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**The serrated neoplasia pathway of colorectal tumours:  
identification of MUC5AC hypomethylation as an early  
marker of high-risk polyps**

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Key Words:	hyperplastic polyp, sessile serrated adenoma, colonic cancer, mucin, methylation

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3 **The serrated neoplasia pathway of colorectal tumours: identification of *MUC5AC***  
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5 **hypomethylation as an early marker of high-risk polyps**  
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9 **Short title: *MUC5AC* hypomethylation in high-risk colorectal serrated polyps**  
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47 **Key words:** hyperplastic polyp, sessile serrated adenoma, colonic cancer, mucin, methylation.  
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52 **Abbreviations:** CA, conventional adenoma; CIMP, CpG island methylation phenotype;  
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54 CIMP-H, CIMP-high; CIMP-L, CIMP-low; CRC, colorectal carcinoma; GCHP, goblet cell-  
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56 rich hyperplastic polyp; MSI, microsatellite instability; MSS, microsatellite stable; MVHP,  
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3 microvesicular hyperplastic polyp; SSA, sessile serrated adenoma; SSA/D, sessile serrated  
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5 adenoma with dysplasia; TSA, traditional serrated adenoma.  
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**ABSTRACT**

The serrated neoplasia pathway accounts for 20 to 30% of colorectal cancers (CRC), which are characterised by extensive methylation (CpG Island Methylation Phenotype, CIMP), frequent *BRAF* mutation and high microsatellite instability (MSI). We recently identified *MUC5AC* mucin gene hypomethylation as a specific marker of MSI CRC. The early identification of preneoplastic lesions among serrated polyps is currently challenging. Here we performed a detailed pathological and molecular analysis of a large series of colorectal serrated polyps and evaluated the usefulness of mucin genes *MUC2* and *MUC5AC* to differentiate serrated polyps and to identify high-risk lesions. A series of 330 colorectal polyps including 218 serrated polyps (42 goblet cell-rich hyperplastic polyps (GCHP), 68 microvesicular hyperplastic polyps (MVHP), 100 sessile serrated adenoma (SSA), 8 traditional serrated adenoma (TSA)) and 112 non-serrated polyps was analysed for *BRAF/KRAS* mutations, MSI, CIMP, *MLH1* and *MGMT* methylation, and *MUC2* and *MUC5AC* expression and methylation. We show that *MUC5AC* hypomethylation is an early event in the serrated neoplasia pathway, and specifically detects MVHP and SSA, arguing for a filiation between MVHP, SSA, and CIMP-H/MSI CRC, whereas GCHP and TSA arise from a distinct pathway. Moreover, *MUC5AC* hypomethylation specifically identified serrated lesions with *BRAF* mutation, CIMP-H or MSI, suggesting that it may be useful to identify serrated neoplasia pathway-related precursor lesions. Our data suggest that MVHP and SSA should benefit from the same therapeutic and surveillance strategies.

**What's new?**

The identification of high-risk colorectal serrated polyps is challenging since benign hyperplastic polyps and sessile serrated adenoma may share the same morphology and since carcinogenesis in this pathway is faster than in the conventional adenoma-carcinoma pathway. We identified *MUC5AC* hypomethylation as a new early marker of serrated neoplasia pathway, specifically detecting CIMP-H/MSI CRC precursors MVHP and SSA, suggesting they should benefit from the same surveillance for accurate management of patients.

For Peer Review

## INTRODUCTION

Most colorectal cancers (CRC) develop through a conventional adenoma (CA)-carcinoma sequence. However, 20 to 30% of CRC develop through the 'serrated neoplasia pathway', named as for the serrated appearance of crypt in the precursor polyps.<sup>1-3</sup>

Serrated lesions represent a heterogeneous group of polyps, divided into hyperplastic polyps (HP), sessile serrated adenomas (SSA) and traditional serrated adenomas (TSA) by the latest World Health Organisation (WHO) classification of tumors of the digestive system (fourth edition 2010).<sup>4</sup> However a diagnostic 'grey-zone' exists between HP and SSA, with a wide inter-observer variability due to the lack of consensus diagnostic criteria.<sup>5-9</sup> In addition, contradictory guidelines were given for the diagnosis of serrated polyps by the WHO and then by an expert panel,<sup>10</sup> resulting in some confusion among pathologists.<sup>9</sup> Yet, it is important to distinguish SSA from HP, since the former are associated with a malignant potential whereas the later are commonly considered to be benign. Furthermore, neoplastic progression through this pathway has been reported to be faster than within the conventional adenoma-carcinoma pathway. Underdiagnosis of serrated neoplasia pathway precursors in pathologic practice may result in inadequate surveillance and thus contribute to interval carcinomas.<sup>9</sup>

SSA were underdiagnosed for many years due to their resemblance to HP and it currently remains unanswered whether SSA arise *de novo* or derive from pre-existing HP.<sup>11,12</sup>

Molecular characterization has improved our understanding of serrated polyps and associated-risk of malignant progression. SSA are mainly observed in the proximal colon and are associated with frequent *BRAF* mutation and high CpG island methylation phenotype (CIMP-H), suggesting that SSA are precursors of sporadic CIMP-H CRC, most of which display high microsatellite instability (MSI) through epigenetic inactivation of *MLH1*.<sup>2,11,13</sup> TSA seem to be less strongly associated with the serrated neoplasia pathway, but they are

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3 associated with frequent *KRAS* or *BRAF* mutations, low CIMP (CIMP-L) and are supposed to  
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5 be precursors of CIMP-L microsatellite stable (MSS) CRC.  
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8 Moreover, serrated pathway-related polyps and CIMP-H/MSI CRC have similar  
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10 phenotype, including frequent mucinous pattern with increased expression of the intestinal  
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12 mucin *MUC2* and aberrant expression of the gastric mucin *MUC5AC*.<sup>14,15</sup> We recently  
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14 showed that abnormal expression of *MUC2* and *MUC5AC* in CIMP-H/MSI CRC was closely  
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16 related to altered methylation of their promoter.<sup>16</sup> Interestingly, *MUC5AC* demethylation was  
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18 specific to MSI CRC, suggesting that it may serve as a specific marker of the serrated  
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20 neoplasia pathway.  
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26 In this study, 330 polyps including 218 serrated polyps were selected in order to (1)  
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28 further characterise molecular features of serrated polyps and (2) evaluate the potential  
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30 interest of mucin genes *MUC2* and *MUC5AC* to discriminate serrated polyps and to identify  
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32 high-risk lesions. We demonstrate that *MUC5AC* hypomethylation is an early and specific  
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34 marker of preneoplastic lesions in the serrated neoplasia pathway of the colorectum.  
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## MATERIAL AND METHODS

