Detection of EGFR Activating and Resistance Mutations by Droplet Digital PCR in Sputum of EGFR-Mutated NSCLC Patients

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ABSTRACT

BACKGROUND: Proof of the T790M resistance mutation is mandatory if patients with EGFR-mutated non-small cell lung cancer (NSCLC) progress under first- or second-generation tyrosine kinase inhibitor therapy. In addition to rebiopsy, analysis of plasma circulating tumor DNA is used to detect T790M resistance mutation. We studied whether sputum is another feasible specimen for detection of EGFR mutations.

METHODS: Twenty-eight patients with advanced EGFR-mutated NSCLC were included during stable and/or progressive disease. The initial activating EGFR mutations (exon 19 deletions or L858R mutations) at stable disease and at progressive disease (together with T790M) were assessed in simultaneously collected plasma and sputum samples and detected by droplet digital polymerase chain reaction (ddPCR).

RESULTS: Activating EGFR mutations were detected in 47% of the plasma samples and 41% of sputum samples during stable disease, and in 57% of plasma samples and 64% of sputum samples during progressive disease. T790M was detected in 44% of the plasma samples and 66% of the sputum samples at progressive disease. In ddPCR T790M-negative results for both specimens (plasma and sputum), negativity was confirmed by rebiopsy in 5 samples. Concordance rate of plasma and sputum for T790M was 0.86, with a positive percent agreement of 1.0 and a negative percent agreement of 0.80.

CONCLUSIONS: We demonstrated that EGFR mutation analysis with ddPCR is feasible in sputum samples. Combination of plasma and sputum analyses for detection of T790M in NSCLC patients with progressive disease increases the diagnostic yield compared with molecular plasma analysis alone.

KEYWORDS: Sputum, EGFR, NSCLC, ddPCR, T790M

Introduction

First-, second- or third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have become standard treatment for patients with advanced EGFR-mutated non-small cell lung cancer (NSCLC).1,2 Deletions in exon 19 and the L858R point mutation in exon 21 account for about 90% of EGFR activating mutations and confer response to EGFR TKIs.3 However, approximately 50% to 75% of patients treated with first- or second-generation TKIs will develop resistance caused by EGFR p.Thr790Met point mutation (T790M) leading to disease progression.4-7 The third-generation EGFR TKI osimertinib targets EGFR activating mutations and the T790M resistance mutation.8 Hence, EGFR mutation analysis at the time of progression under first- or second-generation EGFR TKIs is fundamental for decision-making of subsequent therapy.

Rebiopsy is not always feasible, and recently, the analysis of circulating tumor DNA (ctDNA) from plasma samples (liquid biopsy) has proven to be a clinically useful alternative.9 The
A recent study by Hubers et al. evaluated the feasibility of tumor samples among the 65 included patients. To improve mutations in EGFR sputum cell-free DNA (cfDNA). They reported that sputum EGFR mutation in Wang et al. used a super-ARMS to detect confirmed tumor cells in the specimens. Another recent study by sputum of 35 patients, but with the prerequisite of cytologically EGFR mutations in 91%, with 84% in plasma and 63% in sputum. Other analyses of EGFR mutations in sputum cell-free DNA (cfDNA). They reported that sputum results matched in 46.2% with confirmed EGFR mutations in tumor samples among the 65 included patients. To improve the detection rate of EGFR, Wu et al. used combined plasma, sputum, and urine samples and used a next-generation-sequencing platform in 50 patients. They reported a combined sensitivity of 91%, with 84% in plasma and 63% in sputum. However, comparisons of sputum testing with rebiopsy in progressive disease or with results from plasma ctDNA analysis are currently lacking. Other analyses of EGFR mutations in sputum as 1 part of other cytological samples (eg, obtained by needle-aspiration, or bronchial brushing) did not compare the outcome with the tissue biopsy or detailed information for spu-

**Methods**

**Patients**

Patients with advanced EGFR-mutated lung adenocarcinoma were recruited at the Department of Pneumology, University Hospital Krems, Karl Landsteiner University of Health Sciences, and at the Department of Respiratory and Critical Care Medicine, and Ludwig Boltzmann Institute of COPD and Respiratory Epidemiology, Otto Wagner Hospital, Vienna, between July 2017 and April 2019. All patients had histologically confirmed lung adenocarcinoma with EGFR mutations in their initial tissue biopsy at diagnosis.

