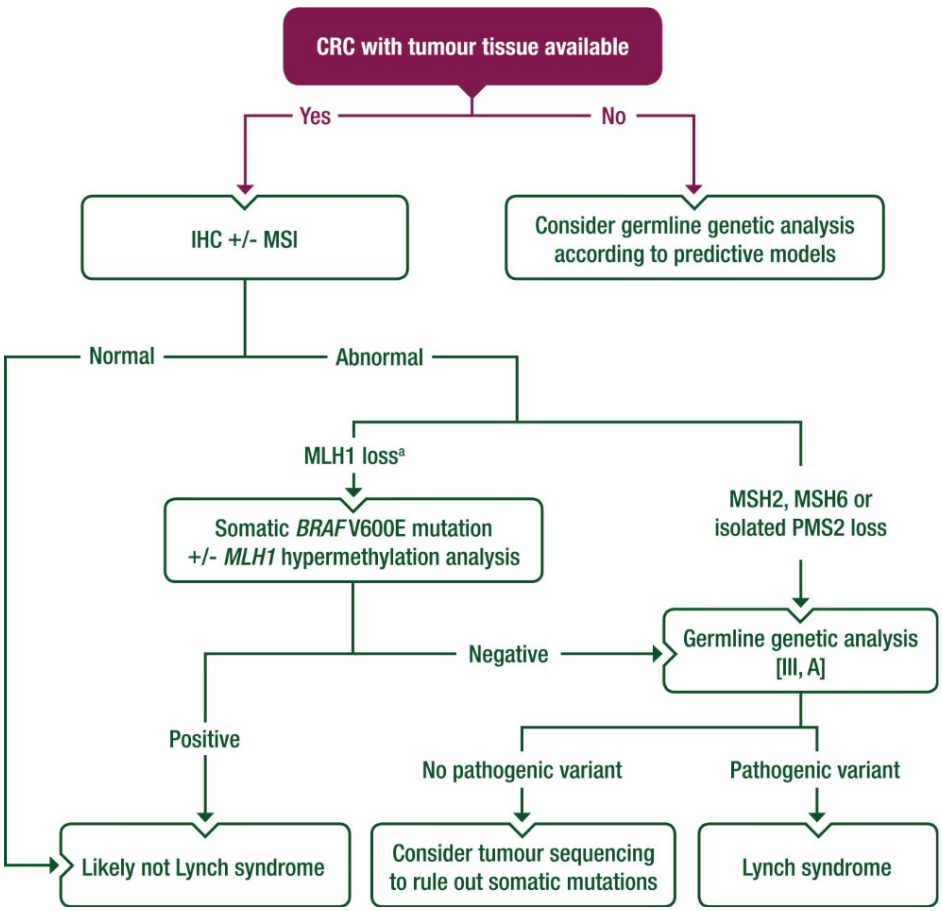


Figure 2. Algorithm for Lynch syndrome diagnosis [(Stjepanovic et al., 2019) *permission to use granted].

In 1990s the era of genetics began, which among other things revolutionized the diagnostics of LS. All four key MMR genes were discovered, and in addition the PMS1 homologue 1 (*PMS1* gene) and later, the MutL homologue 3 (*MLH3* gene) on 14q24 were identified (*GeneCards: The Human Gene Database webpage*; Lynch et al., 2015). To date there is no certain evidence to link these two latter MMR genes to LS-associated cancers.

MLH1 gene (OMIM #609310). The *MLH1* gene is one of the most important predisposing genes for LS, constituting 41% of all MMR gene variants. It is so because *MLH1* gene product is obligatory in every type of MMR protein heterodimer (Peltomaki et al., 2023). In 1994, the 3p21–23 locus was cloned and mapped as the second LS locus by Papadopoulos et al. (Papadopoulos et al., 1994*)* and Bronner et al. (Bronner et al., 1994) and another MMR gene, MutL homologue 1 or *MLH1*, was linked to the 3p21 chromosomal region, and disease-causing variants were identified in HNPCC pedigrees, referring to its linkage to the disease (Papadopoulos et al., 1994). Most of the *MLH1* disease-causing variants are truncating (mostly nonsense or frameshift); missense variants constitute around 40% (Peltomaki, 2016). According to ClinVar (Landrum et al., 2018) (accessed May 2023), there are presently 5,027 variants reported in the *MLH1* gene, and 1,782 of them are either likely pathogenic or pathogenic (LP/P) (*ClinVar database webpage*). Niessen et al. showed in 2009 that ~9% of LS cases can be caused by germline *MLH1* hypermethylation (Niessen et al., 2009). Constitutional intragenic disease-causing variants as well as *MLH1* epimutations cause a severe LS phenotype, including young age of cancer onset and high risk for multiple primary tumours (Pinto et al., 2018). The general population prevalence of pathogenic *MLH1* variants has been estimated to be 0.051% (1:1946) (Biller et al., 2019). In the NCCN Guidelines the cumulative CRC risk throughout life is then estimated to be 46–61% with an average age at diagnosis of 44 years (NCCN-guidelines, 2023).

MSH2 gene (OMIM #120435). This gene is the second most important predisposing gene for LS, its disease-causing variants constituting 36% of the variants in MMR genes (Peltomaki et al., 2023). In 1993, the first genetic locus responsible for LS was mapped to chromosome 2p21 (Lynch et al., 2015), and that verified for the first time the genetic background of LS. Later, Leach et al. mapped the MutS homologue 2 (*MSH2* gene) to that region and noted its segregation in LS cancer families, therefore concluding that MutS is probably responsible for the then-called HNPCC (Leach et al., 1993). Similarly to *MLH1*, most of the *MSH2* disease-causing variants are truncating; missense variants constitute 31% (Peltomaki, 2016). According to ClinVar (Landrum et al., 2018) (accessed May 2023), there are presently 6,849 variants with clinical evaluation in the *MSH2* gene, and 2,064 of them are either likely pathogenic or pathogenic *(ClinVar database webpage)*. *MSH2* disease-causing variants are associated with the highest risk for extracolonic cancers (for example: EC, ovarian cancer (OC) and urological tract cancers) (Idos & Valle, 1993; Lin-Hurtubise et al., 2013). Likely pathogenic and pathogenic variants of *MSH2* have been reported more commonly than pathogenic variants of the other three MMR genes in

individuals with MTS (Jessup et al., 2016). The general population prevalence of pathogenic *MSH2* variants has been estimated to be 0.035% (1:2841) (Biller et al., 2019). In the NCCN Guidelines cumulative CRC risk throughout life is then estimated to be 33–52% with an average age at diagnosis comparable to those with *MLH1* variants (NCCN-guidelines, 2023).

MSH6 gene (OMIM #614350). In 1997, Miyaki et al. discovered the MutS homologue 6 (*MSH6* gene) as the fourth MMR gene connected to HNPCC. As it was discovered in a family not meeting the Amsterdam I criteria, it was initially hypothesized that it was due to phenotypic heterogeneity (Miyaki et al., 1997). *MSH6* constitutes 18% of all MMR gene variants (Peltomaki et al., 2023). At first it was thought that *MSH6* disease-causing variants lead to MSI-H tumours, but this might not be the case in germline *MSH6*-associated cancers (Peltomaki, 2005). Similarly to *MLH1* and *MSH2* genes, most of the *MSH6* disease-causing variants are truncating; missense variants make up 49% according to Peltomäki (Peltomaki, 2016). According to ClinVar (Landrum et al., 2018) (accessed May 2023), there are presently 8,467 variants with clinical assessment in the *MSH6* gene, and 1,819 of them are either likely pathogenic or pathogenic *(ClinVar database webpage*). The population prevalence of pathogenic *MSH6* variants has been estimated to be 0.132% (1:758) (Biller et al., 2019). In the NCCN Guidelines the cumulative lifetime risk of CRC is estimated to be 10–44% and the average age of presentation 42–69 years (NCCN-guidelines, 2023).

PMS2 gene (OMIM #614337). In 1994, Nicolaides et al. mapped and linked the Postmeiotic Segregation Increased (S. Cerevesiae) 1 (*PMS1* gene) and the PMS1 homologue 2 (*PMS2* gene*)* to HNPCC (Nicolaides et al., 1994). *PMS2* constitutes ~5% of all MMR gene variants (Peltomaki et al., 2023). Today we know that there are four key MMR genes, and *PMS1* is not one of them. Disease-causing variants in the *PMS2* gene are predominantly missense, constituting 62% of all variants (Peltomaki, 2016). According to ClinVar (Landrum et al., 2018) (accessed May 2023), there are presently 4,768 variants with clinical rating in the *PMS2* gene, and 909 of them are either likely pathogenic or pathogenic (*ClinVar database webpage*). In the NCCN Guidelines the cumulative lifetime risk of CRC is estimated to be 8.7–20% and the average age of presentation 61–66 years (NCCN-guidelines, 2023). The general population prevalence of pathogenic *PMS2* pathogenic variants has been estimated to be the highest of MMR genes at 0.140% (1:714) (Biller et al., 2019). Nevertheless, the proportion of *PMS2* pathogenic variants in cancer patients is the lowest and they are associated with the lowest risk for any LS-related cancer (Seppala, Dominguez-Valentin, et al., 2021). Regardless, there are data suggesting that cancers could still be diagnosed at an early age: a review of 234 individuals with a *PMS2* pathogenic variant found that 8% were diagnosed before age 30 (Goodenberger et al., 2016).

EPCAM gene (OMIM #613244). Its disease-causing variants are rare (Peltomaki et al., 2023). Niessen et al. showed in 2009 that ~6% of LS can be caused by *EPCAM* deletions (Niessen et al., 2009). Germline *EPCAM* deletions result

in methylation of the surrounding genomic region, affecting the *MSH2* promoter located 18 Kb downstream (Martinez-Roca et al., 2022). Therefore, they cause LS by inactivation of structurally normal *MSH2* genes through an epigenetic mechanism (Peltomaki et al., 2023). Individuals with *EPCAM* deletion typically have early-onset CRC and a CRC cumulative risk of up to 75%. Compared to individuals with *MSH2* disease-causing variants, they rarely develop extracolonic tumours (Kempers et al., 2011). *EPCAM* deletions of the 3' region only have been shown to carry a lower risk for extracolonic cancers, whereas deletions that extend into *MSH2* confer extracolonic cancer risks similar to intragenic *MSH2* disease-causing variants (Tutlewska et al., 2013). According to ClinVar (Landrum et al., 2018) (accessed May 2023), there are presently 686 variants with clinical estimation in *EPCAM* gene, and 135 of them are either likely pathogenic or pathogenic (*ClinVar database webpage*).

2.2.2 Other hereditary colorectal cancer syndromes

2.2.2.1 *AXIN2*-related oligodontia-colorectal cancer syndrome

Disease-causing variants in the *AXIN2* gene are the genetic cause of oligodontia-colorectal cancer syndrome (OMIM #608615) or oligodontia-cancer predisposition syndrome (ORPHA 300576). This syndrome has AD inheritance and its prevalence according to the Orphanet database is ~1:1,000,000. Germline loss-of-function (LoF) variants in *AXIN2* have been known to be associated with oligodontia and ectodermal dysplasia for quite some time (Chan et al., 2022). The absence of one or more teeth, excluding wisdom teeth, is known as tooth agenesis. It can further be divided into hypodontia (up to six teeth missing) and oligodontia (six or more teeth missing) (Bilgin, 2018). Ectodermal dysplasia manifests as abnormal development of the skin, hair, nails, or sweat glands (Beard et al., 2019). It has been shown in animal models that genes associated with dental development or odontogenesis can be involved in cancer development, but clinically it might be surprising (Cobourne, 2009).

The Axin2 protein encoded by the *AXIN2* gene consists of 843 amino acids and is part of the canonical Wnt or Wnt/β-catenin pathway, being its feedback inhibitor. Wnt pathway signalling is important in cell proliferation, differentiation and homeostatic self-renewal and if dysregulated, is associated with colorectal cancer development. Liu et al. were the first to describe this association; furthermore *AXIN2* somatic variants have been reported in microsatellite instable colorectal tumours (Liu, 2000). A few years later, Lammi et al. reported a large family with colon polyposis and CRC in Finland carrying the disease-causing variant c.1966C>T, p.Arg656* in *AXIN2* (Lammi et al., 2004). The polyposis resembles that of attenuated FAP, but there are limited data on the molecular features of the polyps and CRC that develop.

AXIN2 disease-causing variants particularly inhibit the development of permanent teeth and specifically posterior molars, but do not significantly affect the development of deciduous or primary teeth. During tooth development,

AXIN2 is expressed in enamel knots and dental mesenchyme. The failure of tooth development is probably linked to increased signalling of Wnt/β-catenin pathway in the mesenchyme when carrying an *AXIN2* pathogenic variant (Jarvinen et al., 2018). As the Wnt pathway is also important in craniofacial morphogenesis, *AXIN2* variants have also been associated with facial clefts in humans (Letra et al., 2012). Furthermore, *AXIN2* polymorphisms have been found to be associated with oral clefts in two independent population studies by Letra et al. (Letra, 2009; Letra et al., 2012). These authors also provided some biological evidence in support of *AXIN2*'s role in clefting. They observed co-localization of Axin2 with cleft-associated Irf6 protein in epithelium and demonstrated *AXIN2* gene expression during murine palatogenesis (Letra et al., 2012).

Knowing all these associations, one question still remains, how to detect and diagnose families with this rare cancer predisposition syndrome. In this era of genomics different NGS panels and exome sequencing are used for genetic testing. In our *AXIN2* publication (Roht, Hyldebrandt, et al., 2023), we also concluded that awareness on that subject is important. Jensen et al. also stated, that *AXIN2* should be included in NGS panels for detection of hereditary cancer predisposition or CRC, in the context of tooth agenesis/development or oligodontia and ectodermal dysplasia (Jensen et al., 2022).

2.3 Genetic counselling and surveillance of colorectal cancer syndrome patients and their family members

In 2003, the Council of the European Union strongly recommended implementation of population-wide CRC screening for all individuals aged 50–74 years, with annual or biannual faecal occult blood tests (FOBTs) followed by colonoscopy when the test result was positive (Navarro et al., 2017). In 2021, a publication on CRC screening in Europe was published in the Lancet Oncology by Cardoso et al., a study based on the data of national cancer registries of 21 European countries (Cardoso et al., 2021). Long-term screening programmes and coverage of more than 50% of the population of interest, were seen to significantly reduce colorectal cancer incidence; in general reduced cancer mortality was also observed (Gogenur & Qvortrup, 2021).

European countries have not yet implemented population-wide screening for detecting hereditary cancer syndromes, probably mostly due to the many challenges, including lack of resources, little experience of general population level DNA screening, and reporting-related issues (VUS etc.). Outside Europe, the USA and Australia have been rather courageous in implementing population-wide DNA screening for some hereditary diseases. Based on their cohort of 26,906 participants the Healthy Nevada Project (HNP) included *BRCA*-related hereditary breast and ovarian cancer (HBOC), LS and familial hypercholesterolemia (FH). They found a combined disease-associated gene alteration carrier rate of 1.33% for all three diseases; furthermore, 90% of individuals had not been previously identified, only ~20% had documented information on their

genetic risk and/or family history, and only a quarter (25.2%) reported a disease history in their family (Grzymski et al., 2020). In a large Australian study, preventative DNA screening for *BRCA1, BRCA2, PALB2, MLH1, MSH2, MSH6*, *LDLR*, *APOB* and *PCSK9* genes was offered to 10,000 Australians aged 18–40. It is estimated that compared to current rates of clinical DNA testing, DNA screening for HBOC and LS in adults aged 18–25 years would prevent 2,411 cancers and save 1,270 lives (Lacaze et al., 2022).

Genetic counselling as a term was first suggested in 1947 by Sheldon Reed. Later on two main professional societies, the American Society of Human Genetics (ASHG) founded in 1948 and the National Society of Genetic Counsellors (NSGC) founded in 1979, started to manage the field of genetics and genetic counselling (Raymond & Everett, 2009). In Europe, the European Society on Human Genetics (ESHG) is leading the field.

In general, the process of genetic counselling should include collecting detailed medical history of individual cancers as well as cancers in family members, drawing the pedigree of at least two, preferably three generations, pre-test counselling for discussing advantages and disadvantages of genetic testing, and explaining and choosing the best method for genetic testing (Kim & Byeon, 2019). Genetic counselling and testing allows identification of individuals at risk to undergo surveillance and prophylactic surgery, which in turn reduces cancer morbidity and mortality. The process of genetic counselling has been shown to increase personal discipline and adherence to surveillance, whereas it has not been associated with long-term anxiety or distress in those interested in genetic testing (Raymond & Everett, 2009). An Estonian study from 2022, piloting a genotype-first approach for breast cancer screening, also found that the majority of participants (74–88%) felt calm after receiving their genetic risk information, only a minority (11–22%) reported feeling worried, upset or tense (Jurgens et al., 2022).

Identifying individuals or families to investigate for hereditary cancer syndromes, relies mainly on clinical guidelines and clinical judgement. In the 1990s the Amsterdam Criteria and Bethesda Guidelines, which were later on updated, mostly led the way in clinical thinking. The most important points of these guidelines were covered in the LS diagnostics section above (section 2.2.1.5).

2.3.1 Lynch syndrome genetic counselling, surveillance and treatment

This section will mostly concentrate on management of LS from the CRC point of view: to compare knowledge and recommendations of European and American leading societies in the field of cancer genetics, the European Society of Medical Oncology (ESMO) (Stjepanovic et al., 2019) and NCCN Guidelines (NCCN-guidelines, 2023) are used, respectively. In addition, the joint British Society of Gastroenterology (BSG), Association of Coloproctology of Great Britain and Ireland (ACPGBI), United Kingdom Cancer Genetics Group

(UKCGG) guidelines on management of hereditary CRC are discussed (Monahan et al., 2020). On the subject of LS, in addition, the Mallorca guidelines of the European Hereditary Tumour Group (EHTG) together with European Society of Coloproctology (ESCP) are used (Seppala, Latchford, et al., 2021). Both of the European guidelines are approved by the European Reference Network for patients with Genetic Tumour Risk Syndromes (ERN GENTURIS). Estonia is also an affiliated member of ERN GENTURIS.

