# A simple article template

## 1 Introduction

### 1.1 Mismatch Repair (MMR) System

The MMR system is one of the many evolutionarily conserved pathways involved in the repair and maintenance of DNA. In Humans, the MMR system works via a 4 step process :

1. **Error Recognition**. Subtle changes in the DNA helical structure, typically as a result of base-mismatches, insertion/deletion (indel) loops, and DNA adducts formed by mutagens, are recognized in newly synthesized DNA, for example during DNA replication or recombination.
2. **Excision**. The error-containing region in the daughter strand is degraded by EXO1.
3. **Resynthesis**. DNA is resynthesized by DNA Pol $\delta$ or $\epsilon$ along the single-strand region left by EXO1, essentially allowing the cell to "try again".
4. **Ligation**. DNA Ligase I seals any leftover nicks in the DNA.

The initial error recognition step is mediated by proteins of the MSH, MLH, and PMS2 families which which function as heterodimeric complexes (Figure 1). Specifically, the MutS$\alpha$ (MSH2-MSH6) and MutS$\beta$ (MSH2-MSH3) complexes recognize and bind to errors with differing affinities. Upon binding to the error site, these MutS complexes recruit accessory factors which mediate the downstream steps of the MMR process. These include MutL$\alpha$ (MLH1-PMS2), MutL$\beta$ (MLH1-PMS1), and MutL$\gamma$ (MLH1-MLH3). Specifically, these MutL complexes introduce multiple nicks 5' of the error in the daughter strand, these serve as entry points for EXO1.

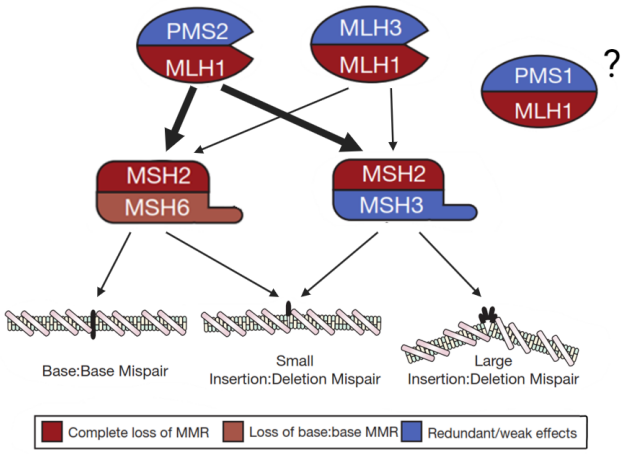


Figure 1: **Error Recognition in MMR.** While both MutS complexes are able to bind small (1-2 nt) indel loops, MutS$\alpha$ uniquely binds base mismatches while MutS$\beta$ uniquely binds larger (2-13 nt) indel loops. MutL$\alpha$ is the main accessory factor that is recruited, but can be substituted for by MutL$\beta$ while not much is known about MutL$\gamma$. Color indicates effect of protein loss.

If left unrepaired, mismatches and indel loops may develop into base and indel mutations respectively during the next cell cycle. Thus, MMR minimizes the frequency of these mutations by preventing the formation of its precursors. Loss of function in MMR genes leads to an MMR deficient (MMRd)

phenotype that is characterized by elevated mutation rates. Indeed, MMRd mice had a significantly higher indel rate (up to ∼72 fold in MLH1$^{-/-}$) compared to WT, MMR proficient (MMRp) mice [1].

### 1.1.1 Other MMR functions

Outside of its classical role, the MMR system is also involved in suppressing recombination, trinucleotide repeat (TNR) expansion, the DNA damage response, and many more. We discuss these briefly here :

1. **TNR Expansion.** Various neurological disorders are thought to be caused by TNR expansion []. For example, the severity and penetrance of Huntington's Disease (HD) correlates with the number of CAG repeats in the HTT gene. Interestingly, in transgenic HD mice, CAG expansion was observed in MMRp, but not MMRd mice [2]. This is thought to be due to the stabilization of CAG hairpins by MutS$\beta$ during DNA replication [3].

2. **DNA Damage Response.** Alkylating agents are often used as chemotherapeutic agents. They work by forming alkylating lesions in the DNA which trigger double-strand break (DSB) formation in an MMR-dependent manner, leading to apoptosis. For example, $O^6$-methylguanine is a cytotoxic alkylating lesion that mispairs with thymine during DNA replication. Since $O^6$-methylguanine is located in the template strand, resynthesis of the daughter strand by MMR will regenerate the mismatch, creating a "futile repair loop" [4]. This process leads to the accumulation of single-strand gaps, which can lead to the formation of DSBs. If these DSBs become excessive and irreparable, they trigger apoptosis.

