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Somatic POLE exonuclease domain mutations in endometrial cancer : Insights into the biology of POLE-mutant tumors

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Insights into the biology of *POLE*-mutant tumors

Inge Charlotte Van Gool

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Insights into the biology of *POLE*-mutant tumors

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Gelukkig kunnen we erbij zitten
Opa Van Gool, † 2013

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Chapter 1

Introduction

INTRODUCTION

Personalized medicine concerns the tailoring of prevention, diagnosis, and treatment of disease to the individual's characteristics, needs, and preferences. The concept of personalized medicine is not new. From the time of Hippocrates (~400 B.C.E.), doctors have been aware that patients with similar symptoms may suffer from different diseases; and similarly, that patients with the same disease may respond differently to given treatments.¹ In cancer care, clinicians have long used the location of the tumor and the stage of disease with which a patient presents to direct initial and adjuvant treatment. The stage of disease can be summarized by the extent to which the tumor has spread beyond its original site. For patients with a localized or low-stage tumor, surgical resection can be curative. In contrast, when the cancer has spread throughout the body, surgery alone is not effective, whereas systemic therapy such as chemotherapy may be. Over time, tumor location and disease stage have been combined with multiple other clinicopathological features to provide an increasingly comprehensive profile of a specific tumor. Such features include the histological tumor type and grade (a measure of the appearance and growth patterns of tumor cells), the invasion of cancer cells into lymph and/or blood vessels, and the expression of specific proteins as determined by immunohistochemical analyses. The resulting clinicopathological profile has since been used to predict how a particular cancer will behave and consequently, to assess how best to treat an individual patient.

While the concept of personalized medicine based on clinicopathological characteristics in cancer is not new, the genomic data that can be incorporated into clinical decision making is a rapidly expanding field. Sanger sequencing has been the traditional method of genomic analysis: with this method, 500 to 600 base pairs of DNA can be sequenced per reaction (to put this into perspective, the human genome consists of three billion base pairs). In the past two decades, newer methods of genomic analysis have been introduced that can sequence longer fragments of DNA in a single run. As multiple DNA sequencing platforms were developed around the same time, fierce competition between manufacturers arose to increase the length and output of the sequences, and at the same time, to lower the costs. These new and cheaper sequencing technologies enabled an unprecedented expansion of our knowledge of the human genome.^{2,3}

Cancer can, in essence, be considered a disease of the genome in a specific organ or tissue. Much of the contemporary research on cancer has focused on using the genomic data to identify and understand the molecular basis of this disease. This has led to a more advanced form of personalized medicine, now often also termed precision medicine, in which the individual's tumor characteristics not only include clinicopathological

data but also the molecular profile of the specific cancer. Such a combination can even more accurately predict a patient's prognosis and the likelihood of response of the tumor to conventional treatment (e.g. chemo- or radiotherapy). Moreover, it may predict response to specific targeted therapies, directed against molecular alterations identified in the patient's tumor.

The benefits through the application of precision medicine have been clearly demonstrated in breast cancer. In this tumor type, molecular profiling has been implemented to provide more precise estimates of patient outcomes. Together with clinical and morphological features (including age, tumor size, grade, and lymph node status), determination of hormone receptor status and of human epidermal growth factor 2 (HER2) expression (overexpression as surrogate marker for gene amplification) are the standard of care. Both markers are used to predict a patient's prognosis and to stratify for conventional and targeted (adjuvant) treatments.⁴ Moreover, this integrated molecular approach has been further refined by the use of gene expression profiles such as the MammaPrint or OncotypeDX. These profiles provide additional prognostic information, and, in certain patients, allow for tailored treatments.⁵⁻⁹

In summary, the use of molecular biomarkers has the potential to improve treatment response and prognostic forecasting. Although for some cancers, molecular profiling has already been implemented in clinical practice, for other cancer types, including endometrial cancer, this is not yet the standard of care. This chapter describes the epidemiology, diagnosis, and (adjuvant) treatment options in endometrial cancer. Moreover, it describes our current knowledge of molecular alterations and how these may improve personalized medicine in endometrial cancer.

Endometrial cancer

Epidemiology

Endometrial cancer arises from the epithelial lining of the uterus, called the endometrium. It is the most common gynecological malignancy in the Western world, accounting for approximately 1900 new cases in the Netherlands each year.⁴ Due to the increasing obesity and ageing of the population, two important risk factors for the development of endometrial cancer, the incidence of endometrial cancer has been rising rapidly (36% increase since 2000 in the Netherlands, Figure 1).¹⁰⁻¹³ This disease typically afflicts older women - incidence peaks between 65 and 75 years of age - who present with postmenopausal bleeding.^{4,11} This symptom is recognized by both patients and physicians as an indication for prompt referral and investigation, and leads to diagnosis of endometrial cancer in an early stage of disease in the majority (~75%) of patients.^{11,12} Prognosis for these patients is favorable, with a five-year overall survival of 85-90%. For women with

recurrent or metastatic disease, however, outcomes are poor, with a five-year overall survival of ~20% for patients with distant metastases.¹² Because of the favorable prognosis of early-stage disease, the overall mortality rate is relatively low: approximately 460 women die of endometrial cancer in the Netherlands each year. This contrasts with other gynecological malignancies such as ovarian cancer, where ~75% of patients die of the disease.¹¹

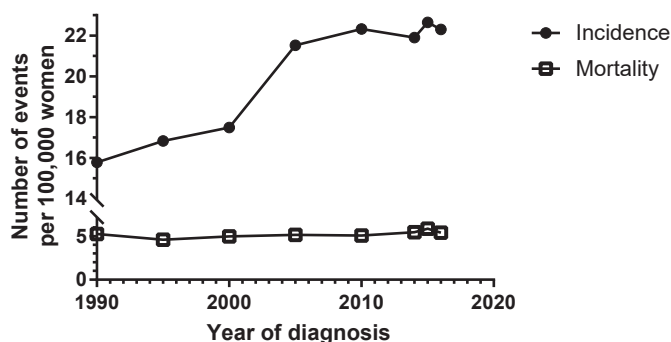


Figure 1. Incidence and mortality of endometrial cancer in the Netherlands per 100,000 women in the period 1990 – 2016.¹¹

Diagnosis and primary treatment

Endometrial cancer is diagnosed by histopathological assessment of endometrial tissue obtained through sampling of the endometrium by biopsy or curettage. After diagnosis, standard treatment consists of surgery, during which the uterus, both ovaries and fallopian tubes are removed (abdominal or laparoscopic total hysterectomy and bilateral salpingo-oophorectomy).⁴ The indication for adjuvant treatment is currently based on a patient's risk of disease recurrence as estimated by clinicopathological risk factors. Risk factors include tumor type, tumor grade, stage of disease, age of the patient, and lymphovascular space invasion. These factors are described in more detail below.

Clinicopathological risk factors

Histological type

Historically, endometrial carcinomas can be divided morphologically into two main tumor types: endometrioid and non-endometrioid endometrial cancers (Figure 2).¹⁴ Endometrioid carcinomas, the most common subtype (~80% of cases), are cancers that morphologically resemble the normal endometrium. This subtype is associated with unopposed estrogen stimulation and usually arises in the background of endometrial hyperplasia. In contrast, non-endometrioid endometrial cancers are estrogen-independent tumors that often arise in atrophic endometrium and have various histological subtypes, including serous and clear cell carcinomas.^{14,15} Hamilton et al., among others,

reported that while 13% of endometrial cancers showed non-endometrioid histology in their study, these cases accounted for 47% of endometrial cancer deaths.¹⁶ Thus, compared to endometrioid-type tumors, non-endometrioid endometrial cancers have an aggressive clinical course and account for a disproportionate number of endometrial cancer deaths.

FIGO grade

Endometrioid endometrial cancers are graded according to the International Federation of Gynecology and Obstetrics (FIGO) classification based on the percentage of non-squamous solid growth and on the degree of nuclear atypia in comparison to normal endometrium (Figure 2A-B).^{12,15,17} An endometrioid endometrial cancer consisting of a predominantly glandular architecture with no or minimal solid growth ($\leq 5\%$) is considered grade 1, whereas endometrial cancers with 6-50% or $>50\%$ solid growth are designated grade 2 and 3, respectively. When nuclear atypia is notable (e.g. nuclei differ greatly in size and shape) and when this atypia does not correspond to the architectural

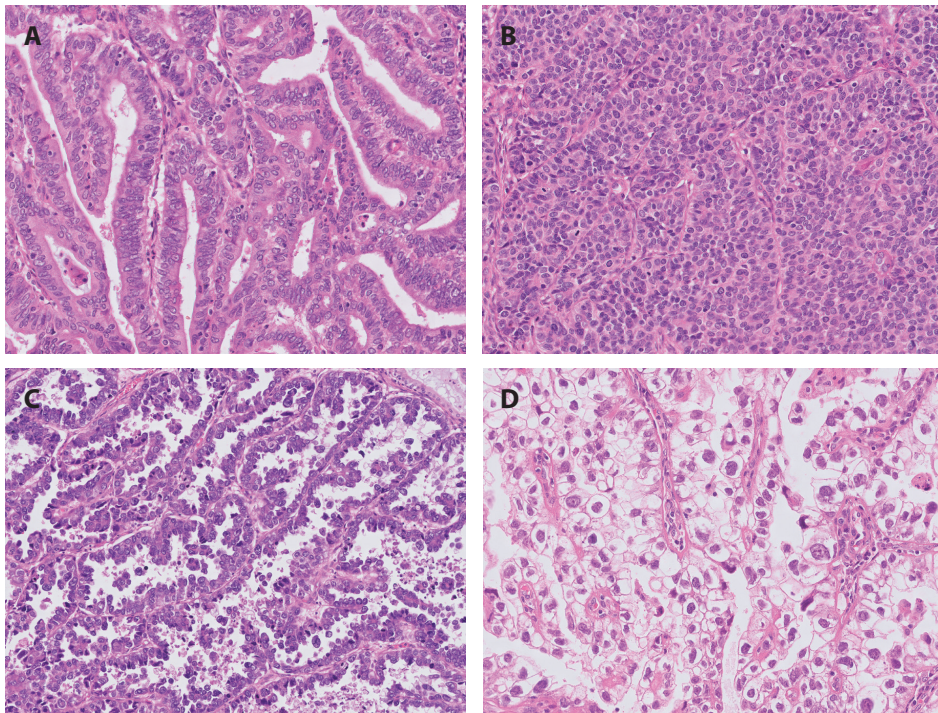


Figure 2. Histological classification of endometrial cancers.

Common histological subtypes of endometrial cancer are endometrioid-type (A and B), serous (C), and clear cell (D) carcinomas. Furthermore, endometrioid endometrial cancers are graded based on the presence of solid growth and nuclear atypia: examples of a grade 1 (A) and grade 3 (B) endometrioid endometrial cancer are shown. Figures A, B, and D are at x20 magnification, C at x10.

grade, the grade of the endometrial cancer can be raised by one. For every stage of disease, high-grade endometrial cancers (grade 3) have a poorer clinical outcome.^{12,18}

FIGO stage

The FIGO staging system uses clinical, surgical and pathological findings to classify endometrial cancers into four stages based on the extent of tumor growth (Table 1). This classification takes invasion of the tumor in the myometrium into account, invasion of tumor in cervical stroma and/or serosa, involvement of the adnexae, of pelvic and/or para-aortic lymph nodes, and of surrounding or distant organs and tissues. Evidently, survival decreases with increasing stage.¹²

Table 1. FIGO 2009 staging of endometrial cancer.^{12,17}

Stage	Description
Stage I	Tumor confined to the corpus uteri
IA	No or less than one half myometrial invasion
IB	Invasion equal to or greater than the outer one-half of the myometrium
Stage II	Tumor invades the cervical stroma but does not extend beyond the uterus
Stage III	Local and/or regional spread of the tumor
IIIA	Tumor invades the serosa and/or adnexae
IIIB	Vaginal and/or parametrial involvement
IIIC	Metastases to pelvic and/or para-aortic lymph nodes
IIIC1	Metastases to pelvic lymph nodes
IIIC2	Metastases to para-aortic lymph nodes with or without positive pelvic lymph nodes
Stage IV	Tumor invades bladder and/or bowel mucosa, and/or distant metastases
IVA	Tumor invades the bladder or bowel mucosa
IVB	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

Age at diagnosis

The age of the patient is another determinant of prognosis in endometrial cancer. Age is associated with other prognostic factors such as tumor type: endometrial cancers of elderly women are more often of non-endometrioid histology. Despite this association, although it remains to be clarified how this is reflected in the underlying tumor biology, age is an independent prognostic factor in endometrial cancer. Patients with an age at diagnosis of 60 years or more have a higher risk of both locoregional (vaginal or pelvic) and distant recurrences and more often succumb to endometrial cancer than those younger than 60 years at diagnosis.¹⁹⁻²²

Lymphovascular space invasion

Finally, over the past three decades, many independent studies have shown the prognostic significance of lymphovascular space invasion in endometrial cancer.²³⁻³⁰ Lymphovascular space invasion can be defined as the presence of cancerous cells in a space lined by endothelial cells outside the immediate invasive border.²⁴ The presence of substantial lymphovascular space invasion is an important risk factor for lymph node involvement, local recurrence (even in the absence of lymph node metastases) and distant metastases.²³⁻³⁰ Although a standard definition and an optimal scoring method of lymphovascular space invasion remain to be developed, a comparison of different scoring methods determined a three-tiered scoring system to be the most straightforward and to have the strongest prognostic power.²⁴

Adjuvant therapy

Together, these clinicopathological risk factors are used to group patients according to their risk of endometrial cancer recurrence with consequent implications for adjuvant treatment (Table 2).³¹ In this risk assessment, patients with early-stage, endometrioid-type endometrial cancers are stratified into low-, (high-)intermediate-, and high-risk groups. Patients with more advanced-stage disease and women with non-endometrioid-type cancers are all classified as being at high risk. For patients with low-risk endometrial cancer, no additional treatment is recommended as their risk of recurrence after hysterectomy is low.^{19,32-34} Therapeutic options considered in the adjuvant setting for (high-)intermediate and high-risk disease are radiotherapy (either vaginal brachytherapy or external beam radiotherapy), chemotherapy or a combination.

In patients with intermediate-risk endometrial cancer, large randomized controlled trials showed that external beam radiotherapy significantly decreases the risk of locoregional recurrence (vaginal and/or pelvic recurrences): in the PORTEC-1 trial, the five-year risk was 14% without additional treatment and 4% with radiotherapy. However, radiotherapy does not improve survival in early-stage disease: overall survival is approximately 80-85% after five years, both with and without adjuvant treatment. In addition, most recurrences are local (predominantly found at the vaginal vault), and the majority can be effectively salvaged.^{19,33,34} In the PORTEC-2 trial, vaginal brachytherapy has been shown to reduce the risk of vaginal recurrence as effectively as external beam radiotherapy: five-year recurrence rates were less than 2% for both vaginal brachytherapy and external beam radiotherapy.³⁵ However, vaginal brachytherapy causes significantly less bowel toxicity than external beam radiotherapy, and quality of life remains high, with rates similar to a norm population matched for age and gender.^{36,37} Therefore, vaginal brachytherapy is currently the standard adjuvant treatment for patients with (high-)intermediate-risk endometrial cancer.^{35,38}

Table 2. Risk groups in endometrial cancer to guide the use of adjuvant treatment according to the ESMO-ESGO-ESTRO consensus guidelines 2016.³¹

Risk group	Description	Adjuvant treatment recommendations
Low	Stage I endometrioid, grade 1-2, <50% myometrial invasion, LVSI negative	No adjuvant treatment recommended
Intermediate	Stage I endometrioid, grade 1-2, ≥50% myometrial invasion, LVSI negative	Vaginal brachytherapy No adjuvant treatment is an option, especially for patients aged <60 years
High-intermediate	Stage I endometrioid, grade 3, <50% myometrial invasion, regardless of LVSI status	External beam radiotherapy when LVSI positive Vaginal brachytherapy when grade 3 and LVSI negative
High	Stage I endometrioid, 1-2, LVSI unequivocally positive, regardless of depth of myometrial invasion	
	Stage I endometrioid, grade 3, ≥50% myometrial invasion, regardless of LVSI status	External beam radiotherapy Consider adjuvant and concurrent chemotherapy (standard: carboplatin and paclitaxel)
	Stage II endometrioid	
	Stage III endometrioid, no residual disease	Consider vaginal brachytherapy boost when stage II
	Non-endometrioid (serous, clear cell, undifferentiated, carcinosarcoma)	

In a pooled analysis of long-term outcomes of the PORTEC-1 and -2 trials, patients with early-stage grade 3 disease and/or with substantial lymphovascular space invasion had significantly increased pelvic regional and distant recurrence rates. For the 5% of patients who had substantial lymphovascular space invasion, the risk of pelvic regional recurrence was 15% after five years, compared to 2% for those without lymphovascular space invasion.²⁴ These patients are therefore considered at high-intermediate risk in the current risk classification according to the recent ESMO-ESGO-ESTRO consensus guidelines. For patients with high-intermediate-risk endometrial cancer with substantial lymphovascular space invasion, external beam radiotherapy is recommended to maximize pelvic control.³¹

Controversy remains concerning the optimal adjuvant therapy for patients with high-risk endometrial cancer, which includes those with early-stage grade 3 disease with deep invasion, those with more advanced stages, and those with non-endometrioid-type cancers. Two trials compared pelvic external beam radiotherapy to chemotherapy (consisting of three to five cycles of cyclophosphamide, doxorubicin and cisplatin) and found no difference between the arms regarding overall and progression-free survival.^{20,21} In a multicenter randomized trial, sequential external beam radiotherapy and different re-

gimes of chemotherapy (predominantly platinum-based) was compared to radiotherapy alone; the combination resulted in a significantly higher five-year progression-free survival (78% vs. 69%).³⁹ Two randomized controlled trials in which combined radiotherapy and chemotherapy is compared to either external beam radiotherapy (PORTEC-3) or chemotherapy (GOG-0258) alone, have recently presented preliminary results. The GOG-0258 trial for stage III-IVA endometrial cancer found that, although chemotherapy combined with radiotherapy reduced the risk of vaginal, pelvic, and para-aortic recurrences, it did not increase the recurrence-free survival compared to chemotherapy alone.⁴⁰ In the PORTEC-3 trial, which included stage I-III high-risk disease, a trend towards an improved five-year failure-free survival (i.e. relapse or endometrial cancer-related death) was found with concurrent chemotherapy and radiotherapy. In women with stage III disease, the addition of chemotherapy significantly improved the failure-free survival by 11% after five years.⁴¹ While awaiting the long-term outcomes of these studies, external beam radiotherapy is recommended to maximize locoregional control. To decrease the rate of distant metastases, adjuvant platinum-based chemotherapy can be considered, especially for stage III disease and for those with non-endometrioid-type cancers. Current literature supports the use of combined radiotherapy and chemotherapy rather than either modality alone.³¹

Despite the use of this clinicopathological risk assessment, over- and undertreatment remain an important clinical problem. On one hand, approximately seven endometrial cancer patients need to be treated with vaginal brachytherapy to prevent one vaginal recurrence. This results in unnecessary treatment-associated costs and toxicities: six of out of the seven patients treated would not have had a recurrence regardless of the vaginal brachytherapy.^{32,35,42} On the other hand, approximately 7% of patients with intermediate-risk endometrial cancer develop distant metastases within five years that might have been prevented with tailored adjuvant treatment.³² Similarly to the model used in breast cancer described at the beginning of this chapter, recent studies proposed the use of molecular biomarkers in an integrated risk model to more accurately predict the clinical outcome of endometrial cancer patients.

Molecular (integrated) classification

In 2013, The Cancer Genome Atlas (TCGA) proposed a new classification of endometrial cancer based on genomic, transcriptomic and proteomic analysis of 373 endometrial cancers (Figure 3A-B). This classification included four distinct molecular subgroups with prognostic significance: (1) a group characterized by mutations in the exonuclease domain of the gene encoding the catalytic subunit of DNA polymerase epsilon (*POLE*), which have a very high mutational burden ('ultramutated'), a specific mutational signature, but also a very good clinical outcome; (2) a microsatellite-unstable (MSI) group,

which shows a hypermutated phenotype, many insertions and deletions due to defects in mismatch repair, and has an intermediate prognosis; (3) a copy-number low group with a lower mutational burden, a microsatellite-stable phenotype, and an intermediate to favorable outcome; and (4) a copy-number high group, which contains the majority of non-endometrioid (mostly serous) cancers, has a low mutation rate, is associated with *TP53* mutations, and has the poorest clinical outcome.⁴³ The clinical follow-up data in the TCGA study were, however, limited. Furthermore, while the employed methodologies were useful to identify the molecular subgroups, they are costly and cannot easily be implemented in routine clinical practice. Therefore, different studies validated this molecular classification and its prognostic implications in independent cohorts using surrogate markers, which can be analyzed on formalin-fixed paraffin-embedded (FFPE) tissues as described below.^{42,44-46}

Stelloo et al. defined the TCGA molecular subgroups on FFPE tumor samples by clinically applicable methods: *POLE*-mutant cases were identified by Sanger sequencing of a part of the *POLE* exonuclease domain, microsatellite-unstable cases by microsatellite instability assay and immunohistochemistry of the mismatch repair proteins, and copy-number high cases by immunohistochemical analysis of p53.^{42,45} Identification of these cases leaves a *POLE*-wild-type, microsatellite-stable, p53-wild-type group that was called 'copy-number low' in the TCGA study or 'no specific molecular profile' (NSMP), as no driver mutation has been identified for this group.⁴⁵ Similarly to the TCGA study, Stelloo et al. showed highly significant differences in recurrence and survival rates between the molecular subgroups (Figure 3C).⁴² The use of surrogate markers in FFPE samples to establish the molecular classification was further supported in subsequent studies, which also replicated its prognostic impact.^{44,46}

Both Stelloo et al. and Talhouk et al. compared risk models based on this molecular classification to models based on clinicopathological or combined molecular and clinicopathological characteristics and assessed their ability to predict clinical outcomes.^{42,44} Both studies showed that a model integrating both molecular alterations and clinicopathological risk factors most strongly improved the risk assessment of endometrial cancer patients.^{42,44} The study from Stelloo et al., based on the pooled PORTEC-1 and -2 trials, proposed the inclusion of lymphovascular space invasion status, expression of L1 cell adhesion molecule, and *CTNNB1* mutational status in addition to the TCGA molecular subgroups in the integrated risk classification. L1 cell adhesion molecule (L1CAM; CD171) is a membrane glycoprotein of the immunoglobulin superfamily, whose expression in endometrial cancer is strongly related to disease progression and recurrence.⁴⁷⁻⁵¹ *CTNNB1* exon 3 mutations lead to activation of Wnt/ β -catenin signaling, and characterize an aggressive subset of low-grade early-stage endometrial cancers.^{52,53} In the resulting

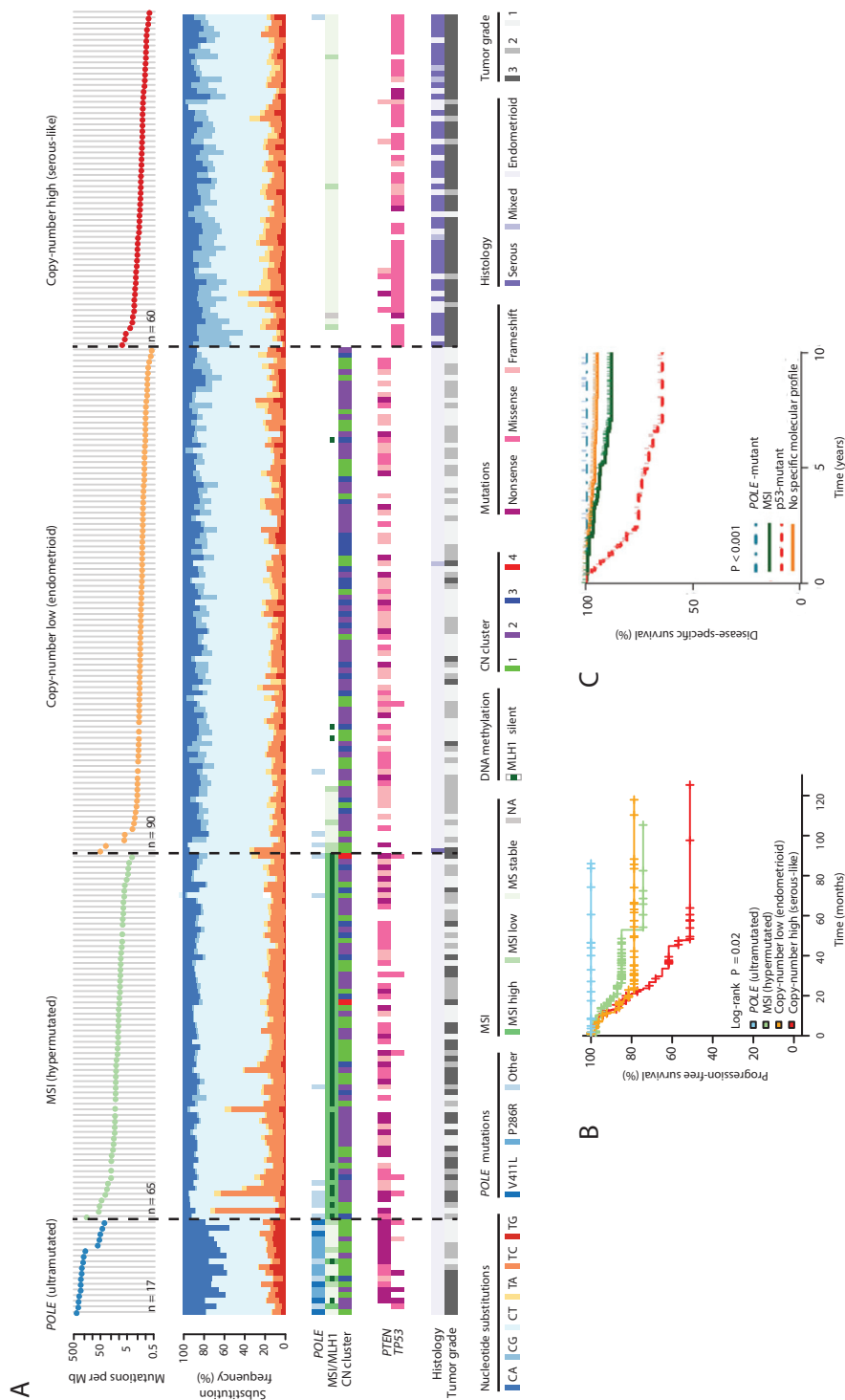


Figure 3. Molecular characterization of endometrial cancer and its prognostic significance in two independent studies.

(Figure 3 continued) (A) Genomic, transcriptomic, and proteomic analysis of 373 endometrial cancers by The Cancer Genome Atlas (TCGA) identified four distinct molecular subgroups. Molecular and clinicopathological characteristics are depicted for each molecular subgroup. (B) Progression-free survival for the different molecular subgroups from the TCGA study is shown. (C) Disease-specific survival for the four molecular subgroups, identified with clinically applicable surrogate markers, in a pooled analysis of two large randomized controlled trials (PORTEC-1 and PORTEC-2) of endometrial cancer is depicted. Figures A and B are adapted from TCGA, Nature 2013 (reprinted under the Creative Commons license).⁴³ Figure C is adapted from Stelloo et al., Clin Can Res 2016 with permission from the American Association for Cancer Research.⁴² Abbreviations: CN, copy number.

molecular integrated risk model, stage I endometrial cancers at (high-)intermediate risk could be reclassified to favorable, intermediate, or unfavorable risk groups. This lead to 55% of patients at intermediate risk of recurrence being reassigned to the favorable risk group: these patients may not benefit from adjuvant treatment. Moreover, 15% of patients were reclassified to the unfavorable group: these patients may benefit from more extensive adjuvant treatment.⁴² This model is currently prospectively evaluated in the multicenter randomized PORTEC-4a trial and may substantially reduce unnecessary adjuvant treatment in endometrial cancer.⁵⁴

SUBJECT, AIMS AND OUTLINE OF THIS THESIS

Personalized medicine in cancer care can be improved upon by combining molecular biomarkers with clinicopathological characteristics to better predict a patient's prognosis and/or response to treatment. The Cancer Genome Atlas paved the way towards such an even more personalized approach in endometrial cancer: a molecular classification was proposed in which four distinct subgroups were identified with prognostic significance.⁴³ One of the molecular subgroups in endometrial cancer as proposed by The Cancer Genome Atlas was novel, and was characterized by mutations in the *POLE* proofreading exonuclease domain.^{43,55-57} Moreover, The Cancer Genome Atlas found that *POLE* exonuclease domain-mutant cancers are characterized by a very high mutational burden, which is amongst the highest found in human cancers.^{43,55,58} Genomic instability and mutability are central to cancer development and progression.⁵⁹ Therefore, it was striking that, despite this so-called 'ultramutated' phenotype, multiple independent studies showed that patients with *POLE* exonuclease domain-mutant endometrial cancers have a very good clinical outcome.^{42-46,60-62}

The aims of this thesis were to gain insight into somatic *POLE* exonuclease domain mutations in endometrial cancer and especially into the underlying mechanism(s) by which these *POLE* mutations contribute to the observed favorable clinical outcome. These insights may facilitate the implementation of *POLE* exonuclease domain mutations as an

important prognostic biomarker in routine clinical practice. Moreover, it may provide a deeper understanding of molecular mechanisms underlying endometrial cancer, which may lead to the discovery of new therapeutic options. Finally, as *POLE* exonuclease domain mutations are found in a wide variety of human malignancies (for example colorectal cancer), these findings may be generalizable to other cancer types as well.

In **chapter 2**, the current understanding of the mechanisms and the consequences of *POLE* exonuclease domain mutations in human cancers is reviewed. Furthermore, chapter 2 reports on the potential usefulness of these mutations as novel cancer biomarkers and therapeutic targets. **Chapter 3** describes a histopathological and immunohistochemical characterization of *POLE* exonuclease domain-mutant endometrial cancers to aid their detection in routine clinical practice. **Chapter 4** shows how enhanced immunogenicity may contribute to the favorable clinical outcome of *POLE* exonuclease domain-mutant endometrial cancers. **Chapter 5** focuses on validation of the results obtained from chapter 3 and on the possible utility of immunotherapeutic strategies in a series of high-risk endometrial cancer. **Chapter 6** describes the sensitivity to adjuvant treatment strategies of *POLE* exonuclease domain-mutant cancers and its impact on clinical outcome. **Chapter 7** focuses on the timing of *POLE* exonuclease domain mutations in carcinogenesis. Finally, **chapter 8** provides a general discussion of the results of this thesis, focusing on future perspectives.

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Chapter 2

A panoply of errors: polymerase proofreading domain mutations in cancer

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ABSTRACT

Although it has long been recognized that the exonucleolytic proofreading activity intrinsic to the replicative DNA polymerases Pol δ and Pol ϵ is essential for faithful replication of DNA, evidence that defective DNA polymerase proofreading contributes to human malignancy has been limited. However, recent studies have shown that germline mutations in the proofreading domains of Pol δ and Pol ϵ predispose to cancer, and that somatic Pol ϵ proofreading domain mutations occur in multiple sporadic tumors, where they underlie a phenotype of 'ultramutation' and favorable prognosis. In this review, we summarize the current understanding of the mechanisms and consequences of polymerase proofreading domain mutations in human malignancies, and highlight the potential utility of these variants as novel cancer biomarkers and therapeutic targets.

N.b. Italicized words or phrases are defined in the Glossary following the main text and references.

A PANOPLY OF ERRORS: POLYMERASE PROOFREADING DOMAIN MUTATIONS IN CANCER

Accurate replication of DNA before cell division is a prerequisite for the suppression of mutagenesis and tumor development. The remarkable fidelity of eukaryotic DNA replication – estimated at one incorrect base for every 10^9 to 10^{10} nucleotides replicated¹ – results from a combination of highly accurate base incorporation and exonuclease proofreading by the replicative DNA polymerases Pol δ and Pol ϵ , and post-replication surveillance by the DNA *mismatch repair* (MMR) apparatus.^{2,3} Defects in either polymerase proofreading or MMR increase the mutation rate in *Saccharomyces cerevisiae* and cause tumors in mice.⁴⁻⁸ Although the importance of MMR deficiency (MMR-D) in human cancer has been recognized for more than two decades,^{9,10} until recently, evidence that defective polymerase proofreading contributes to human malignancy has been scarce.¹¹ However, over the last three years, studies have shown that germline mutations in the proofreading domains of *POLD1* and *POLE* (which encode the catalytic subunits of Pol δ and Pol ϵ , respectively, in humans) predispose to colorectal cancer (CRC) and other malignancies.¹² Somatic *POLE* proofreading domain mutations are found in 7-12% of endometrial cancers (ECs), in 1-2% of CRCs, and occasionally in tumors of the breast, stomach, pancreas and brain, where they define a distinct, *ultramutated* tumor subgroup.¹³⁻¹⁹ *POLE* proofreading domain mutations predict favorable prognosis in EC,²⁰⁻²⁴ and may also do so in glioblastoma,¹⁴ possibly because the exceptional number of mutations in these cancers causes an enrichment of antigenic *neopeptides*, leading to an enhanced antitumor immune response.^{25,26}

In this review, we summarize the current understanding of the mechanisms and consequences of replicative DNA polymerase proofreading domain mutations in human malignancies. Although we provide an outline of the organization and function of replicative DNA polymerases as a background, we do not cover this subject in detail as it has been described comprehensively in several excellent reviews.²⁷⁻³⁰ Instead, we highlight the distinctive clinicopathological and molecular characteristics of replicative DNA polymerase proofreading domain-mutant tumors, and focus on the potential utility of these variants as novel cancer biomarkers and targets for therapy.

DNA polymerase proofreading

Pol δ and Pol ϵ are the principal eukaryotic DNA replicases, and together are responsible for the bulk of DNA replication, following priming by Pol α .³¹⁻³⁴ They are *B family polymerases* and, unlike Pol α , have a 3'-5' exonuclease activity that proofreads the newly synthesized DNA strand.^{3,35} Both Pol δ and Pol ϵ comprise four subunits in humans, the largest of which contains the catalytic and proofreading exonuclease active sites, and

is encoded in humans by *POLD1* and *POLE*, respectively.³⁶ The other smaller subunits (encoded in humans by *POLD2*, *POLD3* and *POLD4*, and by *POLE2*, *POLE3* and *POLE4*) also perform several important or essential roles. In the case of Pol δ , they stabilize the holoenzyme complex and stimulate DNA polymerase activity via interactions with the replication processivity factor proliferating cell nuclear antigen (PCNA).³⁷⁻³⁹ The essential second subunit of Pol ϵ mediates the interaction with *GIN5* and may help to target the Pol ϵ holoenzyme to the leading strand during the initiation of DNA replication,⁴⁰⁻⁴² whereas the non-essential third and fourth subunits are critical for binding double-stranded DNA and for processive DNA synthesis and processive 3'-5' exonuclease degradation.⁴³

Studies of mutant Pol ϵ and Pol δ polymerases with particular error signatures in *S. cerevisiae* and *Schizosaccharomyces pombe* have suggested a model of DNA replication in which Pol δ replicates the lagging strand following priming by Pol1 (the functional equivalent of the catalytic subunit of human Pol α), whereas Pol ϵ replicates the leading strand.⁴⁴⁻⁴⁶ This division of labor has been corroborated by analyses of yeast mutants engineered to misincorporate ribonucleoside triphosphates (rNTPs; reviewed in ⁴⁷) and by biochemical reconstitution experiments,^{48,49} and is broadly accepted. Indeed, further support for this model – albeit indirect – was provided by the crystal structure of *S. cerevisiae* Pol ϵ catalytic subunit (Pol2), which revealed the existence of a domain absent in the corresponding Pol δ subunit (Pol3) that could explain its enhanced processivity.⁵⁰ However, these roles have been questioned by a very recent publication, which suggests that previous results may have been confused by differential MMR and proposes that Pol δ replicates both the leading and lagging strands, whereas the functions of Pol ϵ are limited to repair synthesis and proofreading of the leading strand.⁵¹ Although the precise contribution of Pol ϵ to leading strand replication awaits definitive clarification, current data are concordant in indicating that its exonuclease domain preferentially proofreads the leading strand.^{27,51} In addition to their roles in DNA replication, in both yeast and humans both Pol δ and Pol ϵ also function in base excision repair (BER),^{52,53} nucleotide excision repair (NER),^{54,55} MMR⁵⁶⁻⁵⁸ and double-strand break repair.^{59,60} Pol ϵ has also been implicated in cell cycle checkpoint regulation and propagation of chromatin modification states (reviewed in ⁶¹), thus mutations affecting this protein could potentially affect a wider range of cellular activities than just replication fidelity.

The proofreading function of both Pol δ and Pol ϵ requires several highly conserved exo motifs in their exonuclease domains, within which lie the catalytic site residues that are essential for exonuclease activity (D316 and E318 in Pol δ , and D275 and E277 in Pol ϵ , in humans; Figure 1).^{27,28} Misincorporation of a base into the primer strand results in pausing of the polymerase (due to reduced efficiency in extending a mispaired primer terminus) and a switch from the catalytic to the exonuclease domain.⁶² The incorrect

base is then excised and the correct base inserted before DNA synthesis continues.⁶² Multiple studies in model organisms have confirmed the essential role of DNA polymerase proofreading in the maintenance of genomic stability. In *S. cerevisiae*, mutants of Pol δ and Pol ϵ containing alanine substitutions of the exonuclease active site residues have no exonuclease activity, and cells expressing these variants show a ~100-fold increase in mutation rate.^{4,63,64} Mice harboring the equivalent substitutions show an

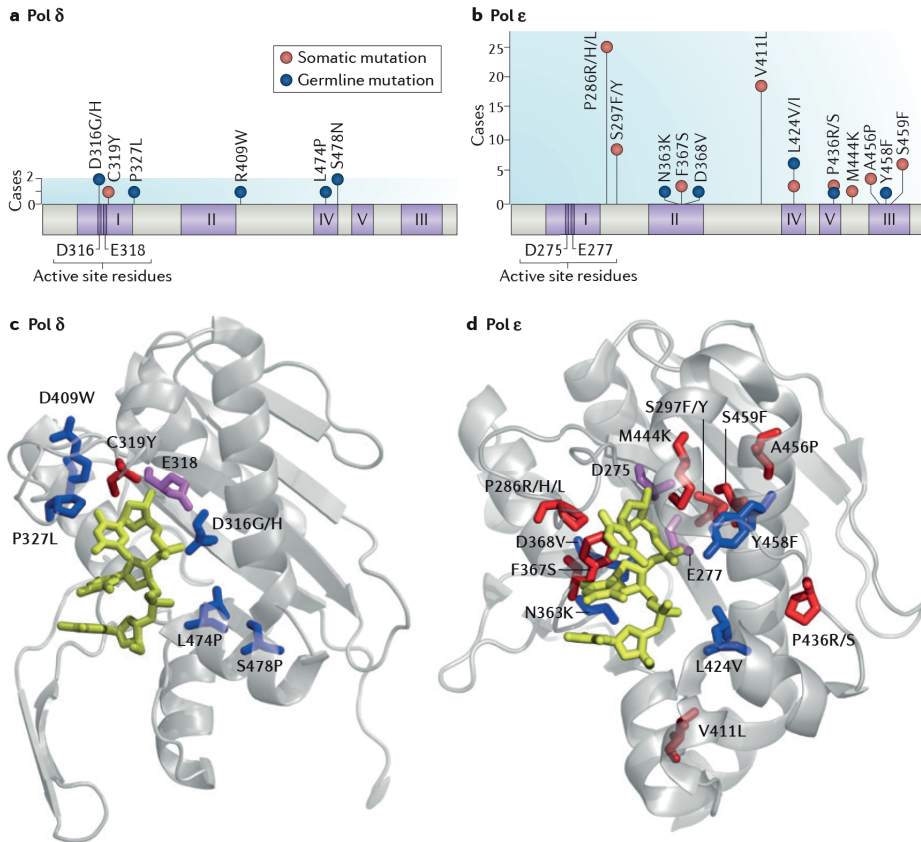


Figure 1. Frequency and location of germline and somatic Pol δ and Pol ϵ proofreading exonuclease domain mutations in cancers.

A schematic representation of the exonuclease domains of Pol δ (A) and Pol ϵ (B) showing conserved exo motifs (I-V), exo I active site residues, and the position and frequency of germline and somatic mutations. In the lower panels, the positions of germline (blue) and somatic (red) proofreading domain mutations are mapped to the *Saccharomyces cerevisiae* Pol δ (C, Protein Data Bank identifier (PDB ID) 3IAY¹¹⁶) and Pol ϵ (D, PDB ID 4M8O⁵⁰) exonuclease domain structures. Single-stranded DNA from the aligned bacteriophage T4 polymerase complex (PDB ID 1NOY¹¹⁸) is shown in yellow. The exo I motif active site residues are highlighted in magenta (with exception of mutated Pol δ active site residue D316, which is also the site of germline mutations). Note that Pol ϵ residues L424V and P436R/S are the site of both germline and somatic mutations and are colored according to which alteration is more frequent.

increased mutation rate and develop tumors.⁶⁻⁸ Notably, cancers only develop in animals homozygous for proofreading-null *Pold1* or *Pole* alleles (the mouse orthologues of *POLD1* and *POLE*),⁶⁻⁸ and the tumor spectrum that they develop differs between the two: *Pold1*-mutant mice develop lymphomas and carcinomas of the skin and lung,⁷ whereas *Pole* mutants are prone to intestinal tumors and histiocytic sarcomas.⁶ A simple explanation for these phenotypes is that defective proofreading leads to an increased mutation rate, as some misincorporated nucleotides escape subsequent correction by MMR, but the reality may be more complicated. For instance, studies in *S. cerevisiae* indicate that elevation of deoxyribonucleoside triphosphate (dNTP) levels by cell cycle checkpoint activation is responsible for the *mutator phenotype* of proofreading-defective DNA polymerases.^{65,66}

Proofreading domain mutations in cancer

In 2012, exome sequencing of 224 sporadic CRCs by The Cancer Genome Atlas (TCGA), and a similar analysis of 72 CRC exomes, revealed a subset of ultramutated, yet *microsatellite-stable* tumors with recurrent somatic mutations within the *POLE* exonuclease domain.^{13,16} The most common of these involved the replacement of proline by either arginine or histidine at codon 286 (*POLE* P286R/H), and recurring substitutions were also found at codons 411 (*POLE* V411L) and 459 (*POLE* S459F; Table 1).^{13,16} Subsequently, two studies detected heterozygous somatic *POLE* proofreading domain mutations, including the P286R and V411L substitutions, in ~7% of sporadic ECs, where they were also associated with ultramutation and microsatellite stability (Table 1).^{15,17} These mutations localized to highly conserved or invariant residues within, or close to, the exo motifs that are essential for proofreading activity, and they were predicted to perturb DNA binding by structural mapping (Figure 1B,D).¹⁵ In parallel with these reports, an independent study used linkage analysis and whole genome sequencing to show that families with multiple colorectal tumors but without known predisposition mutations carried heterozygous germline mutations that caused substitutions in the proofreading domains of *POLD1* (*POLD1* S478N) and *POLE* (*POLE* L424V; Table 2).¹² Interestingly, the *POLD1* mutation was also associated with EC. Similar to the somatic *POLE* variants, these mutations affected highly conserved residues in or adjacent to the exo motifs at the DNA-binding interface (Figure 1A,C).¹² Furthermore, the germline variant alleles have been shown to reduce proofreading activity and cause a mutator phenotype in yeast.^{12,64}

Current data suggest that germline *POLE* and *POLD1* mutations are present in 0.5-2% of patients in *intestinal polyposis* and CRC cohorts enriched for familial disease.^{12,67-69} The *POLE* L424V mutation seems to be the most common deleterious germline variant, with 21 independent carriers identified to date.^{68,70,71} Although predominantly associated with CRC, this variant also predisposes to EC, and it may confer moderately increased

Table 1. Pathogenic somatic Pol δ and Pol ϵ proofreading exonuclease domain mutations that occur in sporadic cancers.

Protein change (nucleotide substitution)	Evidence in support of pathogenicity				Molecular characteristics*			
	Structural mapping	PhyloP score [†]	Yeast fluctuation assay	Biochemical evidence for functional effect [‡]	Median number of mutations per exome (range)	Percentage MSS	Percentage of tumors with $\geq 20\%$ C>A substitutions	Tumor type(s)
Pol δ								
C319Y (956G→A)	Within exo I motif	2.38	Yes ⁶⁴	NR	7,349 (44–14,654)	100	NA	GBM ⁷⁷ and MM ⁷⁸
Pol ϵ								
P286R/H/L (857C→G/A/T)	Flanking exo I motif	2.57	Yes ⁸²	Yes ¹⁸	5,147 (738–16,248)	100	93	CRC, ^{13,16,82} EC, ^{15,17,20,21,24} GBM, ¹⁴ EOC, ¹¹⁸ and BrC ¹¹⁹
S297F/Y (890C→T/A)	Flanking exo I motif	2.67	NR	NR	5,429 (4,918–15,545)	50	66	EC, ^{15,17,20,21,24} GBM, ^{14,77} EOC, ⁷⁵ and CC ¹¹⁹
F367S (1100T→C)	Exo II active site	2.19	NR	Yes ^{18,120}	2,934	100	100	CRC ^{11,13}
V411L (1231G→C/T)	Flanking exo IV motif	2.66	NR	Yes ¹⁸	6,294 (955–14,074)	100	88	CRC, ¹³ EC, ^{15,17,20,21,24} GBM, ¹⁴ EOC, ¹¹⁸ and GC ⁷⁹
L424V/I (1270C→G/A)	Exo IV active site	2.66	Yes ⁶⁴	Yes ^{18,120}	163 (85–6,724)	50	100	EC ¹⁷ and BrC ¹¹⁹
P436R (1307C→G)	Exo V motif	3.53	NR	Yes ¹²⁰	6,131	100	100	CRC ¹³ and EC ²¹
M444K (1331T→A)	Flanking exo V motif	2.15	NR	NR	1,204	100	100	EC ¹⁷
A456P (1366G→C)	Within exo III motif	2.61	NR	NR	5,968	100	100	CRC ^{18,121} and EC ^{15,17,21}
S459F (1376C→T)	Within exo III motif	2.52	NR	Yes ^{18,120}	4,780 (1,868–9,907)	100	75	CRC, ^{13,18,121} GBM, ¹⁴ and AA ⁷⁷

*Data from exome sequencing studies.

[†]PhyloP (phylogenetic conservation) scores were calculated per nucleotide using the alignment of 46 vertebrates dbNSFPv23. For P436R the variant mapped to the third position of a codon so the average PhyloP score for the codon is displayed.[‡]Data from studies of B family polymerases.^{||}Number of exomes sequenced: *POLD1* C319Y, 2; *POLE* P286R/H/L, 16; *POLE* S297F/Y, 5; *POLE* F367S, 1; *POLE* V411L, 10; *POLE* L424V/I, 3; *POLE* P436R, 1; *POLE* M444K, 1; *POLE* A456P, 1; *POLE* S459F, 4.

Abbreviations: AA, anaplastic astrocytoma; BrC, breast cancer; CC, squamous cell cervical carcinoma; CRC, colorectal cancer; EC, endometrial cancer; EOC, endometrioid ovarian carcinoma; GBM, glioblastoma; GC, gastric cancer; MM, multiple myeloma; MSS, microsatellite stable; NA, not applicable; NR, not reported.

Table 2. Pathogenic germline Pol δ and Pol ϵ proofreading exonuclease domain mutations.

Evidence to support pathogenicity of variant		Percentage of variant carriers with indicated phenotype													
		Structural mapping	PhyloP score ^a	Yeast fluctuation assays	Biochemical evidence ^b	Segregates with affection status	Number of carriers and unrelated carriers (total) ^c	Mean age at diagnosis (range) ^{d,e}	Percentage of variant carriers with indicated phenotype						
CRC	EC								B/C	Du/C	OC	GBM	Duodenal A or P	Colonic A or P	Other cancers reporter in carriers
Pol δ															
D316G (947A→G)	Exo I motif active site	1.93	Yes ⁶⁴	Yes ¹²⁰	Yes	2 and 1 (3)	51 (44–57)	50	100	50	0	50	0	67	
D316H (946G→C)	Exo I motif active site	1.16	Yes ⁶⁴	Yes ¹²⁰	Yes	2 and 1 (3)	61 (58–64)	50	0	50	0	0	100	Mesothelioma 67	
P327L (981C→G)	Flanking exo I motif	2.16	Yes ^{82*}	Yes ^{18*}	NR	1 and 1 (2)	70	0	0	0	0	0	100	0 12	
R409W (1225C→T)	Flanking exo II motif	2.26	NR	NR	NR	1 and 1 (2)	32	100	0	0	0	0	100	0 67	
L474P (1421T→C)	Exo IV motif	1.92	Yes ⁶⁴	Yes ^{18*}	Yes	6 and 2 (8)	40 (23–52)	67	33	0	0	0	17	0 67, 70	
S478N (1433G→A)	Exo IV motif	1.19	Yes ¹²	NR	Yes	11 and 3 (14)	35 (26–52)	45	36	0	0	0	91	Astrocytoma 12	
Pol ε															
W347C (1041G→T)	Outside exo motifs	2.71	Yes ⁷⁴	NR	No [#]	11 and 1 (12)	49 (14–70)	0	0	0	0	0	NR	Prostate, cutaneous and uveal melanoma 74	
N363K (1089C→A)	Exo II motif active site	4.9	NR	NR	Yes	12 and 1 (13)	41 (28–56)	75	17	0	0	25	0	NR 83	Pancreatic 72
D368V (103A→T)	Exo II motif active site	5.13	NR	Yes ¹²⁰	NR	1 and 1 (2)	47	100	0	0	0	0	0	0	69

Table 2. (continued) Pathogenic germline Pol δ and Pol ε proofreading exonuclease domain mutations.

Protein change (nucleotide substitution)	Structural mapping	Evidence to support pathogenicity of variant				Percentage of variant carriers with indicated phenotype										Other cancers reporter in carriers	Refs
		PhyloP score [*]	Yeast fluctuation assays	Biochemical evidence [†]	Segregates with affection status	Number of carriers and unrelated carriers (total) [§]	Mean age at diagnosis (range)	CRC	EC	BrC	DuC	OC	GBM	Duodenal A or P	Colonic A or P		
L242V (1270C→G)	Exo IV motif active site	2.66	Yes ⁶⁴	Yes ¹⁸	Yes, 2 <i>de novo</i> carriers	48 and 21 (69)	39 (16–64)	61	2	2	2	2	4	19	92	ODG and neuro-endocrine carcinoma	12, 68–71
P436S (1306C→T)	Within exo V motif	3.53	NR	NR	<i>De novo</i>	1 and 1 (2)	31	100	0	0	0	0	0	100	100	0	68
Y458F (1373A→T)	Exo III motif active site	4.97	NR	Yes ¹²⁰	Yes	13 and 2 (15)	48 (38–63)	62	0	0	15	8	0	NR	62	Pancreatic	73

^{*}PhyloP (phylogenetic conservation) scores were calculated per nucleotide using alignment of 46 vertebrates dbNSFPv23. If a variant mapped to the third position of a codon the average PhyloP score for the codon is displayed.

[†]Data from functional studies of B family polymerases.

[§]Reported as of August 2015.

^{||}Mean age at diagnosis in years refers to cancer or adenoma diagnosis, whichever was earliest.

^{*}Functional studies of the corresponding residue in Pol ε.

[†]Six mutation carriers were unaffected.

Abbreviations: A or P, adenomas or polyps; BrC, breast cancer; CRC, colorectal cancer; DuC, duodenal carcinoma; EC, endometrial cancer; GBM, glioblastoma; NR, not reported; OC, ovarian cancer; ODG, oligodendroglioma.

risk of many other tumor types (including carcinomas of the breast, stomach and ovary, brain tumors, and duodenal adenomas and carcinomas; Table 2). The demonstration of *de novo* *POLE* L424V mutations^{70,71} and the absence of a detectable haplotype shared among apparently unrelated families¹² suggests that this is a *mutational hot spot*. Other recently reported germline *POLE* mutations include N363K, which maps to the exo II motif active site and is associated with CRC and pancreatic cancer;⁷² Y458F, which affects the exo III motif active site and predisposes to multiple tumor types;⁷³ and W347C, which lies outside the exo motifs and has been associated with melanoma (Figure 1B,D; Table 2).⁷⁴ *POLE* N363K and *POLE* Y458F are highly penetrant, being associated with malignancy in 11 of 12 and 9 of 13 carriers, respectively (Table 2).^{72,73} The importance of the W347C variant is uncertain, as it seems to display lower penetrance, with six unaffected carriers in the family, and it does not seem to confer a strong risk of CRC (<10% cases), despite evidence of pathogenicity in *S. pombe*.⁷⁴

Although no additional *POLD1* S478N carriers have been reported since 2012, five other pathogenic germline *POLD1* proofreading domain mutations have been identified (Figure 1A,C; Table 2). One of these, a recurrent mutation at codon 474 (*POLD1* L474P),^{67,70} affects the equivalent residue to *POLE* L424V, and another, *POLD1* D316G, affects the exo I motif active site.⁶⁷ The cancer risk in carriers of germline *POLD1* proofreading domain mutations seems to be limited to CRC and EC, with no evidence of a predisposition to duodenal or ovarian malignancies.^{67,70} However, caution is required, as the modest number of germline *POLD1* variant carriers makes phenotypic characterization less certain than it is for *POLE* variant carriers. Interestingly, similar to the *POLE* active site variants, the germline *POLD1* D316G mutation appears to be highly penetrant (malignancy in 4 of 4 carriers).⁶⁷

Somatic *POLE* proofreading domain mutations occur in ~1-2% of CRCs^{13,16} and 7-12% of ECs,^{15,17,20-23} and they have also been detected in ultramutated tumors of the brain, pancreas, ovary, breast and stomach,^{14,18,75} as well as in uterine carcinosarcomas.⁷⁶ The most common *POLE* variants are the P286R and V411L substitutions (Figure 1B,D; Table 1); other recurrent substitutions include S297F, A456P and S459F. Most of these occur within, or close to, the exo motifs; however, unlike the germline mutations, the catalytic sites themselves are seldom affected.^{13,15} In contrast to *POLE*, to date only two cases of possibly pathogenic somatic *POLD1* proofreading domain mutations have been identified. The first is a C319Y variant detected in an ultramutated pediatric glioblastoma from a patient with biallelic congenital MMR-D (CMMR-D) due to a compound heterozygous mutation in mutS homolog 6 (*MSH6*: c.3984_3987dupGTCA;c.3959_3962delCAAG).⁷⁷ The *POLD1* C319Y mutation has also been detected in a myeloma with a normal mutation burden,⁷⁸ and its functional effect is uncertain at present. The second is a D316N

substitution in a highly mutated sporadic gastric cancer.⁷⁹ This tumor displays a high proportion of insertions and deletions (indels), and as the *POLD1* variant allele fraction is only 0.13, it is unclear to what extent this substitution has contributed to the mutation burden in this tumor.

The difference in the frequency of somatic proofreading domain mutations between *POLD1* and *POLE* is both notable and unexplained. It seems unlikely to be due to bias in the tumor types analyzed to date: the exomes of more than 400 lung adenocarcinomas – a common tumor type in *Pold1*-proofreading-null mice – have been sequenced at the time of writing.^{80,81} Given the current uncertainty regarding the roles of Pol δ and Pol ϵ at the replication fork,⁵¹ it will be intriguing to see whether future studies reveal to what extent, if any, this discrepancy reflects differential contributions of the two polymerases to DNA replication and repair, or other processes.

Functional insights into pathogenicity

The deleterious consequences of cancer-associated polymerase proofreading domain mutations on exonuclease activity and mutation rate have been confirmed by functional studies. In cell-free assays, the somatically occurring *POLE* P286R/H and *POLE* S459F mutations reduce exonuclease activity to levels similar to those of a proofreading-null *POLE* construct with substitution of both exo I active site residues, whereas the somatic *POLE* V411L and *POLE* F367S variants and the *POLE* L424V variant (which can be either somatic or germline) substantially reduce, but do not eliminate, exonuclease function.¹⁸ Interestingly, the substitution equivalent to human *POLE* P286R was recently shown to confer an exceptionally strong mutator phenotype in *S. cerevisiae*.⁸² This far exceeded that of the proofreading-null allele, suggesting that *POLE* P286R may exert effects beyond proofreading alone.⁸² Importantly, in this study the mutation rate was also substantially increased in diploid strains heterozygous for the *POLE* P286R analogue.⁸² These results provide a possible explanation for why mice with proofreading-null *Pole* alleles only develop tumors in the homozygous state,⁶ yet somatic *POLE* proofreading domain mutations in human cancers seem to be mostly, if not all, heterozygous changes.^{15,18} This may also explain the paucity of exonuclease active site mutations in sporadic cancers. Given these exciting data, the results of similar analyses of other cancer-associated mutations are eagerly awaited.