The NCCN guidelines are a useful and quick way to keep up to date with cancer risks depending on the MMR gene affected, but there are many other factors modifying the cancer risks of LS carriers. Penetrance and expression of LS depends on many different factors: personal characteristics of the individual, lifestyle factors, the variant found in the MMR gene, and others (International Mismatch Repair, 2021). Dominguez-Valentin et al. published a paper in 2020 presenting findings from PLSD on cancer risks by gene, age and gender (Dominguez-Valentin et al., 2020). They conducted an international, multicentre prospective observational study with test and validation cohorts, resulting in 6,350 participants. In conclusion they found, that the lifetime CRC incidence for *MLH1* and *MSH2* carriers is ~50%, and that female carriers of *MLH1*, *MSH2* or *MSH6* disease-causing variants have a rapid increase in gynaecological cancer risk after the age of 40 years. Concerning urothelial and upper gastrointestinal tract cancers, *MLH1* and *MSH2* carriers are at higher risk at older ages (in their 50s and 60s). *MSH2* carriers in particular are at increased risk for prostate cancer. *MSH6* carrier females have a high penetrance for CRC risk, whereas *MSH6* carrier males have a lifetime risk of 18% for CRC, due to which they can be lost for genetic testing, surveillance and segregation analysis. *PMS2* carriers do not have increased risk of CRC, EC, or ovarian cancer (OC) before age 50, and may only have slightly higher risks at older ages (Dominguez-Valentin et al., 2020). Win et al. included more than 5,000 LS carriers in their study to estimate the differences in CRC risk within and between sexes, and across different geographical regions (Win et al., 2017). The penetrance was highest for *MLH1* and *MSH2* variants and lowest for *PMS2*. The wide variation in the CRC risk between carriers is dependent on many factors, for example sex, MMR gene affected and geographical location, and therefore the mean cumulative risk probably only applies to a minority. Therefore, existing guidelines might not be applicable to most of the carriers, and at the moment it is hard to determine personalized risk, mostly due to familial risk factors modifying CRC risk (International Mismatch Repair, 2021). Data from the UK Biobank (454,712 participants) also emphasize that cancer risks depend significantly on family history. They found that penetrance to age 60 was higher for carriers of a pathogenic *MLH1* or *MSH2* variant in those with a family history (27.1% and 25.2%, respectively) than in those without (15.2% and 3.2%, respectively), but this has yet to be evaluated (Jackson et al., 2022). In 2022, the PLSD group published comparisons of CRC incidence risk between PLSD and International Mismatch Repair Consortium (IMRC) cohorts. They found, that the average cumulative incidences of CRC in *MLH1* carriers at 70

years of age were 52% in males and 41% in females; for *MSH2* carriers 50% and 39%; for *MSH6* carriers 13% and 17% and for *PMS2* carriers 11% and 8%. In the IMRC cohort, the risks for males and females carrying *MLH1*, *MSH2*, *MSH6* or *PMS2* disease-causing variants were as follows: 40% and 27%; 34% and 23%; 16% and 8% and 7% and 6%, respectively. This could be related to colonoscopic surveillance of all carriers in the PLSD group, fidelity of CRC recording in prospective versus retrospective cohorts, or factors in carcinogenesis independent of surveillance (Moller et al., 2022). Data from the UK Biobank also suggest that family history significantly increases cancer risk in LS carriers: individuals with a pathogenic *MLH1, MSH2* or *MSH6* variant had an increased risk of bowel cancer that was significantly higher in those with family history (relative hazard 63.7, 68.4 and 12.1, respectively) than those without (relative hazard 20.9, 18.6 and 5.9, respectively), but again, this information needs to be evaluated and validated before guiding clinical practice (Jackson et al., 2022).

Among other things, genetic consultation in cancer genetics includes recommendations on modifying factors. For example, the Mallorca guidelines advise that LS individuals should be made aware during counselling that alcohol consumption, smoking and obesity all increase the risk of CRC, whereas physical activity decreases it. In addition, LS individuals should be recommended to take daily acetylsalicylic acid (aspirin), known also as chemoprevention, with a minimum dose of 75–100 mg, for CRC risk reduction (Seppala, Latchford, et al., 2021). Discussions on the optimal dose of aspirin are continuing. Results from the double-blind, randomized, placebo-controlled CAPP2 study supported prevention of colorectal cancer with aspirin in LS when using it daily at a dosage of 600 mg for 2–4 years (Burn et al., 2020). In the meantime, there are data from earlier studies indicating that aspirin could be sufficiently effective also at lower doses, therefore other groups also suggest using an aspirin dose of 100 mg until further information becomes available (Cook et al., 2013; Serrano et al., 2022). An ongoing CAPP3 study will probably provide better answers, but this will take several years. Concerning gynaecological cancers, there have been suggestions of using oral contraceptives, especially progestogen-only contraceptives, as chemoprevention, as these reduce the risk of gynaecological cancers (Llach et al., 2022).

Genetic post-test counselling is of critical value. It has been suggested that due to its complexity and diversity, in-depth counselling should be multi-disciplinary, encompassing a team of different clinicians from genetic nurse or counsellor to surgeon, oncologist, gastroenterologist and others (Kim & Byeon, 2019). Recommendations on management of LS are in constant change, and there are many different guidelines. For example, the NCCN Guidelines are updated annually, and therefore are very popular among clinicians. In time, the guidelines have become more specific concerning cancer risks and surveillance dependent on the MMR gene affected. Järvinen et al. showed already in 2000, that early colonoscopic evaluation of healthy individuals with LS every three years can substantially reduce CRC incidence, CRC-associated and overall

mortality (Jarvinen et al., 2000). A recent paper by Llach et al. published in 2022, showed that regular surveillance decreases CRC incidence (6% versus 16%) and mortality (8% versus 22%), and that the main benefit is detection of early CRCs (Llach et al., 2022). Guidelines from ASCO, ESMO, NCCN, the Multi-Society Task Force on Colorectal Cancer, the American College of Gastroenterology and others recommend colonoscopies with an interval of 1–2 years for healthy LS carriers (Yurgelun & Hampel, 2018). Guidelines on management of hereditary CRC by the ACPGBI and UKCGG (Monahan et al., 2020) state that for all LS patients, colonoscopic surveillance should be assigned every two years, whereas the Mallorca guidelines (Seppala, Latchford, et al., 2021) differentiate between different MMR genes: for *MLH1*, *MSH2* and *MSH6* carriers every 2–3 years (if CRC has occurred before, then biannually), for *PMS2* carriers every five years. Both of the latter guidelines recommend surveillance starting from 25 years for *MLH1* and *MSH2* carriers, but for *MSH6* and *PMS2* carriers from 35 years, and state that there is no difference between genders (Monahan et al., 2020; Seppala, Latchford, et al., 2021). The NCCN Guidelines give similar recommendations (NCCN-guidelines, 2023). ESMO clearly states that in all cases, the age of cancer onset in the youngest member of the family is to be considered and that surveillance should be started five years earlier (Stjepanovic et al., 2019). There are different opinions on which kind of colonoscopy is the best: chromo-endoscopy (indigo carmine dye spraying added to the standard colonoscopy) or high-definition white-light endoscopy, concluding that when carried out in high quality centres, they are probably equivalent, at least in those specialist settings (Monahan et al., 2020; Seppala, Latchford, et al., 2021). A meta-analysis of three studies supports this opinion: concerning adenoma detection among individuals with LS, dye-assisted chromo-endoscopy was not superior to white-light examination alone (Houwen et al., 2021). There is no effective screening for EC or OC, but the risk-reducing surgery of hysterectomy together with bilateral salpingo-oophorectomy has been shown to be effective in preventing these cancers (Yurgelun & Hampel, 2018). The 2019 Manchester consensus conference strongly recommended that risk-reducing surgery should be offered to females at the age of 35–40, but not earlier, and for *MLH1*, *MSH2* and *MSH6* carriers only (Crosbie et al., 2019). Concerning management of other LS-associated cancers besides CRC, guidelines on management of hereditary CRC, both by ACPGBI and UKCGG and by ESMO, recommend that *Helicobacter pylori*, should be excluded, or treated when found (Monahan et al., 2020; Stjepanovic et al., 2019). The guidelines on management of hereditary CRC by ACPGBI and UKCGG recommend that surveillance of gastric, small bowel and pancreatic cancers should only be performed in the context of clinical trials (Monahan et al., 2020). Although ESMO guidelines recommend considering screening for pancreatic cancer by magnetic resonance imaging (MRI) and/or endoscopic ultrasound (EUS) annually, it generally agrees on the matter of gastric and small bowel cancer screening for all LS individuals, and recommends screening of gastric cancer only when there is a high incidence of gastric cancer in that region or a

positive family history (Stjepanovic et al., 2019). Although, in LS, only CRC surveillance has proven value so far, there are other surveillance schemes recommended by health care professionals (Seppala, Dominguez-Valentin, et al., 2021). Llach et al. also claim that presently, except for CRC, there is no surveillance of proven value for other extracolonic cancers (Llach et al., 2022).

From a treatment perspective, in the event of a first CRC, extended surgery (subtotal colectomy with ileosigmoid anastomosis or total colectomy with ileorectal anastomosis) is preferred for *MLH1* and *MSH2* carriers, while for *MSH6* and *PMS2* carriers there are insufficient data to support this. One should assess and take into account patient-specific factors (age, comorbidities, compliance with screening, etc.), when deciding between segmental and more extended colonic resection in LS-associated CRC (Yurgelun & Hampel, 2018). The Mallorca guidelines further broaden this subject with consideration of prophylactic colorectal surgery, and conclude that based on CRC risk alone, this should not be recommended (Seppala, Latchford, et al., 2021). Llach et al. also suggest acting accordingly in the case of CRC, and in terms of rectal cancer, that there is no requirement for specific LS-associated management. They also state that stage II CRC should be treated by surgery only, since these patients do not benefit from adjuvant 5-fluorouracil (5-FU) or capecitabine chemotherapy. Any difference in the benefit from standard adjuvant chemotherapy with FOLFOX/CAPOX in stage III CRCs is not yet well established, therefore patients should receive it until further data are available (Llach et al., 2022).

The immune system has a critical role as a gatekeeper preventing carcinogenesis in LS (Llach et al., 2022). Immune checkpoint inhibitors (ICI) have altered the treatment of LS by extending survival of patients, although at the moment this only applies to metastatic CRC. The PD-1 antibody pembrolizumab is therefore at the moment standard of care for patients with MMR-D metastatic CRC, as compared to chemotherapy as first-line treatment it has shown significantly longer progression-free survival and fewer adverse effects. An important ongoing question, though, is why MMR-D metastatic CRCs frequently exhibit resistance to ICIs; future studies are needed to resolve this (Jin & Sinicrope, 2022).

For years, development of cancer vaccines has been an important topic. Cancers with a defect in DNA MMR genes, such as LS, have a particularly high mutational burden and predictable neoantigenesis (Hernandez-Sanchez et al., 2022). The first clinical study with a frameshift peptide (FSP) neoantigen-based cancer vaccine was more than a decade ago. So far it is known that vaccination with MSI-induced FSP neoantigens is well tolerated and induces adequately both cellular and humoral immune responses (Kloor et al., 2020). Although a lot of work has been done, there are still many questions and further studies are needed.

Last, but not least, counselling of family members is also an important part of genetic counselling. Educating individuals at risk is critical for their decision-making. Among other things, relationships between family members are of utmost importance. There are data suggesting that it might be even more

significant than professional counselling in terms of who undergoes genetic testing. There is no consensus on family planning (prenatal testing and preimplantation diagnostics) yet, but the risks for future children should be discussed (Kim & Byeon, 2019). Concerning cancer risks and colonoscopic surveillance, a PLSD calculator for cumulative risk for cancer by age, genetic variant, and gender, is available for patients as well as healthy family members (*Prospective Lynch Syndrome Database (PLSD) risk calculator*).

2.3.2 Genetic counselling of *AXIN2* carriers

There is little knowledge on management of *AXIN2* carriers, unfortunately. We still do not have any official and published European guidelines for this syndrome. Given the rareness of the syndrome, clinical recommendations with respect to cancer risk management are expert-opinion based and typically modelled on guidelines for other colorectal cancer and polyposis syndromes. In the NCCN Guidelines, colonoscopy is suggested from the age of 25–30 years every 2–3 years, if negative. If polyps are found, every 1–2 years. Surgery is indicated if the polyp burden becomes unmanageable with colonoscopy (NCCN-guidelines, 2023). Experts from ERN GENTURIS suggested performing colonoscopy every two years and to add gastro-duodenoscopy every three years, or more frequently, depending on endoscopic findings, to the surveillance scheme (personal communication with ERN GENTURIS team). The reason for the latter was the known involvement of Axin2 in the Wnt pathway, that is also dysregulated in *APC*-associated FAP, where such surveillance is recommended (Roht, Hyldebrandt, et al., 2023). Jensen et al. also concluded that recommendations similar to those for AFAP are suggested (Jensen et al., 2022). In 2019, Beard et al. recommended the following management: annual colonoscopy from ages 18–20; colectomy for those individuals, whose polyp burden is not manageable by endoscopy, followed by annual surveillance of residual rectum or ileal pouch; upper gastrointestinal endoscopy from age 25 with interval dependent on polyp burden (Beard et al., 2019). In 2023, Leclerc et al. suggested surveillance similarly to that for *MUTYH* biallelic variant carriers (Leclerc et al., 2023). Clearly, further information and collaboration is needed to establish European guidelines for the management of *AXIN2* patients.

2.4 Summary of the literature

CRC is one of the most common cancers and the second leading cause of cancer-related death in the world (*World Cancer Research Fund International webpage*). According to NCCN Guidelines, the life-time risk of CRC in the general population is 4.1% (NCCN-guidelines, 2023). In addition to known risk factors like environment, age, sex etc., genetics also plays an important role in individual CRC risk. Altogether, 5–10% of CRC cases are caused by high or moderate penetrance gene variants associated with different hereditary cancer syndromes (Kastrinos et al., 2020). The most common hereditary CRC pre-

disposition syndrome is LS, constituting 1–5%, of all CRC cases (Menahem et al., 2019; Peltomaki, 2016). MMR genes (*MLH1, MSH2, MSH6* and *PMS2*) secure the genomic stability by correcting base-base mismatches and small indels resulting from DNA replication and recombination errors. Due to MMR system deficiency, alterations in the sequence length of microsatellites also emerge, resulting in MSI (Baretti & Le, 2018). Historically, different clinical guidelines have been used to diagnose LS, but as most of the patients would not have been found, nowadays two approaches are mostly used. First, molecular screening of CRC and EC for MSI-H or MMR deficiency by IHC to identify individuals who need germline testing of MMR genes (Yurgelun & Hampel, 2018), and secondly, direct germline testing of MMR genes if the individual and/or family history is suggestive of LS. Genetic counselling is an important and helpful tool for educating patients, finding individuals at risk, and reducing cancer risks and cancer-related death rates by surveillance. Recommendations for treatment and surveillance guidelines are updated constantly, providing necessary knowledge for best clinical outcomes. In addition, different cancer risk calculators have been composed for LS individuals (*PREMM5 model; Prospective Lynch Syndrome Database (PLSD) risk calculator*). There are also rarer, but still significant hereditary CRC syndromes, for example the oligodontia-colorectal cancer syndrome (OMIM #608615) caused by *AXIN2* gene pathogenic variants. In this case, we only have expert-based opinions for surveillance and management at the moment, thus further research and collaboration is needed to establish guidelines to lead clinical thinking. In summary, the key factors for success in hereditary colorectal cancer syndromes are as follows: finding healthy individuals at risk, surveillance and/or risk-reducing surgery and treatment, all according to our knowledge. The main aim of this study was to gather epidemiologic and genetic data on hereditary colorectal cancer syndromes in Estonia. From clinical point of view, our aim was to test if we could find more Lynch syndrome patients with our approach, which would result in better management and surveillance of these individuals.

3. AIMS OF THE PRESENT STUDY

The aims of the present study are:

1. To analyse the estimated prevalence of pathogenic/likely pathogenic germline variants in Estonian colorectal cancer patients using NGS in a routine clinical setting (Paper I).
2. To estimate the prevalence of Lynch syndrome in the general adult population and to specify the genotypes/phenotypes of Lynch syndrome (Paper II).
3. To analyse the effectiveness of immunohistochemistry tests of MMR genes amongst colorectal cancer patients over 50 years of age (Paper II).
4. To specify the clinical phenotype and associated cancer risk of *AXIN2* carriers (Paper III).

4. MATERIAL AND METHODS

4.1 Study subjects

4.1.1 Study subjects of Paper I

We retrospectively reviewed the clinical and laboratory data of all CRC and polyposis cases sent for genetic testing between July 2016 and July 2021 at the Genetics and Personalized Medicine Clinic (GPM Clinic) of Tartu University Hospital (TUH). This is the only genetic service centre in Estonia and almost all molecular diagnostic tests in the country are performed there. CRC was determined from C18-C21 codes from the International Classification of Diseases 11th Revision (ICD-11). For accuracy, it has to be mentioned that anal cancer is part of these ICD-11 codes, but it is different etiologically and from treatment perspective from CRC. In Estonia we do not have separate statistics for anal cancer, so it is included in this statistics.

We defined polyposis as the development of numerous polyps (growths that protrude from a mucous membrane), and excluded cases of solitary polyps if genetic findings did not identify a known polyposis syndrome and/or if the family history was negative for CRC or polyposis. During the five-year study period, 5705 Illumina TruSight One (TSO) and Illumina TruSight One Expanded (TSOE) and 3704 Cancer panel analyses were done. Due to CRC or polyposis in the anamnesis and/or family anamnesis 314 NGS analyses were carried out: of these, 126 individuals had CRC and 44 had colorectal polyposis. The remaining 144 were either healthy family members (113 individuals) or had other types of cancer or solitary polyps with a family history (31 individuals).

4.1.2 Study subjects of Paper II

We collected data on LS patients and pre-symptomatic carriers for ten years, from 2012–2022, both retro- and prospectively, using databases of different diagnostic laboratories and referral visits to the TUH GPM Clinic in Estonia. We first reviewed the molecular database of GPM Clinic (more than 5,000 samples analysed for germline hereditary cancer risk) and our collaborators' databases (Asper Biogene, EstBB), then collected clinical data and formed two cohorts: the diagnostic cohort and the EstBB cohort representing the Estonian general population.

Diagnostic cohort. The diagnostic cohort consisted of 119 individuals. Index cases (n=71) were individuals with either cancer in their case history or healthy carriers consulted by a clinical geneticist as index patients in the Lynch family, diagnosed in the routine clinical setting in GPM Clinic. The remainder (n=48) were family members. Details are provided in Table 5.

Table 5. Details of the diagnostic cohort (index cases and family members).