   Given the presence of endogeneous alkylating agents [5], it is reasonable to assume that MMR-dependent DSB formation also occurs in healthy humans under a normal context.

3. **Suppressing Recombination.** One of the pathways for DSB repair involves homologous recombination (HR). However, recombination between two divergent strands (homeologous recombination) is generally unwanted since it may lead to genomic instability in the form of chromosomal rearrangements, etc... [6]. Fortunately, the MMR system suppresses homeologous recombination through rejection of the resulting heteroduplex DNA. This occurs through the detection and binding of mismatches that arise when the two divergent DNA strands attempt to recombine [].

   Homologous chromosomes differ by about 1 in 1000 base pairs on average, just by virtue of genetic diversity between parents []. Moreover, it was shown that it only takes a single nucleotide mismatch to reduce recombination by 2.5 fold while a pair of mismatches can lead to as high as a 175-fold in reduction [7]. Thus, the MMR system may suppresses recombination, and hence the frequency of DSB repair, even in cases where it may be unnecessary to do so. Indeed, the frequency of DSB-induced recombination was increased in MSH2 deficient cell lines compared to WT [8].

MMR is also involved in : somatic hypermutation [], VDJ recombination [], and meiotic crossing-over [9]. All of the above highlight the mutagenic potential of the MMR system, contrary to its classical role. In other words, DNA repair is inherently mutagenic, and this is true for other repair systems as well [].

## 1.2 MMRd in Human Disease

Given the diverse roles of the MMR system, any disruption or malfunction in its components can have significant biological implications. Studies in MMR knockout mice reveal that the main detriments of MMRd are sterility [10] and increased cancer susceptibility [11]. This is expected, given the role of MMR in meiotic crossing-over and in limiting mutational burden. Of course, the accumulation of mutations can often lead to the deactivation of tumor suppressor genes, ultimately driving cancer progression [].

In humans, common SNP variants in MLH1 and PMS2 are linked with increased risk of sperm damage and male infertility [12]. Furthermore, defects in MLH1, MSH2, MSH6, PMS2, POLD, and POLE are associated with increased cancer risk. The prevalence of MMRd varies widely across cancers, ranging from as low as ∼1% (prostate, bladder, etc..) to as high as ∼15% in colorectal (CRC) and ∼30% in endometrial cancer (EC) []. These defects may either be constitutional (germline) or sporadically generated. Evidence for the involvement of other MMR genes (MSH3, EXO1) are either lacking [13] or conflicting [14, 15].

### 1.2.1 Germline MMR defects

Germline mutations in various MMR genes underlie heritable cancer predisposition diseases : Lynch syndrome (LS) in the case of MLH1, MSH2, MSH6, and PMS2 [16] and polymerase proofreading-associated polyposis (PPAP) in the case of POLD and POLE [17]. The most prevalent type of cancers associated with LS and PPAP are CRC and ECs, accounting for ∼3% of cases in LS [16] and ∼0.3-0.7% in PPAP [18]. LS and PPAP tumors present with different mutational signatures; while indel mutations, specifically microsatellite instability (MSI), are more prevalent in LS [], base substitutions are more prevalent in PPAP [17]. Additionally, while colorectal polyps are present in PPAP, there are close to none in LS [19].

A biallelic germline mutation in the LS genes, most commonly PMS2, results in constitutional mismatch repair deficiency (CMMRD). This rare condition is characterized by the early-onset (childhood) development of haemotological, brain, and/or colorectal cancers [20]. CMMRD patients may also exhibit slight immunodeficiency, though it is not clinically significant [21]. However, there is evidence of lymphocyte deficiency in patients with biallelic POLE mutations, which contributes to an increased susceptibility to respiratory infections [22].

### 1.2.2 Sporadic MMR defects

Approximately 70% of MMRd in CRC and EC arise sporadically []. Epigenetic mechanisms are mostly responsible for these cases. For example, an acquired hypermethylation of the MLH1 promoter explains almost all sporadic MSI cases in CRC [23]. Somatic bi-allelic mutations in MLH1 or MSH2 are also possible, but much rarer [24, 25]. Meanwhile, a subset of sporadic cases with stable microsatellites (MSS) can be attributed to somatic bi-allelic POLE mutations in both CRC [26] and EC [27].

