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1 **A tumor focused approach to resolving the etiology of DNA mismatch repair deficient**
2 **tumors classified as suspected Lynch syndrome**
3

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77 **Running Title: A novel genomic approach to resolve Suspected Lynch Syndrome**

78

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92 The authors declare no potential conflicts of interest.

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105 **Abstract**

106 Routine screening of tumors for DNA mismatch repair (MMR) deficiency (dMMR) in colorectal
107 (CRC), endometrial (EC) and sebaceous skin (SST) tumors leads to a significant proportion of
108 unresolved cases classified as suspected Lynch syndrome (SLS). SLS cases (n=135) were recruited
109 from Family Cancer Clinics across Australia and New Zealand. Targeted panel sequencing was
110 performed on tumor (n=137; 80xCRCs, 33xECs and 24xSSTs) and matched blood-derived DNA
111 to assess for microsatellite instability status, tumor mutation burden, COSMIC tumor mutational
112 signatures and to identify germline and somatic MMR gene variants. MMR immunohistochemistry
113 (IHC) and *MLH1* promoter methylation were repeated. In total, 86.9% of the 137 SLS tumors
114 could be resolved into established subtypes. For 22.6% of these resolved SLS cases, primary
115 *MLH1* epimutations (2.2%) as well as previously undetected germline MMR pathogenic variants
116 (1.5%), tumor *MLH1* methylation (13.1%) or false positive dMMR IHC (5.8%) results were
117 identified. Double somatic MMR gene mutations were the major cause of dMMR identified across
118 each tumor type (73.9% of resolved cases, 64.2% overall, 70% of CRC, 45.5% of ECs and 70.8%
119 of SSTs). The unresolved SLS tumors (13.1%) comprised tumors with only a single somatic
120 (7.3%) or no somatic (5.8%) MMR gene mutations. A tumor-focused testing approach reclassified
121 86.9% of SLS into Lynch syndrome, sporadic dMMR or MMR-proficient cases. These findings
122 support the incorporation of tumor sequencing and alternate *MLH1* methylation assays into clinical
123 diagnostics to reduce the number of SLS patients and provide more appropriate surveillance and
124 screening recommendations.

125

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126 **Introduction**

127 The current diagnostic strategy for identifying Lynch syndrome, the most common inherited
128 cancer syndrome, as recommended by the National Comprehensive Cancer Network
129 (www.nccn.org, last accessed date: November 8th, 2022) and the Evaluation of Genomic
130 Applications in Practice and Prevention group (1), involves screening tumours for evidence of
131 DNA mismatch repair (MMR)-deficiency (dMMR) via immunohistochemical staining for loss of
132 expression of one or more of the MMR proteins (MMR IHC) and/or for microsatellite instability
133 (MSI). Loss of MLH1/PMS2 protein expression necessitates testing for *MLH1* promoter
134 methylation (or *BRAF* V600E in colorectal cancers (CRCs)) and if negative, germline MMR gene
135 testing. For other patterns of loss of expression, germline MMR testing is undertaken. This
136 approach, while effective at identifying people with Lynch syndrome, still results in a significant
137 proportion of dMMR tumors without identified *MLH1* methylation or germline MMR pathogenic
138 variant, referred to as Lynch-like or suspected Lynch syndrome (SLS) (2). A diagnosis of SLS
139 presents challenges for the clinician with regards to recommendations for ongoing cancer risk
140 management and for screening for first-degree relatives. For the patient, an SLS diagnosis results
141 in variable psychosocial and behavioral responses related to the interpretation of their diagnosis
142 (3,4).

143

144 Previous studies have shown the SLS group to be etiologically heterogeneous,
145 encompassing both inherited and sporadic causes of dMMR (2,5,6). Furthermore, the risk of cancer
146 in SLS patients and their relatives requires clarification (2,7,8). These uncertainties make the
147 clinical management of an SLS diagnosis challenging. Complex or cryptic germline MMR gene
148 pathogenic variants that are more difficult to detect with current methodology, including those

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149 within intronic or regulatory regions, have been described (9–19). In addition, somatic mosaicism
150 of MMR gene pathogenic variants (20,21) or germline pathogenic variants in non-MMR genes,
151 including *POLE*, *POLD1* or *MUTYH* that somatically inactivate one of the MMR genes (15,22),
152 are rare causes of tumor dMMR. The most commonly reported cause of SLS in CRC and
153 endometrial cancer (EC) is biallelic somatic MMR gene mutations (often referred to as double
154 MMR somatics) (23–27), where each of the two mutations inactivate an allele in the same MMR
155 gene that is shown to be defective by the pattern of MMR protein loss of expression observed in
156 the tumor. Biallelic somatic MMR gene mutations have also been reported in dMMR sebaceous
157 skin tumors (SSTs) in the absence of germline MMR gene pathogenic variants (28). Furthermore,
158 the possibility that an SLS diagnosis has arisen due to a false positive tumor MMR IHC result or
159 false negative *MLH1* methylation test result has been previously described (25). The ability to
160 stratify people with an SLS diagnosis into those with an incorrect screening test result or an
161 inherited or sporadic etiology, is of clinical importance for risk appropriate clinical management
162 of the patient and their relatives.
163

164 CRCs, ECs and SSTs are tumor types that demonstrate the highest frequencies of dMMR,
165 where up to 26% (29), 31% (29,30) and 31% (31) of these tumor types respectively, present with
166 dMMR. The aim of this study was to investigate both inherited and somatic causes of 135 CRC-,
167 EC-, or SST-affected people with an SLS diagnosis referred from Family Cancer Clinics across
168 Australia and New Zealand. The findings from this large cohort with SLS will inform future
169 diagnostic approaches that will improve the stratification of patients into those with a definite
170 diagnosis of Lynch syndrome and those with somatic causes of dMMR. It will also eliminate the

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171 genetic counselling uncertainty of the finding of dMMR tumor where a somatic causation is
172 demonstrable.
173

174 **Methods**

175 *Study Cohort*

176 The study participants were people diagnosed with SLS during clinical work-up. SLS was
177 defined as: 1) having tumor dMMR as determined by MMR IHC where germline testing of the
178 MMR genes did not find a pathogenic variant, 2) for tumors that showed loss of MLH1/PMS2
179 expression, tumor *MLH1* methylation testing returned a negative or inconclusive result, or 3) for
180 CRC, where *MLH1* methylation testing was not completed, the tumor tested negative for the
181 *BRAF* V600E mutation. Participants meeting the SLS criteria and with tumor tissue and blood-
182 derived DNA available for testing were identified for this analysis. In total, 140 participants with
183 SLS were identified for testing from two studies:

184 1) the ANGELS study (*Applying Novel Genomic approaches to Early-onset and suspected*
185 *Lynch Syndrome colorectal and endometrial cancers*) recruited SLS patients diagnosed
186 with CRC and/or EC between 2014 – 2021 from Family Cancer Clinics across Australia
187 and New Zealand (32) (n=124);
188 2) the Muir-Torre Syndrome Study (MTS) recruited people diagnosed with one or more SSTs
189 between July 2016 and September 2021 from Sullivan Nicolaides Pathology in Brisbane,
190 Australia (31) or from Family Cancer Clinics across Australia (n=26).