	Total	Not enough detailed information	Healthy or benign changes	One cancer in health history	Two cancers at different time or site in health history	Three or more cancers in health history	Most frequent cancer site	Mean age of first cancer	Mean age of LS diagnosis	MMR IHC done
Diagnostic cohort; index cases	71	3	18	29	15	6	CRC	44.8 years	45.9 years	26/50 (52%) for cancer cases; 10/26 (38.5%) MLH1 and PMS2 negative expression
Diagnostic cohort; family members	48	None	40	7	1	None	CRC	43.5 years	36.8 years	2/8 (25%) for cancer cases

EstBB cohort. The EstBB is a population-based biobank with genetic and health data from over 200,000 participants. The EstBB represents more than 20% of Estonia's adult population. All participants in the EstBB have been genotyped using the Illumina Infinum Global Screening Array (GSA, Illumina, Inc.). A subset of participants has high-coverage sequencing data (genome or exome sequencing). All EstBB participants have provided broad written consent, allowing EstBB to continuously update their health data through periodical linking to national electronic databases and re-contact participants as appropriate. In the EstBB cohort, LS-associated LP and P variants were identified from high-coverage genome sequencing (n=2,420) and exome sequencing (n=2,356) data. High-coverage sequencing data includes 4,776 individuals, of whom 47% were female and 53% male. In addition, P/LP variants of MMR genes were searched from 201,134 EstBB participants with imputed genotyping array data. Altogether 63% (n=131,769) of EstBB participants were female.

Initially, we detected LS-associated disease-causing gene variants in 69 individuals from the EstBB cohort, which was further divided into two groups. The first group consisted of 30 individuals, 17 of whom were included in our study, referred for consultation with a clinical geneticist and molecular findings validated in the GPM Clinic. During consultations, we detected that three of those 17 cases were family members. The remaining cases (13/30) were excluded from this study as a clinical geneticist had already consulted with them,

they were not reachable by mail or phone or refused to participate. The second group of 39 individuals from the EstBB cohort carrying LS-associated disease-causing gene variants were excluded from this study as they had not consulted with a clinical geneticist by the time of submitting the article, and thus molecular findings had not been validated in the GPM Clinic laboratory. Recalling of these individuals is planned in the near future. It has been shown in previous EstBB studies that approximately 60% of EstBB participants are interested in further investigations and will come to genetic counselling (Jurgens et al., 2022).

Finally, for reference and comparing clinical data, we used detailed information from 23 individuals (14 index cases and additional nine family members) from the EstBB cohort, who had all consulted with a clinical geneticist, similarly to individuals seen in the clinical setting in the GPM Clinic, and molecular findings were validated in our molecular diagnostic laboratory from new blood samples.

MMR IHC pilot study. We also conducted a pilot study on IHC testing of MMR genes in the Pathology Service of TUH, which started in January 2018 and lasted until December 2021 (included). It covered CRC patients in South Estonia aged up to 70 years. Altogether, 464 MMR IHC analyses (one MMR IHC test contained four stainings) were performed during four years (100–128 per year). As a result, 19 individuals were called back for genetic consultation due to changes in MMR IHC and tested for LS.

4.1.3 Study subjects of Paper III

Data were collected via a structured questionnaire, which was sent to all the clinicians known to be caring for *AXIN2* gene carriers in hospitals involved in the ERN GENTURIS network as well as from the Mayo Clinic and the Boston Children's Hospital. In our cohort of 13 carriers, we had eight males and five females from 4 to 95 years of age. All the patients were Caucasian origin, eleven of them from Europe and two from North America.

4.2 Methods

4.2.1 Methods of the study on germline pathogenic variants in Estonian colorectal cancer patients (Paper I)

Individuals in the cohort were sequenced using the TruSight Cancer panel (TSC, 94 genes, Illumina, Inc.) from 2015 until mid-2020, and with the TruSight Hereditary Cancer panel (TSHC, 113 genes, Illumina, Inc.) from mid-2020. For CRC relevant genes, it is notable that the TSHC panel includes the *POLE* and *POLD1* genes which are absent from the TSC panel. Detection of CNVs of *BRCA1, BRCA2, TP53, CHEK2* and *CDH1* genes was carried out using the DECoN program (Fowler et al., 2016). In a few cases, where a

hereditary cancer syndrome was suspected, but the gene was not covered on TruSight Cancer panels, or there was no finding, we also used TruSight One (TSO) and TruSight One Expanded (TSOE) panels (Illumina, Inc.). These platforms cover ~4800 and ~6700 genes respectively, which are associated with known genetic disorders or clinical phenotypes. CNV analysis performed with TSO and TSOE panels for genes *BRCA1*, *BRCA2, TP53, CHEK2,* and *CDH1,* was carried out using CoNIFER (Krumm, 2012). We also report incidental findings in our routine setting based on criteria published by the American College of Medical Genetics (ACMG), if the patient has given his/her consent (Miller, 2021).

All TruSight panels were sequenced on the Illumina MiniSeq or NextSeq platform in a clinical setting. Raw sequencing reads were aligned to the hg19 reference genome using BWA MEM alignment algorithm, and variant calling was performed with the Genome Analysis Toolkit (GATK) (McKenna et al., 2010). Variants were classified according to the criteria published by ACMG (Richards et al., 2015). The clinical relevance of all genetic variants was assessed using ClinVar (Landrum et al., 2018), InSight (*InSiGHT DNA Variant Database webpage*) and HGMD Pro databases, as well as the Genome Aggregation Database, gnomAD (Karczewski et al., 2020; Stenson et al., 2003). The pathogenicity of previously undescribed findings was evaluated using the Varsome Clinical platform and/or other *in silico* protein functionality prediction programs (Kopanos et al., 2019). CNVs were detected for NGS data using either CoNIFER (v0.2.2) or DECoN (v1.0.2) software, or using the MLPA method. The following genes were tested by MLPA: *MLH1, MSH2, EPCAM, MSH6, MUTYH, PMS2, APC*, using their respective MRC HOLLAND (The Netherlands) MLPA® kits: Probemix P003 MLH1/MSH2; Probemix P072 MSH6-MUTYH; Probemix P008 PMS2; and Probemix P043 APC.

Sanger sequencing was used to test family members if the disease-causing variant had already been detected.

4.2.2 Methods of the study on the prevalence and molecular landscape of Lynch syndrome in the affected and general population (Paper II)

Molecular methods. Until 2016, LS molecular testing in Estonia was performed in different laboratories using Sanger and/or NGS and MLPA diagnostics for MMR genes. From 2016 onward, most of the diagnostic cohort cases were investigated at the GPM Clinic by the NGS TSC panel (94 genes, Illumina, Inc.) and since July 2020 with TSHC panel (113 genes, Illumina, Inc.). Sanger sequencing was used to test family members if the disease-causing variant had already been detected in the family. Both TSC, as well as TSHC panels were prepared and sequenced on Illumina MiniSeq or NextSeq platform in a clinical setting according to the manufacturers' instructions. Raw sequencing reads were aligned to the hg19 reference genome using the BWA-MEM alignment algorithm, and variant calling was performed by GATK (McKenna et

al., 2010). Variants were classified according to the criteria published by ACMG (Richards et al., 2015). The clinical relevance of all genetic variants was assessed using ClinVar (Landrum et al., 2018), InSight (InSight database), VarSome Premium (Saphetor SA) and HGMD Pro databases (HGMD Professional database, Qiagen), as well as the gnomAD (Karczewski et al., 2020; Kopanos et al., 2019; Stenson et al., 2003). Until 2021, to detect CNVs in MMR genes, MLPA (Schouten et al., 2002) diagnostics for *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* genes (MRC-Holland) were routinely used if the NGS panel did not reveal any P/LP LS variants. From 2021 onwards the DECoN program was used to detect possible CNVs for MMR+*EPCAM* genes from NGS data (Fowler et al., 2016). Our previous work showed that around 400 MLPA analyses were done during the five-year study period, and no new disease-causing variants were found. All deletions were first discovered by the NGS method and specific DECoN program and then confirmed by MLPA analysis (Roht et al., 2022). Based on this knowledge, MLPA is only used to confirm pathogenic CNV findings in MMR genes in our current practice. Exceptionally due to the presence of pseudogene (*PMS2CL*), MLPA testing is still routinely used for detecting CNVs in the *PMS2* gene.

For the EstBB cohort, the library preparation methods, quality control and array genotyping have been described elsewhere (Leitsalu et al., 2021). Genotype calling for sequencing, array data, and quality control has also been described previously (Jurgens et al., 2022).

A custom, in-house pipeline was used to annotate variants in all LS-associated genes from sequencing, array genotyping and imputed data. All identified variants in LS-associated genes were cross-referenced with the ClinVar database (Landrum et al., 2018), and only variants annotated as pathogenic or likely pathogenic were retrieved. All selected candidate variants were confirmed by Sanger sequencing prior to participant re-contact using DNA samples stored in the EstBB. We identified 38 probands, who carry a total of five distinct heterozygous variants in LS-associated genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*).

Statistical methods. The prevalence of LS (for each year) was defined as the total number of patients with this syndrome diagnosed from 2012 to 2022, divided by the number of inhabitants living in Estonia within the same period. The birth prevalence of LS was calculated by dividing the total number of adult patients with this syndrome born during the period from 1930 to 2003 by the recorded number of live births (LB) for the same period. According to the database of Statistics Estonia (*Statistics Estonia webpage*), there were 1,433,916 LB from 1930 to 2003. The 95% confidence interval for birth prevalence was calculated based on the Poisson distribution (Begaud et al., 2005).

The prevalence and LB prevalence of LS were estimated using a general linear model (GLM) analysis using R version 4.2.0 (Team, 2020). A Poisson distribution was assumed for the prevalence cases, and the default logarithmic link function was used. The only variable in the prevalence model was the observation year, and in the LB prevalence model, it was the birth year. The mean (expected) prevalence or LB prevalence rate for a given year and the

corresponding 95% confidence limits were calculated using R. Differences were considered statistically significant if the p-value was less than 0.05. To estimate the differences in the mean age of LS diagnosis and of first cancer between the diagnostic index cases and family member cases, the Wilcoxon rank-sum test was applied.

MMR IHC. Immunohistochemistry of MMR genes is a widely used method to identify MMR status, which can either be IHC positive (protein expression is normal and therefore nuclear staining in tumour cells is detected) or negative (loss of protein expression and no staining is detected in tumour cells) (McCarthy et al., 2019). The TUH Department of Pathology uses MMR IHC, and one test encompasses four stains (MutL Protein Homolog 1 (MLH1), Clone ES05, Agilent Technologies, Inc.; Ventana anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody, Roche; Ventana anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody, Roche; Ventana anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody, Roche). Our pilot study was based on TUH patients, so MMR IHC technology was used for LS screening.

4.2.3 Methods of the study of *AXIN2*-related oligodontia-colorectal cancer syndrome with cleft palate as a possible new feature (Paper III)

We gathered 13 *AXIN2* disease-causing variant carriers. Seven of the *AXIN2* carriers (Cases number 1, 3, 4, 6, 11, 12, 13) were identified by the NGS method. Sequencing was performed in these patients mostly for diagnostic purposes: hypo- or oligodontia, polyps, or cancer, and due to cleft palate seen in Family 2. The remaining patients were family members of patients with a known *AXIN2* variant in whom the *AXIN2* variant was detected by Sanger sequencing. For NGS library preparation different capture-based or amplicon-based protocols, for example NexteraFlex for Enrichment (Illumina, Inc.) or Haloplex Target Enrichment System (Agilent Technologies, Inc.), were used. In Case 1, the *AXIN2* variant was detected through Whole Genome Sequencing (WGS) in a research project. In Cases 3 and 4, the TSOE panel (Illumina, Inc.) with 6700 genes was used, and in Case 6 the ectodermal dysplasia and hypodontia panel of 34 genes was used. In Cases 11, 12 the diagnosis was made using commercially available NGS cancer panels (70 and 84 genes respectively), and in Case 13 the *AXIN2* variant was found with a WES-based colorectal polyposis panel, which included 13 known colorectal polyposis syndromes.

Sequencing was performed on NextSeq500, NovaSeq6000 or HiSeq X Ten (Illumina, Inc.). All targeted regions were sequenced with ≥50x depth. WGS was sequenced with a mean coverage of ≥30x depth.

Most of the patients were detected in a routine clinical setting. Cases 3 and 4 were found in the cohort of 4,704 Estonian patients investigated by Illumina's TSOE panel for different clinical reasons, revealing a frameshift variant; no other disease-causing indels, duplications, deletions, stop or missense variants were found in *AXIN2* gene in this cohort. Case 12 was found on screening 3,000

unselected cancer patients. In other cases, we do not have additional information about cohort sizes. Sanger validation was done in Cases 1, 3 and 13.

Paper IV was more of a practical introduction into genetic counselling of cancer patients and their family members, due to which methodology is not discussed separately.

4.3 Ethics

The studies were approved by the Research Ethics Committee of the University of Tartu (274T-5, 14.11.2017 and 292/M-13, 15.04.2019), for Paper II also 372M-9, 4.01.2023. In addition, for Paper III, the study was approved by the Mayo Clinic's institutional review board. In Norway, patients signed the Oslo University Hospital's declaration of consent, which was approved by the data protection officer at Oslo University Hospital.

5. RESULTS

5.1 Study of Estonian colorectal cancer patients investigated in a routine clinical setting (Paper I)

Our retrospective study included 314 analyses carried out due to CRC or polyposis in the anamnesis and/or family anamnesis. We identified 55 LP/P variant positive individuals among those screened using any of the platforms described in the Methods section over the five-year study period (Table 6). One hundred and seventy CRC and polyposis cases were investigated during the five-year study period, and the remaining 144 cases were either healthy family members, patients with non-CRC cancers, or individuals with solitary polyps with a relevant family history. Pathogenic or likely pathogenic germline variants were found in 38 out of the 170 patients, which constitutes 22.3% of CRC and polyposis cases.

Table 6. Diagnostic yield of disease-causing variants in different clinical groups and healthy family members.

Group studied (number of individuals)	Number of findings	Diagnostic yield (%)
CRC cases (126)	27	21.4
Polyposis cases (44)	11	25.0
Healthy family members (113)	12	10.6
Other cases (other type of cancer or solitary polyps with family anamnesis) (31)	5	16.1

As shown in Figure 3, only 2.7–6.1% of CRC cases were genetically tested in Estonia during the years 2016 to 2019. While this number is increasing, CRC cases are still genetically tested approximately ten times less often than, e.g., breast cancer patients and their family members in Estonia.

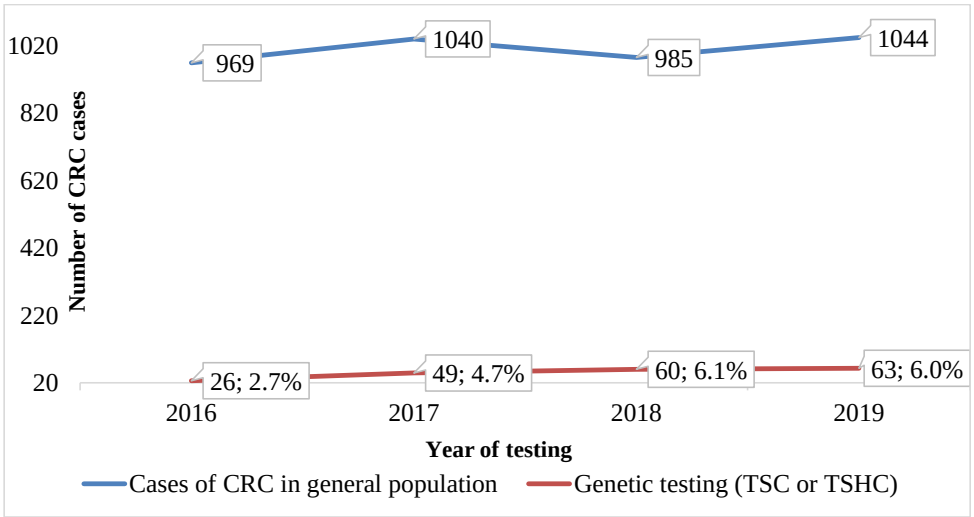


Figure 3. Proportion of individuals NGS tested for CRC (ICD-11 C18-C21) among total cases in the general population.

Of all cases tested in our cohort, there were 27 cases of CRC and 11 polyposis cases in which a LP/P germline variant was identified. Altogether, we detected 12 LP/P variants in healthy family members (Table 6). Among the known or classic CRC-related genes (*APC, AXIN2, BMPR1A, CHEK2, EPCAM, GREM1, MLH1, MSH2, MSH3, MSH6, MUTYH, NTHL1, PMS2, POLD1, POLE, PTEN, RPS20, SMAD4, STK11* and *TP53* gene), MMR genes disease-causing variants were the most prevalent (16/20) in our cohort, accounting for 80.0% of all cases. A LP/P variant in the *MLH1* gene was identified in one third of the cases (Table 7).

We also identified several cases of CRC or polyposis in which non-classical CRC genes were affected. These included two cases of *BRCA1* disease-causing variants, and additional variants in the genes *SPINK1, VHL, FANCM*, *BLM*, *APC*, *MUTYH, STK11, BMPR1A,* and *DSPP*. Further details about these cases are provided in Table 8.

Table 7. Likely pathogenic/pathogenic germline variants in typical and/or potential CRC- associated genes.

Gene	Variant	Clinical significance	Previously described	No of patients
MLH1	NM_000249.3(MLH1):c.1976G>C, p.(Arg659Pro)	Pathogenic	Yes	3
MLH1	NM_000249.3(MLH1):c.1918C>T, p.(Pro640Ser)	Likely pathogenic	Yes	1
MLH1	NM_000249.3(MLH1):c.1668–1G>T, p.?	Likely pathogenic	Yes	1
MLH1	NM_000249.3(MLH1):c.146T>A, p.(Val49Glu)	Pathogenic	Yes	1
MLH1	NM_000249.3(MLH1):c.92C>A, (p.Ala31Asp)	Likely pathogenic	No (this study)	1
MLH1	NM_000249.3(MLH1):c.55A>T, p.(Ile19Phe)	Pathogenic	Yes	1
MSH2	NM_000251.2(MSH2):c.1164_1165delinsGT, p.(Asn388_Arg389delinsLys*)	Pathogenic	No (this study)	1
MSH2	NM_000251.2(MSH2):c.1283_1284del, p.(His428Profs*14)	Pathogenic	No (this study)	1
MSH2	NM_000251.2(MSH2):c.1661+5G>A, p.?	Likely pathogenic	No (this study)	1
MSH2	NM_000251.2(MSH2):c.793-1G>A, p.?	Pathogenic	Yes	1
MSH2#	MSH2 exon 1–3 deletion and EPCAM exon 9 deletion	Pathogenic	Yes	1
MSH2#	MSH2 exon 1–6 and EPCAM exon 8-9 deletion	Pathogenic	Yes	1
CHEK2	NM_007194.4(CHEK2):c.319+2T>A, p.?	Likely pathogenic	Yes	2
CHEK2	NM_007194.4(CHEK2):c.1189del, p.(Val397Phefs*17) mosaic (12%)	Likely pathogenic	No (this study)	1
CHEK2	NM_007194.4(CHEK2):c.908+1540_1095+330del, p.(Met304Leufs*16)	Pathogenic	Yes	1
MSH6	NM_000179.2(MSH6):c.3725G>A, p.(Arg1242His)	Pathogenic	Yes	1
MSH6	NM_000179.2(MSH6):c.3514dup, p.(Arg1172Lysfs*5)	Pathogenic	Yes	1

legacy name of the deletion involving two genes (*MSH2* and *EPCAM*)

Table 8. Genetic findings in patients with likely pathogenic or pathogenic variants in genes other than known or classic CRC-related genes.

