Research Article

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# Targeted Cancer Next-Generation Sequencing as a Primary Screening Tool for Microsatellite Instability and Lynch Syndrome in Upper Gastrointestinal Tract Cancers

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## Abstract

**Background:** No consensus guideline has been established for microsatellite instability testing in upper gastrointestinal tract cancers. This study aims to determine whether targeted cancer next-generation sequencing can accurately detect microsatellite instability in upper gastrointestinal tract cancers and screen for patients with Lynch syndrome.

**Methods:** In a cohort of 645 upper gastrointestinal tract cancers, targeted next-generation sequencing assessed microsatellite instability by identifying characteristic insertion and deletion mutations. Sequencing classification was compared with mismatch repair protein IHC. Cancers with microsatellite instability by sequencing were analyzed using a testing protocol to identify patients with Lynch syndrome.

**Results:** Sequencing identified microsatellite instability in 3.6% (23/645) of upper gastrointestinal tract cancers, including 28% (8/29) of small intestinal and 9% (9/97) of gastric carcinomas. In 20 cancers classified as having microsatellite instability, 19 demonstrated loss of expression of MLH1, PMS2, MSH2, or MSH6, and one cancer was indeterminate by IHC. In contrast, 52 control cancers demonstrated retained expression of all mismatch repair proteins. Using targeted sequencing as the initial screening test, 1.1% (7/645) of patients were identified to have pathogenic germline variants confirming a diagnosis of Lynch syndrome.

**Conclusions:** Targeted cancer next-generation sequencing is an accurate first-line test to detect microsatellite instability in upper gastrointestinal tract cancers.

**Impact:** This study provides a proof of concept for the use of targeted next-generation sequencing to detect microsatellite instability and screen for Lynch syndrome.

## Introduction

Microsatellite instability defines a characteristic genomic phenotype of insertion and deletion mutations in DNA repeat regions (1). Microsatellite instability is caused by the genetic or epigenetic inactivation of DNA mismatch repair genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* and is a feature of some sporadic cancers and cancers associated with Lynch syndrome (2).

Microsatellite instability is most commonly observed in colorectal and endometrial cancers. Although the overall incidence of upper gastrointestinal tract cancers is lower than that of colorectal and endometrial cancers, recent studies evaluating large cancer datasets have identified significant rates of microsatellite instability in cancers of the stomach and small intestine (3–5). Clinical guidelines recommend universal screening for Lynch syndrome in colorectal cancers (6), but no consensus guidelines currently exist for microsatellite instability testing in upper gastrointestinal tract cancers. The accurate identification of microsatellite instability in upper gastrointestinal tract cancers may directly benefit patients in two ways. Microsatellite instability serves as a biomarker to predict response to immune checkpoint inhibitor therapy in solid tumors, including upper gastrointestinal tract cancers (5, 7). Patients who have cancers with microsatellite instability may be at increased risk for Lynch syndrome, and patients and affected family members may benefit from genetic counseling and germline testing (8).

As next-generation sequencing technology becomes clinically available for cancer genotyping to identify actionable driver mutations, we hypothesize that targeted cancer sequencing panels can also detect microsatellite instability and be used as a primary screening tool for Lynch syndrome in upper gastrointestinal tract cancers.

## Materials and Methods

Patients were prospectively enrolled in an institutional cohort study for cancer genotyping. All participants provided written informed consent for tumor sequencing. This study was approved

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Targeted next-generation sequencing on tumor tissue was performed as described previously (9). DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tissue with at least 20% tumor nuclei. Indexed sequencing libraries were enriched for exons of 275 genes (102 specimens) or 298 genes (543 specimens), encompassing 757,787 base pairs or 831,033 base pairs of targeted genome, respectively, using solution-based hybrid capture (Agilent SureSelect; Agilent Technologies). Massively parallel sequencing was performed using Illumina HiSeq2500 (Illumina, Inc.). Data analysis was performed using a custom pipeline, including Indelocator (GATK; Broad Institute) for calling insertion and deletion variants.