Clinicopathological characteristics

Intestinal polyposis seems to be more severe in carriers of germline *POLE* proofreading domain mutations than in those with *POLD1* proofreading domain variants (10–50 compared with <20 colonic polyps per patient, respectively); however, in both cases the polyps themselves are typically of unremarkable histology.¹² Similarly, with the ex-

ception of young age at onset (typically <50 years), CRCs and other malignancies in these patients do not seem to display any distinguishing clinicopathological features, although the number of cases studied to date is limited.¹² Tumors with somatic *POLE* proofreading domain mutations also show a strong tendency to occur in young patients and, when examined, typically exhibit several other notable characteristics, including an association with high tumor grade, a prominent lymphocytic infiltrate and the presence of multiple bizarre *multinucleated giant cells*.^{14,25,83}

An association of somatic *POLE* proofreading domain mutations with good prognosis was first suggested by the EC study from TCGA.¹⁷ This has since been confirmed in EC by several groups²⁰⁻²⁴ and was also proposed for glioblastomas by a recent report.¹⁴ As many patients in these series received adjuvant radiotherapy, either alone or in combination with chemotherapy, it is currently not possible to draw definitive conclusions on whether *POLE* proofreading domain mutation status is a prognostic or predictive biomarker, or indeed both. However, the lack of recurrences of high-grade *POLE* proofreading domain-mutant ECs in two large clinical trials in which chemotherapy was not used, and in a subset of patients who did not receive radiotherapy in one of these studies,²⁰ suggests that *POLE* proofreading domain mutations may portend a good prognosis in the absence of postoperative treatment. While this is reminiscent of the favorable clinical outcome of hypermutated early-stage, MMR-D CRCs,^{84,85} it should be noted that the impact of MMR-D on EC prognosis is unclear,^{22,86,87} and the prognostic import of *POLE* proofreading domain mutations in CRC awaits confirmation.

Molecular characteristics

Although the number of tumors from germline *POLD1* and *POLE* proofreading domain mutations analyzed to date is relatively modest, most have been found to be microsatellite stable;¹² however, *microsatellite instability* (MSI) has been noted in some cases.⁷¹ Analysis of the mutation spectrum has been limited to the tumors from *POLE* L424V and *POLD1* S478N carriers, which revealed a phenotype of base substitutions and missense substitutions with relatively few frameshift mutations.¹² As noted above, the most striking feature of tumors with somatic *POLE* proofreading domain mutations is their ultramutation.^{13,16,18,19} Similar to the tumors from germline *POLE* variant carriers, these are predominantly base substitution mutations,^{13,15,16,18,19} and they occur within a unique mutation signature with a 100-fold increase in C→A transversions in the context TCT and a 30-fold increase in C→T transitions in the context of TCG.^{15,18,19} This results in a strong bias for particular amino acid changes, with an overrepresentation of serine to tyrosine or leucine, and arginine to isoleucine or glutamine substitutions, and a substantial increase in glutamic acid to stop codon mutations.¹⁸ These manifest as a distinctive pattern of missense and truncation mutations in oncoproteins and tumor suppressors, including:

PI3K catalytic subunit- α (*PIK3CA*) R88Q; *PTEN* R130Q; adenomatous polyposis coli (*APC*) R1114X and *APC* Q1338X; *MSH6* E946X and *MSH6* E1322X; F-box and WD repeat domain-containing 7 (*FBXW7*) E369X; and *TP53* R213X.^{12,13,15,16,18,88}

Sporadic *POLE* proofreading domain-mutant cancers display few copy number alterations (CNAs) and, similar to tumors in germline variant carriers, a strong tendency to microsatellite stability, despite frequent mutations in MMR genes.^{13,15-17} Accumulating evidence suggests that the interaction between defective DNA polymerase proofreading and MMR is complex and may depend on the extent to which the function of each activity is compromised. For example, whereas the combination of mutator DNA polymerases and partial MMR function causes attenuated growth in *S. cerevisiae*,⁸⁹ the combination with complete MMR loss is *synthetically lethal* in *S. cerevisiae* and mice.^{6,89,90} Interestingly, a recent study of patients with CMMR-D who developed glioblastomas showed that these tumors occurred following the acquisition of DNA polymerase proofreading domain mutations and were ultramutated, yet predominantly microsatellite stable.⁷⁷ Most of the germline MMR mutations in these cases involved either PMS1 homolog 2 (*PMS2*) or *MSH6*, whereas sporadic DNA polymerase proofreading domain-mutant tumors tend to acquire *MSH6* mutations.^{13,17,77} Loss of *MSH6* or *PMS2* function generally causes a milder mutator phenotype, sometimes lacking MSI, than loss of other MMR components such as mutS homolog 2 (*MSH2*) and mutL homolog 1 (*MLH1*).^{89,91} Although it is tempting to speculate that the combination of defective proofreading and profound MMR deficiency may result in a mutation rate that exceeds the optimum for tumor fitness,^{92,93} empirical verification of this is currently lacking, and it must be noted that although they are rare, tumors with DNA polymerase proofreading domain mutations and MSI have been reported.^{21,71,77} It will be of particular interest to determine the timing and clonality of both events in these cancers, and to examine whether these tumors harbor secondary *antimutator mutations* that permit continued viability, as has been demonstrated in yeast.^{89,94}

Challenges in determining pathogenicity

Although the number of confirmed germline *POLD1* and *POLE* proofreading domain mutations has grown since their initial report in 2012 (Table 2; also curated in the Leiden Open Variation Database (LOVD)), differentiation of pathogenic from non-pathogenic variants in a patient with clinical features that suggest the possibility of DNA polymerase proofreading domain mutations and family history remains challenging. The Exome Aggregation Consortium (ExAC) database currently lists 56 missense or loss-of-function variants mapping to the exonuclease domains of *POLD1* and 75 such variants mapping to the *POLE* exonuclease domain, all of which have a population frequency of <1%. Most of these are likely to have no impact on the proofreading function of these enzymes. In-

deed, of the germline variants reviewed here, only *POLD1* D316H is present in the ExAC database, at a frequency of 0.001% (being present in only 1 of 98,012 alleles genotyped). Therefore, filtering variants according to their presence in ExAC and similar databases will be of limited utility, as it will only exclude variants with relatively high frequencies, such as those with a frequency of >0.1%. Instead, we suggest that the following criteria should ideally be satisfied to prove pathogenicity of a variant: first, segregation with affection status in pedigrees; second, conservation of the affected residue between human DNA polymerases and those from other species; third, evidence of functional effect from at least one of the following: analysis of corresponding residue in model organisms, cell-free exonuclease assays or other polymerase assays;^{18,95} and fourth, demonstration of elevated base substitution frequency by sequencing of tumor DNA.

Confirming the pathogenicity of somatic DNA polymerase proofreading domain mutations may also be difficult. Both *POLD1* and *POLE* are large genes, and they are likely to acquire somatic mutations secondary to other causes of increased mutation burden, such as MMR-D. Given the association of somatic *POLE* proofreading domain mutations with prognosis,^{14,17,20-24} it is important to differentiate these pathogenic variants from passenger mutations that are of no functional consequence, particularly given examples in the recent literature where a functional role has been inferred for *POLD1* and *POLE* variants of uncertain pathogenicity.⁹⁶

On the basis of their analysis of TCGA data, Shinbrot and colleagues¹⁸ have proposed criteria to identify bona fide pathogenic somatic *POLE* proofreading domain mutations. These include: ultramutation (often exceeding 100 mutations per megabase); an increased proportion of C→A transversions, exceeding 20% of all substitutions (although it should be noted that the most common substitutions are typically C→T transitions); and *POLE* mutation at a residue that is recurrently mutated in cancer (Figure 1A; Table 1). While most *POLE* proofreading domain-mutant cancers display these characteristics, we advocate a degree of flexibility in their application, as the predominant mutations caused by proofreading-deficient human Pol ε *in vitro* are T→A transversions,⁹⁷ and it is possible that novel pathogenic *POLE* proofreading domain mutations could cause a different mutational signature. We also suggest that consideration should be given to other features that may indicate a pathogenic variant including: a preponderance of missense mutations (that is, a relative absence of indels); the presence of characteristic flanking nucleotide bias;^{15,18,19} and a relative lack of CNA.^{15,17} Although the coexistence of MSI does not preclude the presence of a deleterious DNA polymerase proofreading variant, it should prompt careful evaluation of its pathogenicity, ideally using bioinformatic predictors (for example, MutationTaster,⁹⁸ SIFT⁹⁹ and Polyphen¹⁰⁰) and structural mapping using the conserved yeast Pol ε structure.⁵⁰ Although some tumors carrying

bona fide pathogenic *POLE* proofreading domain mutations will fall outside this classification,²¹ we believe that it represents a reasonable starting point that can be refined and improved as further data are accumulated in the future.

A role in early tumorigenesis?

The association of germline variants with malignancy¹² suggests that DNA polymerase proofreading domain mutations are able to initiate cancer, and current data, although limited, are consistent with somatic *POLE* proofreading domain mutations occurring as an early event in sporadic tumors. Analysis of the variant allele fraction in glioblastomas suggests that *POLE* proofreading domain mutations are present in the earliest persisting clone,^{14,77} and genes in which driver mutations are known to occur early in CRC and EC frequently display evidence of the *POLE* proofreading domain mutation signature.¹³⁻¹⁸ Interestingly, analysis of tumors from patients with CMMR-D suggests that the acquisition of somatic pathogenic DNA polymerase proofreading domain mutations is associated with rapid tumor growth,⁷⁷ consistent with the prediction of mathematical models that a strong mutator phenotype confers a preferential advantage in early tumorigenesis.^{1,101} Further insights into the timing and consequences of polymerase proofreading domain mutations may be provided by analysis of pre-cancers and multiregion sequencing of tumors carrying these variants.

Explaining associations with prognosis

As noted previously, in addition to ultramutation and good prognosis, *POLE* proofreading domain-mutant tumors frequently display a prominent lymphocytic infiltrate,^{14,25,83} similar to that observed in MMR-D CRCs.¹⁰² Recent characterization of this in EC has shown that this infiltrate comprises a population of CD8⁺ cytotoxic lymphocytes and is accompanied by increased expression of cytotoxic lymphocyte differentiation markers (such as T-box 21 (*TBX21*) and eomesodermin (*EOMES*)), and effectors (such as interferon- γ (*IFNG*), perforin 1 (*PRF1*) and granzyme B (*GZMB*)) compared with other ECs.²⁵ Bioinformatics analysis revealed that the number of 'antigenic mutations' – that is, mutations in expressed genes that encode neopeptides predicted to bind major histocompatibility complex (MHC) molecules – is substantially greater in *POLE* proofreading domain-mutant tumors than in other ECs, providing a potential explanation for these results.^{25,26} Interestingly, *POLE* proofreading domain-mutant tumors displayed increased expression of genes encoding multiple immunosuppressive checkpoints — including programmed cell death protein 1 (*PDCD1*), PD-1 ligand 1 (*PDL1*), cytotoxic T lymphocyte-associated antigen 4 (*CTLA4*), lymphocyte activation gene 3 protein (*LAG3*), T cell immunoglobulin mucin receptor 3 (*TIM3*; also known as *HAVCR3*) and T cell immunoreceptor with immunoglobulin and ITIM domains (*TIGIT*)²⁵ — suggesting that up-regulation of these molecules may be required for tumor growth. It will be important to

investigate whether these findings are observed in *POLE* proofreading domain-mutant tumors of other tissues and to investigate whether tumors from carriers of germline DNA polymerase proofreading variants display evidence of enhanced immunogenicity that may affect their prognosis.

Another possible contributor to the favorable outcome of DNA polymerase proofreading domain-mutant tumors relates directly to their mutator phenotype. Studies of mutator Pol ϵ and Pol δ variants in yeast have demonstrated the existence of an ‘error threshold’ that, if exceeded, results in reduced viability.^{89,90,94} The combination of defective DNA polymerase proofreading and complete loss of MMR is lethal as it exceeds this threshold.^{89,94} Interestingly, sequential biopsy of cancers in patients with CMMR-D demonstrated that although the acquisition of somatic DNA polymerase proofreading domain mutations was associated with the dramatic accumulation of mutations and rapid tumor growth, the total mutation burden subsequently seemed to plateau.⁷⁷ The upper limit of 10,000–20,000 exonic mutations suggested by this study is highly concordant with the number of mutations observed in sporadic *POLE* proofreading domain-mutant adult cancers.¹⁹ Tumor mutation burden is not simply a reflection of mutation rate (it also reflects the number of cell divisions during tumor development), and the relationship between the two in DNA polymerase-mutant tumors is uncertain. Nevertheless, it may be speculated that while the proofreading-deficient mutator phenotype confers a growth advantage in early tumorigenesis, it compromises fitness in later-stage cancers. This may be explained by an intriguing recent study that used mathematical modeling to predict the effect of passenger mutations on the fitness of (non-hypermutated) tumors.¹⁰³ This showed that whereas strongly deleterious passenger mutations are subject to negative selection, those with individually moderate negative effects are able to fixate (become fixed into the genome), and collectively are predicted to exert a negative impact on tumor growth.¹⁰³ As this model was based on tumors with only relatively small numbers of protein-coding mutations (an average of 10–366 per tumor type), it will be interesting to examine the effect of passenger mutations on the fitness of DNA polymerase proofreading domain-mutant tumors.

Clinical management

Diagnosis

The *polymerase proofreading-associated polyposis* (PPAP) phenotype overlaps with that of *Lynch syndrome* and *MUTYH-associated polyposis*, and screening and management algorithms are broadly similar. Valle and colleagues⁷⁰ have proposed that screening of the *POLD1* and *POLE* exonuclease domains is indicated in patients with polyposis (10–100 adenomas) and/or those with early-onset CRC (diagnosis before the age of 50), who lack germline mutations in MMR genes, *APC* or *MUTYH*. The concomitant presence of

extracolonic tumors (Table 2), particularly EC and stomach or duodenal tumors, should increase clinical suspicion of germline *POLD1* or *POLE* proofreading domain mutations. However, at present there is insufficient evidence to recommend screening of patients who lack colonic phenotypes. Similar to patients with Lynch syndrome,¹⁰⁴ carriers of germline *POLD1* and *POLE* proofreading domain mutations should be offered colonoscopies at one- to two-year intervals from the age of 25 and upper gastrointestinal endoscopy to check for duodenal tumors (particularly in carriers of *POLE* proofreading domain variants). Although EC screening is not of proven benefit for this cohort, women with germline *POLD1* and *POLE* proofreading domain mutations might pragmatically be offered this from the age of 40, and clinicians should be aware of the potential increased risk of ovarian, brain and breast cancers in these patients, with consideration given to preventive measures if available.

The distinctive characteristics of tumors with somatic *POLE* proofreading domain mutations also have potential implications for patient management. Most notably, the association with good prognosis in EC^{17,20-24} suggests that *POLE* proofreading domain mutations might identify a group of patients who are less likely to benefit from adjuvant treatment following surgery. This is clinically relevant, as *POLE* mutations are more common in tumors defined as 'high risk' by conventional criteria,^{20,22} for which postoperative radiotherapy and chemotherapy are often recommended. However, as noted earlier, the possibility that the favorable outcome of these tumors reflects increased sensitivity to treatment cannot be excluded at present, and further preclinical and clinical studies will be required before *POLE* proofreading domain mutations can be used to guide management in EC. Similar studies will also be needed to confirm the utility of *POLE* mutation status as a biomarker in other cancer types.

Therapeutic targeting

The remarkable mutation burden of *POLE* proofreading domain-mutant tumors also raises the possibility that they may be particularly sensitive to specific therapeutic strategies. Perhaps the most obvious of these is the use of immune checkpoint inhibitors that target immunosuppressive molecules such as PD-1 and PD-L1. These agents have recently demonstrated striking activity against highly mutated MMR-D CRCs, melanomas and non-small cell lung cancers (NSCLCs),¹⁰⁵⁻¹⁰⁸ in which response seems to correlate with increased tumor antigenic mutation burden^{96,109} and the density of pre-treatment intratumoral infiltration by CD8⁺, PD-1⁺ and PD-L1⁺ lymphocytes.¹¹⁰ These are all prominent features of *POLE* proofreading domain-mutant tumors,^{25,26} suggesting that these cancers may be excellent candidates for drugs targeting PD-1-PD-L1 signaling. Furthermore, the recent demonstration that immune checkpoint inhibition may be potentiated by radiotherapy¹¹¹ suggests that investigation of these combinations against

DNA polymerase proofreading domain-mutant cancers in preclinical models and clinical trials may be worthwhile.

Another potential therapeutic strategy against DNA polymerase proofreading domain-mutant cancers relates to the concept of the error threshold discussed earlier. In theory, agents such as mutagenic nucleosides or inhibitors of DNA repair¹¹² could be used to increase the mutation rate in these tumors to a level that exceeds this threshold, resulting in lethal mutagenesis and loss of viability.⁹³ Clearly, in the first instance such a strategy would be appropriate only in patients with incurable disease who lack other treatment options, although in selected cases this may be worthy of exploration.⁹³ Preclinical studies suggest that a similar effect may result from modification of the dNTP pool;^{67,113} however, differences in nucleotide synthesis and the DNA damage response between yeast and humans mean that further work is required before the possible utility of this approach in humans can be predicted.

Given the modest frequency of DNA polymerase proofreading domain mutations overall, any therapeutic study is likely to have to recruit patients with multiple tumor types (a design frequently referred to as a *basket trial*¹¹⁴) or to combine proofreading-deficient tumors with other hypermutated cancers, such as those with defective MMR.

Conclusions and future directions

Evidence has only recently emerged to support the longstanding postulate that defective DNA polymerase proofreading may contribute to human cancer.¹¹⁵ Nevertheless, it is now clear that germline mutations in the exonuclease domains of *POLD1* and *POLE* predispose to polyposis, CRC and other malignancies,^{12,67-73} and that somatic *POLE* proofreading domain mutations cause ultramutation in sporadic ECs, CRCs and several other cancers.¹³⁻¹⁹ The exceptional mutation load in somatic *POLE* proofreading domain-mutant ECs is associated with an enhanced immune response^{25,26} and an excellent prognosis.^{17,20-24} The possibility that mutation rate in these tumors approaches the maximum compatible with continued viability is an intriguing one, the investigation of which may provide novel insights into the consequences of a mutator phenotype in cancer. It will also be of interest to determine whether the ultramutator phenotype in DNA polymerase proofreading domain-mutant tumors represents an Achilles' heel that can be exploited for therapy, as has recently been suggested.⁷⁷

From a clinical perspective, PPAP should be considered and tested for in patients with unexplained polyposis and/or early-onset CRC, particularly if family members have EC or other extracolonic cancers suggestive of germline *POLD1* or *POLE* proofreading domain mutations.⁷⁰ Meanwhile, somatic DNA polymerase proofreading domain mutations

exemplify the challenge of implementing precision medicine. For example, the modest frequency of *POLE* proofreading domain mutations in EC (7-12%) has limited the power of some studies to evaluate their impact on prognosis,²¹ a problem that is likely to prove even greater in other tumors in which these mutations are less common. As most novel cancer variants occur at a similarly low frequency (<10%), evaluation of these as prognostic and predictive biomarkers will require large-scale collaborations and coordinated analysis. At a more basic level, the results of studies performed to date pose several fundamental questions. For example, why are somatic mutations largely restricted to *POLE*, whereas germline variants of both *POLE* and *POLD1* cause cancer? Why are substitutions of the exo motif catalytic sites proportionally more common among germline than among somatic proofreading domain mutations? Why does the mutator phenotype associated with *POLE* P286R exceed that associated with the exonuclease-null allele? Determining the answers to these questions will be a priority for future studies.

During the past three years, defects in DNA polymerase proofreading have been recognized to drive tumorigenesis in a small but important fraction of common cancers. Given the rapid progress in the field, we are optimistic that the next three years will see similar advances in our understanding of this novel tumor subgroup, with consequent benefits for patients.

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GLOSSARY

Antimutator mutations – mutations that result in an increase in the fidelity of DNA replication; examples have been reported in both *Escherichia coli* and *Saccharomyces cerevisiae*, although their occurrence in human cancer is currently not proved.

B family polymerases – replicative and repair DNA polymerases that are grouped on the basis of sequence and structural similarities, and include the prokaryotic DNA polymerase Pol II, and the eukaryotic DNA polymerases Pol ζ, Pol α, Pol δ and Pol ε.

Basket trial – also known as a bucket trial. A study in which patients with a specific molecular marker are recruited for treatment with a corresponding targeted therapy, irrespective of tumor histology or tissue of origin.

GIN5 – a multiprotein complex essential for eukaryotic DNA replication. The name is derived from the Japanese go, ichi, ni and san (meaning five, one, two and three), and refers to the constituent components in budding yeast: Sld5, Psf1, Psf2 and Psf3.

Intestinal polyposis – a phenotype of multiple colonic polyps and increased risk of colorectal cancer that may be due to a germline mutation in a known predisposition gene (for example, adenomatous polyposis coli (APC) or MUTYH) or may lack an obvious genetic cause.

Lynch syndrome – a tumor predisposition syndrome caused by germline mutation of mismatch repair genes, which manifests as increased risk of carcinomas of the colon and rectum, endometrium, ovary, stomach and several other organs.

Microsatellite instability (MSI) – the presence of small insertions and deletions (indels) at repetitive DNA microsatellites that result from defective DNA mismatch repair.

Microsatellite-stable – absence of small insertions and deletions (indels) at repetitive DNA microsatellites as a result of proficient DNA mismatch repair.

Mismatch repair (MMR) – a mechanism of post-replicative DNA repair that removes mismatched bases and small insertions and deletions (indels) from the newly synthesized DNA strand.

Multinucleated giant cells – abnormally large cells with multiple nuclei and typically bizarre morphology that are observed in association with chronic inflammation and some

types of malignancy.

Mutational hot spot – a genomic region that is the site of mutations in cancers at a frequency greater than that expected by chance: for example, codon 600 in the serine/threonine kinase BRAF.

Mutator phenotype – elevation of the mutation rate above that expected for normally dividing cells. It has been argued that the acquisition of a mutator phenotype is a characteristic of many cancers.

MUTYH-associated polyposis – a syndrome characterized by multiple intestinal adenomas and an increased risk of colorectal carcinoma. It is caused by germline mutations in the base excision repair gene *MUTYH*.

Neoepitope – novel epitopes, created by somatic mutations in tumors, that are capable of eliciting an antitumor immune response.

Polymerase proofreading-associated polyposis (PPAP) – a high-penetrance, dominantly inherited condition characterized by multiple colorectal adenomas and carcinomas, caused by germline mutations in the genes encoding the catalytic subunits of the DNA polymerases Pol δ and Pol ϵ (*POLD1* and *POLE*, respectively).

Synthetically lethal – a type of genetic interaction in which the combination of mutations in two genes results in cell death, whereas cells harboring only one of the mutations are viable.

Ultramutated – describes tumors with an exceptional frequency of mutations, often exceeding 100 mutations per megabase and often caused by mutations in the gene encoding the catalytic subunit of DNA polymerase ϵ (*POLE*).



Chapter 3

Blinded histopathological characterization of *POLE* exonuclease domain-mutant endometrial cancers: sheep in wolf's clothing

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ABSTRACT

Objective

POLE exonuclease domain mutations identify a subset of endometrial cancer (EC) patients with an excellent prognosis. The use of this biomarker has been suggested to refine adjuvant treatment decisions, but the necessary sequencing is not widely performed and is relatively expensive. Therefore, we aimed to identify histopathological and immunohistochemical characteristics to aid the detection of *POLE*-mutant ECs.

Methods

Fifty-one *POLE*-mutant endometrioid, 67 *POLE*-wild-type endometrioid and 15 *POLE*-wild-type serous ECs were included (total N=133). An expert gynecological pathologist, blinded to molecular features, evaluated each case (two or more slides) for sixteen morphological characteristics. Immunohistochemistry was performed for p53, p16, MLH1, MSH2, MSH6, and PMS2.

Results

POLE-mutant ECs were characterized by a prominent immune infiltrate: 80% showed peritumoral lymphocytes and 59% tumor-infiltrating lymphocytes, as compared with 43% and 28% of *POLE*-wild-type endometrioid ECs, and 27% and 13% of serous counterparts ($P<0.01$, all comparisons). Of *POLE*-mutant ECs, 33% contained tumor giant cells; this proportion was significantly higher than that in *POLE*-wild-type endometrioid ECs (10%, $P=0.003$), but not significantly different from that in serous ECs (53%). Serous-like features were as often (focally) present in *POLE*-mutant as in *POLE*-wild-type endometrioid ECs (6-24%, depending on the feature). The majority of *POLE*-mutant ECs showed wild-type p53 (86%), negative/focal p16 (82%) and normal mismatch repair protein expression (90%).

Conclusions

A simple combination of morphological and immunohistochemical characteristics (tumor type, grade, peritumoral lymphocytes, MLH1, p53 expression) can assist in pre-screening for *POLE* exonuclease domain mutations in EC, increasing the probability of a mutation being detected from 7% to 33%. This facilitates the use of this important prognostic biomarker in routine pathology.

INTRODUCTION

An integrated clinicopathological and molecular classification has the potential to refine the clinical risk prediction of endometrial cancer (EC) patients and to provide more tailored treatment recommendations.¹⁻³ One of the important prognostic subgroups included in this classification, constituting 7-12% of ECs, is characterized by somatic mutations in the exonuclease domain of the *POLE* gene.⁴⁻⁶ *POLE* encodes the catalytic subunit of DNA polymerase ϵ .⁷ Mutations in the exonuclease domain of *POLE* (severely) reduce proofreading activity during DNA replication, and thereby contribute to a very high mutational burden, which is among the highest found in human cancers (a so-called 'ultramutator' phenotype).^{6,8} Moreover, *POLE*-mutant ECs occur at a relatively young age and are often high-grade tumors.⁹ It is thus not surprising that, on the basis of current guidelines, the majority of patients with *POLE*-mutant ECs would be offered adjuvant radiotherapy, and approximately one-third would be offered some form of chemotherapy.¹⁰

Despite the aggressive appearance of these tumors, multiple independent studies have shown that patients with *POLE*-mutant ECs have an excellent prognosis, with a five-year disease-specific survival rate of 98-100%.^{2,5,6,9,11} As this prognostic benefit is suggested to be independent of adjuvant therapy, no or less intensive adjuvant treatment may suffice for early-stage *POLE*-mutant ECs.^{9,12} This potential of *POLE* mutations to reduce overtreatment may spare a relatively young patient group from the toxicities of adjuvant therapy. In addition to the prognostic importance, recent studies have demonstrated that *POLE*-mutant ECs show a strong antitumor immune response, but also upregulation of markers involved in immune suppression, including programmed death 1 and programmed death-ligand 1.¹³⁻¹⁶ Therefore, although *POLE*-mutant ECs rarely recur, patients with (recurring or metastatic) *POLE*-mutant ECs may be good candidates for immune checkpoint inhibition, as demonstrated in recent case reports.^{17,18} These prognostic and potentially predictive implications make the identification of *POLE* exonuclease domain mutations in EC essential.

Screening for *POLE* mutations is performed by sequencing of the exonuclease domain (exons 9-14). This technique is not widely available in routine pathology laboratories and is relatively expensive, hampering the clinical use of this important biomarker. Two previous studies aimed to identify clinicopathological and/or morphological features of ECs with *POLE* mutations.^{10,19} Hussein et al., for example, noted the presence of 'bizarre/giant tumor cell nuclei' in *POLE*-mutant ECs, and of foci with severe nuclear atypia, suggesting that this could lead to concerns about serous carcinomas.¹⁹ However, in both studies, the pathologists evaluated only *POLE*-mutant ECs: scoring for the morphological features was

not performed blindly. Also, for the majority of cases, the number of slides assessed for each case was unknown. Therefore, we performed a blinded in-depth characterization of a large series of *POLE*-mutant ECs, with multiple slides available for each case. In this study, we sought to identify histopathological and immunohistochemical characteristics of ECs carrying *POLE* exonuclease domain mutations, to aid their detection in EC.

MATERIALS AND METHODS

Patient selection

ECs from the PORTEC-1 and PORTEC-2 studies, and from an EC series of the Leiden University Medical Center archives (2001-2015), were collected after approval by the institutional review board.^{20,21} For all ECs, histological type, tumor grade and stage of disease (classified according to the World Health Organization and FIGO classifications) were reviewed by an expert gynecological pathologist.²⁰⁻²⁴ Cases were considered for inclusion in this study when a minimum of two different hematoxylin and eosin (H&E)-stained slides of the EC were available. Moreover, molecular analyses - *POLE* mutational status, the presence of microsatellite instability, and p53 expression - were conducted prior to this study (details in Supplementary Methods and Supplementary Table 1).^{1,9,13,25}

From these EC cohorts, by use of the available data as described above, cases were selected for the current study. First, all previously identified ECs with pathogenic *POLE* exonuclease domain mutations were included, resulting in 51 *POLE*-mutant tumors for analysis (details on the included substitutions in Supplementary Table 2). To address any morphological similarities between *POLE*-mutant and serous ECs, a control group of *POLE*-wild-type serous ECs was included (N=15). Furthermore, *POLE*-wild-type endometrioid ECs were included to represent the other, previously identified molecular subgroups.⁶ These cases were proportionally matched on tumor grade with the *POLE*-mutant group, allowing reliable comparison of morphological characteristics between the groups. This resulted in the inclusion of 35 microsatellite-unstable and 32 microsatellite-stable endometrial cancers (four p53-mutant and 28 with no specific molecular profile).

Morphological characteristics

For all included cases, the presence of papillary growth, slit-like spaces and hobnailing was evaluated. In addition, when such a feature was present, the percentage of the tumor displaying the characteristic was noted, estimated over all available slides of a case. Cytonuclear atypia was classified as marked, rather than mild or moderate, when the majority of the tumor cells displayed strongly pleomorphic nuclei. When, at scanning magnification (x5), easily identifiable mitotic figures or apoptotic bodies were present, this was noted

as brisk mitotic activity and brisk apoptotic activity, respectively. Tumor giant cells were identified in ECs containing mononuclear or multinucleated cells (size range 10–40 times the size of a lymphocyte) with large, bizarre, often hyperchromatic nuclei in at least one slide of the tumor. As with brisk mitotic and apoptotic activity, tumor-infiltrating lymphocytes (TILs) and peritumoral lymphocytes were present when these features were readily detectable upon scanning of the slides (magnification: x5). TILs are found intraepithelially within the tumor, whereas peritumoral lymphocytes appear as lymphoid aggregates adjacent or close to the invasive margin of the tumor. The predominant invasion type was categorized into no invasion, broad front invasion, microcystic elongated and fragmented invasion, adenomyosis-like, or infiltrating glands.^{26,27}

ECs were examined for the presence of necrosis and for the presence (isolated, focal or scattered) of squamous and eosinophilic metaplasia. Squamous metaplasia presenting as morules (circumscribed, solid round, intraglandular nests composed of cells with (im)mature squamous differentiation) were denoted separately.²⁸ The percentage of solid growth was estimated over all available slides for each case, and was categorized as $\leq 5\%$, 6–50%, and $> 50\%$ according to the FIGO classification.^{22,23} Myometrial invasion, dichotomized with a cut-off of 50%, was either evaluated as part of the inclusion criteria of the PORTEC-1 and PORTEC-2 trials or extracted from the pathology reports.^{20,21} Lymphovascular space invasion was defined as the presence of tumor cells in a space lined by endothelial cells outside the immediate invasive border of the tumor, and was classified as absent, focal or substantial, as described previously.²⁹ All slides were blindly scored for each morphological characteristic by an expert gynecological pathologist (T.B.).

Immunohistochemical analyses

One formalin-fixed paraffin-embedded tissue block containing representative tumor tissue was selected for each case for immunohistochemistry. Immunohistochemistry for p53, MLH1, MSH2, MSH6, PMS2, and p16 was performed as described previously; procedures, primary antibodies, and scoring methods are shown in the Supplementary Methods.^{1,30–32}

Statistical analyses

Continuous variables were compared by use of the Kruskal-Wallis and Mann-Whitney U tests (with Bonferroni correction for multiple comparisons). Associations between categorical variables were tested by use of χ^2 statistics or Fisher's exact test. Logistic regression was performed on all morphological and immunohistochemical features to identify a combination of characteristics that best predict *POLE* exonuclease domain mutational status using the R statistical software package LogicReg version 1.5.9 (further details are given in the Supplementary Methods).^{33,34}

RESULTS

The clinicopathological characteristics of the 133 included ECs are summarized in Table 1. All *POLE*-mutant cases were classified as endometrioid ECs after central review, despite not being selected for tumor type. In the original pathology reports, 49 of 51 (96.1%) *POLE*-mutant ECs were considered to be endometrioid-type as well, while two high-grade ECs were originally classified as serous and clear cell, respectively. A median of four slides (range 2–13) of each case was blindly assessed for all morphological characteristics.

Table 1. Clinicopathological characteristics of the study population and of the included molecular/histopathological subgroups of endometrial cancer.

	Total N=133	<i>POLE</i> -mu- tant* N=51	<i>POLE</i> -wild- type endometrioid* N=67	<i>POLE</i> -wild- type serous* N=15	<i>P</i> value <i>POLE</i> versus endometrioid [‡]	<i>P</i> value <i>POLE</i> ver- sus serous [‡]
Age, median (range), years	64.8 (36 – 85)	62.1 (36 – 81)	65 (40 – 84)	73.7 (61 – 85)	0.497 [§]	0.001 [§]
FIGO 2009, n (%)						
I-II	119 (89.5)	49 (96.1)	61 (91.0)	9 (60.0)	0.463	0.001
III-IV	14 (10.5)	2 (3.9)	6 (9.0)	6 (40.0)		
Tumor type, n (%)						
Endometrioid	117 (88.0)	51 (100)	66 (98.5)	0 (0.0)	1	<0.001
Serous	15 (11.3)	0 (0.0)	0 (0.0)	15 (100)		
Mixed epithelial (endometrioid – clear cell)	1 (0.8)	0 (0.0)	1 (1.5)	0 (0.0)		
Tumor grade, n (%)						
Low (grade 1 or 2)	88 (66.2)	35 (68.6)	53 (79.1)	0 (0.0)	0.195	<0.001
High (grade 3)	45 (33.8)	16 (31.4)	14 (20.9)	15 (100)		
Slides assessed, median (range)	4 (2 – 13)	4 (2 – 11)	4 (2 – 10)	3 (2 – 13)	NA	NA

*Clinicopathological characteristics are shown for the total study population as well as for each molecular/histopathological subgroup. The included subgroups are: *POLE*-mutant, *POLE*-wild-type endometrioid (microsatellite-unstable, p53-mutant or with no specific molecular profile), and *POLE*-wild-type serous endometrial cancers. The *POLE*-wild-type endometrioid group is denominated 'endometrioid' throughout this article, despite the single mixed epithelial case.

[‡]*P* values are calculated over *POLE*-mutant and *POLE*-wild-type endometrioid (*POLE* vs. endometrioid) or over *POLE*-mutant and *POLE*-wild-type serous (*POLE* vs. serous) endometrial cancers, respectively.

[§]Bonferroni adjusted *P*<0.0167 to indicate statistical significance.

^{||}The non-parametric Kruskal-Wallis test revealed no significant differences in the number of slides assessed between the three subgroups (*P*=0.384). Therefore, no pairwise comparisons were performed.

Abbreviations: NA, not applicable.

POLE-mutant ECs showed peritumoral lymphocytes in 80% of cases (Figure 1A-C; Table 2). The presence of peritumoral lymphocytes was strongly associated with *POLE* mutation both in comparison with both *POLE*-wild-type serous ECs (27%, $P<0.001$) and *POLE*-wild-type endometrioid ECs (43%, $P<0.001$). Similarly, an increased presence of TILs was found in *POLE*-mutant ECs (59%) in comparison with *POLE*-wild-type endometrioid ECs (28%) and serous ECs (13%, $P=0.001$ and $P=0.003$ respectively; Figure 1D-E). Moreover, peritumoral lymphocytes and TILs were present significantly more often in *POLE*-mutant ECs than in microsatellite-unstable cases included in the *POLE*-wild-type endometrioid EC group (respectively: 80% vs. 57%, $P=0.020$; and 59% vs. 34%, $P=0.025$).

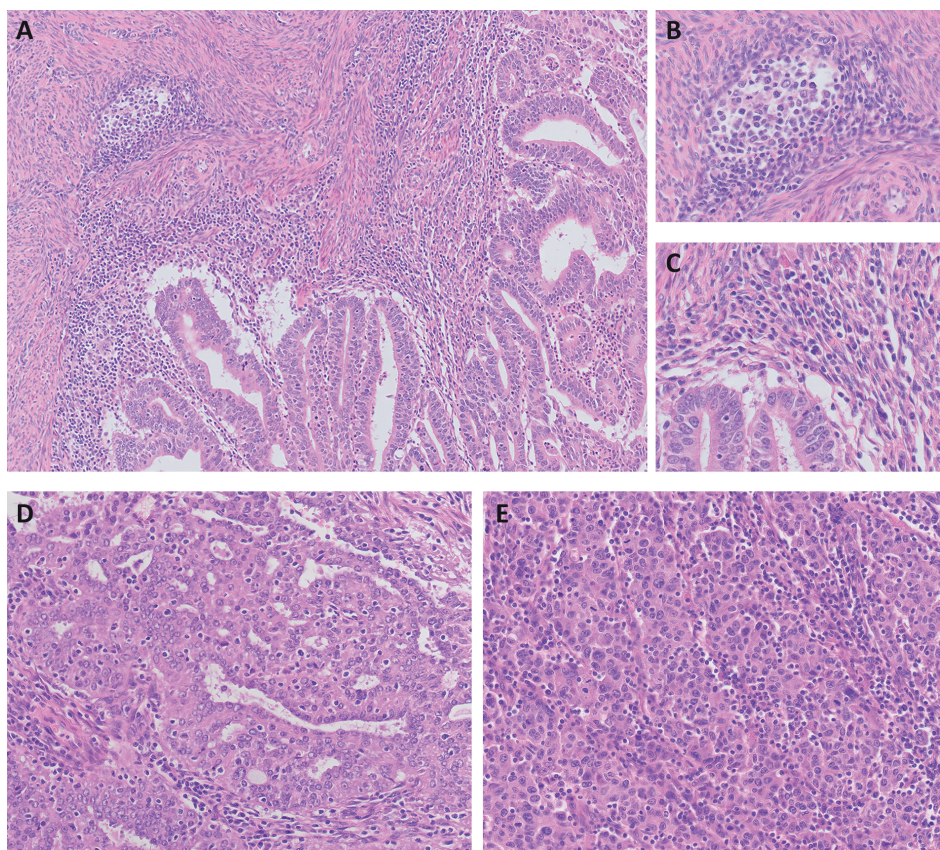


Figure 1. Peritumoral and tumor-infiltrating lymphocytes in *POLE*-mutant endometrial cancers. The presence of a prominent peritumoral lymphocytic infiltrate in a low-grade (grade 2) endometrioid-type *POLE*-mutant endometrial cancer is shown (A, magnification: x10), with insets of Crohn's-like infiltrate (B, magnification: x40) and of the peritumoral lymphocytes (C, magnification: x40). Examples of tumor-infiltrating lymphocytes in *POLE*-mutant endometrial cancers are shown (D, low-grade endometrioid-type; and E, high-grade endometrioid-type; both magnification: x20).

Although tumor giant cells were most frequently found in *POLE*-wild-type serous ECs (53%), 33% of *POLE*-mutant ECs also contained these cells ($P=0.227$; Figure 2; Table 2). Among *POLE*-mutant and *POLE*-wild-type endometrioid ECs, however, the presence of tumor giant cells was significantly associated with *POLE* mutation (33% vs. 10%, $P=0.003$). Tumor giant cells showed keratin positivity, while being negative for β -human chorionic gonadotropin (β -hCG; Supplementary Figure 1).

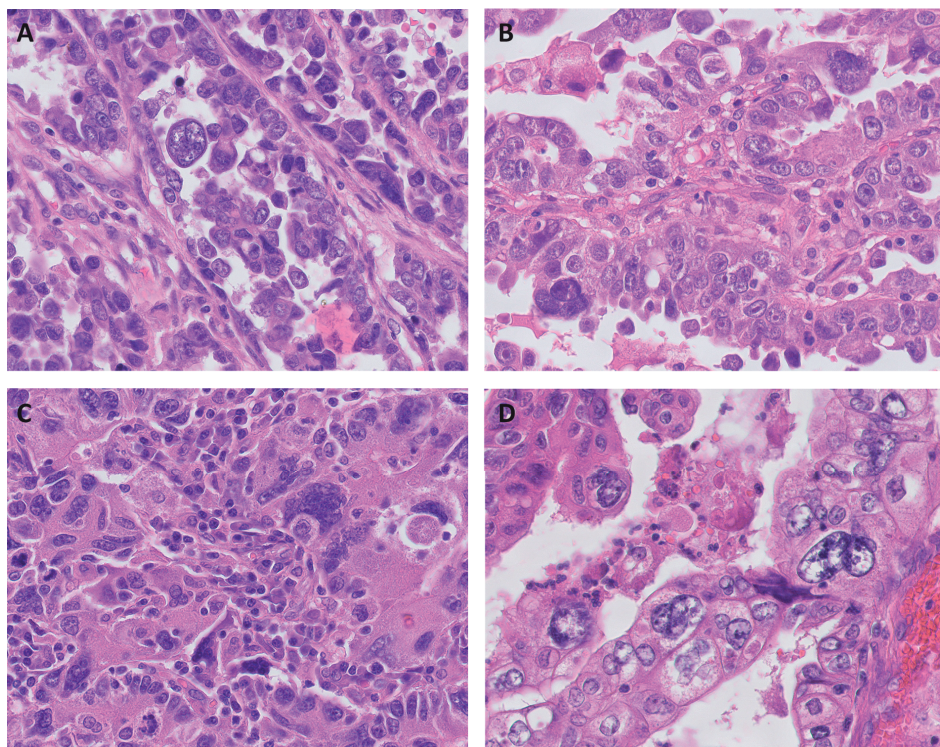


Figure 2. Tumor giant cells in *POLE*-wild-type serous and *POLE*-mutant endometrial cancers. Examples of tumor giant cells in two different (*POLE*-wild-type) serous endometrial cancers are shown (A and B), and examples of tumor giant cells in two different *POLE*-mutant endometrial cancers (C and D, all magnification: x40).

Among the endometrioid ECs, no differences were found between *POLE*-mutant and *POLE*-wild-type cases with regard to serous-like features (i.e. papillary growth, slit-like spaces, hobnailing, marked cytonuclear atypia, and brisk mitotic activity): there were no differences in either the presence of each feature separately (Table 2), or the percentage of tumor showing a feature (data not shown), or the presence of three or more features in the same case (12% of *POLE*-mutant ECs vs. 9% of *POLE*-wild-type ECs, $P=0.761$). In contrast, the presence of serous-like features, the percentage at which they are seen in the cancer, as well as their co-occurrence correlated strongly with *POLE*-wild-type serous

ECs as compared with *POLE*-mutant ECs ($P < 0.05$ for all associations; Table 2).

Table 2. Presence of morphological characteristics in *POLE* exonuclease domain-mutant endometrial cancers compared to endometrial cancers of other molecular/histopathological subtypes.

	<i>POLE</i> -mutant N=51, n (%)	<i>POLE</i> -wild-type endometrioid N=67, n (%)	<i>POLE</i> -wild-type serous N=15, n (%)	<i>P</i> value <i>POLE</i> vs. endometrioid*	<i>P</i> value <i>POLE</i> vs. serous*
Peritumoral lymphocytes	41 (80.4)	29 (43.3)	4 (26.7)	<0.001	<0.001
Tumor-infiltrating lymphocytes	30 (58.8)	19 (28.4)	2 (13.3)	0.001	0.003
Tumor giant cells	17 (33.3)	7 (10.4)	8 (53.3)	0.003	0.227
Papillary growth	7 (13.7)	7 (10.4)	13 (86.7)	0.775	<0.001
Hobnailing	6 (11.8)	4 (6.0)	10 (66.7)	0.325	<0.001
Slit-like spaces	11 (21.6)	11 (16.4)	13 (86.7)	0.477	<0.001
Marked cytonuclear atypia	12 (23.5)	12 (17.9)	10 (66.7)	0.453	0.004
Brisk mitotic activity	12 (23.5)	14 (20.9)	9 (60.0)	0.732	0.012
Brisk apoptotic activity	6 (11.8)	10 (14.9)	0 (0.0)	0.787	0.323
Broad front invasion type	26 (51.0)	27 (40.3)	3 (20.0)	0.248	0.042
Squamous metaplasia	15 (29.4)	20 (29.9)	0 (0.0)	0.959	0.015
Solid growth ($\geq 5\%$)	31 (60.8)	32 (47.8)	7 (46.7)	0.160	0.382
Necrosis	21 (41.2)	29 (43.3)	8 (53.3)	0.819	0.555
Myometrial invasion ($> 50\%$)	30 (58.8)	34 (50.7)	8 (53.3)	0.383	0.771
Substantial lymphovascular space invasion	7 (13.7)	9 (13.4)	4 (26.7)	1	0.254

The number and percentage of cases in which a morphological characteristic is present is indicated for each molecular/histopathological subgroup (for definitions of each characteristic, see Materials and methods).

**P* values are calculated over *POLE*-mutant and *POLE*-wild-type endometrioid (*POLE* vs. endometrioid) or over *POLE*-mutant and *POLE*-wild-type serous (*POLE* vs. serous) endometrial cancers, respectively.

With regard to the invasion type, broad front invasion was associated with *POLE*-mutant ECs, in contrast to *POLE*-wild-type serous ECs (51% vs. 20%, $P = 0.042$), but did not differ among the endometrioid ECs (Table 2; Supplementary Table 3). Similarly, squamous metaplasia was associated with *POLE*-mutant ECs in comparison with *POLE*-wild-type serous ECs (29% vs. 0%, $P = 0.015$), but did not differ between *POLE*-mutant and *POLE*-wild-type endometrioid ECs (Table 2).

We next sought to determine whether immunohistochemistry for markers that are commonly used in diagnostic pathology could aid in the detection of *POLE* mutations in EC. Approximately 16% of *POLE*-mutant ECs showed p53-mutant-like expression (five of eight cases showed subclonal p53-mutant-like expression), as compared with 6% of *POLE*-wild-type endometrioid ECs ($P = 0.123$) and 80% of *POLE*-wild-type serous ECs ($P < 0.001$; Table 3). Immunohistochemistry for p16 revealed similar results: strong and

diffuse p16 expression did not differ between *POLE*-mutant ECs and *POLE*-wild-type endometrioid ECs (18% vs. 13% respectively, $P=0.609$), but it was strongly associated with serous ECs when *POLE*-mutant ECs were compared with *POLE*-wild-type serous ECs (18% vs. 73% respectively, $P<0.001$). Moreover, *POLE*-mutant ECs showing three or more serous-like features ($n=6$) all showed p53-wild-type expression and negative or focal p16 expression (Figure 3), whereas the serous ECs with a less prototypical serous morphology (showing fewer than three serous-like features, $n=4$) all showed p53-mutant-like expression and/or strong and diffuse p16 expression.

Table 3. Results of immunohistochemistry for p53 and p16 on *POLE* exonuclease domain-mutant compared *POLE*-wild-type endometrioid and serous endometrial cancers.

	<i>POLE</i> -mutant N=51, n (%)	<i>POLE</i> -wild-type endometrioid N=67, n (%)	<i>POLE</i> -wild-type serous N=15, n (%)	<i>P</i> value <i>POLE</i> vs. endometrioid*	<i>P</i> value <i>POLE</i> vs. serous*
p53 [‡]					
Wild-type	43 (84.3)	63 (94.0)	3 (20.0)	0.123	<0.001
Mutant	8 (15.7)	4 (6.0)	12 (80.0)		
p16					
Negative/focal	42 (82.4)	58 (86.6)	4 (26.7)	0.609	<0.001
Diffuse	9 (17.6)	9 (13.4)	11 (73.3)		

**P* values are calculated over *POLE*-mutant and *POLE*-wild-type endometrioid (*POLE* vs. endometrioid) or over *POLE*-mutant and *POLE*-wild-type serous (*POLE* vs. serous) endometrial cancers, respectively.

[‡]p53 mutational status was based on immunohistochemistry, except for three cases (one *POLE*-mutant and two *POLE*-wild-type endometrioid) in which p53 expression was scored as indefinite. Sanger sequencing revealed no mutation in these cases.

With regard to DNA mismatch repair status, 90% (46/51) of *POLE*-mutant ECs were mismatch repair-proficient as suggested by normal expression of the four mismatch repair proteins (MLH1, PMS2, MSH2, and MSH6; Supplementary Table 4). Five *POLE*-mutant ECs showed abnormalities in mismatch repair protein expression: one showed loss of MLH1 and PMS2, one loss of PMS2, two subclonal loss of MLH1 and PMS2 (deficiency of the two proteins found in the same tumor area), and one subclonal loss of MLH1 and complete loss of PMS2. All serous ECs showed normal expression of the mismatch repair proteins. One *POLE*-wild-type p53-mutant EC showed subclonal loss of MLH1 and PMS2 (3%).

A combination of morphological and immunohistochemical characteristics that best predicts *POLE* mutational status was identified by performing logic regression. We found that a logic model including five such characteristics had the best predictive capability (lowest misclassification rate). With the use of this number and a simulated annealing algorithm, the best Boolean combination of characteristics to predict *POLE* mutational status was as follows: (MLH1 wild-type expression or p53-wild-type expression) and

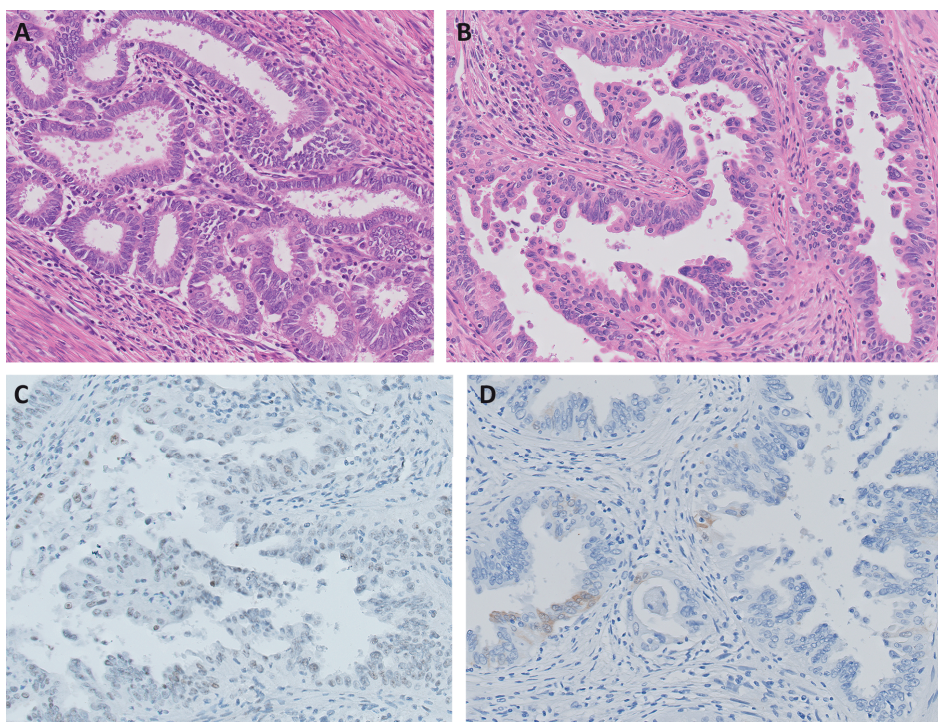


Figure 3. Immunohistochemistry of p53 and p16 in a *POLE*-mutant endometrioid endometrial cancer with serous-like features.

This figure shows one *POLE*-mutant tumor classified as an endometrioid endometrial cancer (A) that also demonstrates serous-like features (B, papillary growth, hobnailing and slit-like spaces). Despite these serous-like features, immunohistochemistry reveals p53-wild-type expression (C) and focal p16 positivity (D, all images magnification: x20).

(endometrioid-type EC) and (peritumoral lymphocytes present or tumor grade 3). This model has an estimated sensitivity of 80% and specificity of 88%. With a prevalence of *POLE* exonuclease domain mutations in EC of 7%, this yields a positive predictive value of 33%.

DISCUSSION

We performed a blinded histopathological and immunohistochemical characterization of the largest series of *POLE* exonuclease domain-mutant ECs to date, also including *POLE*-wild-type endometrioid and serous EC control groups. Our study shows that *POLE*-mutant ECs are characterized by the presence of a striking immune infiltrate, consisting of peritumoral and TILs, and that one-third contains tumor giant cells. All *POLE*-mutant cases were classified as endometrioid ECs, but, similarly to *POLE*-wild-type endome-

trioid ECs, features suggestive of serous morphology can (focally) be present. On immunohistochemical investigation, the majority of *POLE*-mutant ECs show wild-type p53 expression, negative or focal p16 expression, and normal expression of the mismatch repair proteins. A combination of these morphological and immunohistochemical characteristics can be used to assist pre-screening for *POLE* exonuclease domain mutations when sequencing is not directly available.

POLE-mutant ECs show evidence of a prominent immune infiltrate, as illustrated by the remarkably high percentages of cases in which peritumoral lymphocytes or TILs were found (80% and 59%, respectively). Similarly, Bakhsh et al. found peritumoral lymphocytes in 79% of *POLE*-mutant ECs, and Hussein et al. described peritumoral lymphocytes and/or TILs in 84% of *POLE*-mutant ECs (as compared with 84% of *POLE*-mutant ECs in the current study).^{10,19} In addition, an increased lymphocytic infiltrate was found to be present in colorectal cancers with somatic *POLE* exonuclease domain mutations.³⁵ This has been described for *POLE*-mutant glioblastomas as well, suggesting this infiltrate is a characteristic of *POLE*-mutant cancers regardless of tumor type.³⁶ These findings are in line with the proposed mechanism by which *POLE* mutations contribute to the excellent clinical outcome of these patients: ultramutation results in the presentation of antigenic neopeptides by tumor cells, stimulating a cytolytic antitumor immune response.^{13,14,16} The differences in the presence of peritumoral lymphocytes and TILs between *POLE*-mutant ECs and *POLE*-wild-type serous and endometrioid ECs (including all proposed prognostic molecular subgroups) were strongly significant. Thus, the presence of a prominent immune infiltrate, readily identifiable on hematoxylin and eosin-stained slides, is a clear characteristic of *POLE*-mutant (endometrial) cancers.

Tumor giant cells were most frequently found in *POLE*-wild-type serous ECs (53%). This is in agreement with previous literature describing anaplastic tumor giant cells in approximately half of serous-type cancers of the uterus, but also of the Fallopian tube and ovary.³⁷ The presence of tumor giant cells in *POLE*-mutant ECs was first described by Hussein et al. (40% of cases). These cells were reported to be present in *POLE*-mutant glioblastomas as well.³⁶ The current study is, however, the first to date to associate tumor giant cells in a blinded fashion with *POLE* mutation status among endometrioid-type ECs (33% of *POLE*-mutant ECs vs. 10% of *POLE*-wild-type ECs).¹⁹ This study has demonstrated that these tumor giant cells do not represent osteoclast-like giant cells (no abundant eosinophilic cytoplasm and keratin-positive) or trophoblastic cells (β -hCG-negative) but that they are of epithelial origin, as indicated by their keratin positivity. A role for the markedly increased somatic mutational burden in the formation of tumor giant cells in *POLE*-mutant tumors seems plausible: the resultant genetic instability may interfere with normal cell cycle progression. This is, however, contradicted by the relatively infrequent

presence of tumor giant cells in mismatch repair-deficient ECs, which are expected to be hypermutated: only 6% of *POLE*-wild-type mismatch repair-deficient ECs contained tumor giant cells in this study, as compared with 16% of *POLE*-wild-type mismatch repair-proficient tumors.⁶ Tumor giant cells have been described in a limited number of ECs.³⁸⁻⁴² These studies included cases with a high-grade endometrioid component, abundant immune infiltrate, and intact mismatch repair protein expression. On the basis of the current and earlier studies, these cases may, in part, represent unidentified *POLE*-mutant ECs.^{9,19}

All *POLE*-mutant ECs included in this study were classified as endometrioid cancers. Although the exclusion criteria of the PORTEC-2 trial (exclusion of non-endometrioid ECs) may, in part, have influenced this, it is in agreement with previous literature that the vast majority of *POLE*-mutant endometrial cancers are of the endometrioid type.^{2,4-6,9-11,15,43} However, *POLE*-mutant ECs with a pure non-endometrioid morphology have also been described.^{2,4,5,10,43-48} Our study shows that features suggestive of serous morphology can, usually focally, be present in *POLE*-mutant endometrioid ECs, similarly to *POLE*-wild-type endometrioid ECs. Features of pseudo-glandular serous carcinoma, such as pseudo-stratification and scalloped luminal borders, were not evaluated in this study: although they overlap partly with the assessed serous-like features, this may be worthy of further exploration. Our results are supported by the study of Hussein et al. who, despite intratumoral heterogeneity, reported an endometrioid component in all but one of the included *POLE*-mutant cases (24/25).¹⁹ Whether *POLE* mutations do occur in pure non-endometrioid ECs or whether these represent misclassified endometrioid ECs with non-endometrioid-like features remains to be elucidated.

Our study has some limitations. First, all slides were evaluated by one gynecological pathologist. Therefore, the issue of interobserver variability was not addressed. However, this study, to a large degree, validated previous findings in a blinded manner. For the majority of features analyzed, we found similar results, suggesting a low degree of variability. Moreover, as described above, the inclusion criteria of the PORTEC trials may have influenced our case selection, resulting in predominantly low-stage, endometrioid ECs.^{20,21} However, arguably, low-stage disease and an endometrioid-type morphology may be characteristics of *POLE*-mutant ECs. Despite these limitations, this histopathological characterization contains the largest number of *POLE*-mutant ECs to date, is the first to characterize these tumors in a blinded fashion, and is strengthened by the availability of multiple slides for each case.

