Germline variants in the mismatch repair genes: Detection and phenotype

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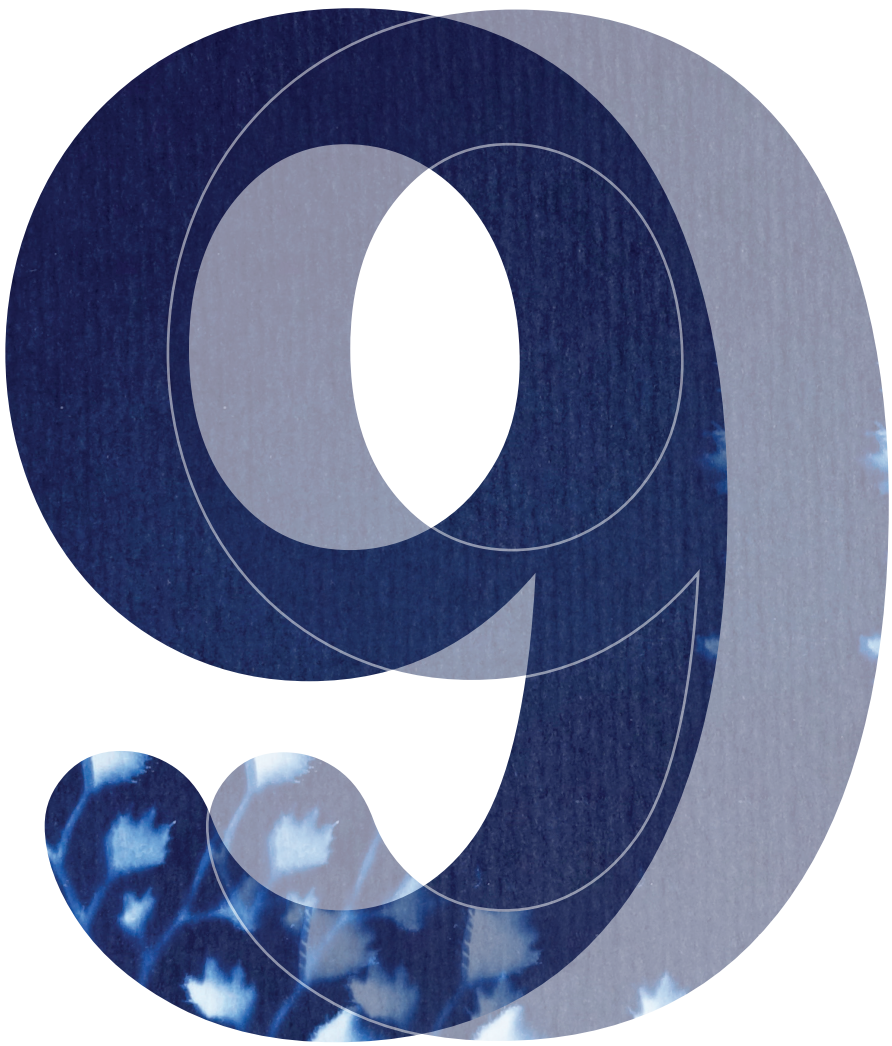

Discussion

ABSTRACT

The studies in this thesis aimed at exploring strategies to improve the detection of pathogenic germline variants in the mismatch repair (MMR) genes (**Part I, chapters 2-5**) as well as elucidating the phenotype of these variants once identified (**Part II, chapters 6-8**). Part I discusses when testing for Constitutional Mismatch Repair Deficiency (CMMRD) should be considered in children without cancer but with an NF1-like phenotype. Furthermore, the prevalence of MMR deficiency and Lynch syndrome in small bowel cancer and serous ovarian cancer is described. Part II explores a new method to establish unbiased colorectal cancer risk for Lynch syndrome patients, which is vital information to define (new) surveillance protocols. In addition, the effect of genotype and parent-of-origin on phenotype is determined in a cohort of *PMS2* variant carriers. Lastly, the prevalence of adenomas and incident colorectal cancers is described for the largest cohort of *PMS2* variant carriers to date.
In the following chapter, the main findings are briefly summarized and further discussed in the context of current literature.