### Patients and tissues

Specimens of colorectal serrated lesions and conventional adenoma were retrieved from the archives of the Department of Pathology of the Lille University Hospital. All cases were collected from patients who underwent endoscopic mucosal resection, polypectomy or colectomy at Lille University Hospital from 2009 to 2013. Polyps were selected on the basis that they fulfilled the diagnostic criteria, were well oriented, and had sufficient available tissue for both immunohistochemical and molecular analyses. Clinical and endoscopic data were available for all patients. Clinical information retrieved included sex and age, colonic localisation (classified as proximal or distal relative to the splenic flexure) and size of the polyp. Informed consent was obtained from all patients. Approval of this study was obtained from the Institutional Review Board of Lille University Hospital.

### Histological analysis

All specimens were reviewed by three experienced gastrointestinal pathologists (EL, AW, FR) blinded to the clinical and molecular information. Serrated lesions (HP, SSA and TSA) were classified on the basis of the criteria from the latest WHO classification.<sup>4</sup> Representative serrated polyps included in this study are shown in Figure 1. Mixed polyps or cases with hyperplastic polyposis were excluded from this study. HP were defined as serrated polyps with simple tubular architecture composed of serrated crypts in the upper half of the lesion, narrow and straight crypts at the base. HP were subdivided into two groups: microvesicular HP (MVHP) and goblet cell-rich HP (GCHP) defined as per previously published criteria.<sup>4</sup> MVHP were characterized by elongated and relatively straight crypts, with serration that are visible mainly near the luminal end of the crypts, epithelial cells with microvesicular (small

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3 droplet) mucin and absence of cytologic dysplasia (Figure 1A). GCHP were characterized by  
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5 elongated and slightly serpiginous crypts mostly composed of mucin-rich goblet cells (Figure  
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7 1B). SSA were defined as serrated polyps with basal crypt dilatation, crypt branching,  
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9 horizontal extension of crypts at the base, prominent serrations, mitoses in the upper half of  
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11 crypts and dystrophic goblet cells (Figure 1C). To classify a polyp as SSA, there should be at  
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13 least two or three contiguous SSA-type crypts. Dysplasia was defined by the presence of  
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15 narrow elongated hyperchromatic nuclei, nuclei stratification and basophilic cytoplasm  
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17 (Figure 1D). TSA were defined as serrated polyps composed of a uniform population of cells  
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19 showing abundant eosinophilic cytoplasm, nuclear elongation, ectopic crypt formations and  
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21 nuclear hyperchromasia with a tubular or villous architecture and mild crypt or surface slit-  
22  
23 like serration (Figures 1E and 1F).  
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27 In total, 330 polyps from 330 patients were included in the study. They contained 218  
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29 serrated polyps with 110 HP (68 MVHP, 42 GCHP), 100 SSA (41 SSA with dysplasia), 8  
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31 TSA (3 with dysplasia). Non-serrated adenomas (112 CA including 17 tubular, 18 villous, 77  
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33 tubulovillous) diagnosed during the same period were also included as controls.  
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### 37 38 **Immunohistochemistry**

39  
40 Immunohistochemical studies were carried out on 4- $\mu$ m-thick formalin-fixed, paraffin-  
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42 embedded whole tissue sections using antibodies against mucins MUC2 and MUC5AC as  
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44 previously described.<sup>17</sup> The average percentage of stained tumour cells and intensity of  
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46 staining were calculated using a semiquantitative histological scoring method (Hscore).<sup>18</sup>  
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48 Hscore above or equal the threshold of 20 was considered as positive expression.  
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### 52 53 **DNA methylation analysis**

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3 The promoter methylation status of 6 genes commonly used to define CIMP status (*CDKN2A*  
4 (*p16*), *RUNX3*, *CACNA1G*, *SOCS1*, *NEUROG1*, and *IGF2*), DNA repair genes *MLH1* and  
5 *MGMT*, and mucin genes *MUC2* and *MUC5AC* was determined by quantitative bisulfite  
6 pyrosequencing assays as previously described.<sup>16</sup> Amplification of *MGMT* and *MLH1* was  
7 performed using the PyroMark kits (Qiagen). The percentage of methylation for a given gene  
8 was calculated as the mean methylation levels of all CpG sites analysed. Tumours with a  
9 methylation level above or equal to the threshold of 10% were considered as methylation-  
10 positive. Tumours were defined as CIMP-H when  $\geq 3/6$  of the CIMP-specific gene markers  
11 were methylated. Tumours with 1 or 2 methylated markers and tumours with no methylated  
12 marker were considered as CIMP-L and CIMP-negative, respectively. Hypomethylation was  
13 defined by a methylation level below the thresholds of 70% for *MUC5AC* and 60% for  
14 *MUC2*, as previously described.<sup>16</sup>

### 31 **MSI and mutation analysis**

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33 MSI was determined using a six-marker panel as previously described.<sup>19</sup> Mutations in codons  
34 12 and 13 of *KRAS* and in codon 600 of *BRAF* were identified by pyrosequencing as  
35 previously described.<sup>16</sup>

### 42 **Statistical analysis**

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44 SPSS version 19.0 software (SPSS, Chicago, IL) and GraphPad Prism version 6.02 were used  
45 for statistical analyses. Discrete variables were compared using the Chi square test or the  
46 Fischer exact test as appropriate. Continuous variables were compared using the Mann-  
47 Whitney *U* test. A 2-tailed probability value (*P*) was calculated. A *P* value < 0.05 was  
48 considered statistically significant. CIMP-L and CIMP-negative tumours were grouped *vs*  
49 CIMP-H tumours for statistical analyses.