191 The study was approved by The University of Melbourne human research ethics committee
192 (HREC#1750748 and HREC#1648355) and at certain Familial Cancer Clinic institutional review

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193 boards. All ANGELS and MTS study participants provided informed consent and a peripheral
194 blood sample. Biopsy or resection tumor tissue blocks/slides were collected where possible.

195

196 *DNA Mismatch Repair Protein Immunohistochemistry*

197 Pre-study MMR IHC testing to categorize the tumor as dMMR as part of the SLS diagnosis was
198 performed by various diagnostic pathology services across Australia and New Zealand. For this
199 study, MMR IHC was repeated as described in **Supplementary material** if tissue was available.

200

201 *Tumor MLH1 Methylation Testing*

202 Pre-study tumor *MLH1* methylation testing was performed using the methylation sensitive-
203 multiplex ligation probe dependent amplification (MS-MLPA) assay at various diagnostic
204 pathology services across Australia. For this study, *MLH1* methylation testing employed a
205 MethyLight assay (33,34) and a methylation-sensitive high resolution melting assay (MS-HRM)
206 (35), performed on the same tumor DNA sample from SLS cases that showed loss of MLH1/PMS2
207 expression or solitary loss of PMS2 expression (46,47). These independent assays targeted seven
208 overlapping CpG sites within the C-region of the *MLH1* gene promoter and were run with a set of
209 DNA standards (0% - 100% methylation) and no-template (negative) controls. Bisulfite
210 conversion of tumor and blood-derived DNA was performed using the EZ DNA Methylation-
211 Lightning^{TM} Kit (Zymo Research, Irvine, USA). For MethyLight, *MLH1* methylation was
212 quantitatively reported based on the percentage of methylated reference (PMR) calculations (34),
213 where tumors with a PMR ≥10% were considered "positive" (33,34). For MS-HRM, the
214 MeltDoctor^{TM} HRM Reagent Kit (Thermo Fisher Scientific, Massachusetts, USA) was used where

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215 tumors demonstrating ≥5% were considered *MLH1* methylation "positive". For each tumor
216 positive for *MLH1* methylation, the matched blood-derived DNA sample was tested in people with
217 tumors diagnosed <50 years or with multiple tumors using these two assays for evidence of
218 constitutional *MLH1* methylation (*MLH1* epimutation).

219

220 *Targeted Multi-Gene Panel Testing*

221 All tumors and matched blood-derived DNA samples from the n=135 SLS cases underwent multi-
222 gene panel sequencing assay, modified from the assay described in Zaidi *et al.* (36), which captured
223 297 genes (2.005 megabases (Mb)). The panel comprised the MMR and *EPCAM* genes as well as
224 other established hereditary CRC and EC genes including *POLE, POLD1,* and *MUTYH*. Details
225 of the capture design and sequencing are provided in the **Supplementary Material.** Details of the
226 bioinformatic pipeline for variant calling as well as methodology for calculation of tumor
227 mutational burden (TMB) and tumor mutational signatures (TMS) are provided in the
228 **Supplementary Material**.

229

230 *Determining Tumor DNA Mismatch Repair Deficiency from Panel Sequencing Data*

231 Overall tumor dMMR status was determined from the panel sequencing data by applying the
232 additive feature combination approach described in Walker *et al.*, (37) (**Supplementary Table**
233 **S1)**. Briefly, six dMMR predictive features, namely MSMuTect, MANTIS, MSIseq, MSISensor,
234 INDEL count and TMS ID2+ID7 (32) were derived for each tumor with thresholds for classifying
235 dMMR determined previously (37) (see **Supplementary Table S1**). Tumors were considered
236 dMMR overall when ≥3/6 of the features were positive for dMMR.

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237

238 *Statistical Analysis*

239 All statistical analyses were done using the R programming language (v. 4.1.0) (38). Correlation
240 scores for categorical values between multiple groups were estimated using the *chi-square* test. *p*-
241 values <0.05 were considered statistically significant.

242

243 **Results**

244 *Characteristics of the SLS Study Participants*

245 An overview of the study design is shown in **Figure 1** and includes the categorization of the SLS
246 cases using the results from tumor sequencing as well as re-testing of *MLH1* methylation and
247 MMR IHC which is described in detail below. The clinicopathological characteristics of the 137
248 tumors with sufficient DNA for testing from 135 study participants meeting the SLS criteria,
249 overall and by tumor type, are presented in **Table 1**. Of note, two participants each had a CRC that
250 showed loss of all four MMR proteins, where one tumor (ID018) was from a carrier of a germline
251 *MSH2* pathogenic variant explaining the loss of MSH2/MSH6 expression but where the loss of
252 MLH1/PMS2 expression was unexplained, while the other tumor (ID046) had no cause for loss of
253 expression of all four MMR proteins during pre-study clinical investigations.

254

255 *Determining Tumor dMMR Status*

256 For the SLS tumors, firstly, confirmation of dMMR status was assessed using both the additive
257 feature approach combining the results from MSMuTect, MANTIS, MSIseq, MSISensor, INDEL
258 count and TMS ID2+ID7 as described in Walker *et al.*, (37) and by repeating MMR IHC where
259 possible. The results of the additive feature approach, overall and for each tumor type, are shown

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260 in **Supplementary Figure S1**, where 85.4% (117/137) were predicted to be dMMR having ≥3/6
261 tumor features, including 87.5% (70/80) of the CRCs, 69.7% (23/33) of the ECs and all the SSTs
262 (100%, 24/24). Of these 117 dMMR predicted tumors, 81.2% had all six tumor features positive
263 for dMMR.
264
265 MMR IHC was repeated internally for 65/137 (47.4%) SLS tumors. Discordant MMR IHC
266 results between the pattern of loss reported prior to the study entry compared with testing
267 completed during the study were observed in 20% (13/65) of the SLS tumors (**Supplementary**
268 **Table S2**). For 8/13 (61.5%) of these SLS tumors (7 CRCs and 1 EC) retained/normal expression
269 of the MMR proteins was observed when repeated. All eight were predicted to be pMMR results
270 by the additive feature combination approach. Furthermore, no tumor *MLH1* methylation or double
271 somatic MMR mutations were identified in this group from internal testing, supporting a final
272 categorization of pMMR. Five SLS tumors showed a different pattern of MMR protein loss
273 compared with the pre-study result (5/13, 38.5%) (**Supplementary Table S2**). In each case, the
274 new pattern of loss was consistent with cause of dMMR identified by this study. For example,
275 ID009 showed solitary loss of MSH6 expression initially and when repeated internally showed
276 loss of MLH1/PMS2 that was related to tumor *MLH1* methylation. There were 12 tumors that were
277 classified as dMMR by MMR IHC but determined to be pMMR by the additive feature
278 combination approach giving an overall accuracy between tumor panel sequencing derived dMMR
279 status and the MMR IHC status of 92% (95% confidence intervals, CI: 86.5%-92%)
280 (**Supplementary Table S3**).
281
282 *Evidence of Tumor MLH1 Methylation*