LYNCH SYNDROME

Detection (Part I)

Given the estimated carrier frequency of 1 in 279 (1 in 1,946 for *MLH1*, 1 in 2,841 for *MSH2*, 1 in 758 for *MSH6* and 1 in 714 for *PMS2)* in the general population for pathogenic variants in the MMR genes,[1] it is clear that with current strategies many individuals with Lynch syndrome remain unidentified. This is likely partly due to the relatively mild phenotype associated with *PMS2* and *MSH6* (colorectal cancer risk estimates between 11 and 69%), compared to the phenotype associated with *MLH1* and *MSH2* (colorectal cancer risks estimates between 52 and 97%).[2-6] With the introduction of universal screening for MMR deficiency in all colorectal and endometrial cancers diagnosed before age 70, increasing numbers of these more mildly affected families are identified. Nonetheless, room for improvement in the identification of new Lynch syndrome families remains. In this thesis (chapter 4) we showed that the prevalence of Lynch syndrome (6.2%) and MMR deficiency in general (22.3% and 2.1% for respectively complete and subclonal MMR deficiency) in resected adenocarcinomas of the small bowel is high. Introducing universal (i.e. reflex analysis by pathologists regardless of family history) MMR deficiency screening in these tumors would therefore be an efficient way to identify more Lynch syndrome families. In particular since small

bowel tumors are relatively rare (300 cases in the Netherlands in 2018 according to www.cijfersoverkanker.nl). Hence, performing immunohistochemistry and subsequent molecular diagnostics would be a relatively low burden for pathology departments. Since a large range in age at diagnosis of the Lynch syndrome related tumors was observed (range 35-77 years, *this thesis*) we suggest not to put age restrictions or any other prerequisites on the universal screening of these tumors.

Introducing universal screening for MMR deficiency in small bowel carcinomas and the subsequent identification of Lynch syndrome families through an index patient with small bowel cancer is going to pose questions and challenges during the counselling process. While this strategy is a good opportunity to offer newly identified Lynch syndrome carriers colonoscopic and gynecologic surveillance, family members of an index patient with small bowel cancer may feel worried about developing small bowel cancer themselves. This may be particularly distressing since surveillance of the small bowel is currently not offered due to lack of evidence for its effectiveness.[7] Reassurance of these family members will require knowledge and skills from any clinical geneticist or genetic counsellor that counsels these families. Furthermore, this demonstrates that the prevention and detection of small bowel cancer (in the context of Lynch syndrome) can be improved. To address prevention and treatment of Lynch syndrome-associated small bowel cancer, future research should focus on unraveling the molecular pathways and mechanisms that lead to the development of these rare tumors. Recent research efforts have identified different molecular pathways in the development of colorectal cancer in Lynch syndrome for different MMR genes.[8-10] Evidence is accumulating that carriers of *MLH1* variants can develop colorectal cancer directly from mismatch repair deficient crypts, predisposing them to an increased risk of developing incident colorectal cancer (i.e. cancer in between two surveillance colonoscopies) and is associated with somatic *CTNNB1* variants.[8,10] This direct pathway to cancer without a benign precursor seems to be lacking in *PMS2* carriers. It is still unknown why different (molecular) pathways exist for the different MMR genes. *CTNNB1*-hotspot variants were also analyzed in the small bowel cancer cohort in this thesis. The number of Lynch syndrome related small bowel cancers was too small to power a reliable analysis of the molecular pathways, but the only tumor carrying a *CTNNB1* pathogenic variant was from an *MLH1* patient (unpublished data). Further insights into the pathogenesis of small bowel cancer may provide us with clues to identify those individuals at greater risk who may indeed benefit from surveillance measures. Known risk factors for the development of small bowel cancer in the general population are the presence of Crohn's disease and celiac disease.[11] Lifestyle related

factors that influence the risk of sporadic small bowel cancers are similar to those for cancer of the colon and include alcohol consumption, smoking and the consumption of red meat.[11] Should these lifestyle factors influence Lynch syndrome and the risk of small bowel cancer, they might act as a way to preselect patients with higher a-priori risks. However, currently, it is unknown whether these are also risks factors in Lynch syndrome-related small bowel cancer.

Our findings in small bowel cancer are also relevant for therapy purposes. Since MMR deficient colorectal cancers are highly immunogenic, they are a good target for immune checkpoint inhibition through treatment with PD1/PD-L1 blockers[12-14] and evidence is emerging that this is also the case for other MMR deficient tumors, including small bowel cancer.[15] Hence, patients may benefit from the knowledge that their tumor is MMR deficient, regardless of its etiology (*i.e.* sporadic due to two somatic mutations or Lynch syndrome-related).