Gene	Variant	Clinical significance	Previously described	No of patients
PPM1D	NM_003620.3(PPM1D):c.1602del, p.(Phe534Leufs*5)	Pathogenic	Yes	1
BRCA1	NM_007294.3(BRCA1):c.5266dup, p.(Gln1756Profs*74)	Pathogenic	Yes	1
BRCA1	NM_007294.3(BRCA1):c.181T>G, p.(Cys61Gly)	Pathogenic	Yes	1
SPINK1	NM_001354966.1(SPINK1):c.198A>C, p.(Lys66Asn)	Pathogenic	Yes	1
VHL	NM_000551.3(VHL):c.598C>T, p.(Arg200Trp)	Pathogenic	Yes	1
FANCM	NM_020937.2(FANCM):c.5791C>T p.Arg1931*	Likely pathogenic	Yes	1
BLM	NM_000057.4(BLM):c.1083_1084del p.(Cys361*)	Pathogenic	Yes	1

In our CRC patients, MMR-associated LP/P variants were the most common, making up 37.2% (16/43) of all the findings. Among MMR genes, *MLH1* was the gene most frequently affected, with variants identified in around 50% of MMR cases and 8/43 (18.6%) of all the cases with any genetic finding. The next most common gene with CRC-related disease causing variants was *CHEK2*, which was detected in five cases (11.6%). We identified three novel disease-causing gene variants in *MSH2* and one in both *MLH1* and *CHEK2* genes (Table 7). In other traditional or non-traditional CRC genes, including *APC, BLM, BMPR1A, BRCA1, FANCM, MSH6, MUTYH, PMS2, SMAD4, SPINK1* and *VHL,* novel variants were not found (Table 8).

As described in the methods section, several different techniques were employed during the five-year study period. In our cohort, the diagnostic yield for TSC was 33/238 (13.9%) and for TSHC 8/66 (12.1%). There was no statistical significance between the diagnostic yield of different cancer panels applied in clinical workflow. We did not find any disease-causing alterations in genes included exclusively in the TSHC panel, for example *POLE* and *POLD1* genes. The combined diagnostic yield for TSC and TSHC panels was 13.4% (Table 9). On rare occasions, a larger panel (such as TSO or TSOE) than TruSight Cancer panels was used in a clinical setting for cancer diagnostics; the TSO diagnostic yield was 2/10 (20.0%), and we did not include incidental findings in this study. All patients referred to a TSO or TSOE panel had leading medical problems other than cancer in their anamnesis or family anamnesis. In a few cases, data reanalysis was necessary with the bigger cancer panel (TSHC, 113 genes).

Table 9. Diagnostic yield of different methods used in hereditary cancer diagnostics.

Method	Total no. of analyses in five years	No. of analyses for CRC or polyposis in anamnesis	No. of analyses with a genetic finding	Diagnostic yield of the method (%)
TruSight Cancer panel (94 genes)	2805	238	33	13.9%
TruSight Hereditary Cancer panel (113 genes)	899	66	8	12.1%
TruSight One panel	5075	10	2	20%

From the clinical point of view, we report several remarkable findings rarely associated with CRC. We detected a *FANCM* disease-causing stop-gain variant NM_020937.4 (FANCM):c.5791C>T in a CRC case. Until recently, *FANCM* variants had only been reported in association with BC. We also report one

mono-allelic *MUTYH* variant in a case in which a woman had more than ten adenomatous polyps (found at the age of 64) in her colon and had had EC. The patient's mother had had rectal cancer at the age of 44. *MUTYH* heterozygous variants are associated with a ~2.5-fold-increased risk of CRC compared to the general population (Win et al., 2014). In two cases, a genetic cause of disease was found using either TSO or TSOE. First, a 64-year-old man with colorectal polyposis and *dentinogenesis imperfecta* with a finding of NM_014208.3(DSPP): c.52G>T p.(Val18Phe). The *DSPP* gene has previously been linked to oral cancers (Joshi et al., 2010), but MMP20-DSPP co-localization and interaction has been observed in breast, colorectal and other cancers as well (Aseervatham et al., 2019). TSO/TSOE was also used to diagnose a 72-year-old woman who had had ascending colon adenocarcinoma at the age of 64 and had a family history of muscle disease; a known familial *CHEK2* variant NM_007194.3(CHEK2): c.319+2T>A p.? was confirmed in the patient as well. The TSO panel was used to identify a possible cause of muscle disease.

Altogether, around 400 MLPA analyses for different genes (*MLH1, MSH2, MSH6, PMS2, EPCAM, APC, MUTYH*) were done during the five-year study period. New LP/P variants were not discovered by MLPA analysis. Therefore, after 2019 we only use MLPA testing in Lynch syndrome diagnostics for *PMS2* gene CNV detection.

5.2 Lynch syndrome's prevalence and molecular genetics in the affected and general population (Paper II)

We collected data from 181 LS carriers in Estonia during ten years, of whom 105 were females and 76 males, aged 2–92, born between 1930 and 2020. We have detailed clinical information on 139 individuals. Of these 139 individuals, 116 were part of the diagnostic cohort, and the EstBB cohort consisted of 23 individuals. An additional 39 individuals from the EstBB cohort were put on hold primarily because they had not consulted a clinical geneticist at the time of writing this thesis; recalling and counselling of these individuals is planned in the near future.

Results of the detailed clinical information group. From the group with detailed information (139 individuals), there were 82 index cases (studied first in the family but not all had cancer) and 57 family members. This cohort included 79 females and 60 males, aged 2–92, born between 1930 and 2020. In the diagnostic cohort, the mean age of LS diagnosis was 41.5 years, and the mean age of first cancer diagnosis was 44.4 years. Most individuals [20.9% (29/139)] with a cancer history had had at least one malignant tumour, further 10.8% (15/139) had had two and 4.3% (6/139) had suffered from three or more in their health history. For three individuals, we lacked data and, therefore, they were excluded from the cohort with detailed clinical data. As predicted, the most frequent tumour was CRC. From an oncological point of view, 64.0% (89/139) were healthy or had benign changes (polyps in the colon or, in one

case, mandibular osteomas). For the EstBB cohort, the mean age of LS diagnosis was 46.3 years, and all of them are healthy from an oncological perspective.

LS prevalence in Estonia. In our calculations, we counted only adult individuals, as LS is not clinically significant in childhood. Therefore, LS birth prevalence between 1930 and 2003 in Estonia was estimated at 1:8638 (95% CI: 1:9859–7588) or 11.58 (95% CI: 10.14–13.18) for 100,000 LBs. Figure 4 illustrates the prevalence of 100,000 LBs for both cohorts. The maximum of 14.35 cases falls to the year 1972, which was expected as LS usually manifests in middle age. The CI widened from 2015 onward, probably because of a few children who were diagnosed due to reporting incidental findings. Otherwise, they would have been diagnosed later in life.

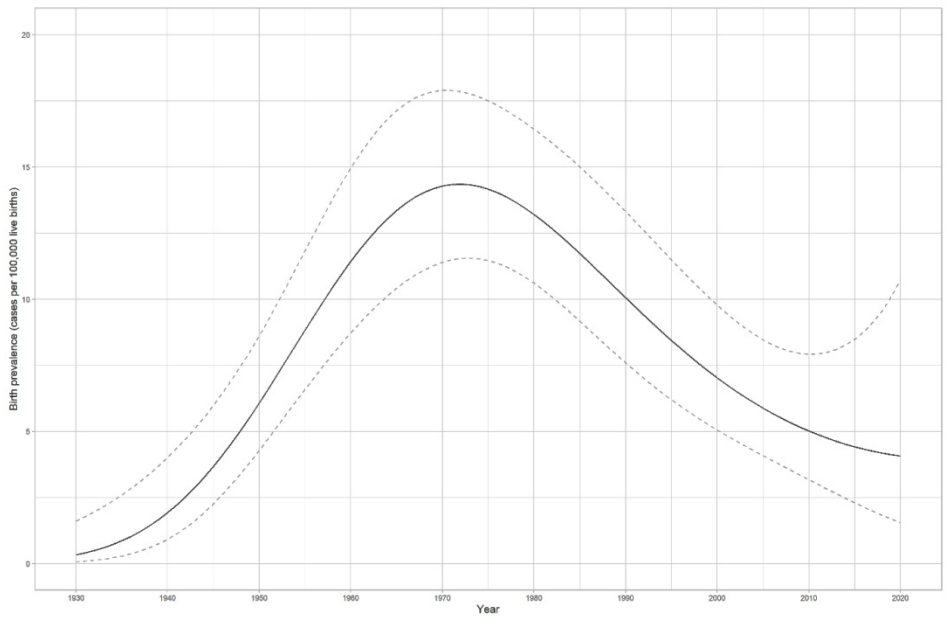


Figure 4. Lynch syndrome's birth prevalence (cases per 100,000 live births) in the diagnostic and Estonian Biobank cohorts during the years 1930–2020 in Estonia. Confidence interval (95% CI) is shown in dotted lines.

The prevalence of LS (cases per 100,000 persons) has risen by almost six times in ten years (2012–2022), from 0.34 (95% CI 0.22–0.52) to 1.99 (95% CI 1.54–2.57) ($p < 0.0001$) (Figure 5).

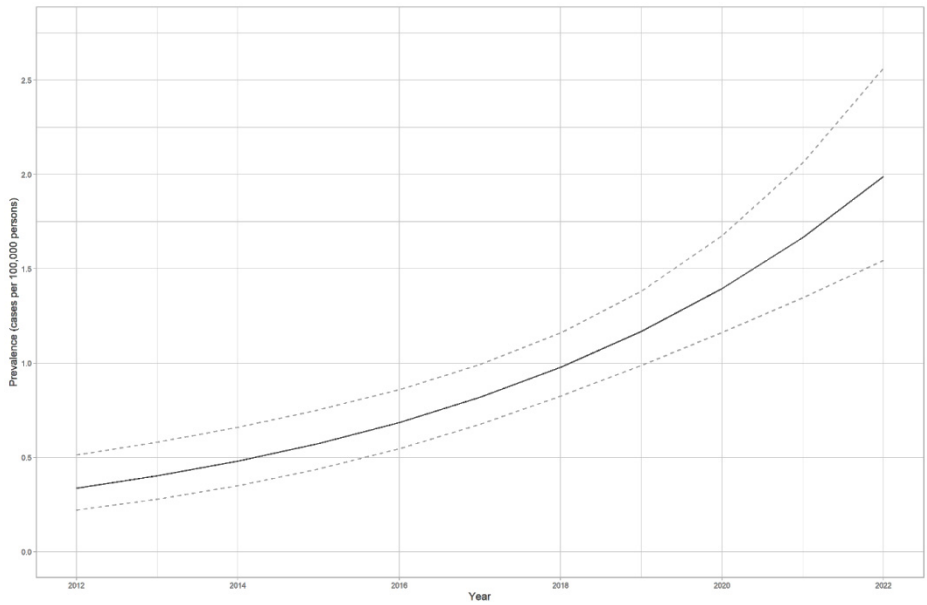
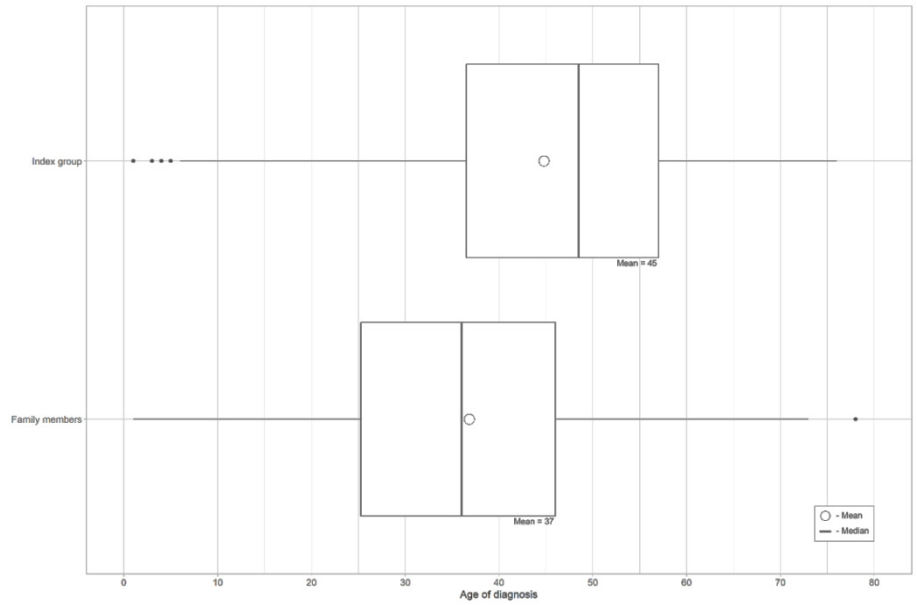


Figure 5. Lynch syndrome's prevalence (cases per 100,000 persons) in Estonia from 2012 to 2022. Confidence interval (95% CI) is shown in dotted lines.

Clinical aspects. The diagnostic cohort consisted of 71 index cases and 48 family members. We had 35 male and 36 female index cases born between 1943 and 2020. Altogether, 50 out of 71 index cases had had at least one cancer in their health history, 18 were healthy carriers and seven of them were diagnosed because of reporting incidental findings (children and young people investigated due to other health problems). In all families with incidental findings, informed consent was signed, and pre-test counselling about incidental findings and their consequences was carried out. The mean age of the first cancer was 44.8 years, and the most frequent cancer types were colorectal, prostate, breast, endometrial and bladder cancer. In our diagnostic cohort six individuals had had three or more cancers. Five out of six patients with three or more cancers had either *MLH1* or *MSH2* and/or *EPCAM* disease-causing variants. The sixth case, the only known constitutional mismatch repair deficiency (CMMRD) patient in Estonia, had compound-heterozygous variants in *MSH6* gene. She is currently 25 years old. At the age of 10, she had acute T-cell lymphoblastic leukaemia. At the age of 17 years, she was diagnosed with severe colorectal polyposis, and laparoscopic total proctocolectomy with end ileostomy was performed. Microscopically, the polyps were mostly tubular adenomas exhibiting high-grade dysplasia. In the biggest adenoma (50 mm) from the sigmoid colon, intraepithelial adenocarcinoma was found (Soplepmann & Laidre, 2016). At the age of 21 years she was diagnosed with diffuse astrocytoma of the frontal lobe and at the age of 24 years with acute myeloid leukaemia (personal communication with Dr P. Laidre and Dr J. Soplepmann).

The mean age of LS diagnosis was 44.6 years. Almost half (52%) of the index cases, who had suffered from cancer, had been screened for LS by MMR IHC. Altogether, in 38.5% of these cases, MLH1 and PMS2 expression were negative, which can either refer to sporadic cancer of different causes or disease-causing germline variants in the *MLH1* or *PMS2* gene. Of the 48 family members, there were 27 females and 21 males. Forty (83.3%) were healthy from an oncological point of view, and eight (16.7%) had cancer in their case history: in seven cases only one cancer, and in one case two cancers. Six of those eight individuals had had colorectal cancer at some point in their life. The mean age at the first cancer diagnosis was 43.5 years, which is quite similar to that of the index cases, and it was also statistically insignificant ($p = 0.5422$). All individuals in the family member group had a family history of cancer. The mean age at LS diagnosis was 36.8 years, which is 8 years earlier than for the index cases group; this difference was statistically significant ($p = 0.0035$). This is probably the result of communication and awareness of potential cancer risks in the family, which brings them to the medical system. We did not find a statistically significant difference in the mean age of the first cancer diagnosis between the diagnostic cohort's index cases and family members, which was an expected outcome. In the diagnostic cohort, on average, 2.1 family members were tested per index case. Details of both index and family members' cancer cases are covered in Table 5, Figures 6 and 7.

A



B

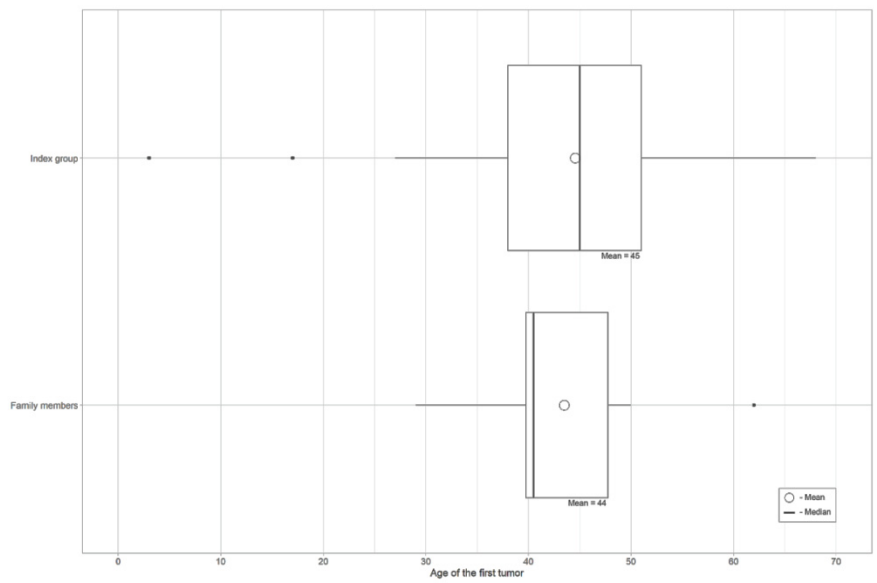


Figure 6. (**A**) Mean and median age of Lynch syndrome diagnosis in the diagnostic cohort. (**B**) Mean and median age of first tumour diagnosis in the diagnostic cohort.