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283 The dual MethyLight and MS-HRM *MLH1* methylation assay approach was performed on 77 SLS
284 tumors, including all 47 tumors which had pre-study clinical *MLH1* methylation testing. Tumor
285 *MLH1* methylation was detected in 23 tumors from 22 SLS cases where the concordance between
286 the two internal assays was 100% and, in all but one of the tumors, there was loss of expression of
287 MLH1 protein by IHC (a single *MLH1* methylation positive tumor ID031 showed solitary loss of
288 PMS2). Five of these tumors had pre-study clinical *MLH1* methylation testing reporting no *MLH1*
289 methylation detected (4/5 were EC tumors) (**Supplementary Table S4**). There were six SLS
290 tumors that reported inconclusive *MLH1* methylation results from pre-study clinical testing that
291 were found to be positive for *MLH1* methylation, although at low levels, by this study
292 (**Supplementary Table S4**). Two of the SLS cases were identified by the study as a primary *MLH1*
293 epimutation carrier (ID033 and ID013; dMMR-PriEpi) showing *MLH1* methylation in their SST-
294 and peripheral blood-derived DNA, and in the case of ID013 in their CRC tissue-derived DNA as
295 well. Two SLS cases showed tumor *MLH1* methylation while also being a carrier of a germline
296 MMR pathogenic variant (ID018 and ID034) demonstrating two concurrent mechanisms that
297 accounted for the unique patterns of MMR protein loss observed in both (**Supplementary Table**
298 **S4**). Therefore, 18/23 *MLH1* methylation positive tumors were re-categorized from SLS to
299 sporadic *MLH1* methylated tumors (dMMR-MLH1me). Of all the *MLH1* methylation positive
300 cases identified in this study, 55.6% (5/9) of the CRCs were diagnosed ≤50 years of age, whereas
301 all *MLH1* methylation positive ECs (n=9) were diagnosis >50 years of age.
302
303 *Determining a Germline Cause of dMMR in SLS*
304 The germline pathogenic variants and variants of uncertain significance (VUS) identified in the
305 DNA MMR genes, *MUTYH*, and the exonuclease domain of *POLE* genes are shown in

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306 **Supplementary Table S4**. There were no germline pathogenic variants or VUS's identified inside
307 the exonuclease domain of the *POLD1* gene. Two germline MMR gene pathogenic variant carriers
308 were identified (dMMR-LS). One, an *MSH2* deletion of exon 7 was known prior to study entry
309 (ID018) with the CRC tumor showing loss of all four MMR proteins and was positive for *MLH1*
310 methylation. The second carrier (*MSH6* c.3834_3849dup p.Thr1284Glnfs*10) was identified in
311 ID034 who had MLH1/PMS2 and MSH6 loss in EC diagnosed at 55-60 years that was not reported
312 in previous clinical testing. The tumor showed a somatic *MSH6* mutation (*MSH6*: c.3261del
313 p.Phe1088Serfs*2) and was positive for *MLH1* methylation accounting for the loss of
314 MLH1/PMS2. The third case harbored an *MLH1* VUS (*MLH1* c.400A>G p.Lys134Glu in ID028)
315 identified in an SST tumor showing loss of MLH1/PMS2 and two somatic *MLH1* mutations. A
316 further six VUS variants were identified in MMR genes which did not match the defective MMR
317 gene identified by the pattern of MMR IHC loss. No biallelic *MUTYH* carriers were identified.
318 However, two germline *POLE* variants within the exonuclease domain were observed, c.825C>G
319 p.Asp275Glu and c.861T>A p.Asp287Glu, both of which are considered to be VUS
320 (**Supplementary Table S5**).
321
322 *Determining Double Somatic MMR Mutations as a Cause of dMMR in SLS*
323 For the remaining 105 tumors not categorized as pMMR, dMMR-MLH1me, dMMR-PriEpi or
324 dMMR-LS, two somatic mutations in the MMR gene indicated to be defective by the pattern of
325 MMR IHC loss were identified in 87/105 (82.9%) tumors (**Table 2**). The presence of two or more
326 somatic MMR mutations in each tumor was specific to the double somatic MMR mutations
327 (dMMR-DS) tumors compared with the other tumor subtypes (**Supplementary Figure S2**). The
328 somatic mutations comprised either two single nucleotide/small indel mutations or a single

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329 nucleotide/small indel mutation combined with a large deletion in the wildtype allele (loss of
330 heterozygosity, LOH) (**Supplementary Figure S3**). When the tumors were stratified by their
331 revised pattern of protein loss by IHC, >80% of tumors for each pattern were dMMR-DS across
332 all tumor types (**Table 2**). Single somatic MMR gene mutations (dMMR-SS) occurred in 9.5% of
333 the SLS tumors while no somatic MMR mutations (dMMR-SLS) were found in 7.6% (**Table 2**).
334
335 For the dMMR-DS tumors, it was not possible to determine whether the double somatic
336 mutations in the same MMR gene were in *cis* or *trans*. To address this, the number of somatic
337 MMR mutations identified in each tumor across all four MMR genes were mapped to the pattern
338 of MMR protein loss by IHC (**Figure 2**). Two or more somatic MMR mutations were rarely found
339 in an MMR gene not considered to have the primary defect by IHC. For example, in tumors that
340 showed loss of MLH1/PMS2 expression, multiple somatic mutations were observed in *MLH1* but
341 rarely in the *MSH2*, *MSH6* or *PMS2* genes (**Figure 2A**), suggesting that when multiple mutations
342 occur in the gene with loss of expression, they are acting in *trans* to inactivate both alleles. Multiple
343 somatic MMR mutations rarely occurred in the dMMR-MLH1me or pMMR tumors (**Figure 2B**
344 **& 2C**).
345
346 **Table 3** provides a summary of the categorization of all 137 SLS tumors overall and by
347 tumor type. The cause for the dMMR phenotype, whether related to incorrect pre-study MMR IHC
348 or *MLH1* methylation test result or identified germline or somatic cause, could be identified in
349 119/137 (86.9%) of the SLS cases and, therefore, considered resolved. The SLS tumors that were
350 considered unresolved in terms of their dMMR etiology were those classified as dMMR-SS (7.3%,
351 10/137) and dMMR-SLS (5.8%, 8/137) (**Table 3**).

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352

353 *Characteristics of the dMMR-DS Tumors*

354 The characteristics of the participants with dMMR-DS tumors including the sex, age at tumor
355 diagnosis, PREMM5 scores and tumor site are shown in **Supplementary Table S6**. Two-thirds of
356 the CRC dMMR-DS tumors were in the proximal colon (**Supplementary Table S6,**
357 **Supplementary Figure S4**). The mean age at CRC diagnosis was 46.6 ± 13.1 years with 50% of
358 the tumors diagnosed before age 50 years, in contrast to the EC and SST dMMR-DS tumors had
359 an older mean age at diagnosis (**Supplementary Table S6**). The dMMR-DS CRCs located in the
360 proximal colon had an older age at diagnosis compared with the dMMR-DS distal CRCs (p-
361 value=0.043, *t-test*; **Supplementary Figure S5**). A PREMM5 score was calculated on each of the
362 dMMR-DS categorized participants with the distribution of scores overall and by tumor type
363 shown in **Supplementary Figure S6**. Over 80% of the dMMR-DS CRCs had a PREMM5 score
364 greater than the 2.5 threshold, however, this proportion was much lower for the EC and SST groups
365 (**Supplementary Table S6**).