While a convincing argument can be made to start universal screening for MMR deficiency in all small bowel carcinomas, this is different for ovarian cancers. In our case series (n=54) of high-grade serous ovarian cancer, there we no cases of MMR deficiency. These results corroborate the guidelines as suggested by, among others, Chui et al.[16] and Zeimet et al.[17] to only perform MMR deficiency screening in specific histological subtypes of ovarian cancer (*i.e.* endometroid and clear-cell ovarian cancer). Although MMR deficiency has been described by others in relatively high frequencies in serous ovarian cancers,[18] this is potentially due to misclassification of the histological subtype of these cancers in the past. Classification of the histological subtypes in ovarian cancer is known to be challenging and inter-observer variability has been described.[19] Over the recent years significant improvements have been made in histological subtyping of ovarian cancers which may influence the conclusions drawn in previous studies.[20,21] The tumors in our cohort have undergone central pathology review according to the latest World Health Organisation (WHO) guidelines, while many of the previously reported cases series have either been published before the most recent guidelines and/or do not specifically state that central pathology review was performed on their samples. This hypothesis also explains why in Lynch syndrome cohorts with ovarian cancer still a relatively large proportion (22% - 36%) of serous ovarian cancers is described.[22-24] An interesting follow-up study to provide further support to restrict screening for MMR deficiency to non-serous ovarian cancers would be to reclassify Lynch syndrome-associated ovarian tumors from previous studies according to current standards. Another angle would be to evaluate MMR deficiency status of serous ovarian cancers

in Lynch syndrome patients, since they could also be sporadic ovarian cancers that occurred by chance in a Lynch syndrome patient and are not related to the germline mutation.[24] Drawing firm conclusions from these results regarding an association with the germline MMR mutation will however be challenging. A similar discussion has been going on for the possible association of breast cancer with Lynch syndrome. Although quite a large proportion (65%) of breast cancers that have occurred in Lynch syndrome patients show MMR deficiency,[25] this has still not put the debate to rest. Presence of MMR deficiency is still not iron-clad proof that it is causally related to the development of the tumour.
In daily clinical practice caution is warranted when excluding Lynch syndrome as a differential diagnosis in a patient with serous ovarian cancer. Particularly if the diagnosis was made several years ago, histopathological review according to current standards should be considered.

Phenotype (Part II)

While improving the detection of Lynch syndrome through strategies such as universal tumor testing is an important field of investigation, it is equally important to gain further insight in cancer risk, surveillance strategies and molecular pathways that are involved in the development of Lynch syndrome-associated tumors. All these elements are crucial to be able to offer adequate surveillance programs to a newly identified carrier of a pathogenic MMR variant.

The cancer risk analyses as published in chapter 6 of this thesis provide important evidence by using a novel risk estimation approach that supports previous publications on the low cancer risks in *PMS2*- and *MSH6*-associated Lynch syndrome.[2,3,5,6,26-28] In older publications, colorectal cancer risks were estimated to be as high as 70% in Lynch syndrome patients[5] and up until recently the same cancer risks for all four genes were communicated to patients. Recent literature, however, shows that these early studies may have overestimated true cancer risks in general due to bias.[29] Also, cancer risks in *PMS2* and *MSH6* carriers are lower than those in *MLH1* and *MSH2*.[2,5,6,26] The more recent publications on risk estimation for Lynch syndrome, use statistical approaches (such as modified segregation analysis) to correct for ascertainment bias.[2,6,26] The downside of these statistical approaches is potential overcorrection. In chapter 6 of this thesis we describe a cohort of families that were ascertained through the CMMRD phenotype of the index patient instead of a family history suspect for Lynch syndrome, thus circumventing the need for complicated statistical approaches to correct for ascertainment bias. The results from this study show that cumulative

colorectal cancer risk at age 70 lies between 4.3 and 12.7% for PMS2 and between 4.5 and 22.7% for MSH6.[30] Together with previous reports that used statistical approaches, these estimates can be used to adapt surveillance guidelines in Lynch syndrome by making them gene-specific.