Sputum collection

Sputum collection was conducted using the “Sputum DNA Collection, Preservation, and Isolation Kit” (Norgen Biotek Corp, Thorold, Canada) according to the manufacturer’s instructions. Sputum collection was performed after a minimum of 30 minutes of fasting. Patients were instructed to inhale 25 mL of a 3% sodium chloride solution from a nebulizer with oxygen flow. Subsequently, a minimum of 2 mL of the expectoration was collected in a sputum collection tube provided by the kit. Another sputum sample was collected with cell-free sputum collection tubes (VACUETTE tube Z no additive) for cytological examination. Clinicopathological data were retrieved from the patient’s medical records.

Sample collection and analysis was performed with approval and in accordance with the local ethics authorities of both study sites (Ethic Committee of the Federal State Lower Austria, GS1-EK-4/479-2017; Ethic Committee of the City of Vienna, EK 18-172-0918). All patients gave their written informed consent for providing blood and sputum samples for genotyping and data analysis.

**Plasma preparation**

For blood sampling, ctDNA blood collection tubes (Roche, Pleasanton, CA) were used and each blood sample had a volume of about 8 mL. To isolate the plasma, whole blood was centrifuged at 200 g for 10 minutes and subsequently at 1600 g for 10 minutes. The supernatant was collected and centrifuged at 1900 g for 10 minutes, as previously described. DNA isolation from sputum

Sputum collection tubes containing the respective sputum samples were incubated in a water bath at 56°C before they were transferred into 50-mL tubes for further processing. Circulating tumor DNA was extracted from the sputum specimens using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer with the following 3 exceptions:

1. Instead of adding 100 µL of protease K per milliliter of sample, 120 µL were added per milliliter of sample.
2. Buffer ACL (Qiagen, Hilden, Germany) for sample lysis was replaced by sputum liquefier provided in the “Sputum DNA Collection, Preservation, and Isolation Kit” (Norgen Biotek Corp, Thorold, Canada). The volume of added sputum liquefier corresponded to the volume of the respective sputum sample (1:1 ratio).
3. The incubation step at 60°C was performed for 60 minutes instead of 30 minutes.

Circulating tumor DNA from sputum was finally eluted in 40-µL buffer and stored at −20°C until analysis was performed.
DNA isolation from plasma

Circulating tumor DNA was isolated from 2 mL of plasma using the QIAamp circulating nucleic acid kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions as previously described.11

Droplet digital analysis

Primers and probes for the detection of EGFR exon 19 deletions, L858R mutations, and T790M mutations were custom-made by Life Technologies (Carlsbad, CA). Primer sequences and ddPCR cycling conditions were previously described.10 For ddPCR, the QX-200 ddPCR system (Bio-Rad, Hercules, CA) was used according to the manufacturer’s instructions.

Analysis of ddPCR data was performed with QuantaSoft analysis software (Bio-Rad). Results were reported as copies of mutant allele per milliliter of plasma/sputum. The threshold for positivity was >1 copy/mL for all assays. As the actual copy-number of a sample varies with the amount of background wild-type cfDNA, at least 2 positive droplets for EGFR exon 19 deletions, L858R mutations, and T790M mutations in a triplicate had to be present for calling a sample “mutation positive.”11

Cytological examination of sputum samples

For cytological examination, sputum samples were Papanicolaou stained. Alveolar macrophages, bronchial epithelial cells, squamous metaplasia, and presence of tumor cells were scored. Sputum samples were considered representative when alveolar macrophages and/or bronchial epithelial cells were present.

Statistical analysis

All statistical data were analyzed with IBM SPSS Statistics software, version 23 (SPSS, IBM Corporation, Armonk, NY).

The distributions of parameters were tested for normal distribution using the Shapiro-Wilk test. Concordance rate, negative percent agreement (NPA; specificity), and positive percent agreement (PPA; sensitivity) for plasma and sputum samples were calculated via contingency table (Table 1). Differences were considered statistically significant if P was <.05.