366

367 **Discussion**

368 In this study, we investigated both germline and somatic causes of dMMR using a custom-
369 designed, multi-gene panel sequencing assay, and additionally investigated the potential of
370 incorrect MMR IHC and tumor *MLH1* methylation results, in a large series of people diagnosed
371 with SLS across CRC, EC and SST tumor types. Using this approach, we could resolve the
372 diagnosis for 86.9% of the SLS tumors into recognized clinically actionable subtypes. The largest
373 subtype of SLS tumors were those with double somatic MMR mutations (dMMR-DS, 64.2%) that
374 are thought to be related to a low risk of second primary cancers and a low risk of cancer in

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375 relatives. Furthermore, 13.1% and 5.8% of SLS tumors were related to incorrect *MLH1*
376 methylation and MMR IHC results, respectively, during pre-study clinical work-up. These results
377 provide an important evidence base to improve tumor testing approaches for Lynch syndrome.
378 Furthermore, our results highlight the added benefit to resolving an SLS diagnosis from deriving
379 dMMR-associated features and tumor mutational signatures from tumor sequencing assay to
380 confirm dMMR status and provide insights into tumor etiology.
381

382 The predominant cause of dMMR in the SLS CRC, EC and SST tumors was double somatic MMR
383 mutations, resulting in somatic biallelic inactivation of the MMR gene, which is reflected in the
384 pattern of protein loss identified by MMR IHC. After excluding tumors incorrectly categorized as
385 SLS, 90.2% of CRCs, 68.2% of ECs and 77.3% of SSTs were identified as dMMR-DS (**Table 2**).
386 Previous studies investigating SLS dMMR CRC and EC tumors have reported similarly high
387 proportions with double somatic MMR mutations ranging from 52.5%-100% (5,6,23–26,39–41).
388 Elze *et al.* (23), reported 88.8% (182/205) of dMMR CRCs and 80.9% (38/47) of dMMR ECs with
389 two somatic inactivating events. Pearlman *et al.* (40) and Hampel *et al.* (24) identified double
390 somatic MMR mutations in 88.4% (76/86) of dMMR SLS CRCs and in all of the 12 SLS ECs
391 tested in the Ohio Colorectal Cancer Prevention Initiative study, respectively. For SSTs, Joly *et al.*
392 (28) reported 53.8% (7/13) of the dMMR SLS tumors tested had likely double somatic MMR
393 mutations. A study by Lefol *et al.* (6) investigated the prevalence of double somatic MMR
394 mutations in multiple tumor types including CRC, EC and SST tumors observing 69.6%, 65% and
395 50%, respectively. Our study adds further confirmation that double somatic MMR mutations
396 underlie the majority of the SLS dMMR subtype and supports the importance of incorporating
397 tumor sequencing to resolve an SLS diagnosis. Furthermore, we have screened the largest group

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398 of SLS SSTs to date, demonstrating that double somatic MMR mutations are the most likely cause
399 for dMMR after exclusion of Lynch syndrome.
400 The identification of only a single germline MMR pathogenic variant in *MSH6* that was
401 missed by previous clinical germline testing was reassuring. Arnold *et al.* (9) reported 7% (9/128)
402 of SLS cases had germline pathogenic variants identified that were missed by prior testing. The
403 hotspot *MSH2* c.942+3A>T pathogenic variant (42) can be missed because it resides within a low
404 DNA complexity region. The *MLH1* c.400A>G p.Lys134Glu VUS identified, occurred in a tumor
405 with loss of MLH1/PMS2 expression and with two somatic *MLH1* mutations, where one of these
406 may function as the "second hit" on the wildtype allele, however, further characterization of this
407 variant is needed to determine whether this person has Lynch syndrome or double somatic MMR
408 mutation-related dMMR. The other six MMR VUS occurred in genes that did not match the pattern
409 of protein loss by MMR IHC and, therefore, this reduces their likelihood of being pathogenic. In
410 addition, we investigated germline pathogenic variants in non-MMR genes namely, *MUTYH*,
411 *POLE* and *POLD1*, as these have been previously shown to result in a double MMR somatic
412 mutation dMMR phenotype (15,22). We did not find germline biallelic *MUTYH* pathogenic
413 variants nor did we see strong evidence for the tumor mutational signature profile, SBS18 and
414 SBS36, that is strongly associated with germline biallelic inactivation of *MUTYH* gene (43) in any
415 of the SLS tumors suggesting biallelic *MUTYH* inactivation is a rare cause of dMMR in SLS.
416 Although we found only a single germline MMR and no non-MMR pathogenic variants in our
417 SLS cases, the presence of a personal and/or family cancer history of Lynch syndrome spectrum
418 tumors may provide cause for further investigation of these genes with alternate technology such
419 as whole genome sequencing (41) or long-read genome sequencing (18), which have had success
420 at identifying structural rearrangements and intronic pathogenic variants in the MMR genes.

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421

422 Our approach to re-test tumor *MLH1* methylation and MMR IHC resulted in the identification
423 of 18.9% of cases incorrectly classified as SLS, being either *MLH1* methylation positive tumors
424 or being pMMR tumors. The study by Pearlman et al (27) found 13.7% of non-methylated CRCs
425 had an incorrect MMR IHC result. There are recognized challenges with MMR IHC testing due to
426 technical artefacts and inherent variability in the interpretation of the staining by different
427 pathologists (44,45). The pre-study MMR IHC was performed at multiple different private and
428 public pathology laboratories across the country which may have led to the false positive IHC
429 results we observed. The addition of our additive feature combination approach for predicting
430 dMMR status from tumor sequencing data supported the reclassification of IHC results to pMMR
431 in all eight cases. This highlights the value in applying alternate methodologies to confirm dMMR
432 status when a diagnosis of SLS is made. Different patterns of loss were also observed in five SLS
433 tumors, including four indicating loss of MLH1 which resulted in a further four SLS cases being
434 tested for *MLH1* methylation, two of which were positive.

435

436 In addition to the false positive MMR IHC results, our study found 13.1% of the SLS
437 tumors were indeed positive for tumor *MLH1* methylation indicating a large proportion was missed
438 by pre-study clinical testing, particularly for the EC tumor type, which resulted in an incorrect SLS
439 diagnosis. Of note, one SLS case with solitary loss of PMS2 expression was positive for *MLH1*
440 methylation. *MHL1* methylation in tumors showing solitary PMS2 loss have been described
441 previously (46,47). Although the reason for these false negative results is difficult to definitively
442 determine, potential reasons include: 1) intratumoral heterogeneity of *MLH1* methylation where
443 different areas of the tumor were tested by the pathology labs and by the study, and 2) the

