Chapter 6

General discussion
Although Lynch syndrome was one of the first cancer syndromes described, to date it is still underdiagnosed. To aid the identification of Lynch syndrome patients, several sets of criteria were developed. Unfortunately, even the most recent set, the revised Bethesda criteria lacks specificity and is therefore insufficient for use in the clinic. Therefore, the Mallorca group recommends investigation of all CRCs (in individuals <70 years) by immunohistochemistry of the four main MMR proteins or MSI. A recent study showed this recommended screening to be cost effective. This will increase the number of detected tumors with MMR abnormalities and as a result MMR genes will be sequenced more often. In addition, considering the advancements in sequencing technology especially next generation sequencing (Tafe (2015) and references therein), it is not unlikely that direct sequencing of the four main MMR genes without prescreening for MSI and IHC will become cost efficient and therefore more widely applied in a diagnostic setting. With increased sequencing of the MMR genes, the number of VUS that are found will also go up. This increase may even be disproportionately larger than the increase of clear pathogenic mutations. With the early, more stringent criteria, the large majority of families screened for LS were families with a prominent phenotype, conferring a bias towards overt deleterious mutations. When the threshold for sequencing is lowered, families with a milder cancer predisposition will more frequently undergo genetic testing. One may expect the number of VUS found in these families to be relatively large because, as we have shown in chapter 4, some missense mutations result in a partial MMR defect which can be reflected by a reduced penetrance and hence a less obvious family history of cancer. Also, CMMRD patients often carry a combination of weak alleles, that predispose to LS-related cancers at later age. As heterozygous inheritance of such mutations often does not manifest as LS, functional testing is imperative to diagnose CMMRD.

**Implications of a VUS**

When a VUS is found in one of the MMR genes, the implications on counselling and screening are significant. As long as the effect of the VUS on MMR functionality is unclear, there is nothing to be gained from testing other healthy family members for this VUS: as non-carriers cannot be safely excluded from screening, all family members are advised to undergo regular screening for CRC and EC. This screening is done by colonoscopy every 1-2 years starting from age 20-25 years and annual gynaecological surveillance for women. These screening programs have shown their merits through a 62% reduction in CRC incidence and a 65-70% decreased mortality. However the methods used for screening are fairly intrusive and cause both physical and mental distress. This is illustrated by the study
of Petersen\textsuperscript{12} in which some LS patients reported a consideration not to undergo colonoscopies because of pain and discomfort although most did not question the recommendation of surveillance. Similarly, both a Dutch\textsuperscript{13} and a Finnish study\textsuperscript{14} found significant delays (more than 1 year) between two colonoscopies for 25\% and 17\% of LS mutation carriers, respectively, in spite of the generally high screening compliance.

Several studies have shown that 6-30\% of individuals who undergo genetic counselling for CRC experience serious cancer-specific worries or clinically relevant levels of distress (\textsuperscript{15}and references therein). However, the extent of distress depends on whether the individual is a carrier or a non-carrier of a pathogenic mutation and on whether they have been personally affected by CRC or EC. When a mutation can be classified as pathogenic, non-carriers can be safely excluded from the burdensome screening. Furthermore, it has been shown that genetic testing decreases short- and long-term colon cancer anxiety in unaffected (\textit{i.e.} no history of CRC) non-carriers. For unaffected carriers of a pathogenic LS mutation on the other hand, studies have shown varying results. Many report only short-term increases or no change in distress upon genetic testing (reviewed by \textsuperscript{15}). A recent Danish study however, showed that the majority of unaffected carriers feels optimistic about the future, mainly because of the available surveillance programs\textsuperscript{12}. To date only very little is known about the psychological impact of genetic testing among LS cancer patients who received either positive (\textit{i.e.} carrying a pathogenic mutation) or uninformative results, which includes patients in which a VUS was found in one of the MMR genes\textsuperscript{15}.