On the basis of the current and previous studies, a classical *POLE*-mutant EC will present at a relatively young age, as a low-stage high-grade endometrioid-type tumor with a

striking immune infiltrate, scattered tumor giant cells and a broad front invasion pattern. Immunohistochemistry of p53, p16 and the mismatch repair proteins can aid the detection of *POLE* mutations and will typically show wild-type p53, negative or focal p16, and normal mismatch repair protein expression. Because this approach is not conclusive, sequencing of the *POLE* exonuclease domain will remain the gold standard. However, in this study, we have shown that a certain combination of tumor type, grade, presence of peritumoral lymphocytes, and immunohistochemistry for MLH1 and p53 can increase the prior probability of detection of a *POLE* mutation almost five-fold. This underlines the potential of these morphological and immunohistochemical characteristics to assist in pre-screening for *POLE* exonuclease domain mutations in EC, facilitating the use of this important prognostic biomarker in routine pathology. Moreover, as some of these traits have also been described in other tumor types carrying *POLE* exonuclease domain mutations, the proposed combination of characteristics to aid the detection of these *POLE* mutations may be generalizable to other cancers.

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SUPPLEMENTARY METHODS

Immunohistochemical analyses

Slides (4µm) from formalin-fixed paraffin-embedded tissue blocks were deparaffinized in xylene, and rehydrated in a graded ethanol series. Endogenous peroxidases were inactivated in 0.3% H₂O₂/methanol. Antigen retrieval was performed by microwave oven treatment in 10 mmol/L Tris-EDTA pH 9.0 (p53, MLH1, MSH2, MSH6, PMS2) or in 10 mmol/L citrate buffer pH 6.0 (p16). Slides were incubated overnight with primary monoclonal antibodies against p53 (clone DO-7, 1:2000, NeoMarkers), MLH1 (clone ES05, 1:100, DAKO), MSH2 (clone FE11, 1:200, DAKO), MSH6 (clone EPR3945, 1:800, Genetex), p16 (clone E6H4, 1:50, Roche) at room temperature and against PMS2 (clone EP51, 1:75, DAKO) at 4°C. For the mismatch repair proteins, sections were incubated for 15 minutes with Envision FLEX+ Linker Rabbit (DAKO, K8009; MSH6, PMS2) or Mouse (DAKO, K8021; MLH1, MSH2), followed by incubation with the secondary antibody. For all antibodies, BrightVision Poly-HRP-GAM/R/R (DPV0110HRP, ImmunoLogic) was used as secondary antibody (30 minutes incubation). A selected number of cases was automatically stained with beta-hCG (A0231, 1:10000, DAKO) or with cytokeratin (AE1/AE3, 1:100, DAKO) using Dako Autostainer Link 48 (Dako, Glostrup, Denmark). Slides were counterstained with hematoxylin, dehydrated and mounted.

All immunohistochemical stainings were evaluated by two observers (I.G., E.S.) independently, blinded for patient and tumor characteristics. Discrepancies were resolved under a multihead microscope. P53 was scored mutant-like when >50% of the tumor cells showed strong positive nuclear staining, or when a discrete geographical area showed >50% tumor cell positivity (diffuse and subclonal mutant-like expression respectively).^{30,31} If p53 was evaluated as 'indefinite', i.e. no p53 staining of the tumor, Sanger sequencing was performed (we acknowledge that with this method larger deletions may be missed). Expression of MLH1, MSH2, MSH6, PMS2 was classified as wild-type when the tumor cells showed nuclear protein expression, and as (subclonal) loss when (discrete geographical sheets of) tumor cells were negative but stromal cells and/or immune infiltrate (internal controls) showed positive nuclear staining. P16 was scored as negative when <10% of tumor cells showed nuclear and/or cytoplasmic staining, focal/patchy when 10-30% of tumor cells were positive, and diffuse in case of nuclear and/or cytoplasmic expression in >30% of tumor cells.³²

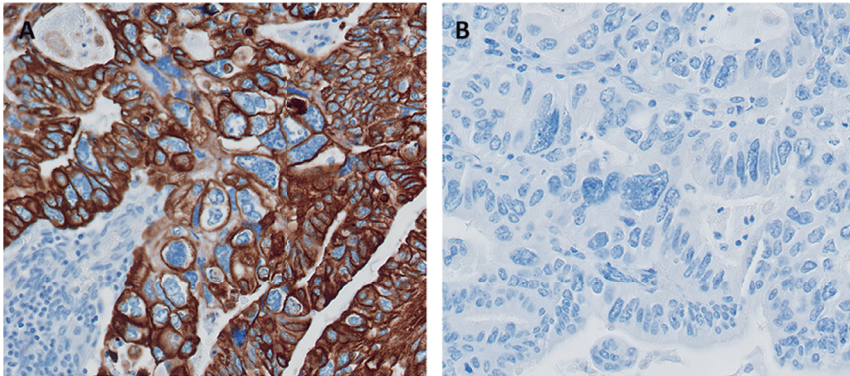
DNA analyses

The prognostic molecular subgroups were assessed as described previously.^{1,5} In brief, tumor DNA was isolated from formalin-fixed paraffin-embedded tissue blocks by using a tissue microarrayer (Beecher Instruments). *POLE* exonuclease domain mutations were detected by Sanger sequencing of exons 9, 13, and 14 (primer details in Supplementary

Table 1). Microsatellite instability (MSI) was determined using the Promega MSI analysis system (version 1.2). Tumors with instability in two or more markers were defined as being microsatellite-unstable, whereas those with instability in one marker or less were classified as microsatellite-stable (MSS). *TP53* sequencing of exons 5-8 was performed for those cases in which p53 was scored indefinite in immunohistochemistry.

Statistical analyses

Continuous variables were compared using the Kruskal-Wallis and Mann-Whitney U tests (with Bonferroni correction for multiple comparisons). Associations between categorical variables were tested using χ^2 statistics or Fisher's exact test. Logic regression was performed on all morphological and immunohistochemical features to identify a combination of characteristics that best predict *POLE* exonuclease domain mutational status using the R statistical software package LogicReg version 1.5.9.^{33,34} For this analysis, morphological characteristics solid growth, invasion type and lymphovascular space were made binary (solid growth: <5% vs. $\geq$ 5%; invasion type: broad front vs. other; lymphovascular space invasion: absent or focal vs. substantial). All included endometrial cancers were randomly split into a training (N=88) and test set (N=45). Leave-one-out cross-validation was performed on the training set to determine the optimal size of the logic model (number of leaves with the lowest misclassification rate). This number and a simulated annealing search algorithm were used to find the best Boolean combination of characteristics. The performance of the identified model was measured using sensitivity and specificity. All reported *P* values were two-sided with a *P* value of <0.05 indicating statistical significance. Statistical tests were performed using IBM SPSS Statistics software (version 23) and R (R Core Team (2013) – <http://R-project.org/>).



Supplementary Figure 1. Immunohistochemistry for keratin and β -hCG on a *POLE*-mutant endometrial cancer.

The tumor giant cells are keratin-positive, indicating an epithelial origin (A) and β -hCG-negative, suggesting they are not syncytiotrophoblastic cells (B, both magnification: x20).

Supplementary Table 1. PCR primer details of *POLE* exon 9, 13 and 14 Sanger sequencing.

Target (residues)	Primer name	Primer sequence	Primer used for sequencing
Exon 9	<i>POLE</i> -Ex9Fw	5'– tgcttatTTgtccccacag – 3'	Forward
(268–303)	<i>POLE</i> -Ex9Rv	5'– tacttcccagaagccacctg – 3'	Reverse
Exon 13	<i>POLE</i> -Ex13Fw	5'– tctgttctcattctccttcag – 3'	Forward
(410–453)	<i>POLE</i> -Ex13Rv	5'– cgggatgtggcttacgtg – 3'	Reverse
Exon 14	<i>POLE</i> -Ex14Fw	5'– tctggcgttctctcctcag – 3'	Forward
(454–491)	<i>POLE</i> -Ex14Rv	5'– cgacaggacagataatgctcac – 3'	Reverse

Supplementary Table 2. *POLE* exonuclease domain mutations identified in the study cohort.

<i>POLE</i> exonuclease domain mutation	Frequency N=51, n (%)
P286R	31 (60.8)
V411L	17 (33.3)
S297F	1 (2.0)
A456P	2 (3.9)

Supplementary Table 3. Invasion type, presence of eosinophilic metaplasia and morule in *POLE*-mutant endometrial cancers compared to endometrial cancers of other molecular/histopathological subtypes.

	<i>POLE</i> -mutant* N=51, n (%)	<i>POLE</i> -wild-type endometrioid* N=67, n (%)	<i>POLE</i> -wild-type serous* N=15, n (%)	<i>P</i> value <i>POLE</i> vs. endometrioid [‡]	<i>P</i> value <i>POLE</i> vs. serous [‡]
Invasion type				0.325	0.162
No infiltration	0 (0.0)	1 (1.5)	0 (0.0)	1	NA
Broad front invasion	26 (51.0)	27 (40.3)	3 (20.0)	0.248	0.042
Infiltrating glands	20 (39.2)	36 (53.7)	10 (66.7)	0.118	0.080
MELF	4 (7.8)	3 (4.5)	2 (13.3)	0.464	0.612
Adenomyosis-like	1 (2.0)	0 (0.0)	0 (0.0)	0.432	1
Eosinophilic metaplasia					
Absent	49 (96.1)	65 (97.0)	15 (100)	1	1
Present	2 (3.9)	2 (3.0)	0 (0.0)		
Morules					
Absent	51 (100)	64 (95.5)	15 (100)	0.257	NA
Present	0 (0.0)	3 (4.5)	0 (0.0)		

*For the morphological characteristics, the number and percentage of cases in each category are shown for the included molecular/histopathological subgroups. The included subgroups are: *POLE*-mutant, *POLE*-wild-type endometrioid (microsatellite-unstable, p53-mutant or with no specific molecular profile), and *POLE*-wild-type serous endometrial cancer.

[‡]*P* values are calculated over *POLE*-mutant and *POLE*-wild-type endometrioid (*POLE* vs. endometrioid) or over *POLE*-mutant and *POLE*-wild-type serous (*POLE* vs. serous) endometrial cancers, respectively.

Abbreviations: MELF – microcystic elongated and fragmented, NA – not applicable.

Supplementary Table 4. Mismatch repair protein expression based on immunohistochemistry in the molecular and histopathological endometrial cancer subgroups.

	<i>POLE</i> -mutant N=51, n (%)	<i>POLE</i> -wild-type microsatellite- unstable N=35, n (%)	<i>POLE</i> -wild-type microsatellite- stable endometrioid N=32, n (%)	<i>POLE</i> -wild-type microsatellite- stable serous N=15, n (%)
MLH1				
Wild-type	47 (92.2)	4 (11.4)	31 (96.9)	15 (100)
Subclonal loss	3 (5.9)	1 (2.9)	1 (3.1)	0 (0.0)
Loss	1 (2.0)	30 (85.7)	0 (0.0)	0 (0.0)
PMS2				
Wild-type	46 (90.2)	4 (11.4)	31 (96.9)	15 (100)
Subclonal loss	2 (3.9)	2 (5.7)	1 (3.1)	0 (0.0)
Loss	3 (5.9)	29 (82.9)	0 (0.0)	0 (0.0)
MSH2				
Wild-type	51 (100)	30 (85.7)	32 (100)	15 (100)
Subclonal loss	0 (0.0)	1 (2.9)	0 (0.0)	0 (0.0)
Loss	0 (0.0)	4 (11.4)	0 (0.0)	0 (0.0)
MSH6				
Wild-type	51 (100)	29 (82.9)	32 (100)	15 (100)
Subclonal loss	0 (0.0)	2 (5.7)	0 (0.0)	0 (0.0)
Loss	0 (0.0)	4 (11.4)	0 (0.0)	0 (0.0)
MMR status				
Wild-type	46 (90.2)	1 (2.9)*	31 (96.9)	15 (100)
MLH1 & PMS2 (subclonal) loss	5 (9.8)	30 (85.7)	1 (3.1) [‡]	0 (0.0)
MSH2 & MSH6 (subclonal) loss	0 (0.0)	4 (11.4)	0 (0.0)	0 (0.0)

*This endometrial cancer with positive expression in immunohistochemistry of the mismatch repair proteins is considered microsatellite-unstable based on the presence of an MSI-high phenotype (Promega MSI analysis system).

[‡]Microsatellite instability assay (Promega MSI analysis system) showed a microsatellite-stable phenotype in areas with and without MLH1 protein expression, resulting in the classification of this case as p53-mutant, microsatellite-stable.



Chapter 4

POLE proofreading mutations elicit an antitumor immune response in endometrial cancer

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ABSTRACT

Objective

Recent studies have shown that 7 to 12% of endometrial cancers are ultramutated due to somatic mutation in the proofreading exonuclease domain of the DNA replicase *POLE*. Interestingly, these tumors have an excellent prognosis. In view of the emerging data linking mutation burden, immune response and clinical outcome in cancer, we investigated whether *POLE*-mutant endometrial cancers showed evidence of increased immunogenicity.

Methods

We examined immune infiltration and activation according to tumor *POLE* proofreading mutation in a molecularly defined endometrial cancer cohort including 47 *POLE*-mutant tumors. We sought to confirm our results by analysis of RNAseq data from the TCGA endometrial cancer series and used the same series to examine whether differences in immune infiltration could be explained by an enrichment of immunogenic neoepitopes in *POLE*-mutant endometrial cancers.

Results

Compared to other endometrial cancers, *POLE* mutants displayed an enhanced cytotoxic T cell response, evidenced by increased numbers of CD8⁺ tumor-infiltrating lymphocytes and CD8A expression, enrichment for a tumor-infiltrating T cell gene signature, and strong upregulation of the T cell cytotoxic differentiation and effector markers T-bet, Eomes, IFNG, PRF and granzyme B. This was accompanied by upregulation of T cell exhaustion markers, consistent with chronic antigen exposure. *In silico* analysis confirmed that *POLE*-mutant cancers are predicted to display more antigenic neoepitopes than other endometrial cancers, providing a potential explanation for our findings.

Conclusions

Ultramutated *POLE* proofreading-mutant endometrial cancers are characterized by a robust intratumoral T cell response, which correlates with, and may be caused by an enrichment of antigenic neopeptides. Our study provides a plausible mechanism for the excellent prognosis of these cancers.

INTRODUCTION

Endometrial cancer is the commonest gynecological malignancy in the Western world, and affects approximately 150,000 women each year in Europe and the US combined.¹ Endometrial cancers have traditionally been classified into endometrioid and non-endometrioid tumors according to clinical and histopathological criteria. However, recent work has shown that this dualistic model can be improved upon by a molecular classification into subgroups that more accurately reflect underlying tumor biology and clinical outcome.^{2,3}

One interesting subgroup is the 7-12% of endometrial cancers with somatic mutation in the proofreading exonuclease domain of the DNA replicase *POLE*.^{2,4-6} Polymerase proofreading is essential for ensuring fidelity of DNA replication,⁷ and in keeping with its dysfunction, *POLE* proofreading-mutant cancers have a frequency of base substitution mutation among the highest in human cancer.^{2,8,9} *POLE*-mutant endometrial cancers display other distinctive features, including a characteristic mutation signature, with a preponderance of C→A transversions and bias for particular amino acid substitutions, and strong associations with endometrioid histology, high grade, and microsatellite stability (MSS).^{2,4-6,10} We and others have recently shown that, despite the association with high grade, *POLE*-mutant endometrial cancers have an excellent prognosis.^{5,6,11} However, the reasons for this were unclear.

Although the ability of the immune system to suppress malignant disease has long been recognized,¹² the last few years have seen a remarkable increase in our understanding of the complex and dynamic interplay between cancers and the host immune response. For example, preclinical and translational studies have confirmed that tumor missense mutations can lead to presentation of antigenic neoepitopes by MHC class I molecules, resulting in activation of T cell-mediated cytotoxicity.¹³⁻¹⁶ Consequently, mutations that cause strongly antigenic epitopes are likely to undergo negative selection in developing tumors.^{13,14} Cancers also demonstrate multiple alternative mechanisms of immune escape, including downregulation of HLA class I expression, and upregulation of immunosuppressive molecules including PD-1/PD-L1, TIM3, LAG3 and TIGIT in a phenomenon referred to as adaptive immune resistance.^{17,18} Despite this, it is clear that in many cancers, the immune system retains a degree of control over tumor growth – illustrated by the association between increased density of tumor-infiltrating lymphocytes (TILs), particularly CD8⁺ cytotoxic T cells, and favorable outcome in multiple cancer types, including endometrial cancer.¹⁹⁻²² Interestingly, a recent study has shown that CD8⁺ cell infiltration correlates strongly with the number of predicted antigenic mutations in tumors,²³ suggesting that the immunogenicity of cancers is determined at least partly by their mutational

burden.²⁴ These data are consistent with the observations that hypermutated, microsatellite-unstable (MSI) endometrial and colorectal cancers typically display greater TIL density than other tumors.^{25,26}

During our previous studies,^{4,6} we noted that *POLE*-mutant endometrial cancers frequently displayed strikingly high TIL density, often accompanied by a Crohn-like reaction. Similar observations have recently been reported following pathological review of *POLE*-mutant TCGA endometrial cancers.²⁷ We hypothesized that this may represent infiltration by cytotoxic T lymphocytes, which could in turn contribute to the favorable prognosis of these tumors. We also speculated that this might relate to an increase in antigenic neoepitopes in *POLE*-mutant endometrial cancers secondary to ultramutation. We tested this using a molecularly defined cohort of endometrial cancers, including 47 *POLE*-mutant tumors, and the recently published TCGA series.²

MATERIALS AND METHODS

Patients and tumors

Tumors were selected from the PORTEC-1 and PORTEC-2 studies (n=57^{28,29}) and endometrial cancer series from the Leiden University Medical Center (LUMC, Leiden, the Netherlands; n=67) and the University Medical Center Groningen (UMCG, Groningen, the Netherlands; n=26), to provide similar numbers of low- (grade 1/2) and high-grade (grade 3) tumors of the common molecular subtypes: *POLE*-wild-type, microsatellite-stable (MSS); *POLE*-wild-type, MSI; and *POLE* (proofreading)-mutant (Supplementary Table 1). All cases were of endometrioid histology (EEC), and all *POLE*-mutant tumors were MSS. Of 373 cases reported in the TCGA series, 245 had paired whole exome and RNAseq data and were informative for this analysis.² Of these, 197 were EECs, four mixed EEC/non-endometrioid endometrial cancers (NEECs), and 44 serous (NEEC) cancers. Ethical approval for tumor molecular analysis was granted at the LUMC, UMCG, and by Oxfordshire Research Ethics Committee B (Approval No. 05\Q1605\66).

POLE mutation and microsatellite instability status

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) blocks and sequencing of *POLE* hot spot exons 9 and 13 (which contain around 90% of pathogenic proofreading mutations) was performed in tumors from the PORTEC, LUMC, and UMCG series as previously reported.^{4,5} All mutations were confirmed in at least duplicate PCR reactions. Details of whole exome sequencing performed by the TCGA have been previously reported.² Pathogenic *POLE* proofreading mutations were defined as somatic variants within the exonuclease domain associated with ultramutation and a frequency of C→A

transversions of $\geq 20\%$.¹⁰

MSI status was determined in the PORTEC, LUMC, and UMCG cases by a five-marker panel of microsatellites as reported previously,³⁰ with exception of four cases in which this failed, where status was determined by MLH1, MSH2, MSH6, and PMS2 immunohistochemistry (IHC).⁶ Determination of MSI in the TCGA series was by a seven-marker panel, with MSI-High defined as alteration at three or more markers.² Classification of *POLE*-mutant TCGA endometrial cancers was also informed by results of recent analysis of mononucleotide repeats in 48 genes.¹⁰ In our analysis, only MSI-high cases were classified as MSI - we excluded one TCGA case for which MSI status could not be determined. The single tumor that displayed both *POLE* proofreading mutation and MSI by these criteria was assigned to the *POLE*-mutant group on the basis of its characteristic mutation signature, in accordance with a recent report.¹⁰

Histological assessment

Tumors were evaluated for the presence/absence of TILs and for Crohn-like reaction by a gynecological pathologist (T.B.) blinded to other clinicopathological data.

IHC and cell quantification

Following deparaffinization, antigen retrieval, and blocking of peroxidase activity, whole slides were incubated overnight at 4°C (CD8, CD3) or room temperature (TIA-1, HLA, FoxP3) with primary antibodies against CD8 (1:50, clone C8/144B, DAKO, Agilent technologies), CD3 (1:25, clone F7.2.38, DAKO), HCA2 and HC10 (both 1:800, kindly provided by Prof. Dr. J. Neefjes, the Netherlands Cancer Institute), FoxP3 (1:100, mAbcam 450, Abcam), and TIA-1 (1:400, clone 2G9A10F5, Beckman Coulter). Sections were subsequently incubated with either anti-mouse Envision+ reagent (K4000, DAKO) for 30 minutes (CD8 primary), RAMpo (1:100) and GARpo (1:100) secondary and tertiary antibodies (CD3 primary), or BrightVision-Poly/HRP (Poly-HRP-GAM/R/R; DPV0110HRP; ImmunoLogic; HCA2, HC10, FoxP3, and TIA-1) before 3,3-diaminobenzidine (DAB) treatment and hematoxylin counterstaining. Slides were then dehydrated and mounted before digitalization (ScanScope, Aperio Technologies, or Ultra Fast Scanner 1.6 RA. Philips) and analysis.

CD8⁺, CD3⁺, FOXP3⁺, and TIA-1⁺ cell numbers were quantified in intraepithelial and intrastromal regions in the center of the tumor (CT) and the invasive margin (IM) as previously reported.^{19,22} For each region, the mean number of positive cells in eight high-power fields (HPF; 200µm x 200µm) was calculated. For analysis of HLA expression, the percentage of tumor cells with membranous HCA2 and HC10 staining was quantified as previously described.³¹ In each case, scoring was performed independently by two observers, blinded to other clinicopathological data.

Immunofluorescence

Following deparaffinization, antigen retrieval, and blocking of peroxidase activity, whole slides were stained overnight at 4°C with primary antibody against TIA-1 (1:50, ab2712, Abcam). Sections were subsequently incubated with anti-mouse Envision+ reagent (K4000, DAKO) for 30 minutes and HRP visualized using cyanine 5 tyramide signal amplification (TSA) according to the manufacturer's instructions (PerkinElmer). Next, whole slides were stained overnight with primary antibody against CD8 (1:25, clone C8/144B, DAKO) and a biotinylated antibody against fibronectin (1:50, ab6584, abcam). Slides were incubated with goat anti-mouse AF555 (1:150 Life Technologies) and streptavidin-dylight488 (1:150, Thermo Scientific), counterstained with DAPI (Life Technologies) and mounted in prolong gold mounting medium (Life Technologies). Immunofluorescent slides were scanned using a TissueFAXs imaging system (TissueGnostics). Processed channels were merged using Adobe Photoshop CS5 (Adobe).

Leukocyte methylation scores

Leukocyte methylation scores (syn1809223^{32,33}) were downloaded from Synapse (<https://www.synapse.org/>) and annotated according to MSI and *POLE* status.

TCGA RNAseq data

Details of the TCGA RNA sequencing analysis have been previously reported.² RSEM normalized³⁴ and raw RNAseq count data were downloaded from FireBrowse (http://firebrowse.org/?cohort=UCEC&download_dialog=true) accessed November 11, 2014. After removal of normal tissue controls and technical duplicates, 245 samples with RSEM normalized and 231 samples with raw count data were informative for analysis.

Gene set enrichment analysis

TCGA raw counts were annotated by molecular subtype before normalization and ranking of genes differentially expressed between *POLE*-mutant (n=16) and other (n=205) endometrial cancers using DESeq.³⁵ Gene set enrichment analysis (GSEA³⁶) was then performed with the PreRanking setting, using GO Biological Processes and C7 Immunologic Signatures sets from the Molecular Signatures Database (MSigDB; <http://www.broadinstitute.org/gsea/msigdb/genesets.jsp?collection=BP>), and a published 200-gene T cell tumor infiltration gene signature.¹⁸

Prediction of antigenic neoepitopes

We created an algorithm to estimate the immunogenicity of individual tumors taking into account the following considerations: (i) to generate a functional neoepitope, a missense mutation must be expressed; (ii) most functional neoepitopes identified to date are predicted to bind MHC class I molecules (IC₅₀<500 nmol/L) by NetMHCPan;^{23,37,38} and (iii)

the likelihood that a neoepitope is antigenic is reduced if the corresponding wild-type peptide also binds the MHC with similar affinity as T cells to the epitope may be centrally deleted or tolerized.³⁹ Our strategy was similar to others reported recently.^{15,23,38,40} For each tumor, we calculated all possible 9mers for every missense mutation in expressed genes (defined as nonzero reads from RNAseq) and calculated the binding affinity of the mutant and corresponding wild-type peptide for HLA-A*02:01 (as a single model example HLA allele) using NetMHCPan 2.8.³⁷ In the event that several peptides had an $IC_{50} < 500$ nmol/L, the strongest binder was used for analysis. We defined antigenic mutations as neoepitopes predicted to bind MHC molecules ($IC_{50} < 500$ nmol/L) for which the corresponding wild-type peptide was not predicted to bind MHC ($IC_{50} > 500$ nmol/L).

Statistical analyses

We used the nonparametric Mann–Whitney U test for all comparisons of continuous data and Spearman rho to analyze correlation between variables. Categorical variables were compared using the Fisher exact test. All statistical tests were two sided, with a *P* value of < 0.05 taken to indicate significance. Except where indicated, statistical tests were unadjusted. Statistical analyses were performed using STATA and Prism 6.0 (GraphPad).

RESULTS

***POLE* proofreading-mutant endometrial cancers show increased lymphocytic infiltrate**

Preliminary analysis of hematoxylin and eosin-stained sections suggested that *POLE* proofreading-mutant endometrial cancers frequently displayed a prominent lymphocytic infiltrate and Crohn-like lymphocytic reaction (Supplementary Figure 1A–B). Formal quantification of this in a set of 150 endometrial cancers comprising approximately equal numbers of *POLE* proofreading-mutant/microsatellite stable (*POLE*-mutant), *POLE*-wild-type/MSI, and *POLE*-wild-type/MSS subtypes of low and high grade (Supplementary Table 1), confirmed that TILs were more frequent in *POLE*-mutant (22/47) than in both MSS (8/54; $P = 0.0009$, Fisher exact test), and MSI (10/49; $P = 0.009$) subtypes (Supplementary Figure 1C). Crohn-like reaction was also significantly more common in *POLE*-mutant than other tumors ($P < 0.001$ both comparisons; Supplementary Figure 1D).

Increased density of intratumoral CD8⁺ lymphocytes in *POLE*-mutant endometrial cancers

Mindful of the relationship between cytotoxic T cell infiltrate and favorable cancer outcome,^{19–22} and the excellent prognosis of *POLE*-mutant endometrial cancers,^{5,6,11} we next examined whether *POLE* mutants showed evidence of increased T cell infiltrate in

our endometrial cancer cohort. While as anticipated,²⁵ CD8⁺ cell numbers in intraepithelial and intrastromal compartments in the CT and the IM were higher in MSI than MSS endometrial cancers ($P < 0.0001$ for all comparisons, Mann–Whitney U test), in *POLE*-mutant tumors, the density of CD8⁺ infiltrate was frequently striking (Figure 1A), and significantly exceeded that of both MSS ($P < 0.0001$ for all four regions) and MSI cancers in the CT (median 5.9 vs. 2.6 intraepithelial CD8⁺ cells per HPF, $P = 0.001$; 26.0 vs. 13.5 intrastromal CD8⁺ cells, $P = 0.002$; Figure 1B). Furthermore, the proportion of tumors with numbers of CD8⁺ cells exceeding the median in all four regions was substantially higher in *POLE*-mutant (60.0%) than MSI (31.3%, $P = 0.007$, Fisher exact test) and MSS tumors (7.2%, $P < 0.0001$). Staining for CD3 and the cytolytic marker TIA-1 in a subset of cases confirmed increased T cell density in *POLE*-mutant tumors (Supplementary Figure 2A–B) and suggested that the infiltrate contained lymphocytes capable of cytotoxic activity (Supplementary Figure 3A–B). Co-immunofluorescence confirmed co-expression of TIA-1 in the CD8⁺ lymphocytes comprising the *POLE*-mutant tumor infiltrate (Figure 2A–F), further supporting the conclusion that these cells were capable of mediating an antitumor effect.

Interestingly, in light of the correlation previously reported between B cell and T cell subsets at the IM,⁴¹ we found that dense CD20⁺ stromal infiltrate in this region was more common in *POLE* mutants (Supplementary Figure 4A), while a tendency to increased numbers of FOXP3⁺ cells in both MSI and *POLE*-mutant tumors (Supplementary Figure 4B) was also notable, given that this has been associated with favorable cancer prognosis in some studies.⁴¹

Cytotoxic T cell infiltration and activation in *POLE*-mutant endometrial cancers in TCGA series

We sought to confirm our results using the TCGA endometrial cancer series,² in which the improved clinical outcome of *POLE*-mutant tumors was first suggested (Figure 3A). Of 244 informative tumors in this study, 157 were MSS, 69 MSI, and 18 *POLE* proofreading-mutant (the single tumor with both *POLE* proofreading mutation and MSI was categorized as *POLE*-mutant according to its mutation spectrum, in keeping with a recent report¹⁰). Forty-three of the MSS tumors were NEECs, while all MSI and *POLE*-mutant endometrial cancers cases were EECs (Supplementary Table 1).

We first examined leukocyte methylation scores, which estimate the proportion of a heterogeneous tumor sample that consists of leukocytes.^{32,33} After confirming that scores correlated strongly with CD8A expression ($\rho = 0.65$, $P < 0.0001$), we noted that, following exclusion of MSI and *POLE*-mutant tumors, both leukocyte methylation scores and CD8A expression did not differ between EECs and NEECs ($P = 0.52$ and $P = 0.16$ respectively,

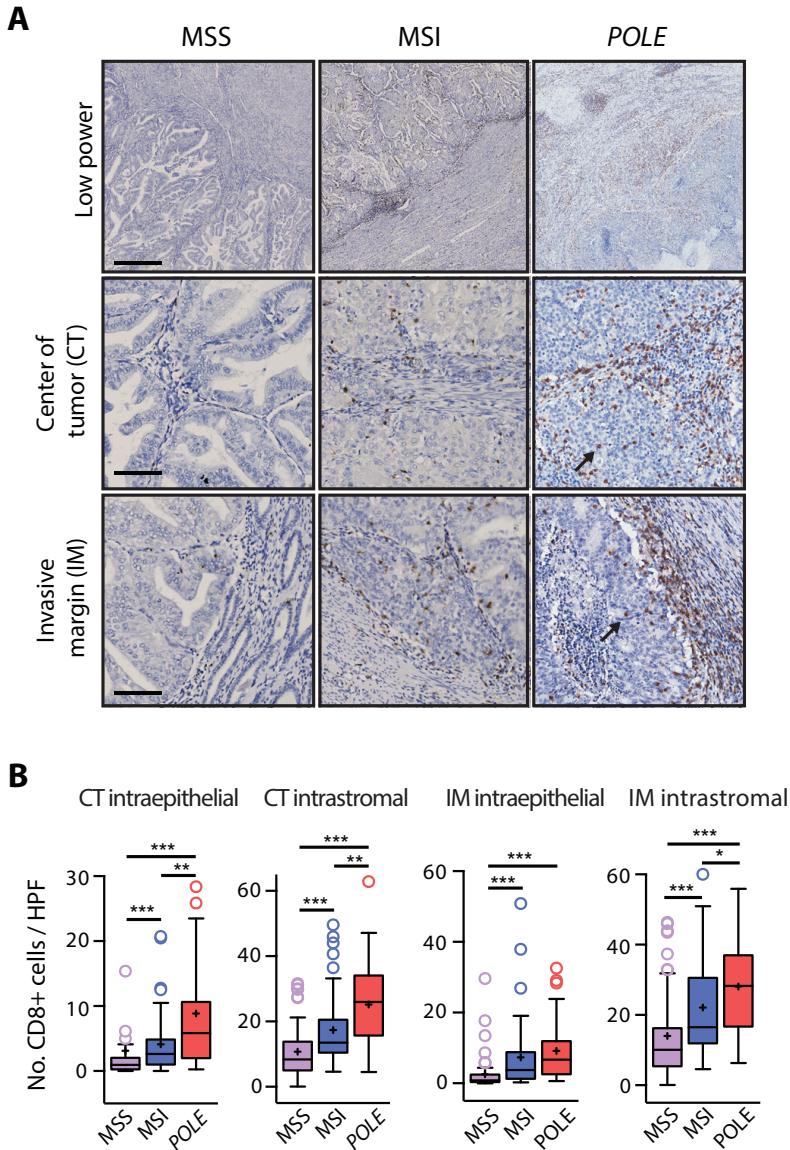


Figure 1. Increased CD8⁺ lymphocyte infiltration in *POLE*-mutant endometrial cancers.

(A) Results of CD8 IHC by endometrial cancer molecular subtype shown at low magnification (top) and high power views of the center of the tumor (CT) and invasive margin (IM). Arrows highlight intraepithelial CD8⁺ cells in *POLE*-mutant tumor. Scale bars, 500 μ m (upper panel in A) and 100 μ m (middle and bottom panels in A). (B) Quantification of CD8⁺ cell number in intraepithelial and intrastromal compartments in the CT and IM by endometrial cancer molecular subtype. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile plus 1.5xIQR, and the lower whisker the 25th percentile minus 1.5xIQR. The median and mean values are indicated by a horizontal line and cross, respectively. Statistical comparison between groups was made by the unadjusted two-sided Mann–Whitney U test, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

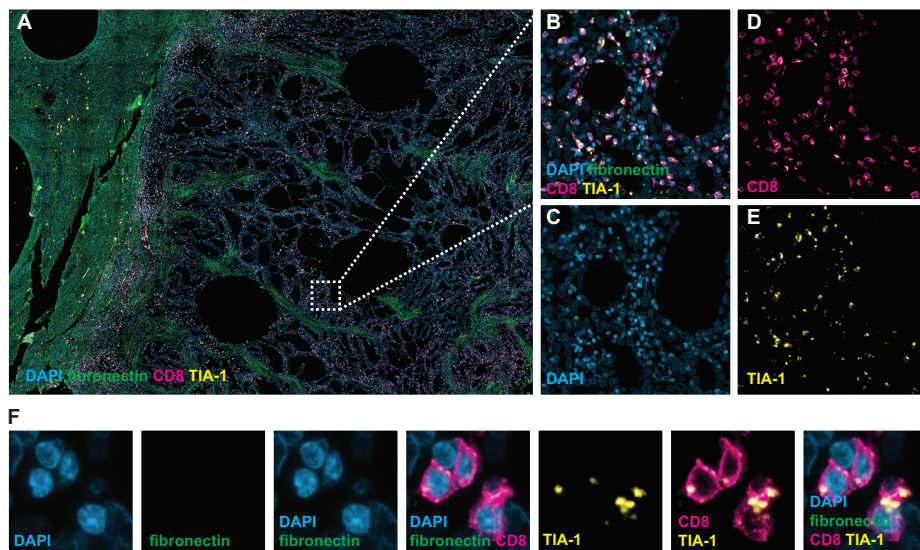


Figure 2. CD8⁺ TILs in *POLE*-mutant tumors show cytolytic potential.

(A) Low-magnification image of *POLE*-mutant tumor following co-immunofluorescence (co-IF) staining for DAPI (nuclei), fibronectin (extracellular matrix), CD8, and the cytolytic marker TIA-1. (B-F) Co-IF images of the tumor center following staining for all markers (B), DAPI (C), CD8 (D), and TIA-1 (E) confirming co-expression of TIA-1 by tumor-infiltrating CD8⁺ lymphocytes, also shown at high magnification (F).

Mann–Whitney U test). We therefore included tumors of both histologies in the MSS cohort in all subsequent analyses. Leukocyte methylation scores were similar in MSI and MSS tumors (median 15.5% vs. 14.2%, $P=0.2$), in contrast with a significant increase in *POLE* mutants (median 23.3%; $P=0.006$ vs. MSS, $P=0.07$ vs. MSI; Figure 3B), concordant with our previous results. Given the biological differences between NEECs and EECs, we formally confirmed that these results were essentially unaltered following exclusion of the former from the MSS group (median 14.7%, $P=0.008$ vs. *POLE*-mutant endometrial cancers).

We proceeded to explore whether infiltration of *POLE*-mutant endometrial cancers by cytotoxic T cells was manifest as immune expression signatures and/or increased expression of key immunological genes. Agnostic pathway analysis of TCGA RNAseq data by GSEA demonstrated significant enrichment of immune-related pathways in *POLE*-mutant endometrial cancers compared with other tumors, including Immune Response (normalized enrichment score (NES) 4.12 FDR $q<0.0001$) and Immune System Process (NES 3.77, $q<0.0001$). GSEA also confirmed that *POLE*-mutant cancers showed striking enrichment of a recently reported, highly specific 200-gene signature corresponding to tumor T cell infiltration (Figure 3C).¹⁸

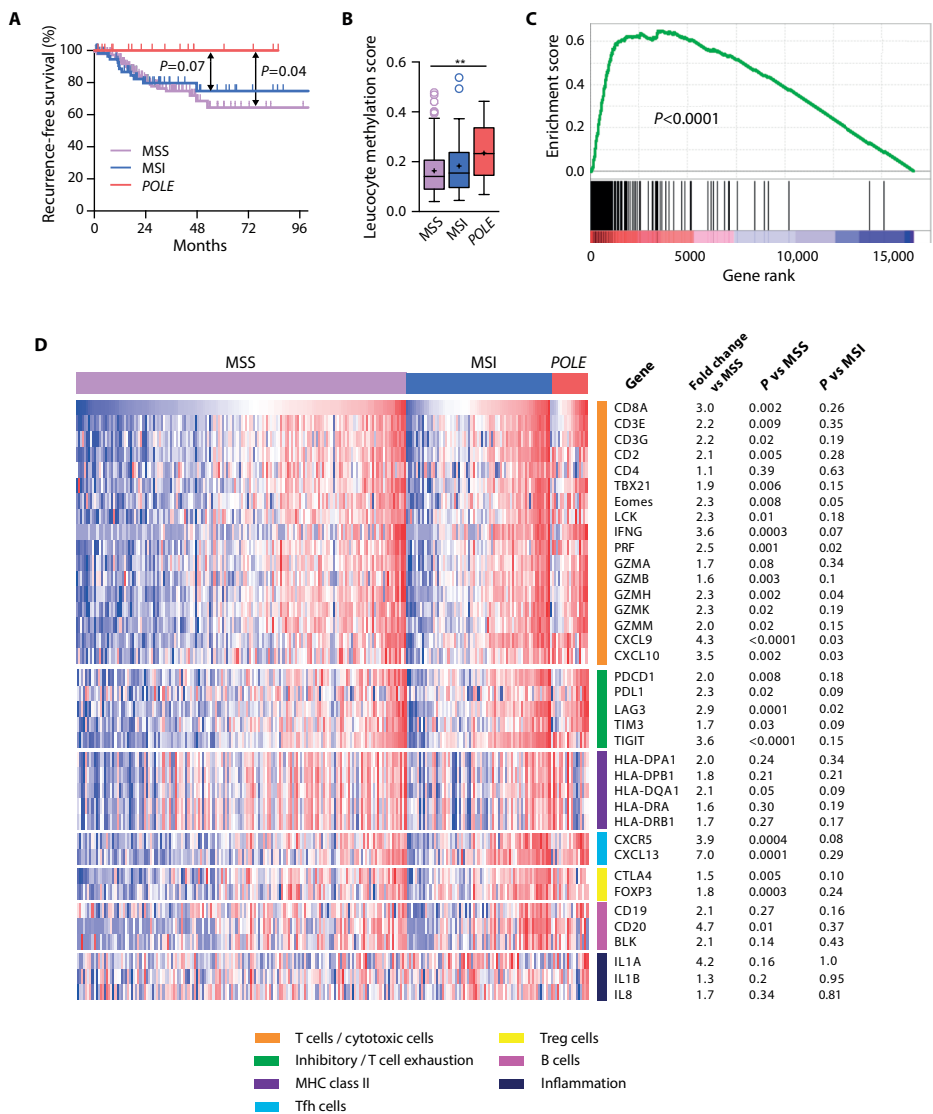


Figure 3. Clinical outcome and T cell response according to tumor molecular subtype in TCGA endometrial cancers.

(A) Kaplan–Meier curves demonstrating recurrence-free survival of *POLE* wild-type, MSS (N=147), MSI (N=63), and *POLE* proofreading domain-mutant (*POLE*, N=18) endometrial cancers in the TCGA series (note that survival data were not available for all cases). (B) Comparison between subgroups was made by the two-sided log-rank test. Leukocyte methylation scores according to endometrial cancer molecular subtype. Unadjusted comparison between groups was made by the two-sided Mann–Whitney U test. (C) GSEA of 200-gene tumor-associated T-cell signature¹⁸ in *POLE*-mutant endometrial cancers compared with other tumors. Raw RNAseq counts were normalized and ranked using DESeq before GSEA analysis with PreRanking. (D) Heatmap showing expression of immunologic genes according to endometrial cancer molecular subtype. RSEM-normalized RNAseq expression data were log₂ transformed, zero centered and assigned unit variance. For each gene, the mean fold change in expression in *POLE* mutants relative to MSS endome-

trial cancers was calculated and expression compared between *POLE* mutants, MSS, and MSI endometrial cancers by the unadjusted, two-sided Mann–Whitney U test; ** $P < 0.01$.

Focused analysis of genes involved in T cell-mediated cytotoxicity confirmed that, compared with MSS tumors, MSI endometrial cancers had higher expression of CD8A (2.1-fold, $P = 0.0005$) and IFN γ (2.1 fold, $P = 0.0006$) though expression of the cytotoxic differentiation and activation markers T-bet (TBX21), Eomes, perforin, and granzymes B, H, K and M was either essentially unchanged (≤ 1.1 -fold) or not significantly increased. In contrast, and once again consistent with our previous results, *POLE* mutants demonstrated substantial upregulation of CD8A (3.0-fold vs. all MSS tumors, $P = 0.002$; 3.2-fold vs. MSS EECs only, $P = 0.004$), accompanied by significant increases in T-bet (1.9-fold, $P = 0.006$), Eomes (2.3-fold, $P = 0.008$), IFNG (3.6-fold, $P = 0.0003$), PRF (2.5-fold, $P = 0.001$), granzymes B, H, K, and M (1.6- to 2.3-fold, $P = 0.002$ – 0.02), and the IFN γ -induced cytokines CXCL9 (4.3-fold, $P < 0.0001$) and CXCL10 (3.5-fold, $P = 0.002$; Figure 3D and Supplementary Figure 5). Upregulation of most of these genes in tumors has been shown to predict good prognosis.^{19,41} *POLE* mutants also demonstrated striking upregulation of the T follicular helper genes CXCL13 (7.0-fold, $P = 0.0001$) and CXCR5 (3.9-fold, $P = 0.0004$), which have recently been shown to strongly predict favorable outcome in colorectal cancer.⁴¹ Notably, despite limited numbers, in several cases, expression of cytotoxic markers and cytokines exemplifying effector status in *POLE* mutants significantly exceeded that in MSI tumors (e.g., PRF, $P = 0.02$; GZMH $P = 0.04$; CXCL9/10 both $P = 0.03$; Figure 3D and Supplementary Figure 5). Collectively, these data not only corroborated our previous finding that *POLE*-mutant tumors had greater T lymphocyte infiltration than other endometrial cancers, but also strengthened the conclusion that these lymphocytes were capable of exerting antitumor activity.

Mechanisms of immune escape in *POLE*-mutant endometrial cancers

POLE-mutant cancers have significantly better prognosis than other endometrial cancers,^{5,6,11} as evidenced by the absence of recurrences in the TCGA series.² However, the presentation of all patients in this study with clinically detectable tumors, in some cases with lymphatic spread, indicates that any immune response was, at best, only partially successful in suppressing *POLE*-mutant endometrial cancer growth. We therefore explored potential mechanisms of immune escape in these tumors.

We first considered the possibility that *POLE*-mutant endometrial cancers may escape from immune surveillance by loss or inactivation of components required for antigen presentation. Although 31.8% of *POLE*-mutant endometrial cancers in our study set of 150 endometrial cancers showed loss of HLA class I protein expression by IHC, this was not significantly different to that observed in MSI (28.6%, $P = 0.82$, Fisher exact test)

or MSS tumors (20.0%, $P=0.24$) and was not reflected in increased CD8⁺ cell numbers. Similarly, although we found a tendency to overrepresentation of *POLE* mutants among TCGA endometrial cancers with HLA class I gene expression in the lowest quartile, this was also not significantly different from other molecular subtypes ($P=0.07$ vs. MSS).

Interestingly, although mutations in MHC pathway components were common in *POLE*-mutant tumors in the TCGA series, most were of unlikely pathogenicity, with exception of two tumors with potentially functional variants. The first, a $\beta 2$ -microglobulin R117* mutation also detected in a *POLE*-mutant colorectal cancer,⁹ had a variant allele fraction of 0.59 and is likely to affect stability of the MHC complex by disruption of the interaction with the HLA heavy chain.⁴² The second, an HLA-B S112R substitution with variant allele fraction 0.71, lies near the F pocket essential for peptide display, and is predicted to be deleterious by both Mutation Assessor and SIFT (Supplementary Table 2). However, the effect of each variant on antigen presentation is, at present, uncertain.

We proceeded to examine whether the cytotoxic T cell response in *POLE*-mutant endometrial cancers may be attenuated by upregulation of immunosuppressive mediators - a phenomenon termed adaptive immune resistance.¹⁷ We found that the T cell exhaustion markers LAG3, TIM-3, and TIGIT, and the T cell inhibitors PD-1 and CTLA-4, were strongly correlated with CD8A expression across all endometrial cancers of all molecular subtypes ($\rho=0.65$ to 0.87 ; $P<0.0001$ for all cases), though the correlation with PD-L1 was more modest ($\rho=0.34$, $P<0.0001$; Supplementary Figure 6A). Interestingly, although expression of these markers in MSI compared with MSS endometrial cancers was either unchanged/minimally altered (TIM-3, CTLA4, PD-L1) or moderately increased (LAG3 1.9-fold, $P<0.002$; TIGIT 2.2-fold, $P<0.0001$), in *POLE* mutants all were significantly and substantially upregulated (e.g., LAG3 2.9-fold, $P<0.0001$ vs. MSS, $P<0.02$ vs. MSI; TIGIT 3.6-fold, $P<0.0001$ vs. MSS, $P<0.15$ vs. MSI; Figure 3D and Supplementary Figure 6B), consistent with prolonged antigen stimulation. However, as noted above, the overall increase in expression of cytotoxic effector markers suggested that this upregulation was insufficient to fully suppress the T cell response in *POLE* mutants.

***POLE* proofreading-mutant endometrial cancers are likely to display increased numbers of antigenic neoepitopes**

We hypothesized that the T cell response in *POLE*-mutant endometrial cancers might be due to an excess of antigenic neoepitopes as a consequence of ultramutation. To quantify this, we analyzed the TCGA endometrial cancer series using a methodology similar to several recent studies.^{23,40} Our algorithm was based on three assumptions: first, that a mutation must be in an expressed gene to exert an effect; second, for a neoepitope to act as an antigen, it must bind MHC class I molecules ($IC_{50}<500$ nmol/L); and third, that

neopeptides for which the corresponding wild-type peptide also binds MHC molecules are less likely to be immunogenic due to central deletion or tolerization.³⁹

Applying these criteria, we found that 5.9% (7,880/134,473) of the total number of missense mutations in TCGA endometrial cancers were predicted to be potentially antigenic. Of these, 73% (5767) occurred in *POLE*-mutant tumors, reflected in a significantly higher number of antigenic mutations per cancer compared with both MSI and MSS subtypes (median 365.5 vs. 16 vs. 2 respectively, $P < 0.0001$ for all comparisons, Mann–Whitney U test; Figure 4A), though this is likely to underestimate the number of antigenic mutations in MSI tumors as frameshift mutations were not included in our analysis. A substantial majority of antigenic mutations were in tumors with greater than median CD8A expression in both the whole series (78.4%), and the *POLE*-mutant subgroup (83.9%), though the strength of correlation between the two variables was modest, possibly as a result of immune escape mechanisms (Figure 4B).

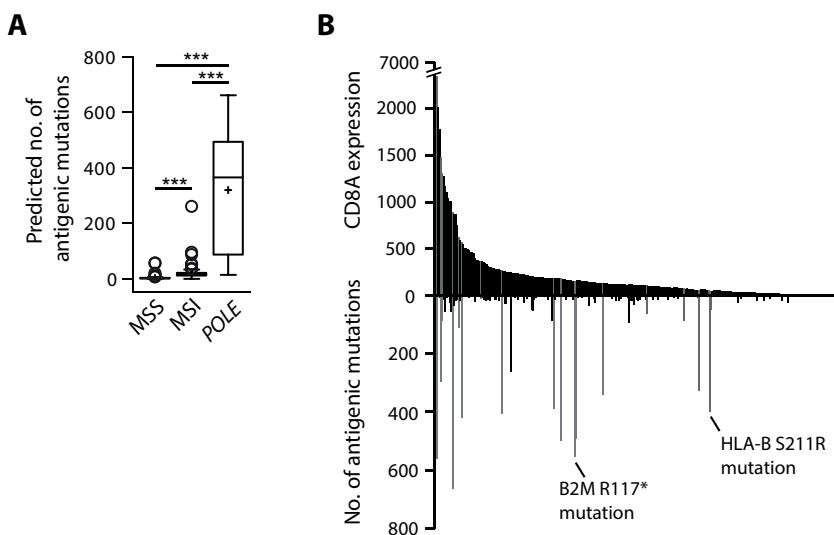


Figure 4. Antigenic mutation burden and tumor CD8A expression according to *POLE* mutation.

(A) The number of mutations predicted to generate antigenic neopeptides in individual TCGA tumors was calculated using exome and RNAseq data (see Materials and methods). Box and whisker plots signify $IQR \pm 1.5 \times IQR$, mean and median as previously. Comparison between groups was made by the unadjusted two-sided Mann–Whitney U test. (B) Relationship between number of antigenic missense tumor mutations and tumor CD8A expression. *POLE*-mutant samples are highlighted in gray, together with possible mechanisms of immune escape in two cases; *** $P < 0.0001$.

DISCUSSION

By complementary analysis of two independent series totaling nearly 400 patients, and including over 60 *POLE* proofreading-mutant tumors, we have shown that *POLE*-mutant endometrial cancers are characterized by a striking CD8⁺ lymphocytic infiltrate, a gene signature of T cell infiltration, and marked upregulation of cytotoxic T cell effector markers. Furthermore, we show that, as a consequence of their remarkable mutation burden, *POLE* proofreading-mutant cancers are predicted to display substantially more antigenic peptides than other tumors, providing a possible explanation for our findings. Although our data demonstrate correlation rather than causation, the strong association between cytotoxic lymphocyte infiltration and favorable outcome in multiple cancers^{19-22,41} leads us to speculate that an enhanced T cell antitumor response may contribute to the excellent prognosis of *POLE*-mutant endometrial cancers.

During the last few years, a combination of next generation sequencing technology, improved *in silico* peptide-MHC-binding prediction,³⁷ and the clinical application of immune checkpoint inhibitors⁴³⁻⁴⁵ have helped facilitate remarkable insights into the mechanisms of tumor immunoediting and immune escape. The intriguing observation that clinical benefit from CTLA-4, PD-1, and PD-L1 inhibition is greater in melanoma and cigarette smoking-associated lung cancer than most other malignancies can now be interpreted in light of the understanding that in these highly mutated tumors, adaptive immune resistance is a key enabler of disease progression,¹⁷ and its inhibition can restore the ability of T cells to respond to antigenic peptides presented by these cancers.⁴³ In keeping with this, in melanoma response to checkpoint inhibitors has very recently been shown to correlate both with the number of predicted antigenic tumor mutations,⁴⁰ and with the degree of cytotoxic T lymphocyte tumor infiltration before treatment.⁴⁶ In light of these data, the association between the number of predicted antigenic peptides and T cell response in *POLE*-mutant endometrial cancers in our study is noteworthy, as is the marked increase in T cell exhaustion markers, as these have recently been shown to identify tumor neoantigen-specific CD8⁺ cells in patients with cancer.^{47,48} Despite upregulation of these immunosuppressors in *POLE*-mutant endometrial cancers, we also found substantial increases in cytotoxic differentiation markers and effectors suggesting that the degree of adaptive immune resistance in these cancers may be insufficient to fully suppress CD8⁺ T cell cytotoxicity.⁴⁹ Collectively, our data suggest a complex interaction between the antigenic landscape of *POLE*-mutant endometrial cancers and the immune response. In this regard, molecular analysis of recurrences from the few patients with *POLE*-mutant endometrial cancers who do experience relapse may provide insights into mechanisms of immune escape. Finally, these patients may be good candidates for immune checkpoint inhibitor therapy, as might those patients

with *POLE* proofreading-mutant tumors of other histologies, for which outcomes are more uncertain.

Interestingly, while as anticipated we observed moderately increased T cell infiltration in MSI tumors,²⁵ this was not associated with the marked increase in cytotoxic effector markers seen in *POLE* mutants. Although some studies have reported improved prognosis of MSI endometrial cancers, this is inconsistent,⁵⁰ in contrast with the clear association of MSI with favorable outcome in early colorectal cancer.²⁶ Comparison of the immune response between MSI tumors of both types may provide insights into this discordance.

Our study has limitations. Because of differing sample preservation (FFPE vs. fresh frozen), we were unable to validate either the IHC or RNAseq analysis between series, although the results from both analyses were highly concordant. Furthermore, the retrospective nature of our study meant that we were unable to investigate the repertoire and antigen response of T cells in patients with *POLE*-mutant cancers. This and other functional analyses will require prospective investigation.

In summary, we have demonstrated that ultramutated *POLE* proofreading-mutant endometrial cancers are characterized by a robust intratumoral T cell response, which correlates with, and may be caused by an enrichment of antigenic neopeptides. Our study provides a plausible mechanism for the excellent prognosis of these cancers, and further evidence of the link between somatic mutation and immunoediting in cancers.