## RESULTS

### Clinicopathological characteristics of the cohort

Table 1 summarises the clinical and endoscopic findings of the patients and their polyps.

Among the 330 polyps, 153 were from women and 177 from men, with overall median age of 65 years (range, 32 to 88). Patients with TSA were significantly older than patients with other polyps ( $P = 0.04$ ). There were no significant different sex ratio between the subtypes of polyps or between patients with serrated polyps (HP, SSA, TSA) and those with CA.

Overall, the median size of polyps was 10 mm (range, 3 to 35 mm) (Table 1). Among serrated polyps, HP were significantly smaller (median, 4.9 mm) than SSA (median, 9 mm), SSA/D (median, 10) and TSA (median, 20 mm) ( $P < 0.0001$ ,  $P < 0.0001$  and  $P < 0.0001$ , respectively).

In terms of tumour site, 44% were proximal and 56% were distal (Table 1). Compared to CA, serrated polyps were more frequently found in the proximal colon ( $P < 0.001$ ). Among serrated polyps, 53% of MVHP, 69% of SSA, and 71% of SSA/D were proximal, whereas only 5% of GCHP and 25% of TSA were proximal.

### Mutational and methylation characteristics of polyp subtypes

Activating mutations in *BRAF* or *KRAS*, MSI and abnormal methylation of a number of genes are common events in preneoplastic colorectal lesions. All cases were successfully analysed for these molecular events. Molecular alterations within the different polyp types are shown in Figure 2.

Compared to CA, serrated polyps displayed higher frequency of *BRAF* V600E mutation (21.6% vs 1.8%), MSI (17.9% vs 0%) and CIMP-H (24.8% vs 5.4%) and lower frequency of

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3 *KRAS* mutations (11.5% vs 33.9%). *BRAF* and *KRAS* mutations were mutually exclusive in all  
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5 types of polyps.

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7 Among GCHP, 0% displayed *BRAF* mutation, whereas 47.6% displayed *KRAS* mutation,  
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9 and 0% was MSI. Only 2.4% were CIMP-H, but 11.9% were CIMP-L and 9.5% displayed  
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11 abnormal methylation of *MGMT*. Conversely, among MVHP, 33.8% displayed *BRAF*  
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13 mutation, 0% *KRAS* mutation, 8.8% were MSI, and 13.2% were CIMP-H. *BRAF* mutations,  
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15 MSI and CIMP were more common in proximal MVHP compared to distal MVHP (47.2% vs  
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17 18.7%,  $P = 0.02$ , 16.7% vs 0%,  $P = 0.03$ , and 25.0% vs 0%,  $P = 0.0025$ , respectively).

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20 In SSA and SSA/D, *BRAF* mutations were significantly more frequent than *KRAS*  
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22 mutations (22.0% vs 2.0%), 33.0% were MSI with methylation of *MLH1*. 41.0% of SSA were  
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24 CIMP-H and 8.0% displayed methylation of *MGMT*. *BRAF* mutations, MSI and CIMP were  
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26 more common in proximal SSA compared to distal SSA (28.0% vs 9.7%,  $P = 0.06$ , 43.0% vs  
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28 9.0%,  $P = 0.001$ , and 59.5% vs 4.0%,  $P < 0.0001$ , respectively).

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31 In TSA, *KRAS* mutations were detected in 37.5% of cases, *BRAF* in 25.0% and all were  
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33 MSS. TSA were essentially CIMP-negative (50.0%) or CIMP-L (37.5%), and were  
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35 particularly prone to *MGMT* methylation (37.5%). Notably, CIMP-L, detected in 7.3% of all  
36  
37 polyps, was significantly associated with *MGMT* methylation ( $P < 0.0001$ ).

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40 Taken together, our findings argue for a relationship between MVHP and SSA, GCHP  
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42 and TSA being apart. Moreover, comparison of methylation patterns between the different  
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44 sub-groups showed an increase in the number of methylation-positive CIMP-target genes and  
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46 in the methylation levels from MVHP to SSA to SSA/D (Figure 2 and Figure 3A).

#### 51 52 **Mucin expression in subtypes of polyps**

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54 Immunohistochemistry for the intestinal mucin MUC2 and the gastric mucin MUC5AC was  
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56 performed and analysed in all polyps. Frequencies and representative images of expression  
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3 within the different types of polyps are shown in Figure 4 and Additional supporting  
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5 information.

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7 MUC2 and MUC5AC expressions were more frequent in serrated polyps than in CA  
8 (95% vs 63%,  $P < 0.0001$  and 96% vs 59%,  $P < 0.0001$ , respectively). Moreover, in HP  
9 (GCHP and MVHP), SSA, SSA/D and TSA, MUC2 and MUC5AC staining intensity was  
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11 generally strong to moderate and homogeneously distributed along the epithelium, whereas it  
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13 was more heterogeneous in CA.  
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### 18 19 20 21 **Mucin gene methylation in subtypes of polyps and association with clinicopathologic** 22 **features**

