Title: A multiplex assay of variant effect (MAVE) of MSH6 enables accurate, prospective Lynch Syndrome clinical variant interpretation

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Abstract: Pathogenic germline variants in DNA mismatch repair factor genes cause Lynch Syndrome (LS), a dominant, hereditary cancer predisposition syndrome affecting more than 1:300 individuals worldwide. However, the large and growing burden of variants of uncertain significance (VUS) complicates genetic counseling for this condition. Missense variants make up the majority of VUSs, reflecting both their sheer number and the difficulty of assigning their pathogenicity. Incomplete penetrance and variable age of onset compound this challenge for *MSH6*, making family history difficult to interpret. To systematically and prospectively provide functional evidence in Lynch Syndrome, we have established and validated a mismatch repair gene multiplex assay of variant effect (MAVE), which we apply here to the key C-terminus of the mismatch repair factor *MSH6*. Our variant-to-function map accurately recapitulates standing clinical variant interpretations across a large validation set of previously classified benign (n=33) and pathogenic (n=26) variants (ROC AUC = 0.9779). Therefore, under the OddsPath framework, our *MSH6* MAVE scores may be considered strong evidence for or against *MSH6* missense variant pathogenicity. To apply this resource in a heterogenous, real-world clinical setting, we intersected our MAVE scores with a large clinical database of genetic test orders. We identified 3,527 individuals who carry an *MSH6* missense variant in this region, and looked up their variants in the MAVE results. Individuals carrying a missense variant indicated by the MAVE dataset to be functionally abnormal showed a higher risk for colorectal cancer (hazard ratio [HR]=2.4, 95% CI 1.3-4.6; both sexes) and uterine cancer (HR 3.4, 95% CI 1.42-7.0; females only), as compared to individuals who carried a functionally normal missense variant (p=0.00593and 0.00458, respectively). These results can be used to guide the VUS resolution. Of the 535 *MSH6* missense VUS found in this cohort, 17% were functionally abnormal. Ongoing reclassification of these variants as pathogenic or likely

pathogenic will result in new diagnoses, enabling optimal prevention and treatment for those individuals and family members who carry the same variants.