To further improve cancer risk estimations, also for the rarer types of Lynch syndrome-associated cancer, initiatives were developed to gather large amounts of data on Lynch syndrome families. In the near future these initiatives, such as the Colon Cancer Family Registry (CCFR, https://www.coloncfr.org/)[31] and the Prospective Lynch Syndrome Database (PLSD, https://www.plsd.eu) are expected to provide us with detailed data and risk estimations and indeed, some of the first results have already been published.[3,27,28,32] Although these initiatives are large enough to stratify risk estimations, not only per gene, gender and other factors such as country, much knowledge is also still to be gained on 1) why cancer risks are so different for the different genes and 2) why cancer risks can be so different even for carriers of mutations within the same gene. The genotype-phenotype study presented in chapter 7 of this thesis suggests that part of the explanation of risk differences within one gene (in this case *PMS2)* may lie within the type of mutation (genotype). Although no significant differences in colorectal cancer risk were identified between the genotype groups (hazard ratio: 1.31, P = 0.38), there was a lower age at colorectal cancer diagnosis in those with a variant that results in loss of RNA expression compared to those with a variant with retained RNA expression (mean age at colorectal cancer diagnosis of 51.1 versus 60.0 years).[33] However, further confirmation of any such correlation is yet needed since our results are limited by the amount of patients that could be included in the analyses at that time. Furthermore, a recent paper on genotype-phenotype associations in *MLH1*-related Lynch syndrome suggested a different type of phenotype-genotype correlation.[34] Based on their data, Ryan *et al*. suggest that the age of onset of endometrial cancer for *MLH1* is later for those with a truncation mutation versus those with a missense mutation, potentially indicating a dominant negative effect of missense *MLH1* mutations.[34] While this is an interesting finding, even leading the authors to propose genotype-specific gynecological surveillance, these data are in striking contrast to the genotype-phenotype correlation that we identified in our cohort for colorectal cancer (with a later age at onset for carriers of a mutation that shows retained RNA expression). A possible explanation for the discrepancy between the results in chapter 7 of this thesis and the study by Ryan et al.[34] could be a bias, due to the analysis of index and non-index patients together.[35] Index patients tend to be patients with the most severe phenotype in the family. If there is a relative overrepresentation of index

patients in either one of the genotype-subgroups as presented by Ryan et al., then this could explain any differences found between genotype-subgroups. Similarly, it can be debated whether the way mutations were grouped according to genotype (missense versus truncation mutations) is the correct method. Careful consideration should be given to categorizing mutations into different genotype groups. Although it may seem logical and intuitive to group mutations according to missense versus truncating variants, the truth is likely more complicated since certain truncating mutations may indeed result in nonsense mediated decay, while others (e.g. in frame exon deletions in *PMS2*) may still result in a protein with potential residual activity. On the other hand, missense variants within a specific domain of the gene or with an effect on splicing may be just as detrimental to protein function (or even cause a dominant negative effect as suggested by Ryan et al)[34] as a truncating mutation. Therefore, to suggest that genotype-phenotype correlations can be implemented in screening guidelines is preliminary. Further evidence is first needed to substantiate any genotype-phenotypes correlations. The aforementioned databases (CCFR and PLSD) may provide a good dataset to perform such studies.

Other mechanisms that have been suggested to explain risk differences within the same MMR gene are parent of origin effect,[36] anticipation,[37] SNPs,[38-40], gut microbiome[41] and lifestyle factors such as smoking and body mass index.[42-45] Since a parent of origin effect could not be identified in our *PMS2* cohort (chapter 7, this thesis*)* and there is no biological mechanism that could explain such an effect this is a factor that is unlikely to truly influence cancer risk. Anticipation is an unlikely factor for similar reasons; there is lack of a biological explanation for an anticipation effect and, as demonstrated recently by our research group, apparent anticipation effects in previous publications are more likely to be caused by a form of bias or a cohort effect.[46]

The genotype-phenotype manuscript in this thesis focused on any such correlations for the *PMS2* gene, but much knowledge is also still to be gained on genotype-phenotype correlations between the different MMR genes. For a long time these genotype-phenotype studies mainly focused on clinical phenotype (i.e. cancer risk). However, it is now generally accepted that the clinical phenotype is very different for the different genes (with higher cancer risks for *MLH1* and *MSH2,* moderate to low cancer risks for *MSH6* and low risks for *PMS2*). As briefly discussed above in the context of small bowel cancer; an exciting and relatively new research field that focusses more on the etiology behind these risk differences, is the molecular tumor analysis. Data generated by these molecular analyses are being used to understand the different pathways that