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444 sensitivity of *MLH1* methylation detection is likely different between different assays. The 100%
445 concordance between the MethyLight and MS-HRM assay results while reassuring, also suggests
446 these two assays may have increased sensitivity over MS-MLPA. This may be in part related to
447 methodological differences relating to the need for bisulfite conversion for the MethyLight and
448 MS-HRM assays compared with methylation-sensitive restriction enzyme for MS-MLPA. Our
449 findings support the use of an alternate *MLH1* methylation assay when an SLS case with loss of
450 MLH1/PMS2 is identified. A recent study that integrated *MLH1* methylation and targeted tumor
451 sequencing is a promising approach to triage for Lynch syndrome where a single test would be
452 more efficient and perhaps overcome some of the limitations of current MMR IHC and *MLH1*
453 methylation testing (48).
454
455 Defective MMR gene function and loss of protein expression relies on the two-hit hypothesis
456 requiring both alleles to be inactivated to drive tumorigenesis. The identification in our study, and
457 reported in other studies using tumor sequencing to resolve SLS (6,25,28), that identification of
458 only a single somatic MMR mutation presents a conundrum to the interpretation of dMMR
459 etiology. The possibility that there is a second somatic mutation that has not been identified by our
460 experimental approach e.g., intronic somatic mutation, or that there is an undetected germline
461 MMR pathogenic variant (18,25), is plausible given the dMMR tumor status, although each would
462 have a different outcome for clinical management. The observation in this study that single somatic
463 MMR mutations occurred in MMR genes not considered defective by the pattern of protein loss
464 by IHC (**Figure 2**) and that single somatic MMR mutations occurred in *MLH1* methylation
465 positive tumors and even in pMMR tumors (**Supplementary Figure S2**) suggests a single somatic

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466 MMR mutation can occur unrelated to the dMMR etiology, hence our categorization of the
467 dMMR-SS tumors as unresolved.
468
469 The strengths of this study include the large number of cases diagnosed with SLS based on
470 prior clinical work-up identified from family cancer clinics across each state of Australia and from
471 New Zealand, representing the real-world heterogeneity of cases, diagnostic laboratory
472 methodology and nuanced approaches to triaging for Lynch syndrome. Furthermore, tumor types
473 representing those with the highest prevalence of dMMR, CRC, EC, and SST, were studied where
474 the diagnosis of SLS is more likely to occur. The decision to repeat *MLH1* methylation and MMR
475 IHC testing with different methodology resolved a larger number of SLS cases. Our custom-
476 designed tumor sequencing assay enabled the investigation of multiple causes of dMMR
477 simultaneously including SLS cases with unusual patterns of protein loss by IHC, including an
478 SLS case with loss of all four MMR proteins that harbored double somatic mutations in *MLH1*
479 and in *MSH6*. Furthermore, evaluation of multiple NGS-derived tumor features namely TMB,
480 INDEL count, multiple MSI calling tools and COSMIC TMS enabled accurate dMMR prediction
481 to support the MMR IHC result. Lastly, screening for *MLH1* epimutations in blood-derived DNA
482 in SLS tumors with loss of MLH1/PMS2 diagnosed <50 years and in all six SST tumors with loss
483 of MLH1/PMS2 identified two primary epimutation carriers, both in SST.
484
485 The identification of double somatic MMR mutations implies the dMMR tumor has a sporadic
486 etiology, however, there remains some uncertainty that this is truly the case. This is in part due to
487 previous reports showing that in rare cases a germline MMR pathogenic variant that is difficult to
488 detect with current sequencing technology, including intronic pathogenic variants or a cryptic or

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489 complex germline variant may underlie the dMMR tumor phenotype (9,10,17,18,49). Although
490 our capture was designed to include probes to cover non-coding regions of the MMR genes, not
491 all these regions could be probed due to low sequencing complexity. We have previously tried to
492 address the idea of missing intronic and complex MMR pathogenic variants using whole genome
493 sequencing but found no viable germline MMR gene candidates in familial and/or early-onset SLS
494 cases (41). Furthermore, this study did not include screening for potential somatic mosaicism of
495 MMR variants in the dMMR-DS group, which would require deep sequencing analysis to detect
496 low level mosaic mutations and screening of other distinct DNA sources. Somatic MMR
497 mosaicism has been previously described (20,21) although is rare. Follow-up studies of this
498 potential mechanism are needed as the identification of post-zygotic mosaicism of an MMR
499 pathogenic variant would have implications for future cancer risk and potentially for the carrier's
500 offspring. The unresolved group dMMR-SS and dMMR-SLS tumors, comprising 13.5% of the
501 SLS tumors, remain categorized as SLS and will require further investigation to determine a
502 somatic, germline or technical cause for their dMMR tumor. Finally, we were not able to
503 investigate the original MMR IHC result/slides for the 20% of tumors that were identified as
504 misclassified and, therefore, could not determine the basis, whether technical or from staining
505 interpretation, for the pre-study MMR IHC result. Further engagement of quality assurance
506 programs for MMR IHC and training for Pathologists may be needed to minimize the number of
507 false positive / negative MMR IHC results and to trigger further laboratory investigations before
508 reporting when unusual patterns of loss e.g. MSH6 and PMS2 loss are observed, as was reported
509 pre-study for two SLS cases in this study.
510

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511 **Conclusion**

512 This study demonstrated a tumor-focused approach that incorporated multiple pieces of evidence,
513 including contemporary NGS-derived tumor features and somatic screening of the MMR genes to
514 resolve 86.9% of the SLS cases into clinically actionable subtypes. These findings provide an
515 evidence base to reduce the number of patients diagnosed with SLS and improve triaging for Lynch
516 syndrome. The increased implementation of tumor sequencing to identify double somatic MMR
517 mutations will improve risk appropriate clinical management of the patient and their relatives.
518 Further studies are needed to elucidate the non-coding regions of the MMR genes and to clarify
519 the cancer risks for first degree relatives associated with people with double somatic MMR
520 mutation tumor as currently the evidence is limited and focused on the heterogeneous SLS subtype
521 (7). A large and systematic study of somatic mosaicism is needed in double somatic MMR
522 mutation tumors to understand the true prevalence. Finally, efficient triage of cancer-affected
523 people for Lynch syndrome should start with tumor and matched germline sequencing of the MMR
524 genes (among others), for the determination of dMMR status, identification of double somatic
525 MMR mutations and germline MMR pathogenic variants, while capturing therapeutic targets,
526 although supporting cost-effectiveness evidence would be needed.

527

528

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531

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722 **Table Legends**

723 **Table 1.** Overview of the study participants and their clinicopathological features overall and by
724 tumor type. Abbreviations: colorectal cancer, CRC; endometrial cancer, EC; sebaceous skin tumor,
725 SST; Applying Novel Genomic approaches to Early-onset and suspected Lynch Syndrome
726 colorectal and endometrial cancers, ANGELS; Muir-Torre Syndrome, MTS; standard deviation,
727 SD; Amsterdam II criteria, AM II; International Federation of Gynecology and Obstetrics, FIGO.