## 1.3 Clinical Implications?

## 1.4 MMRd detection

Detection of MMRd in tumors is crucial step in the identification of cases associated with germline MMR defects such as LS, PPAP, and CMMRD. Identification of patients with these conditions is important since they can benefit from genetic counselling and cascade testing in relatives to enable early detection and intervention. For example, in the case of LS, intensive colonoscopic surveillance [] and daily aspirin intake [] have been shown to be effective intervention strategies.

The most direct method for diagnosing these conditions is germline sequencing of the relevant MMR genes to identify pathogenic variants. However, this approach is often inefficient and cost-prohibitive. Instead, tumors are typically tested for MMRd first, with genetic confirmation reserved for positive cases [28]. Current MMRd testing methods focus on detecting defects in the MLH, MSH, and PMS gene families; in fact, when academics refer to MMRd, they often specifically mean defects in these particular families, excluding EXO1, POLD, and POLE. We shall adhere to this convention from now on. Below, we outline some of these methods :

1. **Methylation tolerance.** Since cytotoxicity to alkylating agents is dependent on MMR function, MMRd cells display tolerance towards these agents. Tolerance to N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) in lymphoblastoid cells has been used to develop a functional MMR assay to identify patients with CMMRD [29]. The same assay has also been used to assess the effects of variants of unknown significance (VUS) in MLH1 and MSH2 towards MMR capacity [30].
2. **Immunohistochemistry (IHC).** This involves using antibodies against MLH1, MSH2, MSH6, and PMS2 to measure their expression, a lack of which would indicate MMRd [31]. However, some mutations can lead to the production of nonfunctional proteins that remain antigenic, potentially resulting in false negatives. It is estimated that around 6% of MMRd cases, particularly those related to MLH1, escape IHC detection due to this issue [32].
3. **MSI testing.** See section 1.5.

After the confirmation of MMRd in a tumor, features such as the lack of MLH1 methylation [] and absence of the somatic BRAF 1799T¿A mutation [] are strong indicators of a hereditary (as opposed to sporadic) etiology. Patients without these features are strongly recommended for germline testing [28].

### 1.5 MSI testing

Microsatellites are genomic regions composed of short sequence motifs (1–6 bp) repeated in tandem []. These regions are particularly prone to indel loop formations caused by strand slippage during DNA replication, resulting in an increased occurrence of alleles with altered repeat lengths []. In tumors, MSI is defined by the presence of alternative-length alleles that cannot be observed in the corresponding germline DNA [33]. Alternatively, quasi-monomorphic microsatellites may be used. These refer to microsatellites with minimal allelic variation within a population []. This approach eliminates the need for paired tumor-germline sample collection since the expected germline allele length (reference allele) can be reliably inferred from a human reference genome library like hg19.

Given the role of MMR in correcting indel mutations, MMRd tumors very often present with high levels of MSI []. The sensitivity of MSI tests towards detecting MMRd varies greatly depending on the affected gene and tumor type In MSH6-mutant tumors, the sensitivity is lower (77%) compared to MLH1 and MSH2 mutants (90%) [28, 34]. MSI is also harder to detect in non-CRC tumors. The proportion of unstable microsatellites and average allelic shifts are lower in MSI-positive EC when compared to MSI-positive CRC (0.27 vs 0.45, and 5.1bp vs 9.3bp respectively) [35]. Moreover, MSI is only detected in 20% of brain tumors from LS patients [28].

Traditionally, microsatellite length determination proceeds via PCR amplification of a microsatellite marker panel with fluorescent primers, followed by capillary gel electrophoresis (CGE) where the amplification products migrate, and hence separate based on their size (length) [33]. The results are displayed on an electropherogram which shows distinct peaks based on allele sizes. CGE has been increasingly replaced by next-generation sequencing (NGS) since it is cheaper and allows the examination of a greater range of markers, enabling genome-wide MSI analysis [36].

One significant challenge with MSI testing is that the PCR amplification step introduces additional indel mutations through the same "slippage" process that causes indels to occur naturally in vivo. This introduces a degree of noise in the PCR products, which is particularly concerning for data analysis involving rare (<1% frequency) allelic variants, as it becomes difficult to distinguish true in vivo mutations from artefacts introduced during amplification.