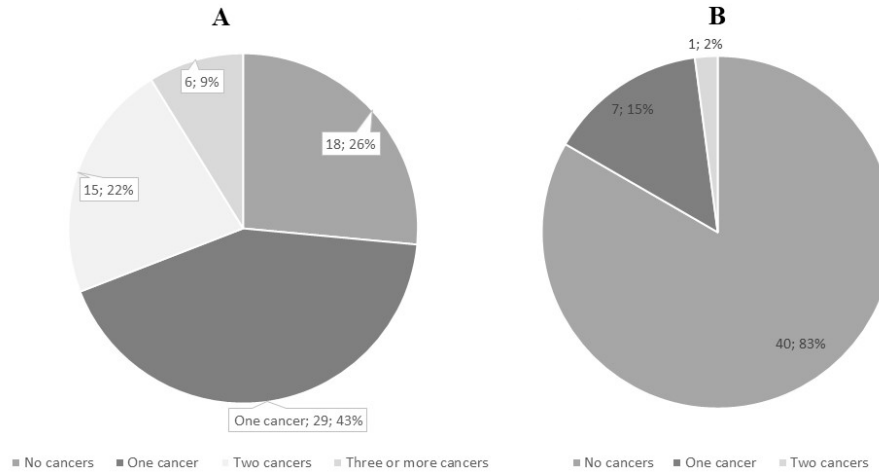


Figure 7. Cancer cases in health history in diagnostic cohorts' index cases (A) vs family members (B).

The EstBB cohort consisted of 14 index cases and nine family members. Of 14 index cases, 12 were females, and two were males. All 23 individuals were healthy from an oncological point of view. The mean age at LS diagnosis for the whole EstBB cohort was 46.45 years. The EstBB cohort had 1.07 family members for one index case. Details of the EstBB cohorts epidemiological and molecular data are covered in Table 10 and Table 11. Both tables also consist data on EstBB cohort being recalled at the moment, and nine *MLH1* carriers, most of whom had already been added to the diagnostic cohort.

MMR genes pathogenic variants in the Estonian population. In the diagnostic cohort's index cases, 28.2% (20/71) carried an *MLH1* disease-causing variant; 28.2% (20/71) had *MSH2* disease-causing variants either alone, or together with *EPCAM* deletion in five cases; *MSH6* variants made up 19.7% (14/71) and *PMS2* pathogenic variants 23.9% (17/71) of all the cases. *MLH1* gene variant NM_000249.4: c.1976G>C p.(Arg659Pro) was the most frequent in the diagnostic cohort. Most of the variants identified frequently in the diagnostic cohort were also the most common in EstBB cohorts (Table 11). All of our findings in the diagnostic cohort were first discovered on the NGS panel. No exon deletions nor duplications were found with MLPA analysis, so we no longer use MLPA diagnostics routinely for LS diagnostics (except for the *PMS2* gene because of its pseudogene). In the EstBB cohort, the initial recalled cohort consisted of 32 individuals (two of them deceased) (Table 10). Twenty-three of them were counselled by a clinical geneticist, 14 of whom were probands, and nine were family members. In this cohort of 14 EstBB probands, *PMS2* disease-causing variants were the most common, making up more than half (57.2%; 8/14), *MSH6* variants constituted 35.7% (5/14), and there was only one case of an *MSH2* disease-causing variant (7.1%). *PMS2* variant NM_000535.7: c.861_864del, p.(Arg287Serfs*19) was the most common variant found.

Table 10. Details of EstBB cohorts.

	Total	Not enough detailed data	Healthy or benign changes	One cancer in health history	Two cancers in health history	Three or more cancers in health history	Most frequent cancer site	Mean age of first cancer	Mean age of LS diagnosis	MMR IHC done
EstBB cohort, not yet recalled (n=37), deceased (n=2)	39	0	32	2	1	4	Skin	52.1 years	NA	NA
EstBB cohort, recalled (n=30), deceased (n=2)	32	0	24	3	2	3	Skin	52.1 years	NA	NA
EstBB cohort *MLH1* carriers, recalled (n=8), deceased (n=1)	9	0	4	1	1	3	Colon	43.0 years	NA	NA

Table 11. MMR genes variants represented in EstBB cohorts.

Gene	Variant	No. of individuals carrying this variant	Exon/Intron position	Class of variant based on ACMG Criteria
		NM_000249.4(MLH1)		
MLH1	c.1976G>C, p.(Arg659Pro)	9	17	Pathogenic
MLH1	c.1668-1G>T, p.?	2	intron 14	Likely pathogenic
		NM_000251.3(MSH2)		
MSH2	c.793-1G>A, p.?	8	intron 4	Pathogenic/likely pathogenic
MSH2	c.181C>T, p.(Gln61*)	1	1	Pathogenic
MSH2	c.2131C>T, p.(Arg711*)	2	13	Pathogenic
		NM_000535.7(PMS2)		
PMS2	c.943C>T, p.(Arg315*)	16	9	Pathogenic
PMS2	c.825A>G, p.(Gln275=)	13	8	Pathogenic/likely pathogenic
PMS2	c.861_864del, p.(Arg287fs)	14	8	Pathogenic
		NM_000179.3(MSH6)		
MSH6	c.1095G>A, p.(Trp365*)	1	4	Pathogenic
MSH6	c.3226C>T, p.(Arg1076Cys)	14	5	Likely pathogenic

In this study, we identified ten new variants in MMR genes: four in *MSH2*, two of which were together with *EPCAM* deletion, three in *MSH6,* two in *MLH1*, and one in the *PMS2* gene (Table 12). Reporting new variants in genetic databases is important for spreading knowledge and from practical point of view in decision-making when reporting these back to clinicians.

Table 12. Pathogenic and likely pathogenic variants in MMR genes detected in our Lynch syndrome cohort.

Gene	Variant	No. of Individuals (%)	Exon/Intron Position	Class of Variant Based on ACMG Criteria
		NM_000249.4(MLH1)		
MLH1	c.1976G>C, p.(Arg659Pro) ‡	13 (31.7%)	17	Pathogenic
MLH1	c.1668-1G>T, p.? ‡	4 (9.6%)	Intron 14	Likely pathogenic
MLH1	c.55A>T, p.(I19F)	4 (9.6%)	1	Pathogenic
MLH1	c.92C>T, p.(Ala31Asp)	3 (7.32%)	1	Likely Pathogenic
MLH1	c.751del, p.(Tyr251Thrfs*3)	3 (7.32%)	9	Pathogenic
MLH1	c.1168delG, p.(Glu390Asnfs*11) †	2 (4.8%)	12	New in this study Likely pathogenic
MLH1	c.1918C>T, p.(Pro640Ser)	2 (4.8%)	17	Likely pathogenic
MLH1	c.2128_2131dupAACT, p.(Ser711*) †	1 (2.4%)	19	New in this study Likely pathogenic
MLH1	c.146T>A, p.(Val49Glu)	1 (2.4%)	2	Pathogenic
MLH1	c.840T>G, p.(Tyr280*)	1 (2.4%)	10	Pathogenic
MLH1	**c.1685A>C,** p.(Gln562Pro)	1 (2.4%)	15	Likely pathogenic
		NM_000251.3(MSH2)		
MSH2	c.1283_1284delAC, p.(His428Profs*14)	4 (14.8%)	8	Likely pathogenic
MSH2	*MSH2* exon 9–15 deletion NC_000002.11:g.(?_47690170)_(47708011_?)del †	4 (14.8%)	Deletion ex 9–15	New in this study Likely pathogenic
MSH2	c.793-1G>A, p.? ‡	3 (11.1%)	Intron 4	Likely pathogenic
MSH2	*MSH2* exon 8 deletion NC_000002.11:g.(?_47669476)_(47710098_?)del	2 (7.4%)	Deletion ex 8	Pathogenic
MSH2	c.1164_1165delinsGT, p.(Asn388_Arg389delinsLys*)	2 (7.4%)	7	Pathogenic
MSH2	c.289C>T, p.(Gln97*)	2 (7.4%)	2	Pathogenic
MSH2	c.1661+5G>A, p.?	1 (3.7%)	10	Likely pathogenic
MSH2	*MSH2* exon 11–14 deletion NC_000002.12:g.(?_47698104)_(47705658_?)del	1 (3.7%)	Deletion ex 11–14	Pathogenic
MSH2	c.942+3A>T, p.?	1 (3.7%)	Intron 5	Pathogenic
MSH2	c.942+1G>T, p.?	1 (3.7%)	Intron 5	Likely pathogenic
MSH2	c.181C>T, p.(Gln61*)	1 (3.7%)	1	Pathogenic
MSH2	c.2131C>T, p.(Arg711*) ‡	1 (3.7%)	13	Pathogenic
MSH2	c.1942dupA, p.(Ile648Asn*fs6) †	1 (3.7%)	12	New in this study Likely pathogenic

Gene	Variant	No. of Individuals (%)	Exon/Intron Position	Class of Variant Based on ACMG Criteria
MSH2+EPCAM	*MSH2* exon 1–7 and EPCAM exon 9 deletion NC_000002.12:g.(?_47614711)_(47657080_?)del †	2 (7.4%)	*MSH2* ex. 1–7 *EPCAM* ex. 9	New in this study Likely pathogenic
MSH2+EPCAM	MSH2 exon 1–6 and EPCAM exon 8–9 deletion NC_000002.12:g.(?_47612305)_(47643568_?)del †	(3.7%)	*MSH2* ex. 1–6 *EPCAM* ex. 8–9	New in this study Likely pathogenic
		NM_000179.3(MSH6)		
MSH6	c.3226C>T, p.(Arg1076Cys)	14 (43.75%)	5	Likely pathogenic
MSH6	c.3514dupA, p.(Arg1172Lysfs*5)	4 (12.5%)	6	Pathogenic
MSH6	c.2419G>T, p.(Glu807*)	3 (9.4%)	4	Pathogenic
MSH6	c.1998dupT, p.(Asp667*) †	3 (9.4%)	4	New in this study Likely pathogenic
MSH6	c.3725G>A, p.(Arg1242His)	1 (3.1%)	8	Pathogenic/Likely pathogenic
MSH6	c.3522dup, p.(Thr1175Tyrfs*2) †	1 (3.1%)	6	New in this study Likely pathogenic
MSH6	c.2308G>T, p.(Gly770Cys) †	1 (3.1%)	4	New in this study Likely pathogenic
MSH6	c.3261del, p.(Phe1088fs)	1 (3.1%)	5	Pathogenic
MSH6	c.3195_3199del, p.(Asn1065Lysfs*5)	1 (3.1%)	5	Pathogenic
MSH6	c.2569_2572del, p.(Asp857Phefs*10)	1 (3.1%)	4	Pathogenic
		NM_000535.7(PMS2)		
PMS2	c.861_864del, p.(Arg287Serfs*19)	11 (26.2%)	8	Pathogenic
PMS2	c.1666del, p.(Glu556Lysfs*39) †	8 (19%)	11	New in this study Likely pathogenic
PMS2	c.703C>T, p.(Gln235*)	4 (9.5%)	6	Pathogenic
PMS2	c.2413C>T, p.(Q805*)	4 (9.5%)	14	Pathogenic
PMS2	c.1939A>T, p.(Lys647*)	3 (7.14%)	11	Pathogenic
PMS2	c.2506del, p.(Glu836Argfs*15)	2 (4.76%)	15	Pathogenic
PMS2	c.2445+1G>T,	2 (4.76%)	14	Pathogenic/Likely pathogenic
PMS2	c.2192_2196del, p.(Leu731Cysfs*3) in mosaic level 10%	2 (4.76%)	13	Pathogenic
PMS2	c.2T>A, (p.Met1?)	1 (2.4%)	1	Pathogenic
PMS2	c.634C>T, p.(Gln212*)	1 (2.4%)	6	Pathogenic
PMS2	c.137G>T, p.(Ser46Ile)	1 (2.4%)	2	Likely pathogenic
PMS2	c.319C>T, p.(Arg107Trp)	1 (2.4%)	4	Likely pathogenic
PMS2	c.1588C>T, p.(Gln530*)	1 (2.4%)	11	Pathogenic

† new, detected in this study. ‡ found in both diagnostic and Estonian Biobank cohorts.

To compare, we found novel MMR pathogenic gene variants also in the routine clinical setting NGS study published in 2022 (Paper I). We detected four new unpublished variants: two in *MLH1* and two in the *MSH2* gene. These have been entered into ClinVar (Landrum et al., 2018), and details are covered in Paper I (Roht et al., 2022).

MSH2 gene carrier case report: Highest number of cancers in health history

The highest number of cancers per person in the diagnostic cohort was more than ten. This was a female proband from southern Estonia. She was 77 years at the time of LS diagnosis. She had had 14 cancers altogether in four organ systems: three CRCs, one small intestine cancer, three urothelial system cancers, three basal cell carcinomas (basaliomas) and four of two other types of skin cancer. The first cancer was CRC at the age of 44 years. From the family history, it is known that her mother had died of EC at the age of 41 years, two of her mother's sisters had gastric cancers in their 60s, and the mother's third sister had CRC at the age of 72 years. Her son, who was a carrier, had a malignant gastrointestinal stromal tumour (GIST) in the small intestine at the age of 40 years. Her two daughters were healthy non-carriers. The pedigree is depicted in Figure 8. MMR IHC from the colorectal adenocarcinoma showed a loss of MSH2 and MSH6 expression, and the NGS TSC panel discovered a disease-causing (Class 5) variant in *MSH2* NM_000251.2:c.1283_1284del p.(His428 Profs*14), which had not been described in the databases at the time the analysis was performed (in 2018). As she also had different skin tumours (basaliomas, keratoacanthomas, etc.), the final diagnosis was Muir–Torre syndrome, a subtype of LS.

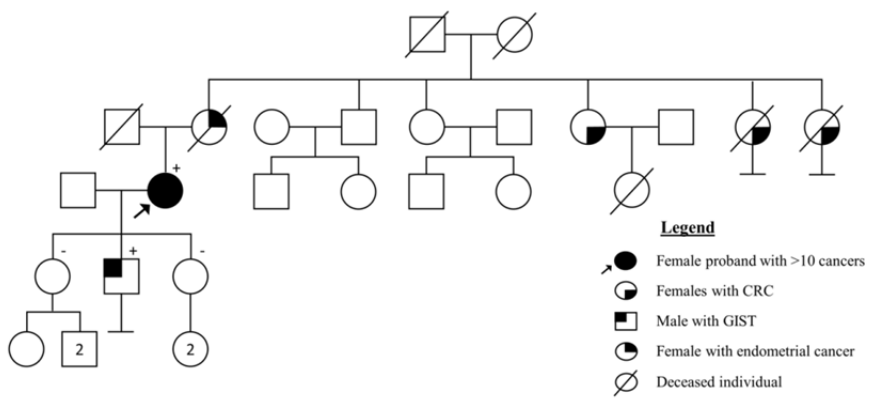


Figure 8. Pedigree of the case report.

MMR IHC pilot study. The MMR IHC pilot study resulted in 52 IHC-negative individuals (11.2%) being filtered out from 464 analyses. MLH1 and PMS2 negative expression was the most common finding (36.5%). Out of 52 indivi-

duals, 19 (36.5%) were called out for germline testing, 12 (23.1%) had previously consulted a clinical geneticist in routine clinical work, and 21 (40.4%) had not been consulted due to different reasons (not reached, did not want to attend, tested for somatic variants only etc.). Eleven individuals out of 31 had variants in MMR genes: in nine cases a P/LP variant, and in two cases, VUS. Six individuals out of 11 (54.5%) were in the group of CRC presenting from 50 to 70 years, and where raising the age limit of MMR IHC testing from 50 to 70 was expected to improve the diagnosis of LS. *MSH2* gene disease-causing variants alone or with *EPCAM* deletion were the most frequent, constituting two-thirds of the findings. Details are covered in Figure 9.

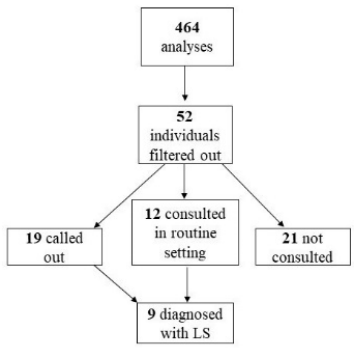


Figure 9. MMR genes immunohistochemistry pilot study groups.

5.3 *AXIN2*-related oligodontia-colorectal cancer syndrome study (Paper III)

Thirteen *AXIN2* P/LP variant carriers from six families were included in the study and their data are shown in Table 13 and Figure 10. Eight were male and five were female with ages ranging from 4–95 years. Among them four were children (≤ 18 years), who were tested due to clinical indications [failure to thrive, hypodontia and/or cleft palate (CP)]. Eleven cases were from Europe and two from North America. The North American patients were thought to be of European descent. Probably, in most cases *AXIN2* variants were inherited, although we have no clear evidence; we are not aware of any *de novo* cases.

Table 13A. Clinical and molecular data of *AXIN2* Cases 1–7.

Type of data	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Family number	Family 1	Family 1	Family 2	Family 2	Family 2	Family 3	Family 3
Demographics							
Sex	M	M	F	M	F	M	F
Current age (y)	10y	41y	6y	4y	26y	20y	50y
Ethnicity	Estonian	Estonian	Estonian	Estonian	Estonian	Norwegian	Norwegian
Testing indication	Research setting (SRS)	Segregation analysis	Diagnostic setting (Pierre-Robin sequence)	Diagnostic setting (cleft palate)	Segregation analysis	Diagnostic setting (oligodontia)	Segregation analysis
Clinical features							
Phenotype (dysmorphology)	N	N	Pierre-Robin sequence	Bilateral cleft palate	Bilateral cleft palate	N	N
Oligodontia	+	+	-	-	-	+	-
No. of missing teeth	8	14	2	-	1 impacted tooth	22	2
Eyes	N	N	N	N	N	N	N
Ectodermal dysplasia	-	-	-	-	-	-	-
Gastrointestinal polyps	n.d.	+	n.d.	n.d.	n.d.	+	+
Type and no of polyps	n.d.	1 tubular adenoma	n.d.	n.d.	n.d.	2 tubular adenomas, 1SSP, 1HP	Many tubular adenomas and HPs
Colorectal cancer	-	-	-	-	-	-	-
Other cancers	-	-	-	-	-	-	-
Other health problems	-	-	Epilepsy	-	-	-	-
Molecular genetic finding							
cDNA (NM_004655.4)	c.1882C>T	c.1882C>T	c.1214_1215dup	c.1214_1215dup	c.1214_1215dup	c.2023dupC	c.2023dupC
Protein change	p.(Arg628Trp)	p.(Arg628Trp)	p.(Gly406Argfs*53)	p.(Gly406Argfs*53)	p.(Gly406Argfs*53)	p.Arg675ProfsTer32	p.Arg675ProfsTer32
Detection method	WGS	Sanger	NGS	NGS	Sanger	NGS	Sanger

Type of data	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Type of mutation	Missense	Missense	Frameshift	Frameshift	Frameshift	Frameshift	Frameshift
Clinical significance	VUS	VUS	Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic
Other genetic findings	-	-	-	-	-	-	-
Follow-up and treatment							
Colonoscopy interval	From 18y	Once a year	From 18y	From 18y	Follow-up not started yet	Once a year	Once in two years
Other investigations	-	-	-	-	-	Gastroscopy at 25y	-
Specific treatment	-	-	-	-	-	-	-

Table 13B. Clinical and molecular data of *AXIN2* Cases 8–13 and summary of the most important publications.