One small but recent study by Katz \textit{et al.}\textsuperscript{16} reported on screening adherence and cancer risk perceptions in patients with CRC that displayed LS-like characteristics, being MSI-high status and negative IHC for at least one of the four main MMR genes, \textit{MLH1}, \textit{PMS2}, \textit{MSH2} or \textit{MSH6}. Genetic testing in these individuals had revealed no pathogenic MMR mutation and \textit{MLH1} promoter methylation was excluded. Patients with an MMR VUS were also included in this group. These patients had received the same screening recommendations as patients with proven LS. Screening compliance in this group was high, just as it is for LS patients. However, only 41\% had correctly understood the results of the genetic test as uninformative negative (no mutation found) or VUS. Due to the very limited data available, it is unclear if carriers of a MMR VUS in general experience the same lack of understanding of their genetic tests results. If so, this is particularly worrisome as the patient is primarily responsible for informing family members, although he/she is supported in this process by clinical geneticists and/or genetic counsellors\textsuperscript{17}. In the study by Katz \textit{et al.}\textsuperscript{16}, most patients did inform family to undergo CRC screening (\textit{i.e.}
colonoscopy) but only one third advised relatives to undergo genetic counselling (i.e. consulting a clinical geneticist, mutation testing) and EC screening was only advised in 50% of the cases.

A perceived lack of understanding of the implications of a VUS was also reported by genetic counsellors for BRCA VUS carriers in the US. They felt confident about their own understanding of VUS results but less certain about the level of understanding achieved in their patients\textsuperscript{18}. This difficulty from a patient’s perspective to understand what a VUS means has also been reported in another study, especially in patients with lower educational background\textsuperscript{19}. It has also been shown that patients find genetic counselling less reassuring and informative when a VUS is found compared to an uninformative negative (i.e. no mutation found). Furthermore, the group of VUS patients reported a smaller reduction in cancer distress compared to the uninformative negative group\textsuperscript{20}. Other studies have looked at risk-reducing strategies among women with a BRCA VUS compared to woman with a BRCA deleterious mutation. They found that women with a VUS less often undergo risk-reducing surgery\textsuperscript{21-23} and furthermore saw a lower screening compliancy for the VUS group in the first year after diagnosis\textsuperscript{21}. If we assume that these differences are largely due to the uncertain effect the VUS has on gene function, it is likely that a VUS found in one of the MMR genes would exert similar effects on the patient’s mental wellbeing and screening compliance. This seems contradictory to the results of the Katz study, which showed high screening compliance. However, in their study, patients with uninformative negative results were in the same group as patients with a VUS which would dilute possible differences between VUS carriers and carriers of a deleterious mutation.

Taken together, some of the burdens that come with hereditary cancer will remain in spite of knowing the nature of the defect found, but if a VUS can be classified as pathogenic, it will make a great difference for unaffected non-carriers and they can safely be excluded from further screening. For carriers, the effect of pathogenic classification may vary but they will be able to make better-informed decisions, which may benefit screening compliance. For woman, proper classification may enable them to decide to undergo a prophylactic hysterectomy once they feel their family is complete. Furthermore, one study reported that 20% of women undergoing genetic testing for LS would consider having children at a younger age in order to undergo this surgery earlier\textsuperscript{24}.

**Variant classification**

Reliable classification of VUS is clearly extremely important. Unfortunately, it has also proven to be very challenging, especially because the high level of certainty that has to be
achieved in order to safely exclude non-carriers from screening. Many efforts have been taken using different approaches and a system of five classes was developed that provides an estimation of the chance that a MMR gene variant is pathogenic or not, combined with clinical recommendations for each class. The classes in this system are: class 5 (> 99% probability of pathogenicity), class 4 (likely pathogenic, 95-99% probability of pathogenicity), class 3 (uncertain, 5-95% probability of pathogenicity), class 2 (likely neutral, 0.1-5% probability of pathogenicity) and class 1 (neutral < 0.1% probability of pathogenicity). Recently, the Variant Interpretation Committee from the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) used clinical data and data from functional assays, both published and unpublished, to classify many variants using this system of five classes. In spite of this massive effort, about one third of variants still had to be assigned to class 3 and therefore retained the VUS status. This remaining class 3 group largely consists of missense mutations.

The data used for classification generally comes from five different categories:
- Family history, e.g. co-segregation of the variant with disease
- Clinical data, e.g. MSI status of the tumor and IHC staining for the four main MMR genes
- In silico assays which predict the effect of a mutation based on physiochemical characteristics and conservation between species
- In vitro assays which study the effect of in vitro produced variant protein in cell-free systems
- In vivo assays in which the variant is produced either exogenously from a plasmid or endogenously by modifying the gene. These assays are done using a variety of different systems such as yeast, E. coli, human cancer cell lines and mouse embryonic stem cells (ESCs) (this thesis).