Results

Patient characteristics

A total of 28 patients (24 women and 4 men) were included in the present study. The median age at inclusion was 66 years, with 46.4% of the patients being younger than 65 years (n = 13). All patients were diagnosed with NSCLC, had adenocarcinoma histology, and an activating EGFR mutation in their initial tumor biopsy at diagnosis: 19 patients (68%) had EGFR exon 19 deletions and 9 (32%) had EGFR (exon 21) L858R mutations. Seventeen samples were derived from patients who, at the time of sample collection, had stable disease or response under EGFR TKI therapy, and 14 samples were obtained from patients showing progressive disease at the time of sample collection. In 3 patients, samples were collected at the time of stable disease as well as at the time of progression under EGFR TKI: 1 of these patients received gefitinib and the other 2 patients received afatinib. The median time between the sputum collection during stable disease and progressive disease was 4 months (3-5 months). Corresponding radiological images for these 3 patients are added as Supplemental Material.

Overall TKI therapy at the time of sample collection consisted of afatinib (n = 20), gefitinib (n = 5), and osimertinib (n = 2). Four patients (12.9%) were TKI treatment-naïve at sample collection, due to a prior complete surgical resection of early-stage NSCLC.

In total, 80.6% of the patients showed a positive bronchus sign at the time of sample collection, which refers to the presence of a bronchus leading directly to 1 or multiple lung lesions (i.e., tumor masses) on a computer tomography of the lungs. A summary of the baseline characteristics of the study population is shown in Table 2.

Activating EGFR mutations detected in plasma and sputum

The mean volume of collected sputum samples was 2.93 mL. Table 3 provides an overview of EGFR mutation results in plasma and sputum of the complete study population. Of 17 patients presenting with stable disease at the time of sample collection, 4 patients were positive for the initial activating EGFR mutation in both, plasma and sputum sample. In 4 patients only the plasma sample, and in 3 patients only the sputum sample showed the initial activating EGFR mutation. In 6 patients, both specimens were EGFR mutation negative. The median copy number of initial activating EGFR mutation during stable disease was 17.6 copies/mL (1.5-128.3) in

| Table 1. Calculation of concordance rate, positive percent agreement, and negative percent agreement. |
|--------------------------------------------------------|---------------------------------------------------------------|
| PLASMA | TOTAL |
| NEUTRAL | POSITIVE |
| Sputum | | |
| Negative | a* | b | a+b |
| Positive | c | d | c+d |
| Total | a+c | b+d | N |

*a the number of plasma negative, sputum negative.

*b the number of plasma positive, sputum negative.

*c the number of plasma negative, sputum positive.

*d the number of plasma positive, sputum positive.

Concordance rate = 100% x (a + d) / (a + b + c + d).

Sensitivity (positive percent agreement) = 100% x d / (b + d).

Specificity (negative percent agreement) = 100% x a / (a + c).
the plasma samples and 27.1 copies/mL (1.3-503.8) in the sputum samples. Among 14 patients with progressive disease, the initial activating EGFR mutation was detectable in both specimens (plasma and sputum) in 5 patients, in plasma-only in 3 patients, and in sputum-only in 4 patients. In 2 patients, the initial activating EGFR mutation was not detectable in both specimens. The median copy number of the activating EGFR mutation during progressive disease in plasma samples was 124.6 copies/mL (20.5-15 608.0) and 125.0 copies/mL (5.4-3818.2) in sputum samples. In 3 patients, samples were collected during stable disease and later again at progressive disease. Therefore, they were included in both disease activity subgroups.

T790M mutations detected in plasma and sputum

Due to progressive disease in 14 patients, subsequent analysis for T790M resistance mutation was performed in both plasma and sputum samples. In 4 of these patients, T790M mutation was detected by plasma analysis and all of them were positive for T790M in the respective sputum sample. Two patients showed positive results for T790M only in the sputum sample. In 8 patients, T790M was not detectable by ddPCR, so rebiopsy was performed in 5 of the 8 patients who were consistently T790M negative in plasma and sputum. In all the 5 patients, the biopsy specimens were negative for T790M, which reflects a “true negativity” for a negative sputum result. Of the 3 patients in whom a rebiopsy was not possible, 1 patient refused the intervention, 1 patient had a poor performance status, and 1 patient was lost to follow-up.