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24 Methylation of *MUC2* and *MUC5AC* mucin genes was analysed in all polyps. Results are  
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26 shown in Figure 2 and Figure 3B.  
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30 *MUC2* mean methylation level was 59.2% in serrated polyps and 63.8% in CA. *MUC2*  
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32 hypomethylation was more frequent in serrated polyps than in CA (36.7% vs 18.8%,  
33  
34  $P < 0.0001$ ). Among serrated polyps, *MUC2* hypomethylation was detected in 50.0% of  
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36 GCHP, 20.6% of MVHP, 44.1% of SSA, 43.9% of SSA/D and 12.5% of TSA. In GCHP,  
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38 *MUC2* hypomethylation was significantly associated with *KRAS* mutation ( $P = 0.006$ ). In  
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40 MVHP, *MUC2* hypomethylation was associated with proximal location ( $P = 0.001$ ) and  
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42 CIMP ( $P = 0.002$ ). In SSA, *MUC2* hypomethylation was associated with proximal location ( $P$   
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44  $< 0.0001$ ), *BRAF* mutation ( $P < 0.0001$ ), MSI ( $P < 0.0001$ ), and CIMP ( $P < 0.0001$ ).  
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48 *MUC5AC* mean methylation level was 82.5% in serrated polyps and 92.3% in CA.  
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50 *MUC5AC* hypomethylation was detected in 18.8% of serrated polyps and none of the CA,  
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52 indicating that *MUC5AC* hypomethylation is specific of the serrated pathway ( $P < 0.0001$ ).  
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54 Furthermore, in serrated polyps, *MUC5AC* hypomethylation was detected in 8.8% MVHP,  
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56 25.4% SSA, and 43.9% SSA/D, but in none of GCHP or TSA. In MVHP, *MUC5AC*  
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3 hypomethylation was associated with proximal location ( $P = 0.011$ ), *BRAF* mutation  
4 ( $P = 0.017$ ), MSI ( $P < 0.0001$ ), *MLH1* methylation ( $P < 0.0001$ ), and CIMP-H ( $P < 0.0001$ ).  
5  
6  
7 In SSA (with or without dysplasia), *MUC5AC* hypomethylation was associated with proximal  
8 location ( $P < 0.0001$ ), *BRAF* mutation ( $P < 0.0001$ ), MSI ( $P < 0.0001$ ), *MLH1* methylation  
9 ( $P < 0.0001$ ), and CIMP ( $P < 0.0001$ ).  
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### 16 ***MUC5AC* hypomethylation is predictive of high-risk serrated lesions**

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18 We further evaluated the performances of *MUC5AC* hypomethylation for the identification of  
19 lesions harbouring molecular alterations, which should thus be considered as at high risk of  
20 progression to carcinoma.  
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25 *MUC5AC* hypomethylation was detected in 52.7% (39/74) of serrated polyps harbouring  
26 *BRAF* mutation, CIMP-H, or MSI and in none (0/144) of the other serrated polyps, which  
27 corresponds to a positive predictive value of 100% and a negative predictive value of 81.4%  
28 for the identification of high-risk serrated lesions.  
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34 By comparison, *MUC2* hypomethylation was detected in 52.7% (39/74) of serrated  
35 polyps harbouring *BRAF* mutation, CIMP-H or MSI, but also in 27.8% (40/144) of the others,  
36 which corresponds to a positive predictive value of 49.4% and a negative predictive value of  
37 75.9% for the identification of high-risk serrated lesions.  
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## DISCUSSION

Despite the improved recognition of the clinicopathological significance of serrated lesions of the colorectum, some questions remain to be resolved, such as the differential diagnosis of serrated polyp subtypes and especially the identification of those which require close surveillance. In this study, we performed a detailed pathological and molecular analysis of secreted mucin genes *MUC2* and *MUC5AC*. We show that *MUC5AC* hypomethylation is specific of high-risk serrated polyps, suggesting that *MUC5AC* hypomethylation may serve as a marker to identify serrated pathway neoplasia-related precursors.

Clinicopathological and molecular features were characterized in our series of polyps. Clinicopathological data were in agreement with previous reports,<sup>20-23</sup> confirming that most SSA and MVHP are proximal, whereas most TSA and GCHP are distal. In line with recent reports,<sup>11,13,24,25</sup> we found that SSA, especially proximal SSA/D, harboured frequent *BRAF* mutation, were often CIMP-H and MSI due to *MLH1* methylation. Moreover, although the number of TSA in our series was small, we confirmed that they may display *KRAS* or *BRAF* mutations,<sup>26-28</sup> were MSS, and often CIMP-L with methylation of *MGMT*. Regarding HP, MVHP differed from GCHP in that they harboured frequent *BRAF* mutation, some of them being CIMP-H and MSI. Conversely, GCHP showed exclusively *KRAS* mutations, were MSS, CIMP-negative or CIMP-L, but prone to *MGMT* methylation.

A common feature of serrated pathway-related polyps and CIMP-H/MSI CRC is frequent mucin-rich/mucinous pattern with increased expression of secreted mucins. We analysed the expression of the secreted mucins *MUC2* and *MUC5AC* in our series of polyps. According to prior studies,<sup>29,30</sup> increased expression of the intestinal mucin *MUC2* and aberrant expression of the gastric mucin *MUC5AC* was observed in most serrated polyps, especially in MVHP