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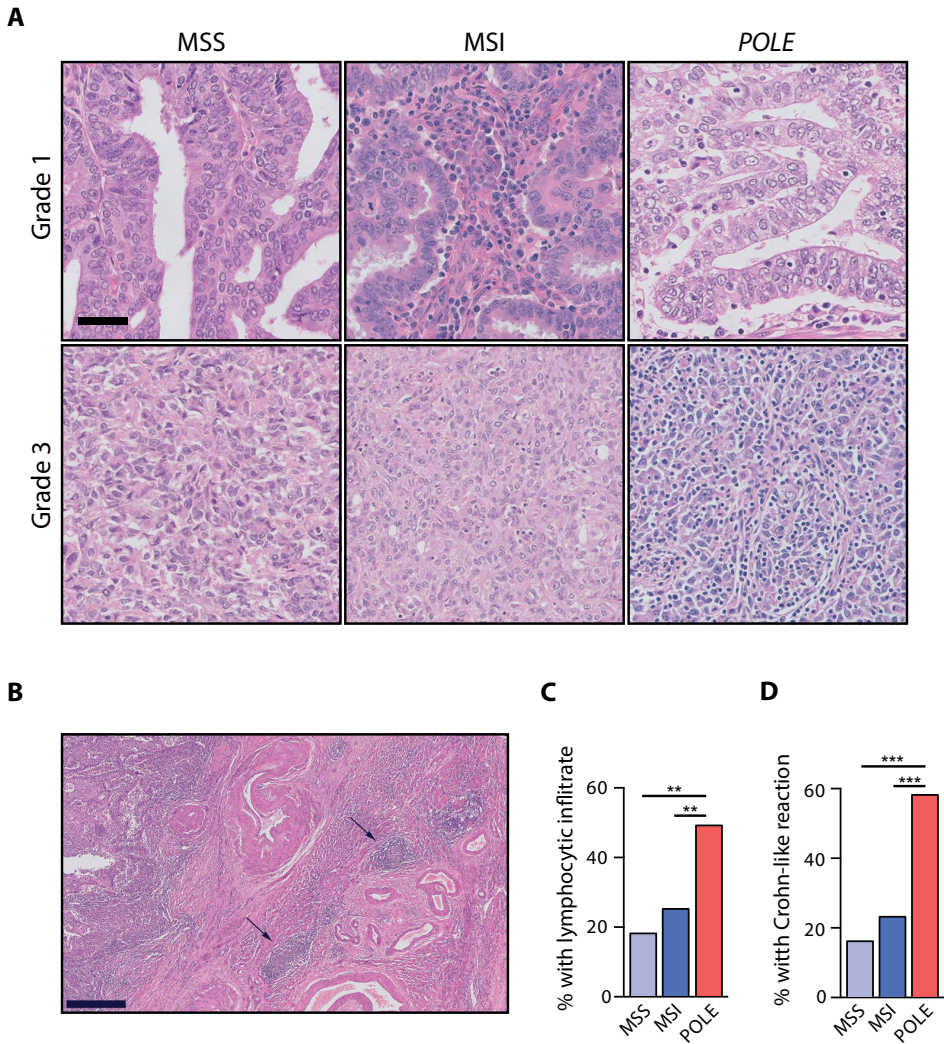
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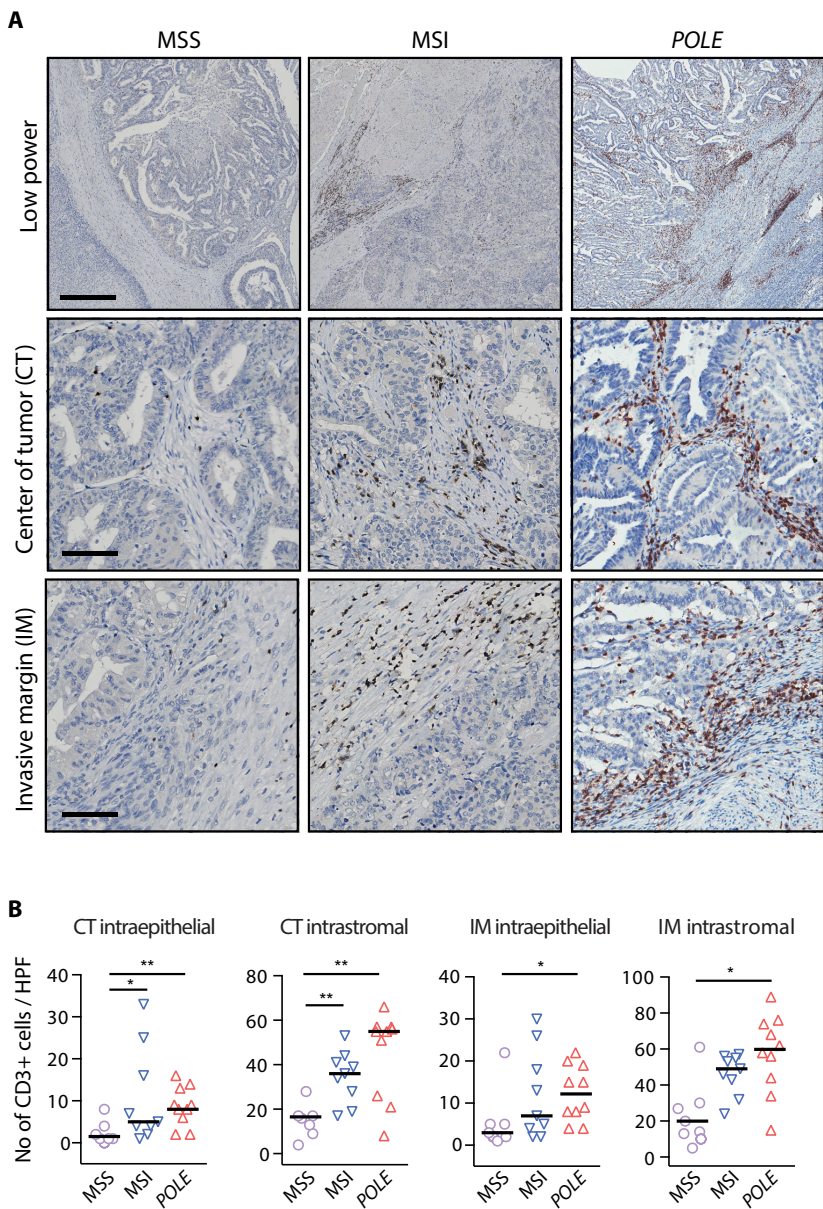
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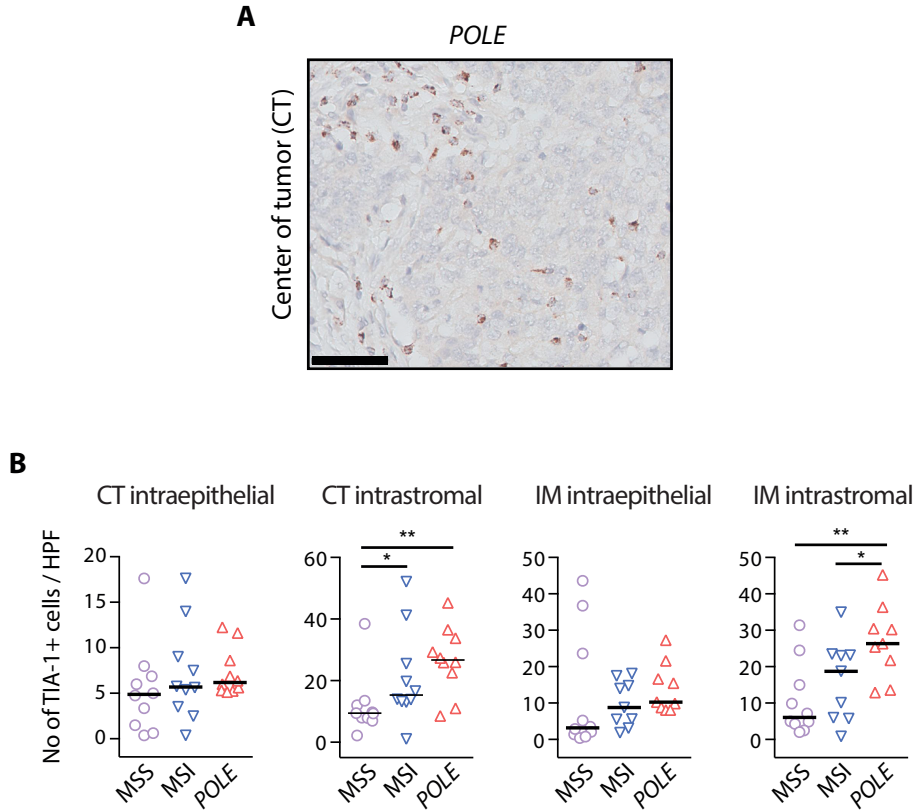
Supplementary Figure 1. Tumor-infiltrating lymphocytes and Crohn-like reaction in *POLE*-mutant endometrioid endometrial cancers.

(A) Representative H&E-stained sections demonstrating tumor-infiltrating lymphocytes (TILs) in *POLE*-wild-type, microsatellite-stable (MSS), *POLE*-wild-type, microsatellite-unstable (MSI) and *POLE* proofreading domain-mutant (*POLE*) ECs in both low- and high-grade tumors. Scale bar corresponds to 50µm. (B) Representative H&E stained section demonstrating Crohn-like reaction (indicated by arrows) in a *POLE*-mutant tumor. Scale bar corresponds to 500µm. (C) Frequency of lymphocytic infiltrate by EC molecular subtype. (D) Frequency of Crohn-like reaction by EC molecular subtype. Comparison between groups in C and D was made by two-sided Fisher's exact test; ** and *** indicate $P < 0.01$, and $P < 0.001$, respectively.



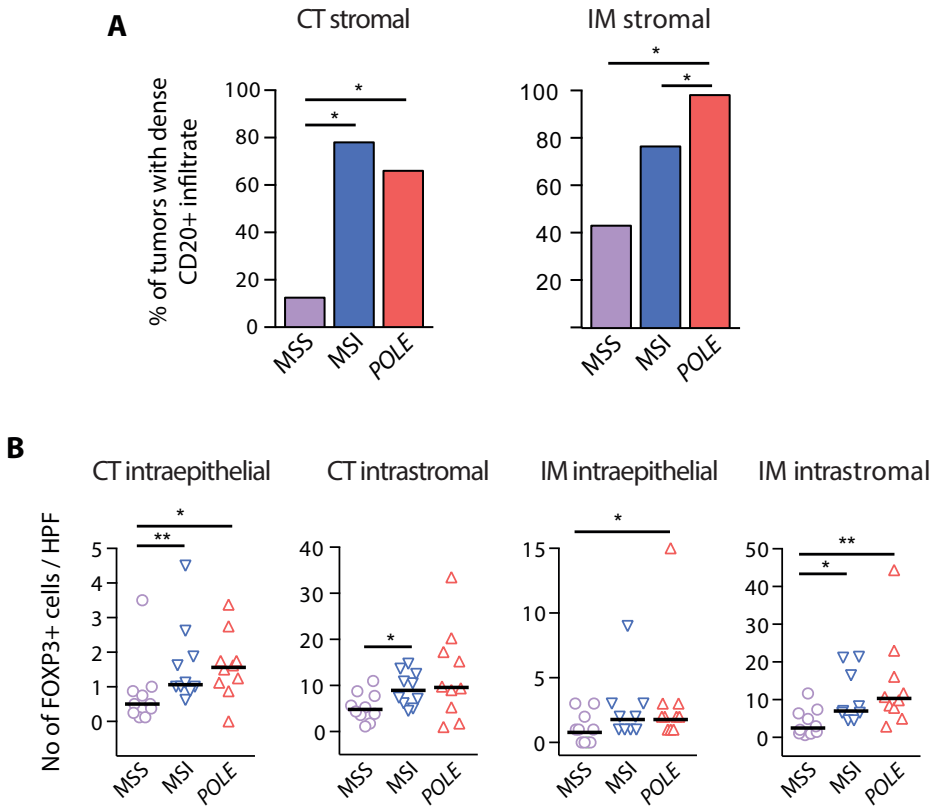
Supplementary Figure 2. CD3⁺ tumor infiltrate according to EC molecular subtype.

(A) Representative images of CD3⁺ staining in microsatellite-stable and -unstable *POLE*-wild-type cancers (MSS & MSI) compared to a *POLE* proofreading domain-mutant (*POLE*) tumor at low magnification (upper panels) and high magnification in the center of the tumor (CT) and invasive margin (IM) (middle and lower panels). Scale bar in upper panel indicates 500μm, bar in middle and lower panels indicates 100μm. (B) Quantification of CD3⁺ cell numbers per high power (200 x 200μm) field in intraepithelial and intrastromal compartments within the center of the tumor (CT) and invasive margin (IM) according to EC molecular subtype. Horizontal bars indicate the median. Comparison between groups was made by unadjusted Mann-Whitney test; * and ** indicate $P < 0.05$, and $P < 0.01$, respectively.



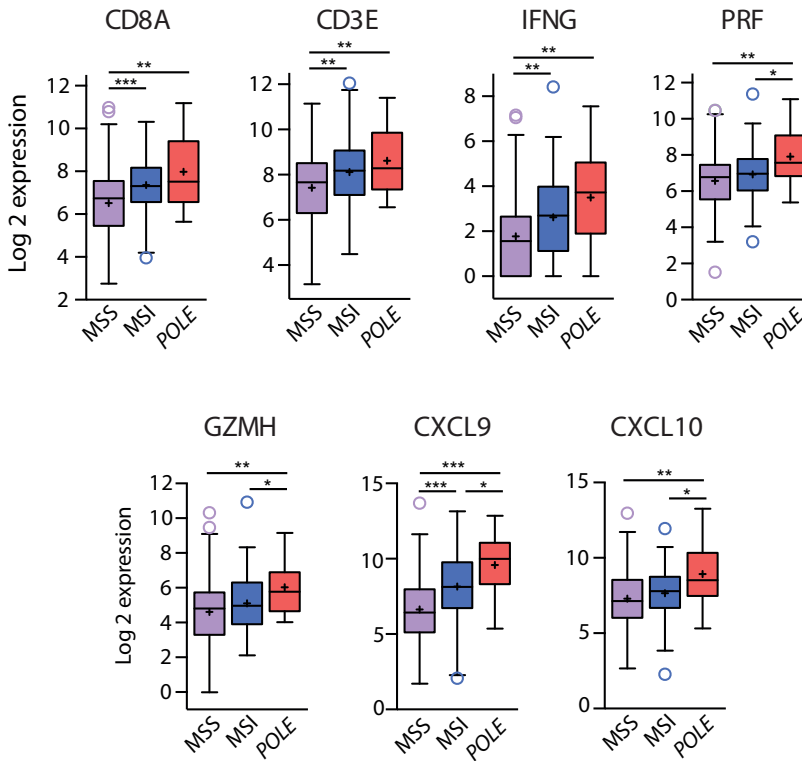
Supplementary Figure 3. Number of TIA-1⁺ cytolytic T cells according to molecular subtype.

(A) Representative high magnification image of TIA-1⁺ staining in a *POLE* proofreading domain-mutant (*POLE*) tumor. Scale bar indicates 50μm. (B) Quantification of TIA-1⁺ cell numbers per high power (200x200μm) field in intraepithelial and intrastromal compartments within the center of the tumor (CT) and invasive margin (IM) for MSS, MSI and *POLE*-mutant tumors. Horizontal bars indicate the median. Comparison between groups was made by unadjusted Mann-Whitney test; * and ** indicate $P < 0.05$, and $P < 0.01$, respectively.



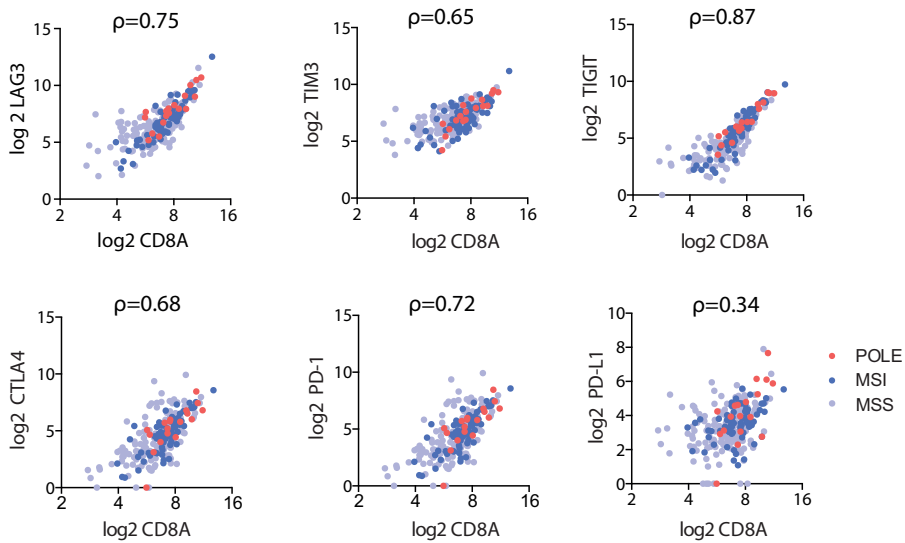
Supplementary Figure 4. CD20⁺ and FOXP3⁺ tumor infiltrate according to EC molecular subtype.

(A) Percentages of tumors displaying dense intrastromal CD20⁺ infiltrate in the center of the tumor (CT) and invasive margin (IM). (B) Quantification of number of FOXP3⁺ cells in tumor regions by EC molecular subtype. Horizontal bars indicate the median. Statistical comparisons in A and B were made by unadjusted two-sided Mann-Whitney test; * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.

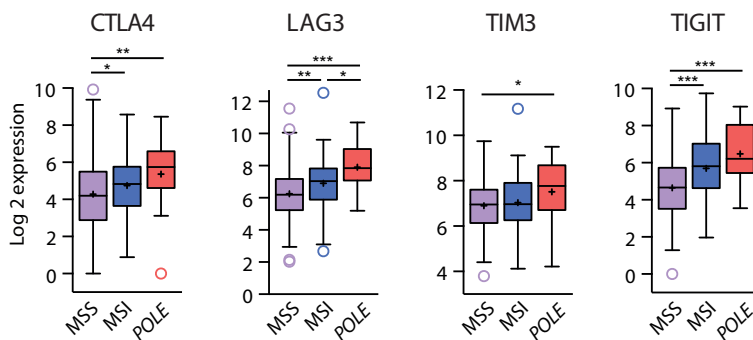


Supplementary Figure 5. Expression of T cell markers and cytotoxic effectors by EC molecular subtype. Expression of genes encoding T cell subunits CD8 α (CD8A), CD3 ϵ (CD3E), cytotoxic effector molecules interferon γ (IFN), perforin (PRF) and granzyme H (GZMH), and IFN γ -induced cytokines CXCL9 and CXCL10 was compared between molecular subtypes using RSEM normalized TCGA RNAseq data. Box and whiskers signify IQR $\pm$ 1.5xIQR, mean and median as previously. Statistical comparison between groups was made by unadjusted two-sided Mann-Whitney test; *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

A



B



Supplementary Figure 6. T cell exhaustion markers according to tumor CD8A expression and EC molecular subtype.

(A) Correlation between CD8A expression and T cell exhaustion markers ($P<0.0001$, all cases). (B) T cell exhaustion makers according to EC molecular subtype. Box and whiskers signify $IQR \pm 1.5 \times IQR$, mean and median as previously. Statistical comparison between groups was made by unadjusted Mann-Whitney test; *, **, and *** correspond to $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

Supplementary Table 1. Demographic and clinicopathological characteristics of cases by EC molecular subtype.

	MSS	MSI	POLE	Total
PORTEC, LUMC & UMCG cases				
Age, median (range), years	62.5 (36-84)	68.8 (35-89)	63.5 (46-82)	65.0 (35-89)
FIGO stage (2009), n				
I/II	48	39	45	132
III/IV	6	10	2	18
Grade, n				
1/2	45	39	33	117
3	9	10	14	33
Type, n				
EEC	54	49	47	150
NEEC	0	0	0	0
Total, n	54	49	47	150
TCGA series*				
Age, median (range), years	64 (34-90)	63 (35-88)	57 (33-87)	63 (33-90)
FIGO stage (2009), n				
I/II	116	57	14	187
III/IV	40	12	4	56
Unknown	1	1	0	2
Grade, n				
1/2	97	45	9	151
3	60	24	9	93
Type, n				
EEC	114	69	18	201
NEEC	43	0	0	43
Total, n	157	69	18	244

*TCGA analysis excludes one NEEC for which MSI status could not be determined. Secondly, all *POLE*-proofreading mutant ECs were MSS with exception of one TCGA tumor (TCGA-AP-A051) which was assigned to the *POLE*-mutant subgroup on basis of characteristic mutation signature.

Abbreviations: MSS, microsatellite-stable *POLE*-wild-type; MSI, microsatellite-unstable *POLE*-wild-type; *POLE*, *POLE* proofreading-mutant; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer.

Supplementary Table 2. Antigen presentation pathway mutations in TCGA *POLE* proofreading-mutant ECs.

TCGA ID	<i>POLE</i> mutation	Exonic mutations, n	CD8A expression*	Antigen presentation pathway mutations	Variant allele frequency	Predicted functional effect	
						Mutation assessor	SIFT
TCGA-B5-A0JY	P286R	8890	High	HLA-B E253K B2M R117*	0.33 0.59	Medium N/A	Deleterious (0.00) N/A
TCGA-B5-A0TC	M444K	1203	Low	PDIA3 R344C None	0.36 0.30	Medium Neutral	Deleterious (0.00) Deleterious (0.00)
TCGA-D1-A103	A456P	5965	High	PDIA3 R344C	0.30	Medium	Deleterious (0.00)
TCGA-D1-A16X	P286R	1755	High	None			
TCGA-D1-A16Y	V411L	952	Low	None			
TCGA-A5-A0GP	V411L	1235	Low	None			
TCGA-AP-A051	L424I	6716	High	PDIA3 A69T	0.31	Low	Deleterious (0.04)
TCGA-AP-A056	V411L	7226	High	HLA-C E288K CALR S300Y	0.16 0.34	Low Neutral	Tolerated (0.06) Deleterious (0.00)
TCGA-AP-A059	S297F	9187	High	None			
TCGA-AP-A0LM	V411L	10489	High	B2M A6V CANX K182N	0.13 0.20	Low Medium	Tolerated (1.0) Deleterious (0.00)
TCGA-AX-A05Z	P286R	5677	High	None			
TCGA-AX-A0J0	P286R	6913	Low	HLA-B S112R CALR K62R TAPBP R383Q	0.71 0.50 0.57	High Medium Medium	Deleterious (0.00) Deleterious (0.00) Tolerated (0.34)
TCGA-B5-A11E	V411L	8210	High	PDIA3 E384*	0.44	N/A	N/A
TCGA-B5-A11N	P286R	1557	High	None			
TCGA-BG-A0VX	L424V	163	High	None			
TCGA-B5-A0JF	P286R	7391	High	CANX D37N	0.33	Medium	Deleterious (0.00)
TCGA-B5-A0UV	P286R	7801	High	None			

Supplementary Table 2. (continued) Antigen presentation pathway mutations in TCGA POLE proofreading-mutant ECs.

TCGA ID	POLE mutation	Exonic mutations, n	CD8A expression*	Antigen presentation pathway mutations	Variant allele frequency	Predicted functional effect
TCGA-D1-A17Q	P286R	5141	Low	CALRY128C	0.13	High Deleterious (0.00)
				CANX Q508H	0.48	Medium Deleterious (0.00)
				TAPBP R383Q	0.38	Medium Tolerated (0.34)

*Relative to median CD8A expression in all TCGA ECs.

POLE proofreading mutation, immune response and prognosis in endometrial cancer

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ABSTRACT

Endometrial cancers (ECs) with *POLE* proofreading mutations are typified by ultramutation and excellent prognosis. We investigated whether these were related, and found that *POLE*-mutant ECs display a robust T cell response that corresponds to an enrichment of antigenic tumor neopeptides. Enhanced immunogenicity may explain the favorable outcome of *POLE*-mutant ECs.

POLE PROOFREADING MUTATION, IMMUNE RESPONSE AND PROGNOSIS IN ENDOMETRIAL CANCER

The proofreading exonuclease activity intrinsic to the replicative DNA polymerases epsilon and delta (Pols ϵ and δ) is essential to maintain fidelity of DNA replication and prevent mutagenesis. While a role for defective polymerase proofreading in human cancer has long been postulated, this has only recently been confirmed, with the demonstration that germline mutations in the exonuclease domains of *POLE* and *POLD1* (which encode the principal subunits of Pols ϵ and δ respectively) predispose to cancer.¹ Subsequently, we and others have shown that somatic *POLE* proofreading mutations occur in 7-12% endometrial cancers (ECs),^{2,3} 1-2% colorectal cancers (CRCs), as well as cancers of the brain, stomach and pancreas (TCGA unpublished, <http://www.cbiportal.org>, accessed June 2015). In keeping with the essential contribution of polymerase proofreading to replication fidelity, *POLE* proofreading-mutant ECs are ultramutated.³ However, perhaps less predictably, they also have an excellent prognosis.^{3,4} We hypothesized that these two characteristics may be related – more specifically, that tumor neoepitopes caused by ultramutation may stimulate an cytolytic immune response, analogous to previous observations in hypermutated mismatch repair-deficient CRCs.⁵ In a recent study,⁶ we investigated this in two large EC cohorts.

Following the observation that *POLE* proofreading mutants had a higher density of tumor-infiltrating lymphocytes (TILs) than other ECs, we confirmed that this represented a CD8⁺ cytotoxic T cell infiltrate likely to be capable of cytolytic activity, as evidenced by co-staining for the activation marker TIA-1. Consistent with these data, examination of RNAseq data from the independent TCGA EC series confirmed significant enrichment for immune-related pathways and a highly specific 200-gene tumor T cell infiltration signature in *POLE* proofreading-mutant ECs. This analysis also demonstrated that *POLE*-mutant tumors displayed significantly increased expression of CD8A and other T cell cytotoxic differentiation and effector markers known to predict favorable outcome in cancer,⁷ including T-bet, Eomes, IFN- γ , perforin, and granzymes B, H, K and M. Using a bioinformatic approach to investigate the possible contribution of antigenic tumor neoepitopes to the antitumor immune response, we found that *POLE* proofreading-mutant ECs were predicted to display substantially more antigenic peptides than other ECs, providing a potential explanation for our findings.

Taken together, our data suggest that enhanced immunogenicity contributes to the excellent prognosis of *POLE* proofreading-mutant ECs, and are concordant with a recent study, which showed that dendritic cells pulsed by *POLE*-mutant tumor lysates stimulated greater CD4⁺ and CD8⁺ cell proliferation than those pulsed by ECs lacking

POLE mutations.⁸ However, this begs the question of why *POLE*-mutant ECs are not eliminated by this enhanced cytotoxic T cell response? We found no evidence of an increased frequency of loss of HLA class I protein expression in *POLE*-mutant ECs, and functional mutations in the antigen presentation machinery also appeared relatively uncommon (2 of 18 cases). In contrast, we found striking increases in the expression of immunosuppressive checkpoint molecules and regulatory T cell markers, including LAG3, TIM-3, TIGIT, PD-1, CTLA-4 and FOXP3, in *POLE*-mutant ECs, suggesting that this may be the principal mechanism of immune evasion in these tumors.

In short, our data suggest that *POLE* proofreading-mutant ECs evoke a striking antitumor immune response, which is likely to contribute at least partly to their excellent prognosis (Figure 1). In addition to validating our results in further independent EC series, it will be important to determine whether an enhanced cytotoxic T cell reaction also occurs in other *POLE* proofreading-mutant cancers. Interestingly, recent data suggest this may be the case in glioblastomas.⁹ Given the association between benefit from immune checkpoint inhibitors and tumor mutation burden,¹⁰ our study also suggests that the few patients with advanced or recurrent *POLE* proofreading-mutant cancers may be promising candidates for these agents. Finally, a pressing question is why some *POLE*-mutant tumors do not appear to stimulate as potent an immune reaction as others? Is it simply that these tumors are less mutated? Or do they harbor novel mechanisms of immune escape? Thus, while much remains unknown, further study of *POLE*-mutant cancers may provide insights into antitumor immune response and evasion that are generalizable more broadly, with potential benefits for a wide range of cancer patients.

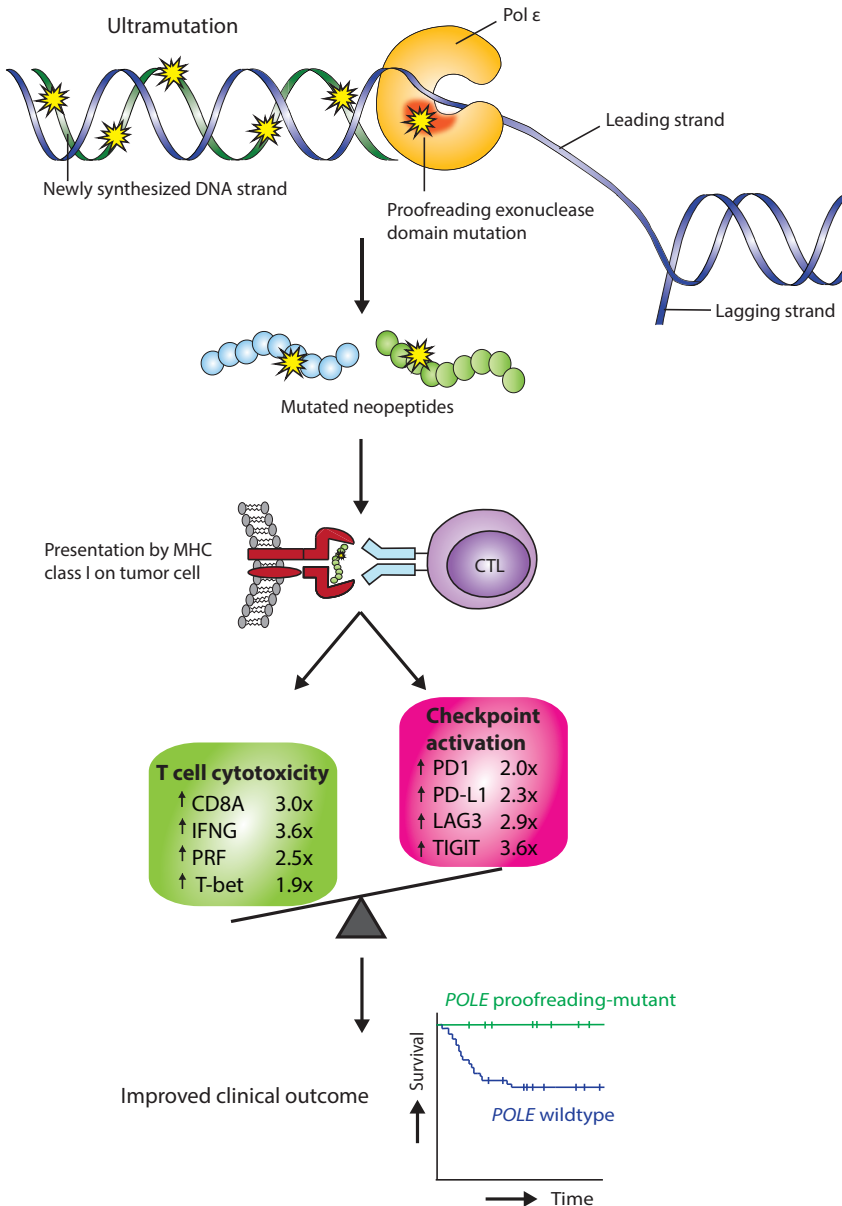


Figure 1. Possible mechanism linking *POLE* proofreading mutation, immune response and favorable endometrial cancer prognosis.

POLE encodes the catalytic and proofreading subunit of DNA polymerase ε (Pol ε), the leading strand replicase in humans. Cancer-associated *POLE* exonuclease domain mutations perturb proofreading activity, resulting in tumor ultramutation. Enhanced presentation of mutated antigenic neopeptides stimulates both a cytolytic T cell response and upregulation of immunosuppressive checkpoints; however, increased effector cytokine expression (not shown) suggests that the T cell response is functional and at least partly contributes to the favorable prognosis of *POLE* proofreading domain-mutant endometrial cancers.

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Chapter 5

Immunological profiling of molecularly classified high-risk endometrial cancers identifies *POLE*-mutant and microsatellite-unstable carcinomas as candidates for checkpoint inhibition

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ABSTRACT

High-risk endometrial cancer (EC) is an aggressive disease for which new therapeutic options are needed. Aims of this study were to validate the enhanced immune response in highly mutated ECs and to explore immune profiles in other EC subgroups. We evaluated immune infiltration in 116 high-risk ECs from the TransPORTEC consortium, previously classified into four molecular subtypes: (i) ultramutated *POLE* exonuclease domain-mutant ECs (*POLE*-mutant); (ii) hypermutated microsatellite-unstable (MSI); (iii) p53-mutant; and (iv) no specific molecular profile (NSMP). Within The Cancer Genome Atlas (TCGA) EC cohort, significantly higher numbers of predicted neoantigens were demonstrated in *POLE*-mutant and MSI tumors compared to NSMP and p53 mutants. This was reflected by enhanced immune expression and infiltration in *POLE*-mutant and MSI tumors in both the TCGA cohort (mRNA expression) and the TransPORTEC cohort (immunohistochemistry) with high infiltration of CD8⁺ (90% and 69%), PD-1⁺ (73% and 69%) and PD-L1⁺ immune cells (100% and 71%). Notably, a subset of p53-mutant and NSMP cancers were characterized by signs of an antitumor immune response (43% and 31% of tumors with high infiltration of CD8⁺ cells, respectively), despite a low number of predicted neoantigens. In conclusion, the presence of enhanced immune infiltration, particularly high numbers of PD-1 and PD-L1 positive cells, in highly mutated, neoantigen-rich *POLE*-mutant and MSI endometrial tumors suggests sensitivity to immune checkpoint inhibitors.

INTRODUCTION

The development of novel immunotherapeutic strategies such as checkpoint inhibitors has the potential to transform the field of oncology. So far, durable responses have been established in subsets of patients, for example with metastatic melanoma, non-small cell lung cancer, and mismatch repair-deficient cancers including two patients with endometrial cancer (EC).¹⁻⁷ While the clinical efficacy of immune checkpoint inhibitors is evident in a subset of patients, selecting the patients who may benefit from this therapy remains challenging. A key mechanism for the benefit of immune checkpoint inhibition in these cancers is the induction of a strong neoantigen-driven T cell response against the tumor. Indeed, comprehensive analysis of large genomic datasets such as The Cancer Genome Atlas (TCGA) have provided a clear link between mutational load and activation of the immune system, implicating the involvement of neoantigens in driving cytotoxic T cell responses in cancer.⁸⁻¹⁰ Furthermore, several clinical trials have shown a strong association between the presence of high numbers of predicted neoantigens, immune infiltration and response to cancer immunotherapy.¹¹⁻¹⁵ In particular, the presence of CD8⁺ cytotoxic T cells and expression of the immune checkpoints PD-1 and PD-L1 have been proposed as important predictors of objective tumor regression.^{3,16}

Characterization of the immune contexture of individual tumors may provide guidance in selecting appropriate immunotherapy for each individual patient, especially when integrated with an analysis of genomic alterations.^{10,17,18} A molecular classification has recently been proposed by The Cancer Genome Atlas (TCGA), which identified four genomically distinct EC subgroups: an ultramutated group characterized by somatic mutations in the exonuclease domain of *POLE* (encoding the catalytic subunit of DNA polymerase epsilon), a microsatellite-unstable (MSI) hypermutated group with many substitutions as well as insertions and deletions due to mismatch repair deficiency, a copy-number high (serous-like) group with frequent *TP53* mutation and a copy-number low (microsatellite-stable, MSS) group with no specific molecular profile (NSMP).¹⁹

In line with this, we, and others, have recently demonstrated high numbers of predicted immunogenic mutations and enhanced antitumor immune infiltration in ultramutated *POLE*-mutant and, to a lesser extent, in hypermutated microsatellite-unstable EC.²⁰⁻²³ These studies combined with the emerging data linking mutational load, immune activation and response to cancer immunotherapy render *POLE*-mutated and MSI cancers plausible candidates for immune checkpoint inhibition.^{3,10-13,24} This is further underlined by recent case reports demonstrating the efficacy of anti-PD-1 inhibitors in advanced *POLE*-mutant or mismatch repair-deficient cancers, including those of endometrial origin.^{7,25,26}

In this study, we aimed to validate our previous findings of an enhanced immune response in *POLE*-mutant and MSI endometrial cancers in a cohort of EC patients at high risk of recurrence. High-risk EC patients are a particularly relevant subgroup, as most have no or only very modest gain from standard local or systemic treatment after surgery. Novel treatment options are therefore urgently needed. The use of a molecularly defined cohort of high-risk endometrial cancer also enabled us to explore the immune profiles of the poorly characterized NSMP and p53-mutant subgroups. With this approach we provide a rationale for the administration of checkpoint inhibition strategies in subsets of *POLE*-mutant and MSI endometrial cancer patients.

MATERIALS AND METHODS

Selection of patients and tissues

A previously described cohort of 116 high-risk EC patients was used in the current study (Table 1).²⁷ In brief, tumor tissues from high-risk EC patients were selected from partner institutions of the TransPORTEC consortium using inclusion criteria of the PORTEC-3 study. Patients included in the PORTEC-3 had EC with one of the following FIGO 2009 stages and grade: 1A grade 3 with myometrial- and lymphovascular space invasion; 1B grade 3; II, IIIA or IIIC; IIIB if only parametrial invasion; stage IA (with invasion), 1B, II or III with serous or clear cell histology.²⁸

Construction of tissue microarray

Morphologically representative paraffin-embedded tissue blocks containing at least 70% tumor cells were selected by two experienced gynecopathologists (V.S. and T.B.). The selected tumor blocks were used to construct (and validate) a Tissue Microarray (TMA) as previously described.²⁷ One millimeter-sized tumor (center of the tumor) and tumor/stroma (invasive margin) cores of each tumor block were randomly distributed on the TMA in triplicate.

Assessment of *POLE*, MSI, p53 and NSMP status

Classification of patients into the four molecular subgroups was performed as previously described.²⁷ In brief, tumor DNA isolation was performed fully automated using the Tissue Preparation System (Siemens Healthcare Diagnostics).²⁹ Bi-directional Sanger sequencing was used to screen exons 9, 13 and 14 of the *POLE* exonuclease domain for somatic mutations. Microsatellite instability and p53 mutational status were determined as previously described.^{27,30}

Table 1. Clinicopathological characteristics of the high-risk endometrial cancer patient cohort.

	All patients N=116	POLE-mutant N=15	MSI N=19	NSMP N=42	P53-mutant N=40	P value
Age, mean (range), years	66 (21-85)	61 (49-80)	65 (49-82)	64 (21-84)	71 (45-85)	0.004
Stage, n (%)						
I	42 (36.2)	8 (53.3)	5 (26.3)	16 (38.1)	13 (32.5)	0.246
II	21 (18.1)	3 (20.0)	2 (10.5)	12 (28.6)	4 (10.0)	
III	41 (35.3)	3 (20.0)	10 (52.6)	11 (26.2)	17 (42.5)	
IV	11 (9.5)	1 (6.7)	2 (10.5)	3 (7.1)	5 (12.5)	
Unknown	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.5)	
Tumor type, n (%)						
Endometrioid	86 (74.1)	14 (93.3)	17 (89.5)	35 (83.3)	20 (50.0)	<0.001
Serous	12 (10.3)	0 (0.0)	0 (0.0)	0 (0.0)	12 (30.0)	
Clear cell	18 (15.5)	1 (6.7)	2 (10.5)	7 (16.7)	8 (20.0)	
Grade, n (%)						
1	13 (11.2)	0 (0.0)	2 (10.5)	8 (19.0)	3 (7.5)	0.036
2	5 (4.3)	1 (6.7)	3 (15.8)	1 (2.4)	0 (0.0)	
3	98 (84.5)	14 (93.3)	14 (73.7)	33 (78.6)	37 (92.5)	
Lymphovascular space invasion, n (%)						
Yes	55 (47.4)	6 (40.0)	15 (78.9)	18 (42.9)	16 (40)	0.103
No	40 (34.5)	9 (60.0)	2 (10.5)	18 (42.9)	11 (27.5)	
Unknown	21 (18.1)	0 (0.0)	2 (10.5)	6 (14.3)	13 (32.5)	
Depth of myometrial invasion, n (%)						
<50%	23 (19.8)	4 (26.7)	2 (10.5)	6 (14.3)	11 (27.5)	0.261
>50%	87 (75.0)	11 (73.3)	17 (89.5)	33 (78.6)	26 (65.0)	
Unknown	6 (5.2)	0 (0.0)	0 (0.0)	3 (7.1)	3 (7.5)	
Adjuvant therapy, n (%)						
Yes	82 (70.7)	14 (93.3)	15 (78.9)	33 (78.6)	20 (50.0)	0.134
No	10 (8.6)	1 (6.7)	1 (5.3)	2 (4.8)	6 (15.0)	
Unknown	24 (20.7)	0 (0.0)	3 (15.8)	7 (16.7)	14 (35.0)	

Characteristics are shown for the whole group, as well as for each of the molecular subgroups analyzed.
Abbreviations: MSI, microsatellite-unstable; NSMP, no specific molecular profile.

Immunohistochemistry

TMA sections were deparaffinized and rehydrated. Antigen retrieval was performed using 0.01M citrate buffer pH 6.0, and endogenous peroxidase activity was blocked. Slides were incubated overnight at room temperature (CD3, TIA-1, T-bet and PD-1), for one hour at room temperature (CD8, CD20) or overnight at 4°C (CD103) with primary antibodies against CD3 (1:100, clone PS-1, Diagnostic BioSystems), CD8 (1:50, clone C8/144B, DAKO), CD20 (1:200, clone L26, DAKO), CD103 (1:200, Integrin αEβ7, Abcam),

TIA-1 (1:200, clone 2G9A10F5, Beckman Coulter), T-bet (1:400 in 10% normal goat serum, sc-21003, Santa Cruz Biotechnology), PD-1 (1:200, AF1086, R&D), and PD-L1 (1 µg/mL, clone E1L3N, Cell Signalling Technology). Slides were incubated with BrightVision Poly-HRP (poly-HRP-GAM/R/R, DPV0110HRP, Immunologic; CD3, TIA-1, T-bet), a goat HRP-polymer kit (GHP516H, Biocare Medical; PD-1), anti-mouse secondary antibody (K4007, DAKO, CD8, CD20) or anti-rabbit secondary antibody (K4011, DAKO, CD103) for 30 minutes. For CD103, a slightly different method using avidin/biotin blocking was used as described previously.³¹ PD-L1 staining was performed using the Ventrana Discovery Ultra Platform for automatic staining, detection was performed using the Discovery Amp-HQ kit (tyramide-based amplification). Antibody binding was visualized with 3,3'-diamino-benzidine-tetrahydrochloride (DAB) and haematoxylin counterstaining. Slides were dehydrated and mounted before digitalization (Ultra Fast Scanner 1.6 RA, Philips or ScanScope, Aperio technologies) and analysis.

Quantification of IHC

Total numbers of CD3⁺, CD8⁺, CD103⁺, CD27⁺, TIA-1⁺, T-bet⁺, CD20⁺, CD45RO⁺ and PD-1⁺ cell numbers were quantified per core. The percentage of tumor and stroma surface area within each core were estimated, and used to extrapolate cell counts to 100% surface area. Cores taken from the tumor center were included in the analysis if at least two out of the three cores contained >20% tumor. Cores from the infiltrative margin were included in the analysis if at least two out of the three cores contained >20% stroma and if there was tumor tissue present. Average cell counts per 100% surface area were recorded for the tumor center and infiltrative margin. Slides were counted manually by two individuals (F.E. and I.G.) that were blinded for other clinicopathological data. Interobserver variation was evaluated by Spearman rank correlation (median R² 0.935, range 0.682-0.988).

Quantification of PD-L1 was evaluated on tumor-infiltrating immune cells and tumor cells as previously described.¹ In brief, the proportion of PD-L1 expressing tumor cells (tumor score) was noted as a percentage of the total number of tumor cells within that core. Due to very low expression of PD-L1 it was decided to consider any expression of PD-L1 on tumor cells as positive. Furthermore, the percentage of tumor-infiltrating immune cells (immune score) with moderate to strong PD-L1 expression was registered. Immune cells were defined positive when cells displayed clearly visible cytoplasmic and/or membranous staining. Patients were included in the analysis if at least two out of three cores were evaluated; the final score was based on the core with the highest PD-L1 expression. For the analyses of the immune score, PD-L1 positivity was defined as >1% (based on the median score in the cohort).

Immunofluorescence

Three combinations of multi-color immunofluorescent stainings were performed as described previously.³² The first combination consisted of anti-CD163 (polyclonal rabbit, ab87099, Abcam), anti-CD68 (monoclonal mouse IgG2a, clone 514H12, ABDserotec) and anti-keratin (monoclonal mouse IgG1, clone AE1/AE3, MAB3412, Milipore). The second combination consisted of anti-PD-L1 (polyclonal rabbit, clone SP142, Roche) and anti-PD-1 (monoclonal mouse IgG1, clone NAT105, Abcam), and the third of anti-CD8 (mouse monoclonal IgG2b, clone 4B11, Novo Castra) and anti-PD-1 (polyclonal goat, R&D systems).

In short, after slides were deparaffinized and rehydrated, antigen retrieval was achieved by microwave oven treatment in a Tris-EDTA buffer at pH 9.0. Slides were incubated with the listed primary antibodies overnight. The following secondary Alexa Fluor labeled antibodies were used for the CD163 – CD68 – keratin and PD-L1 – PD-1 combinations: 647 goat anti-rabbit, 546 goat anti-mouse IgG2a, and 488 goat anti-mouse IgG1 (all from Invitrogen, Life Technologies, Carlsbad, USA). Donkey anti-goat 488 and donkey anti-mouse IgG 647 were used for PD-1/CD8 detection. The slides were counterstained with DAPI and cover-slipped. Immunofluorescent images were acquired with an LSM700 confocal laser scanning microscope equipped with an LCI Plan-Neofluar 25x/0.8 Imm Korr DIC M27 objective (Zeiss, Göttingen, Germany). Double or triple positivity of cells in the center of the tumor as well as at the invasive margin was determined using LSM Image Browser (version 4.2.0.121, Zeiss). Images from the two triple immunofluorescent stainings were merged using Adobe Photoshop CS6.

TCGA RNA sequencing data

TCGA RNAseq analysis was performed as previously reported.^{19,20} Data were downloaded from FireBrowse on November 11, 2014 (http://firebrowse.org/?cohort=UCEC&download_dialog=true). In total, 245 samples with RSEM normalized data were available for analysis.

Prediction of antigenic neoepitopes

Prediction of antigenic neoepitopes was performed as previously reported.²⁰ In brief, an algorithm was developed to estimate the immunogenicity of individual tumors in which the following considerations were taken into account: i) to generate a functional neoepitope a missense mutation must be expressed; ii) most functional neoepitopes are predicted to bind Major Histocompatibility Complex (MHC) class I molecules ($IC_{50} < 500\text{nM}$) by NetMHCpan;^{8,33,34} iii) the likelihood that a neoepitope is antigenic is reduced if the corresponding wild-type peptide also binds the MHC with similar affinity as T cells to the epitope may be centrally deleted or tolerized.³⁵ Our strategy was similar to that

reported by others.^{8,13,34,36} For each tumor all possible 9mers for every missense mutation in expressed genes (defined as non-zero reads from RNAseq) and the binding affinity of the mutant and corresponding wild-type peptide for HLA-A*02:01 were calculated using NetMHCpan 2.8.³³ If several peptides had an $IC_{50} < 500\text{nM}$, the strongest binder was used for analysis. We defined antigenic mutations as neoepitopes predicted to bind MHC molecules ($IC_{50} < 500\text{nM}$) for which the corresponding wild-type peptide was not predicted to bind MHC ($IC_{50} > 500\text{nM}$).

Statistical analyses

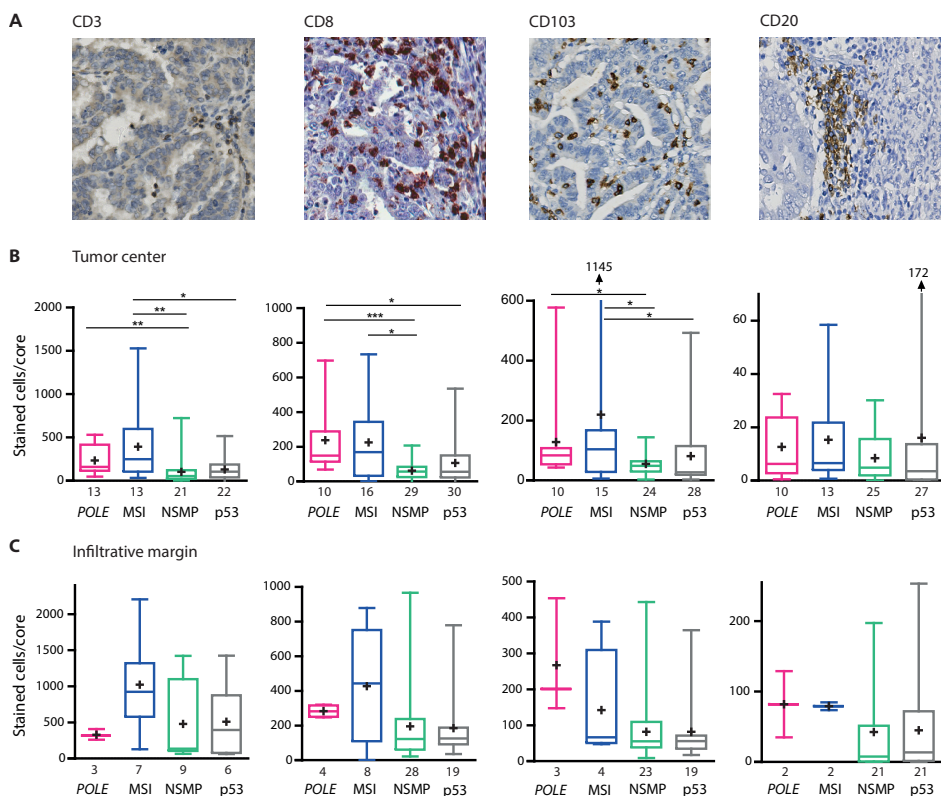
Comparison between clinicopathological characteristics of the four molecular subgroups was made using Kruskal-Wallis followed by Man-Whitney U (for age) and χ^2 tests (for all other variables). Correlations between immunohistochemical stainings and the four molecular subgroups were evaluated using Kruskal-Wallis followed by Mann-Whitney U tests. The same method was used to evaluate correlations between RNA expression from the TCGA cohort of immune-related genes and the four molecular subgroups. Additionally, analyses were performed combining *POLE*-mutant and MSI samples versus NSMP and p53-mutant samples. All tests were performed two-sided. Significance was defined as a *P* value of < 0.05 . Statistical analyses were performed using IBM SPSS version 22 (SPSS Inc., Chicago, USA) and GraphPad Prism (GraphPad Software Inc., CA, USA).

RESULTS

Enhanced infiltration of intratumoral CD3⁺, CD8⁺ and CD103⁺ lymphocytes in *POLE*-mutant and MSI tumors

We first sought to characterize the lymphocytic infiltrate in the four EC molecular subtypes by immunohistochemical analysis of CD3, CD8, CD103 and CD20 (Figure 1A-B). Compared to NSMP and p53-mutant tumors, both *POLE*-mutant and MSI tumors demonstrated increased density of CD3⁺ T lymphocytes within the tumor center (*POLE* vs. NSMP, $P=0.002$; MSI vs. NSMP, $P=0.001$; MSI vs. p53, $P=0.018$). Staining for cytotoxic T lymphocyte marker CD8 and the intraepithelial T lymphocyte marker CD103 revealed similarly increased infiltrate in the tumor center (comparison of CD8⁺ cells: *POLE* vs. NSMP, $P<0.001$; *POLE* vs. p53, $P=0.021$; MSI vs. NSMP, $P=0.016$; comparison of CD103⁺ cells: *POLE* vs. MSI, $P=0.023$; MSI vs. NSMP, $P=0.035$; MSI vs. p53, $P=0.030$). Based on a median of 80.5 CD8⁺ cells/core in the whole cohort, 90% of *POLE*-mutant, 69% of MSI, 31% of NSMP and 43% of p53-mutant tumors were categorized as highly infiltrated with CD8⁺ cells. There was no difference in numbers of CD20⁺ B lymphocytes within the tumor center. A combined analysis in which the two molecular subgroups with a high expected neo-antigen load (*POLE*-mutant and MSI) were compared with the two molecular subgroups

Within the infiltrative margin, CD3⁺, CD8⁺, CD103⁺ or CD20⁺ infiltration did not significantly differ between the four molecular subgroups (Figure 1C). Combined analysis showed a higher infiltration of CD8⁺ and CD103⁺ in *POLE*-mutant and MSI (CD8, $P=0.010$; CD103, $P=0.016$; Supplementary Figure 1B).



(A) Representative immunohistochemical stainings of CD3⁺, CD8⁺, CD103⁺ and CD20⁺ cells. (B) Average number of positively stained intratumoral cells for each of the markers in the above panel, counted per core, corrected for the number of cells present. (C) Average number of positively stained cells for each of the markers in the above panel, counted per core within the infiltrative margin, corrected for the number of cells present. The numbers of cases analyzed for each molecular subgroup are listed below the x-axis. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. **P*<0.05, ***P*<0.01, ****P*<0.001.

Increased infiltration of CD45RO⁺ and TIA-1⁺ lymphocytes in MSI tumors

To analyze the function of the tumors' lymphocytic infiltrate, we performed immunohistochemistry for CD45RO, CD27, T-bet and TIA-1 (Figure 2A-B). Within the tumor center, MSI tumors contained more CD45RO⁺ memory T lymphocytes compared to NSMP and p53-mutant tumors (MSI vs. NSMP, $P=0.029$; MSI vs. p53; $P=0.008$). MSI tumors also harbored more TIA-1⁺ cytolytic lymphocytes within the tumor center (MSI vs. NSMP, $P=0.019$; MSI vs. p53, $P=0.043$). There were no differences in the numbers of CD27⁺ naïve

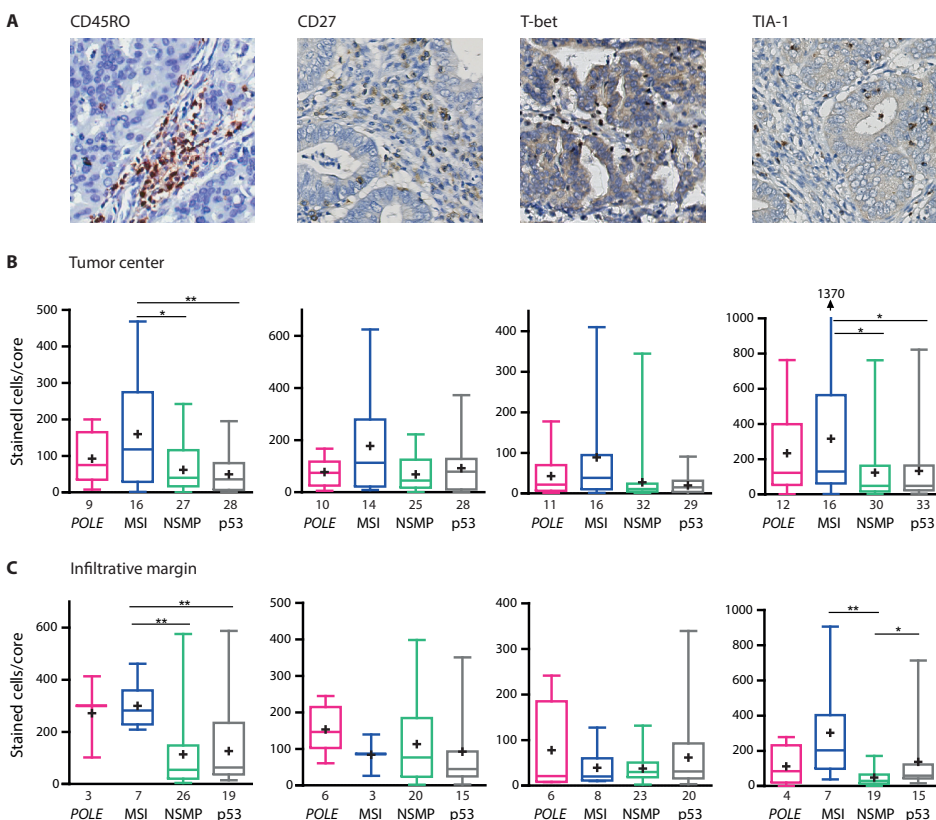


Figure 2. Infiltration of TIA-1⁺, T-bet⁺, CD27⁺ and CD45RO⁺ cells in *POLE*-mutant, MSI, NSMP and p53-mutant endometrial cancers.

(A) Representative immunohistochemical stainings of CD45RO⁺, CD27⁺, T-bet⁺ and TIA-1⁺ cells. (B) Average number of positively stained intratumoral cells for each of the markers in the above panel, counted per core within the tumor center, corrected for the number of cells present. (C) Average number of positively stained cells for each of the markers in the above panel, counted per core within the infiltrative margin, corrected for the number of cells present. The numbers of cases analyzed for each molecular subgroup are listed below the x-axis. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

T cells and T-bet⁺ differentiated cells between the four molecular subgroups. Combined analysis of molecular groups revealed the presence of more CD45RO⁺ and TIA-1⁺ cells in *POLE*-mutant/MSI tumors compared to NSMP/p53-mutant tumors (Supplementary Figure 2A). Moreover, this also demonstrated higher numbers of T-bet⁺ differentiated cells within *POLE*-mutant/MSI tumors compared to NSMP/p53-mutant tumors ($P=0.021$).

Concordant with our findings in the tumor center, the infiltrative margin of MSI tumors contained more CD45RO⁺ lymphocytes (MSI vs. NSMP, $P=0.002$; MSI vs. p53, $P=0.003$) and more TIA-1⁺ cytolytic T lymphocytes (MSI vs. NSMP, $P=0.002$; Figure 2C). NSMP tumors demonstrated more TIA-1⁺ lymphocytes compared to p53-mutant tumors (NSMP vs. p53, $P=0.023$). The numbers of CD27⁺ and T-bet⁺ cells did not significantly differ between the four molecular subgroups. Data from the combined analyses supported the increased density of CD45RO⁺ and TIA-1⁺ cells within *POLE*-mutant/MSI tumors (Supplementary Figure 2B).

Increase in infiltration of PD-1⁺ and PD-L1⁺ lymphocytes in *POLE*-mutant and MSI tumors

The increased lymphocytic infiltrate of *POLE*-mutant and MSI tumors, in combination with their expected ultramutated (*POLE*-mutant tumors) or hypermutated (MSI tumors) status, prompted us to investigate the presence of PD-1⁺ and PD-L1⁺ cells within this cohort (Figure 3A).

The tumor center of *POLE*-mutant and MSI tumors harbored high numbers of PD-1⁺ immune cells (*POLE* vs. NSMP, $P<0.001$; *POLE* vs. p53, $P=0.050$; MSI vs. NSMP, $P=0.003$; Figure 3B). This was supported by the combined analysis (Supplementary Figure 3A). Based on a median of 14.0 PD-1⁺ cells/core in all patients, 73% of *POLE*-mutant, 69% of MSI, 31% of NSMP and 48% of p53-mutant tumors were categorized as highly infiltrated with PD-1⁺ cells.

POLE-mutant and MSI tumors showed markedly increased infiltration of PD-L1⁺ immune cells within the tumor center compared to NSMP and p53-mutant tumors (*POLE* vs. NSMP, $P<0.001$; *POLE* vs. p53, $P<0.001$; MSI vs. NSMP, $P<0.001$; MSI vs. p53, $P=0.002$; Figure 3C). The combined analysis showed similar results (Supplementary Figure 3B). In total, 100% of *POLE*-mutant, 71% of MSI, 18% of NSMP and 29% of p53-mutant tumors were categorized as PD-L1-positive (based on the immune score). Strikingly, only one tumor sample, a p53-mutant EC, contained PD-L1 expressing tumor cells (noted as a positive tumor score, data not shown).