Microsatellite instability as detected by next-generation sequencing was defined as greater than 3 microsatellite indel events per megabase (Mb) pair in the targeted genome, with events defined as single nucleotide insertion or deletion variants in homopolymeric DNA repeats of four or more nucleotides. Cancers with total microsatellite indel events below this threshold were classified as microsatellite stable. This criterion achieved 96% sensitivity and 99% specificity compared with mismatch repair protein IHC in colorectal adenocarcinomas (10).

IHC for MLH1, PMS2, MSH2, and MSH6 protein expression was performed as a case–control study. We tested all samples classified to have microsatellite instability with available pathology material. For each case sample predicted to have microsatellite instability, at least two control samples classified as microsatellite stable, matched by diagnosis, were tested. In addition, IHC was performed on all samples with greater than 2 but fewer than 3 microsatellite indel events per megabase if tissue was available. IHC and *MLH1* promoter methylation analyses were performed per standard laboratory protocol as described previously (11).

Subsequent germline sequencing was performed using the same targeted next-generation sequencing assay on DNA isolated from nonneoplastic, FFPE tissue. The assay included coverage of coding regions of mismatch repair genes *MLH1*, *PMS2*, *MSH2*, and *MSH6*.

## Results

### Patient characteristics

A total of 645 upper gastrointestinal tract carcinomas were sequenced, including 230 from esophagus or gastroesophageal junction, 199 from pancreas, 97 from stomach, 60 from bile duct, 29 from small intestine, 19 from gallbladder, and 11 from ampulla of Vater. A total of 426 patients (66%) were male, and 219 (34%) were female. The median age at the time of testing was 65.3 years (mean 64.2 years, range 19.0–93.6 years).

### Detection of microsatellite instability in upper gastrointestinal tract cancers by targeted sequencing

Microsatellite instability was identified by next-generation sequencing in 23 of 645 (3.6%) upper gastrointestinal tract cancers. Cancers classified to have microsatellite instability had a median of 9.2 microsatellite indel events per megabase (mean 10.9, range 3.4–22.4). A total of 555 of 622 (89.2%) cancers classified as microsatellite stable had 0 microsatellite indel events. Fifty-five of 622 (8.8%) cancers classified as microsatellite stable had between 0 and 2 microsatellite indel events, and 12 of 622 (1.9%) had between 2 and 3 microsatellite indel events (Fig. 1A).