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3 and SSA. Accumulating evidence indicates that serrated pathway-associated colorectal  
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5 tumours may display aberrant gastric-type differentiation. However, MUC5AC expression  
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7 was also observed in CA, although it was less frequent and weaker than in serrated polyps.  
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9 Our findings, in line with recent ones,<sup>30</sup> demonstrate that gastric-type expression is not  
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11 exclusive to serrated pathway-associated lesions.  
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14 Based on our recent data showing that abnormal expression of MUC2 and MUC5AC in  
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16 CRC was closely related to demethylation of their promoters,<sup>16</sup> we determined the  
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18 methylation status of *MUC2* and *MUC5AC* in our series of polyps. Hypomethylation of  
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20 *MUC2* and *MUC5AC* was detected in a proportion of serrated lesions including HP,  
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22 indicating that the demethylation process is an early event in polyp development. This  
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24 mechanism may contribute to increased production of mucus and to acquisition of their  
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26 particular mucin-rich phenotype.  
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31 It has been suggested that SSA may originate from certain HP. The frequent finding of  
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33 MVHP-like areas within large SSA and identification of excessive methylation and *BRAF*  
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35 mutations in both MVHP and SSA are evidence supporting that SSA may represent  
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37 ‘advanced’ forms of MVHP, but the relationship between these two entities has not been  
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39 clearly established.<sup>31-33</sup>  
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43 A key finding of this study was that *MUC5AC* hypomethylation was restricted to MVHP  
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45 and SSA, suggesting it may be useful to discriminate GCHP from MVHP and SSA.  
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47 Moreover, there was a gradual increase in the frequency of *MUC5AC* hypomethylation from  
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49 MVHP to SSA and SSA/D suggesting that SSA arise from MVHP. Interestingly, our recent  
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51 data in CRC showed that *MUC5AC* hypomethylation was specific to MSI/CIMP-H CRC.<sup>16</sup>  
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53 Altogether, our data argue for a progression of MVHP to SSA, SSA/D, and then to  
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55 MSI/CIMP-H CRC (Figure 5). Some authors have questioned the utility of distinguishing  
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3 MVHP and GCHP.<sup>10,34</sup> Our data reinforce the concept that MVHP and GCHP are distinct  
4 morphological but also biological entities, highlighting a true relevance to diagnose MVHP  
5 and to precise the HP subtype in pathological reports.  
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9 We further evaluated the performances of *MUC5AC* hypomethylation in identifying high-  
10 risk lesions. *MUC5AC* hypomethylation accurately identified only serrated lesions with *BRAF*  
11 mutation, CIMP-H or MSI, with a positive predictive value of 100% and a negative predictive  
12 value of 81.4%, validating the utility of *MUC5AC* hypomethylation to assess the malignant  
13 risk of precursor lesions.  
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17 Furthermore, our data suggest that *MUC5AC* demethylation may play a role in the  
18 serrated pathway to colorectal cancer. Understanding the mechanisms leading to *MUC5AC*  
19 demethylation and subsequent aberrant expression of the gastric-type *MUC5AC* mucin could  
20 help to elucidate whether it is directly involved in the progression of tumours through this  
21 pathway.  
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25 Until recently, information regarding TSA was very scarce, probably due to their rarity,  
26 accounting for less than 1% of all colorectal polyps.<sup>4</sup> Recent studies on large series of TSA  
27 have enriched our knowledge regarding their clinicopathological and molecular features.<sup>27,28</sup>  
28 Whereas TSA are morphologically fairly similar, they are characterized by molecular  
29 heterogeneity, showing *BRAF* or *KRAS* mutation and variable CIMP, suggesting at least two  
30 pathways of neoplastic progression. Frequent identification of SSA-type lesions in proximal  
31 and *BRAF* mutant TSA and frequent association with MVHP led to the hypothesis that some  
32 TSA develop from SSA.<sup>28</sup> However, no *MUC5AC* hypomethylation was detected in TSA in  
33 our series, especially in the two proximal TSA-with *BRAF* V600E mutation. Although the  
34 number of TSA was small in our series, our data suggest a pathway distinct from the MVHP-  
35 SSA-CIMP-H CRC pathway, as previously suggested by Tsai *et al.*<sup>27</sup> Our hypothesis is  
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3 supported by recent findings obtained *in vivo* in a *BRAF*-mutant mouse model, which showed  
4 that the resulting mice developed serrated polyps which progressed to TSA, but not to SSA.<sup>35</sup>  
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6 Conversely, we observed similarities between TSA with *KRAS* mutation and some GCHP that  
7 were CIMP-L with *MGMT* methylation, suggesting that some TSA may develop from GCHP  
8 and progress to conventional adenoma and CIMP-negative or CIMP-L MSS CRC (Figure  
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Our data have major implication at the clinical level. The identification of high-risk serrated polyp is currently challenging, but is essential to guide adequate cancer screening and prevention care, especially the neoplastic progression within this pathway has been reported to be faster than within the classical adenoma-carcinoma pathway.<sup>36,37</sup>

In current clinical practice, whereas the diagnosis of SSA is followed by the same colonoscopic surveillance as for patient with CA,<sup>34</sup> the diagnosis of HP including MVHP does not lead to a follow-up. Identification of MVHP as precursor lesions of SSA could impact medical practice.

In conclusion, this study further confirms the importance to accurately diagnose serrated lesions. We have shown that *MUC5AC* hypomethylation may be useful to distinguish serrated polyps and to identify high-risk lesions. Moreover, our findings suggest that some MVHP may progress to SSA, SSA/D, and CIMP-H/MSI CRC. In this respect, MVHP and SSA should benefit from the same therapeutic and surveillance strategies.

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For Peer Review

Table 1. Clinical and endoscopic findings

Polyp type	HP	SSA	SSA/D	TSA	CA
No. of cases	110	59	41	8	112
Male:Female ratio	60:50	35:24	18:23	3:5	61:51
Median age (y) (range)	65.5 (34-88)	61 (32-83)	63 (43-82)	71 (60-85)	65 (45-82)
Median size (mm) (range)	4.9 (3-8)	9 (5-20)	10 (5-30)	20 (15-30)	15 (8-35)
Proximal:Distal colon	38:72	41:18	29:12	2:6	34:78

HP, hyperplastic polyp; SSA, sessile serrated adenoma; SSA/D, sessile serrated adenoma with dysplasia; TSA, traditional serrated adenoma; CA, conventional adenoma

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**Figure Legends.****Figure 1. Representative serrated colorectal polyps included in the study.**

A. Microvesicular hyperplastic polyp (MVHP) located in the proximal colon, 20 x magnification. Relatively straight crypts are mostly composed of microvesicular epithelial cells.

B. Goblet cell-rich hyperplastic polyp (GCHP) located in the distal colon, with slightly serpiginous crypts and numerous mucin-rich goblet cells, 40 x magnification.

C. Sessile serrated adenoma (SSA) located in the proximal colon, 20 x magnification. Note the serrations that extend to the crypt base, basal crypt dilatation, horizontal growth and absence of cytologic dysplasia.

D. SSA with low-grade dysplasia (SSA/D), defined by enlarged hyperchromatic nuclei, nuclear stratification and loss of polarity, 20 x magnification.

E. Traditional serrated adenoma (TSA), with protuberant villous architecture, 5 x magnification.

F. TSA with low-grade dysplasia, 10 x magnification. Note slit-like serrations (arrows), epithelial cells with abundant brightly eosinophilic cytoplasm with central nuclei, ectopic crypts formations.