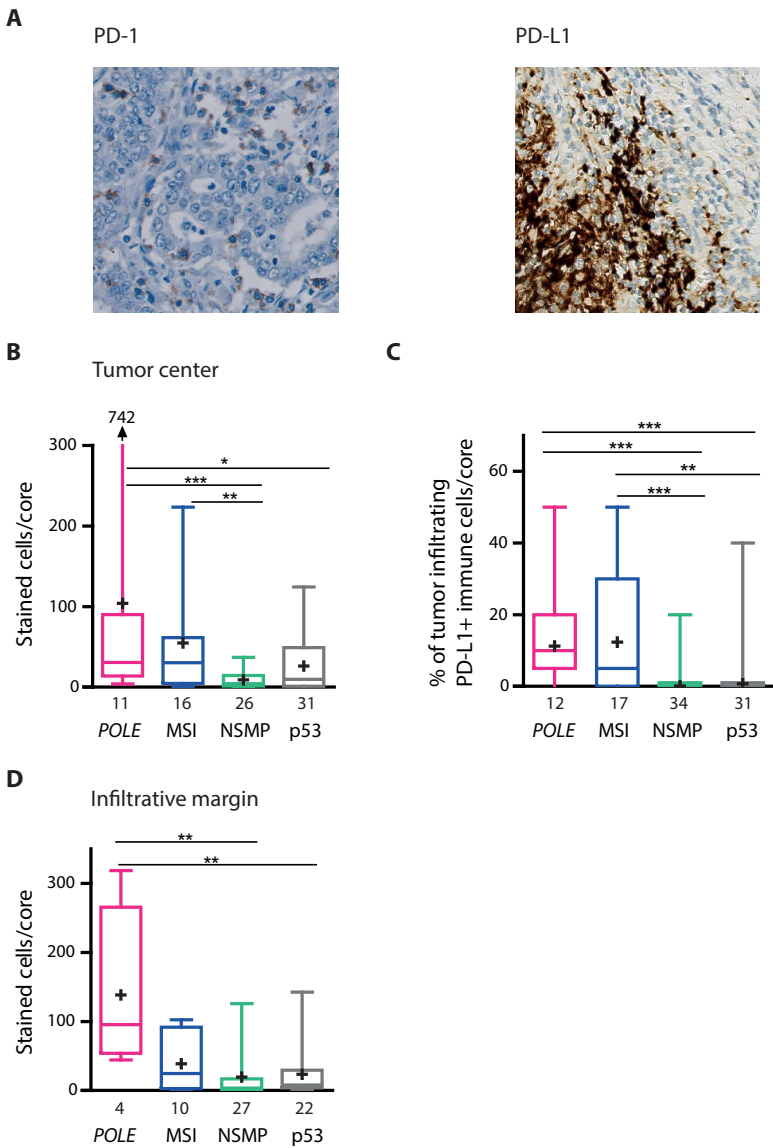


Figure 3. Infiltration of PD-1⁺ and PD-L1⁺ cells in *POLE*-mutant, MSI, NSMP and p53-mutant endometrial cancers.

(A) Representative immunohistochemical stainings of PD-1⁺ and PD-L1⁺ cells. (B) Average number of PD1⁺ cells counted per core within the tumor center, corrected for the number of cells present. (C) Percentage of PD-L1-positive tumor-infiltrating immune cells within the tumor core and infiltrative margin core. (D) Average number of PD-1⁺ stained cells counted per core within the infiltrative margin, corrected for the number of cells present. The numbers of cases analyzed for each molecular subgroup are listed below the x-axis. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Within the infiltrative margin, only the *POLE*-mutant subgroup showed high densities of PD-1⁺ immune cells (*POLE* vs. NSMP, $P=0.008$; *POLE* vs. p53, $P=0.007$; Figure 3D). Combined analysis supported the presence of high numbers of PD-1⁺ cells within the *POLE*-mutant/MSI group compared to the NSMP/p53-mutant group (Supplementary Figure 3C).

PD-L1 is preferentially expressed on myeloid cells

Recently, several studies have shown PD-L1 expression on tumor-associated myeloid cells.^{1,37-40} Therefore, to determine whether this was also the case for our cohort, we performed two multi-color immunofluorescence stainings on consecutive whole slides of a highly infiltrated *POLE*-mutant tumor sample using the following combinations of monoclonal antibodies: CD68 - CD163 – epithelial cell marker cytokeratin, and PD-L1 - PD-1, respectively (Figure 4). CD68⁺ and/or CD163⁺ myeloid cells (including macrophages and myeloid dendritic cells) were found in the stromal regions within the center of the tumor, demarcated by the cytokeratin⁺ tumor cells (Figure 4A). PD-1⁺ and PD-L1⁺ cells were seen in close proximity, also predominantly located in the intratumoral stromal areas (Figure 4B). A co-immunofluorescent staining of PD-1 and CD8 shows frequent co-localization, indicating that PD-1 can be expressed by (cytotoxic) T cells (Supplementary Figure 4). PD-L1 expression co-localized with CD68⁺ and CD163⁺, supporting the idea that in our cohort PD-L1 is not mainly expressed by tumor cells but by myeloid cells (Figure 4C-D).

TCGA RNA sequencing data demonstrates higher expression of CD8A, CD3E, ITGAE (CD103), MS4A1 (CD20), PTPRC (CD45RO), CD27, TBX21 (T-bet) and PDCD1 (PD-1) in *POLE*-mutant and MSI tumors

Next, we compared our data with the expression of above-described immune markers in The Cancer Genome Atlas (TCGA) cancer cohort, which was originally used to devise the molecular classification of EC (Figure 5).¹⁹ Previously we have shown higher expression of, among others, CD3E, CD8A, TBX21 (T-bet) and PDCD1 (PD-1) in *POLE*-mutant compared to MSI and MSS tumors.²⁰ We now extend this analysis to specifically compare the four proposed prognostic subgroups.¹⁹ Of the 244 informative samples, the TCGA cohort included 18 *POLE*-mutant, 69 MSI, 96 NSMP and 62 *TP53*-mutant. Analysis of the RNA sequencing data of this cohort demonstrated higher expression of CD8A, CD3E, ITGAE (CD103), MS4A1 (CD20), PTPRC (CD45RO), CD27, TBX21 (T-bet) and PDCD1 (PD-1) in *POLE*-mutant and MSI tumors compared to NSMP and *TP53*-mutant. TIA-1 expression did not differ between the four molecular subgroups. *POLE*-mutant ECs showed a trend towards increased expression of CD274 (PD-L1; $P=0.057$).

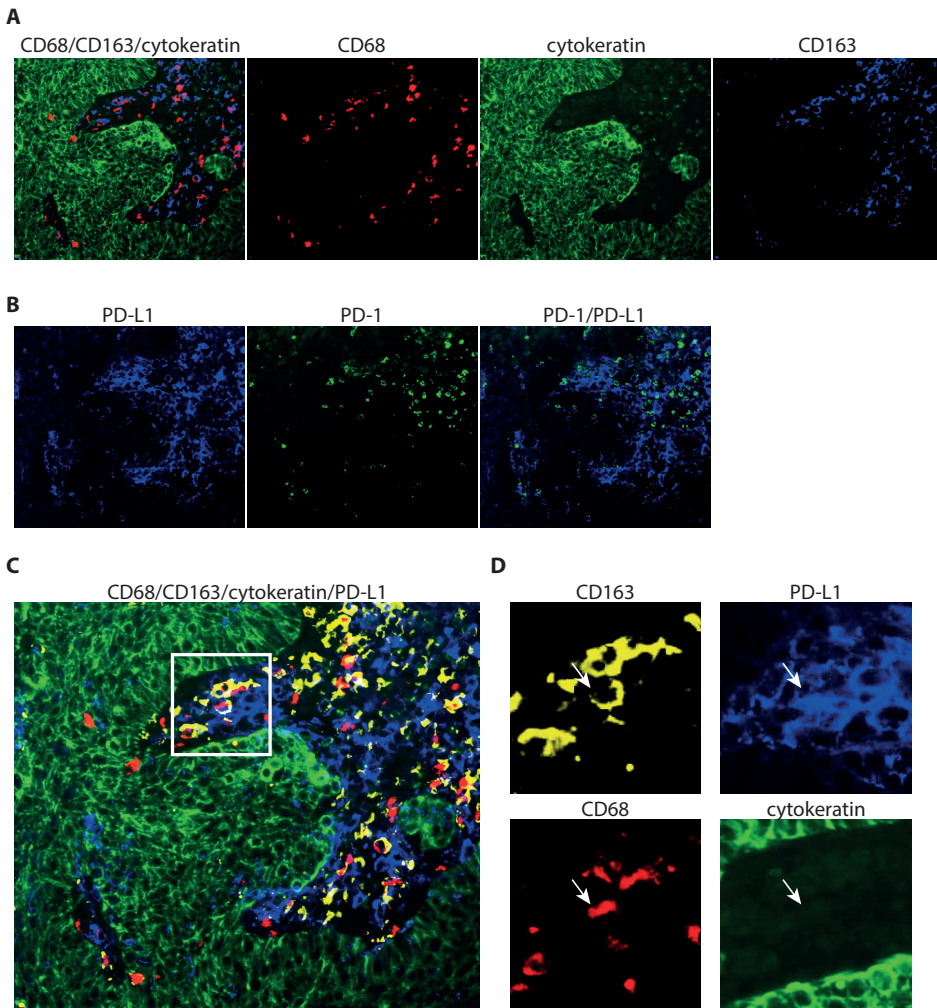


Figure 4. Immunofluorescent stainings of PD-1, PD-L1 and myeloid markers. Representative image of a *POLE*-mutant endometrial cancer stained with keratin (green) - CD163 (blue) - CD68 (red; A), and PD-1 (green) - PD-L1 (blue; B). The two triple immunofluorescent stainings from A and B, performed on sequentially cut slides, are layered (C), with single channel markers for the inset (D), with keratin (green), PD-L1 (blue), CD68 (red) and CD163 (yellow), demonstrating the co-localization of PD-L1 with myeloid markers.

Patients with *POLE*-mutated and MSI tumors have higher numbers of predicted neoantigens, regardless of their immune infiltration status

The presence of a subset of *POLE*-mutant and MSI tumors with a relatively low immune infiltration and NSMP and *TP53*-mutant tumors with a relatively high immune infiltration led us to evaluate the relationship between immune infiltrate and numbers of predicted neoantigens within the TCGA cohort (Figure 6). First of all, we demonstrated the pres-

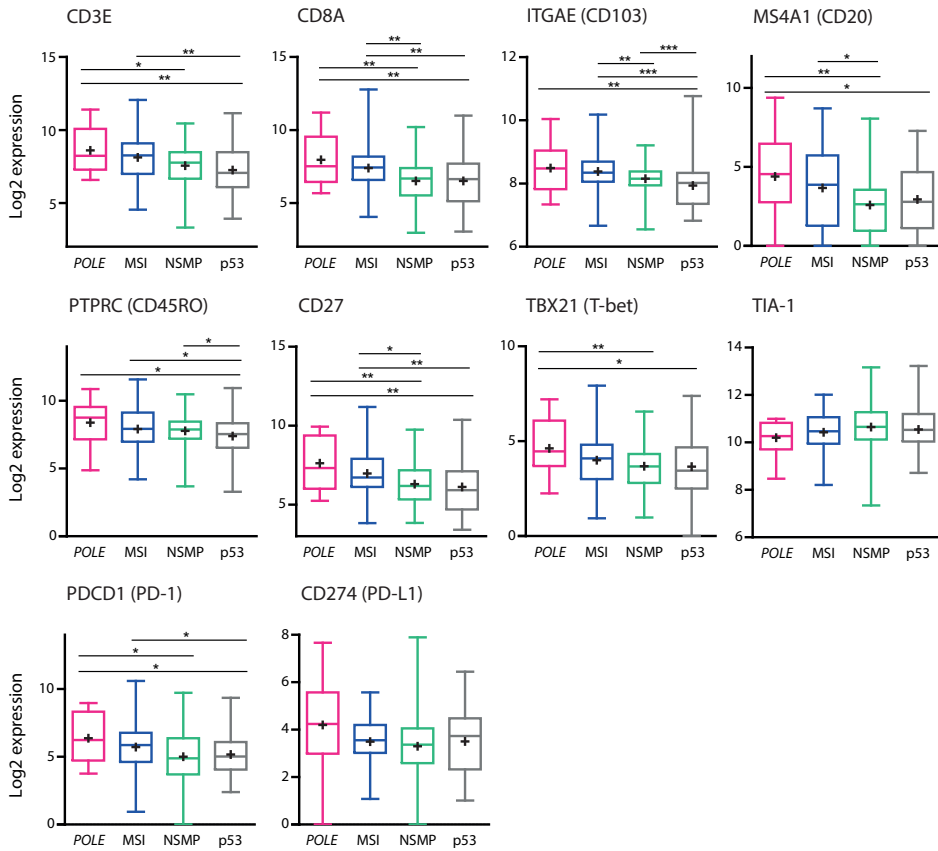


Figure 5. Expression of immune markers according to tumor molecular subtype in TCGA series. RSEM normalized RNAseq data were $\log_2$ transformed and analyzed according to tumor molecular subtype. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ence of higher numbers of expected neoantigens in *POLE*-mutant and MSI tumors compared to NSMP and *TP53*-mutant tumors (Figure 6A). The molecular subgroups were dichotomized according to CD8A expression from RNAseq data, with high infiltration defined as expression above the median of the respective molecular subgroup. Subsequently, we quantified predicted neoantigens for high- and low-infiltrated tumors within the molecular subgroups (Figure 6B). No differences were found in the numbers of predicted neoantigens between samples with high or low CD8A expression within the molecular subgroups.

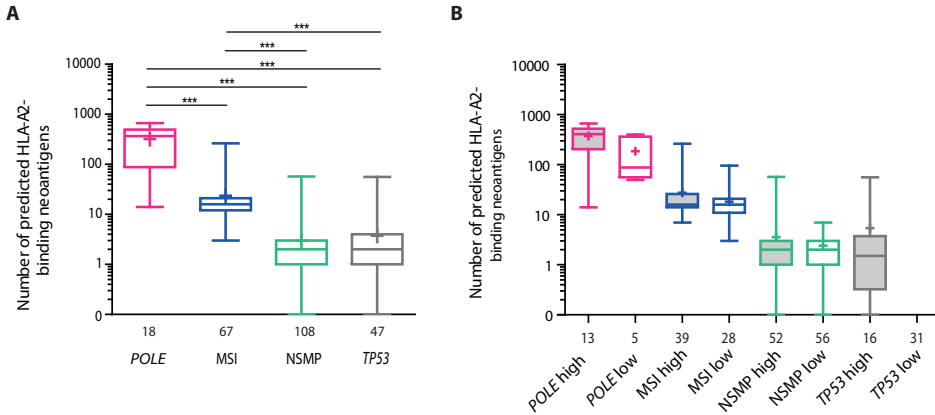


Figure 6. Predicted number of HLA-A2-binding neoantigens across the four molecular subgroups in The Cancer Genome Atlas endometrial cancer cohort.

(A) Comparison between the number of predicted HLA-A2 binding neoantigens in *POLE*-mutant, MSI, NSMP and *TP53*-mutant subgroups based on RNAseq. (B) Comparison between patients with high and low infiltration (based on CD8A expression from RNAseq, relative to median within the group) of lymphocytes within *POLE*-mutant, MSI, NSMP and *TP53*-mutant subgroups. The numbers of cases analyzed for each molecular subgroup are listed below the x-axis. Boxes represent the interquartile range (IQR), with upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

In this study we demonstrate the presence of high numbers of tumor-infiltrating T cells in *POLE*-mutant and MSI tumors, both predicted to be neoantigen-rich, from a clinically relevant cohort of high-risk EC patients. Moreover, these two molecular subtypes harbor high densities of PD-1- and PD-L1-expressing immune cells, rendering them attractive candidates for immune checkpoint inhibition strategies.

The presence of a prominent immune infiltrate in *POLE*-mutant and MSI high-risk EC is in concordance with our previous findings in a preselected cohort including 47 *POLE*-mutant, 49 microsatellite-unstable and 54 microsatellite-stable tumors, in which we demonstrated that *POLE*-mutant tumors, and to a lesser extent MSI tumors, are characterized by a robust intratumoral T cell response.²⁰ These initial findings have recently been extended to other unselected EC cohorts, in which high densities of peritumoral and tumor-infiltrating T lymphocytes have been described in *POLE*-mutant tumors.^{21,22,41} High expression of PD-1 and PD-L1 on intraepithelial immune cells in *POLE*-mutant and MSI ECs has previously been suggested by Howitt et al., albeit in a cohort which included only three *POLE*-mutant cases.²¹ An interesting difference between the data presented by Howitt et al. and the current study, is the expression of PD-L1 on tumor cells. Howitt

et al. describe that 20% of ECs (*POLE*-mutant, MSI and MSS) show PD-L1⁺ tumor cells, whereas within our high-risk cohort only 1 out of 116 tumors showed any expression of PD-L1 on the tumor cells (using the same PD-L1 antibody). Our use of tissue microarrays may have led to an underestimation of PD-L1⁺ tumor cells, as PD-L1 expression is known to be heterogeneously distributed.⁴² Moreover, consecutive full slides of one *POLE*-mutant case were stained using multi-color immunofluorescence: PD-L1 expression was predominantly found in the intratumoral stromal regions in close proximity with PD-1⁺ cells. Furthermore, PD-L1 expression co-localized with CD68⁺ and CD163⁺, suggesting that in this case PD-L1 is primarily expressed by myeloid cells rather than tumor cells. PD-L1⁺ immune cells have previously been described by (among others) Heeren et al. and Herbst et al.; the latter also showed that PD-L1 positivity on immune cells, but not on tumor cells, was associated with response to immune checkpoint inhibition.^{1,38}

Comparisons of outcomes from our immunohistochemical analyses in the TransPORTEC high-risk cohort and analyses of the RNA sequencing data from The Cancer Genome Atlas (TCGA) showed similar results for five out of ten markers, namely CD3, CD8, CD103, CD45RO and PD-1. The immunohistochemical analyses of the TransPORTEC cohort did not reveal significant differences in numbers of CD20⁺ and CD27⁺ cells between the four molecular subgroups, while analysis of the TCGA cohort demonstrated increased expression of CD20⁺ and CD27⁺ cells within the *POLE*-mutant and MSI subgroups. This inconsistency may be attributed to the use of a TMA for immunohistochemical analyses of CD20⁺ and CD27⁺ cells. These immune cells frequently reside in tertiary lymphoid structures in the myometrium, which are frequently seen in *POLE*-mutant tumors.^{20,43-45} The areas containing these structures may not have been present in the TMA. Secondly, outcomes regarding TIA-1-, T-bet- and PD-L1-positivity were discordant. These differences may be due to the known discrepancy between mRNA and protein expression.⁴⁶ Another possible explanation for these discrepancies may be the relatively high proportion of clear cell EC (15.5%) within the TransPORTEC high-risk cohort, while only endometrioid, serous and mixed histologies were included in the TCGA study.

The presence of high numbers of CD8⁺ and PD-1⁺ cells in *POLE*-mutant and MSI tumors may suggest the presence of high numbers of tumor-specific T cells targeting neoantigens within these subgroups of patients. Similarly, our analysis of the TCGA EC cohort demonstrates that *POLE*-mutant and MSI tumors are characterized by a significantly higher number of mutations predicted to result in major histocompatibility complex-binding neoantigens, and a correspondingly higher number of tumor-infiltrating CD8⁺ T cells, as assessed by CD8A mRNA levels. This link between neoantigen accumulation and infiltration by immune cells is supported by a recent genomic characterization of colorectal cancers, in which an association between high neoantigen load, overall lym-

phocytic infiltration, tumor-infiltrating lymphocytes and survival was demonstrated.¹⁰

Surprisingly, the number of predicted immunogenic mutations did not directly reflect the levels of CD8A mRNA expression within each molecular subgroup (Figure 6). Similarly, in our immunohistochemical analysis, we found MSI tumors, expected to be neoantigen-rich, with almost no signs of CD8⁺ T cell infiltration, and p53-mutant tumors, expected to have low numbers of neoantigens, with an enhanced intratumoral immune response. One explanation for this apparent discrepancy between immune infiltration and the number of predicted neoantigens could be that the nature (i.e. clonal versus subclonal) of the neoepitopes, instead of the crude number of predicted neoantigens, determine the degree of immune response.¹³ Another explanation may be that within our analyses only predicted binding to HLA-A*02:01 was taken into account rather than to individual HLA alleles. Furthermore, immune responses may be impeded by impairment of MHC class I expression due to mutations in HLA, β 2-microglobulin and JAK-1 in highly mutated ECs.^{20,47} Therefore, a logical next step in understanding the interaction between neoepitopes and immune response within the four molecular subgroups would be the direct identification and characterization of tumor-specific T cells targeting these neoantigens, as has recently been performed by Gros et al. in melanoma.²⁴

With regard to the p53-mutant tumors with an enhanced antitumor immune response despite low expected neoantigen load, we hypothesize that this response may be aimed at self-antigens or cancer/testis antigens instead of neoepitopes. Taking into account their unfavorable survival outcomes, further investigation of the highly infiltrated p53-mutant subset will be of great interest as this may provide new insight in the selection of candidates for immune checkpoint therapies.

The data on mutational load, neoantigens and immune infiltration reported by us and others suggest that checkpoint inhibition may be a strategy of particular interest for treating advanced stage patients with *POLE*-mutant and MSI tumors. Recent case reports provide proof of principle by demonstrating the efficacy of anti-PD-1 inhibitors in a limited number of advanced stage *POLE*-mutant or mismatch repair-deficient cancers.^{7,25,26} Moreover, a Phase II trial evaluating immune related objective responses to pembrolizumab in patients with or without mismatch repair (MMR) deficiency, demonstrated objective responses in 40% of patients with MMR-deficient colorectal cancer and 71% of patients with MMR-deficient non-colorectal cancers (including two EC). Contrastingly, no objective responses were observed in the MMR-proficient colorectal cancers. Moreover, data from this study adds to the growing body of evidence suggesting that high numbers of somatic mutations (in this case due to MMR deficiency) and high numbers of predicted neoantigens play an important role in the sensitivity to checkpoint inhibi-

tion.^{11-13,15} Furthermore, an in-depth analysis of patients treated with anti-PD-1 therapy prioritized PD-L1 expression as being the most closely associated with objective tumor regression.⁴⁸ Further analyses of non-responders may uncover other mutations affecting epitope presentation, T cell infiltration and response to checkpoint inhibition.

From a clinical point of view, as checkpoint inhibitors are associated with significant costs and potential toxicities, it is essential to select individual patients that will benefit from these therapies. Patients with low/intermediate-risk disease carrying *POLE* mutations have an excellent prognosis under standard treatment, and therefore checkpoint inhibition is unlikely to be appropriate for this group.^{19,49-51} However, (although infrequently occurring) *POLE*-mutant and MSI patients with recurring or metastatic disease are possible candidates.^{7,25,26} Clinical trials, in which high-risk EC patients are grouped according to molecular subtype, will be required to determine clinical benefit of immunotherapy.

Importantly, the data thus far regarding *POLE*-mutant EC may be applicable to other tumor types harboring *POLE* mutations. While *POLE* mutations are found in 7-12% of EC, they are also found in other malignancies including colorectal cancers, cancers of the brain, breast, pancreas and stomach, albeit at lower frequencies.^{19,27,49,52-57} Although a prognostic advantage of this mutation has now been established in glioblastoma and stage II/III colorectal cancer, patients with recurrent or metastatic hypermutated disease may also benefit from immunotherapeutic strategies such as checkpoint inhibitors as proposed for EC.^{7,56,58} Basket trials stratifying patients according to tumor molecular alterations such as *POLE* mutations should be initiated to investigate whether these patients may also benefit from checkpoint inhibition.

In summary, taking into account the strong immune infiltration, high numbers of PD-1⁺ and PD-L1⁺ lymphocytes, large numbers of somatic mutations and neoantigens, and the recently demonstrated clinical efficacy in these cohorts of patients, *POLE*-mutant and MSI tumors are expected to benefit from checkpoint inhibition.^{21,25,59}

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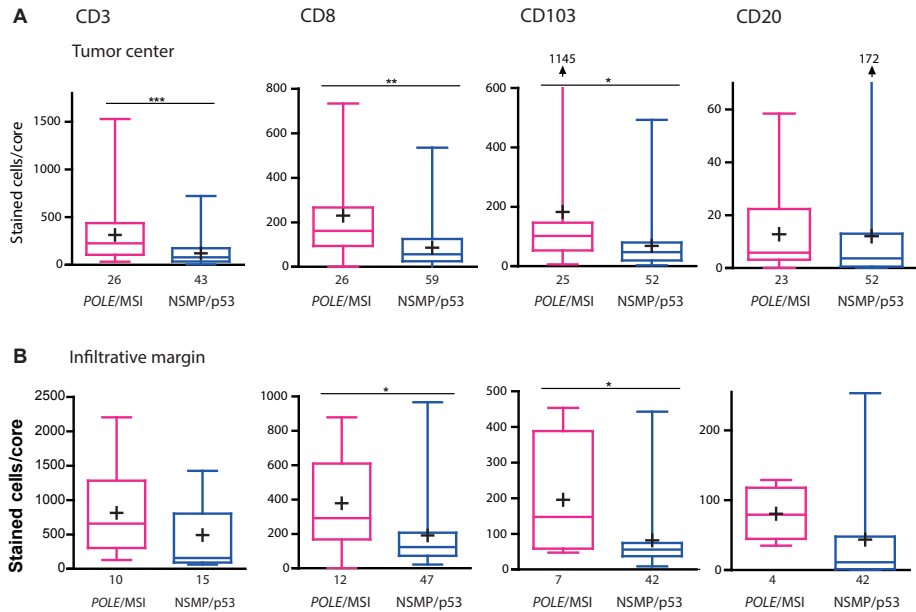
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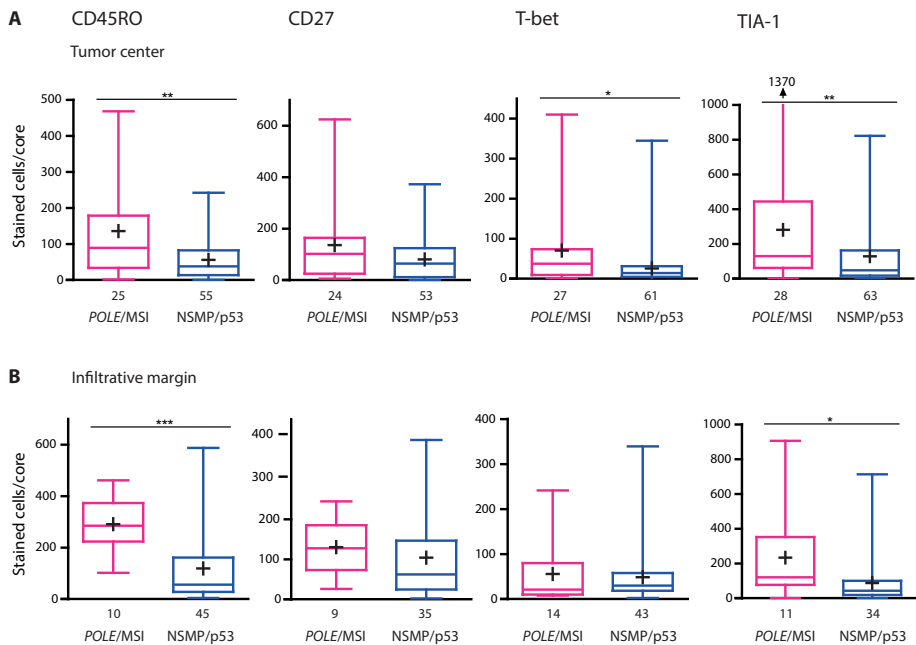
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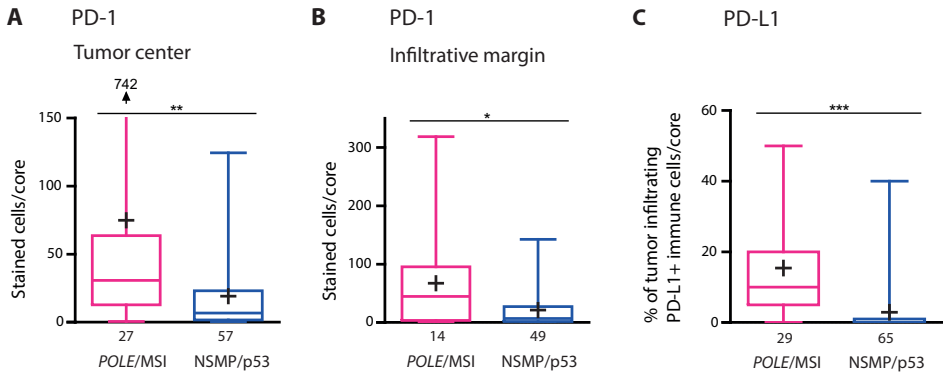
Supplementary Figure 1. Infiltration of CD3⁺, CD8⁺, CD103⁺ and CD20⁺ cells in *POLE*-mutant/MSI compared to NSMP/p53-mutant endometrial cancers.

(A) Average number of positively stained cells for each of the markers in the above panel, counted per core within the tumor center, corrected for the number of cells present. (B) Average number of positively stained cells for each of the markers in the above panel, counted per core within the infiltrative margin, corrected for the number of cells present. The numbers of cases analyzed for each molecular subgroup are listed below the x-axis. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



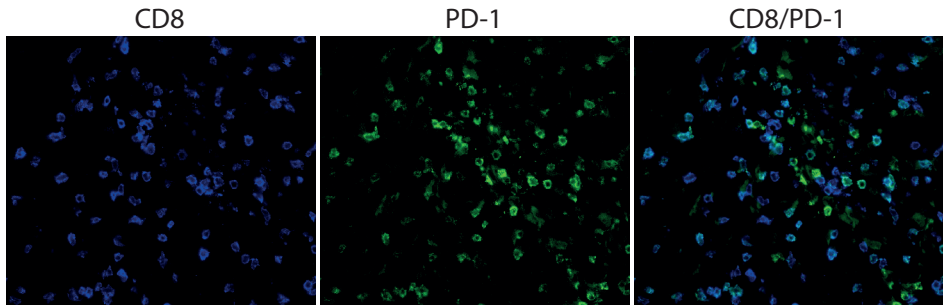
Supplementary Figure 2. Infiltration of CD45RO⁺, CD27⁺, T-Bet⁺ and TIA-1⁺ cells in *POLE*-mutant/MSI compared to NSMP/p53-mutant endometrial cancers.

(A) Average number of positively stained cells for each of the markers in the above panel, counted per core within the tumor center, corrected for the number of cells present. (B) Average number of positively stained cells for each of the markers in the above panel, counted per core within the infiltrative margin, corrected for the number of cells present. The numbers of cases analyzed for each molecular subgroup are listed below the x-axis. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Supplementary Figure 3. Infiltration of PD-1⁺ and PD-L1⁺ cells in *POLE*-mutant/MSI compared to NSMP/p53-mutant endometrial cancers.

(A) Average number of PD-1⁺ stained cells counted per core within the tumor center, corrected for the number of cells present. (B) Average number of PD-1⁺ stained cells counted per core within the infiltrative margin, corrected for the number of cells present. (C) Percentage of tumor-infiltrating immune cells with moderate to strong PD-L1 expression per core within cores taken from the tumor and infiltrative margin, corrected for the number of cells present. The number of cases analyzed for each molecular subgroup are listed below the x-axis. Boxes represent the interquartile range (IQR), with the upper whisker indicating the 75th percentile and the lower whisker the 25th percentile. The median and mean values are indicated by a horizontal line and cross, respectively. Abbreviations: *POLE*, *POLE*-mutant; MSI, microsatellite-unstable; NSMP, no specific molecular profile; p53, p53-mutant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Supplementary Figure 4. Immunofluorescent staining of CD8 and PD-1.

Representative image of a *POLE*-mutant endometrial cancer stained with CD8 and PD-1, demonstrating co-localization of CD8 and PD-1 on immune cells.



Chapter 6

Adjuvant treatment for *POLE* proofreading domain-mutant cancers: sensitivity to radiotherapy, chemotherapy and nucleoside analogs

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ABSTRACT

Objective

Pathogenic *POLE* proofreading domain mutations are found in many malignancies where they are associated with ultramutation and favorable prognosis. The extent to which this prognosis depends on their sensitivity to adjuvant treatment is unknown, as is the optimal therapy for advanced-staged or recurrent *POLE*-mutant cancers.

Methods

We examined the recurrence-free survival of women with *POLE*-mutant and *POLE*-wild-type endometrial cancers in the observation arm of the randomized PORTEC-1 endometrial cancer trial (N=245 patients with stage I endometrial cancer for analysis). Sensitivity to radiotherapy and selected chemotherapeutics was compared between *Pole*-mutant mouse-derived embryonic stem (mES) cells, generated using CRISPR-Cas9 (*Pole* mutations D275A/E275A, and cancer-associated P286R, S297F, V411L) and isogenic wild-type cell lines.

Results

In the observation arm of the PORTEC-1 trial (N=245), women with *POLE*-mutant endometrial cancers (N=16) had an improved recurrence-free survival (10-year recurrence-free survival 100% vs. 80.1% for *POLE*-wild-type; HR, 0.143; 95% confidence interval, 0.001-0.996; $P=0.049$). *Pole* mutations did not increase sensitivity to radiotherapy nor to chemotherapeutics in mES cells. In contrast, *Pole*-mutant cells displayed significantly increased sensitivity to cytarabine and fludarabine (IC_{50} *Pole* P286R-mutant vs. wild-type: 0.05 vs. 0.17 μ M for cytarabine, 4.62 vs. 11.1 μ M for fludarabine; $P<0.001$ for both comparisons).

Conclusions

The favorable prognosis of *POLE*-mutant cancers cannot be explained by increased sensitivity to currently used adjuvant treatments. These results support studies exploring minimization of adjuvant therapy for early-stage *POLE*-mutant cancers, including endometrial and colorectal cancers. Conversely, *POLE* mutations result in hypersensitivity to nucleoside analogs, suggesting the use of these compounds as a potentially effective targeted treatment for advanced-stage *POLE*-mutant cancers.

INTRODUCTION

Somatic mutations in the proofreading (exonuclease) domain of DNA polymerase ϵ (encoded by the *POLE* gene) are found in a wide variety of human malignancies, including 7-12% of endometrial cancers, ~1% of colorectal cancers and sporadically in cancers of the brain, stomach, breast, and pancreas.¹⁻⁴ During DNA replication, the proofreading activity of polymerase ϵ recognizes and excises misincorporated nucleotides, thereby increasing replication fidelity. Pathogenic *POLE* proofreading domain mutations (hereafter referred to as '*POLE* mutations') are associated with an exceptionally high number of single nucleotide variants: cancers with such *POLE* mutations often harbor more than 100 mutations/Mb.^{2,5} Despite this so-called 'ultramutated' phenotype, patients with *POLE*-mutant cancers have a favorable prognosis. This has been shown in early-stage endometrial cancer, where impact was particularly strong in high-grade endometrioid tumors, stage II/III colorectal cancer, and was suggested for glioblastomas.^{1,4,6-12} The majority of patients in these studies received additional treatment after surgery. Therefore, we evaluated in an endometrial cancer series and in a cell-based model whether the favorable prognosis of *POLE*-mutant cancers might be caused by increased sensitivity to adjuvant treatment. *POLE* mutations can also be found in advanced-stage disease, where the prognostic impact remains uncertain, and in cancer types with poor prognosis such as glioblastomas. Because these *POLE* mutations reside in the proofreading domain that excises non-matched nucleotides, we investigated the efficacy of nucleoside analogs as potential targeted therapies for advanced-stage *POLE*-mutant cancers.

MATERIALS AND METHODS

Endometrial cancer patient cohort

Clinical data of women with endometrial cancer who did not receive adjuvant treatment (N=369) were collected from the randomized PORTEC-1 trial, which compared external beam radiotherapy with observation in stage I endometrial cancer.¹³ *POLE* mutational status was determined as described previously⁷ with the addition of screening *POLE* exon 14 using Sanger sequencing (primers available upon request). For 119 endometrial cancers, *POLE* mutational status could not be determined, because screening of exons 9, 13 and 14 failed or because no material was available. Twenty-three cases were designated '*POLE*-wild-type' despite failure of exon 14 sequencing, based on screening of *POLE* exons 9 and 13. Women with endometrial cancers harboring a *POLE* mutation but also another relevant molecular alteration (i.e. p53 mutation or mismatch repair deficiency, based on previous analyses¹⁴) were excluded (*POLE*-mutant & p53-mutant, n=3; *POLE*-mutant, p53-mutant, mismatch repair-deficient, n=2), in accordance with our

previous publication.¹⁴ This resulted in 245 endometrial cancer patients for analysis. Details on the specific *POLE* mutations identified in the endometrial cancers can be found in Supplementary Table 1.

Generation of the cell-based model

Different *POLE* proofreading domain mutations (P286R, S297F, V411L, and D275A/E277A) were recreated in wild-type mouse-derived embryonic stem (mES) cell lines using CRISPR-Cas9. All cell lines in this study were derived from wild-type primary diploid 129/OLA mouse-derived embryonic stem cell line E14.¹⁵ Cell lines were cultured for three to six weeks from thawing to use in experiments. Cell culture was tested every other month for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (LT07-318; Lonza, Switzerland). As this study is limited to experiments with mES cells, no live animals were used, an Institutional Animal Care and Use Committee approval was not required.

In order to make these mES cells *Pole*-mutant, a CRISPR guide RNA was designed for each mutation using the web resources available at <http://crispr.mit.edu>. Pairs of these oligonucleotides (Supplementary Table 2) were ordered from Integrated DNA Technologies (Leuven, Belgium), annealed, and, after BbsI digestion (New England Biolabs), cloned into a modified expression plasmid pX330 (Addgene #42230; now containing the CRISPR-Cas9 system and a puromycin-resistance marker). Plasmids were isolated using an endotoxin-free Midiprep kit (Qiagen), and gRNA inserts were verified by Sanger sequencing. As template for homologous recombination, PCR fragments (~2.4 kbp) containing the specific *Pole* proofreading domain mutations as well as silent mutations in the PAM sequences were generated as described previously (oligonucleotides in Supplementary Table 3).¹⁶ Introduction of the specific substitutions was confirmed by Sanger sequencing.

Wild-type mES cells were transfected with the mutation-specific Cas9- and CRISPR guideRNA-expressing plasmid and PCR-based repair template using Lipofectamine 2000 (#11668019, Thermo Fisher Scientific). For this transfection, the manufacturer's protocol was followed with minor alterations: use of knockout DMEM, more Lipofectamine for each transfection (14.4 µl in 125 µl medium), a higher DNA input (3–4 µg), and a 20 minute incubation of diluted DNA in Lipofectamine at room temperature. Puromycin (1.5 µg/ml for two days; A11138-03, Thermo Fisher Scientific) was added to the medium for positive selection of transfected cells. Drug-resistant cells were seeded at a low density. Independent clones were manually picked, expanded and reseeded as single cells. Isolated clones were again manually picked to ensure purity of the resultant cell line. To identify targeted clones, DNA and RNA were isolated from drug-resistant clones and

regions were amplified by PCR (oligonucleotides sequences in Supplementary Table 4). *Pole* proofreading domain mutations were designed to yield a restriction site; therefore, PCR products amplified from genomic DNA were subjected to restriction site digestion to verify knockin (BtgZI for D275A/E277A, BstUI for P286R, MbolI for S297F, and AclI for V411L; all New England Biolabs). Amplified genomic DNA and cDNA products of clones with an introduced restriction site were purified, and mutations were confirmed by Sanger sequencing analysis.

For each *Pole*-mutant mES cell line, metaphase spreads were made, and fluorescence in situ hybridization was performed using biotin-16-dUTP-labelled or digoxigenin-11-dUTP-labeled (#11093070910 and #11093088910 respectively, Roche) bacterial artificial chromosome clones RP24-154A19 and RP24-388K6 (BACPAC Resource Center, Chori). Chromosome numbers were equal for all cell lines used in subsequent experiments, and all cell lines contained two copies of the *Pole* allele (Supplementary Figure 1). Spontaneous mutant frequencies of the *Pole*-mutant mES cell lines were determined at the Hypoxanthine Phosphorybosyl Transferase (*Hprt*) gene as described previously.¹⁷ Additional details on these experiments can be found in the Supplementary Methods.

Treatment sensitivity of mES cells

Pole proofreading domain-mutant and wild-type mES cells were treated with 5-fluorouracil (F6627, Sigma Aldrich), methotrexate (S1210, Selleckchem), paclitaxel (S1150, Selleckchem), cytarabine (S1648, Selleckchem), gemcitabine (S1714, Selleckchem), fludarabine (S1229, Selleckchem), cladribine (S1199, Selleckchem), clofarabine (S1218, Selleckchem), doxorubicin, cisplatin, or etoposide. To determine the sensitivity to these compounds, mES cells were seeded in 96-well tissue culture plates (5000 cells/well). The following day, cells were treated with increasing concentrations of each compound and incubated for 24–72 hours (24 hours: cytarabine, gemcitabine, clofarabine, paclitaxel, doxorubicin; 48 hours: cisplatin, cladribine, fludarabine, methotrexate, etoposide; 72 hours: 5-fluorouracil). Seventy-two hours after treatment was initiated, cells were detached with 0.05% trypsin-EDTA and resuspended in PBS containing 2% FCS. Adherent cells were quantified using flow cytometry. Sensitivity to ionizing radiation was determined similarly: mES cells were seeded in 6-well tissue culture plates (50,000 cells/well). The following day, cells were irradiated with a single dose of up to 8 Gy. Seventy-two hours after irradiation, cells were detached with 0.05% trypsin-EDTA and manually counted. In each experiment, for every cell line, cell counts were normalized to counts of vehicle-treated cells. At least three independent experiments were conducted per cell line for each treatment.

Statistical analyses

For PORTEC-1 participants in the observation arm, recurrence-free survival (RFS) was calculated, defined as the time from randomization to recurrence, with censoring at date of last contact or death in patients without recurrence. RFS was evaluated using the Kaplan-Meier method and compared between *POLE*-mutant and *POLE*-wild-type endometrial cancers using a Cox proportional hazards model with Firth's correction, owing to the absence of events in the *POLE*-mutant group.¹⁸ To analyze treatment sensitivity in the cell-based model, IC₅₀ values of mES cell lines were calculated for each experiment by non-linear regression analysis (variable slope) of the normalized cell counts. Outliers in IC₅₀ values were identified with Grubbs test for each cell line. For each *Pole*-mutant cell line, IC50's were compared to IC50's of the isogenic wild-type cell line using independent-samples t-tests (with Holm-Bonferroni correction when indicated). Levene's test for equality of variances was used to determine when to assume equal variances. All statistical tests were two-sided, and a *P* value of less than 0.05 was considered statistically significant; this was adjusted downward in case of Holm-Bonferroni correction. All analyses were performed in R (3.3.0 version, package 'coxphf'), Graphpad Prism 7, or SPSS 23.0 software.

RESULTS

Prognosis of patients with *POLE*-mutant endometrial cancers who did not receive adjuvant treatment

The prognostic significance of *POLE* mutations was evaluated in the observation arm of the randomized PORTEC-1 trial, which included 245 stage I endometrial cancers for analysis (Figure 1).^{7,13,14} None of the women with *POLE*-mutant endometrial cancers (0/16) developed a recurrence, compared to 44 recurrences (19.2%) in 229 patients with *POLE*-wild-type tumors; this difference in RFS was statistically significant (10-year RFS 100% vs. 80.1%; HR, 0.143; 95% confidence interval (CI), 0.001-0.996; *P*=0.049 based on Cox regression with Firth's correction). These results suggest a favorable prognosis of early-stage *POLE*-mutant cancers in the absence of adjuvant treatment, indicating that the increased sensitivity to adjuvant treatment does not explain the good clinical outcome of these tumors.

Treatment sensitivity conferred by *POLE* mutations in a model system

To support the PORTEC-1 data, we investigated the effect of *POLE* mutations on treatment sensitivity in a model system. To this end, we recreated three common somatic, cancer-associated *POLE* proofreading domain mutations (P286R, S297F, and V411L),² as well as a proofreading-deficient positive control (double mutation of exonuclease active sites, D275A/E277A)¹⁹ in isogenic mouse-derived embryonic stem (mES) cell lines using

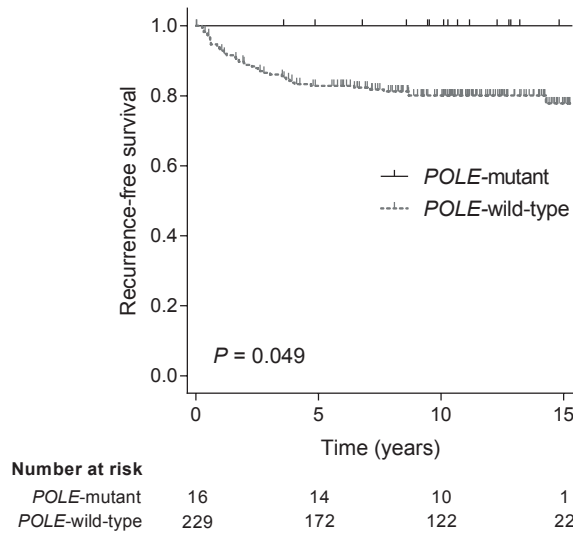


Figure 1. Recurrence-free survival of endometrial cancer patients in the observation arm of the PORTEC-1 study according to *POLE* mutational status.

Among endometrial cancer patients who did not receive adjuvant treatment, none of the women with *POLE*-mutant endometrial cancers developed a recurrence (0/16, 10-year RFS 100%), as compared to 44 recurrences in 229 women with *POLE*-wild-type tumors (19.2%, 10-year RFS 80.1%). This difference in recurrence-free survival was statistically significant (HR, 0.143; 95% CI, 0.001-0.996; $P=0.049$).

CRISPR-Cas9. For each *Pole* mutation, a minimum of two independent homozygous cell lines was used for subsequent experiments. Consistent with the mutational burden in *POLE*-mutant cancers, these *Pole* mutations gave rise to a mutator phenotype in mES cells with the severity of the phenotype depending on the substitution (P286R ~8x higher than wild-type, D275A/E277A, S297F, V411L ~4x; Supplementary Figure 2).

Sensitivity to adjuvant treatments used for cancer types that harbor *POLE* mutations was determined in the mES cells. *Pole* mutations did not result in increased sensitivity to ionizing radiation or to selected chemotherapeutic agents (i.e. 5-fluorouracil, cisplatin, paclitaxel, doxorubicin, etoposide and methotrexate; Table 1, Figures 2 and 3, Supplementary Table 5).

Based on the role of the polymerase ϵ proofreading domain in the recognition and excision of mispaired nucleotides, we investigated nucleoside analogs as potential novel targeted therapies for *POLE*-mutant cancers. Indeed, *Pole* mutations resulted in increased sensitivity to cytarabine and fludarabine: sensitivity to both compounds was significantly increased for the *Pole* D275A/E277A-mutant cell lines, but was even more pronounced in cells with somatic *Pole* hotspot mutations (Figure 4, Table 1, Supplementary Tables 5 and 6). Because somatic *POLE* mutations in cancers are heterozygous, sensitivity to

Table 1. Sensitivity to adjuvant treatment strategies conferred by *POLE* proofreading domain mutations in a cell-based model.

Treatment	Wild-type			<i>Pole</i> D275A/E277A*			<i>Pole</i> P286R*			<i>Pole</i> S297F*			<i>Pole</i> V411L*		
	IC ₅₀ (mean ± SD)	IC ₅₀ (mean ± SD)	P (95% CI)	IC ₅₀ (mean ± SD)	IC ₅₀ (mean ± SD)	P (95% CI)	IC ₅₀ (mean ± SD)	IC ₅₀ (mean ± SD)	P (95% CI)	IC ₅₀ (mean ± SD)	IC ₅₀ (mean ± SD)	P (95% CI)	IC ₅₀ (mean ± SD)	IC ₅₀ (mean ± SD)	P (95% CI)
Ionizing radiation (Gy)	2.96 ± 1.28	2.94 ± 1.02	0.976 (-1.63, 1.67)		3.51 ± 1.36	0.553 (-2.53, 1.45)	2.19 ± 1.21	2.82 ± 0.893	0.390 (-1.16, 2.71)		2.82 ± 0.893	0.866 (-1.70, 1.98)			
5-Fluorouracil(μM)	0.25 ± 0.04	0.23 ± 0.07	0.513 (-0.06, 0.11)		0.31 ± 0.02	0.057 (-0.12, 0.002)	0.30 ± 0.01	0.25 ± 0.01	0.109 (-0.11, 0.01)		0.25 ± 0.01	0.895 (-0.06, 0.06)			
Cisplatin (μM)	0.39 ± 0.17	0.28 ± 0.09	0.265 (-0.10, 0.32)		0.31 ± 0.07	0.403 (-0.12, 0.28)	0.36 ± 0.09	0.19 ± 0.06	0.825 (-0.22, 0.27)		0.19 ± 0.06	0.051 (-0.001, 0.40)			
Doxorubicin (nM)	8.92 ± 4.04	10.7 ± 2.92	0.468 (-7.01, 3.49)		9.21 ± 0.23	0.909 (-5.85, 5.28)	12.0 ± 1.1	7.70 ± 1.73	0.237 (-8.76, 2.51)		7.70 ± 1.73	0.586 (-3.66, 6.11)			
Etoposide (nM)	27.2 ± 15.7	32.7 ± 7.09	0.515 (-23.0, 12.0)		32.8 ± 7.39	0.513 (-23.1, 12.0)	27.5 ± 7.05	26.6 ± 10.6	0.971 (-15.9, 15.4)		26.6 ± 10.6	0.937 (-15.6, 16.9)			
Methotrexate (nM)	60.1 ± 8.48	70.7 ± 28.1	0.401 (-38.0, 16.9)		85.6 ± 24.6	0.043 (-50.1, -0.97)	74.2 ± 11.1	75.7 ± 28.2	0.069 (-29.6, 1.46)		75.7 ± 28.2	0.228 (-43.1, 12.0)			
Paclitaxel (nM)	9.57 ± 2.94	13.7 ± 3.41	0.084 (-9.04, 0.70)		10.9 ± 3.00	0.490 (-5.53, 2.86)	6.64 ± 0.59	9.14 ± 0.70	0.039 (0.19, 5.68)		9.14 ± 0.70	0.719 (-2.30, 3.15)			
Cytarabine (μM)	0.17 ± 0.02	0.13 ± 0.03	0.027 [‡] (0.006, 0.07)		0.05 ± 0.01	<0.001 [‡] (0.09, 0.15)	0.05 ± 0.01	0.04 ± 0.004	<0.001 [‡] (0.09, 0.15)		0.04 ± 0.004	<0.001 [‡] (0.10, 0.16)			
Fludarabine (μM)	11.1 ± 3.98	6.82 ± 0.69	0.012 [‡] (1.23, 7.40)		4.62 ± 1.50	0.001 [‡] (3.35, 9.68)	3.40 ± 0.51	6.29 ± 0.43	<0.001 [‡] (4.66, 10.8)		6.29 ± 0.43	0.006 [‡] (1.77, 7.92)			

*Sensitivity to treatment of mouse embryonic stem cell lines with different homozygous *Pole* proofreading domain mutations (D275A/E277A, P286R, S297F, V411L, respectively) is shown. For each *Pole* mutation, mean IC₅₀ and standard deviation (SD) of one cell line are provided (results of additional cell lines in Supplementary Table 5). IC₅₀s of the *Pole*-mutant cell line are compared to IC₅₀s of the *Pole*-wild-type cell line; the corresponding *P*-values and 95% confidence intervals (CI) are shown.

[‡]Difference remains significant after Holm-Bonferroni correction for multiple comparisons.

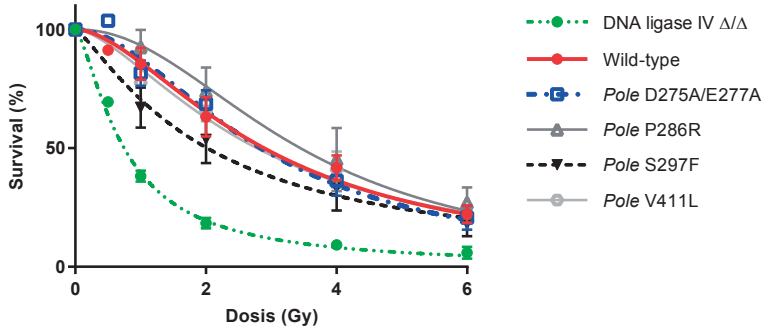


Figure 2. Sensitivity to ionizing radiation in *Pole*-mutant and *Pole*-wild-type cell lines.

Non-linear regression of relative survival after ionizing radiation is shown for homozygous *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *Pole* D275A/E277A double mutation results in proofreading deficiency.¹⁹ The *Pole* P286R, S297F, and V411L mutations are somatic *POLE* hotspot mutations.² As a radiosensitive positive control cell line, a DNA ligase IV knockout mouse-derived embryonic stem cell line (DNA ligase IV Δ/Δ) was used. Sensitivity to ionizing radiation did not significantly differ between *Pole*-mutant and *Pole*-wild-type cells (for details on these comparisons see also Table 1). Dots and error bars indicate mean and standard error of the mean respectively.

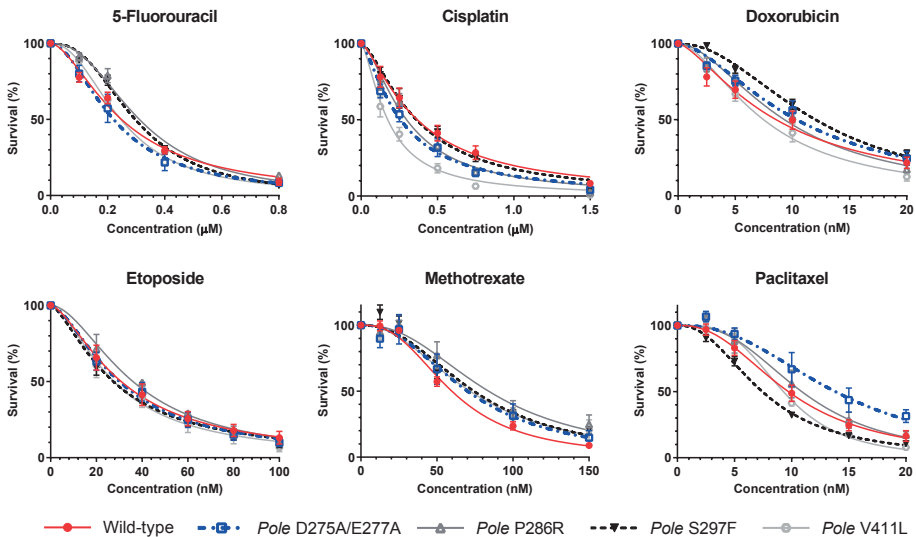


Figure 3. Sensitivity to different chemotherapeutic agents in *Pole*-mutant and *Pole*-wild-type cell lines.

Non-linear regression of relative survival after treatment with chemotherapeutic agents is shown for homozygous *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *Pole* D275A/E277A double mutation results in proofreading deficiency.¹⁹ The *Pole* P286R, S297F, and V411L mutations are somatic *POLE* hotspot mutations.² Sensitivity to 5-fluorouracil, cisplatin, doxorubicin, and etoposide did not significantly differ between *Pole*-mutant and *Pole*-wild-type cells. For methotrexate, a significant difference was found between IC_{50} s of the *Pole* P286R-mutant cell line and the *Pole*-wild-type cell line. For paclitaxel, a significant difference was found between IC_{50} s of the *Pole* S297F-mutant and the *Pole*-wild-type cell lines. These differences in both methotrexate and paclitaxel sensitivity did not remain significant after correction for multiple comparisons (for details on these comparisons, see also Table 1). Dots and error bars indicate mean and standard error of the mean, respectively.

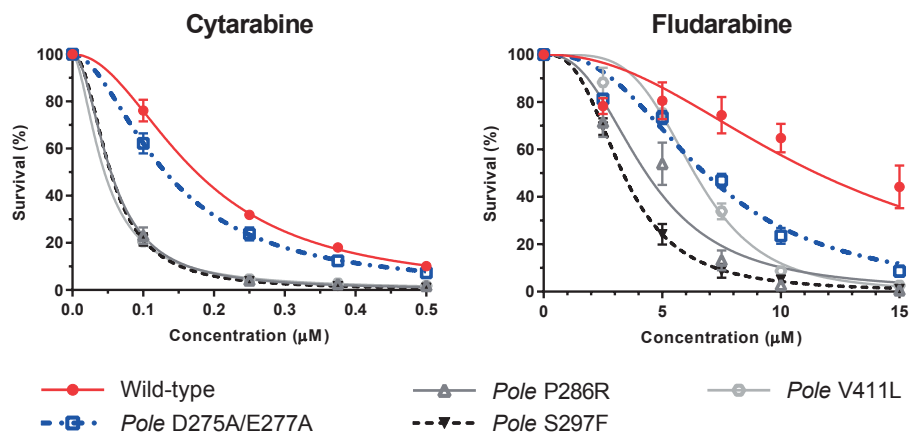


Figure 4. Cytarabine and fludarabine sensitivity in *Pole*-mutant and *Pole*-wild-type cell lines.

Non-linear regression of relative survival after nucleoside analog treatment is shown for *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *POLE* D275A/E277A double mutation results in proofreading deficiency.¹⁹ The *Pole* P286R, S297F, and V411L mutations are somatic *POLE* hotspot mutations.² All homozygous *Pole*-mutant cell lines were significantly more sensitive to cytarabine and fludarabine than the isogenic *Pole*-wild-type cell line (also after multiple comparisons correction, see Table 1). Dots and error bars indicate mean and standard error of the mean, respectively.

cytarabine and fludarabine was also assessed in a heterozygous *Pole* S297F-mutant cell line. This resulted in sensitivity lower than in the homozygous *Pole*-mutant lines, but higher than in the wild-type, reaching significance for cytarabine (mean IC_{50} 0.14 μ M vs. 0.17 μ M; $P=0.025$ for cytarabine; mean IC_{50} 7.58 μ M vs. 11.1 μ M; $P=0.169$ for fludarabine; Supplementary Figure 3).