One approach to account for these errors is the use of unique molecular identifiers (UMIs)—short, nucleotide sequences attached to individual DNA molecules prior to amplification []. The amplified reads are grouped by their UMI, with the idea that each group contains reads derived from the same template molecule. A "consensus" sequence is built from each group, typically by selecting the most commonly occuring base in each position. Using these consensus sequences effectively eliminates the majority of PCR-induced errors, but a large number of reads (sequencing depth) are required to generate a suitable number of consensus sequences for analysis [37]. Despite this, this technique has been explored for accurate microsatellite genotyping in forensic applications [].

### 1.6 Aims

## 2 Methodology

### 2.1 Data

Building on the previous analysis which utilized three datasets (1 CRC, 2 EC), the current study incorporates two new CRC datasets and a CMMRD dataset, all obtained from publicly accessible sources []. Importantly, the CMMRD dataset is derived from blood tissue samples. This gives us some insight into how MMRd affects MSI in a non-tumor environment.

These samples were classified as MSS, MSI-low, or MSI-high by the Promega MSI 1.2 assay []. Following guidelines [38], MSS and MSI-low samples were put in the same group (the MMRp group). Likewise, we refer to the MSI-high samples as MMRd. The data for each sample is characterized by a list of 24 tables corresponding to a 24 quasi-monomorphic mononucleotide marker panel developed by Gallon et al []. Each table contains PCR read counts of its corresponding marker sorted into bins based on their standardized lengths. The "standardized length" of read is given by its length subtracted by the reference allele length. For example, the marker DEPDC2 has a length of $a$, so a corresponding read of length $a - 1$ will be sorted into DEPDC2's -1 bin.

Furthermore, UMI groupings were introduced as follows : For each microsatellite, PCR reads were grouped by UMI. The most commonly occuring microsatellite length in each group was obtained, and these lengths were sorted into bins as described before. This is referred to as the D1 grouping. The exclusion of groups with only one member gives rise to the D2 grouping, which is in theory more accurate than D1. Data without UMI grouping is referred to as D0.

## 2.2 Quality Control

Samples with more than 12 empty tables were excluded. Excluding tables with low total counts (<100) had a negligible impact on the results, so these were kept in. Table 1 presents the number of samples in each dataset after exclusion.

Table 1.

## 2.3 Mutation Frequency Analysis

The goal of this analysis was to determine how MMR status affects the read count distribution in each marker. To do this, for each marker, the frequency of each bin was calculated by dividing its count (the number of reads assigned to the bin) with the total count across all bins. Only the -2, -1, 0, and +1 bins were considered due to the lack of counts in any other bins.

For each marker, frequencies in the relevant bins were grouped by MMR status. The Mann-Whitney U test (two-tailed) was used to check for statistical differences between the MMRp and MMRd groups in each bin. This allows marker-specific differences to be observed, something that was not done in the previous study.

Additionally, the AUC was calculated for each bin. The AUC value ($0 \leq \text{AUC} \leq 1$) represents the probability that a randomly chosen bin frequency in the MMRd group exceeds one randomly chosen from the MMRp group. In other words, it quantifies how effectively bin frequency discriminates between the two groups. This analysis was initially done for the D0 counts (Figure 3) before later moving on to D1 and D2 (Figure 4).

### 2.3.1 Previous Analysis

A similar analysis was performed in the eariler study. [stuff]

# 3 Previous Result

Our previous work showed that bin frequencies corresponding to deletions (-1, -2) were significantly higher in the MMRd group as expected. Surprisingly, the opposite pattern was observed for insertions, that is, the +1 bin frequency was found to be higher in the MMRp group instead (Figure 2).

Figure 2.

This would seem to suggest that, in the context of insertions, microsatellites are more stable in MMRd tumors; a perhaps questionable statement to make considering MMR's role in indel correction. There are two possible explanations for this discrepancy :

1. **PCR errors.** Some reads in the +1 bin may be attributable to PCR artifacts. Given its low frequency (∼1%), the +1 bin is particularly sensitive to PCR noise. Additionally, the larger initial pool of 0-length (reference) DNA in MMRp samples may act as a source for the generation of +1 reads during amplification, potentially explaining the higher +1 bin frequency observed in the MMRp group.
2. **Normal MMR function.** As previously discussed, MMR can have has pro-mutagenic effects, particularly in the context of TNR expansion. Thus, it is possible that MMR activity directly contributed to the higher +1 bin frequency in the MMRp samples.

Assuming the first hypothesis is true, then the +1 bin frequency could potentially be used to estimate the PCR error rate, this can help further normalize the dataset. Assuming the second hypothesis is true, then the +1 bin frequency can be used as a feature to predict MMR function.