**Figure 2. Schematic representation of anatomic, mutational and methylation characteristics of polyps.**

Horizontal rows represent individual polyp. Each column shows the tumour site and the presence or absence of mutation (*KRAS*, *BRAF*), microsatellite instability (MSI), abnormal promoter methylation (*MLH1*, *MGMT*, CIMP-related genes, *MUC2*, *MUC5AC*), CpG island methylator phenotype (CIMP). Dark grey squares indicate proximal site, presence of

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3 mutation, MSI, hypermethylation of *MLH1*, *MGMT*, CIMP markers and high CIMP; light  
4 grey squares indicate low CIMP; white squares indicate distal site, absence of mutation, MSI,  
5 methylation for *MLH1*, *MGMT*, CIMP markers and hypomethylation of *MUC2* or *MUC5AC*  
6 (see materials and methods for details). GCHP, goblet cell-rich hyperplastic polyps; MVHP,  
7 microvesicular hyperplastic polyp; SSA, sessile serrated adenoma; SSA/D, sessile serrated  
8 adenoma with dysplasia; TSA, traditional serrated adenoma; CA, conventional adenoma.  
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### 18 **Figure 3. Methylation profiles in colorectal polyps.**

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20 These graphs show individual methylation levels for CIMP markers (A) and mucin genes (B)  
21 in the different polyp subtypes: goblet cell-rich hyperplastic polyp (GCHP), microvesicular  
22 hyperplastic polyp (MVHP), sessile serrated adenoma (SSA), sessile serrated adenoma with  
23 dysplasia (SSA/D), traditional serrated adenoma (TSA) and conventional adenoma (CA).  
24 Each circle represents an individual specimen; the horizontal bars represent means values.  
25 Indicated comparisons correspond to GCHP vs MVHP, and to each serrated polyp subtype vs  
26 CA (Fisher exact test). Only significant *P*-values are shown. Note the restriction of *MUC5AC*  
27 hypomethylation to MVHP, SSA and SSA/D and the increase in methylation levels from  
28 MVHP to SSA and to SSA/D for CIMP markers and mucin genes.  
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### 43 **Figure 4. Mucin immunohistochemical expression in colorectal polyps.**

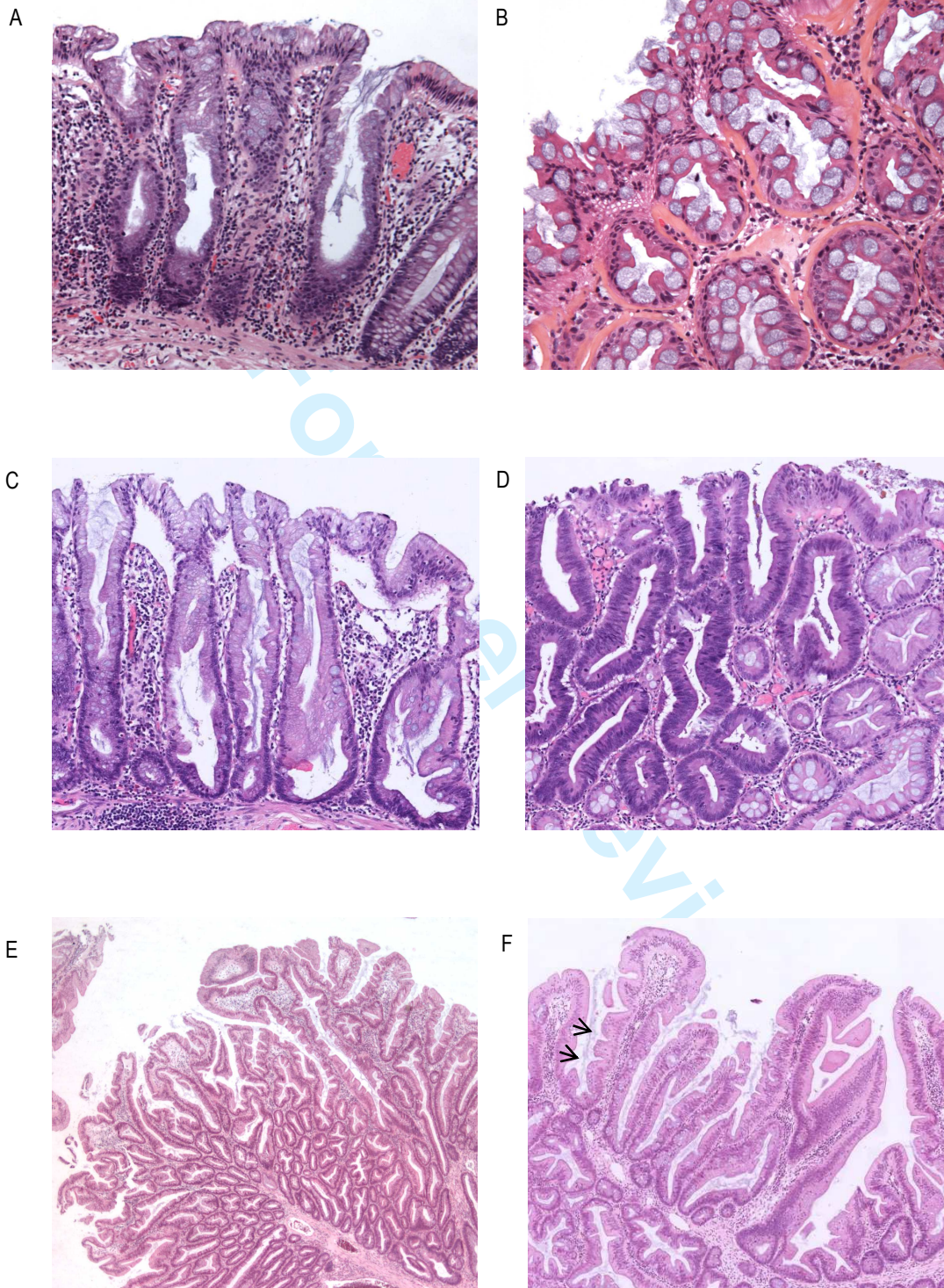
44  
45 Representative photomicrographs of immunohistochemistry for MUC2 and MUC5AC in  
46 colorectal polyps. Note the positivity of MUC2 and MUC5AC in hyperplastic polyps (HP),  
47 sessile serrated adenomas (SSA) and in traditional serrated adenomas (TSA). The staining  
48 was homogeneous along the crypt, with a moderate or high intensity. The staining was more  
49 heterogeneous in conventional adenoma (CA) in terms of frequency, intensity and  
50 distribution.  
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5 **Figure 5. Schematic representation of putative pathways to colorectal cancer.**  
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7 GCHP, goblet cell-rich hyperplastic polyp; MVHP, microvesicular hyperplastic polyp; SSA,  
8 sessile serrated adenoma; SSA/D, sessile serrated adenoma with dysplasia; TSA, traditional  
9 serrated adenoma; CA, conventional adenoma; CIN, chromosomal instability; CIMP, CpG  
10 island methylator phenotype; CIMP-H, CIMP-high; CIMP-L, CIMP-low; MSI, microsatellite  
11 instability; MSS, microsatellite stable.  
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For Peer Review

Figure 1.