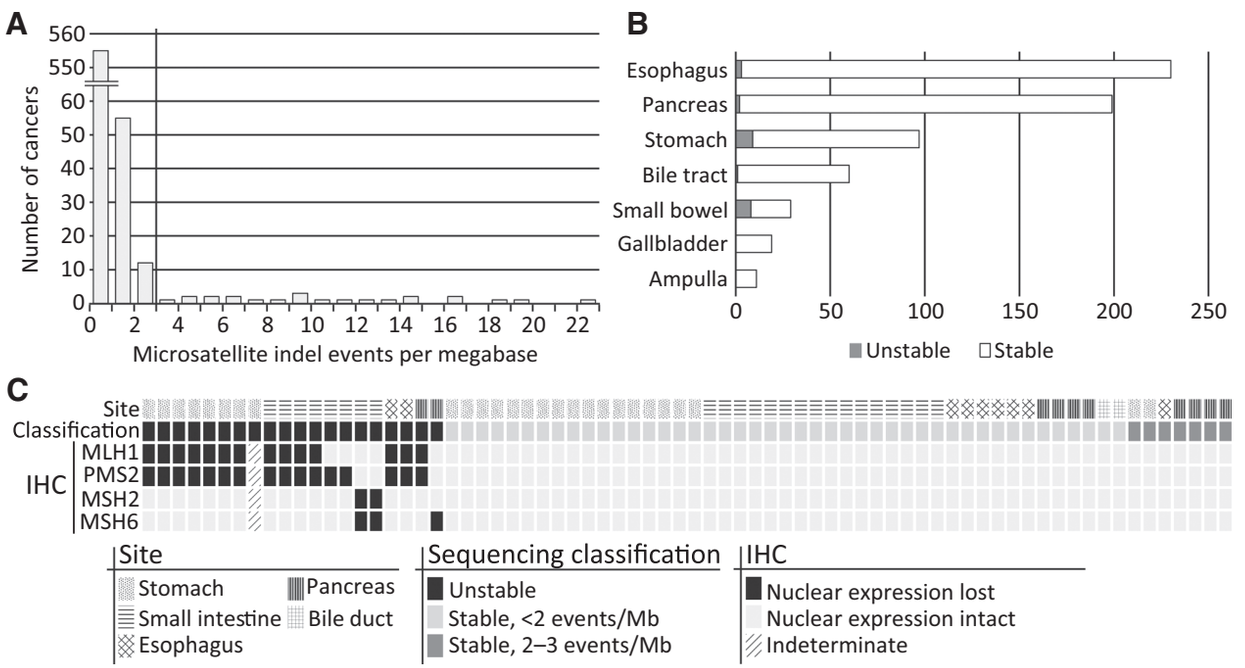


**Figure 1.**

Detection of microsatellite instability by next-generation sequencing. **A,** Number of microsatellite insertion or deletion events per megabase in upper gastrointestinal tract cancers. Cancers with greater than three events per megabase were classified as having microsatellite instability. **B,** Frequency of microsatellite instability in upper gastrointestinal tract cancers, by site. **C,** Validation of microsatellite instability classification by sequencing compared with IHC.