Motivated by this observation, we aim to validate these results in an expanded set of samples, and to also test the above hypotheses.

# 4 Results

## 4.1 Mutation Frequency Analysis

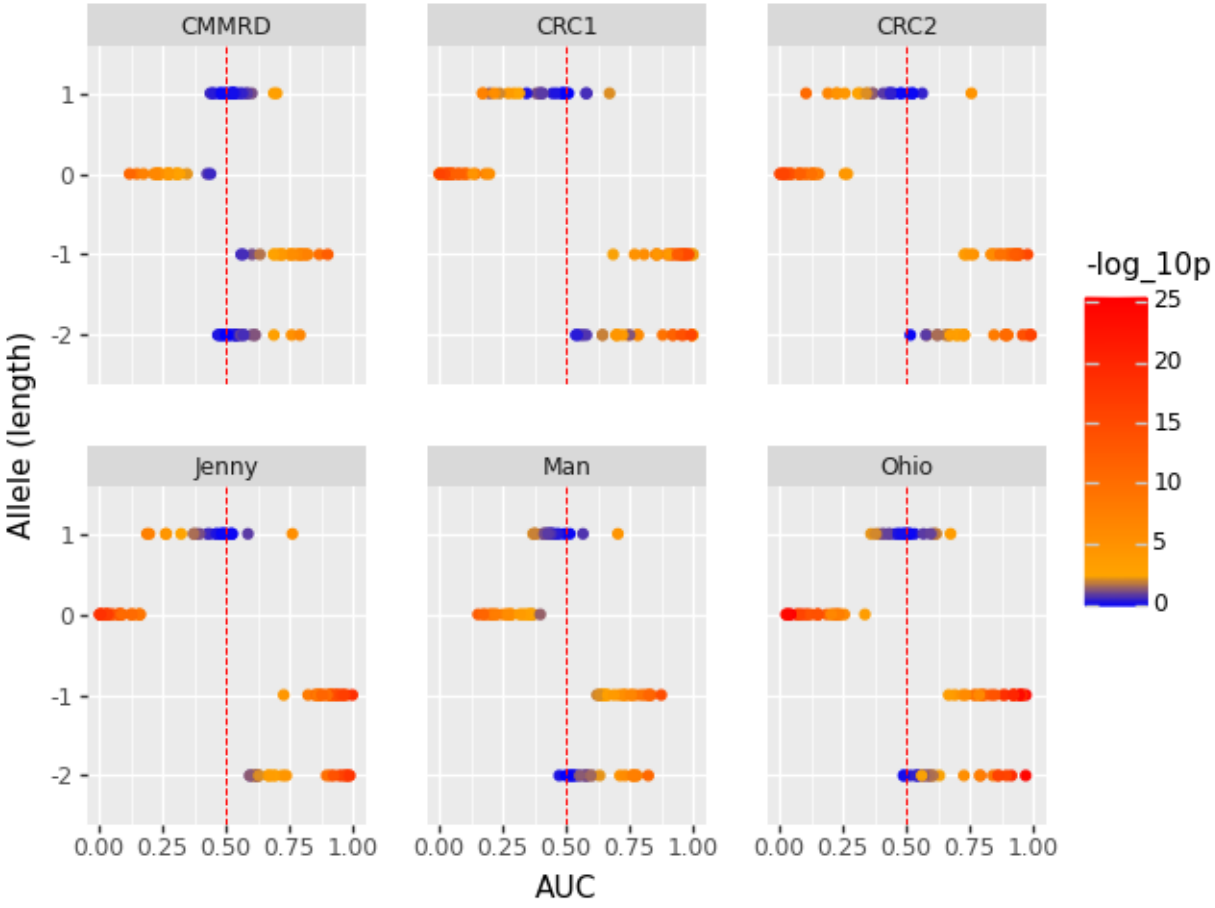


Figure 2: AUC values of different (-2, -1, 0, +1) bin frequencies across all markers and significance thereof (indicated by color) in the context of MMRp and MMRd discrimination, grouped by dataset.

Having markers with left-leaning AUC values in the +1 bin is indicative of insertions occuring more readily in the MMRp group. In general agreement with our previous results, this pattern can be observed in the old and new CRC datasets, very slightly in the EC datasets, but not in the CMMRD dataset. This is the only bin which behaves differently depending on tissue type. Since datasets of the same tissue type behave similarly, this justifies their merging in future analyses.