DISCUSSION

In this study, we present two independent lines of evidence to show that the prognostic benefit of *POLE* proofreading domain mutations is independent of adjuvant treatment. First, we provide clinical data from the PORTEC-1 endometrial cancer trial to show that the favorable outcome associated with *POLE* mutations is also observed in women who did not receive adjuvant therapy. Second, in an *in vitro* model we demonstrate that *POLE* mutations confer increased sensitivity neither to ionizing radiation, nor to commonly used chemotherapeutics. We also show that *Pole* mutations result in significantly increased sensitivity to specific nucleoside analogs currently used to treat hematological malignancies.

This study is limited by the composition of the endometrial cancer cohort: the relatively

favorable outcome of stage I disease made it more challenging to confirm the prognostic effect of *POLE* mutations. Its composition also precluded the joined examination of *POLE* mutational status and other prognostically relevant, clinical factors: this should be addressed in future research. Because *POLE*-mutant cancers are characterized by an antitumor immune response,²⁰⁻²² it should be noted that in the cell-based model, an interaction between the immune system and radiotherapy or chemotherapy could not be assessed. However, the results of the endometrial cancer cohort and the *in vitro* findings are consistent regarding the prognosis of *POLE*-mutant tumors and its independence of adjuvant therapy. These results are supported by previous studies on endometrial and colorectal cancer. McConechy et al. observed no endometrial cancer-related deaths or evidence of recurrent or progressive disease among 14 patients with *POLE*-mutant cancers who did not receive adjuvant treatment.⁹ Moreover, among 23 patients with stage II *POLE*-mutant colorectal cancers, no recurrences were found irrespective of adjuvant chemotherapy.⁴ Our *in vitro* findings are in line with another preclinical study in which no significant difference in sensitivity to paclitaxel was found between *POLE*-mutant and *POLE*-wild-type endometrial cancer cell lines.²² This study reported increased resistance to carboplatin in the *POLE*-mutant cell lines. However, the cell lines used in this study were, contrary to those in our study, non-isogenic, and one carried a *POLE* polymerase domain variant of unknown pathogenicity. Finally, considering the mechanisms of action of the commonly used anticancer treatments and the presumed lack of a role herein of DNA polymerase epsilon's proofreading activity, a *POLE* mutation is unlikely to alter sensitivity to these therapies.

We are the first to study cancer-associated *POLE* proofreading domain mutations in mammalian cells. Remarkably, sensitivity to nucleoside analogs cytarabine and fludarabine was much greater in the cancer-associated *Pole* mutants than in the *Pole* proofreading-deficient cell lines,²³ supporting the presence of defects besides proofreading alone.^{2,5} In contrast, these *POLE* mutations resulted in only a four- to eight-fold increase in the spontaneous mutation frequency at the *Hprt* gene, which seems low compared with the ultramutated phenotype found in *POLE*-mutant cancers.^{2,5} Our mutation data are supported by forward mutation assays with mammalian proteins.⁵ The mutation frequencies in this study were, however, lower than those previously observed in yeast,²⁴ which may be due to different mechanisms of mutability between yeast and mammals. Future studies are needed to unravel the mechanisms behind the ultramutated phenotype of *POLE*-mutant cancers.

In conclusion, these findings suggest that the favorable prognosis of *POLE*-mutant cancers cannot be explained by increased sensitivity to adjuvant therapy but could be due to increased immunogenicity as described in previous studies or to the accumulation of

deleterious mutations ('error catastrophe').^{20-22,25} These results support clinical trials such as the PORTEC-4A trial, which prospectively evaluates observation alone for early-stage *POLE*-mutant cancers; this may provide an opportunity to decrease overtreatment and may spare these patients unnecessary side effects.²⁶ In patients with advanced-stage or aggressive tumors, *POLE* mutations identify candidates for targeted therapies. Recent results with immune checkpoint inhibitors for *POLE*-mutant cancers are promising.^{27,28} This study proposes nucleoside analogs cytarabine and fludarabine as targeted treatment option for *POLE*-mutant cancers, potentially as an alternative to or in combination with immune checkpoint inhibition, which should be the subject of future research. As the prognostic value and therapeutic implications of *POLE* mutations seem generalizable across cancer types, the results of this study support further individualization of treatment for a wide variety of malignancies.

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SUPPLEMENTARY METHODS

Preparation of metaphase spreads

For each *Pole* proofreading domain-mutant mouse embryonic stem cell line, cells were treated with colcemid (0.21 µg/ml for 2.5 hours; #15210040, Thermo Fisher Scientific) to arrest cells in metaphase. After treatment, cells were washed with PBS, detached with 0.05% trypsin-EDTA (Thermo Fisher Scientific), collected and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in hypotonic solution (0.2% sodium citrate and 0.2% KCL). After centrifugation, the hypotonic solution was removed. Cells were resuspended carefully in fixative solution (4:1, methanol:acetic acid) and centrifuged again. This fixation was performed three times before the cells were spread onto glass slides. Slides were mounted with DAPI-containing mounting medium (#H-1200, Vectashield). At least fifty metaphases were examined for each cell line. Chromosome numbers were equal for all cell lines used in subsequent experiments.

Fluorescence in situ hybridization

Bacterial artificial chromosomes (BAC) were ordered from the BACPAC Resource Center (Chori, USA): RP24-154A19 containing the *Pole* gene (5qF), and RP24-388K6 more proximal of the CRISPR/Cas9 target sequences (5qA3). DNA from the BAC clones was isolated using the Nucleobond Xtra Midi kit (MN740410.10, Macherey Nagel). To use the BAC clones as FISH probes, BAC clones were labeled with either biotin-16-dUTP (#11093070910, Roche) or digoxigenin-11-dUTP (#11093088910, Roche) using a nick translation kit (Roche and Enzo Life Sciences) as described previously.¹ Prior to precipitation, Yeast tRNA (#15401011, Invitrogen; ratio yeast tRNA:BAC is 5:1), fish sperm DNA (#1146740001, Roche; ratio fish sperm:BAC is 5:1) and Cot-I (#18440016, Invitrogen; ratio Cot-I:BAC is 10:1) was added to the probes. Probes were then diluted in hybridization mix (50% formamide, 10% dextran sulphate, 2x SSC; 0.3M NaCl, 0.03M sodium citrate, 0.04M sodium phosphate).

Metaphase spreads were treated with RNase (10 µg per slide, diluted in 2xSSC; 0.3M NaCl, 0.03M sodium citrate, pH7) for 10 minutes at 37°C, washed three times in 2xSSC, incubated with 0.005% pepsin/0.02M HCl (#10108057001, Roche) at 37°C for 5 minutes, and washed twice in PBS. Slides were then fixed in 1% formaldehyde/PBS, washed twice in PBS and dehydrated in a graded ethanol series. Ten µl of the probe mix (100ng per probe) was added to each slide, after which slides were placed at 80°C for 75 seconds for denaturation. Hybridization was performed overnight at 37°C in a moist chamber. Slides were washed in stringent wash buffer (K5799, Dako) for 5 minutes at 60°C, twice for 3 minutes in wash buffer, and 3 times 5 minutes in Dig-buffer (0.1M Tris, 0.15M NaCl, pH7.5) with 0.05% Tween20 (TNT) at room temperature. For detection, slides were

incubated with Cy3-labeled streptavidin (1:500, Sigma Aldrich) and FITC-labeled mouse anti-digoxigenin antibody (1:250, Sigma Aldrich) for 10 minutes at 37°C. After dehydration, slides were mounted with mounting medium containing DAPI (Vectashield). A minimum of twenty metaphases was examined for each cell line. All cell lines contained two copies of the *Pole* allele (Supplementary Figure 1).

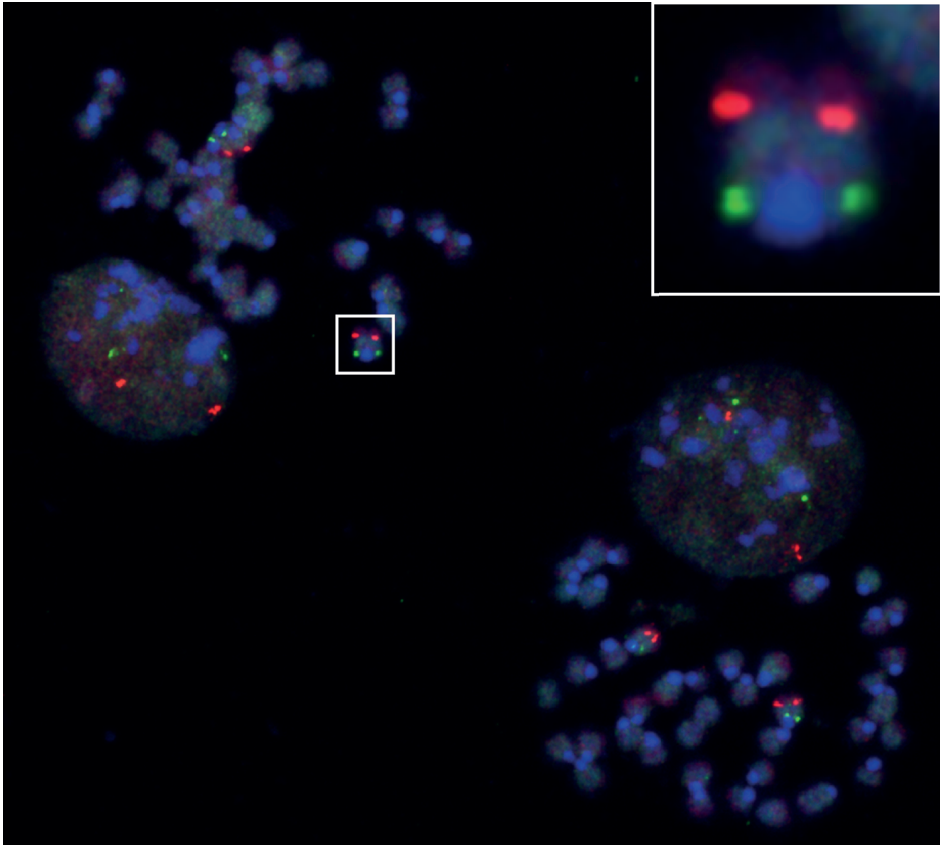
Assessment of mutator phenotypes at the *Hypoxanthine Phosphorybosyl Transferase (Hprt)* gene

Spontaneous mutagenesis at the *Hprt* gene was determined as described previously.² In brief, cells were cultured in medium containing HAT supplement (#21060-017, Gibco) for two consecutive passages to eliminate preexisting *Hprt*-deficient cells. Subsequently, cells were cultured in HT-supplemented medium (#41065-012, Gibco) for one passage, followed by two passages in medium without HAT- or HT-supplement, during which spontaneous *Hprt* mutations can accumulate. Afterwards, cells were continuously grown in medium with 40 µM 6-thioguanine to select for *Hprt*-deficient cells. After approximately ten days, colonies were fixed, stained with methylene blue and counted. These counts were corrected for cloning efficiencies.

As controls for this assay, a wild-type and a mismatch repair-deficient mouse embryonic stem cell line were used. The mismatch repair-deficient cell line resulted from a knock-out of *Msh6* using CRISPR-Cas9 (guide RNA forward 5'-caccgGGAGCCTCCGCTTCCC-GCGG-3', reverse 5'-aaacCCGCGGGAAGCGGAGGCTCC-3', plasmid Addgene #42230), generated similarly to the *Pole*-mutant cell lines. *Msh6* deficiency of the resultant cell line was verified by Sanger sequencing of a PCR product amplified from the CRISPR/Cas9 targeted locus (forward primer 5'-AAAGCACCTTGACAGCTTC-3', reverse primer 5'-GCTTGCCCAATACTCCGAAG-3'), Western Blot for *Msh6* (antibody Abcam #14204) and by verifying its resistance to low doses of 6-thioguanine (A4882, Sigma Aldrich; 4hr 40 µM pulse treatment).

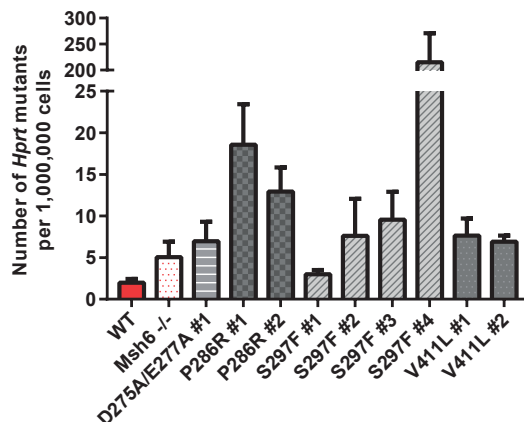
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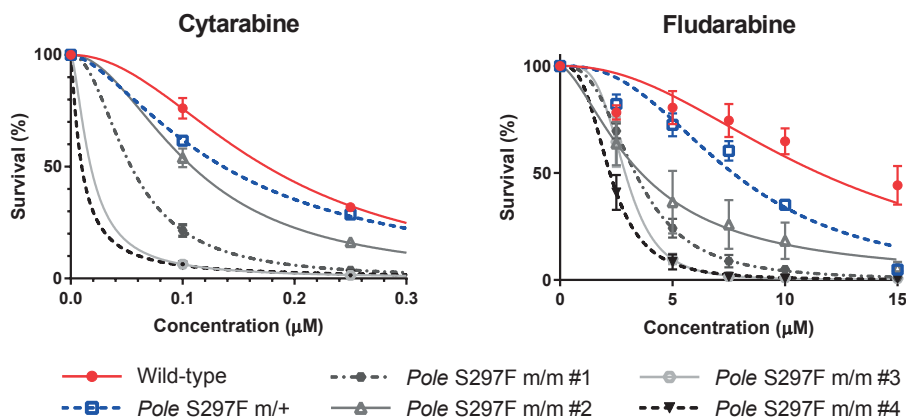
Supplementary Figure 1. Fluorescent In Situ Hybridization of *Pole* on metaphase spreads of *Pole*-mutant cell line.

FISH was performed using a probe containing the *Pole* gene (5qF; biotin-labeled probe detected with Cy3-labeled streptavidin [red]) and a probe proximal of the CRISPR-Cas9 target sequences on the same chromosome (5qA3; digoxigenin-labeled probe detected with FITC-labeled anti-digoxigenin [green]) with DAPI nuclear counterstaining. For each mouse-derived embryonic stem cell line, a minimum of twenty metaphases was analyzed: all cell lines showed normal, comparable intensity of the probes on two chromosome pairs (example of *Pole*-mutant cell line shown, inset of chromosome pair shown).



Supplementary Figure 2. Spontaneous mutant frequency of *Pole*-mutant cell lines.

The spontaneous mutant frequency of different homozygous *Pole* proofreading domain-mutant mouse-derived embryonic stem cells at the Hypoxanthine Phosphorybosyl Transferase (*Hprt*) gene was determined. Wild-type (WT) and *Msh6*-deficient (*Msh6* $-/-$) mouse-derived embryonic stem cell lines were used as controls. D275A/E277A is a *Pole* proofreading-deficient cell line due to double D275A and E277A mutations. P286R, S297F and V411L indicate cell lines with the cancer-associated *POLE* P286R, S297F, or V411L mutations, respectively. For these *Pole* hotspot mutations, a minimum of two independent cell lines was tested (#1 up to #4). The mean and standard error of the mean based on at least two experiments are shown.



Supplementary Figure 3. Sensitivity to nucleoside analogs cytarabine and fludarabine in *Pole* S297F-mutant and *Pole*-wild-type cell lines.

Non-linear regression of relative survival after treatment with nucleoside analogs cytarabine and fludarabine is shown for *Pole* S297F-mutant and *Pole*-wild-type mouse embryonic stem cells. With regard to the *Pole* S297F-mutant cell lines, one heterozygous *Pole* S297F-mutant (m/+) and four homozygous *Pole* S297F-mutant (m/m #1 - #4) cell lines were tested. Sensitivity for the homozygous *Pole* S297F-mutant cell lines was significantly higher compared to *Pole*-wild-type (for details on these comparisons see also Table 1 and Supplementary Table 5). A heterozygous *Pole* S297F mutation resulted in sensitivity lower than the homozygous mutant cell lines, but higher than wild-type, reaching significance for cytarabine (mean IC_{50} 0.14 μ M vs. 0.17 μ M; $P=0.025$ for cytarabine; mean IC_{50} 7.58 μ M vs. 11.1 μ M; $P=0.169$ for fludarabine). Dots and error bars indicate mean and standard error of the mean, respectively.

Supplementary Table 1. *POLE* mutations identified in endometrial cancers in the observation arm of the PORTEC-1 trial.

<i>POLE</i> proofreading domain mutation	Frequency (n=16)
No. (%) with P286R	7 (44)
No. (%) with V411L	5 (31)
No. (%) with S297F	3 (19)
No. (%) with S459F	1 (6)

Supplementary Table 2. Oligonucleotide guide RNA sequences to introduce *Pole* proofreading domain mutations through CRISPR-Cas9.

<i>Pole</i> mutation	Forward/Reverse	Sequence 5'-3'
D275A / E277A	Forward	TCTGATCGGTCTCAGCATCA
	Reverse	TGATGCTGAGACCGATCAGA
P286R	Forward	GGAGATCATCATGATCTGAT
	Reverse	ATCAGATCATGATGATCTCC
S297F	Forward	CTCCTATATGATTGATGGCC
	Reverse	GGCCATCAATCATATAGGAG
V411L	Forward	GATTATGACTGCCCCACAGGA
	Reverse	TCCTGTGGGCAGTCATAATC

Supplementary Table 3. Oligonucleotide primer sequences to generate a template for homologous recombination for transfection*.

Mutation-specific primers[‡]		
Pole mutation	Forward/ Reverse	Sequence 5'-3'[§]
D275A / E277A	Forward	CGACCAA <u>ACTGCCTCTCAAATT</u> <u>TCCTGATGCTGAGACCGATCAG</u>
	Reverse	G <u>AAATTTGAGAGGCAGTTTGGTCGTG</u> <u>GCGATGGCAAATGCCAAAACACAGGGTC</u>
P286R	Forward	CAAATTC <u>CGC</u> GATGCTGAGACAGATCAGATCATG
	Reverse	CTGATC <u>TGTC</u> CAGCATC <u>CGC</u> GGAATTTGAGAGG
S297F	Forward	GATTGATGGCCA <u>AGTGAACAGAATCTC</u>
	Reverse	GGCCATCAATCATATAG <u>AAGATCATCATGATCTGATCGGTCTCAGC</u>
V411L	Forward	GG <u>CTGAAGAGGGACAGTTA</u> <u>CTTCCTGTGGGCAGTCATAATC</u>
	Reverse	GATAACTGTCCCTCTTCAG <u>CCACCTGGAAAAACACAG</u>
Common primers[‡]		
External oligo exon 9	Forward	TCTGCAAGGTGGCAGTGTAATTAC
	Reverse	GGACCAGCCTCATCTGACTT
External oligo exon 13	Forward	GGCCTCTAAGAAACGGCTTTGG
	Reverse	CTTCTCGACTCGTTGCAGCAGG
Joining PCR exon 9	Forward	CCAATTGGACAACATAGTGGAC
	Reverse	GTACATGCTGAGGCCATGAATTG
Joining PCR exon 13	Forward	GGCTTTGGGTCATAGGGATTG
	Reverse	CTAGGATAAGACACCACAGGGC

*Primers were used to generate a template for homologous recombination, to be used with CRISPR-Cas9 in cell transfection, containing *Pole* proofreading domain mutations as well as silent mutations in the PAM sequences. This template was made using two-stage PCR amplification.

[‡]The mutation-specific primers together with the common external oligonucleotides were used to generate overlapping PCR fragments with the different substitutions. Fragments were pooled and amplified with the joining primers in a subsequent PCR.

[§]*Pole* proofreading domain mutations are shown in bold and underlined. Silent mutations in the PAM sequence are underlined.

^{||}Exon 9 contains the D275, E277, P286 and S297 amino acids. Exon 13 contains the V411 amino acid.

Supplementary Table 4. Oligonucleotide sequences to identify targeted colonies after transfection.

Material	<i>Pole</i> proofreading domain exon	Forward/ Reverse	Sequence 5'-3'	Protocol
gDNA	Exon 9 (D275A/E277A, P286R, S297F)	Forward	GTCCCTTGCTAGTGCTGTCC	(95° 30 s, 65° 30 s, 72° 2 min) 34x
		Reverse	TACCTGGGATGCCTACTTGC	
gDNA	Exon 13 (V411L)	Forward	CCCCACGTAGTAGGCAGCTC	(95° 30 s, 63° 30 s, 72° 2 min) 34x
		Reverse	GAGCCAATGGGCTTACGTGCC	
cDNA	Spanning exons 7-14	Forward	GAATCCATGTGCCCCACTGG	(95° 30 s, 59° 30 s, 72° 1 min) 34x
		Reverse	AGTCTGGGGCTGTTCAGTGG	

Supplementary Table 5. Sensitivity to adjuvant treatment strategies conferred by *Pole* proofreading domain mutations in a cell-based model.

Treatment	WT			Pole D275A/E277A			Pole P286R			Pole S297F			Pole V411L		
	IC ₅₀ (mean ± SD)	#2*	P	IC ₅₀ (mean ± SD)	#2*	P	IC ₅₀ (mean ± SD)	#2*	P	IC ₅₀ (mean ± SD)	#3*	P	IC ₅₀ (mean ± SD)	#4*	P
Ionizing radiation (Gy)	2.96 ± 1.28	2.61 ± 0.41	0.654	3.02 ± 1.61	0.938		2.30 ± 1.07	0.446	1.48 ± 0.07	0.013	2.99 ± 0.71	0.973	3.05 ± 0.68	0.920	
5-Fluorouracil (μM)	0.25 ± 0.04	0.22 ± 0.03	0.316	0.20 ± 0.03	0.127		0.26 ± 0.01	0.596	0.28 ± 0.04	0.372	0.18 ± 0.03	0.046	0.25 ± 0.03	0.945	
Cisplatin (μM)	0.39 ± 0.17	0.30 ± 0.11	0.353	0.20 ± 0.04	0.061		0.27 ± 0.03	0.290	0.34 ± 0.13	0.645	0.22 ± 0.06	0.089	0.29 ± 0.03	0.308	
Doxorubicin (nM)	8.92 ± 4.04	9.47 ± 1.94	0.786	7.95 ± 1.97	0.666		8.77 ± 2.61	0.955	10.3 ± 1.20	0.585	6.16 ± 2.03	0.240	9.35 ± 3.02	0.859	
Etoposide (nM)	27.2 ± 15.7	26.3 ± 4.65	0.912	21.1 ± 6.25	0.416		26.4 ± 11.3	0.906	29.9 ± 8.92	0.710	22.8 ± 5.54	0.550	27.3 ± 12.3	0.999	
Methotrexate (nM)	60.1 ± 8.48	94.7 ± 22.2	0.047	84.9 ± 28.4	0.179		81.8 ± 11.9	0.015	75.0 ± 21.2	0.346	66.0 ± 27.6	0.623	78.5 ± 29.2	0.174	
Paclitaxel (nM)	9.57 ± 2.94	7.60 ± 1.87	0.324	11.1 ± 2.06	0.385		6.78 ± 1.13	0.160	7.09 ± 1.14	0.206	9.20 ± 2.64	0.859	11.1 ± 3.72	0.465	
Cytarabine (μM)	0.17 ± 0.02	0.10 ± 0.01	0.001 [‡]	0.05 ± 0.01	<0.001 [‡]		0.11 ± 0.02	0.002 [‡]	0.02 ± 0.01	<0.001 [‡]	0.01 ± 0.01	<0.001 [‡]	0.09 ± 0.02	0.001 [‡]	
Fludarabine (μM)	11.1 ± 3.98	5.29 ± 2.13	0.004 [‡]	3.90 ± 0.81	0.001 [‡]		4.76 ± 2.86	0.005 [‡]	2.59 ± 0.31	<0.001 [‡]	2.29 ± 0.72	<0.001 [‡]	5.10 ± 1.53	0.008 [‡]	

*For each *Pole* proofreading domain mutation (D275A/E277A, P286R, S297F, V411L, respectively) at least two homozygous cell lines were used to determine the treatment sensitivity. In addition to Table 1, this table shows the sensitivity of the other *Pole*-mutant lines (mean IC₅₀ and standard deviation, SD, in table) compared to the sensitivity of the *Pole*-wild-type (WT) mouse embryonic stem cell line; the corresponding *P*-values are shown.

[‡]Difference remains significant after Holm-Bonferroni correction for multiple comparisons.

Supplementary Table 6. Sensitivity to nucleoside analogs gemcitabine, cladribine and clofarabine conferred by *Pole* proofreading domain mutations in a cell-based model.

Cell line	Treatment*					
	Gemcitabine (nM)		Cladribine (μM)		Clofarabine (μM)	
	IC ₅₀ (mean ± SD)	P (95% CI)	IC ₅₀ (mean ± SD)	P (95% CI)	IC ₅₀ (mean ± SD)	P (95% CI)
WT	16.7 ± 5.74	NA	0.16 ± 0.03	NA	0.23 ± 0.08	NA
<i>Pole</i> D275A/E277A						
#1	19.6 ± 6.90	0.379 (-9.69, 3.92)	0.19 ± 0.04	0.049 (-0.06, -0.0002)	0.23 ± 0.06	0.936 (-0.12, 0.13)
#2	18.5 ± 4.12	0.594 (-9.03, 5.46)	0.19 ± 0.05	0.218 (-0.07, 0.02)	0.21 ± 0.05	0.664 (-0.09, 0.14)
<i>Pole</i> P286R						
#1	19.2 ± 4.35	0.465 (-9.81, 4.82)	0.22 ± 0.07	0.197 (-0.16, 0.05)	0.22 ± 0.09	0.893 (-0.13, 0.15)
#2	17.2 ± 0.37	0.901 (-8.20, 7.32)	0.21 ± 0.06	0.046 (-0.09, -0.01)	0.19 ± 0.04	0.384 (-0.07, 0.16)
<i>Pole</i> S297F						
#1	15.9 ± 0.55	0.820 (-6.97, 8.57)	0.19 ± 0.03	0.112 (-0.07, 0.01)	0.15 ± 0.05	0.172 (-0.05, 0.22)
#2	15.3 ± 0.94	0.685 (-6.34, 9.23)	0.13 ± 0.03	0.126 (-0.01, 0.06)	0.12 ± 0.04	0.067 (-0.01, 0.23)
#3	16.5 ± 1.94	0.962 (-7.71, 8.06)	0.13 ± 0.01	0.047 (0.0005, 0.07)	0.13 ± 0.02	0.068 (-0.01, 0.22)
#4	15.4 ± 0.52	0.661 (-5.24, 7.90)	0.13 ± 0.02	0.082 (-0.004, 0.06)	0.15 ± 0.05	0.135 (-0.04, 0.20)
<i>Pole</i> V411L						
#1	17.8 ± 3.12	0.725 (-8.09, 5.83)	0.21 ± 0.03	0.015 (-0.08, -0.01)	0.21 ± 0.06	0.695 (-0.10, 0.14)
#2	17.1 ± 3.88	0.914 (-7.53, 6.81)	0.17 ± 0.03	0.566 (-0.04, 0.02)	0.19 ± 0.09	0.547 (-0.11, 0.19)

*Sensitivity to nucleoside analogs gemcitabine, cladribine and clofarabine was determined for all *Pole* proofreading domain-mutant cell lines and compared to the sensitivity of the *Pole*-wild-type (WT) mouse embryonic stem cell line; the corresponding *P*-values are shown. None of the *P*-values remained statistically significant after Holm-Bonferroni correction for multiple comparisons.

Abbreviations: SD, standard deviation; CI, confidence interval; NA, not applicable.



Chapter 7

Somatic *POLE* exonuclease domain mutations are early events in sporadic endometrial and colorectal carcinogenesis, determining driver mutation landscape, clonal neoantigen burden and immune response

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ABSTRACT

Genomic instability, a hallmark of cancer, is generally thought to occur in the mid to late stages of tumorigenesis, following the acquisition of permissive molecular aberrations such as *TP53* mutation or whole genome doubling. Tumors with somatic *POLE* exonuclease domain mutations are notable for their extreme genomic instability (their mutation burden is among the highest in human cancer), distinct mutational signature, lymphocytic infiltrate and excellent prognosis. To what extent these characteristics are determined by the timing of *POLE* mutations in oncogenesis is unknown. Here, we have shown that pathogenic *POLE* mutations are detectable in non-malignant precursors of endometrial and colorectal cancer. Using genome and exome sequencing, we found that multiple driver mutations in *POLE*-mutant cancers display the characteristic *POLE* mutational signature, including those in genes conventionally regarded as initiators of tumorigenesis. In *POLE*-mutant cancers, the proportion of monoclonal predicted neoantigens was similar to other cancers, but the absolute number was much greater. We also found that the prominent CD8⁺ T cell infiltrate present in *POLE*-mutant cancers was evident in their precursor lesions. Collectively, these data indicate that somatic *POLE* mutations are an early, quite possibly initiating, event in the endometrial and colorectal cancers in which they occur. The resulting early onset of genomic instability may account for the striking immune response and excellent prognosis of these tumors, as well as their early presentation.

INTRODUCTION

Next generation sequencing (NGS) technologies have hugely advanced our understanding of the mechanisms of tumorigenesis. The ability to analyze the entire genome or exome at depth in large numbers of tumors has substantially increased the list of driver genes – that is those which, when mutated, promote tumor growth. It has also revealed that such driver mutations are not always present in the dominant tumor clone.^{1,2} This is clinically relevant, because targeting subclonal drivers is likely to kill only a subpopulation of tumor cells, while successful targeting of clonal variants may lead to tumor eradication. Thus, differentiating early, clonal mutations from late, subclonal ones may not only increase our understanding of the mechanisms of oncogenesis, but also inform the clinical management of patients.²

Fundamentally, all mutations are caused in part by a failure to recognize or repair defects in DNA sequence or chromosome structure. In many cancers, this is a consequence of specific defects in the cellular processes responsible for maintaining genomic integrity.³ One recently described example is the genomic instability caused by missense mutations in the exonuclease (proofreading) domains of the major replicative DNA polymerases *POLE* and *POLD1*.⁴ Polymerase proofreading recognizes and corrects mispaired bases incorporated during DNA replication; its perturbation as a result of these mutations is associated with an exceptional number of SNVs (though not indels), and a distinct mutational signature typified by C:G→A:T transversions where the mutated cytosine is in the context TCT, and C:G→T:A transitions where the mutated cytosine is in the context TCG.⁴⁻⁶ *POLE* and *POLD1* exonuclease domain mutations may occur in the germline, where they cause polymerase proofreading-associated polyposis (PPAP) – a condition characterized by intestinal polyposis and tumors of the colorectum and uterus, among other organs.⁷ Somatic *POLE* exonuclease domain mutations (hereafter simply referred to as *POLE* mutations) occur in sporadic tumors of the endometrium (7-15% cases),^{8,9} colorectum (1-2%),^{10,11} and less commonly in other cancers (although for reasons that are unclear, somatic *POLD1* exonuclease domain mutations are very uncommon). *POLE*-mutant colorectal and endometrial cancers have an excellent prognosis,^{8,11-13} probably owing to a robust antitumor immune response against the multitude of immunogenic neoantigens they are predicted to harbor.^{11,14,15} Very recent reports also suggest that these tumors may be highly responsive to immune checkpoint inhibition.¹⁶

While it is clear that somatic *POLE* mutation causes a mutator phenotype¹⁷ and acts as a cancer driver,^{4,5} several questions about its contribution to tumorigenesis remain unanswered. One of the most important of these relates to the timing of these mutations in cancer development. If *POLE* mutations are late events, their consequences may be

restricted to a subclone of tumor cells, the targeting of which may fail to meaningfully alter tumor behavior. In contrast, if *POLE* mutations occur early, they could rapidly cause a large number of clonal alterations that may alter prognosis or response to therapy. This is particularly pertinent in the light of recent data suggesting that long-term benefit from immune checkpoint inhibition is limited to patients whose cancers harbor neoantigens in the dominant tumor clone.¹⁸ In contrast to germline mutations in DNA repair pathways in rare inherited syndromes (such as the mismatch repair gene variants that cause Lynch syndrome), the acquisition of genomic instability in sporadic cancers has largely been believed to be a mid- to late-stage event during carcinogenesis.¹⁹ For example, in sporadic colorectal cancer – a tumor type in which the molecular progression of precancers (adenomas) to invasive carcinomas has been well-characterized – mismatch repair deficiency (MMR-D) or chromosomal instability (CIN) occur after initiating (epi)mutations in *APC*, *BRAF* or *KRAS*, or other events such as whole genome doubling or loss of chromosome 18q.^{19–24} Thus, in addition to its clinical relevance, the demonstration that the *POLE* mutator phenotype operates from the first stages of tumor initiation would also reveal a novel pathway of sporadic tumorigenesis. A recent case report of a pathogenic *POLE* mutation in an endometrial cancer and its precursor²⁵ suggests that these mutations may occur early in tumor development, but the single case precludes generalization of this result.

In this study, we comprehensively examined the timing of pathogenic somatic *POLE* exonuclease domain mutations in sporadic endometrial and colorectal cancers using tumor whole genome sequencing (WGS), public sequencing data from The Cancer Genome Atlas (TCGA),^{8,10} and targeted sequencing of additional cohorts of cancers and precancers.

MATERIALS AND METHODS

Patients and tumor samples

Details of the cohorts and cases analyzed in this study are shown in Supplementary Tables 1 and 2. Fifty-one formalin-fixed paraffin-embedded (FFPE) endometrial cancers carrying known pathogenic somatic *POLE* exonuclease domain mutations identified in our previous studies^{12,14,26} were reviewed for the presence of a concomitant and spatially discrete area of endometrial intraepithelial neoplasia (EIN) by examination of hematoxylin and eosin (H&E)-stained slides by two expert gynecological pathologists (V.S. & T.B.). 389 FFPE colorectal polyps (tubular adenomas, tubulovillous adenomas and serrated adenomas – hereafter referred to as adenomas), for which *POLE* screening had not previously been performed, were identified from 261 participants in the CORGI study, which

recruited patients with a family history of colorectal cancer and a personal history of a colorectal polyp or colorectal malignancy in the absence of a known tumor predisposition syndrome. Six fresh frozen tumors with pathogenic somatic *POLE* mutations (five endometrial, one colorectal) were identified from a Leuven endometrial cancer cohort used in our previous study,¹² a prospective clinical sequencing program (HICF2) at the University of Oxford, or the University of Birmingham tissue bank. TCGA colorectal (COADREAD)¹⁰ and endometrial (uterine corpus endometrial carcinoma – UCEC)⁸ cancer data were downloaded from the Genomic Data Commons (GDC) Data Portal (<https://portal.gdc.cancer.gov>; June 2017). An additional series of 78 FFPE endometrial cancers including 32 cases with pathogenic somatic *POLE* mutations were identified from the LUMC archives (2001–2015).¹⁴ Further details of the cohorts used in this study are provided in Supplementary Table 1. Molecular analyses were performed on a single tumor or precursor lesion region in each case.

DNA extraction

After review to confirm adequate tumor cellularity, DNA was extracted from fresh frozen or microdissected FFPE tumors and precursors using standard methods (Roche FFPE-T DNA kit, Machery Nagel Nucleospin DNA FFPE XS / FFPE DNA kit or Qiagen Blood and Tissue kit) and resuspended in buffer or water.

DNA sequencing

Full details of the sample preparation and the sequencing methods utilized in this study are provided in the Supplementary Methods. Briefly, endometrial epithelial neoplasias (EIN) and paired carcinomas were sequenced for mutations in 30 cancer genes using molecular inversion probe capture, and a custom version of the 72 gene Ion AmpliSeq Cancer Hotspot panel v2 (including 80 genes; ThermoFisher) (Supplementary Tables 3 and 4). Whole genome sequencing (WGS) of fresh frozen tumors was performed by Illumina HiSeq, and aligned to the reference genome by BWA mem or Isaac.²⁷ FFPE endometrial cancers from the LUMC series were analyzed using the Lifetech/ThermoFisher Ion AmpliSeq Comprehensive Cancer Panel comprising 409 cancer genes (<http://www.lifetechnologies.com/order/catalog/product/4477685>). Mutation calling was performed by LoFreq²⁸ (EINs), Mutect, Mutect2²⁹ or Strelka³⁰ (WGS, TCGA cases), or Ion Torrent variantCaller (EINs, LUMC FFPE tumors). Copy number profiles were derived using Sequenza.³¹ Variant annotation was done using Annovar³² or Variant Effect Predictor.³³

Definition of driver genes

Driver genes were defined using the IntOGen driver gene repository (<https://www.intogen.org/search>) and included both PanCancer (Pooled_driver) and tumor type-specific (perProject_driver) variants (Supplementary Tables 5 and 6).³⁴ High confidence driver

mutations (defined as either truncating mutations in genes likely to be tumor suppressors or recurrent missense mutations in any endometrial or colorectal cancer-specific or pan-cancer gene from the IntOGen set), were determined for a subset of driver genes by manual curation, blinded to tumor molecular characteristics.

Clonality of *POLE* mutations

Most (36 of 38) endometrial and colorectal cancers with pathogenic *POLE* mutations were disomic at the *POLE* locus (chr12q24) and were informative for clonality analysis. Of these, 20 of 22 endometrial cancers, and 12 of 14 colorectal cancers had available copy number annotation. As all 32 of these showed near-diploid genomes (>80% of the genome), we assumed diploid genomes for the four remaining cases.

Mutations were filtered to include only autosomal variants in diploid regions of the genome, called with depth of at least 20x. Mutation allele frequency distributions were generated using the R 'histogram' function, and tumor cellularity inferred as twice the mid-point of the allele frequency bin with highest mutation density, excluding bins with a lower bound below allele frequency 0.1. These values were then subjected to manual curation. The hypothesis that the mutation was present in every tumor cell was tested by a one-sided binomial test, based on the numbers of reference and variant reads at the *POLE* mutation site and the inferred tumor cellularity. Specifically, for a mutation with coverage R , in a tumor with cellularity C , the number of variant reads was modelled as a random variable X , with distribution: $X \sim \text{Binom}(R, C / 2)$. In each case we calculated the probability, p , of finding the observed number of variant reads, v , or fewer, $P(X \leq v)$. Mutations were considered subclonal for $p \leq 0.05$.

Mutational signatures

Previously reported mutational signatures were obtained from <http://cancer.sanger.ac.uk/cosmic/signatures/> on 1st June 2017. The complement of mutational processes active in the life-history of each tumor sample was inferred by classification of mutations into 96 categories following Alexandrov,⁶ and the use of non-negative least squares regression, implemented in the R package 'nnls'. For this analysis, only mutational signatures previously reported as active in that cancer type (endometrial signatures 1, 2, 5, 6, 10, 13, 14 and 26; colorectal signatures 1, 5, 6, and 10) were used for the regression. For cases analyzed by whole exome sequencing, mutational signatures were re-scaled to exomic trinucleotide frequencies. A mutational process was deemed to have been active in the life-history of a tumor if the associated mutational signature had a coefficient of at least 2 percent of the total coefficients in the best-fitting model. Mutations likely to be due to *POLE* exonuclease domain mutation (*POLE*) were identified by considering mutational signatures as multinomial probability distributions caused by specific mutational

processes. The probability of each mutation under all mutational processes active in that tumor was calculated, and mutations were assigned to the “*POLE*” mutational process in cases where the probability under that process was at least twice the probability under any other process.

***POLE* consensus mutational signature scores in driver genes**

Tumor mutations were obtained from calling based on tumor/normal .bam files (*POLE* mutant cases) or TCGA MAF files (MMR-P, MMR-D cases), and classified into 96 categories following Alexandrov.⁶ For each tumor, the distribution of mutations across the 96 types was calculated, and re-scaled to trinucleotide frequencies based on sequencing type, thus obtaining an individual tumor mutational signature. Tumors were then categorized into three groups according to *POLE* mutation and mismatch repair status (i.e. *POLE*-mutant, MMR-P and MMR-D), and a consensus mutational signature was calculated for each group as the average of the individual-tumor signatures among samples in the group, weighted by the number of mutations in each sample. The probability of all non-silent mutations (‘nonsynonymous SNV’, or ‘stopgain’) in driver genes (as defined above) under each of the three consensus mutational signatures was then calculated, and the ratio of the probability of each mutation under the *POLE* consensus mutational signature compared to that under each of the other two consensus mutational signatures was obtained. For each individual gene, a ‘*POLE* score’ was then calculated as the base two logarithm of the minimum value of these ratios across all the non-silent mutations within that gene.

Immunohistochemistry

Immunohistochemistry (IHC) for CD8 was performed as previously reported.¹⁴ The number of CD8⁺ cells was quantified for the epithelial and stromal regions of the EIN. For the final CD8 count per case, the mean of these regions in ten high-power fields (HPF; 625µm x 425µm) was calculated. A similar method was used to quantify CD8 density in colorectal adenomas, although the small lesion size meant that estimates were obtained from the mean of two or three HPFs.

Clonal neoantigen prediction

We estimated the number of clonal neoantigens using a modification of our previously-reported algorithm,¹¹ modified to predict peptide binding against patient-specific HLA molecules (determined from WGS or WES data using OptiType³⁵). Neoantigens were defined as mutations predicted to specify peptides that bound patient HLA molecules with affinity <500nm. Copy number information was obtained from the GDC data portal, as described above. Clonality was determined as described above. Neoantigens were considered clonal if the binomial test *P*-value was over 0.05.

Statistical analysis

Analyses were performed using R (CRAN network) or Prism (GraphPad Software). Statistical comparison between groups was made using the non-parametric Mann-Whitney U test. All *P* values were two sided, unless otherwise specified. Statistical significance was accepted at $P < 0.05$.

Ethical approval

Patient consent for research on tumor tissue was obtained at the recruiting centers under local ethical approval. Molecular analysis of anonymized tissue was performed under Oxford Research Ethics Committee A approval (05/Q1605/66).

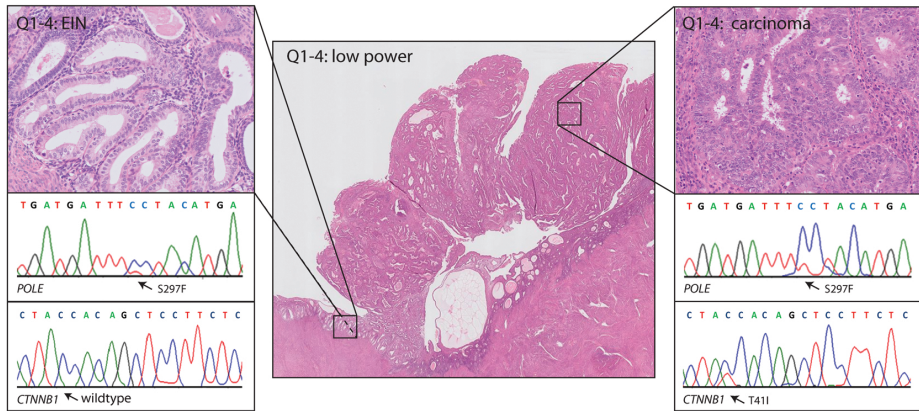
RESULTS

Somatic *POLE* exonuclease domain mutations are detectable in sporadic endometrial and colorectal precancers

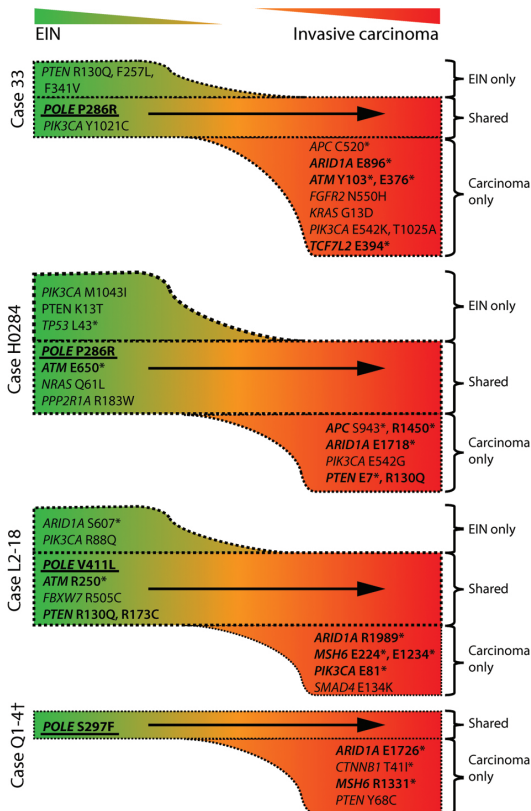
As somatic *POLE* exonuclease domain mutations have been best characterized in endometrial and colorectal cancers, we first examined whether these mutations were present in precursors of these malignancies. Expert histopathological review of 51 *POLE*-mutant endometrial cancers revealed four with a concomitant and spatially discrete area of endometrial intraepithelial neoplasia (EIN), the precursor of endometrioid carcinoma (Supplementary Table 2). Microdissection and targeted sequencing of these lesions by a 30-gene molecular inversion probe capture NGS panel (Supplementary Table 3), a custom 80 gene Ion Ampliseq Cancer Hotspot panel (Supplementary Table 4) and Sanger sequencing revealed that in all cases, the *POLE* mutation present in the carcinoma was also detectable in the paired precursor (Figure 1A-B; Supplementary Table 7). While some other driver mutations were also shared between the precursors and paired cancers (median 4 shared mutations per pair, relative to a median of 7 mutations per EIN and median of 10 mutations per carcinoma), the progression from EIN to malignancy was associated with both the loss (median 3 mutations lost in carcinomas compared to paired EINs) and, more frequently, gain (median 6 mutations gained in carcinomas compared to paired EINs) of driver mutations (Figure 1A-B; Supplementary Table 7). Notably, many of the driver mutations gained were replacements of a glutamic acid or arginine codon with a nonsense codon ($E \rightarrow *$ or $R \rightarrow *$), consistent with the characteristic mutational bias associated with *POLE* mutation (C:G→A:T transversions where the mutated cytosine is in the context TCT, and C:G→T:A transitions where the mutated cytosine is in the context TCG) (Figure 1B; Supplementary Table 7).⁴⁻⁶

We were unable to perform a corresponding analysis of colorectal tumors, because

A



B



C

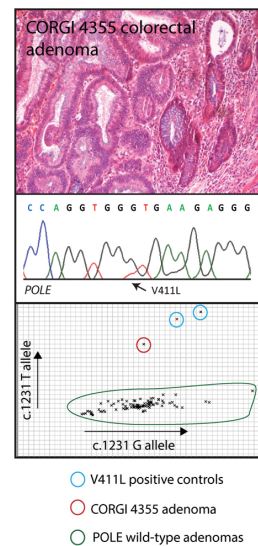


Figure 1. Pathogenic, somatic *POLE* exonuclease domain mutations in precursors of endometrial and colorectal cancers.

Expert histopathological review of 51 endometrial cancers with pathogenic *POLE* exonuclease domain mutations revealed four with concomitant and spatially discrete area of endometrial intraepithelial neoplasia

(EIN). (A) Hematoxylin and eosin (H&E)-stained section from one case with results of Sanger sequencing of the malignant and precursor components. (B) Targeted sequencing of paired endometrial lesions by two orthogonal next generation sequencing panels revealed that *POLE* mutations (bold, underlined) were present in both EIN and carcinomas in all cases (validated by Sanger sequencing in all cases). In each case, progression of EIN to endometrial carcinoma was associated with the gain of driver mutations, several of which were glutamic acid or arginine to stop codon mutations (E→* or R→*) consistent with the *POLE* exonuclease domain-mutant mutational signature (semibold). †The amount of DNA available from the EIN in case Q1-4 was insufficient for molecular inversion probe sequencing. Details of identified driver mutations are provided in Supplementary Table 7. (C) H&E-stained section from colorectal adenoma with the results of Sanger sequencing and allelic discrimination PCR for the wild-type G allele and mutant T allele.

residual precursor is uncommon in colorectal carcinomas. However, screening of 389 colorectal adenomas from 261 patients revealed three (0.8% adenomas, 1.1% patients) with somatic *POLE* mutations (Figure 1C), a frequency concordant with that found in colorectal cancers.¹¹ Unfortunately, the limited amount of DNA available from these lesions precluded analysis of other driver mutations.

Mutational landscape and driver gene alterations suggest that somatic *POLE* mutation is an early event in sporadic endometrial and colorectal cancers

To further investigate the timing and consequences of *POLE* mutations in tumor development, we performed WGS on six cancers (five endometrial, one colorectal), all of which harbored the most common pathogenic *POLE* exonuclease domain variant – a proline to arginine substitution at codon 286 (*POLE* P286R; Figure 2A). Each displayed a substantially elevated mutation burden (122–731 mutations/Mb), and characteristic preponderance of C:G→A:T substitutions in the context TCT (Figure 2A-B; Supplementary Table 8).⁶ In keeping with their early occurrence, both the *POLE* mutations themselves, and other mutations consistent with the known *POLE* mutational signature (see Materials and methods, Mutational signatures) appeared clonal in all six cases (Figure 2C). This was also the case in 17 of 17 endometrial cancers and 12 of 13 colorectal cancers with pathogenic *POLE* exonuclease domain mutations from the TCGA series (Supplementary Figure 1 and 2). This analysis showed that *POLE* mutations were unlikely to occur as late events after the most recent common ancestor in cancer evolution.

We next examined the timing of *POLE* mutations in carcinogenesis in more detail by analysis of driver genes, including some known usually to be mutated early in the pathogenesis of endometrial or colorectal cancer. To assess the likelihood that mutations in these genes were secondary to an earlier *POLE* mutation, we developed a metric to score them according to the probability that they were caused by the mutational process dominant in *POLE*-mutant cancers (presumably caused by the *POLE* mutation itself), rather than the mutational processes operative in other tumors (see Materials and methods, *POLE* consensus mutational signature score for details). For this analysis,

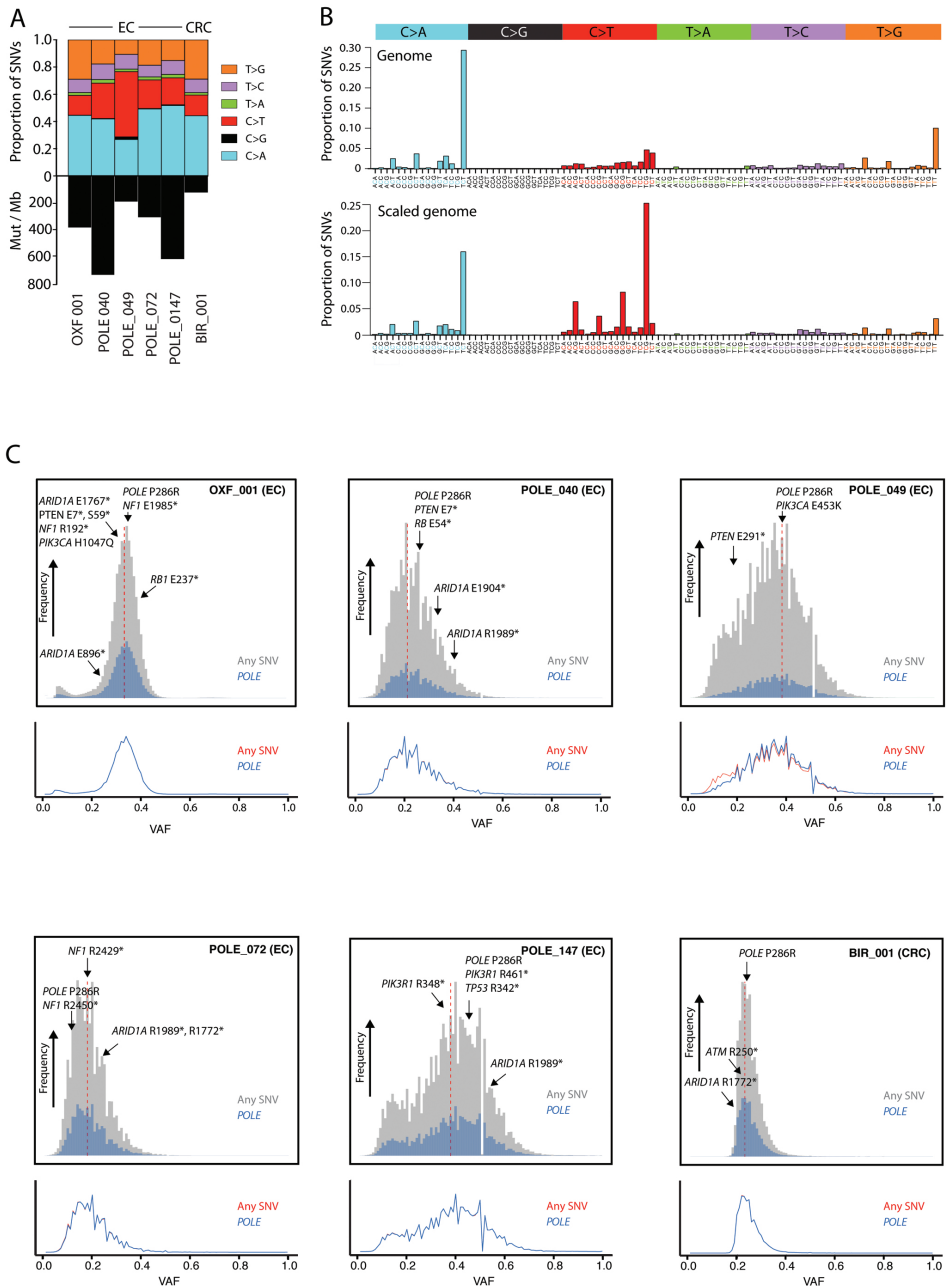


Figure 2. Whole genome sequencing of cancers with *POLE* exonuclease domain mutations. (A) Mutation burden and single nucleotide variant (SNV) type determined by whole genome sequencing (WGS) of five endometrial cancers (EC– Oxf001, POLE_040, POLE_049, POLE_072, POLE_147) and one colorectal cancer (CRC – Bir001) with somatic *POLE* P286R exonuclease domain mutations. (B) Relative proportion of SNV mutations according to trinucleotide context averaged across the six *POLE*-mutant cases.

The upper panel shows the unscaled proportions across the whole genome, while the lower panel shows the inferred mutational signature in a hypothetical genome where all trinculeotide frequencies are represented in equal proportions. (C) Frequency histograms and kernel density plots showing variant allele fraction (VAF) of all SNV mutations, and SNVs likely due to *POLE* exonuclease domain mutation (*POLE*). *POLE* mutations and other driver gene mutations are highlighted by arrows (details provided in Supplementary Table 8). Only mutations in diploid regions of autosomes, and with coverage >20x are shown. The relatively low proportion of SNVs categorized as being due to *POLE* mutation reflects the stringency of the classification used (see Materials and methods, Mutational signatures). Vertical red line indicates clonal peak used to calculate cellularity.

we combined our cohort of *POLE*-mutant tumors with *POLE*-mutant cases from TCGA, using MMR-P and MMR-D TCGA cases as comparators. Strikingly, in *POLE*-mutant tumors, almost all known cancer driver genes displayed evidence of the *POLE* consensus mutational signature, with the notable exception of *POLE* itself (Figures 3 and 4; Supplementary Tables 8–10), consistent with the postulate that the *POLE* signature is a direct effect of the polymerase proofreading mutation. In contrast, MMR-P and MMR-D tumors rarely showed evidence of the *POLE* consensus mutational signature (Figures 3 and 4; Supplementary Tables 8–10). In total, among 206 endometrial and/or colorectal cancer driver genes examined in the cases from the combined endometrial and colorectal cancer cohorts, 50% (1,065/2,118) of those in *POLE*-mutant samples had a *POLE* signature score >0, compared to 14% (628/4,427) in MMR-D and MMR-P cancers ($P < 1 \times 10^{-26}$).

To minimize the possibility of confounding by non-pathogenic mutations in the complete set of driver genes, we repeated these analyses considering only manually curated, high-confidence pathogenic mutations, and obtained similar results ($P < 1 \times 10^{-26}$; Supplementary Figures 3 and 4). As mutation of the tumor suppressors *PTEN* and *APC* are well recognized as early, if not initiating, events in the pathogenesis of endometrial and colorectal cancers respectively, we specifically examined whether somatic variants in these genes varied according to tumor *POLE* mutation status. Among high-confidence pathogenic *PTEN* mutations in endometrial cancers, the proportion with *POLE* consensus mutational signature scores >0 was substantially and significantly greater among *POLE*-mutant cases than among MMR-P and MMR-D tumors (10 of 14 (71.4%) vs. 14 of 82 (17.1%) mutations respectively; $P = 7.8 \times 10^{-3}$, Fisher's Exact Test). Analysis of high-confidence pathogenic *APC* mutations in colorectal cancers revealed similar results (corresponding proportions 9 of 14 (64.3%) vs. 10 of 69 mutations (14.5%); $P = 0.012$, Fisher's Exact Test).