	CRC	EC	SST	TOTAL
Number of individuals, n (%)	78 (57.8%)	33[a] (24.4%)	24[b] (17.8%)	135 (100%)
Number of tumors tested, n (%)	80 (58.4%)	33 (24.1%)	24 (17.5%)	137 (100%)
Study, n (%)				
ANGELS	79 (98.7%)	33 (100%)	0 (0%)	112 (81.8%)
MTS	1 (1.3%)[b]	0 (0%)	24 (100%)	25 (18.2%)
Sex, n (%)				
Male	40 (50%)	0 (0%)	20 (83.3%)	60 (43.8%)
Female	40 (50%)	33 (100%)	4 (16.7%)	77 (56.2%)
Age at diagnosis				
Mean ± SD	47.4 ± 13.2	61.0 ± 9.3	65.2 ± 10.7	53.8 ± 14.1
Min. - Max.	24 - 74	41 - 79	39 - 81	24 - 81
≤ 50 years	46 (57.5%)	3 (9.1%)	4 (16.7%)	53 (38.7%)

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	> 50 years	34 (42.5%)	30 (90.9%)	20 (83.3%)	84 (61.3%)
	Pre-study MMR IHC Pattern of Loss [e]				
	MLH1/PMS2	37 (46.3%)	19 (57.6%)	7 (29.2%)	63 (46%)
	MSH2/MSH6	29 (36.3%)	5 (15.2%)	15 (62.5%)	49 (35.8%)
	MSH6	6 (7.5%)	9 (27.3%)	1 (4.2%)	16 (11.7%)
	PMS2	4 (5%)	0 (0%)	1 (4.2%)	5 (3.6%)
	MSH6 and PMS2	2 (2.5%)	0 (0%)	0 (0%)	2 (1.5%)
	Loss of all four MMR proteins	2 (2.5%)[c,d]	0 (0%)	0 (0%)	2 (1.5%)
COLORECTAL CANCER	**Tumor site, n (%)**				
	Proximal	55 (68.8%)	-	-	-
	Distal	16 (20%)	-	-	-
	Rectum	9 (11.2%)	-	-	-
	Tumor grade, n (%)				
	Well differentiated	8 (10%)	-	-	-
	Moderately differentiated	43 (53.8%)	-	-	-
	Poorly differentiated	26 (32.5%)	-	-	-
	Undifferentiated	1 (1.2%)	-	-	-
	Unknown	2 (2.5%)			

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	Histological type, n (%)				
	Adenocarcinoma	67 (83.8%)	-	-	-
	Mucinous	12 (15%)	-	-	-
	Other	1 (1.2%)	-	-	-
ENDOMETRIAL CANCER	**FIGO staging, n (%)**				
	Stage 1	-	19 (57.6%)	-	-
	Stage 2	-	8 (24.2%)	-	-
	Stage 3	-	6 (18.2%)	-	-
	Histological type, n (%)				
	Endometrioid	-	30 (90.9%)	-	-
	Clear cell	-	2 (6.1%)	-	-
	High-grade serous papillary carcinoma	-	1 (3%)	-	-
SEBACEOUS SKIN TUMOR	**Tumor site, n (%)**				
	Head and neck	-	-	14 (58.3%)	-
	Trunk and limb	-	-	10 (41.7%)	-
	Histological type, n (%)				
	Sebaceoma	-	-	1 (4.2%)	-

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	Sebaceous adenoma	-	-	21 (87.5%)	-
	Sebaceous carcinoma	-	-	2 (8.3%)	-

[a] One individual developed an EC @51-55 and a CRC @56-60 years old (person was counted in ECs because the EC had a younger age at diagnosis).

[b] One individual developed a CRC @51-55 and an SST @61-65 (person counted in SSTs because the person was recruited through the MTS study).

[c] Clinical testing identified a germline MSH2 pathogenic variant resulting in loss of MSH2/MSH6 protein expression. The cause for MLH1/PMS2 loss, however, was unexplained and therefore categorised as SLS.

[d] Total loss of MLH1/PMS2 staining in malignant mass with loss of MSH2/MSH6 staining in less differentiated areas of the tumor.

[e] MMR IHC results determined prior to study.

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729 **Table 2.** Overview of somatic mutation count by tumor type and by observed MMR IHC.
730 Abbreviations: colorectal cancer, CRC; endometrial cancer, EC; sebaceous skin tumor, SST;
731 immunohistochemistry, IHC; DNA mismatch repair, MMR; suspected Lynch syndrome, dMMR-
732 SLS; DNA mismatch repair deficient tumor with double somatic mutations, dMMR-DS; DNA
733 mismatch repair deficient tumor with single somatic mutation, dMMR-SS, DNA mismatch repair
734 deficient tumor with no somatic mutations, dMMR-SLS.

SLS Tumors Tested [a]	***Number of Somatic MMR Mutations***	***CRC (n=61)***	***EC (n=22)***	***SST (n=22)***	***Total (n=105 tumors)*** [g]
Overall	**Double somatic MMR mutations (dMMR-DS)** [b]	**55 (90.2%)**[f]	**15 (68.2%)**	**17 (77.3%)**	***87 (82.9%)***
	Single somatic MMR mutation (dMMR-SS) [c]	3 (4.9%)	4 (18.2%)	3 (13.6%)	10 (9.5%)
	No somatic MMR mutations (dMMR-SLS) [d]	3 (4.9%)	3 (13.6%)	2 (9.1%)	8 (7.6%)
Pattern of MMR IHC Loss [e]					
MLH1/PMS2	**Double somatic mutations in MLH1**	**27 (93.1%)**	**7 (70%)**	**4 (66.7%)**	**38 (84.4%)**
	Single somatic mutation in MLH1	1 (3.4%)	1 (10%)	1 (16.7%)	3 (6.7%)

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	No somatic mutation in MLH1	1 (3.4%)	2 (20%)	1 (16.7%)	4 (8.9%)
	Total	29 (100%)	10 (100%)	6 (100%)	45 (100%)
MSH2/MSH6	**Double somatic mutations in MSH2**	**23 (85.2%)**	**3 (60%)**	**12 (80%)**	**38 (80.9%)**
	Single somatic mutation in MSH2	2 (7.4%)	2 (40%)	2 (13.3%)	6 (12.8%)
	No somatic mutation in MSH2	2 (7.4%)	0 (0%)	1 (6.7%)	3 (6.4%)
	Total	27 (100%)	5 (100%)	15 (100%)	47 (100%)
MSH6	**Double somatic mutations in MSH6**	**4 (100%)**	**5 (71.4%)**	**1 (100%)**	**10 (83.3%)**
	Single somatic mutation in MSH6	0 (0%)	1 (14.3%)	0 (0%)	1 (8.3%)
	No somatic mutation in MSH6	0 (0%)	1 (14.3%)	0 (0%)	1 (8.3%)
	Total	4 (100%)	7 (100%)	1 (100%)	12 (100%)

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PMS2	**Double somatic mutations in PMS2**	**1 (100%)**	**0 (0%)**	**0 (0%)**	**1 (100%)**
	Single somatic mutation in PMS2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	No somatic mutation in PMS2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Total	1 (100%)	0 (0%)	0 (0%)	1 (100%)

[a] For assessment of the presence of somatic mutations fitting to MMR IHC loss, one tumor presenting with loss of all four MMR proteins (ID046) harboring biallelic MLH1 and biallelic MSH6 mutations was excluded from findings presented in **Table 2**.