The large number of variants that still had to be classified as “uncertain” (class 3) indicates that either the above-mentioned methods are insufficient for proper classification or that currently not enough data is available. Most likely it’s a combination of both. Family history can be very helpful in classification but today families are on average smaller and it is hard to obtain reliable data from distant or (long) deceased family members. Because of this, the large amount of family data that is necessary for a reliable classification often cannot be obtained. Clinical data is highly instrumental in detecting full MMR deficiency since this will give rise to MSI and a lack of (nuclear) staining in IHC assays. Deficient MMR can also be caused by promoter methylation of MLH1, which is seen in a small percentage of sporadic CRC but can easily be excluded and should not influence LS diagnosis and VUS classification. There are however other
factors that can hamper reliable assessment of MMR function. As we show in this thesis in chapter 4, both MSI status and IHC staining can vary between tumours from carriers with the same VUS, within the same family. In this case this was most likely due to a partial MMR defect caused by a low-penetrance VUS. If the proband’s tumor shows no MSI or normal IHC staining, such a partial defect may easily be missed.

The advances in in silico analysis in the last decade have been very large. However, the quality of the programs used is still insufficient for them to be used as a standalone classification method. The reliability highly depends on the alignment that is used. As we saw in chapter 4 for the MSH2-Y165D mutation, the use of the protein sequence from the outbred Sprague–Dawley rat strain, which appears to be the only sequenced mammal that has an aspartic acid at this position, caused the YD variant to be classified as polymorphic by the PolyPhen, SIFT and Align-GVGD analysis. This is in stark contrast with the clinical data and our results, which showed a clear, albeit partial, MMR defect (chapter 4). One could use the in silico data to argue against the reliability of our results but a study by Drost et al. 27 showed a complete loss of MMR function for this variant using a cell-free system based on the in vitro produced human MMR protein, thereby confirming the clinical data as well as our results. In spite of the current advancement, in silico programs are still in need of strong validation before they can be used reliably for VUS classification.

In vitro and in vivo assays can be used to closely mimic the human in vivo situation, however it is very challenging to capture all aspects relevant for proper classification in one system. Systems using distantly related species, such as yeast and E. coli, benefit from the relative ease with which they can be manipulated, but often lack sufficient homology to make reliable comparisons between the assay results and the human situation. When the variant protein is ectopically expressed, which is often the case in these systems, expression levels are generally high. These high expression levels may mask partial defects. Furthermore, the post-translational modifications in distantly related species may be different which could, among others, affect protein interaction and hence the outcome of the assay. Systems that use human cell lines for VUS analysis do not suffer from a lack of conservation or differences in post-translational modifications. However, VUS analyses in human cell lines are generally done in MMR-deficient cancer cell lines, complemented with expression vectors for the variant MMR protein. The unstable genetic background of these cancer cell lines could influence the results. In addition, also the human cancer cell line systems are based on ectopically expressed protein, thereby making them unreliable for detecting mild or partial defects.
A recent addition to the spectrum of classification assays is an improved version of an in vitro cell-free MMR assay, in which human variant proteins are used to complement nuclear extracts from cell lines missing a specific MMR protein. This system eliminates the disadvantages of the unpredictable genetic background of the cancer cell lines and is relatively quick. Unfortunately, only a limited number of assays to test MMR function can be performed in a cell-free system and non-physiological protein levels remain a concern. The system can be used to test the repair capacity of the variant protein but not the other functions of the MMR proteins.

Currently, family, clinical and in silico data are combined with data from functional in vitro and in vivo assays in order to come to a classification. However, even with this effort, a large proportion of variants still remained unclassified, possibly also due to the limited amount of functional data available. On top of that, the majority of functional data published to date suffers from one or more of the pitfalls mentioned above, with lack of conservation and non-physiological expression levels being the main concerns, which complicates interpretation of results.

This thesis

The set-up used in this thesis aimed to circumvent both these concerns by using a highly homologous system (mouse ESCs) and physiological expression levels obtained through the oligotargeting technique that allows the variant to be introduced in the endogenous allele. Our study focused on VUS found in MSH2 and MSH6. Because the mouse and human proteins share around 90% identity at amino acid level, this enables us to study the vast majority of VUS found in these two genes. Expression from the endogenous gene ensures physiological expression of the variant protein, and also reveals effects of the VUS on protein expression levels and protein stability. Because our system generates cells expressing the variant protein, we can study not only the mismatch repair capacity of the variant MMR protein but also the ability to prevent recombination between similar but not identical sequences (i.e. homologous recombination). In addition, also the mediation of toxicity of DNA damaging agents, in particular methylating agents, can be studied using our variant ESCs.