Further analysis of these cohorts and of targeted sequencing data from an additional series of endometrial cancers from the Leiden University Medical Center (LUMC), including 32 *POLE*-mutant tumors, confirmed the over-representation of E→*, R→* and arginine to glutamine substitutions (R→Q) among *POLE*-mutant cases, concordant with the results

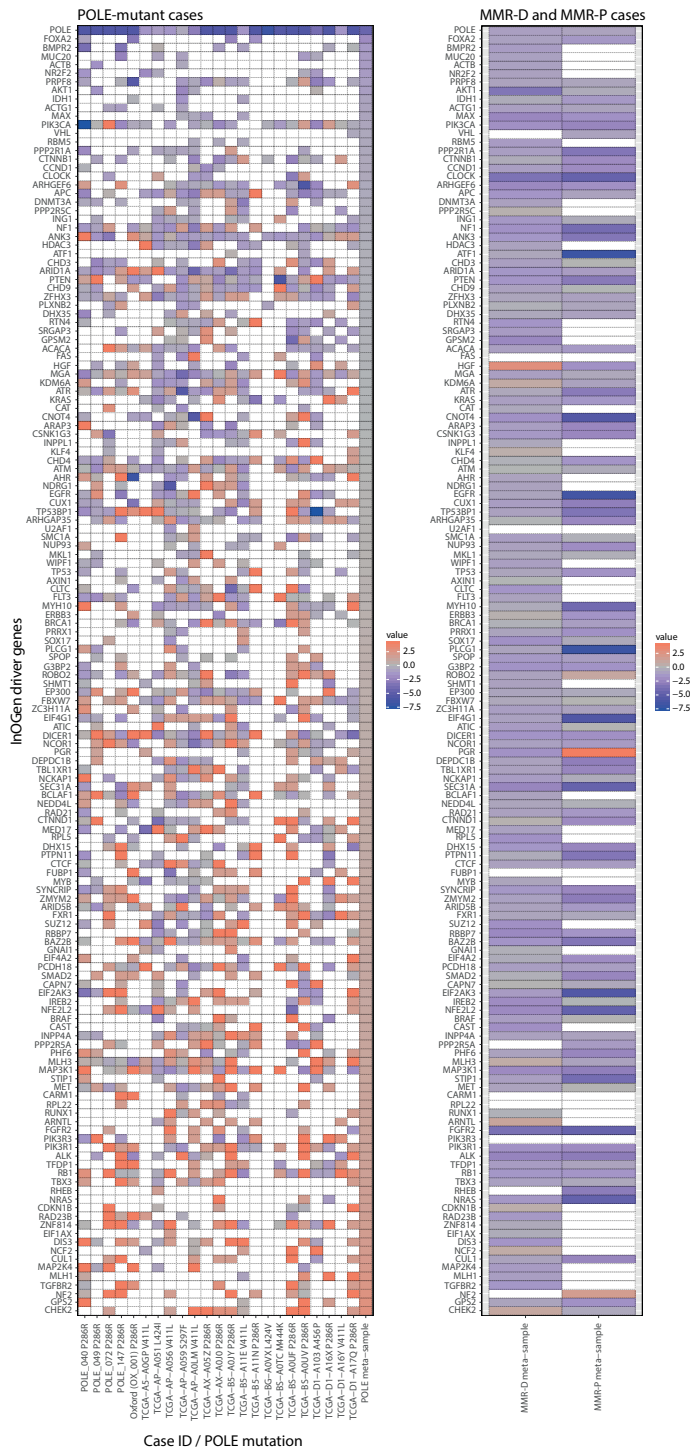


Figure 3.
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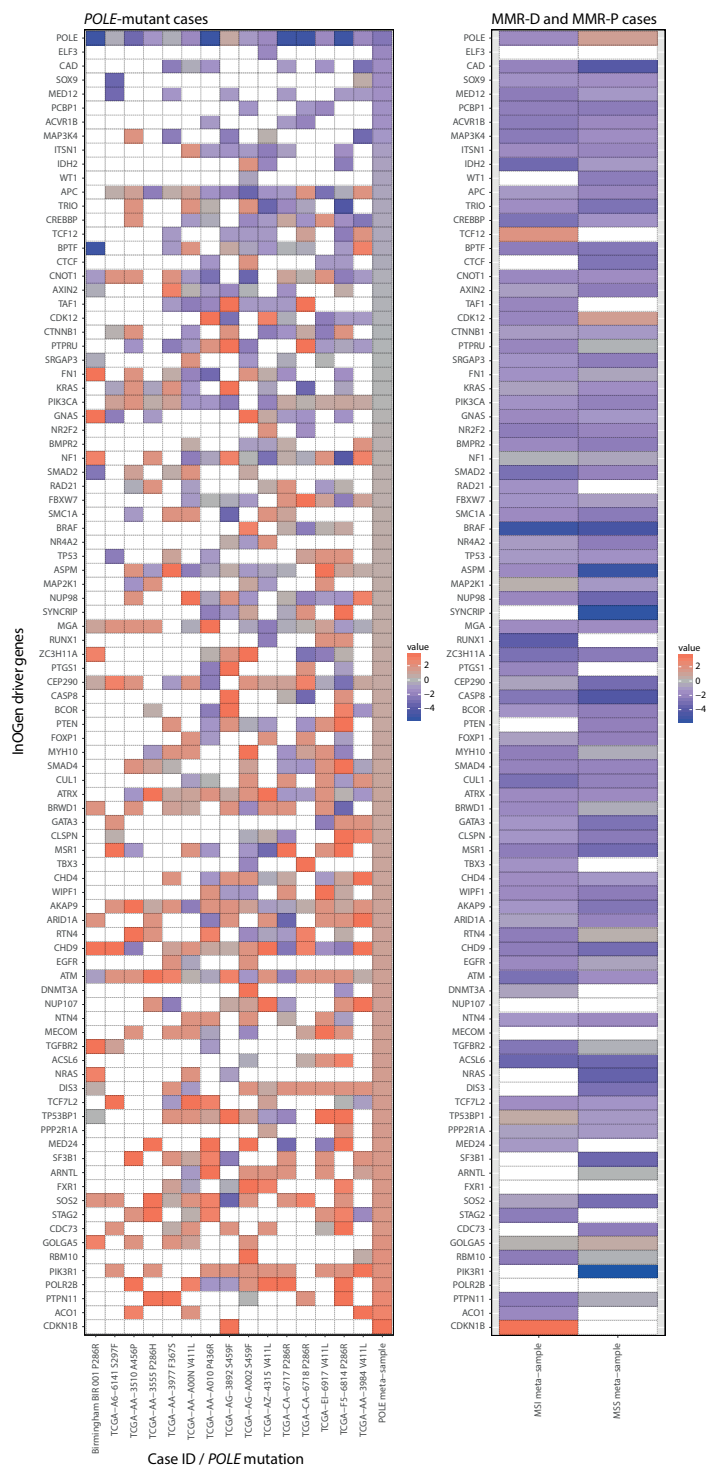


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Figure 3. *POLE* signature mutations in endometrial cancer driver genes.

Heatmap showing modelled probability that mutations in endometrial cancer driver genes (defined based on IntOGen – see Materials and methods, Definition of driver genes; Supplementary Table 5) were due to a prior *POLE* exonuclease domain mutation. Results are shown for samples with a pathogenic *POLE* mutation and MMR-D and MMR-P comparators. Each nonsynonymous mutation in a driver gene was assigned a probability that it was caused by the mutational process that generates the distinct *POLE* mutational signature, rather than by the mutational processes responsible for the consensus mutational signatures of *POLE*-wild-type DNA mismatch repair-proficient (MMR-P) and mismatch repair-deficient (MMR-D) tumors (see Materials and methods, *POLE* consensus mutational signature scores in driver genes, for details). For each gene/sample combination, a 'POLE-score' was then calculated as the minimum value of these ratios, and plotted as a heatmap. Scores are shown for both individual *POLE*-mutant tumors and the combined *POLE*-mutant subgroup; results for tumors within the *POLE*-wild-type, mismatch repair-proficient (MMR-P) and *POLE*-wild-type, mismatch repair-deficient (MMR-D) subgroups are combined for clarity. Scores for *POLE* itself are shown for reference. Details of mutations are provided in Supplementary Tables 8 and 9.

Figure 4. *POLE* signature mutations in colorectal cancer driver genes.

Corresponding heatmap to Figure 3 showing results for known colorectal cancer driver genes, (defined base on IntOGen – see Materials and methods, Definition of driver genes; Supplementary Table 4). Details of mutations are provided in Supplementary Tables 8 and 10.

from the paired endometrial lesions and consistent with the known trinucleotide bias of the *POLE* mutational signature (Supplementary Figures 5-7; Supplementary Tables 7-11). Interestingly, this was evident not only in well-characterized drivers such as *PTEN* in endometrial cancer and *APC* in colorectal cancer as noted above, but also in recurrent, clonal driver mutations found rarely in that tumor type. For example, in the combined TCGA/LUMC endometrial cancer cohorts, truncating mutations in the tumor suppressors *APC*, *NF1* and *RB1* were very rare in *POLE*-wild-type tumors (1.1%, 1.5% and 1.5% respectively), but common among *POLE*-mutant cases (38.8%, 34.7% and 34.7% respectively; $P < 0.001$ for each comparison, Fisher's exact test), where they almost invariably occurred at glutamic acid or arginine codons (Supplementary Figures 5-7; Supplementary Tables 9 and 11).

Collectively, these data suggested that somatic *POLE* mutation occurs early in endometrial and colorectal cancers, and that its attendant mutator phenotype defines a distinct pathway of carcinogenesis from the initial stages of this process.

Somatic *POLE* mutations are associated with a prominent T cell infiltrate in both precancerous and cancerous lesions

Somatic *POLE* exonuclease domain mutations in endometrial and colorectal cancers are associated with enhanced tumor immunogenicity and favorable prognosis.^{11,14,15} We speculated that the early acquisition of somatic *POLE* mutations would cause a rapid acquisition of mutations, some of which would produce neoantigens capable of eliciting

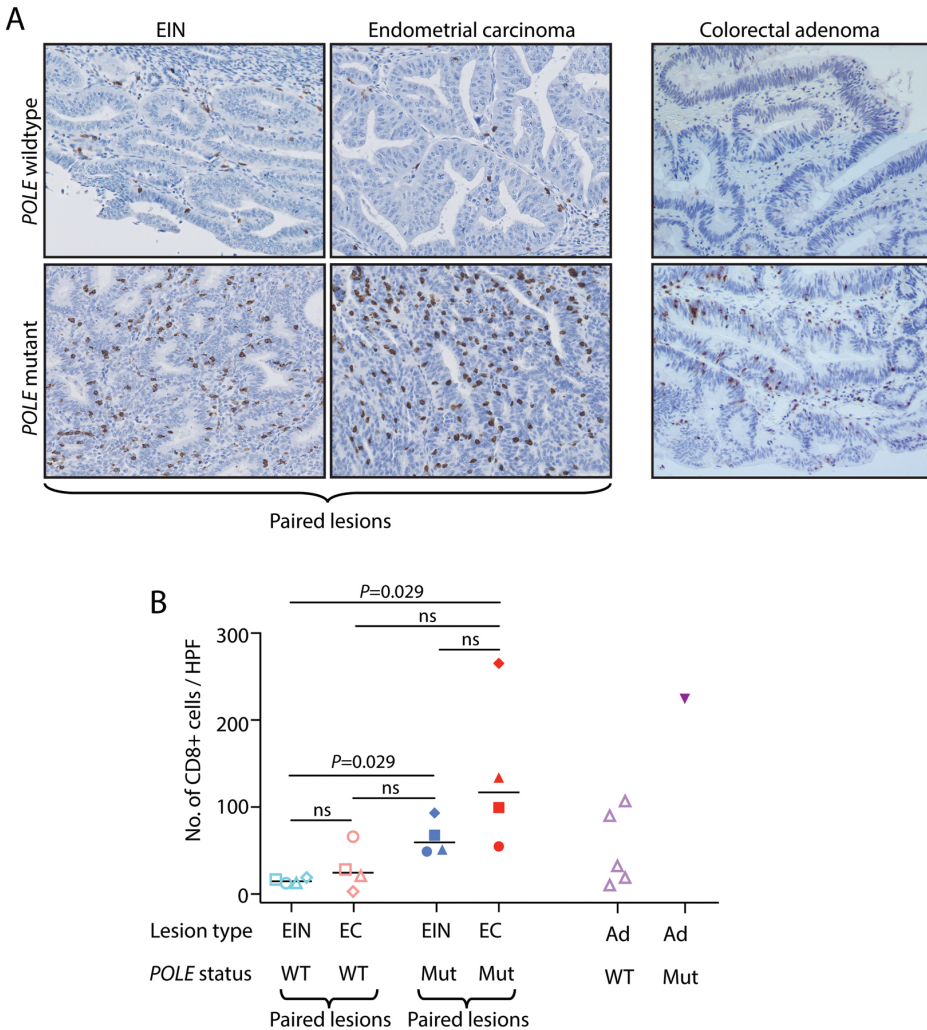


Figure 5. T cell infiltrate in *POLE*-mutant precursor lesions. (A) Representative images of immunohistochemistry (IHC) for the cytotoxic T cell marker CD8 in endometrial intraepithelial neoplasias (EIN) and paired concomitant endometrioid adenocarcinomas and in colorectal adenomas according to *POLE* mutation status. (B) Quantification of CD8⁺ infiltrate density (number of CD8⁺ cells per high power field (HPF) calculated as the mean of 10 high power fields) in *POLE*-wild-type and *POLE*-mutant paired endometrial intraepithelial neoplasia (EIN) and endometrial carcinoma (EC) (n=4 EIN–carcinoma pairs for each genotype) and in *POLE*-wild-type and *POLE*-mutant colorectal adenomas (Ad; n=5 *POLE*-wild-type lesions, and the single *POLE*-mutant adenoma informative for analysis). Symbols (square, circle, triangle and diamond) correspond to paired EIN and endometrial carcinomas for *POLE*-wild-type (open symbols) and *POLE*-mutant (closed symbols) cases. For colorectal adenomas open and closed triangles correspond to unpaired *POLE*-wild-type and *POLE*-mutant adenomas respectively. Statistical comparisons in (B) were performed by unadjusted Mann-Whitney U test.

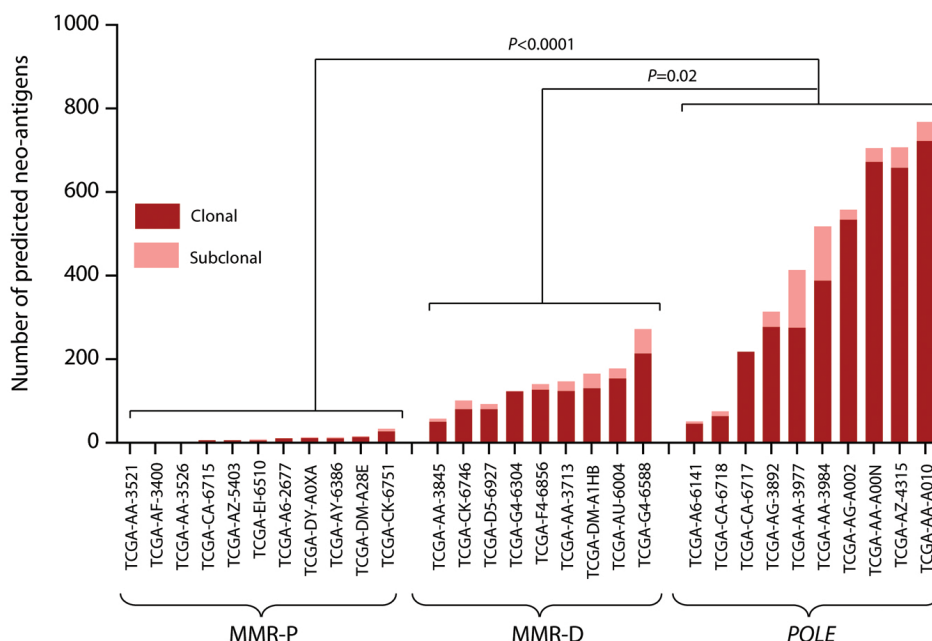


Figure 6. Clonality of predicted neoantigens in *POLE*-mutant colorectal cancers.

Neoantigens were predicted based on the binding affinity of mutant peptides for patient class I HLA molecules, and assigned clonal or subclonal status (see Materials and methods, Clonality of *POLE* mutations). The number of clonal and subclonal neoantigens for *POLE*-wild-type, mismatch repair-proficient (MMR-P), *POLE*-wild-type, mismatch repair-deficient (MMR-D) and *POLE*-mutant colorectal cancers from the TCGA series is shown. Cases in each molecular subgroup were selected to provide broadly similar proportions of disease stages and patient ages: molecular subgroups did not differ significantly in either parameter. Comparison of the number of clonal neoantigen burden between groups was performed by unadjusted Mann-Whitney U test.

ing an antitumor immune response. Consistent with this prediction, all *POLE*-mutant EINs displayed a prominent CD8⁺ infiltrate (Figure 5A), which was significantly greater than that in *POLE*-wild-type EINs (median 59.4 vs. 14.8 CD8⁺ cells per high power field (HPF); $P=0.029$ Mann-Whitney U test), and exceeded that observed in the *POLE*-wild-type endometrial carcinomas, although this difference was not statistically significant (median 59.4 vs. 24.7 CD8⁺ cells per HPF, $P=0.11$; Figure 5B). The increased CD8⁺ cell density in *POLE*-mutant EINs could not obviously be explained by other factors such as patient age, or the stage or grade of the paired carcinoma (Supplementary Table 2). In contrast, the differences in CD8⁺ density between EINs and paired carcinomas among both *POLE*-wild-type and *POLE*-mutant cases were less marked (median 14.8 vs. 24.7; $P=0.34$, and 59.4 vs. 116.9; $P=0.11$ respectively). The single *POLE*-mutant colorectal adenoma for which IHC was possible also demonstrated a dense CD8⁺ infiltrate (154.9 vs. median 34.0 CD8⁺ cells per HPF; Figure 5A-B).

Somatic *POLE* mutations in colorectal cancer are associated with enhanced predicted clonal neoantigen burden

Recent data have shown that the presence of predicted neoantigens within the major tumor clone correlates with benefit from immune checkpoint inhibitor therapy.¹⁸ As the limited amount of FFPE-derived DNA from precursor lesions was inadequate for clonality analysis and neoantigen prediction, we examined predicted neoantigen clonality in a subset of TCGA colorectal cancers including MMR-P, MMR-D and *POLE*-mutant subtypes, broadly matched for patient age and tumor stage. We used an approach similar to our previous reports,^{11,14} modified to incorporate patient-specific HLA haplotypes obtained using OptiType³⁵ and estimates of tumor clonality derived from analysis of variant allele frequencies (see Materials and Methods, Clonal neoantigen prediction). Analysis of our combined cohort by this pipeline confirmed that *POLE*-mutant colorectal cancers harbored a substantially greater number and density of predicted clonal neoantigens (0.12 per Mb) than tumors lacking *POLE* mutations, including both MMR-P (0.0029 per Mb; $P=0.0002$, Mann-Whitney U test) and hypermutated MMR-D cases (0.044 per Mb; $P=0.03$; Figure 6; Supplementary Figure 8).

DISCUSSION

In this study, we have presented multiple lines of evidence to show that pathogenic, somatic *POLE* exonuclease domain mutations are usually early and, as far as we can detect, initiating events in endometrial and colorectal tumorigenesis. We show that the acquisition of *POLE* mutation causes a distinct pattern of mutations in cancer driver genes, substantially increased mutation burden and an enhanced immune response, detectable even in precancerous lesions. Furthermore, we show that early somatic *POLE* exonuclease domain mutations are likely to cause an enrichment of clonal neoantigens that may explain their good prognosis and excellent response to immune checkpoint inhibitors.

APC mutation has traditionally been regarded as the initiating event in sporadic colorectal cancers that develop along the canonical pathway,¹⁹ while mutation of *PTEN* is thought to play a similar role in sporadic endometrioid endometrial cancers.³⁶ Our evidence suggests that in sporadic colorectal and endometrial cancers with pathogenic somatic *POLE* mutations, the *POLE* mutation is antecedent to either of these events. The consequent mutator phenotype it causes influences the type of mutations in these genes and that of the other earliest driver mutations in these cancers, as well as determining their overall mutational landscape.⁶ Whether any of these *POLE*-induced driver mutations represent targetable alterations will be an important topic for future research. Similarly, while the

increased burden of predicted clonal neoantigens in *POLE*-mutant tumors may explain their enhanced immunogenicity, further work is required to understand the molecular factors that determine this and its therapeutic implications. A further intriguing possibility is that the mutator phenotype and mutational bias drives cancers into an evolutionary cul-de-sac of sub-optimal fitness. The presence of *APC* mutations as an alternative to *CTNNB1* mutations in some *POLE*-mutant endometrial cancers is an exemplar, and there are likely to be others, such as *NF1* and *RB1* mutations in endometrial cancer and atypical (Q61P, K117N and A146T) *KRAS* mutations in colorectal cancer. Examination of this hypothesis by comparing the oncogenic effects of these uncommon mutations with those caused by more typical variants in model systems would be of considerable interest.

Our data add to the expanding body of evidence suggesting that the effects of genomic instability in cancer depend upon both its severity and timing. For example, upregulation of APOBEC cytosine deaminase enzymes is common in many types of cancers, resulting in an increased mutation rate and characteristic mutation spectrum.⁶ However, APOBEC overexpression often occurs as a late event in advanced tumors and causes a more modest mutator phenotype than *POLE* mutations.^{2,6} Speculatively, these features may explain why the impact of APOBEC on prognosis appears more variable than that of *POLE* mutation.^{37,38} The early acquisition of somatic *POLE* mutations in sporadic cancers may also help to explain their association with young age at diagnosis, given the prediction that the early gain of a mutator phenotype will accelerate the process of malignant transformation.³⁹

Our study has limitations. The number of precursor lesions informative for detailed analysis was limited, in keeping with the relative rarity of *POLE* mutations in endometrial cancer, and the frequency with which precancerous and cancerous lesions occur in the same tumor section. Moreover, although the spatial separation of the precancerous and cancerous compartments, and the discordance in molecular alterations between the two components in each case suggests otherwise, we cannot exclude the possibility that the apparent precursor lesion is in fact adenocarcinoma colonizing endometrial glands. It will therefore be important to validate our results in additional cohorts, although we note that a very recent study has documented a pathogenic *POLE* mutation in an endometrial cancer precursor.²⁵ Furthermore, all our results are based on the analysis of a single sample of each cancer, meaning that the effects of intratumor heterogeneity on the pattern of driver mutations and clonal neoantigens in *POLE*-mutant tumors requires further definition. However, the absence of multi-region sequencing is unlikely to have confounded the principal conclusions of our study regarding the timing of these pathogenic mutations in cancers.

In summary, we show that pathogenic, somatic *POLE* exonuclease domain mutations

are early, quite possibly initiating, events in sporadic cancers, and strongly shape subsequent tumor evolution. Our observation provides further insights into the distinct biology of these tumors, and may help explain their increased immunogenicity and excellent prognosis.

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SUPPLEMENTARY METHODS

DNA sequencing

Endometrial epithelial neoplasias (EIN) and paired endometrial cancers were analyzed by a custom molecular inversion probe (MIP) panel covering 30 genes commonly mutated in endometrial and colorectal cancers (Supplementary Table 3), and a clinical-grade ThermoFisher Ion AmpliSeq Cancer Hotspot Panel v2, which includes 376 amplicons across 80 genes (Supplementary Table 4). For the MIP panels, libraries were sequenced on an Illumina NextSeq and aligned to GrCh37 using BWA mem. Variants were called using LoFreq¹ and annotated by VEP.² Only variants with VAF >0.1 were used for analysis. For the Ion AmpliSeq panel, library preparation was performed according to the manufacturer's protocol using 50ng of genomic DNA. Libraries were normalized to 60pM, loaded on a PI chip using the Ion Chef System (ThermoFisher), and sequenced with the Ion Proton System (ThermoFisher). The generated reads were aligned to the human reference genome (GRCh37) using the TMAP 5.0.7 software with default parameters (<https://github.com/iontorrent/TS>). The Ion Torrent specific variant caller was used for variant calling (ThermoFisher) and Geneticist Assistant (Softgenetics) for variant interpretation. Variants were removed with a variant allele frequency of <0.1 and/or with a coverage of <100. Called mutations were visually inspected using the Integrative Genomics Viewer (IGV) software (<http://software.broadinstitute.org/software/igv/>). All *POLE* mutations were confirmed by Sanger sequencing as previously reported,³ with the exception of one EIN with low DNA yield, for which competitive allele-specific PCR (LGC Genomics) assay was used (details of primers and reaction conditions available on request). FFPE colorectal adenomas were screened for somatic *POLE* exonuclease domain mutations by a combination of Sanger sequencing and competitive allele-specific PCR as described previously.⁴

DNA from fresh frozen endometrial tumors and paired normal samples for whole genome sequencing (WGS) was quantified using the Qubit® 2.0 fluorometer (Life Technologies, Paisley, UK), and fragmented using the Covaris M220 ultrasonicator (Covaris, Inc., Woburn, MA) to an average fragment size of 250-300bp. Approximately 50ng was used as input for the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, Hitchin, UK). Libraries were prepared as per the manufacturer's guidelines, with size selection for a 250bp insert size, dual indexing and 9 cycles of library amplification. Libraries were sequenced to a median depth of ~50x on Illumina's HiSeq X Ten (150bp paired end reads) at BGI Tech Solutions Ltd, Hong Kong. Somatic mutations were called from DNA sequencing data using Mutect2.⁵ Variants flagged as 'PASS' or 'clustered_events' were accepted as somatic. Variants were annotated using Annovar.⁶ Copy number profiles were derived using Sequenza⁷ for a subset of samples, and manu-

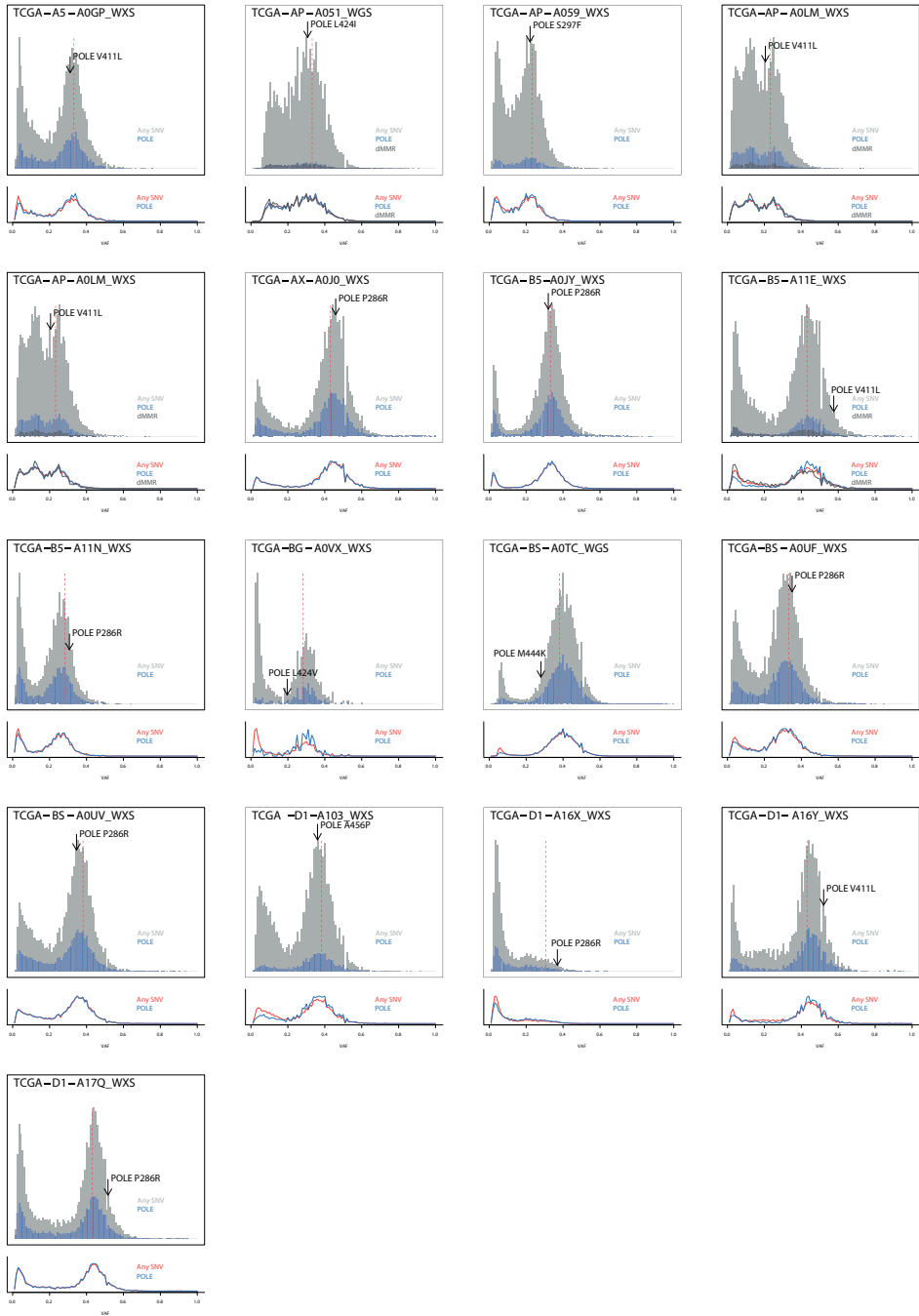
ally curated to remove probable model artefacts. DNA from the colorectal cancer was prepared for WGS using the Truseq PCR-free library preparation kit (Illumina) as per manufacturers guidelines and sequenced on an Illumina HiSeq 2500. Sequenced reads were aligned to the GrCh37 reference genome using the Isaac aligner,⁸ and variant calling performed using Strelka.⁹

FFPE endometrial cancers from the LUMC series were analyzed using the Lifetech/ThermoFisher Ion AmpliSeq Comprehensive Cancer Panel comprising 409 cancer genes (<http://www.lifetechnologies.com/order/catalog/product/4477685>). Extracted genomic DNA was quantified by Qubit and library preparation performed according to the manufacturer's protocol (LifeTechnologies, cat. no. 4477685 and 4480442). Details of PCR conditions and library purification and quantification were as previously reported.¹⁰ Diluted libraries were loaded onto the Ion PI chip v2 BC (cat. no. 4484270) and sequenced on the Ion Proton sequencer (cat. no. 4476610). Signal processing and base calling were performed using the Ion Proton Torrent suite (version 4.4). Variant calling was performed using variantCaller and Ion Reporter software (Thermo). Variant call format (VCF) files were annotated using Variant Effect Predictor (VEP)² and filtered to remove variants with (i) read depth of <50, quality of depth (QD) of <2 or fewer than 10 supporting reads; (ii) flow evaluator alternate allele observation count of <20; (iii) genotype quality (GQ) of <90; (iv) variants in homopolymer runs of >5; (v) variants ≤ 3 bases from the end of an amplicon; (vi) minor allele frequency of >1% in ExAc, 1000 Genomes or UK10K; (vii) a variant allele fraction of <0.1. Data were then manually curated to remove likely artefactual variants (e.g. variant absent from COSMIC, and/or present in more cases than a known endometrial cancer gene such as *PIK3CA* or *PTEN*).

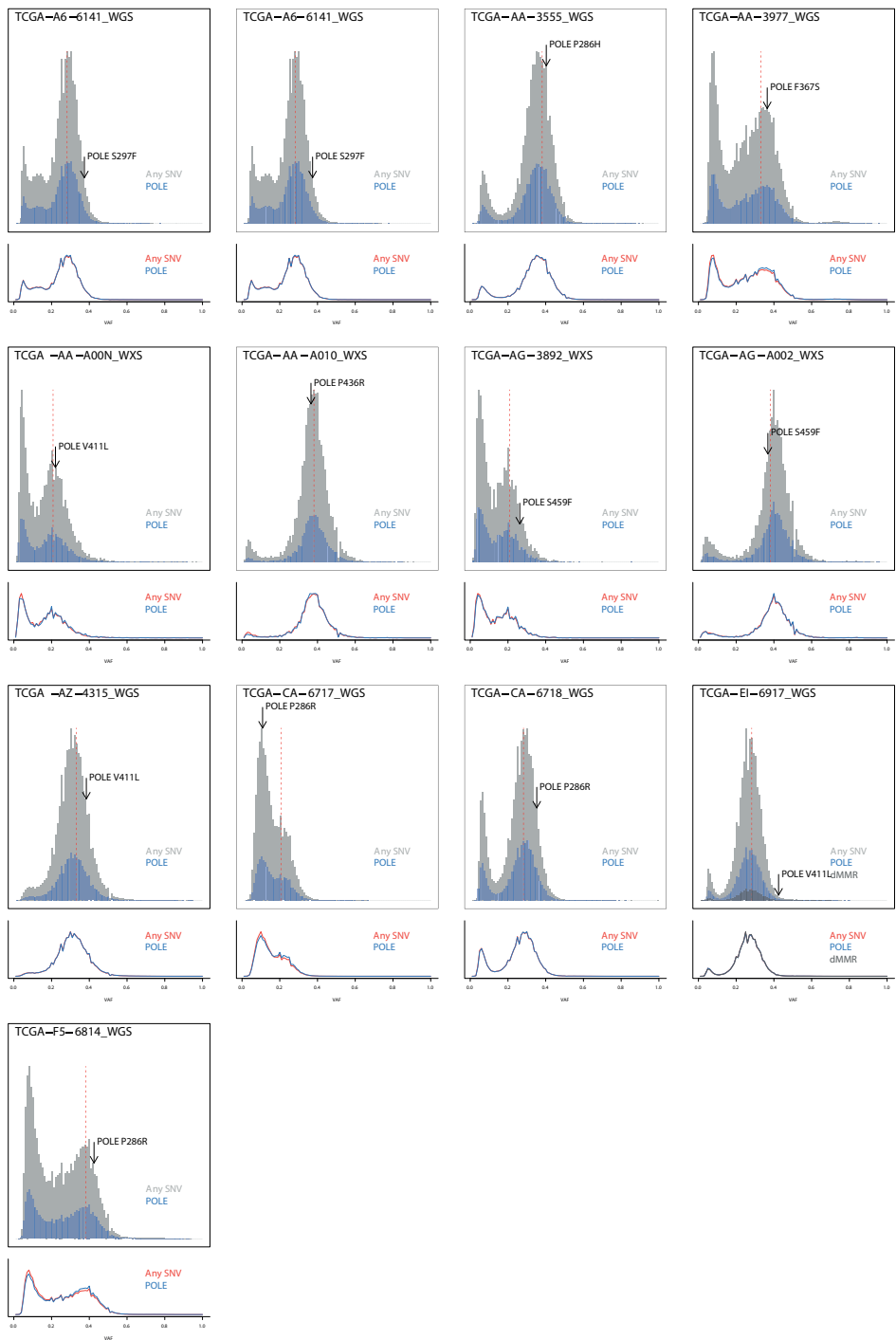
Somatic mutations in TCGA cancers were called from BAMs using Mutect2.⁵ Additional cases from the TCGA COADREAD and UCEC data sets were downloaded as Mutect Mutation Annotation Format (MAF) files from the GDC Data Portal. Variant annotation and model curation was performed as for the WGS cases. Copy number data was downloaded for COADREAD data from the same source. 'Segment Mean' (S) was transformed using the following formula to find copy number index (I): $I = \text{round}((2^S) \times 2)$. Segments outside chr6.p with copy number index equal to 2 were assumed to be diploid.

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Supplementary Figure 1. Legend on page 201



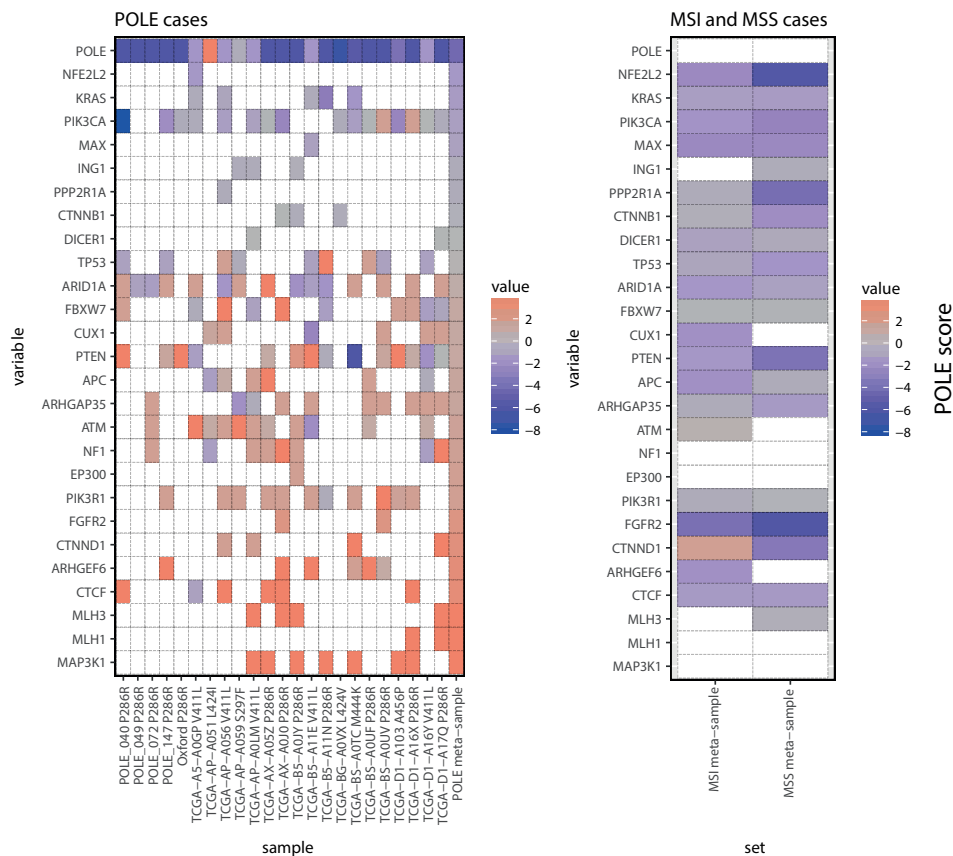
Supplementary Figure 2. Legend on next page

Supplementary Figure 1. Clonality of *POLE* mutations and mutational processes in TCGA endometrial cancers.

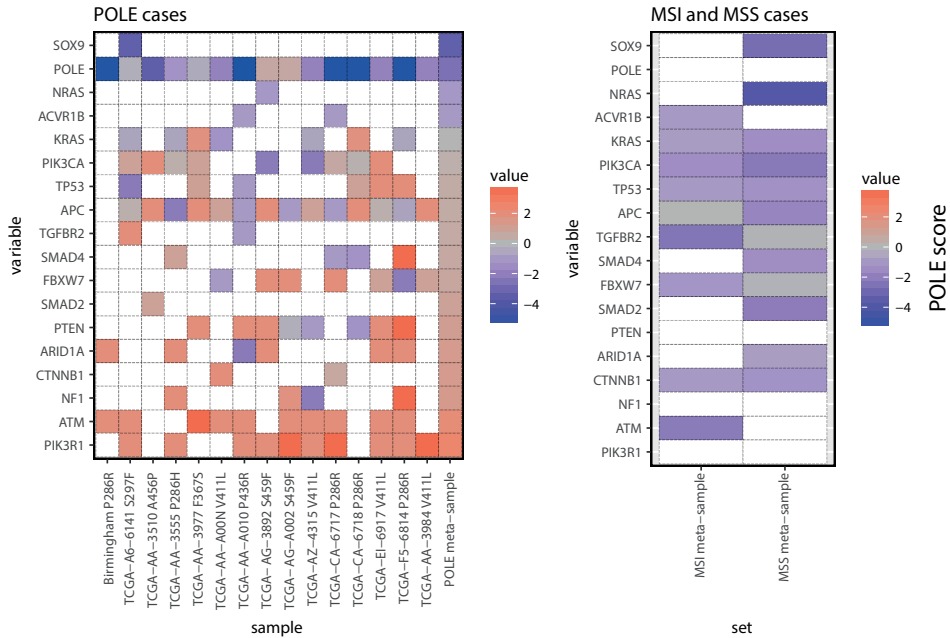
Frequency histograms and kernel density plots showing variant allele fraction (VAF) of all SNV mutations, and SNVs likely due to *POLE* exonuclease domain mutation (*POLE*). Only mutations in diploid regions of autosomes, and with coverage >20x are shown. The relatively low proportion of SNVs categorized as being due to *POLE* mutation reflects the stringency of the classification used (see Materials and methods, Mutational signatures). VAF of *POLE* mutations are highlighted. Vertical red line indicates clonal peak used to calculate cellularity.

Supplementary Figure 2. Clonality of *POLE* mutations and mutational processes in TCGA colorectal cancers.

Frequency histograms and kernel density plots showing variant allele fraction (VAF) of all SNV mutations, and SNVs likely due to *POLE* exonuclease domain mutation (*POLE*). Only mutations in diploid regions of autosomes, and with coverage >20x are shown. The relatively low proportion of SNVs categorized as being due to *POLE* mutation reflects the stringency of the classification used (see Materials and methods, Mutational signatures). VAF of *POLE* mutations are highlighted. Vertical red line indicates clonal peak used to calculate cellularity.

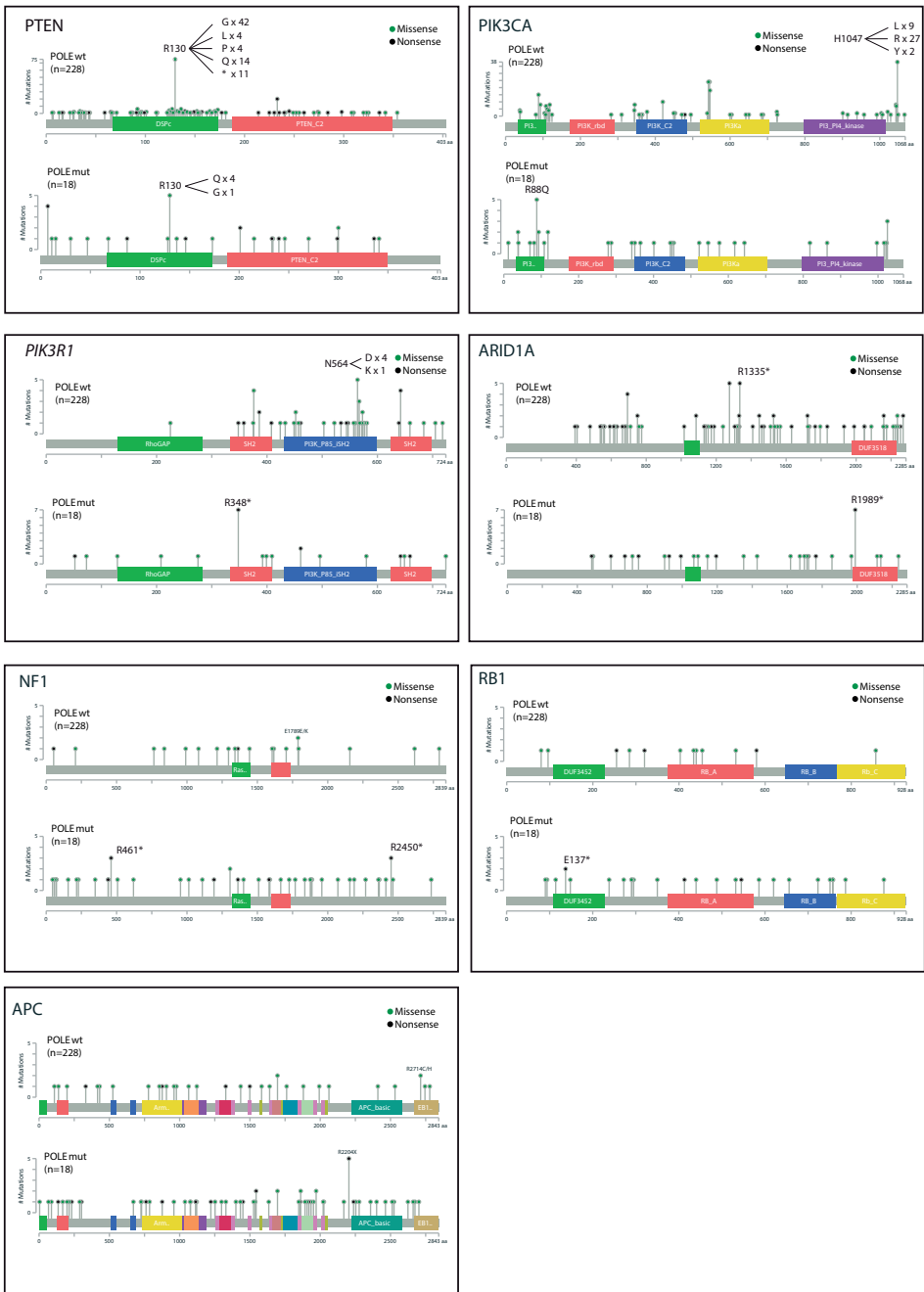


Supplementary Figure 3. *POLE* signature in high-confidence endometrial cancer driver mutations. Corresponding heatmap to Figure 3, limited to high-confidence endometrial cancer driver mutations. High confidence driver mutations were defined as those causing protein truncations known to perturb function of tumor suppressors and missense variants at recurrently-mutated hotspot codons in either tumor suppressors and oncogenes. Each driver gene mutation was assigned a probability that it was caused by the mutational process that generates the distinct *POLE* mutational signature, rather than by the mutational processes responsible for the consensus mutational signatures of *POLE*-wild-type DNA mismatch repair-proficient (MMR-P) and mismatch repair-deficient (MMR-D) tumors (see Materials and methods, *POLE* consensus mutational signature scores in driver genes, for details). For each gene/sample combination, a '*POLE*-score' was then calculated as the base two logarithm of the minimum value of these ratios, and plotted as a heatmap. Scores are shown for both individual *POLE*-mutant tumors and the combined *POLE*-mutant subgroup; results for tumors within the *POLE*-wild-type, mismatch repair-proficient (MMR-P) and *POLE*-wild-type, mismatch repair-deficient (MMR-D) subgroups are combined for clarity.



Supplementary Figure 4. *POLE* signature in high-confidence colorectal cancer driver mutations.

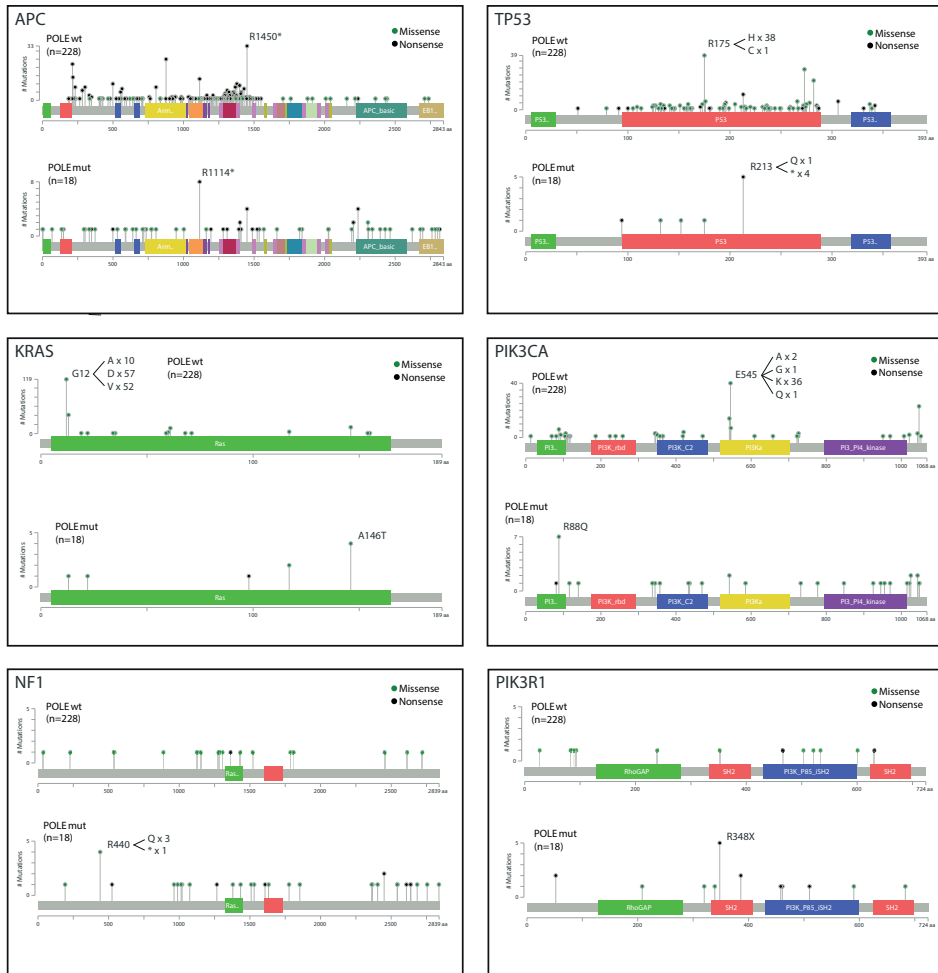
Corresponding heatmap to Figure 4, limited to high-confidence endometrial cancer driver mutations. High confidence driver mutations were defined as those causing protein truncations known to perturb function of tumor suppressors and missense variants at recurrently-mutated hotspot codons in either tumor suppressors and oncogenes. Each driver gene mutation was assigned a probability that it was caused by the mutational process that generates the distinct *POLE* mutational signature, rather than by the mutational processes responsible for the consensus mutational signatures of *POLE*-wild-type DNA mismatch repair-proficient (MMR-P) and mismatch repair-deficient (MMR-D) tumors (see Materials and methods, *POLE* consensus mutational signature scores in driver genes, for details). For each gene/sample combination, a '*POLE*-score' was then calculated as the base two logarithm of the minimum value of these ratios, and plotted as a heatmap. Scores are shown for both individual *POLE*-mutant tumors and the combined *POLE*-mutant subgroup; results for tumors within the *POLE*-wild-type, mismatch repair-proficient (MMR-P) and *POLE*-wild-type, mismatch repair-deficient (MMR-D) subgroups are combined for clarity.



Supplementary Figure 5. Driver mutations in TCGA endometrial cancers.

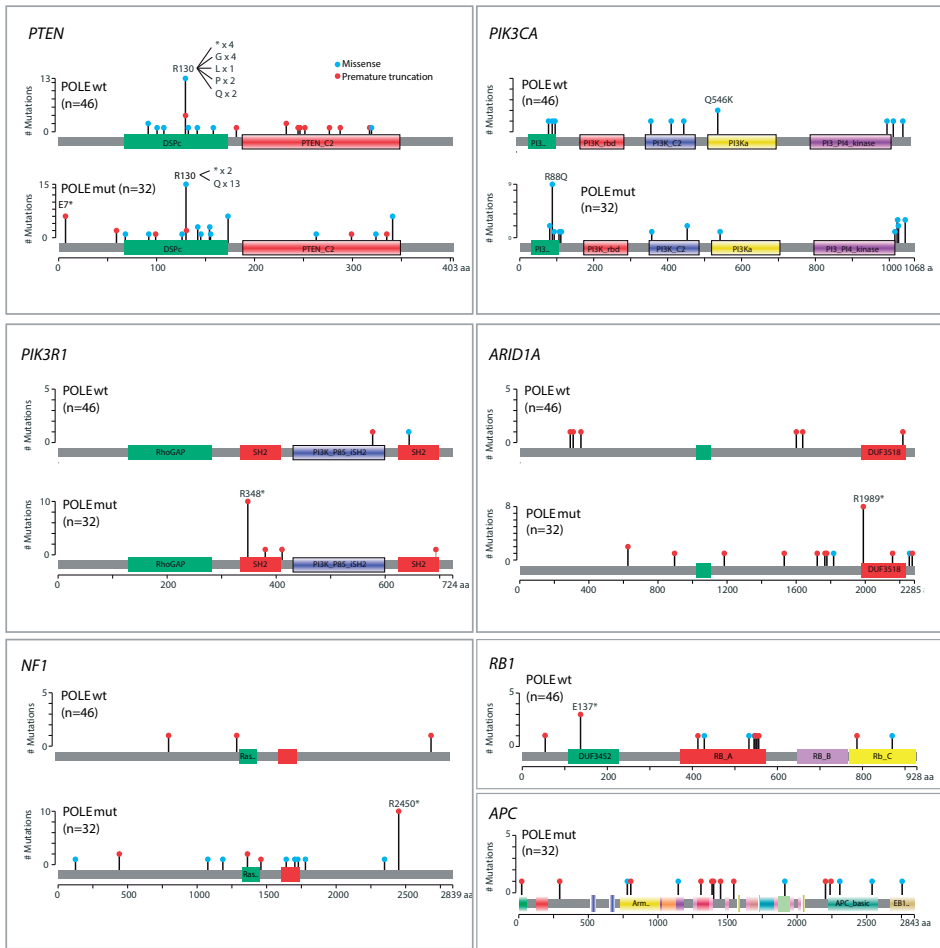
Comparison of mutation type and frequency in selected driver genes according to tumor molecular subtype. *POLE* wt – *POLE*-wild-type group includes tumor irrespective of DNA mismatch repair status. *POLE* mut – pathogenic somatic *POLE* exonuclease domain mutations. Glutamic acid to stop mutations (E→*)

occur when a glutamic acid codon (GAG or GAA) is preceded by an A (e.g. AGAG or AGAA), creating an AGA trinucleotide which is commonly mutated to ATA in *POLE*-mutant tumors, causing a stop codon (TAG or TAA). Arginine to stop mutations (R→*) occur when the *POLE* hotspot trinucleotide TCG is followed by an A, resulting in a TCGA to TTGA mutation. Arginine to glutamine substitutions (R→Q) occur when the reverse complement of this hotspot, CGA is mutated to CAA.



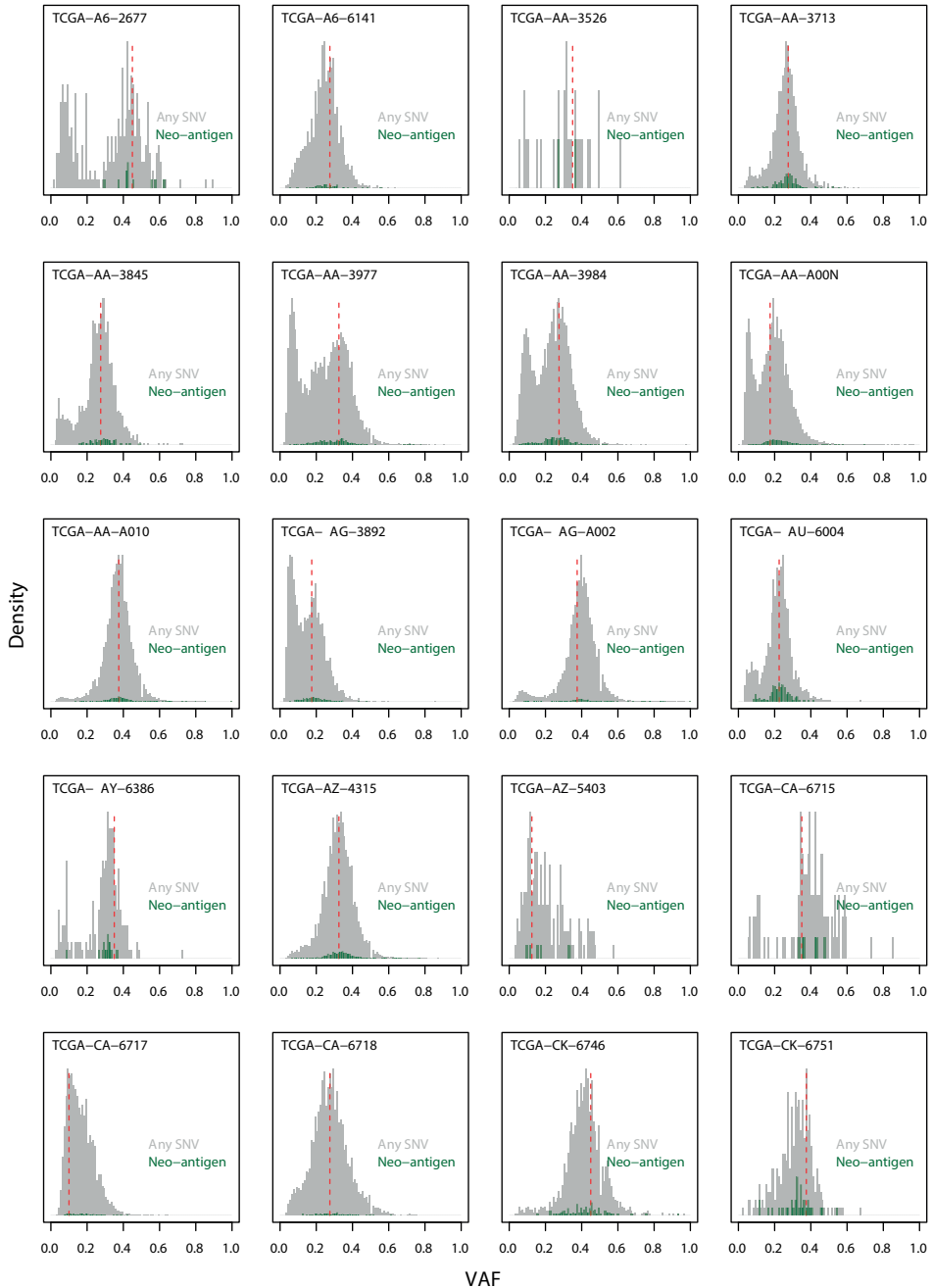
Supplementary Figure 6. Driver mutations in TCGA colorectal cancers.

Comparison of mutation type and frequency in selected driver genes according to tumor molecular sub-type. *POLE* wt – *POLE*-wild-type group includes tumor irrespective of DNA mismatch repair status. *POLE* mut – pathogenic somatic *POLE* exonuclease domain mutations. Glutamic acid to stop mutations (E→*) occur when a glutamic acid codon (GAG or GAA) is preceded by an A (e.g. AGAG or AGAA), creating an AGA trinucleotide which is commonly mutated to ATA in *POLE*-mutant tumors, causing a stop codon (TAG or TAA). Arginine to stop mutations (R→*) occur when the *POLE* hotspot trinucleotide TCG is followed by an A, resulting in a TCGA to TTGA mutation. Arginine to glutamine substitutions (R→Q) occur when the reverse complement of this hotspot, CGA is mutated to CAA.