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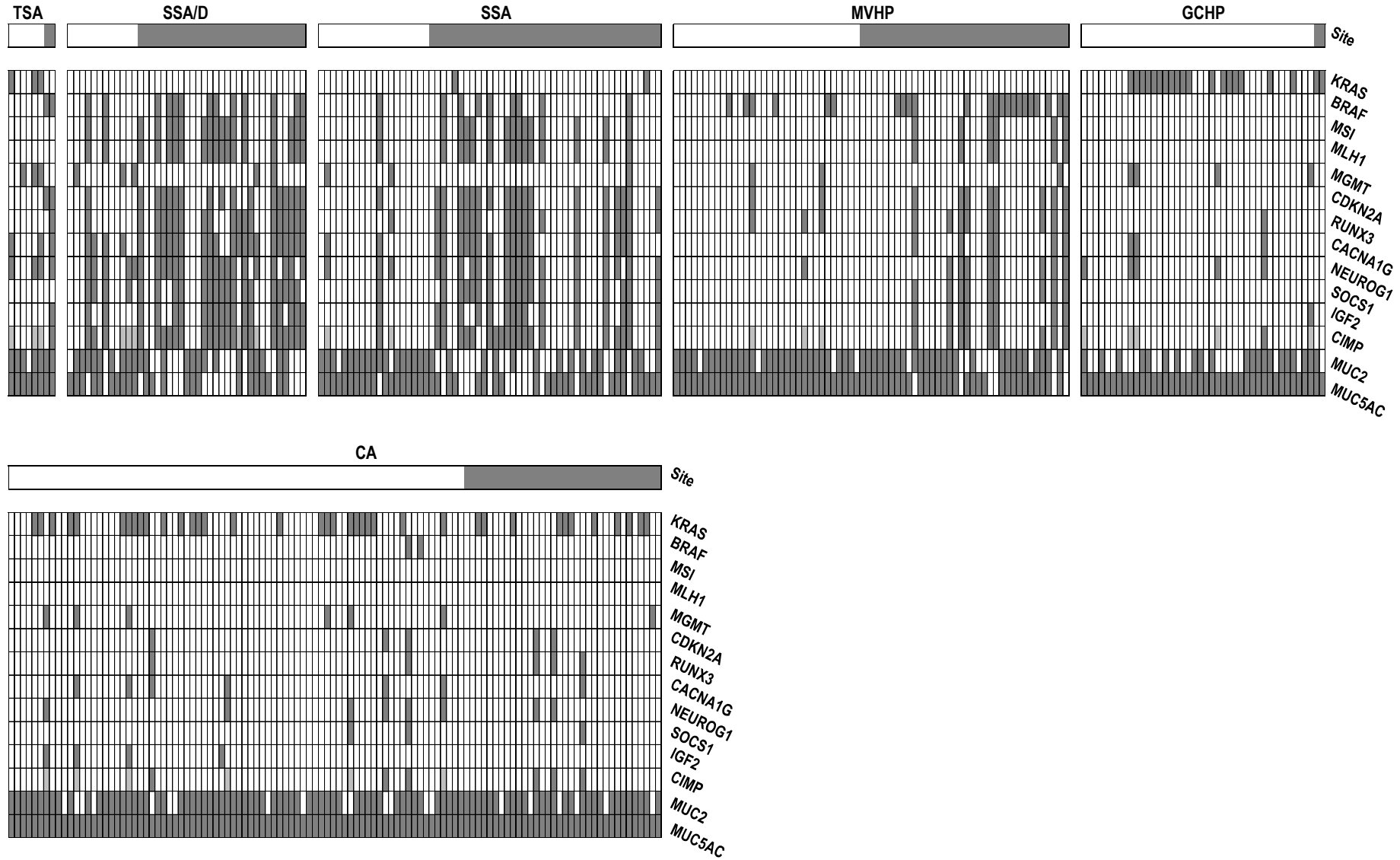
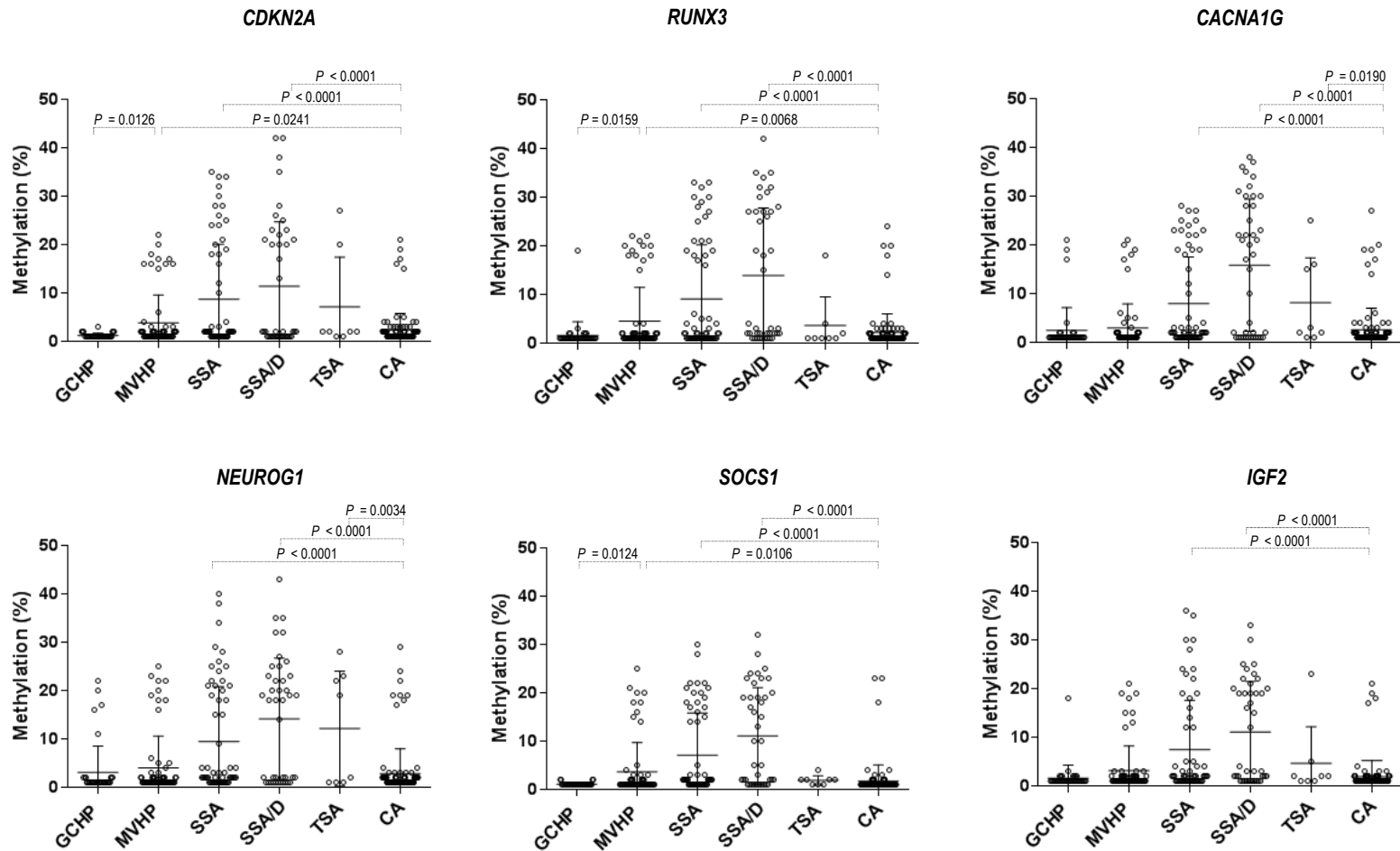