Mouse lines harboring MMR VUS

A major advantage of the use of oligotargeting in mouse ESCs is the possibility to generate mutant mouse lines, which we exploited in chapter 5. Especially in those cases where only a partial defect is found, the generation of a mouse line can be very valuable since it allows studying the effect of the variant on tumor development. The effects of full defects of each of the four main MMR genes on tumor development in mice are already known through the study of conventional knockout lines. It can
be expected that variants which cause complete loss of all MMR functions in our functional cellular assays, (i.e., behave similarly to a full knockout) will exert a similar effect as their conventional knockout counterparts on survival and tumor development in a living animal. But for partial defects, the in vivo effects are much harder to predict. Although our system mimics the human MMR system in many aspects, it still remains a cell-based system and cannot be used to deduce predictions about tumor development. Therefore the ability to make a mutant mouse line of variants with partial MMR defects can be a great help in classification of these variants. Currently, several labs are putting effort in making mouse lines that more closely mimic the situation in LS patients than the conventional full knockout lines. LS is an autosomal dominant disorder (i.e., inheritance of one mutant allele is sufficient to predispose to CRC and EC) although the wild-type allele has to be lost due to a separate event in order for tumor development to take place. In mice it seems this second loss of heterozygosity event hardly takes place, possibly due to their short lifespan, since Msh2+/− and Mlh1+/− mice are not or hardly cancer prone in contrast to their full knockout littersmates. In addition, full MMR knockout mice mainly develop lymphomas early in life contrasting the predisposition to CRC and EC seen in LS patients. Mouse models that utilize a cre-lox system to ensure MMR function is only lost in the intestines avoid this issue and have been developed. More recently, an improved cre-lox based mouse model was published in which MMR loss is activated in only a small (±5%) percentage of the intestinal crypts. This closely mimics the human situation and combining this model with our variant mouse lines would even further improve the added value of a mouse line for classification.

**Classification of the studied VUS**

In this thesis we have employed the oligotargeting technique to study the effects of eight MSH2 and four MSH6 VUS. In chapters 2 and 3 we started with variants we selected from the literature and included known polymorphic and pathogenic variants as a proof of principle. We showed our results to be consistent with clinical data. In silico data generally came to the same classification with the exception of the MSH6-P1087R variant which based on in silico data was classified as pathogenic, while our in vivo data indicated a fully functional variant protein in spite of the reduced protein levels. Full MMR functionality of this variant was also found in a study using a cell-free human expression system. Our classifications of the MSH2-Y103C and MSH2-M688I as polymorphic was in accordance with in silico data and furthermore confirmed by the study of Houlleberghs et al. which is described in more detail below. Surprisingly, these two MSH2 variants and all three MSH6 variants studied in these chapters are still listed as class 3
in the InSiGHT database (http://insight-group.org/). In case of the MSH6 variants this may be explained because the paper describing these variants was published after the listed classification date, which may suggest that a reclassification could alter their status. For both MSH2 variants however, this was not the case and the paper describing our result was mentioned under the available data. In spite of this, the “summary justification” was listed as insufficient evidence.

In chapter 4 we aimed to classify VUS found in the clinic in collaboration with clinical geneticist and counsellors from three clinical genetics centres in the Netherlands. We analysed one MSH2 VUS from each centre and found our results to be consistent with the clinical data. While one variant showed full MMR functionality (MSH2-M813V), the two other variants showed a partial defect, which, to our knowledge, has not been reported for a MMR VUS before. The InSiGHT database assigns the MSH2-M813V variant to class 2, likely polymorphic. Most likely, our data did not contribute to this classification as the classification date precedes the publication of our data and the paper is not mentioned. This is also the case for the MSH2-Q690E variant, which showed a clear albeit partial MMR defect in our assays. To date this variant is listed as class 3 but a re-classification may change this to class 4 (likely pathogenic). This reclassification has already taken place for the MSH2-Y165D variant that was first listed as class 3 but is now a class 4 variant. It can be assumed that this reclassification is at least in part due to our results as our paper and our conclusions are mentioned in this listing.

A critical note

Although we believe our method yields reliable results, a downside is that it is time consuming and labour intensive. Furthermore, it can be difficult to implement our method in clinical labs due to the specific technologies involved, in particular ESC culture and oligo targeting. A possible caveat of our approach is that embryonic stem cells exhibit high levels of MMR gene expression. However, we feel that it is unlikely that this will mask partial defects since (i) we detected partially defective MSH2 variants, and (ii) MNNG sensitivity was already reduced in Msh2+/− (chapter 2) and Msh6+/− (chapter 3) cells, which is indicative for a (mild) haplo-insufficiency of Msh2 and Msh6 in this assay despite high protein levels. Furthermore, MSH2 levels seem also high in the Lgr5-expressing stem cells in the intestinal crypt 40.