Comparison of plasma and sputum test results

Results of plasma and corresponding sputum analyses matched in 29 of the 45 samples (64%; plasma and sputum). Concurrent mutation detection in plasma and the corresponding sputum sample was observed in 13 of the 45 plasma/sputum samples and no mutation was detected neither in sputum nor in the corresponding plasma sample in 16 of the 45 plasma/sputum samples. In patients with stable disease, plasma analysis was slightly more sensitive in detecting the initial activating EGFR mutation (8 of 17; 47%) compared with sputum analysis (7 of 17; 41%) with positive results by both methods (overlap) in 4 patients.

However, in patients with progressive disease, detection of the initial activating mutation was slightly more sensitive in sputum analysis in 9 of the 14 (64%) compared with 8 of the 14 (57%) with plasma analysis (overlap in 5 patients). Furthermore, for the detection of T790M resistance mutation, plasma analysis was less sensitive than sputum analysis: in 4 patients, T790M mutation was found by plasma analysis and all of them were also positive in sputum analysis, but 2 additional T790M-positive patients detected by sputum collection were missed with plasma analysis. Of the remaining 8 patients with progressive disease and negative results for T790M in sputum analysis, 5 were “true negative,” confirmed by rebiopsy and tissue analysis. In the remaining 3 patients, tissue acquisition was not possible and their “true mutation status” remains unknown.

Table 4 shows comparisons of ddPCR results for plasma and sputum samples for EGFR T790M, exon 19 deletions, and L858R. The highest concordance rate was reached in T790M (0.86), with a PPA of 1.0 and an NPA of 0.80. A similar high
The concordance rate was reached in L858R (0.78), with a PPA of 1.0 and a NPA of 0.5. For exon 19 deletions, the concordance rate was lowest (0.45), and the PPA was 0.36 and the NPA was 0.55. A combination of both plasma and sputum analysis shows a detection rate of 78% in L858R, 73% in exon 19 deletions, and 100% for T790M (Figure 1).

Table 3. Details of EGFR mutation analysis in plasma and sputum using ddPCR.

| PAT NO. | AGE | SMOKING STATUS (PACK-YEARS) | SITE OF PROGRESSION | EGFR MUTATION | STABLE DISEASE | PROGRESSIVE DISEASE |
|---------|-----|------------------------------|---------------------|---------------|---------------|---------------------|
|         |     |                              |                     |               | PLASMA COPY # | SPUTUM COPY # | INITIAL EGFR MUTATION | PLASMA COPY # | SPUTUM COPY # | T790M | REBIOPSY |
| 1       | 75  | Ex (30)                       | Del19               |               |              | 3.09 |               |               |               |       |
| 2       | 75  | Never                         | L858R               |               |              |     |               |               |               |       |
| 3       | 58  | Never                         | Del19               | 91.55         | 26.33 |     |               |               |               |       |
| 4       | 63  | Never                         | Del19               |               |              |     |               |               |               |       |
| 5       | 79  | Never                         | Del19               |               |              | 68.57 |     |               |               |               |       |
| 6       | 83  | Never                         | Del19               | 13.17         | 27.11 |     |               |               |               |       |
| 7       | 78  | Never                         | L858R               | 1.45          |     |     |               |               |               |       |
| 8       | 56  | Ex (10)                       | Del19               |               |              |     |               |               |               |       |
| 9       | 67  | Ex (15)                       | Del19               |               |              | 10.28 |     |               |               |               |       |
| 10      | 72  | Never                         | Del19               | 57.16         |     |     |               |               |               |       |
| 11      | 82  | Never                         | Del19               |               |              |     |               |               |               |       |
| 12      | 67  | Never                         | L858R               | 128.33        | 443.37 |     |               |               |               |       |
| 13      | 63  | Never                         | L858R               | 22.11         | 503.08 |     |               |               |               |       |
| 14      | 77  | Never                         | Del19               | 4.91          |     |     |               |               |               |       |
| 15      | 51  | Never                         | Lung, liver         | Del19         |     |     |               |               | 84.0         | 21.21 | 36.25 |
| 16      | 61  | Ex (5)                        | Lung, pleura        | Del19         | 4.02  |     |               |               | 24.56        |     |       |
| 17      | 66  | Never                         | Pleura              | Del19         |     |     |               |               | 25.45        |     |       |
| 18      | 56  | Never                         | Bone                | L858R         |     |     |               |               | 28.67         |     |       |
| 19      | 65  | Never                         | Lung, lymph node    | Del19         |     |     |               |               | 12.67         |     |       |
| 20      | 78  | Ex (5)                        | Lung                | Del19         |     |     |               |               |              |     | Negative |
| 21      | 64  | Ex (15)                       | Brain               | Del19         |     |     |               |               | 5.41          |     | 15.57 |
| 22      | 73  | Never                         | Lung, lymph node    | L858R         |     |     |               |               | 2888.0        | 3818.18 | 3.88  | 59.40 |
| 23      | 80  | Never                         | Lung, bone          | L858R         |     |     |               |               | 20.45         | 125.0 |     | 20.45 |
| 24      | 62  | Never                         | Liver, brain        | L858R         |     |     |               |               |              |     |       |
| 25      | 61  | Never                         | Brain, lung         | Del19         |     |     |               |               | 16.30         |     | 222.22 |
| 26      | 62  | Never                         | Lung                | Del19         |     |     |               |               | 7984.70       | 660.0 | 2438.30 | 656.67 |
| 27      | 58  | Never                         | Lung, liver         | Del19         |     |     |               |               | 165.23        | 2489.96 |     |       |
| 28      | 70  | Ex (50)                       | Lung, bone          | L858R         |     |     |               |               | 15608.0       | 306.51 |     | n.p.  |