Case 8	Case 9	Case 10	Case 11 [12]	Case 12 [11]	Case 13	Summary	Lammi et al., 2004	Beard et al., 2019
Family 3	Family 3	Family 3	Family 4	Family 5	Family 6			
							11 cases/17 individuals	4 cases/4 individuals
F	M	M	M	F	M			
22y	15y	95y	65y	51y	65y			
Norwegian	Norwegian	Norwegian	American	American	Dutch			
Segregation analysis	Segregation analysis	Segregation analysis	Diagnostic setting	Diagnostic setting (cancer)	Diagnostic setting			
N	N	N	N	N	N			
+	-	-	-	-	-	**4 out of 13 (30.8%)**	11 out of 11 (100%)	4 out of 4 (100%)
8	2	4	3#	4#	4		8 and more	n.d.
N	N	N	N	N	Sparse eyebrows		n.d.	n.d.
-	-	-	-	-	-		n.d.	n.d.
+	n.d.	+	+	+	+	**8 out of 13 (61.5%)**	6 out of 12 (50%)	4 out of 4 (100%)
1 tubular adenoma, 1SSP, 1HP	n.d.	Polyposis of the colon	57 polyps in colon	1 Gastric adenoma, 1 HP in colon	Polyps in the sigmoid colon and rectum		Different types, numbers and parts of intestine	Different types, numbers and parts of intestine
-	-	-	-	-	Adenocarcinoma coecum at 62y	**1 out of 13 (7.7%)**	1 case (54y) had adenocarcinoma of the hepatic flexure (5.9%)	1 case (43y) had transverse colon cancer (25%)
-	-	-	Skin cancer at the age of 60y, prostate adeno-carcinoma at 62y	Olfactory neuroblastoma at 49y	Abdominal superficial melanoma at 61y		n.d.	n.d.
IBS	-	-	-	-	Rheumatoid arthritis, glaucoma, hypertension, obstructive sleep apnea		n.d.	n.d.

Case 8	Case 9	Case 10	Case 11 [12]	Case 12 [11]	Case 13	Summary	Lammi et al., 2004	Beard et al., 2019
c.2023dupC p.Arg675ProfsTer32	c.2023dupC p.Arg675ProfsTer32	c.2023dupC p.Arg675ProfsTer32	c.1994dupG p.Asn666fs	c.1822del p.Leu608Phefs*81	c.1999dupA p.Ser667Lysfs*40		c.1966C>T p.Arg565*	c.1972delA p.Ser658Alafs*31
Sanger	Sanger	Sanger	NGS	NGS	NGS			
Frameshift	Frameshift	Frameshift	Frameshift	Frameshift	Frameshift		Stop-mutation	Frameshift
Likely pathogenic	Likely pathogenic	Likely pathogenic	Likely pathogenic/ pathogenic	VUS	Pathogenic		Pathogenic	Likely pathogenetic
-	-	-	-	*NF1* VUS	-			
Once in two years	From 18y	Last at 70y	n.d.	Once in two years	Once in two years			
Gastroscopy at 25y	-	Last rectoscopy at 89y	-	-	Gastroscopy once in three years			
-	-	Subtotal colectomy at 70y	Radiation therapy	Radiation therapy and surgery	Subtotal colectomy at 62y, pembrolizumab due to metastasis			

N-normal; n.d.- no data; SRS- Silver Russell syndrome; SSP-sessile serrated polyp; HP-hyperplastic polyp; VUS-variant of unknown significance
estimate

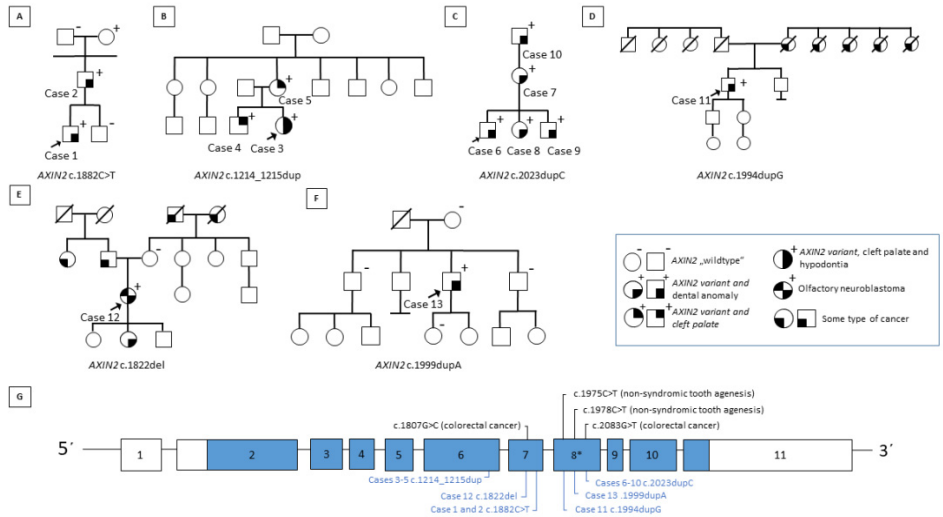


Figure 10. Pedigrees of all investigated families. **(A)** Family 1 (Estonia) – Cases 1 and 2 carrying *AXIN2* c.1882C>T variant; **(B)** Family 2 (Estonia) – Cases 3–5 carrying c.1214_1215dup variant; **(C)** Family 3 (Norway) – Cases 6–10 carrying c.2023dupC variant; **(D)** Family 4 (US) – Case 11 carrying c.1994dupG variant; **(E)** Family 5 (US) – Case 12 carrying c.1822del variant; **(F)** Family 6 (The Netherlands) – Case 13 carrying c.1999dupA variant. **(G)** *AXIN2* gene structure. Our cohort Cases are marked in blue (below the gene exons) and 4 missense variants of unknown significance (VUS) are marked in black above the gene exons. * Variant hot spot region.

The most common clinical feature was either hypo- or oligodontia ranging from 2–22 missing teeth (Table 13 and Figure 11). Four patients (30.8%) had at least eight permanent teeth missing, eight (61.5%) had had gastrointestinal polyps, three (23.1%) had had some type of cancer. Cancer types involved melanoma, unknown type of skin cancer, olfactory neuroblastoma, and prostate and caecal adenocarcinoma. Olfactory neuroblastoma has recently been linked to the *AXIN2* phenotype (Macklin-Mantia & Riegert-Johnson, 2020). Unfortunately, we did not have specific information about some family members in the context of cancer. In a few cases, we saw a dysmorphic phenotype. Case 1 had a clinical diagnosis of Silver-Russell syndrome (SRS, OMIM #186860), which is not associated with the found *AXIN2* variant. Case 3 had (operated) bilateral CP, microretrognathia, microglossia with glossoptosis, and two missing teeth. In addition, Case 3 had epilepsy, which is probably not associated with the found *AXIN2* variant. The younger brother of Case 3 (Case 4) and her mother (Case 5) had CP (operated), and the mother had one impacted tooth.

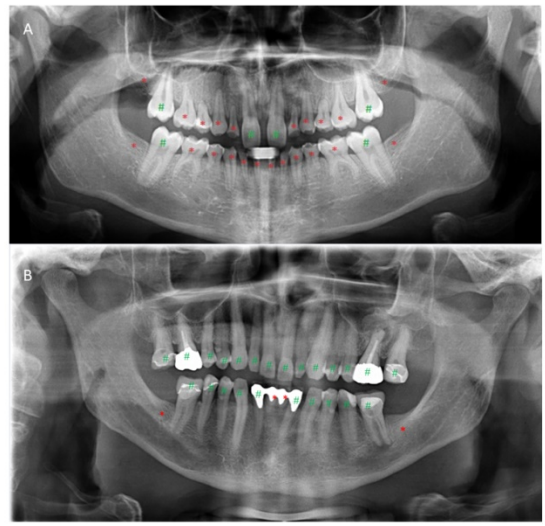


Figure 11. (A) Panoramic image of Case 6 at the age of 14 years. He shows congenital absence of 22 permanent teeth (*). As a consequence, deciduous teeth have not been exfoliated. Only six permanent teeth are present (#). (B) Panoramic image of Case 13 at the age of 64 years. He shows congenital absence of four permanent teeth (*), 31 and 41 (lower central incisors), as well 37 and 47 (posterior molars in the lower jaw). The persistent deciduous central incisors have been extracted and replaced by a bridge, including teeth 42-41-31-32. Present permanent teeth are also shown (#).

Our results of *AXIN2* disease-causing variant carriers' cohort confirms the variations in phenotype between families and individual patients.

The *AXIN2* gene variants reported were almost all pathogenic or likely pathogenic frameshift variants: three of these had not been described before in the ClinVar (Landrum et al., 2018), and HGMD Professional databases (c.1214_1215dup, c.1999dupA, c.2023dupC) (ClinVar database, HGMD Professional database). Most of the variants were located in exon 8, where the mutation hotspot also lies (Figure 10G). Only one missense variant, c.1882C>T, was detected in our cohort (Cases 1 and 2; Figure 10A). This variant has an extremely low allele frequency in normal populations worldwide (gnomAD All VAF 0,017%; 48 alleles out of 282,360). Some *in silico* prediction programs (SIFT, MutationTaster, Provean) predict this variant as damaging while others (PolyPhen2, Align GVGD) assess this as benign. It is likely that this variant is not completely penetrant, and in the absence of proteomic data or functional studies, the actual effect is unknown. As the patient and his father (Cases 1 and 2) have oligodontia and because no other potential disease causing variants were detected using WGS, this missense variant could still be associated with the disease, but at the moment the clinical significance remains unknown until further data are available. The interpretation of variant c.1822del has recently been changed from pathogenic to VUS. At the moment repeated genetic testing with clinical RNA testing for further investigation is pending.

We also gathered information about the clinical management and surveillance that was advised to the patients to propose a surveillance schedule for these individuals.

5.4 Oncological consultations in clinical genetics (Paper IV)

In this paper, the focus has been on oncogenetic consultations in general: what are the criteria for genetic consultation, the possibilities of genetic testing in Estonia, potential outcomes and usefulness for patients and their family members etc. The work has been published in the Estonian language, in the journal *Eesti Arst* (Estonian Physician), which is a well-known medical journal for physicians with diverse medical backgrounds, it was an excellent platform for sharing knowledge and reaching the audience of interest in Estonia.

At first, we discussed the principles of oncogenetic consultation. In Estonia, clinical geneticists and oncologists mostly depend on the NCCN and ESMO guidelines in clinical decision-making, although we have guidelines of our own as well, for example for consulting on hereditary syndromes connected to BC or LS, but these are mainly based on the international guidelines mentioned earlier (Padrik et al., 2022; Roht et al., 2020). In cases of rare hereditary tumour syndromes, but not only then, the ERN GENTURIS guidelines are also used (*ERN GENTURIS webpage*). In Estonia, a digital e-consultation platform, where physicians can ask advice from each other, has been available for some time already. Since 2020, clinical geneticists also can ask and advise their colleagues. It is a great way to select the patients that need genetic consultation, and recent data show that finally it has been implemented and is in use. Another important point or principle is who should be the first individual tested in the family. It is more of a general rule in genetic consultation, that if possible, the first individual tested in the family should be the one with the disease, since with this strategy, the probability of finding the cause is the highest. Of course, it does not apply if the individual with the disease cannot be reached for whatever reason or has passed away. As an exception, in case of LS, if possible, sometimes MMR IHC testing of family members' cancer tissue is done to decide further diagnostic steps for the index family member. Should we test every patient with cancer? As most cancers are sporadic, the answer is no, but there are criteria, which can vary from guideline to guideline, when germline testing for hereditary cancer syndrome detection is indicated.

The most frequent oncological indications in general for genetic consultation are as follows:

1. breast, colorectal or endometrial cancer < 50 years of age
2. ovarian cancer irrespective of age
3. male breast cancer irrespective of age
4. same type of cancer in multiple family members
5. two or more cancers in an individual < 60 years of age
6. childhood cancer

These are rather based on our clinical experience than on scientific guidelines alone. In childhood cancer cases, the general opinion is that all children should be tested.

The publication also covered possibilities and methodologies used for genetic testing. Generally speaking, for solitary tumours independent of specific tumour site or histology a "cancer panel" for germline testing has been available since 2015: at first the TSC panel covering 94 genes, and from July 2020 onwards the TSHC panel covering 113 cancer-related genes. Until 2019, MLPA testing was also routinely used for *BRCA1, BRCA2, CHEK2* and for MMR genes and *EPCAM* testing. Today, through extensive use of the CNV detection program DECoN, MLPA testing can be skipped in most cases, which enables us either to confirm or exclude most of the hereditary cancer syndromes in only 1–2 months. From a clinical point of view, DECoN works rather efficiently, so we use different MLPA analyses primarily to confirm the alteration found, not for initial diagnosis. One important exception to this approach is identification of variants in the pseudogene, *PMS2*, for which we still need MLPA analysis to detect exon deletions. In our current clinical framework, TSO/TSOE panels are rarely used for cancer diagnostics, but we have seen that these panels can also provide important incidental findings in the context of hereditary cancer and polyposis syndromes (Roht et al., 2022). In addition, we gave an overview of possibilities for somatic mutation testing, which can affect treatment choices and is valuable information for family members. Since 2020, TruSight Oncology 500 (Illumina, Inc.) has been available for somatic mutation testing in tumour tissue.

We compared statistics and the diagnostic yield of TSHC panel analysis done in 2020 for BC, CRC and other cancer groups. It showed that, compared to CRC, BC cases are genetically tested around ten times more frequently. In 2021 and 2022, the ratio of genetic testing in BC and CRC had remained the same, but family members of CRC patients were tested more frequently than earlier. We also looked at the statistics of molecular genetic analyses done for BC and OC patients and their family members from 2008–2019. In 2008 very few *BRCA1/BRCA2* sequencing analyses were done, but in 2019 there were already more than 700 analyses per year, and this has steadily increased ever since (Figure 12). The mean diagnostic yield for all cancer groups in the case of cancer patients was 23.4%, and in the case of healthy family members 10.7%.

In the last part of the paper, we focused on the purpose and pros of genetic counselling from different perspectives. First, from the cancer patient's perspective: it helps to distinguish sporadic and hereditary cancers and can sometimes even change tactics of treatment and/or extent of surgery. One example, in terms of the latter, is poly (ADP-ribose) polymerase (PARP) inhibitors as gene specific treatment, which are used in certain cases of OC, BC, pancreatic and prostate cancers, and dependent on tumour localization, *BRCA1* or *BRCA2* disease-causing variants can either be germline only or germline and/or somatic (*Summary of PARP inhibitor Olaparib characteristics*). Other examples, especially from somatic point of view, are *BRAF, NRAS, KRAS* and *EGFR* gene

alterations, all of which affect treatment tactics and course in different cancers (CRC, lung cancer, melanoma etc.).

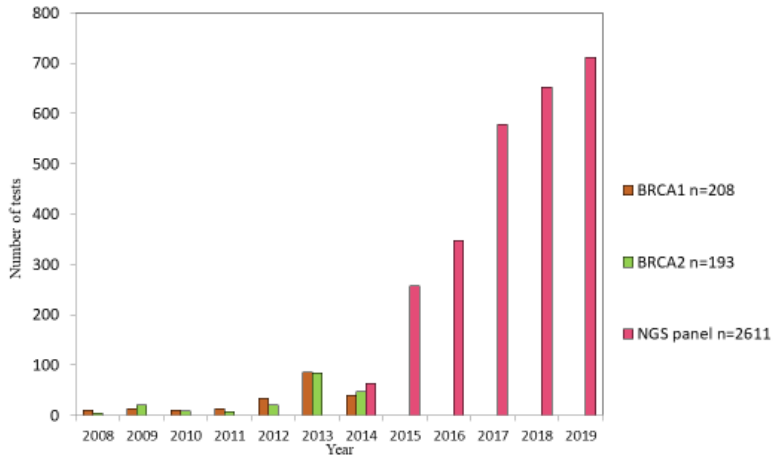


Figure 12. Molecular genetic testing in numbers from 2008–2019 for breast and ovarian cancer cases and their healthy family members.

In the case of a negative germline test result, meaning that disease-causing variants are not found, dependent on the family history we still survey these patients, and re-consult with them usually 3–5 years later for an update. If a hereditary cancer syndrome is confirmed in the pedigree, we offer genetic testing for family members, and it is up to the patient to share information between family members. If individuals test positive, they are offered surveillance and/or risk-reducing surgery, dependent on the finding. For relatives with a negative test result, recommendations on participation in cancer screening programs are given with an emphasis on the fact that they do not have increased cancer risk associated with the specific cancer syndrome in their family, but they still carry a general population risk.

In summary of this paper, the number of oncogenetic consultations already has and will increase in the near future. Thus, it is very important to educate doctors working in this field, keep oneself updated with the newest scientific knowledge and regularly upgrade methodologies and machinery for genetic testing. Genetics already is, and will remain one of the pillars of contemporary and personalized management of oncological patients.

6. DISCUSSION

Colorectal cancer is one of the most common cancers that also plays an important part in cancer-related death. This dissertation provides data on epidemiological and molecular genetics aspects as well as a clinical overview of hereditary colorectal cancer syndromes in Estonia. Population-based data are the foundation for improving diagnostic methods and treatment, prevention and surveillance methods.

6.1 The study of Estonian colorectal cancer patients investigated in a routine clinical setting (Paper I)

We aimed to study CRC and polyposis patients in a routine clinical setting to describe the molecular landscape, diagnostic coverage and yield of these patients. In our CRC patients, LP/P variants in MMR genes were the most common and among them *MLH1* was the gene most frequently affected.

In our study, pathogenic or likely pathogenic germline variants were found in 22.3% of CRC and polyposis cases; for CRC cases only it was 21.4%. An American study from 2017 showed that 9.9% of CRC patients carry at least one germline pathogenic variant in a cancer susceptibility gene (Yurgelun et al., 2017). In their cohort, they found that 3.1% of CRC patients carried a pathogenic MMR gene variant, with *MLH1* being the most prevalent, while 7% carried non-Lynch syndrome gene pathogenic variants. According to Valle et al., 2–8% of all CRC cases carry a pathogenic germline variant in high risk cancer genes, and for individuals <50 years this rises to even 1 in 5 (Valle, de Voer, et al., 2019). A study published in 2022 showed similar results (Uson et al., 2022). We attribute the discordance of our findings with previously published studies to the fact that other studies were much larger, involving thousands of patients compared to our study of 314 individuals. We do not suggest that LS is much more prevalent among CRC patients in Estonia than in other regions.