Another problem may be that by substituting 3-4 nucleotides, codons may be created with low usage that could affect protein levels. We studied this for the MSH2-P622L mutant. Originally, the PL mutant was created using a leucine codon with a relatively low codon usage (13.3). We therefore recreated this variant using the leucine codon with the highest codon usage (40.0). We found no difference in
expression levels, which makes potential effects of codon usage on our assay results unlikely. In addition, the reproducibility of our approach has been shown by the fact that recreating the MSH2-YD and MSH2-QE variants in another study using a slightly different setup that allowed substitution of a single base-pair, showed similar results in the functional assays.

**Room for improvement**

Due to advancements in technology, a main downside of our approach, the time consuming and labour intensive aspects, can now be significantly improved while maintaining the major plus side, the endogenous expression in a highly homologous system that enables the creation of a mutant mouse line. Houlleberghs et al. have accelerated the targeting procedure by creating a hemizygous cell line, which circumvents the need to inactivate the wild-type allele. They also used an improved version of oligotargeting that increases the efficiency through the use of modified oligonucleotides and a different transfection agent. Furthermore, their set-up uses a pre-selection system based on exposure to 6-TG, which is able to select for MMR deficient cell lines and is sensitive enough to still detect variants that give a partial resistance to 6-TG such as MSH2-Y165D and MSH2-Q690E. All of this combined greatly reduces the time and effort involved in the making and analysis of MMR variant ES cell lines, which has a large impact on the applicability of the method.

Next to these important improvements, it would be interesting to implement the recently developed CRISPR/Cas9 technology as an alternative for oligotargeting, to speed up the targeting procedure. This technology makes use of the Cas9 enzyme which has site-specific dsDNA nuclease activity. The Cas9 protein can be targeted to specific genomic loci by a 20-nucleotide guide RNA molecule that hybridises to the DNA and forms a complex with the Cas9 protein. Delivering the Cas9 and the guide RNA into the cells causes a site-specific double-strand break (DSB) at the target locus, which activates the endogenous DSB repair machinery. By simultaneously providing donor DNA, e.g. a single-stranded oligonucleotide, with homology to the target locus the DSB will be repaired by the homology directed repair pathway, thereby allowing the efficient introduction of precise mutations instructed by the donor DNA. Improved delivery methods have increased the efficiency of this method to 70% in mouse ESCs, and 64% and 87% in human ESCs and iPSCs, respectively.

Not only the process of making the variant cell lines can be improved using the most recent technologies, also the assays used for functional analysis leave room for improvement. The MSI analysis as conducted in this thesis is also fairly time consuming since it involves culturing a single cell for a specific number of divisions and subsequent analysis of the length of several MSI markers in a large number
of single cell clones derived from the expanded culture. Use of a reporter gene could facilitate MSI analysis. This could be a fluorescent reporter such as green fluorescent protein (GFP) rendered out of frame due to a microsatellite sequence preceding its ORF. Instability of this microsatellite in MMR-deficient cells may restore the GFP open reading frame. Hence the frequency of GFP-proficient cells reflects the MMR capacity of the variant cell line. In chapter 5 of this thesis we used a slippage reporter composed of a neomycin-resistance gene that was rendered out of frame by a (CA)$_{15}$C repeat, whereas Houleberghs et al. used a neo reporter with a (G)$_{10}$ repeat, which allowed rapid and quantitative assessment of di- and mononucleotide repeat instability, respectively.

Concluding remarks

The Thompson effort classified many MMR VUS by combining clinical and in silico data with functional analysis. In general, the first two datasets are obtained in the clinic while functional assays are mainly performed in research labs. Because of this, clinicians may not always be aware of the data obtained in the research labs, and vice versa, researchers may not always take sufficient notion of clinical data. Also, both approaches require their own specific set of skills and knowledge and their value may therefore not always be fully understood or appreciated by the other party.

We believe our approach is a valuable addendum to existing functional assays and can aid VUS classification and counselling. However, we also feel its full value can best be obtained through collaboration between the clinic and the research lab as shown in chapter 4. We therefore hope this thesis will help to lower the barrier to the clinical implementation of functional tests, which will help resolve the increasing problem of VUS found in suspected LS patients.
References