Abbreviations: ddPCR, droplet digital polymerase chain reaction; n.p., not performed.
Pat nos. 17 to 19 were included twice (at time of stable and progressive disease), they are, therefore, shaded.
Cytological examination of sputum was performed in 72% of samples. In none of the samples, tumor cells could be detected by conventional cytological assessment, although ctDNA was detected.

Table 4. Contingency tables comparing test results of ddPCR for plasma and sputum.

|        | PLASMA | TOTAL |        |
|--------|--------|-------|--------|
|        | NEGATIVE | POSITIVE |        |
| (a) T790M |        |        | Concordance rate 0.86 |
| Sputum |        |        |        |
| Negative | 8 (57%) | 0 | 8 (57%) |
| Positive | 2 (14%) | 4 (29%) | 6 (43%) |
| Total | 10 (71%) | 4 (29%) | 14 (100%) |

| (b) Exon 19 del |        |        | Concordance rate 0.45 |
| Sputum |        |        |        |
| Negative | 6 (27%) | 7 (32%) | 13 (59%) |
| Positive | 5 (23%) | 4 (18%) | 9 (41%) |
| Total | 11 (50%) | 11 (50%) | 22 (100%) |

| (c) L858R |        |        | Concordance rate 0.78 |
| Sputum |        |        |        |
| Negative | 2 (22%) | 0 | 2 (22%) |
| Positive | 2 (22%) | 5 (56%) | 7 (78%) |
| Total | 4 (44%) | 5 (56%) | 9 (100%) |

Abbreviation: ddPCR, droplet digital polymerase chain reaction.

Figure 1. Percentage of detection rate for plasma and sputum analysis by ddPCR and combination of both specimen for EGFR L858R, exon 19 deletions, and T790M. ddPCR indicates droplet digital polymerase chain reaction.