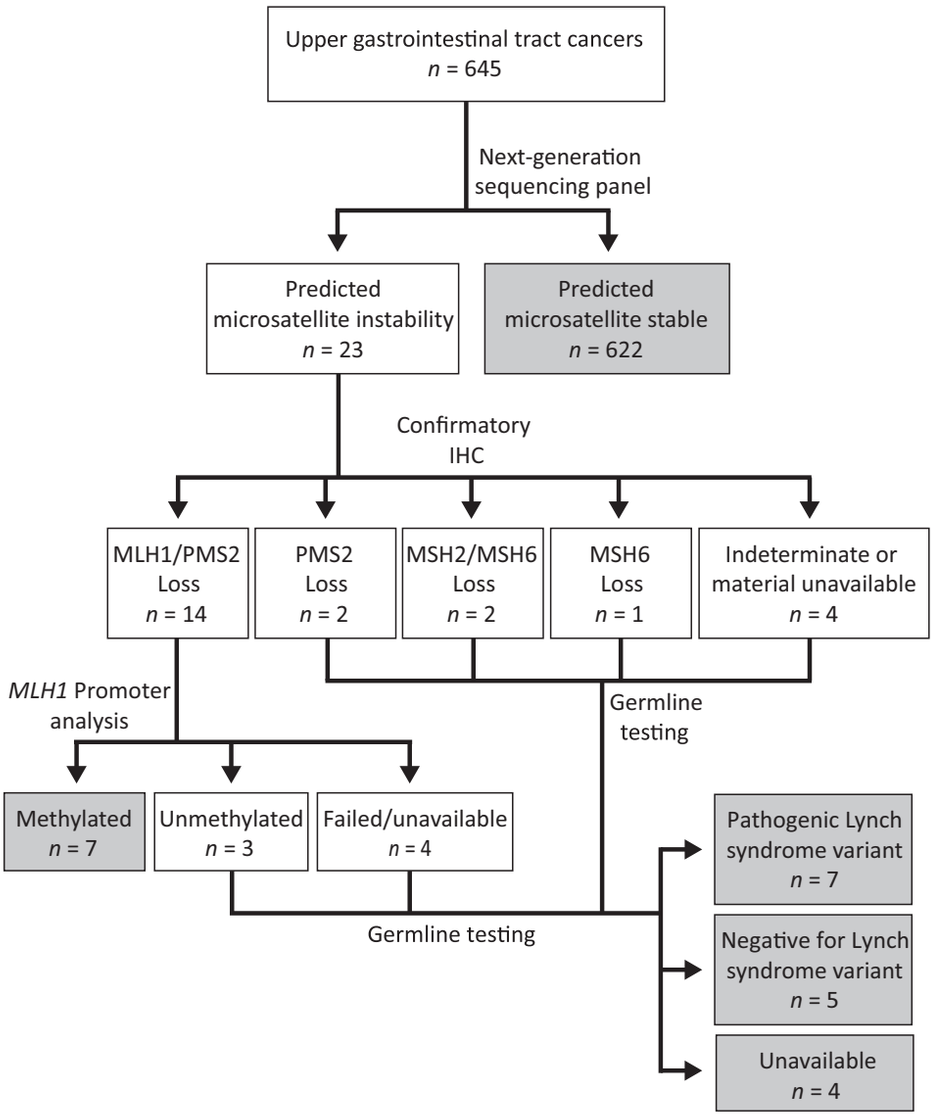


**Figure 2.**
Study protocol for the evaluation of upper gastrointestinal tract cancers to identify patients with Lynch syndrome. Targeted next-generation sequencing was performed on 645 upper gastrointestinal tract cancers. Confirmatory IHC was performed on cancers classified as having microsatellite instability, and *MLH1* promoter methylation analysis was tested for cancers with loss of MLH1 and PMS2 expression. Germline testing was performed on cases suspicious for Lynch syndrome–associated cancers, where nonneoplastic tissue was available.

Microsatellite instability was seen in 8 of 29 small intestinal (28%), 9 of 97 gastric (9%), 1 of 60 biliary tract (2%), 3 of 230 esophageal or gastroesophageal junction (1.3%), 2 of 199 pancreatic (1.0%), 0 of 19 gallbladder, and 0 of 11 ampullary cancers (Fig. 1B).

### Validation of results with mismatch repair protein IHC

Twenty of 23 cases classified to have microsatellite instability had available tumor tissue for IHC. Nineteen of 20 (95%) showed loss of expression of at least one mismatch repair protein including 14 with loss of MLH1 and PMS2, 2 with loss of PMS2, 2 with loss of MSH2 and MSH6, and 1 with loss of MSH6. The final case showed an unusual pattern, with nuclear staining of 5% of tumor nuclei for all four mismatch repair proteins, and was interpreted as indeterminate.

In contrast, all 45 controls classified as microsatellite stable by sequencing showed intact staining for MLH1, PMS2, MSH2, and MSH6 (Fig. 1C). In addition, all 7 cases with between 2 and 3 microsatellite indel events per megabase (classified as microsatellite stable but near the threshold) and with available tissue showed intact staining for MLH1, PMS2, MSH2, and MSH6.

### Identification of Lynch syndrome in patients with upper gastrointestinal tract cancers

For cases with microsatellite instability and available matched normal tissue, we followed a testing algorithm commonly applied to colorectal cancers to identify patients with Lynch syndrome (Fig. 2). For cancers with loss of MLH1 and PMS2 expression, *MLH1* promoter methylation analysis was performed. Of 10 neoplasms with MLH1 and PMS2 loss and successful promoter methylation analysis, *MLH1* promoter methylation was identified in 7.

Nonneoplastic tissue for germline testing was available for 12 of 16 specimens with microsatellite instability and without *MLH1* promoter methylation. Pathogenic Lynch syndrome variants were

**Table 1.** A total of 23 upper gastrointestinal tract carcinomas classified as mismatch repair deficient by targeted panel sequencing, validation with IHC, and identification of pathogenic germline variants to diagnose Lynch syndrome