Figure 2.



Figure 3.

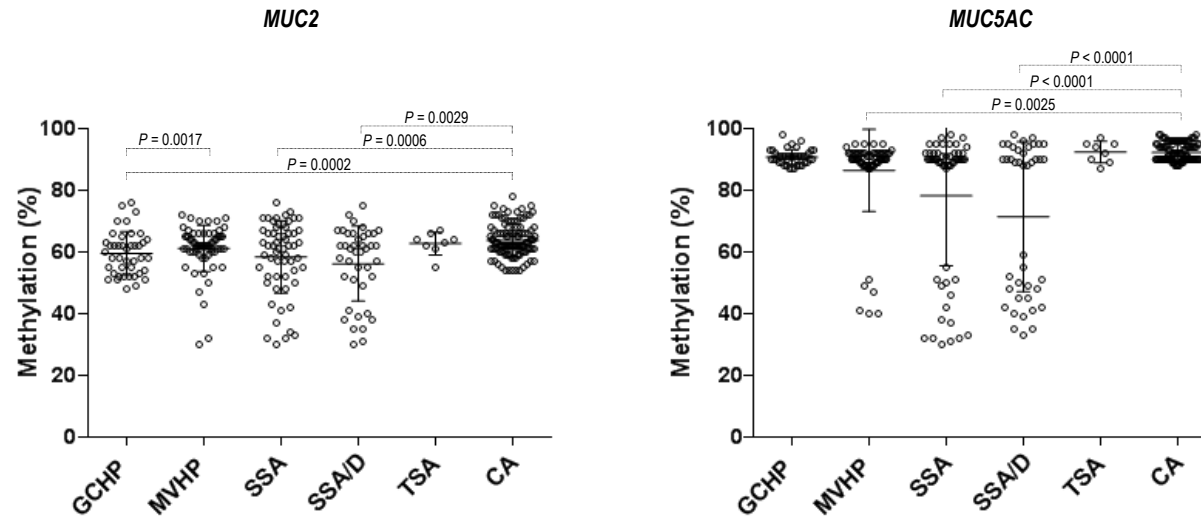
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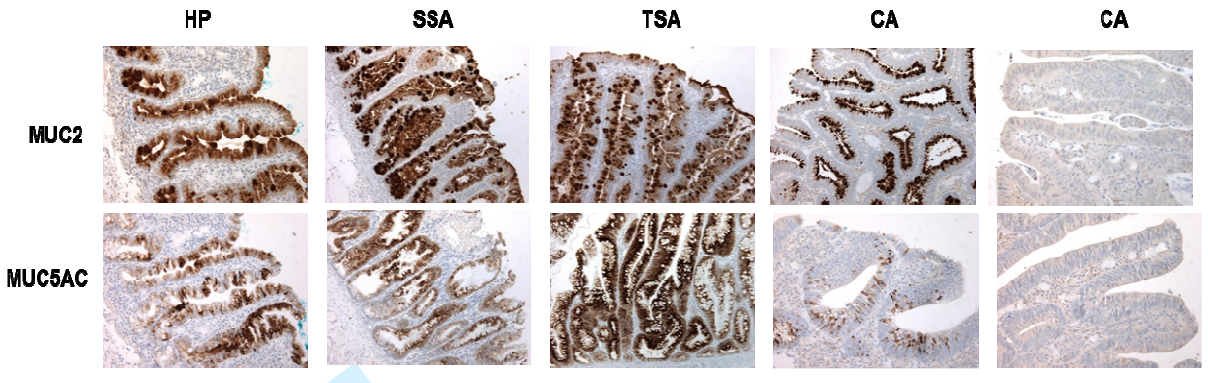
Figure 3. (continued)

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Figure 4.



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Figure 5. Schematic representation of putative pathways to colorectal cancer