Additionally, new variants in all of the four MMR genes were identified and reported to the ClinVar database (Landrum et al., 2018). It is essential to report new genetic variants in international molecular genetics databases about different populations, including Estonians, as various genetic variants databases like ClinVar (Landrum et al., 2018), HGMD (Stenson et al., 2003), gnomAD (Karczewski et al., 2020) and others are used in routine clinical practice by molecular diagnostic laboratories.

We had some interesting findings concerning *FANCM* and *MUTYH* genes in our cohort. One of them was the identification of a *FANCM* disease-causing variant in a CRC case. Until recently, *FANCM* variants had only been associated with breast cancer. However, a recent study confirmed that it is also a risk factor for familial CRC (Cannon-Albright et al., 2020). Today, we have

more than one patient with this *FANCM* disease-causing variant, and indeed, it seems to segregate in CRC families. We suggest that this variant should be included in risk surveillance in association with CRC and considered when planning surveillance for healthy family members. We also report one monoallelic *MUTYH* variant in a case in which a woman had more than ten adenomatous polyps (found at the age of 64) in her colon and had had EC. The patient's mother had had rectal cancer at the age of 44. *MUTYH* heterozygous variants are associated with a ~2.5-fold-increased risk of CRC compared to the general population (Win et al., 2014). Previously, in a large meta-analysis, they had been associated with lower risk estimates (OR 1.16) (Theodoratou et al., 2010). Therefore, laboratories all over the world now report heterozygous variants to ensure early and appropriate surveillance. The NCCN Guidelines state that *MUTYH* heterozygotes should be screened by colonoscopy every 5 years from age 40, or from 10 years earlier than a first degree relative developed CRC. If there are no CRC cases in the family history, the guidance is currently unclear (NCCN-guidelines, 2023).

In our cohort of CRC and polyposis patients, the most used diagnostic methods were Illumina's TruSight Cancer panels and Sanger sequencing of the familial variant in cases with known variants in a close family member. Concerning diagnostic yield, we found that the combined diagnostic yield of TruSight cancer panels in our study was 13.4%. In 2022 a paper was published by Ceyhan-Birsoy et al., stating that the diagnostic yield of expanded genetic testing of CRC patients (more than 2000) was 15.3%, which is quite similar to our results (Ceyhan-Birsoy et al., 2022).

Although effective diagnostic methods including NGS panels are available in Estonia, physicians still perform genetic testing for relatively few cases of CRC and gastrointestinal polyposis, whilst a high proportion of BC cases are genetically tested. To improve genetic testing in CRC and gastrointestinal polyposis patients, raising awareness, collaborating with specialists and encouraging them to follow international guidelines are the key factors. It will remain essential to continue practical lectures and seminars for general practitioners and oncological specialists about the importance of genetic testing in CRC patients. We can detect healthy high-risk family members and implement surveillance measures, which decreases cancer-related death. Cancer treatment is expensive; therefore, prevention and treatment at earlier stages can help reduce the overall cost and burden on the healthcare system. Our study found that through genetic testing, family members are diagnosed with Lynch syndrome approximately eight years earlier than affected individuals in unrecognized families (Roht, Laidre, et al., 2023). Consequently, it might not prevent them from getting a cancer diagnosis, but it enables them to start prevention when they are still healthy.

6.2 The study of Lynch syndrome's prevalence and molecular genetics in the affected and general population (Paper II)

This dissertation's second and third aim were connected to Lynch syndrome studies. LS has a broad spectrum of cancers, and the risk estimates depend on the gene affected. According to the NCCN Guidelines, the highest cancer risks for females are for CRC and EC, at 8.7–61.0% and 13.0–57.0%, respectively. For males, CRC is similarly the highest cancer risk, but other cancer risks are not significantly high (NCCN-guidelines, 2023). In our study, besides CRC, which was the most frequent, other cancer types represented were breast, endometrial, prostate and bladder cancer. There were also individuals with multiple cancers. We had altogether six cases with more than three cancers in their health history: five of these individuals were either carriers of *MLH1* or *MSH2* disease-causing variants, and one was a CMMR-D case with compound-heterozygous variants in the *MSH6* gene previously mentioned. This confirms that *MLH1* and *MSH2* give the highest cancer risks, mainly but not only limited to CRC incidence, and as presented in the clinical case earlier in this thesis, *MSH2* is known to be more connected to extra-intestinal presentations than other LS genes (Lin-Hurtubise et al., 2013; Moller et al., 2022).

When we planned this study, only a few reports about the prevalence of Lynch syndrome in different populations were available. It is not very easy to perform epidemiological studies in large populations; however, Estonia is in a unique situation: we can cover the whole population for epidemiological studies, as the whole population is approximately 1.3 million inhabitants. Including only adults, as LS usually presents in adulthood, the Lynch syndrome birth prevalence between 1930 and 2003 in Estonia was estimated at 1:8638 (95% CI: 1:9859–7588) or 11.58 (95% CI: 10.14–13.18) for 100,000 LBs. We also showed that the prevalence of LS (cases per 100,000 persons) had risen by almost six times in ten years (2012–2022), from 0.34 (95% CI 0.22–0.52) to 1.99 (95% CI 1.54–2.57) ($p < 0.0001$), which is probably the result of better diagnostic opportunities and awareness amongst doctors. Estonia also has its own biobank, the Estonian Biobank that includes ~20% of the adult population, and these data can be used for population studies. Based on the EstBB data, in 2020, our estimated prevalence of MMR genes disease-causing variants was calculated to be 1:485 (0.20%) in the general population. Our estimated prevalence of disease-causing variants in MMR genes in the general population is lower than that reported in the world population. It has been estimated to be 1:100–1:180 in a 2020 study (Cerretelli et al., 2020) and at 1:279 in an earlier study (Win et al., 2017). There are yet to be any recent data in the literature for European populations. The latest work of Zhang et al. (Zhang et al., 2022) estimated the prevalence of MMR disease-causing variants in the Chinese general population to be 0.18%, similar to our estimation from 2020.

We are aware of some limitations in our Lynch syndrome epidemiological studies: probably we have not managed to include all potential LS patients in this study, as before 2015 testing was done in different labs not directly collaborating with our study. Secondly, some patients will have died before testing was readily available and finally a lack of awareness among medical staff concerning the availability and importance of genetic testing. The biggest systematic concern is the lack of a national or hospital-based registry of LS in Estonia, nor do we have a different diagnosis code in the health system. Fortunately, this is soon about to change. In 2023, the Genetics and Personalized Medicine Clinic and the Competence Centre of Rare Diseases of Tartu University Hospital have started to create a rare diseases registry, which, among other diseases, will include all hereditary tumour syndrome cases.

Our LS study also helped us to optimize our diagnostic workflow in oncogenetic testing. Before 2019, our diagnostic workflow for CRC and polyposis consisted of TSC or TSHC together with MLPA analyses, depending on the hypothesis. This meant that the standard workflow could take up to 4–5 months for molecular diagnosis. Today, through extensive use of the CNV detection program DECoN, we can either confirm or exclude most of the hereditary cancer syndromes in only 1–2 months. From a clinical point of view, DECoN works rather efficiently, so we use different MLPA analyses primarily to confirm the alteration found, not for initial diagnosis. One important exception to that rule is the identification of CNVs in the *PMS2* gene in which the presence of a pseudogene makes it difficult to determine the copy number using a DECoN program. For *PMS2* we still need MLPA analysis to detect exon deletions.

In our current clinical framework, TSO/TSOE panels are rarely used for cancer diagnostics, but we have seen, that they can also provide important incidental findings in the context of hereditary cancer and polyposis syndromes. However, an important issue is that there is still no consensus in Europe about whether it is appropriate to provide patient feedback about incidental findings on hereditary cancer syndromes (de Wert et al., 2021). In Estonia, standard practice is to ask whether the patient or the patient's guardian wishes to learn about findings on disease-causing alterations in the ACMG gene list (Miller, 2021). From a public health standpoint, giving feedback is cost-effective and makes the best use of available Health Insurance Funding, which covers all diagnostics used in workup, if indicated. Yet another challenging aspect is whether to report to clinicians and patients findings of variants in genes not (yet) known to be associated with CRC or polyposis, or of variants in known CRC/polyposis genes which are not yet known to be disease-causing. In an era of dramatic increases in application of NGS technologies and discoveries in disease genetics it is however very important to share even variants of unknown significance through databases like ClinVar (Landrum et al., 2018), HGMD Pro and others, that help to assess the clinical relevance of variants found. Sharing these kinds of data supports laboratory specialists and clinicians in reporting findings and decision-making, and therefore should be encouraged.

One of our main interests in LS diagnostics was whether changing the age limits from 50 up to 70 years in MMR genes IHC testing of CRC patients would result in finding more patients. In a recent systematic review and meta-analysis of almost 60,000 colorectal carcinomas, it was found that with age restriction when performing MMR genes IHC under 50 years or 70 years, the main problem is that older patients will be missed (Eikenboom et al., 2022). Therefore, their healthy family members at risk will be lost for surveillance. In our study group, most cases were diagnosed between 50 and 70. The question is no longer, whether we should screen all CRC cancers by MMR IHC or other equivalent methods, but rather who should be tested for germline disease-causing variants after the screening. Therefore, in Estonia, the working group of Lynch syndrome, including specialists caring for these patients and their family members, has recently decided that testing by immunohistochemistry or other equivalent methods will be conducted for all CRC patients irrespective of age. These results are one of the most essential practical implementations of the study at Tartu University Hospital. Depending on the result, these individuals are further studied by germline and/or somatic genetic testing by a clinical geneticist or other specialists if this syndrome is suspected. In the near future, we would like to apply this workflow and management to all colorectal and endometrial cancer patients in other Estonian centralized hospitals.

6.3 The study of *AXIN2*-related oligodontia-colorectal cancer syndrome (Paper III)

The last aim of this dissertation was to specify the phenotype and align surveillance of *AXIN2* disease-causing variant carriers. We gathered thirteen *AXIN2* carriers. In the international community, very few patients with disease-causing *AXIN2* gene variants were reported; therefore, there was an urgent need to broaden the clinical phenotype and describe new features. In our study, we observed three cases of cleft palate in one of the families. Although CP can run in families as a separate trait and could be unrelated to the *AXIN2* variant, the Wnt pathway is known to be important in craniofacial morphogenesis in animal models (Liu et al., 2010). Therefore, we concluded that CP might be one of the new features of *AXIN2*-related oligodontia-colorectal cancer syndrome, but this needs further investigations of more patients. However, this knowledge gives practical insight to clinicians in evaluating and treating these patients. Oligodontia is one feature of *AXIN2*-related oligodontia-colorectal cancer syndrome. During this study, we have actively communicated with orthodontists of Tartu University Hospital and educated them on this rare syndrome. In the case of oligodontia, they can directly order genetic testing or send the patient for genetic counselling. We aim to communicate this further on a broader level to dentists and orthodontists in Estonia and internationally. We also suggest that the *AXIN2* gene be included in CRC molecular profiling panels, and it is also important to gather family history from an oncological point of view in the case

of cleft palate families. Concerning surveillance, we still do not have any official published European guidelines for this syndrome. Colonoscopy every 1–3 years is suggested by most authors, and as the Wnt pathway is also dysregulated in *APC*-gene-associated FAP, most authors also suggest regular gastro-duodenoscopy.

6.4 Oncological consultations in clinical genetics (Paper IV)

Lastly, this dissertation also includes one article published in the journal *Eesti Arst* (Estonian Physician) in the Estonian language. This journal is the only well-known medical journal in our national language for physicians with diverse medical backgrounds. We realized that it is essential to educate medical audiences on oncological consultations in clinical genetics in our national language. The focus of this paper was entirely practical. The most important aspect is to think about hereditary cancer syndromes, due to which we also gave some practical insights in which cases to test individuals genetically. Guidelines play an important part in clinical decision-making, but we must not forget the role of *Ars medicinae.* Samadder et al. published a paper, where they showed that only 52% of all individuals with pathogenic gene variant would have been detected using standard guidelines; almost half (48%) would not have been found. Furthermore, in almost 1/3 (28%) of individuals carrying pathogenic variants in high-penetrance genes, clinical management or treatment changed due to universal genetic testing (Samadder et al., 2021). In the case of hereditary cancer syndromes presenting in adulthood, the general view is that, it is unethical to test children but to delay this until they reach adult age. This view is mostly due to concerns of violating their autonomy in decision-making. Although, as always, there are exceptions to that rule, too, for example in the case of the already discussed *AXIN2*-associated oligodontia-colorectal cancer syndrome. As it can present with different clinical problems from dental anomalies and ectodermal dysplasia to gastrointestinal polyposis and cancer, these patients may be tested already in childhood, and it is still debatable, and depends on family history, at what age exactly the screening should be started. From the practical point of view, we also plan to create information sheets on Lynch syndrome and *AXIN2*-related oligodontia-colorectal cancer syndrome for patients and their family members.

The main practical implications of this dissertation are:

1. One part of this study (Paper I) gathered information on the epidemiological and molecular genetic data on CRC, and more specifically Lynch syndrome, the most common hereditary colorectal cancer syndrome. These data are the basis for other studies on this field in the Estonian population.
2. Genetic testing has improved in recent years; however, it is essential to constantly raise awareness, give lectures and seminars on the topic, publish articles in local professional journals and collaborate with specialists in the relevant field.

3. It is important to mention that in our Lynch syndrome study we found that family members are diagnosed with Lynch syndrome approximately eight years earlier than affected individuals are. Consequently, molecular diagnosis may not avoid progression to a cancer diagnosis, but it enables them to start surveillance while they are still healthy, possibly reducing cancer-related mortality. The diagnostic algorithm of Lynch syndrome was made more cost-efficient as the workflow was changed so that instead of 4–5 months, it takes a total of 1–2 months.
4. Before our study (Paper II) started, MMR genes IHC was used to screen patients <50 years of age for Lynch syndrome. In our study we raised the age-limit to 70 years and it resulted in finding more patients. The working group of Lynch syndrome in Tartu University Hospital has decided that all CRC and EC patients irrespective of their age, are screened for Lynch syndrome. Now, this approach of testing by MMR IHC analysis is only used in Tartu University Hospital, but we would like to apply this to all Estonian patients.
5. One of our aims was also to specify the phenotype of oligodontia-colorectal cancer syndrome associated with *AXIN2* (Paper III). We hypothesize that cleft palate is one of the symptoms of this syndrome, but it needs further studies to be certain. It is important to think about that syndrome. Therefore, we have informed the orthodontists and dentists of Tartu University Hospital about the new symptom, who can send patients for consultation or arrange genetic testing themselves. In addition, it is also important for orthodontists to clarify about cancer cases in the family history. In our opinion, *AXIN2* gene should also be included on CRC gene panels. Furthermore, we plan to produce patient information sheets on Lynch syndrome and *AXIN2*-associated syndrome.

In summary, genetics is and will remain, one of the foundations of contemporary and personalized approach in oncology. Thus, it is crucial to educate medical staff working in this field, update methodologies and equipment for genetic testing, and provide scientific knowledge for better diagnostics, treatment and prevention.

7. CONCLUSIONS

1. The first aim of the study was to estimate the prevalence of pathogenic/likely pathogenic germline variants in Estonian colorectal cancer patients using NGS in a routine clinical setting.
   - Pathogenic or likely pathogenic germline variants were found in 38 out of 170 patients, which constitutes 22.3% of CRC and polyposis cases.
   - In 80% of them, the genetic diagnosis was confirmed to be from disease-causing variants in MMR genes.
   - Only 2.7% of CRC cases were genetically tested in Estonia in 2016, rising to 6.1% in 2019.
2. The second aim was to estimate the prevalence of Lynch syndrome in general adult population and to specify the genotypes/phenotypes of Lynch syndrome.
   - The birth prevalence of LS in 1930–2003 in Estonia was 1:8,638 (95% CI: 1:9,859-7,588) or 11.58 (95% CI: 10.14-13.18) for 100,000 persons.
   - The prevalence of LS (cases per 100,000 persons) has risen from 0.34 (95% CI 0.22–0.52) to 1.99 (95% CI 1.54–2.57) in ten years (2012–2022) ($p < 0.0001$). The increase is almost six times in ten years, probably due to better diagnostic opportunities and health awareness among doctors.
   - The estimated prevalence of LS associated variants in the EstBB cohort in 2020 was 1:485 (Poisson 95% CI: 1:263–1:1009).
   - *MLH1* disease-causing variants together with *MSH2* and/or *EPCAM* disease-causing variants were the most frequent finding in the diagnostic cohort (both accounted for 28.2 %).
   - The *MLH1* gene variant NM_000249.4: c.1976G>C p.(Arg659Pro) was the most frequent in the diagnostic cohort. The *MLH1* associated phenotype was mainly either colorectal or recurrent colorectal cancer and/or endometrial cancer at an early age.
   - The mean age at LS diagnosis of an index cohort's family members was 36.8 years, which is eight years earlier than for the index cases themselves ($p = 0.0035$).
3. The third aim was to analyse the effectiveness of immunohistochemistry analyses among colorectal cancer patients over 50 years of age.
   - From 2018 until 2021 (included), 464 analyses were performed and 52 (11.2%) filtered out due to changes in MMR IHC. In nine out of 31(29%), a definite diagnosis of LS was confirmed. In two cases, the diagnosis stayed probable.
   - LS was diagnosed in 54.5% of cases in the group of colorectal cancer patients aged 50–70 years.

4. The fourth aim was to specify the *AXIN2*-related oligodontia-colorectal cancer syndrome phenotype and the associated cancer risk of *AXIN2* carriers.
   - Cleft palate might be a new feature of *AXIN2*-related oligodontia-colorectal cancer syndrome.
   - Information about the advised surveillance was collected, which might support clinical management of these patients.
   - More clarity about oligodontia-colorectal cancer syndrome, about its variable expression, and associated cancer risks are needed to improve clinical management and to establish guidelines for surveillance.