| | | IHC | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | Microsatellite events per Mb | MLH1 | PMS2 | MSH2 | MSH6 | *MLH1* Promoter | Germline variant | Germline VAF or $log_2$ ratio | Cancer VAF or $log_2$ ratio | Somatic variant | Cancer VAF or $log_2$ ratio |
| Stomach | 12.6 | Loss | Loss | Intact | Intact | Methylated | Not indicated | | | | |
| Stomach | 9.2 | Loss | Loss | Intact | Intact | Methylated | Not indicated | | | | |
| Stomach | 6.9 | Loss | Loss | Intact | Intact | Methylated | Not indicated | | | | |
| Stomach | 3.4 | Loss | Loss | Intact | Intact | Methylated | Not indicated | | | | |
| Stomach | 10.3 | Loss | Loss | Intact | Intact | Unmethylated | Wild-type | | | ***MLH1* deletion** | −0.7 |
| Stomach | 4.6 | Loss | Loss | Intact | Intact | Unmethylated | ***MLH1* exon 13 del** | −0.7 | −1.6 | | |
| Stomach | 19.5 | Loss | Loss | Intact | Intact | Failed | Wild-type | | | | |
| Stomach | 9.2 | Indeterminate | Indeterminate | Indeterminate | Indeterminate | Unmethylated | Wild-type | | | | |
| Stomach | 13.7 | Not available | Not available | Not available | Not available | Not available | Not available | | | | |
| Small intestine | 18.3 | Loss | Loss | Intact | Intact | Methylated | Not indicated | | | | |
| Small intestine | 16.0 | Loss | Loss | Intact | Intact | Methylated | Not indicated | | | | |
| Small intestine | 5.7 | Loss | Loss | Intact | Intact | Unmethylated | ***MLH1* c.2194_2197dup (p.H733Qfs*14)** | 45% | 43% | | |
| Small intestine | 11.5 | Intact | Loss | Intact | Intact | Not indicated | ***PMS2* c.137G>T (p.S46I)** | 51% | 45% | ***PMS2* c.23+1G>T** | 22% |
| Small intestine | 8.0 | Intact | Loss | Intact | Intact | Not indicated | ***PMS2* c.1500dup (p.V501Rfs*4)** | 58% | 45% | | |
| Small intestine | 22.4 | Intact | Intact | Loss | Loss | Not indicated | Wild-type | | | ***MSH2* c.2581C>T (p.Q861*)** | 77% |
| Small intestine | 7.9 | Intact | Intact | Loss | Loss | Not indicated | ***MSH2* c.1786_1788del (p.N596del)** | 48% | 43% | | |
| Small intestine | 6.9 | Loss | Loss | Intact | Intact | Not available | Not available | | | | |
| Pancreas | 5.7 | Intact | Intact | Intact | Loss | Not indicated | ***MSH2* c.1906G>C (p.A636P)** | 48% | 54% | ***MSH2* c.622G>T (p.G208*)** | 8% |
| Pancreas | 9.2 | Loss | Loss | Intact | Intact | Not available | Not available | | | | |
| Esophagus | 16.0 | Loss | Loss | Intact | Intact | Methylated | Not indicated | | | | |
| Esophagus | 14.9 | Loss | Loss | Intact | Intact | Failed | Wild-type | | | | |
| Esophagus | 14.9 | Not available | Not available | Not available | Not available | Not available | ***MLH1* c.2059C>T (p.R687W)** | 47% | 51% | ***MLH1* c.298C>T (p.R100*)** | 40% |
| Bile duct | 4.6 | Not available | Not available | Not available | Not available | Not available | Not available | | | | |

NOTE: $Log_2$ ratio, relative copy number, where 0 represents copy number neutral and −1 represents one copy loss in a specimen where 100% of cells are affected.
Abbreviation: VAF, variant allele fraction. NOTE: Pathogenic variants identified by germline or somatic sequencing are highlighted in bold.

identified in 7 patients, representing 1.1% of upper gastrointestinal tract cancers in our cohort, including 4 small intestinal, 1 esophageal, 1 stomach, and 1 pancreatic cancers (Table 1; Supplementary Data). Patients with Lynch syndrome had a median age of 69.6 years (range 49.8–74.6 years). Upper gastrointestinal tract cancer was the first clinical manifestation of Lynch syndrome in 6 of 7 patients. Colonic adenocarcinoma was the first Lynch syndrome–associated cancer in the other patient.

**Somatic and second hit mutations in upper gastrointestinal tract cancers with microsatellite instability**

We evaluated the tumor sequencing data to identify somatic second hit mutations in mismatch repair genes in patients with Lynch syndrome. In 3 of 7 patients with Lynch syndrome, a second loss-of-function mutation was identified in the tumor specimen. These mutations included a splice site variant and two nonsense variants. In 1 patient with an isolated *MLH1* exon 13 germline deletion, the tumor specimen showed exon 13 deletion involving both copies of *MLH1*, and the second hit in this cancer was most likely due to loss of heterozygosity of the *MLH1* gene locus. In the other 3 patients with Lynch syndrome, a second somatic mutation was not identified, and the observed variant allele fractions in tumor specimens did not support LOH. This result might be due to limitations of the study, including possibly limited ability to detect loss of heterozygosity in relatively low tumor purity conditions or the presence of sequence or structural alterations in noncoding regions of the gene, which were not assessed by this assay. In two upper gastrointestinal tract cancers with microsatellite instability, the full testing algorithm was completed, and germline testing did not identify a pathogenic Lynch syndrome variant. Tumor testing showed somatic inactivation of mismatch repair genes by mutation or focal gene deletion (Table 1).