[b] *Double somatic MMR mutations describes the presence of two or more somatic mutations in the same MMR gene where the pattern of protein loss by IHC indicates that same gene e.g. two MSH2 somatic mutations in a tumor showing loss of MSH2/MSH6 expression.*

[c] *Single somatic MMR mutation describes the presence of only one somatic mutation in the same MMR gene where the pattern of protein loss by IHC indicates that same gene e.g. single MSH2 somatic mutation in a tumor showing loss of MSH2/MSH6 expression.*

[d] *No somatic MMR mutations describes the absence of any somatic mutations in the same MMR gene where the pattern of protein loss by IHC indicates a defective gene e.g., no somatic mutations observed in MSH2 in a tumor showing loss of MSH2/MSH6 expression by IHC.*

[e] *The updated pattern of MMR IHC loss from internal MMR IHC testing was used in this table.*

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[f] *One CRC tumor (ID046) showed loss of all four MMR proteins by IHC and had double somatic MLH1 mutations and double somatic MSH6 mutations was not included in this table.*

[g] *These 105 SLS tumors excluded tumors re-categorized as dMMR-LS, dMMR-MLH1me, dMMR-PriEpi and pMMR by re-testing MLH1 methylation, MMR IHC and deriving dMMR status from panel sequencing and identification of germline MMR pathogenic variants.*

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736 **Table 3.** Summary of the categorization of the SLS tumors, overall and by tumor type, based on
737 the results from tumor panel sequencing, MLH1 methylation and DNA mismatch repair (MMR)
738 immunohistochemistry (IHC) results. Table shows breakdown of sequencing results by tissue type
739 and by cancer subtype. Abbreviations: colorectal cancer, CRC; endometrial cancer, EC; sebaceous
740 skin tumor, SST; suspected Lynch syndrome, dMMR-SLS; DNA mismatch repair, MMR; DNA
741 mismatch repair deficient, dMMR; DNA mismatch repair proficient, pMMR; DNA mismatch
742 repair deficient tumor with double somatic mutations, dMMR-DS; DNA mismatch repair deficient
743 tumor presenting with MLH1 methylation, dMMR-MLH1me; DNA mismatch repair deficient
744 tumor with a primary MLH1 epimutation, dMMR-PriEpi; DNA mismatch repair deficient tumor
745 with a germline pathogenic variant, dMMR-LS; DNA mismatch repair deficient tumor with single
746 somatic mutation, dMMR-SS, DNA mismatch repair deficient tumor with no somatic mutations,
747 dMMR-SLS.

Category	**CRC**	**EC**	**SST**	**Total**
Total Tumors Tested	80	33	24	137
Resolved				
dMMR-DS - Double somatic MMR mutations	56 (70%)[a]	15 (45.5%)	17 (70.8%)	88 (64.2%)
dMMR-MLH1me - *MLH1* methylated[b]	9 (11.3%)	9 (27.3%)	0 (0%)	18 (13.1%)
dMMR-PriEpi - Primary *MLH1* epimutation	1 (1.2%)	0 (0%)	2 (8.3%)	3 (2.2%)[e]

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dMMR-LS - Lynch syndrome	1 (1.2%)[c]	1 (3%)[d]	0 (0%)	2 (1.5%)
pMMR - MMR-proficient	7 (8.8%)	1 (3%)	0 (0%)	8 (5.8%)
Total resolved	**74/80 (92.5%)**	**26/33 (78.8%)**	**19/24 (79.2%)**	**119/137 (86.9%)**
Unresolved				
dMMR-SS - Single somatic mutation	3 (3.8%)	4 (12.1%)	3 (12.5%)	10 (7.3%)
dMMR-SLS - remain as SLS	3 (3.8%)	3 (9.1%)	2 (8.3%)	8 (5.8%)

[a] One tumor carried double somatic mutations in MLH1 and double somatic mutations in MSH6 presenting with loss of all four MMR proteins by IHC.

[b] All tumors positive for tumor MLH1 methylation demonstrated loss of MLH1/PMS2 by MMR IHC except for one CRC tumor showing solitary PMS2 loss by MMR IHC (confirmed by internal testing).

[c] This person carried a germline pathogenic variant in MSH2 (known prior to entering the study) with a somatic MSH2 mutation (2nd hit) and was also positive for tumor MLH1 methylation which accounted for the loss of all four MMR protein expression by IHC.

[d] This person carried a germline pathogenic variant in MSH6 (missed by prior clinical testing) with a somatic MSH6 mutation (2nd hit) as well as presenting with tumor MLH1 methylation accounting for the observed pattern of loss MLH1/PMS2 and MSH6 by MMR IHC.

[e] Two people identified as MLH1 epimutation carriers with one carrier developing an SST and the other developed a CRC and an SST.

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749 **Figure Legends**

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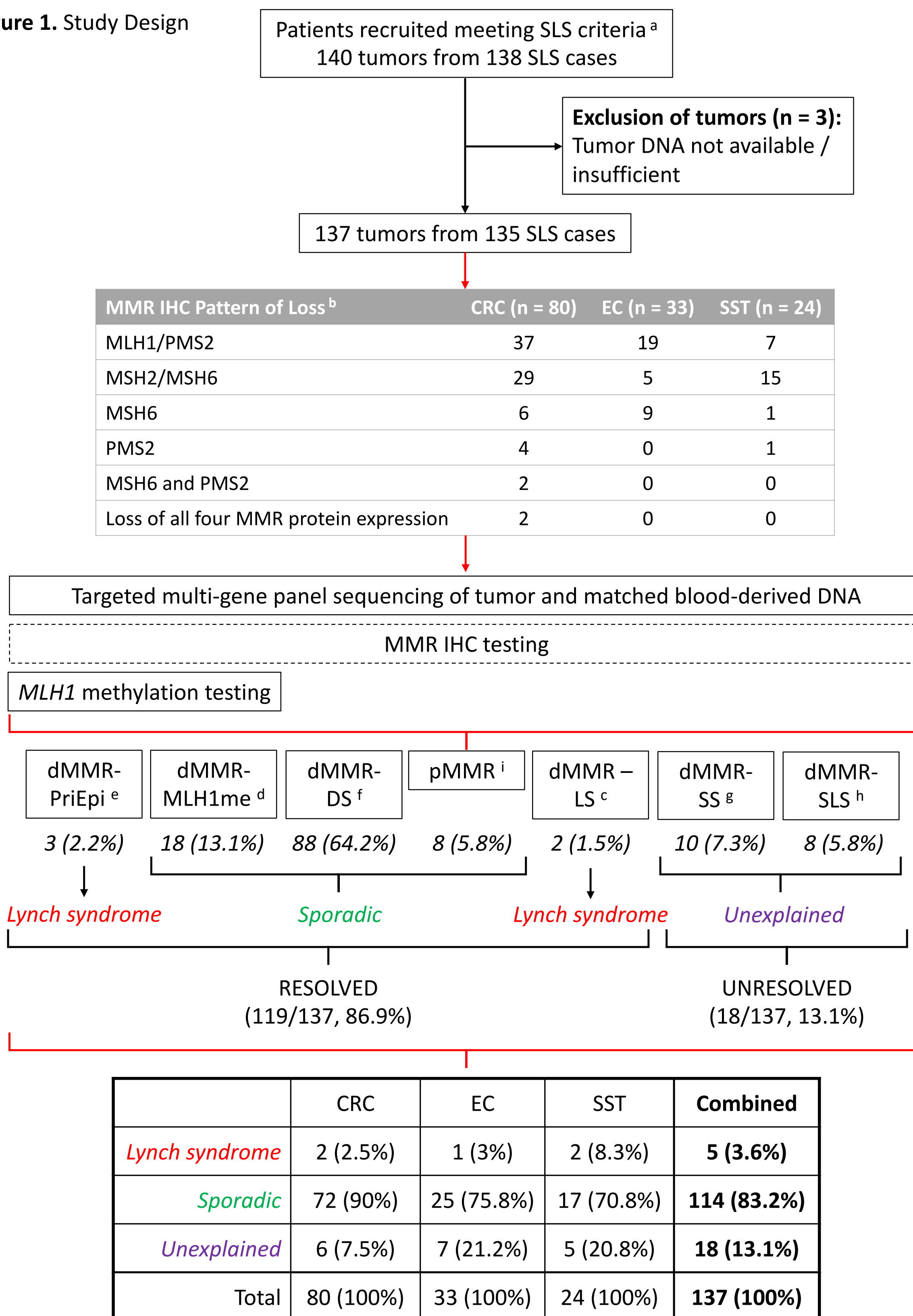
Figure 1. Study Design