It is also interesting to note the existence of a sole significant (yellow) right-leaning marker (DEPDC2) for the +1 bin in each of the datasets.

## 4.2 With UMI

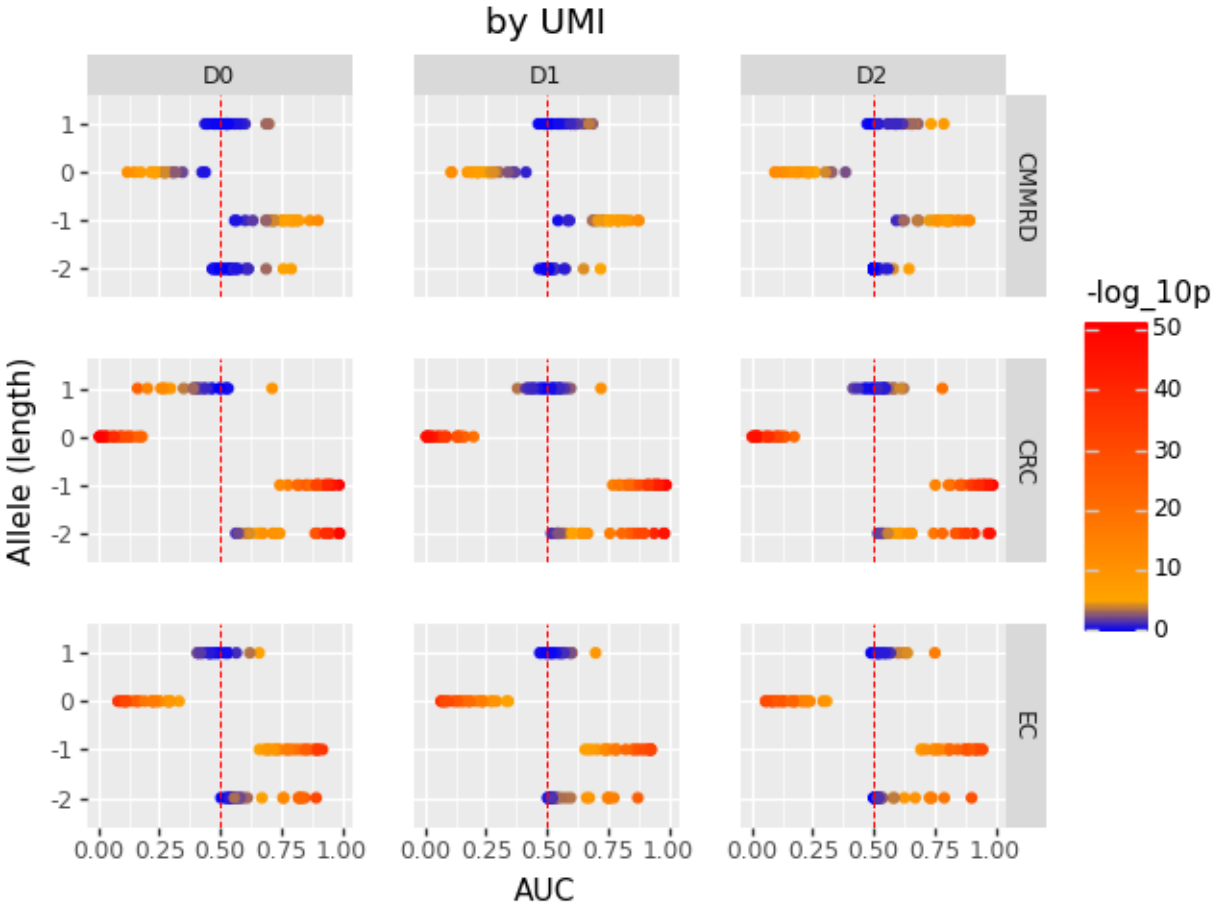


Figure 3: The same as in Figure 2 with UMI groupings (D1, D2) and datasets merged based on tissue type.

UMI groupings were introduced to test the "PCR error" hypothesis. As predicted, the +1 bin—being the most sensitive to PCR noise—is the most affected by UMI grouping; marker significance in the left direction is considerably diminished when D1 and D2 counts were used. This supports the PCR error hypothesis, but the question of why this pattern is more readily observed in CRC still remains to be answered.

We hypothesize that a greater "frequency gap" in the 0 bin between MMRp and MMRd groups (and this frequency gap is high in CRC) may contribute to a greater "frequency gap" in the +1 bin, and this will possibility will be looked at in the next analysis.

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