Age, sex, initial EGFR mutation, ongoing TKI therapy, positive bronchus sign, smoking status, and quantity of the sputum sample showed no significant correlation for a positive detection of EGFR mutation in the sputum sample.
Discussion
Because 50% to 75% of patients with EGFR-mutated NSCLC will develop the T790M resistance mutation, it still remains critically important to test patients for T790M at the time they present progressive disease under first- or second-generation TKI therapy. Next to rebiopsy and molecular analysis of tissue samples, “liquid biopsy” with molecular testing of plasma ctDNA has become an important diagnostic tool. This study describes the high value of the use of sputum for molecular testing with ddPCR for EGFR L858R and exon 19 deletions, and, in particular, T790M mutation. Usually, the tumor offers connection to the airways, and it is likely that tumor tissue components are distributed in the alveoli, bronchioli, and even the bronchial tubes due to mucociliary clearance. As a consequence, in this study, a “positive bronchus sign” was no prerequisite to find ctDNA in the sputum. Our results confirm earlier studies that sputum is a useful specimen for EGFR mutation analysis, but also show that ddPCR is able to detect ctDNA in sputum samples, although cytological examinations do not identify cancer cells in the specimen. This finding is an important milestone to support further studies on the use of sputum for other molecular targets in NSCLC and, most likely, even beyond. Wu et al\(^\text{21}\) reported EGFR findings in sputum using next-generation sequencing (NGS). The advantages of ddPCR compared with NGS are the higher analytical sensitivity, possibility of quantitative analyses, the lower costs for reagents, the shorter duration for performing the tests, and limited bioinformatics. Our results support molecular diagnostics of sputum even in the absence of tumor cells. Therefore, further studies on advanced molecular testing strategies, such as NGS, might help to improve detection of other target mutations in sputum.

The sputum positivity for T790M of 66% is consistent with other reports describing disease recurrence under TKI therapy driven by T790M mutation in 50% to 75%.\(^\text{4,7,10-12,26}\)

Sensitivity for detection of different EGFR mutation was different between plasma and sputum at stable disease and progressive disease. Especially at progressive disease, the distribution of ctDNA in plasma and the bronchial system is increased due to the advancing disease. Hypothetically, distinct specimen such as plasma, urine, or sputum may contain varying amounts of ctDNA. Thus, simultaneous molecular testing of different samples for a target mutation such as EGFR seems rational.

Based on this, a remarkable finding is the detection of T790M in sputum but not in blood in 2 patients with progressive disease under first-line TKI. Therefore, T790M detection in sputum showed higher overall sensitivity than plasma analysis, but this may also be due to the low sample size. Because of the sputum result, both patients received osimertinib and both patients showed a radiological response to therapy. Without sputum examination, a tissue rebiopsy for examination of T790M would have been indicated for both patients. Therefore, an invasive examination (such as bronchoscopy) was prevented with the use of sputum analysis.

Our study has several limitations. First, results of plasma and sputum analyses have not been confirmed with molecular genetic testing of rebiopsied tumor tissue in all patients. Only in 5 patients with progressive disease and negative results for T790M in plasma and sputum, a tissue rebiopsy was performed. Second, we only used ddPCR for EGFR mutation analysis and did not compare it with other analytical platforms, such as Cycleave PCR, COLD-PCR, PangaeaBiotech SL Technology, and others. On the other side, ddPCR has been proven to be valuable for detecting activating EGFR mutations as well as resistance mutations such as T790M.\(^\text{6,11,12}\)

Third, although EGFR is currently the most common drugable NSCLC target mutation, sample size is low in this study and larger confirmation studies would be of value.

In the shades of a coronavirus pandemic, it must be addressed that producing induced sputum increases the risk of infections with airborne viruses such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for anyone close by without proper personal safety equipment (PSE). Sputum should therefore only be obtained with precautions (eg, pretesting for SARS-CoV-2) and use of PSE. All sputum collections for this study were performed before the COVID-19 pandemic.

Based on our results, the molecular genetic analysis of the sputum of patients with cancer opens new opportunities and is of high value, especially when rebiopsy for additional tumor material for genetic analysis is indicated.

Conclusions
This study demonstrated that EGFR mutation analysis with ddPCR is feasible in sputum samples from patients with NSCLC, even in the cytological defined absence of tumor cells in the specimen. Combination of plasma and sputum analyses for detection of T790M in NSCLC patients with progressive disease increases the diagnostic yield compared with molecular plasma analysis alone.

Authors’ Note
Parts of this study have been presented at the ESMO 2019 Congress, 27 September to 1 October 2019, Barcelona, Spain, as poster presentation.

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Author Contributions
KH, AB, PE, and MF provided the overall conception and design of the study. KH and MJH provided the patients. MS, CG, and HF assisted with administrative support. AB, CG, and...
MF collected and assembled the data. KH, AB, MJH, OCB, PE, and MF performed all data analysis. KH, AB, and MF wrote the article. All authors read and approved the final article.

Availability of Data and Material
The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

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Supplemental Material
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