MMR IHC Pattern of Loss [b]	CRC (n = 80)	EC (n = 33)	SST (n = 24)
MLH1/PMS2	37	19	7
MSH2/MSH6	29	5	15
MSH6	6	9	1
PMS2	4	0	1
MSH6 and PMS2	2	0	0
Loss of all four MMR protein expression	2	0	0

	CRC	EC	SST	**Combined**
Lynch syndrome	2 (2.5%)	1 (3%)	2 (8.3%)	**5 (3.6%)**
Sporadic	72 (90%)	25 (75.8%)	17 (70.8%)	**114 (83.2%)**
Unexplained	6 (7.5%)	7 (21.2%)	5 (20.8%)	**18 (13.1%)**
Total	80 (100%)	33 (100%)	24 (100%)	**137 (100%)**

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751 **Figure 1.** Overview of study design. Schema presenting the study inclusion criteria, the breakdown
752 of the clinical MMR IHC results, the testing assays applied and the final study results, separated
753 by tissue type and combined. Abbreviations: suspected Lynch syndrome, SLS; colorectal cancer,
754 CRC; endometrial cancer, EC; sebaceous skin tumor, SST; DNA mismatch repair, MMR;
755 immunohistochemistry, IHC; primary epimutation, dMMR-PriEpi; positive *MLH1* methylation,
756 dMMR-MLH1me; double somatic mutations, dMMR-DS; DNA mismatch repair proficient,
757 pMMR; Lynch syndrome, dMMR-LS; single somatic mutation, dMMR-SS.
758 [a] SLS criteria: individuals diagnosed with a DNA mismatch repair deficient CRC, EC and/or SST
759 with previous negative testing results.
760 [b] Breakdown of clinical MMR IHC results when first entering the study.
761 [c] dMMR with a germline pathogenic variant identified (Lynch syndrome, "dMMR-LS")
762 [d] dMMR with tumor MLH1 methylation (MLH1 methylated, "dMMR-MLH1me")
763 [e] dMMR with tumor and blood MLH1 methylation (primary epimutation, "dMMR-PriEpi")
764 [f] dMMR with double somatic MMR variants in the same MMR gene (double somatic mutation,
765 "dMMR-DS")
766 [g] dMMR with a single somatic MMR variant (single somatic mutation, "dMMR-SS")
767 [h] dMMR with no germline or somatic variants (suspected Lynch syndrome, "dMMR-SLS")
768 [i] pMMR tumors with neither germline or somatic mutations nor hypermethylation of the MLH1
769 gene (DNA mismatch repair proficient, "pMMR")

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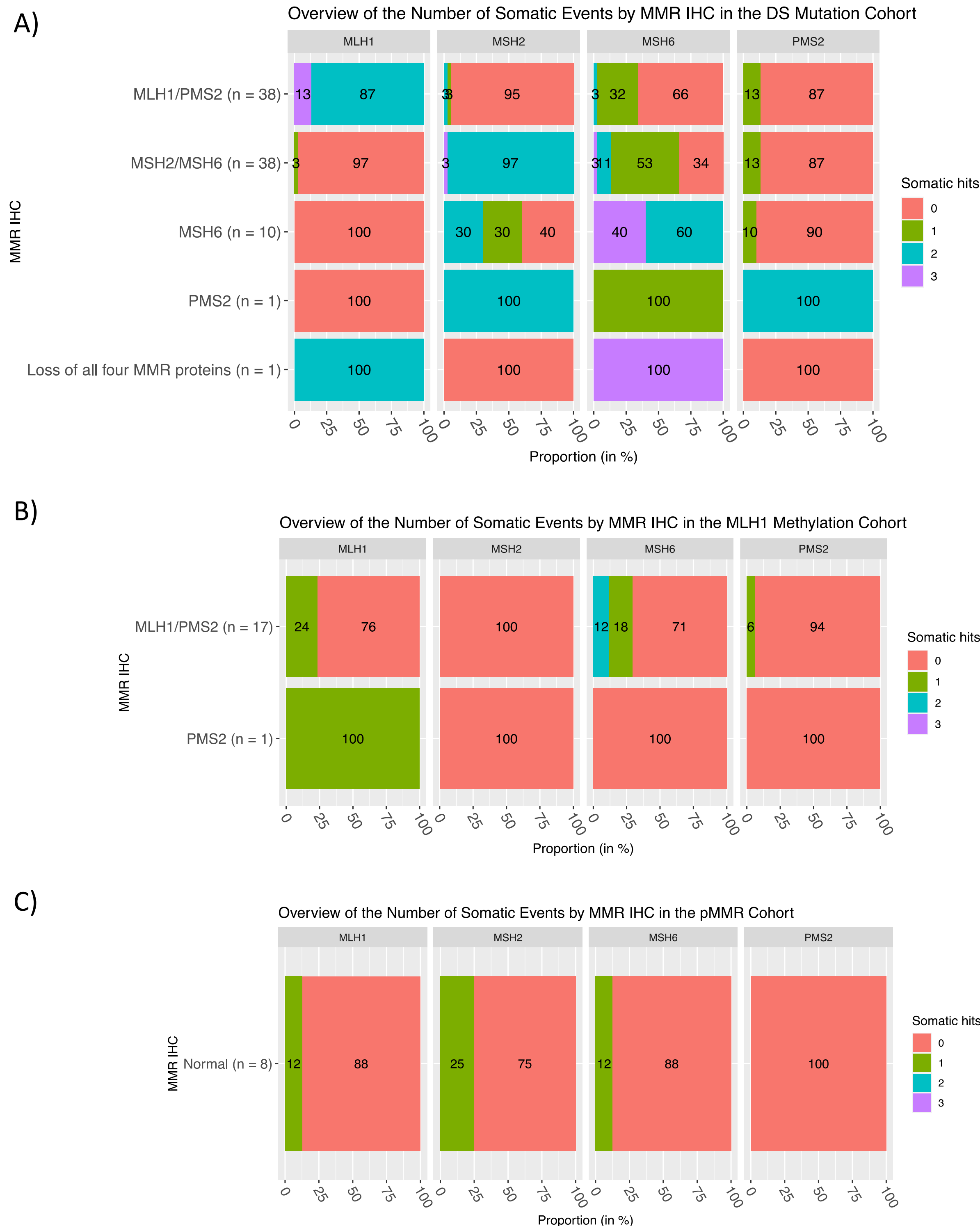
Figure 2



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771 **Figure 2:** Overview of the number of somatic events (somatic mutation and LOH) by MMR IHC
772 in the A) double somatic and B) positive *MLH1* methylation cohorts. Abbreviations: DNA
773 mismatch repair, MMR; immunohistochemistry, IHC.