Supplementary Figure 7. Driver mutations in LUMC endometrial cancers.

Comparison of mutation type and frequency in selected driver genes according to tumor molecular subtype in a cohort of FFPE tumors from the Leiden University Medical Center (LUMC). *POLE* wt – *POLE*-wild-type group includes tumor irrespective of DNA mismatch repair status. *POLE* mut – pathogenic somatic *POLE* exonuclease domain mutations. Glutamic acid to stop mutations (E→*) occur when a glutamic acid codon (GAG or GAA) is preceded by an A (e.g. AGAG or AGAA), creating an AGA trinucleotide which is commonly mutated to ATA in *POLE*-mutant tumors, causing a stop codon (TAG or TAA). Arginine to stop mutations (R→*) occur when the *POLE* hotspot trinucleotide TCG is followed by an A, resulting in a TCGA to TTGA mutation. Arginine to glutamine substitutions (R→Q) occur when the reverse complement of this hotspot, CGA is mutated to CAA.



Supplementary Figure 8. Clonality of neoantigens in TCGA colorectal cancers.

Frequency histograms for cases corresponding to Figure 6 showing variant allele fraction (VAF) of all SNV mutations and predicted neo-antigens. Only mutations in diploid regions of autosomes, and with coverage >20x were considered. Vertical red line indicates inferred clonal peak used to calculate cellularity.

Supplementary Table 1. Cohorts analyzed and molecular analyses performed.

Cohort	Sample type	Analyses performed	Previous reports
Paired endometrial cancers and EINs	FFPE tumor and paired precursor lesions	Molecular inversion probes capture panel sequencing Custom Ion Torrent panel sequencing Sanger sequencing CD8 IHC	<i>POLE</i> -mutant endometrial cancers identified in Refs 12, 14, 26
Colorectal adenomas	FFPE adenomas	Sanger sequencing Competitive allele-specific PCR (Taqman)	Previously unscreened
<i>POLE</i> -mutant endometrial and colorectal cancers	Fresh frozen tumors	Whole genome sequencing	<i>POLE</i> mutant invasive endometrial cancers from Leuven cohort identified in Ref 12. Other cases previously unreported.
TCGA endometrial cancers	Fresh frozen tumors	Re-analysis of genome and exome sequencing	TCGA (Ref 8)
TCGA colorectal cancers	Fresh frozen tumors	Re-analysis of genome and exome sequencing	TCGA (Ref 10)
Endometrial cancers	FFPE tumors	Thermo Comprehensive Cancer Panel	Cases identified in Refs 14, 26

Abbreviations: EIN, endometrial intraepithelial neoplasia; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry.

Supplementary Table 2. Details of cases used for molecular analyses.

Case	Patient age	Sample type	Pathological characteristics	Tumor cell fraction
LUMC 33	61	EIN		60%
		EEC	FIGO stage I, grade 2, LVSI negative	70%
LUMC H0284	46	EIN		50%
		EEC	FIGO stage III, grade 3, substantial LVSI	70%
LUMC L2-18	49	EIN		70%
		EEC	FIGO stage I, grade 1, LVSI negative	70%
LUMC Q1-4	61	EIN		60%
		EEC	FIGO stage I, grade 1, LVSI negative	70%
OXF 001	55	EEC	FIGO stage I, grade 2, LVSI negative	70%
POLE 040	60	Mixed EEC-serous	FIGO stage I, grade 3, LVSI negative	60%
POLE 049	57	Mixed-EEC-serous	FIGO stage I, grade 3, LVSI negative	75%
POLE 072	55	EEC	FIGO stage II, grade 3, LVSI negative	70%
POLE 147	53	EEC	FIGO stage I, grade 3, LVSI negative	60%
BIR 001	61	CRC	T4, N1 adenocarcinoma of descending colon	75%

Abbreviations: EIN, endometrial intraepithelial neoplasia; EEC, endometrioid endometrial adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space invasion.

SUPPLEMENTARY TABLES AVAILABLE ONLINE

The following supplementary tables are available online at: <https://onlinelibrary.wiley.com/doi/abs/10.1002/path.5081>.

Supplementary Table 3. Genes included in custom molecular inversion probe panel.

Supplementary Table 4. Genes included in custom Ion AmpliSeq Cancer Hotspot Panel.

Supplementary Table 5. List of IntOGen endometrial cancer driver genes used in study.

Supplementary Table 6. List of IntOGen colorectal cancer driver genes used in study.

Supplementary Table 7. Driver mutations detected in paired endometrial intraepithelial neoplasias (EIN) and endometrial carcinomas.

Supplementary Table 8. Driver mutations in *POLE*-mutant cancers analyzed by whole genome sequencing.

Supplementary Table 9. Driver mutations in TCGA endometrial cancers by tumor molecular subgroup.

Supplementary Table 10. Driver mutations in TCGA colorectal cancers by tumor molecular subgroup.

Supplementary Table 11. Driver mutations in endometrial cancers analyzed by Ion Ampliseq Comprehensive Cancer Panel.



Chapter 8

Discussion and future perspectives

DISCUSSION AND FUTURE PERSPECTIVES

POLE exonuclease domain mutations (hereafter referred to as '*POLE* mutations') are found in a wide variety of human malignancies, including endometrial cancer. In all tumor types, cancers with *POLE* mutations are characterized by an exceptionally high mutational burden and a specific mutational signature.^{1,2} Despite their so-called 'ultra-mutated' phenotype, patients with *POLE*-mutant cancers have significantly better outcomes compared to patients with *POLE*-wild-type tumors. This prognostic benefit has thus far been shown for early-stage endometrial and stage II/III colorectal cancers, and has been suggested for glioblastomas.^{1,3-10} The studies described in this thesis aimed to gain insight into somatic *POLE* exonuclease domain mutations and especially into the underlying mechanism(s) by which these *POLE* mutations contribute to low risk of recurrence. This chapter puts the main findings of this thesis into perspective of current literature, and discusses the implications of these findings for the treatment of patients with *POLE*-mutant cancers, in particular endometrial cancer.

Explaining the favorable clinical outcome of *POLE* exonuclease domain-mutant cancers

Three hypotheses that could explain or contribute to the favorable prognosis of *POLE*-mutant cancers were studied and will be discussed below.

1. Induction of an antitumor immune response

To explain the good prognosis of *POLE*-mutant cancers, we hypothesized that the ultra-mutated phenotype would result in the presentation of neoantigens on the tumor cell surface. These neoantigens would elicit an antitumor immune response, which may contribute to the favorable outcome of these patients. To support this hypothesis, this thesis and work of others provide several lines of evidence that will now be discussed.

POLE-mutant cancers were predicted to display more neoantigens than *POLE*-wild-type cancers (chapters 4 and 5).¹¹ This prediction was based on *in silico* algorithms that assessed the binding affinity to MHC class I of all possible mutant peptides relative to that of the wild-type peptide. Similar prediction algorithms have been used in many reports to date to identify neoantigens.¹²⁻¹⁸ Previous studies also showed, however, that only a small fraction of mutations in expressed genes resulted in the presentation of neoantigens that elicit T cell reactivity.^{13,15,19-21} Therefore, the *in silico* algorithms could more accurately predict reflect the landscape of immunogenic neoantigens in cancers when filtering of the exome or RNA sequencing data would be improved. Still, the observed difference in predicted neoantigen numbers between *POLE*-mutant and *POLE*-wild-type is very large (~100-fold) with the currently employed methods. Therefore, regardless of

more stringent filtering, these differences suggest that *POLE* mutations indeed result in the presentation of immunogenic neoantigens.

An *in vitro* study supported the concept of increased immunogenicity of *POLE*-mutant cancers. In this study, autologous dendritic cells pulsed with lysate from *POLE*-mutant endometrial cancers induced stronger proliferation of cytotoxic T cells and T helper cells than lysate derived from *POLE*-wild-type cancers.²² This suggests that *POLE*-mutant cancers are capable of eliciting an immune response and that this capacity is greater than in their wild-type counterparts. Whether the increased immunogenicity of *POLE*-mutant cancers is indeed caused by a higher neoantigen load remains to be elucidated.

POLE-mutant (endometrial) cancers are characterized by the presence of an enhanced cytotoxic T cell response (chapters 4 and 5).^{9,22,23} In *POLE*-mutant cancers, tumor-infiltrating lymphocytes are present in larger numbers than in *POLE*-wild-type cancers. Moreover, these lymphocytes demonstrate upregulation of activation and effector markers, suggesting the T cells are cytotoxically active. These findings are supportive of increased immunogenicity of *POLE*-mutant cancers. Furthermore, the association between the presence of such an immune infiltrate and prognosis is well-established: a meta-analysis showed in a pooled cohort of over 12,000 patients with various tumor types that tumor-infiltrating lymphocytes have a positive effect on clinical outcomes.²⁴ A similar association has been previously described for endometrial cancers, albeit in a limited number of studies.^{25,26}

For the T cells to contribute to the favorable prognosis, these lymphocytes would have to be tumor-reactive, recognizing the antigen presented by the tumor cell as being foreign. Evidence for this tumor reactivity is provided by the response of *POLE*-mutant cancers to immune checkpoint inhibition. It is important to note that this response is also clinically very relevant (details on therapeutic implications are given below). Through immune checkpoint inhibition, T cells are relieved of certain inhibitory signals (such as PD-1/PD-L1 signaling), leading to a release of their cytotoxic potential. Although the search for a robust biomarker to predict response to checkpoint inhibitors is still ongoing, response rates are higher in cancers with a high mutational load.^{27,28} This is illustrated by the 40-53% objective response rates found in (mostly colorectal) cancers with mismatch repair deficiency, compared to 0% response in non-hypermutated cancers.^{29,30} Following this rationale, the first few women with advanced-stage endometrial cancers carrying *POLE* mutations have been treated with anti-PD-1 antibodies and showed sustained responses.³¹⁻³³ These responses suggest that upon PD-1/PD-L1 blockade, T cells that are tumor-reactive are 'unleashed', resulting in effective antitumor activity contributing to prolonged survival.

Finally, a parallel can be drawn between *POLE*-mutant cancers and melanomas to provide evidence, albeit circumstantial, supportive of this 'antitumor immune response-hypothesis'. In melanomas, another highly mutated tumor type, lymphocytes have been identified in the tumor microenvironment that are reactive to neoantigens presented by the cancer cells.^{15,20} Furthermore, the tumor-reactive T cells expanded in the peripheral blood after immune checkpoint inhibitor treatment, suggesting a role for these T cells in tumor regression.²⁰ Thus, the mutational load in melanomas is apparently sufficient to generate neoantigens that can be recognized by autologous T cells. Compared to melanomas, *POLE*-mutant cancers carry on average ten times as many mutations (10 mutations per Mb for melanomas vs more than 100 mutations per Mb for *POLE*-mutant cancers).³⁴ It can therefore be expected that the presence of neoantigens and their recognition by T cells are also common in *POLE*-mutant cancers.

In summary, we and others showed that the ultramutated *POLE*-mutant (endometrial) cancers display an enhanced cytotoxic T cell response. This T cell response is accompanied by - and most likely caused by - a significant increase in the number of neoantigens presented at the tumor cell surface. Together, this provides a plausible mechanism by which *POLE* exonuclease domain mutations contribute to the good clinical outcome of the majority of patients.

A question worthy of further exploration is why *POLE*-mutant cancers, despite the antitumor immune response, are not eliminated by the immune system. Defects in antigen processing or presentation occurred at a similar frequency in *POLE*-mutant as in *POLE*-wild-type (chapter 4). Immunosuppressive molecules such as PD-1 and CTLA-4 were upregulated in *POLE*-mutant cancers compared to *POLE*-wild-type. However, considering the overall increase in expression of cytotoxic effector markers, the upregulation of immunosuppressive mediators appeared insufficient to fully suppress the T cell response in *POLE*-mutant endometrial cancers (chapter 4). Therefore, further research on immune escape mechanisms in *POLE*-mutant cancers, e.g. on the presence of immunosuppressive cells in the tumor microenvironment, is warranted.

The above-described immune-related mechanism does not explain the high survival rate of patients with *POLE*-mutant cancers completely. In chapter 3, *POLE*-mutant endometrial cancers were evaluated for the presence of tumor-infiltrating and peritumoral lymphocytes. Although these lymphocytes were prominently present in the majority of cases, ~15% of *POLE*-mutant endometrial cancers showed no evidence of a strong T cell response. In colorectal cancer, a lack of lymphocytic infiltrate seems associated with poorer clinical outcome in the 'hypermutated', mismatch repair-deficient cases.^{35,36} The number of *POLE*-mutant endometrial cancers studied to date is too small to per-

form similar within-subgroup analyses. Considering the genomic similarities between *POLE*-mutant and mismatch repair-deficient cancers, the degree of immune response is likely to have prognostic impact in *POLE*-mutant cancers as well. Therefore, also other mechanisms that could (in part) account for the excellent prognosis of *POLE*-mutant cancers were investigated.

2. *Increased sensitivity to adjuvant treatment*

The favorable prognosis of *POLE*-mutant cancers was, as described, found in several independent studies.^{1,3-10,37} In these studies, the majority of patients (~66%) with *POLE*-mutant cancers received some type of adjuvant therapy (i.e. radiotherapy and/or chemotherapy). Therefore, increased sensitivity to adjuvant treatment might also contribute to the good outcome of *POLE*-mutant cancers. Outcomes were, however, consistently favorable for patients in the PORTEC-1 endometrial cancer trial and in the colorectal cancer cohort regardless of adjuvant therapy (chapter 6).^{4,9} Moreover, in a mouse-derived embryonic stem cell model, *POLE* mutations did not result in greater sensitivity to ionizing radiation or to commonly used chemotherapeutics, including cisplatin, paclitaxel, and doxorubicin. This is in line with another preclinical study in which no significant difference in sensitivity to paclitaxel was found between *POLE*-mutant and *POLE*-wild-type endometrial cancer cell lines, while demonstrating increased resistance to carboplatin in their *POLE*-mutant cell line. The cell lines used in this study were, however, non-isogenic.³⁸ Moreover, the exonuclease activity of DNA polymerase epsilon is presumed not to have a role in the mechanisms of actions of the commonly used anti-cancer treatments: a *POLE* mutation is therefore unlikely to alter sensitivity to these therapies, providing further support for the clinical and *in vitro* findings. It should be noted that in the described model systems, an interaction between radiotherapy and the immune system could not be assessed.³⁹ Nevertheless, the clinical data and the *in vitro* data together suggest that the clinical outcome of *POLE*-mutant cancers is independent of the administered adjuvant treatment. Thus, increased sensitivity to adjuvant therapy does not seem to explain the favorable prognosis of *POLE*-mutant cancers.

3. *Error catastrophe*

The third possible explanation for the excellent clinical outcome of *POLE*-mutant cancers may originate from one of their characteristic features: the ultramutator phenotype. This may be the Achilles' heel of *POLE*-mutant cancers. On one hand, the high mutation rate causes cells to rapidly accumulate mutations. This increases the genetic variation, which in turn facilitates adaptation of malignant cells to changing microenvironments, e.g. immune evasion.⁴⁰⁻⁴² On the other hand, rapid accumulation of mutations may compromise tumor viability. Many random mutations can arise that are neither directly beneficial, nor immediately deleterious. These mutations will not be subject to strong positive or

negative selection and can become fixed in the genome. Subsequently, these mutations may collectively exert a negative effect and can result in so-called error catastrophe.^{42,43}

Error catastrophe occurs when the mutational burden exceeds a certain threshold above which the combined negative effect of the acquired mutations prevails, and cellular viability decreases. Considering the exceptionally high mutational load of *POLE*-mutant cancers, error catastrophe may take place and may thus contribute to the favorable outcome of these tumors. In the primary cancer, the mutation rate may have struck a balance between the potential to adapt to new states and maintenance of tumor viability, preventing tumor regression. This is supported by a study of biallelic mismatch repair-deficient brain cancers: after acquisition of a *POLE* mutation, the number of mutations rapidly increased to a certain threshold without surpassing it.⁴⁴ The high mutational burden may, however, render *POLE*-mutant cancer cells unfit to effectively metastasize: this could account for the rare incidence of distant metastases. Following Paget's 'seed and soil'-hypothesis, also in this scenario the immune system plays a pivotal role.⁴⁵

The existence of an 'error threshold' is supported by the infrequency at which *POLE* exonuclease domain mutations co-occur with other defects that induce replication errors. Indeed, in yeast, combined polymerase ϵ proofreading defects and complete mismatch repair deficiency proved synthetically lethal for the majority of cells.⁴⁶ We found similar results in preliminary experiments in the mouse embryonic stem cell model described in chapter 6. When attempting to knock-out *Msh6*, one of the mismatch repair genes, a mismatch repair-deficient clone was often obtained in the wild-type cell line (approximately 1 in 4 cells), while this proved rarely successful in the *POLE* exonuclease domain-mutant cell lines (~1 in 175 cells, Figure 1). These findings suggest that the absence of both DNA repair pathways results in a rapid and catastrophic accumulation of mutations incompatible with cellular survival. This synthetic lethality may also be the reason why *POLE*-mutant cancers generally are microsatellite stable.⁴⁷

To gain further insight into error catastrophe, future studies could investigate cell death in *POLE*-mutant compared to *POLE*-wild-type cancers. We examined levels of apoptosis in the endometrial cancer series described in chapter 4 by performing immunohistochemistry of cleaved caspase-3. Cleaved caspase-3 is the effector marker of both intrinsic and extrinsic apoptotic pathways.⁴⁸ In *POLE*-mutant and *POLE*-wild-type microsatellite-unstable endometrial cancers, cleaved caspase-3⁺ apoptotic cells were present in significantly higher numbers compared to *POLE*-wild-type microsatellite-stable cases (Figure 2). These results are promising and warrant further exploration.

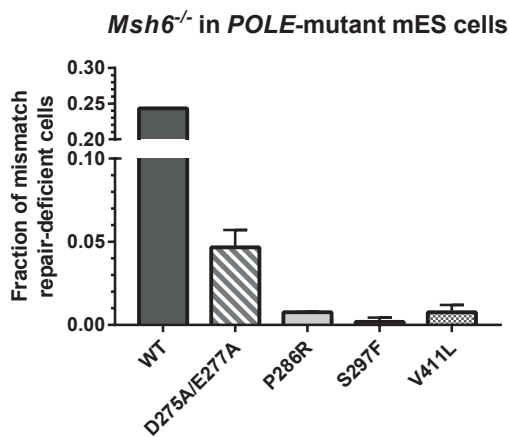


Figure 1. Knockout of *Msh6* in *POLE* exonuclease domain-mutant mouse embryonic stem cells. A knock-out of *Msh6*, one of the mismatch repair genes, was attempted in *POLE*-wild-type (WT) and in *POLE* exonuclease domain-mutant mouse embryonic stem (mES) cells using CRISPR-Cas9. To this end, stem cells were transfected with a guide RNA targeting *Msh6*. Successfully transfected cells were positively selected using puromycin. The number of cells that survived a 6-thioguanine pulse, which selects for loss of mismatch repair, was counted and is shown (box and whisker signify mean and standard deviation, respectively). Results are shown for different homozygous *POLE*-mutant cell lines, namely double-mutant D275A/E277A, and the common cancer-associated *POLE* P286R-, S297F-, and V411L mutants.

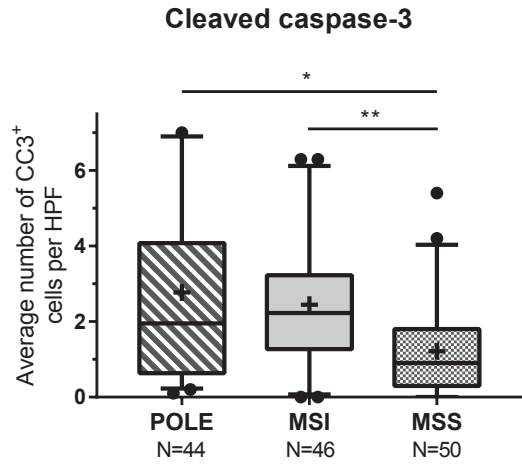


Figure 2. Apoptosis based on immunohistochemistry of cleaved caspase-3 in endometrial cancer according to molecular subgroup. Apoptosis based on cleaved caspase-3 (CC3) counts was compared between *POLE* exonuclease domain-mutant (*POLE*), microsatellite-unstable (MSI), and *POLE*-wildtype, microsatellite-stable (MSS) endometrial cancers. For each endometrial cancer, the number of cleaved caspase-3-positive cells was counted in ten high power fields (HPFs; 325µm×225µm), consisting of at least 70% tumor tissue. The mean of these ten high power fields is used for each case. For each molecular subgroup, boxes represent the interquartile range, with the upper whisker indicating the 95th percentile and the lower whisker the 5th percentile. The median and mean values are indicated by a horizontal line and cross respectively. **P*=0.0005, ***P*=0.0072.

Future studies could also focus on cases that apparently escaped error-induced extinction, e.g. *POLE*-mutant endometrial cancers with combined (partial) mismatch repair deficiency or *POLE*-mutant cancers that present at an advanced stage or that recur. A starting point for such future studies could stem from an interesting finding in the yeast study on polymerase ϵ proofreading and mismatch repair: among the colonies that survived with combined polymerase ϵ proofreading- and mismatch repair defects were colonies that had acquired secondary mutations in *POLE*. These secondary mutations resulted in a slower accumulation of mutations.⁴⁶ It would be of interest to explore whether such ‘antimutator’ variants also occur alongside *POLE* exonuclease domain mutations in human cancers and if present, what their effect is on the mutational burden. At the same time, as many *POLE* mutations outside the exonuclease domain remain ‘variants of unknown significance’, fundamental research is warranted to understand the functional consequences of such variants.

In conclusion, this thesis together with work of others proposes an enhanced antitumor immune response and a catastrophic accumulation of mutations as most likely mechanisms to explain the high survival rate of patients with *POLE*-mutant cancers. While these and other explanations require further research, they may provide a starting point for more tailored (adjuvant) treatment of *POLE*-mutant cancers, as will be discussed below.

Implications for endometrial cancer treatment: current practice and future directions

Early-stage endometrial cancer is treated primarily with surgery; the indication for adjuvant treatment depends on risk factors for recurrence (chapter 1). Despite the use of risk-based indications, vaginal brachytherapy for patients with high-intermediate risk endometrial cancer still yields significant overtreatment: six out of seven patients would not have had a recurrence without vaginal brachytherapy.⁴⁹⁻⁵¹ To reduce overtreatment, screening for *POLE* mutations could be of use. *POLE* mutations predict an excellent prognosis in endometrial cancer.⁵¹ Moreover, as discussed before, this prognostic benefit does not seem to depend on adjuvant treatment. Therefore, it could be argued that for patients with early-stage endometrial cancers carrying *POLE* mutations, surgery alone may suffice, thus reducing overtreatment. For *POLE*-mutant early-stage endometrial cancers, no additional treatment versus vaginal brachytherapy is currently under prospective evaluation in the PORTEC-4A trial.⁵² In this randomized trial, vaginal brachytherapy based on standard clinicopathological indications (chapter 1, Table 2) is compared to molecular profile-based indication for either observation, vaginal brachytherapy or external beam radiotherapy. The PORTEC-4A trial is the first to bring molecular profile-based adjuvant treatment for endometrial cancer into the clinic, and results are eagerly awaited.

This discussion has thus far focused mostly on *POLE* mutations in early-stage endometrial cancer and the high survival rate observed in this patient group. However, as mentioned briefly, *POLE* mutations can also be found in advanced-stage endometrial cancer. The frequency of *POLE* mutations in advanced-stage disease is yet to be determined: a collaborative study by Bosse et al. show 1 (1.6%) *POLE*-mutant cancers among 61 grade 3, stage III-IV endometrial cancers, while this was more common in the TCGA endometrial cancer cohort (7.2%, 4/55 stage III-IV endometrial cancers).^{1,53} Also the prognostic impact of *POLE* mutations in advanced-stage disease remains uncertain. For these advanced-stage endometrial cancers and for other cancer types with relatively poorer prognoses, the mechanisms underlying the good prognosis of *POLE* mutations can be exploited for therapeutic purposes. First of all, the antitumor immune response can be enhanced by immune checkpoint inhibition. Indeed, the first cases of advanced-stage *POLE*-mutant endometrial cancers treated with anti-PD-1 antibodies have been reported, as well as of pediatric, *POLE*-mutant, biallelic mismatch repair-deficient glioblastomas. These studies showed remarkably good responses of at least five months duration (maximum reported duration was 16 months).^{31-33,54} Clinical trials testing the efficacy of immune checkpoint inhibitors in advanced-stage cancers are currently recruiting in which *POLE*-mutant cancers are included.⁵⁵⁻⁵⁸ Whether *POLE* mutational status itself or other factors such as the mutational load, the number or type of predicted neoantigens or the expression of CD8, PD-1 and/or PD-L1 are the strongest markers to predict response to checkpoint inhibition remains to be elucidated. The outcomes of ongoing trials may further advance this field.

If immune checkpoint inhibitors also yield promising results in larger series of *POLE*-mutant cancers, these drugs may be quickly approved for this new indication. How fast this process of approval can be has been shown for the use of pembrolizumab in advanced-stage mismatch repair-deficient solid tumors: while the results of the first phase II study were only published in 2015, pembrolizumab was granted accelerated approval for this indication by the American Food and Drug Administration in May 2017.²⁹ In the Netherlands, PD-1 inhibitors pembrolizumab and nivolumab have been approved thus far only for the treatment of, among others, advanced-stage melanoma and advanced-stage non-small-cell lung carcinoma, although response rates in these tumors are similar to, or even lower than, those observed in mismatch repair-deficient cancers (e.g. for pembrolizumab: ~33% in melanoma, ~45% in PD-L1⁺ non-small-cell lung cancer, 40-53% in mismatch repair-deficient colorectal cancers).^{29,30,59-63} Nonetheless, the use of immune checkpoint inhibitors should be carefully considered because of the high treatment costs. While the exact costs of anti-PD-1 therapy are not known because of confidential price agreements between the Dutch government and the manufacturers, they are likely to approximate or even exceed the threshold value of €80,000 per quality-

adjusted life year, similarly to the anti-CTLA-4 antibody ipilimumab.⁶⁴⁻⁶⁶ These costs raise important ethical and political discussions on how much society is willing - and able - to pay for such treatments. Ongoing trials investigating immune checkpoint blockade for *POLE*-mutant cancers will provide more insight into the response rates, and thereby into the cost-effectiveness of these therapies.

Along with the immune response observed in patients with *POLE*-mutant cancers, the ultramutated phenotype of these tumors may be exploited for treatment. As the mutational burden seems to plateau just below the threshold for cellular viability, drugs that drive the mutation rate past this threshold could be of interest.⁴⁴ The possibility to induce error catastrophe has been demonstrated in RNA viruses by using nucleoside analogs. RNA viruses have remarkably high mutation rates. Nucleoside analogs result in a critical increase of these mutation rates by mispairing after incorporation in the DNA or by preventing the DNA chain from being elongated during replication.^{42,67,68} Taking advantage of their already ultramutated phenotype, a similar approach could be beneficial for cancers with *POLE* mutations. Moreover, the effects of nucleoside analogs may be increased in *POLE*-mutant cells: contrary to their wild-type counterparts, *POLE*-mutant cells may not be able to excise the analog once incorporated.

In mouse embryonic stem cells, we explored the sensitivity conferred by *POLE* exonuclease domain mutations to nucleoside analogs (chapter 6). *POLE*-mutant cells were indeed found to be significantly more sensitive to the nucleoside analogs cytarabine and fludarabine compared to *POLE*-wild-type cells: sensitivity to both compounds was increased approximately three-fold. Increased sensitivity to cytarabine is supported by a previous study in *POLE*-mutant chicken lymphoma and human lymphoblast cell lines, which showed an estimated six-fold increase in sensitivity.⁶⁹

Sensitivity to cytarabine (and fludarabine) was thus far demonstrated in cell lines homozygous for the specific *POLE* mutations. For the S297F hot spot mutation, sensitivity to these two nucleoside analogs was also evaluated in the heterozygous state: sensitivity was lower than in the homozygous *POLE*-mutant lines, but higher than wild-type, reaching significance for cytarabine. Tsuda et al. also showed moderate sensitivity in heterozygous lines.⁶⁹ As the majority of cancers – if not all – are heterozygous for the somatic *POLE* mutations, the effects of cytarabine and fludarabine in *POLE*-mutant cancers remain to be determined. Moreover, while cytarabine and fludarabine are used in the treatment of leukemias and lymphomas, their efficacy as (mono-)treatment for solid tumors appears to be limited, and toxicity can be considerable.⁷⁰⁻⁷⁵ Despite these considerations, the sensitivity conferred by *POLE* mutations to certain nucleoside analogs is substantial and may be very specific for *POLE*-mutant cells. Another advantage of

nucleoside analog treatment may be that by the induction of additional mutations, the probability of neoantigen formation increases. This may further enhance the antitumor immune response. Therefore, studies on the use of nucleoside analogs and other similar treatments in the context of *POLE* mutations are warranted.

To be able to optimally exploit the high mutational burden of *POLE*-mutant cancers, it is important to understand the mechanisms leading to this mutator phenotype. The somatic mutations found in cancers often lie close to, or within, the exonuclease domain motifs, and frequently concern residues that are highly conserved across species (chapter 2). An *in vitro* study showed that *POLE* mutations such as P286R, S459F, and V411L, despite not being located at the exonuclease domain catalytic sites, severely diminished proofreading activity. The degree to which each mutation reduced this activity, however, varied.⁷⁶ Interestingly, when somatic *POLE* hot spot mutations such as P286R were introduced into yeast and mouse embryonic stem cells, they resulted in a higher mutation rate than could be explained by proofreading deficiency alone (chapter 6).^{77,78} dNTP pool imbalances have been put forth by a study in yeast to explain the ultramutator phenotype.⁷⁹ However, this explanation remains to be tested for cancer-associated variants and for mammalian cells, as their dNTP pool regulation differs greatly from yeast. Another explanation for the high mutational load is provided by structural analyses of somatic *POLE* mutations; the *POLE* substitutions found in cancers were predicted to perturb DNA binding.¹¹ Based on these analyses, Barbari et al. suggested that the altered DNA binding may reduce the efficiency of extrinsic proofreading, for example by polymerase δ .⁷⁸ These possible mechanisms, together with their potential utility in treatment of *POLE*-mutant advanced-stage cancers, should be addressed in future studies.

Implementation of *POLE* exonuclease domain mutation screening in clinical practice

In consideration of the prognostic and promising therapeutic implications of *POLE* mutations, implementation of screening for these mutations in clinical practice would be valuable. For which tumor types *POLE* mutation screening should be introduced could be based on the incidence and on prognostic and/or predictive implications. Endometrial cancer would be a logical cancer type to implement screening for: the frequency of *POLE* mutations is relatively high, and the prognostic and predictive implications of these mutations in this cancer type have been described. In early-stage disease, *POLE* as a biomarker is unique in the sense that it may decrease rather than increase costs by reducing indications for adjuvant treatment; this will probably make *POLE* mutational screening cost-effective in this patient group. Moreover, *POLE* mutational screening in patients with advanced-stage disease could help in identifying candidates for targeted therapies.

To determine *POLE* exonuclease domain mutational status, the gold standard is to sequence exons 9 through 14 of the *POLE* gene. Because sequencing is not available in every clinical pathology laboratory, we aimed to identify histopathological and immunohistochemical characteristics to prescreen for *POLE* mutations in endometrial cancer (chapter 3). A combination of tumor type (endometrioid), tumor grade (high), peritumoral lymphocytes, and two routinely performed immunohistochemical stainings (MLH1 and p53) was found to increase the positive predictive value approximately five-fold, from 7% to 33%. Although this prediction model requires validation, the majority of these histopathological features had been ascribed to *POLE*-mutant endometrial cancers in previous studies.^{80,81} Alongside these histopathological features, the age at diagnosis can be taken into account: the likelihood of a *POLE* mutation increases when a patient is diagnosed at a lower than average age, especially below 60 years (average age of an endometrial cancer patient is ~65-67 years).^{3,4} Moreover, although their origin remains to be clarified, the finding of tumor giant cells in an endometrioid-type endometrial cancer may also increase the likelihood of a *POLE* mutation being present. Together, these clinicopathological and immunohistochemical characteristics can aid in selecting cases for pathology consultation at medical centers with a molecular diagnostics laboratory equipped to determine *POLE* mutational status.

Different molecular diagnostic techniques can be used to identify *POLE* exonuclease domain mutations. While Sanger sequencing is a straightforward method, sequencing of all six exonuclease domain exons separately is laborious, especially when taking into account that in only ~1% of malignancies a pathogenic *POLE* mutation will be identified. To make Sanger sequencing more feasible, studies have screened only exons 9, 13 and at times exon 14, since these exons contain the majority of cancer-associated variants thus far identified (chapter 2). However, as massively parallel sequencing of targeted gene panels becomes increasingly available for clinical use, the *POLE* exonuclease domain could also be incorporated in such cancer gene panels. Ideally, these gene panels would be large enough to also establish mutational burden and spectrum. With this approach, *POLE* mutational status would be determined as part of the molecular work-up and would not require an additional laboratory test; this would greatly facilitate its implementation. Moreover, variant identification and pathogenicity testing would be performed in a single test. Through this method, cancers would also be identified with a high mutational burden and/or with the same mutational signature due to other molecular alterations, e.g. *POLD1* exonuclease domain mutations or *POLE/POLD1* polymerase domain variants. As the mutational load of a cancer likely influences tumor immunogenicity and sensitivity to immune checkpoint inhibition, a screening method including the number of mutations may be advantageous.

An important aspect to keep in mind is that after implementation of *POLE* exonuclease domain mutation screening, more *POLE* mutations will be identified. A fraction of these *POLE*-mutant cancers will also harbor other molecular alterations, such as *TP53* mutations. The implications of multiple prognostically relevant alterations for clinical outcome of patients are unknown. Large-scale collaborations are required to assemble a sufficient number of these relatively uncommon cases, and consequently, to determine which molecular alterations have the strongest impact on prognosis.

***POLE* exonuclease domain mutations in personalized medicine**

Somatic *POLE* exonuclease domain mutations have the potential to become one of the success stories of (genomics-based) personalized medicine. After the discovery of these mutations during extensive genomic analyses, not only their prognostic significance was established in subsequent studies, but also their implications for immunotherapies. Moreover, thanks to close collaboration between clinicians, translational and basic scientists, the research that has led to these findings was performed over a relatively short period of approximately six years (Figures 3 and 4). Finally, as the prognostic and predictive value of somatic *POLE* exonuclease domain mutations seems generalizable to other cancer types, these mutations may provide an important step towards implementation of personalized medicine not only in endometrial cancer, but in a wide variety of malignancies.

Journal articles on *POLE* EDMs and cancer

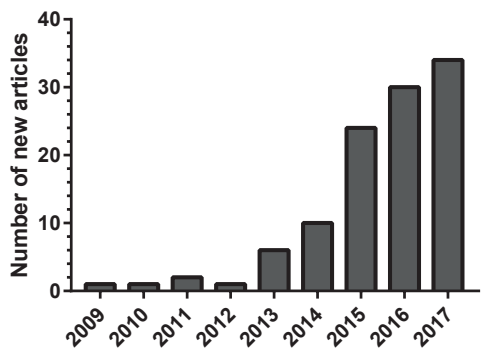


Figure 3. Journal articles on *POLE* exonuclease domain mutations and cancer by year. The graph represents the number of new publications per year on *POLE* exonuclease domain mutations (EDMs) and cancer as referenced by PubMed (through October 2017). Source: PubMed, a division of the US National Library of Medicine and the National Institute of Health (available from: <http://www.ncbi.nlm.nih.gov/pubmed/>).

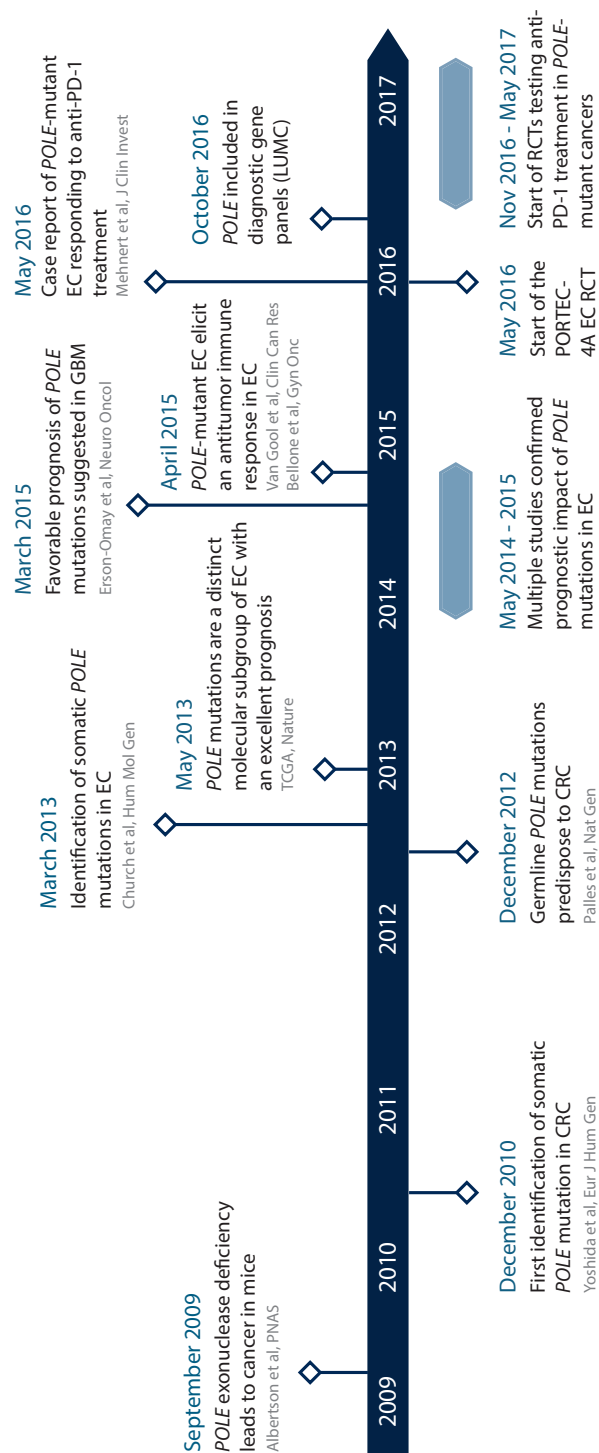


Figure 4. Timeline of important findings and events regarding *POLE* exonuclease domain mutations in cancer.

POLE exonuclease domain mutations are referred to as '*POLE* mutations' in the timeline. Abbreviations: EC, endometrial cancer; CRC, colorectal cancer; GBM, glioblastoma multiforme; TCGA, The Cancer Genome Atlas; LUMC, Leiden University Medical Center; RCT, randomized controlled trial.

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Appendices

Nederlandse samenvatting

Author affiliations

List of publications

Curriculum vitae

Dankwoord

NEDERLANDSE SAMENVATTING

Baarmoederkanker, oftewel endometriumcarcinoom, is een kwaadaardige aandoening van het slijmvlies dat de baarmoeder aan de binnenzijde bekleedt. Het is de meest voorkomende gynaecologische kanker, waarvan de incidentie door toenemende obesitas en vergrijzing van de bevolking stijgt. Chirurgische verwijdering van de baarmoeder vormt de basis van de behandeling. De indicatie voor aanvullende behandeling wordt gesteld op basis van de recidiefkans. Deze kans wordt bepaald met behulp van klinische en pathologische risicofactoren, namelijk de leeftijd van de patiënt, het FIGO-stadium waarin het endometriumcarcinoom zich presenteert, het histologisch tumortype, de mate van differentiatie (uitrijping) van de tumor, aangeduid als tumor graad, de diepte van ingroei in de baarmoederwand, en de aanwezigheid van tumorcellen in bloed- en/of lymfevaten. Voor endometriumcarcinoom patiënten met een laag risico op recidief is de chirurgische behandeling afdoende. Vrouwen met een endometriumcarcinoom met een intermediaire of hoge recidiefkans kunnen adjuvant behandeld worden met radiotherapie (vaginale brachytherapie of uitwendige radiotherapie) en/of chemotherapie. Deze risico-inschatting is echter niet perfect. Er blijft dan ook sprake van overbehandeling bij een deel van de patiënten met een relatief goede prognose, waarbij adjuvante behandeling niet nodig was geweest. Anderzijds is er ook onderbehandeling van patiënten waarbij het endometriumcarcinoom een agressiever klinisch beloop heeft dan op basis van de huidige risicofactoren was voorspeld. Recent is meer bekend geworden over de moleculair genetische basis van het endometriumcarcinoom en zijn vier subgroepen geïdentificeerd met verschillende moleculaire eigenschappen en prognoses. Om het klinische probleem van over- en onderbehandeling te verminderen zijn deze moleculaire veranderingen geïntegreerd in de risicobepaling, waardoor de recidiefkans van een patiënte met endometriumcarcinoom nauwkeuriger kan worden ingeschat. Eén van deze moleculaire veranderingen betreft mutaties in het exonuclease domein van DNA polymerase epsilon (ϵ).

Polymerase ϵ is een DNA-polymerase dat een belangrijke rol speelt bij de verdubbeling van het DNA voorafgaand aan celdeling. Naast de betrokkenheid bij de DNA replicatie kent polymerase ϵ ook exonuclease activiteit. Met dit exonuclease kan polymerase ϵ na het inbouwen van nieuwe nucleotiden de nieuwe DNA sequentie controleren op fouten en, indien aanwezig, ook de verkeerde nucleotide(n) verwijderen. Deze exonuclease activiteit, ook wel polymerase proofreading genoemd, levert dus een belangrijke bijdrage aan de nauwkeurigheid van de DNA replicatie en daarmee aan de integriteit van het erfelijk materiaal in de cel. De exonuclease activiteit van polymerase ϵ is gecodeerd in een apart domein in het *POLE* gen. In dit domein kunnen erfelijke en somatische (niet-erfelijke, spontane) mutaties optreden. Personen met erfelijke *POLE* exonuclease

domein mutaties (hierna aangeduid als '*POLE* mutaties') hebben, afhankelijk van de variant, aanleg voor het ontwikkelen van colorectaal carcinoom (darmkanker). Heterozygote, somatische *POLE* mutaties zijn in allerlei soorten kanker gevonden, waaronder in endometriumcarcinomen (frequentie 7-12%), maar ook in colorectaalcarcinomen (1-2%) en sporadisch in tumoren van o.a. de borst, long, hersenen, huid en alveesklier. Deze *POLE*-mutante tumoren komen vaak voor in relatief jonge patiënten. Ze vormen een aparte moleculaire subgroep die zich onder andere kenmerkt door een zogenoemd ultragemuteerd fenotype: het betreft een kanker met een uitzonderlijk hoog aantal mutaties. *POLE*-mutante tumoren kennen daarnaast een specifiek mutatiespectrum dat zich onderscheidt door een sterke toename in C→A en C→T nucleotide veranderingen in bepaalde DNA sequenties. Ondanks het hoge aantal mutaties voorspelt de aanwezigheid van een *POLE* mutatie een gunstig klinisch beloop voor de patiënt: dit is tot nu toe aangetoond in endometrium- en colorectaal carcinomen en is gesuggereerd voor glioblastomen, een agressieve vorm van hersenkanker.

Het onderzoek beschreven in dit proefschrift richt zich op somatische *POLE* exonuclease domein mutaties in het endometriumcarcinoom. Het doel hierbij is om meer inzicht te krijgen in deze mutaties, in het bijzonder in de onderliggende mechanismen waardoor *POLE* mutaties bijdragen aan de gunstige prognose voor de patiënt.

Een overzicht van de literatuur over *POLE* exonuclease activiteit, de gevolgen van *POLE* mutaties voor maligniteiten en de kenmerken van *POLE*-mutante tumoren – zoals hierboven in het kort beschreven – is uiteengezet in **Hoofdstuk 2**.

Bij de behandeling van het endometriumcarcinoom kunnen *POLE* mutaties gebruikt worden om de indicaties voor adjuvante therapie te verfijnen. Om deze mutaties te detecteren is echter een techniek nodig die niet in elk ziekenhuis beschikbaar is, en bovendien is deze techniek relatief duur. In **Hoofdstuk 3** zijn daarom de histopathologische en immunohistochemische kenmerken van *POLE*-mutante endometriumcarcinomen gedetailleerd in kaart gebracht. Het doel hierbij was om kenmerken van deze tumoren te identificeren die aangeven dat de kans groot is dat een *POLE* mutatie aanwezig is. Op deze manier zou de specialistische techniek om *POLE* mutaties te detecteren gerichter kunnen worden ingezet. In totaal werden 133 endometriumcarcinomen, waarvan 51 een *POLE* mutatie droegen, geblindeerd beoordeeld op de aanwezigheid van zestien morfologische kenmerken. *POLE*-mutante endometriumcarcinomen bleken zich te kenmerken door de aanwezigheid van een prominent immuuninfiltraat. Daarnaast bevatte een derde van de *POLE*-mutante tumoren tumorreuscellen (opvallend grote cellen met bizar-uitziende, grote, en soms verscheidene kernen). Het histologisch type van alle *POLE*-mutante endometriumcarcinomen was endometrioïd adenocarcinoom, waarbij

kenmerken van het sereus-type endometriumcarcinoom even vaak (focaal) aanwezig waren in *POLE*-mutante als in *POLE*-wildtype endometrioïde tumoren. Immunohistochemisch onderzoek toonde bij het grootste deel van de *POLE*-mutante endometriumcarcinomen p53-wildtype expressie; negatieve of focale aankleuring van p16; en normale expressie van de mismatch repair eiwitten. Door een combinatie van deze eenvoudig te scoren kenmerken steeg de kans dat een *POLE* mutatie aanwezig was van 7% naar 33%. Gezamenlijk kunnen deze histopathologische en immunohistochemische karakteristieken dus bijdragen aan het vooraf selecteren van endometriumcarcinomen waarbij het bepalen van *POLE* mutaties nuttig is. Dit faciliteert de implementatie van *POLE* mutatie screening in de klinische praktijk.

Om de gunstige prognose van patiënten met *POLE*-mutante maligniteiten te verklaren, zijn twee hypothesen onderzocht die beschreven worden in de Hoofdstukken 4, 5 en 6. Deze hypothesen betreffen de mogelijkheid van een toegenomen anti-tumor immuunreactie tegen *POLE*-mutante tumoren, en de mogelijkheid van verhoogde gevoeligheid van deze tumoren voor de aanvullende behandeling met radiotherapie of chemotherapie.

De anti-tumor immuunreactie in *POLE*-mutante tumoren is allereerst onderzocht in een cohort van 150 endometriumcarcinomen, waaronder 47 met een *POLE* mutatie (**Hoofdstuk 4**). Met behulp van immunohistochemie was in *POLE*-mutante endometriumcarcinomen, in vergelijking met *POLE*-wildtype tumoren, een sterke toename in het aantal cytotoxische tumor-infiltrerende T-cellen te zien. Deze bevinding is in de endometriumcarcinoomserie van The Cancer Genome Atlas (TCGA, n=244) gevalideerd en verder uitgewerkt. Ook in het TCGA cohort waren er bij analyse van RNA-sequentie data aanwijzingen voor een toegenomen cytotoxische T-cel reactie in *POLE*-mutante endometriumcarcinomen. Deze T-cel reactie toonde tekenen van differentiatie en activatie, maar ook van uitputting, passend bij chronische blootstelling aan antigenen (eiwitfragmenten die door (tumor)cellen aan het immuunsysteem worden gepresenteerd). Bij verdere analyse bleek tevens dat *POLE*-mutante carcinomen meer neoantigenen (antigenen ontstaan als gevolg van tumorspecifieke DNA mutaties) presenteren dan endometriumcarcinomen zonder *POLE* mutaties. *POLE*-mutante endometriumcarcinomen kenmerken zich dus door een sterke intratumorale T-cel reactie die geassocieerd is met, en veroorzaakt kan worden door, een toename in neoantigenen. Deze toegenomen anti-tumor immuunreactie kan (deels) een verklaring bieden voor de gunstige prognose van *POLE*-mutante endometriumcarcinomen.

De bevinding van een versterkte immuunreactie in *POLE*-mutante tumoren is bevestigd in een serie van 116 endometriumcarcinomen met een hoog risico op recidief (**Hoofd-**

stuk 5). In dit cohort waren alle moleculaire subgroepen van het endometriumcarcinoom vertegenwoordigd, namelijk tumoren met een *POLE* mutatie, met microsatelliet instabiliteit ('hypermuteerd'), met p53-mutante expressie en tumoren zonder specifiek moleculair profiel. Zowel *POLE*-mutante als microsatelliet-instabiele endometriumcarcinomen waren sterk geïnfiltrerd door CD8⁺ cytotoxische T-cellen. Ook in een deel van de endometriumcarcinomen met p53-mutante expressie en in sommige tumoren zonder specifiek moleculair profiel bleek een aanzienlijk CD8⁺-immuuninfiltraat aanwezig. In deze studie is ook met immunohistochemie gekeken naar de expressie van immuun checkpoint receptor PD-1 en diens ligand PD-L1, welke na binding de immuunreactie juist kunnen onderdrukken: PD-1⁺ en PD-L1⁺ immuuncellen waren in hoge mate aanwezig in *POLE*-mutante en microsatelliet-instabiele tumoren. De toegenomen anti-tumor immuunreactie, met daarbij ook hoge expressie van PD-1 en PD-L1, suggereert dat *POLE*-mutante en microsatelliet-instabiele endometriumcarcinomen goede kandidaten zijn voor behandeling met immuun checkpoint blokkerende middelen.

Uit diverse studies is gebleken dat *POLE* mutaties bij endometriumcarcinomen en colorectaal carcinomen een gunstig klinisch beloop voor de patiënt voorspellen. Het merendeel van de patiënten in deze studies werd echter aanvullend behandeld. In **Hoofdstuk 6** is daarom onderzocht of de goede prognose van *POLE*-mutante tumoren afhangt van de aanvullende behandeling. Dit is allereerst bekeken in de gerandomiseerde PORTEC-1 studie, welke uitkomsten van patiënten met endometriumcarcinoom vergeleek na uitwendige radiotherapie en na observatie. Ook in de observatie arm hadden patiënten met *POLE*-mutante endometriumcarcinomen significant minder recidieven dan patiënten met *POLE*-wild-type tumoren. Ter ondersteuning van deze klinische data, is de gevoeligheid voor radiotherapie en chemotherapie ook onderzocht in *POLE*-mutante muis embryonale stamcellen. Uit dit modelsysteem bleek dat *POLE*-mutante cellen niet gevoeliger waren dan *POLE*-wild-type cellen voor radiotherapie en ook niet voor verschillende chemotherapieën, waaronder cisplatin, paclitaxel en 5-fluorouracil. Deze resultaten suggereren dat de goede prognose van *POLE*-mutante tumoren niet verklaard kan worden door toegenomen gevoeligheid voor aanvullende behandeling. Dit biedt steun aan vervolgstudies waarin onderzocht zal worden of patiënten met (vroeg stadium) *POLE*-mutante tumoren voldoende behandeld zijn met alleen chirurgie. Dit kan leiden tot vermindering van overbehandeling en voor patiënten kan dit de kans op nadelige bijwerkingen verminderen.

Maligniteiten met *POLE* mutaties staan bekend om hun ultragemuteerde fenotype, hun specifieke mutatie spectrum, hun opvallend aanwezige immuuninfiltraat en een uitstekende prognose voor de patiënt. Het is onbekend in hoeverre deze kenmerken samenhangen met het moment waarop *POLE* mutaties optreden tijdens het ontstaan van

de tumor. **Hoofdstuk 7** beschrijft dat *POLE* mutaties al aanwezig zijn in (niet-maligne) voorloperlaesies van het endometrium- en colorectaalcarcinoom. Uit genoom- of exoomsequencing bleek bovendien dat bijna alle mutaties in *POLE*-mutante tumoren het karakteristieke *POLE* mutatie spectrum volgen. Dit was ook het geval voor mutaties in genen waarvan gedacht wordt dat ze betrokken zijn bij het ontstaan van de tumor. Daarbij bleek dat ook *POLE*-mutante precursorlaesies geassocieerd waren met een toegenomen CD8⁺ immuunreactie, wat een vroeg ontstaan van het ultragemuteerde fenotype suggereert. Daarnaast was er in *POLE*-mutante carcinomen een substantieel hoger aantal clonale immunogene mutaties aanwezig. Deze resultaten duiden erop dat *POLE* mutaties vroege, en mogelijk initiërende, gebeurtenissen zijn in sporadische (niet-erfelijke, verworven) tumoren. Als gevolg hiervan zal genominstabiliteit ook vroeg optreden: dit kan een verklaring zijn voor de uitzonderlijke immuunreactie en de goede prognose van deze tumoren, maar ook voor het voorkomen van *POLE*-mutante tumoren op relatief jonge leeftijd.

De belangrijkste bevindingen uit de vorige hoofdstukken worden in **Hoofdstuk 8** in perspectief van de bestaande literatuur geplaatst. Daarnaast worden in dit hoofdstuk de openstaande vragen met betrekking tot *POLE* mutaties besproken, de mogelijke implicaties voor de behandeling van het endometriumcarcinoom en de mogelijkheden tot implementatie van *POLE* mutatie screening. Naast de in dit proefschrift onderzochte hypothesen om de gunstige uitkomst van tumoren met *POLE* mutaties te verklaren, zou ook de mogelijkheid van 'error catastrophe', oftewel het optreden van dusdanig veel mutaties dat dit verdere tumorgroei negatief beïnvloedt, nader bestudeerd kunnen worden. Voor patiënten met *POLE*-mutante maligniteiten in een vroeg stadium is het belangrijk dat vervolgstudies zullen uitwijzen of aanvullende behandeling hen bespaard kan blijven zonder dat hun recidiefkans stijgt. Voor de zeldzame patiënten met *POLE*-mutante tumoren in een gevorderd stadium en voor patiënten met *POLE* mutaties in agressieve tumoren als glioblastomen zijn juist specifieke, doelgerichte therapieën gewenst. Immuun checkpoint blokkers en mogelijk ook nucleoside analogen zijn kandidaten voor een dergelijke doelgerichte behandeling van *POLE*-mutante tumoren. Detectie van *POLE* mutaties in de klinische praktijk zou kunnen plaatsvinden met behulp van genpanels waarbij, bij voorkeur, ook het aantal mutaties in een tumor en diens spectrum bepaald worden. Toekomstige studies zullen moeten uitwijzen of deze methode in de klinische praktijk haalbaar en kosteneffectief is.

Samenvattend kunnen somatische *POLE* exonuclease domein mutaties, gezien hun implicaties voor de prognose en zeer waarschijnlijk ook voor de behandeling van de patiënt, een belangrijke stap vormen naar een meer geïndividualiseerde behandeling van het endometriumcarcinoom. Aangezien de bevindingen tot nu toe ook van toepassing

lijken op andere tumortypen, zou deze stap richting meer 'therapie op maat' niet alleen voor het endometriumcarcinoom kunnen gelden, maar ook voor colorectaal carcinoom en diverse andere maligniteiten.

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CURRICULUM VITAE

Inge Charlotte Van Gool werd geboren op 29 december 1992 in Zwijndrecht. In 2010 behaalde zij cum laude haar Gymnasium diploma aan het Walburg College te Zwijndrecht. Via het Campus Scholarship Program van het Fulbright Center in Amsterdam studeerde zij vervolgens een jaar Liberal Arts & Sciences aan Wittenberg University, Springfield, Ohio, Verenigde Staten. In 2011 begon Inge met de Bachelor Geneeskunde aan de Universiteit Leiden, die zij in 2014 cum laude afrondde. Gedurende deze periode volgde ze tevens de 'Geneeskunde track' van het Honours College van de Universiteit Leiden, waar ze in 2014 het Honours certificaat van ontving. De Master Geneeskunde onderbrak zij in januari 2015 om fulltime het onderzoek beschreven in dit proefschrift te doen onder begeleiding van prof. dr. V.T.H.B.M. Smit, prof. dr. C.L. Creutzberg en dr. T. Bosse op de afdeling Pathologie van het Leids Universitair Medisch Centrum. In 2016 werd ze aangesteld als PhD-student in het kader van het MD/PhD excellente studenten traject van het Honours College Geneeskunde. Eén van de studies in dit proefschrift is tot stand gekomen in samenwerking met de afdeling Humane Genetica van het Leids Universitair Medisch Centrum; aan deze studie werkte Inge in de periode 2015-2017 onder begeleiding van dr. N. de Wind en dr. M. Drost. Een andere belangrijke, voortgaande samenwerking binnen diverse studies in dit proefschrift is met de onderzoeksgroep van prof. dr. I.P.M. Tomlinson en dr. D.N. Church van het Wellcome Trust Centre for Human Genetics in Oxford, Verenigd Koninkrijk. Bij deze groep liep Inge in 2014 stage, waarvoor ze de René Vogels reisbeurs ontving. In het kader van de studies in dit proefschrift bezocht ze diverse congressen in het binnen- en buitenland waar ze meerdere presentaties en posterpresentaties gaf. In 2016 ontving zij een reisbeurs van het Leids Universitair Fonds voor het Europees Pathologie congres te Keulen, Duitsland waar zij de tweede prijs kreeg voor beste presentatie. Vanaf september 2018 hoopt Inge te starten met haar co-schappen om zo de studie Geneeskunde af te ronden.

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