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SUMMARY IN ESTONIAN

Pärilikud kolorektaalvähi sündroomid Eestis

Kolorektaal- ehk jämesoolevähki defineeritakse kui pahaloomulist kasvajat, mis saab alguse käär- või pärasoolest. Tihti käsitletakse neid koos, sest nad on iseloomult sarnased (*American Cancer Society webpage*). Kolorektaalvähk on enam kui kümme aastat olnud kolmas kõige sagedasem vähipaige Eestis ning maailmas. Eesti Tervise Arengu Instituudi statistika andmetel on esinemissageduse tõus olnud kiire: 2010. aastal oli 791 esmast kolorektaalvähi juhtu (C18-C21), kuid 2020. aastal juba 963 juhtu (The National Institute for Health Development webpage). Oluline on ära märkida, et siia kuulub ka anaalkanali vähk, mis erineb kolorektaalvähist nii etioloogia, histoloogia kui ravi poolest, kuid hetkel ei ole Eestis selle kohta eraldi statistikat.

Eestis algas kolorektaalvähi sõeluuring 2016. aastal. Selle raames kutsub Tervisekassa iga kahe aasta tagant uuringule naisi ja mehi vanuses 60-68 eluaastat. Uuringumeetodiks on roojas peitvere määramine. Kui peitvere test on positiivne, suunatakse indiviid edasi koloskoopiale vähi välistamiseks. Dr. Heigo Reima, kes kaitses 2022. aasta sügisel doktorikraadi teemal "Kolorektaalvähi ravi ja tulemite hindamine ning nende parandamise võimalused Eestis" näitas oma töös, et sõeluuringu tulemusena kolorektaalvähi esinemissagedus kasvas ning esimese staadiumi vähkide esinemissagedus tõusis. Elulemus oli küll paranenud, kuid oli siiski ~10% madalam kui Skandinaavia riikides. Kuigi diagnostika ja multimodaalne ravi on ajas väga palju paranenud, siis on endiselt probleemiks hiline diagnoosimine, kõrge metastaatilise kolorektaalvähi juhtude arv ning erakorraliste operatsioonide hulk. Dr. Heigo Reima koos kolleegidega viis läbi ka randomiseeritud uuringu teemal kas operatsioonimaterjali värvimine metüleensinisega aitab paremini tuvastada haaratud lümfisõlmi, mis omakorda aitab paremini määrata haiguse staadiumit ning otsustada adjuvantse kemoteraapia vajaduse üle (Reima, 2022).

Pärilikest kolorektaalvähi sündroomidest rääkides oli Peutz-Jeghersi sündroom (PJS) üks esimesi, mida publitseeriti. Esmalt kirjeldas seda Inglise arst, kirjeldades kaksikutest tüdrukuid, kellel esines suu limaskesta pigmentatsioon. Üks neist suri 20-aastaselt soole invaginatsiooni ja teine 52-aastaselt rinnavähi tõttu. Sündroom sai 1954. aastal nimetuse uurija Peutzi järgi, kes kirjeldas perekonda, kus esines gastrointestinaalne polüpoos ja limaskestade pigmentatsioon ning pärilikkus vastas autosoom-dominantsele (AD) pärilikkusele, ning uurija Jeghersi järgi, kes defineeris mukokutaanse pigmentatsiooni koos gastrointestinaalse polüpoosiga eraldi fenotüübina (Beggs et al., 2010). Lynchi sündroom (LS) on aga kõige sagedamini esinev geneetiliselt determineeritud kolorektaalvähi sündroom, mille põhjuseks on pärilik muutus *mismatch repair* (MMR) ja/või *EPCAM* geenides.

Enne 2012. aastat oli Eestis võimalik teostada vaid üksikuid pärilike kolorektaalvähi sündroomide geneetilisi uuringuid. Samal aastal avaldati Eesti Arsti ajakirjas artikkel päriliku kolorektaalvähiga patsientide geneetilise konsultee-

rimise ning jälgimise kohta. Autorid väitsid, et ~5% kolorektaalvähi juhtudest on tingitud monogeensetest kõrge penetrantsusega geenivariantidega seotud kasvajasündroomidest. Sel hetkel oli MMR geenide immuunhistokeemia (IHK) ning mikrosatelliitide ebastabiilsuse ehk instabiilsuse (MSI) analüüsid ning kõige sagedamini esinevate pärilike kolorektaalvähi sündroomide geneetilised uuringud juba kättesaadavad. Autorite sõnul võimaldaks efektiivne jälgimine parandada elukvaliteeti ning elulemust (Mikita et al., 2012). Eestis ei ole meile teadaolevalt varasemalt pärilike kolorektaalvähi sündroomidega seotud süsteemset ülevaadet publitseeritud.

Käesoleva uuringu eesmärgid

1. Analüüsida rutiinse töö käigus teostatud järgmise põlvkonna sekveneerimise (NGS) analüüside abil hinnangulist patogeensete ning tõenäoliselt patogeensete iduliini geenimuutuste levimust Eesti kolorektaalvähiga patsientide hulgas.
2. Hinnata Lynchi sündroomi levimust täiskasvanute üldpopulatsioonis ja kirjeldada Lynchi sündroomi genotüüp/fenotüübi omavahelisi seoseid.
3. Analüüsida MMR geenide immuunhistokeemiliste analüüside efektiivsust >50-aastaste kolorektaalvähiga patsientide hulgas.
4. Kirjeldada *AXIN2* geenikandjate fenotüüpi ning kasvajate riske.

Patsientide ja meetodite lühikirjeldus

Uuringus (I artikkel), kus rutiinse töö käigus teostati kolorektaalvähi ja polüpoosiga patsientidele NGS analüüs, kogusime me retrospektiivselt vahemikus juuli 2016-juuli 2021 kliinilise ja molekulaargeneetilise info kõikide SA Tartu Ülikooli Kliinikumi (TÜK) Geneetika ja personaalmeditsiini kliinikusse (GPMK) saadetud juhtude kohta. Viie aasta jooksul teostati *TruSight One* (TSO, Illumina, Inc.) *TruSight One Expanded* (TSOE, Illumina, Inc.) analüüse 5705 ja nn. päriliku vähipaneeli analüüse 3704 uuritavale. Alates 2015. aastast kuni 2020. aasta keskpaigani kasutati päriliku vähi testimiseks *TruSight Cancer* paneeli (TSC, 94 geeni, Illumina, Inc.), edaspidi aga *TruSight Hereditary Cancer* paneeli (TSHC, 113 geeni, Illumina, Inc.). Kõik eelpool toodud paneelid sekveneeriti Illumina MiniSeq või NextSeq platvormil. Geenivariandid klassifitseeriti Ameerika Meditsiinigeneetika Kolledži (ACMG) kriteeriumite alusel (Richards et al., 2015). Koopiaarvude muutused detekteeriti NGS analüüsil kasutades kas CoNIFER (v0.2.2) või DECoN (v1.0.2) tarkvara, või MLPA metoodikat. Sangeri sekveneerimist kasutati pereliikmete uurimisel juhul kui konkreetne geenimuutus oli perekonnas varasemalt juba teada. Uuringu perioodil teostati kolorektaalvähi või polüpoosi tõttu anamneesis ja/või pereanamneesis 314 NGS analüüsi. Kolorektaalvähk oli diagnoositud 126 indiviidil 314-st (defineeritud kui C18-C21), 44-l oli diagnoositud polüpoos (defineeritud kui enam kui ühe polüübi esinemine sooles), 113 juhul oli tegemist terve pereliikmega ja 31 juhul oli tegemist muu kasvajapaikmega või üksiku polüübiga indiviidiga, kellel esines kasvajaid või soolepolüüpe ka pereanamneesis.

Lynchi sündroomi uuringusse (II artikkel) kaasati patsiendid ja presümptomaatilised geenimuutuse kandjad, kes diagnoositi vahemikul 2012-2022. Andmed koguti SA TÜK GPMK molekulaardiagnostika labori (>5000 vähipaneeli) ning koostööpartnerite (Asper Biogene ning Eesti Geenivaramu) andmebaase kasutades. Moodustati kaks kohorti: diagnostiline kohort (123 indiviidi, neist 75 indeksjuhud ja 48 terved pereliikmed) ning Eesti Geenivaramu kohort (23 indiviidi), mis esindas üldpopulatsiooni. SA TÜK GPMK-s kasutatav molekulaargeneetiline metoodika on kirjeldatud eelnevas lõigus. Eesti Geenivaramu kasutas geenivariantide annoteerimiseks majasisest töövoogu. Statistiliseks töötluseks kasutati Lynchi sündroomi sünnisageduse ja sageduse hindamiseks lineaarset mudelit, arvutusteks R programmi versiooni 4.2.0 (Team, 2020) ning 95% usaldusvahemiku arvutamiseks kasutati Poissoni jaotust (Begaud et al., 2005). Statistilise olulisuse piiriks oli klassikaliselt 0,05. Diagnostilise kohordi erinevate gruppide võrdluseks (keskmine diagnoosi ja esimese kasvaja tekke vanus) kasutati Wilcoxoni astaksumma testi. Lynchi sündroomi skriinimiseks kasvajakoest teostati kõigile kolorektaalvähiga patsientidele vähikoe MMR geenide immuunhistokeemiline analüüs (4 markeriga), mille tulemuse alusel otsustati edasiste geneetiliste uuringute vajadus.

AXIN2-ga seotud oligodontia ja kolorektaalvähi sündroomi käsitlevas uuringus (III artikkel) koguti info küsimustiku abil, mis saadeti kõigile klinitsistidele ERN GENTURISe võrgustikus, kes teadaolevalt *AXIN2* geenikandjatega tegelevad, lisaks kolleegidele Mayo Kliinikus ja Bostoni Lastehaiglas. Meie kohordis oli 13 haigusseoselise muutuse kandjat, neist kaheksa mehed ja viis naised vanuses 4–95 aastat. Kõik kandjad olid Europiidse rassi esindajad, üksteist neist Euroopast ja kaks Põhja-Ameerikast. Seitse kandjat tuvastati NGS meetodil, mis teostati diagnostilisel põhjusel. Neist kolmel juhul kasutati erinevaid vähipaneele, kahel juhul kasutati TSOE 6700 geeni paneeli (Illumina, Inc.), ühel juhul detekteeriti indiviid eksoomi sekveneerimine analüüsil ning genoomi sekveneerimist kasutades tuvastati samuti üks indiviid.

Peamised tulemused ja järeldused

1. Rutiinse töö käigus teostatud NGS uuringute tulemused kolorektaalvähiga patsientidel näitasid et:
   - Kolorektaalvähi ning polüpoosiga patsientidest 22,3% esines patogeenne või tõenäoliselt patogeenne iduliini ehk pärilik geenimuutus.
   - Kõigist juhtudest leiti 80% muutus MMR geenides.
   - Aastatel 2016–2019 testiti geneetiliselt vastavalt 2,7–6,1% kolorektaalvähiga patsientidest, mis on umbes kümme korda vähem kui näiteks rinnavähi patsientide kohordis. Uuringuperioodi jooksul geneetilise testimise osakaal tõusis.
2. Lynchi sündroomi levimuse ning genotüüp/fenotüüp uuringu tulemused:
   - LS sünnilevimus Eesti aastatel 1930-2003 oli hinnanguliselt 1:8 638 (95% CI 1:9 859-7 588) või 11,58 (95% CI: 10,14-13,18) 100 000 elussünni kohta.

- LS levimus (juhud 100 000 inimese kohta) on tõusnud 10 aasta jooksul (2012-2022) 0,34-lt (95% CI 0,22-0,52) 1,99-ni (95% CI 1,54-2,57) ($p < 0,0001$). Pea kuuekordne tõus on tõenäoliselt eelkõige tingitud diagnostiliste võimaluste paranemisest ning teadlikkuse tõusust arstkonna seas.
- 2020. aastal oli Eesti Geenivaramu andmetel arvutatud LS seotud haigusseoseliste geenivariantide sagedus 1:485 (Poisson 95% CI: 1:263–1:1009).
- *MLH1* geeni ja *MSH2* geeni haigusseoselised muutused koos või ilma *EPCAM* geeni muutusteta olid diagnostilises kohordis kõige sagedasemad (mõlemad moodustasid 28,2%).
- *MLH1* geeni variant NM_000249.4: c.1976G>C p.(Arg659Pro) oli kõige sagedasem geenivariant diagnostilises kohordis. *MLH1* geenimuutuste fenotüüp hõlmas endas peamiselt kolorektaalvähi ühe- või mitmekordset esinemist koos või ilma günekoloogiliste (peamiselt endomeetriumi) pahaloomuliste kasvajateta noores eas.
- Lynchi sündroomi keskmine diagnoosi vanus pereliikmetel oli 36,8 aastat, mis tähendab, et võrreldes indeksjuhtudega saavad pereliikmed diagnoosi umbes kaheksa aastat varem ($p = 0,0035$). See on tõenäoliselt seotud parema terviseteadlikkusega inimeste seas, ning võimaldab alustada jälgimist ja seeläbi varem avastada potentsiaalseid kasvajaid. See on oluline ka tervishoiupoliitilisest aspektist.

3. Eraldi analüüsisime MMR geenide immuunhistokeemilise analüüsi diagnostilist efektiivsust >50-aastaste kolorektaalvähiga patsientide hulgas:
   - Uuringuperioodil teostati kokku 464 analüüsi, millest filtreeriti välja 52 analüüsi (11,2%). Üheksal juhul 31-st (29%) sai indiviid kindla Lynchi sündroomi diagnoosi, kahel juhul on tegemist tõenäolise Lynchi sündroomi diagnoosiga.
   - >50-aastaste kolorektaalvähiga patsientide hulgas diagnoositi Lynchi sündroom 54,5% juhtudest. Seega on MMR geenide immuunhistokeemiline analüüs samuti näidustatud antud vanusegrupis.
4. *AXIN2*-ga seotud oligodontia-kolorektaalvähi sündroomi uuringud näitasid, et:
   - Suulaelõhe võib olla antud sündroomi uus tunnus.
   - Kogusime kokku info praegu kasutusel olevate jälgimissoovituste kohta, mis võib olla abiks nende patsientide käsitlusel.
   - Samas jõudsime järeldusele, et vajalik on täiendava info kogumine ja koostöö tegemine, et selgitada ja täiendada antud sündroomi fenotüüpi ning kasvajariske, et omakorda parandada nende patsientide kliinilist käsitlust ning luua vajalikud jälgimisjuhendid.

Minu doktoritöö tulemusi saab praktilise väljundina kasutada järgnevalt:

1. Antud töö kogus epidemioloogilisi andmeid kolorektaalvähi kohta ning täpsemalt ka Lynchi sündroomi kui kõige sagedamini esineva päriliku kolorektaalvähi sündroomi kohta. Epidemioloogiliste andmete olemasolu ning geneetilised teadmised on alus edasisteks uuringuteks antud valdkonnas Eestis.
2. Kolorektaalvähi sündroomide geneetiline testimine on ajas paranenud, kuid endiselt ja pidevalt on vajalik spetsialistide teadlikkuse tõstmine, mistõttu on oluline kirjutada sel teemal ka eestikeelsetes ajakirjades ning korraldada loenguid ning praktilisi seminare ning arendada kliinilist ja teaduslikku koostööd Eesti siseselt, kuid ka rahvusvaheliselt.
3. Rahvatervise ning terviseökonoomika seisukohalt on oluline, et Lynchi sündroomi uuring leidis, et Lynchi sündroomi peredes said pereliikmed võrreldes probandidega diagnoosi 8 aastat varem. See võimaldab varasemat jälgimist, mis ei langeta küll kasvajasse haigestumise tõenäosust, kuid võimaldab suure tõenäosusega kasvaja varem avastada, mil see on paremini ravitav ning prognoosi ning elulemust seeläbi parandada. Lisaks muutus uuringu tulemusena ka Lynchi sündroomi diagnostiline algoritm geneetiliste uuringute aspektist, mis tähendab, et varasema 4–5 kuu asemel saab geneetilise lõppvastuse 1–2 kuuga ning kokkuvõttes väheneb seega ka uuringute kogumaksumus. Seega muutus diagnostika antud aspektist kuluefektiivsemaks.
4. Varasemalt kasutati MMR geenide immuunhistokeemilist uuringut skriinimaks Lynchi sündroomi kolorektaalvähi patsientidel vanuses kuni 50 eluaastat. Antud uuringus tõstsime me vanusepiiri kuni 70. eluaastani ning nägime, et see õigustas ennast ning nii leiame me rohkem patsiente üles. Lynchi sündroomi töögrupp on otsustanud, et MMR geenide immuunhistokeemiline uuring tehakse nüüd kõigile kolorektaalvähi patsientidele sõltumata vanusest, sama kehtib endomeetriumi vähiga patsientide puhul. Seda hetkel küll vaid Tartu Ülikooli Kliinikumi tasandil, kuid edaspidi sooviksime seda praktikat laiendada kogu Eestile.
5. Üks meie eesmärkidest oli ka *AXIN2* geeniga seotud oligodontia-kolorektaalvähi sündroomi fenotüübi täpsustamine. Me arvame, et suulaelõhe on antud sündroomi üks uutest tunnustest, kuid see vajab veel kinnitamist. Küll aga on oluline oligodontia korral antud sündroomile mõelda, sellest on teavitatud ka Tartu Ülikooli Kliinikumi stomatolooge ja ortodonte, kes saavad kas ise geneetilise uuringu tellida või suunata patsiendi geneetiku konsultatsioonile. Ka on oluline oligodontiaga peredes uurida kasvajate perekondlikku olemasolu. Meie arvates peaks *AXIN2* geen kuuluma ka kolorektaalvähi geenipaneelil uuritavate geenide hulka. Meil on plaanis nii *AXIN2*- seotud sündroomi kui Lynchi sündroomi osas koostada patsientidele ja nende pereliikmetele ka infolehed.

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PUBLICATIONS

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1. **Roht L**, Laidre P, Tooming M, Tõnisson N, Nõukas M, Nurm M, Estonian Biobank Research Team, Roomere H, Rekker K, Toome K, Fjodorova O, Murumets Ü, Šamarina U, Pajusalu S, Aaspõllu A, Salumäe L, Muhu K, Soplepmann J, Õunap K, Kahre T. The prevalence and molecular landscape of Lynch syndrome in the affected and general population. Cancers 2023; 15;3663.
2. **Roht, Laura**; Hyldebrandt, Hanne K.; Stormorken, Astrid T.; Nordgarden, Hilde; Sijmons, Rolf H.; Bos, Dennis K.; Riegert-Johnson, Douglas; Mantia-Macklin, Sarah; Ilves, Pilvi; Muru, Kai; Wojcik, Monica H.; Kahre, Tiina; Õunap, Katrin (2023). AXIN2-related oligodontia-colorectal cancer syndrome with cleft palate as a possible new feature. Molecular Genetics & Genomic Medicine. DOI: 10.1002/mgg3.2157.
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Publikatsioonide nimekiri (esiautorlus):

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