can eventually cause a normal colon mucosa crypt to develop into cancer.[8-10,47,48] These pathways are not only studied to understand carcinogenesis for Lynch syndrome in general, but also to attempt to understand why differences in phenotype between the different genes exist, despite the fact that they are all part of the same MMR complex.[8] In chapter 8 of this thesis, we show that the risk of incident colorectal cancer in *PMS2* carriers is very low, particularly compared to the other MMR genes. We also describe the number of adenomas in our cohort and the 10-year cumulative risk of developing an adenoma or advanced adenoma after start of surveillance. These data are however more difficult to compare to previously published data on the other MMR genes, due to differences in cohort characteristics (age at first colonoscopy) and analyses methods. Absence of MMR deficiency in the 16 adenomas in our cohort together with previously published molecular data,[8] suggest that *PMS2*-associated colorectal cancer mainly develops through the MMR proficient adenoma-carcinoma pathway, while colon cancers in *MLH1*- mutation carriers are thought to develop primarily from MMR deficient crypts without going through an adenoma stage.[8,10] However, we also identified a relatively high 10-year adenoma risk compared to the other MMR genes as published by others.[49] If *PMS2* variants predispose to the development of more adenomas, than this would not fit within this molecular pathway hypothesis. Potentially, this relatively high adenoma risk is (partly) explained by a higher age at colonoscopy in our cohort, but further analyses and collaboration initiatives will have to prove this. Additionally, our *PMS2* cohort may be enriched for adenoma risk factors due to ascertainment bias.

Clinical and molecular evidence brings us closer to understanding what the differences in pathogenesis and tumor development are between the genes, but it still does not explain how these differences fundamentally develop. In other words: it does not explain why mutations in genes from the same MMR machinery result in different molecular pathways in the development of a tumor. It has been hypothesized that the function of the PMS2 and MSH6 proteins within the complex can in part be taken over by other proteins such as MSH3 and PMS1, while MLH1 and MSH2 lack such a back-up system.[8,50,51] In line with the theory of a back-up system, Morak et al. suggest that pathogenic variants in *MSH3* might even aggravate the *MSH6* phenotype, even though *MSH3* heterozygous variants are not enough to cause a phenotype by themselves.[52] While a back-up system seems a plausible explanation for the differences between the different genes, further evidence is still needed to support this by showing that the mutation rate and microsatellite instability are indeed lower in cells from *PMS2*- and *MSH6*-variant carriers. For this purpose, we recently analyzed the microsatellite

instability patterns of *PMS2*-associated colon tumors in coding microsatellites and compared them to the patterns in other MMR deficient cancer, but we did not identify any significant differences (ten Broeke et al, unpublished data). This type of research is however challenged by the fact that tumors, once developed, are likely to show similar mutational patterns due to selection pressure (i.e. only those cells with a sufficient number of mutations in the right combination of genes will become clinically evident as tumors). A follow-up study is therefore needed to also analyze non-coding microsatellites. In addition, an interesting field of research would be the analysis of molecular changes in different tissues from Lynch syndrome patients, from normal mucosa, MMR deficient crypts, low- and high-grade adenomas to invasive cancers.

A completely different challenge that lies ahead is the interpretation of variants of unknown significance (VUS). Molecular geneticists and clinicians are faced with these VUSs and their dilemmas all across the different disciplines within the field of clinical genetics. If a VUS is identified the question remains whether the phenotype in the patient and/or family has been explained by this finding. But, even more importantly in the field of oncogenetics, it also poses the question of how to manage these patients and their family members. Can the variant be used to discern those with an increased cancer risk from those with an average risk? Should variant carriers be following surveillance as if they have Lynch syndrome or is a milder regime more appropriate? Luckily, several in vitro analyses have been developed that can aid in the classification of any such VUS.[53-60] While these functional analyses are very useful, there are also some drawbacks. First, these analyses are labor intensive and time-consuming before results can be used in clinical practice. Furthermore, not all functional tests are suited for all different types of variants (e.g. splice variants)[55]. Compared to interpreting VUS in some other genes, the advantage of the MMR genes is that there are also clues from the tumor that can be used to further interpret the variant.[61] Particularly if several family members are affected, segregation of the variant along with a MMR deficient tumor phenotype can provide a strong clue towards pathogenicity. Unfortunately, segregation is not always possible. An additional and relatively new valuable source of data to help give some direction in classifying a VUS is the molecular analyses of the tumor of the index patient. If a second hit has occurred in the tumor on top of the VUS, this may be a clue that the VUS is actually pathogenic, while if molecular analysis of the tumor shows two additional pathogenic somatic hits that explain the MMR deficient phenotype this may be a strong argument against pathogenicity of the VUS.[62] While conceptionally this seems like a straight forward principle, more research is needed to establish how much weight can be given to evidence such as this.[62] For example, while loss of heterozygosity is a common second hit that could explain the

MMR deficiency if it occurs on top of a potentially pathogenic VUS, it could also be a consequence of a more generalized, non-specific chromosomal event.[62] Furthermore, tumor heterogeneity may cause different parts of the tumor to have different second hits, which would mean the presence of three variants (the VUS plus two additional pathogenic variants) in the tumor does not necessarily argue against pathogenicity of the VUS.