## Discussion

Next-generation sequencing has emerged as an effective diagnostic tool in cancer care and is being rapidly adopted into clinical practice (12). In addition to the identification of oncogenic driver mutations, sequencing can identify patterns of passenger mutations associated with microsatellite instability. Multiple algorithms have been developed to identify microsatellite instability from sequencing assays (13–15), including from targeted panel sequencing of tumor only specimens (10, 16). Recently, tumor sequencing has been suggested as a replacement for traditional Lynch syndrome screening methods in colorectal cancer (17).

Compared with colorectal cancer, Lynch syndrome screening in the upper gastrointestinal tract faces practical and diagnostic challenges. Many upper gastrointestinal tract cancers are diagnosed from fine needle aspiration cytologic preparations or small biopsies, and patients are frequently treated with neoadjuvant therapy before surgical resection. These diagnostic and treatment patterns limit the availability of diagnostic tumor tissue and make accurate interpretation by IHC methods more difficult. In this setting, targeted cancer sequencing is an appealing alternative for microsatellite instability evaluation. The next-generation sequencing assay used in this study has been validated to be performed on as little as 50 ng of tumor-enriched input DNA.

Our findings demonstrate accurate microsatellite instability assessment in upper gastrointestinal tract cancers by sequencing, and our protocol identifies microsatellite instability in 3.6% and pathogenic germline Lynch syndrome variants in 1.1% of upper gastrointestinal tract cancers in this cohort. Although the frequency of microsatellite instability is lower than that of colorectal cancers, the overall rates of microsatellite instability and Lynch syndrome in upper gastrointestinal tract cancers are clinically significant. Microsatellite instability is a biomarker for response of solid tumors to immune checkpoint inhibitor therapy (5, 18), and the identification of microsatellite instability provides a treatment option for patients who have failed other systemic therapies. In addition, the ability to screen for microsatellite instability has implications for a subset of patients with upper gastrointestinal tract cancers and Lynch syndrome. Our study has identified 7 patients with Lynch syndrome in an unselected cohort. These patients are of similar age compared with patients with sporadic upper gastrointestinal tract cancers. Notably, three of seven pathogenic Lynch syndrome variants are missense variants, and two variants involve *PMS2*. Genetic alterations involving *PMS2* have been associated with a moderately increased risk of colorectal and endometrial cancers, and the significance of *PMS2*-associated Lynch syndrome in cancer risk at other sites is controversial (19).

Patients with Lynch syndrome and affected family members may benefit from enhanced surveillance for the prevention for additional primary cancers, including increased frequency of colonoscopy screening, consideration of prophylactic hysterectomy for women older than 40 years, and other emerging screening strategies (20). These potential benefits are in addition to the detection of driver oncogenic alterations, which may provide druggable targets in upper gastrointestinal tract cancers or determine eligibility in clinical trials (21, 22).

Our findings support the use of targeted cancer sequencing as a first-line screening test in upper gastrointestinal tract cancers to identify microsatellite instability and patients with Lynch syndrome. In laboratories already performing panel sequencing to identify driver mutations, the adoption of a similar protocol may benefit patients with upper gastrointestinal tract cancers.

### Disclosure of Potential Conflicts of Interest

M.B. Yurgelun reports receiving commercial research grant from Myriad Genetic Laboratories Inc. L.M. Sholl is a consultant/advisory board member for AstraZeneca, LOXO Oncology, and Foghorn Therapeutics. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

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**Development of methodology:** A.G. Christakis, N.I. Lindeman, L.E. MacConaill, F. Dong

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A.G. Christakis, D.J. Papke, N.I. Lindeman, L.E. MacConaill, F. Dong

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A.G. Christakis, D.J. Papke, M.B. Yurgelun, A.T. Agoston, N.I. Lindeman, L.E. MacConaill, L.M. Sholl, F. Dong

**Writing, review, and/or revision of the manuscript:** A.G. Christakis, D.J. Papke, J.A. Nowak, M.B. Yurgelun, N.I. Lindeman, L.E. MacConaill, L.M. Sholl, F. Dong

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**Study supervision:** F. Dong

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