CONSTITUTIONAL MISMATCH REPAIR DEFICIENCY (CMMRD)

While it seems logical to improve detection of germline heterozygous MMR variants because of clear consequences for clinical management, much more discussion can be held on improving the detection of individuals with CMMRD. In chapter 2 and 3 of this thesis, relevant considerations and literature are discussed to come to appropriate testing guidelines to improve the detection of CMMRD in healthy individuals. However, as also indicated in these chapters, much of the literature that was used to base these guidelines on is still limited by publication bias and selection bias. Furthermore, surveillance guidelines are yet to be proven to be effective. One step forward to providing more evidence in support of the testing criteria has already been made since their publication. When formulating the guidelines, it was estimated that the prevalence of CMMRD in children suspected of NF1, but without a germline *NF1* pathogenic variant, is 0.4%. Recently, this estimation was confirmed by analyzing the prevalence of CMMRD in a large cohort (n=735) of children suspected of NF1 but lacking an *NF1/SPRED1* pathogenic variant. The prevalence of CMMRD in this cohort was 0.41%.[63]

More research is still needed to evaluate whether these newly diagnosed patients and their parents indeed benefit from such a diagnosis.

There are two large, international research consortia that are focused on CMMRD: the International Biallelic Mismatch Repair Deficiency Consortium, which is in an initiative from Canada, and the European 'Care for CMMRD' (C4CMMRD) consortium. The guidelines as outlined in chapter 3 are supported by the C4CMMRD consortium and at their latest meeting a study proposal was presented to prospectively evaluate the guidelines in order to establish how many CMMRD diagnoses are being made based on these guidelines and whether there is room for improvement of the testing criteria.[64]

Whether an early diagnosis is actually beneficial for the patient and their family members is perhaps more difficult to establish. Any such answer should not only take into

account whether surveillance measures are indeed effective, but should also consider quality of life of the patient and his/her parents. Both the Canadian consortium and the C4CMMRD consortium are evaluating the outcomes of their surveillance programs.[64-68] One of the difficulties is that there are also attenuated forms of CMMRD where cancer does not tend to develop until adulthood.[69] As a consequence, surveillance programs may be appropriate for one patient, while they may be overkill for another and cause unnecessary medicalization and stress. In the future genotype-phenotype correlations, as researched in Lynch syndrome, will hopefully provide clinicians with some guidance to predict phenotype severity also in CMMRD.

An area of study that has not been explored up to now in CMMRD is the psychological impact of the diagnosis and subsequent surveillance measures. While some lessons can be learned from other cancer predisposition syndromes such as Li-Fraumeni syndrome, which is also characterized by high cancer risks and may become manifest through a childhood malignancy,[70,71] the situation is still not completely comparable; CMMRD presents itself predominantly during childhood and has a recessive, rather than a dominant inheritance pattern.[72,73] Future studies should therefore map the psychological burden and quality of life of CMMRD patients with a diagnosis, comparing those diagnosed after they have developed cancer versus those that were diagnosed when they were still healthy. Data from these studies can then be taken into account in testing strategies and surveillance programs.[64]

In addition, while one of the arguments for an early CMMRD diagnosis is the possibility for parents to think about family planning and use the opportunity to use preimplantation genetic diagnostics (PGD), it remains to be seen whether parents will indeed use PGD. While PGD is available for Li-Fraumeni families in the Netherlands, thus far only six couples have gone through the process of using this technique to prevent a germline *TP53* mutation in their offspring.[74]

Concluding remarks

In conclusion, the work described in this thesis explores opportunities to further improve detection of germline pathogenic variants in the MMR genes, both in the setting of Lynch syndrome and CMMRD. Furthermore, an effort has been made to learn more about the phenotype of these germline variants, since identification of germline variants will only be of help to the patient if evidence based surveillance guidelines are available. Future research should focus on providing evidence for further tailoring of surveillance guidelines (ideally on an individual level) and improvement of ways to classify variants of unknown significance.

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