IKZF1 Deletions with COBL Breakpoints Are Not Driven by RAG-Mediated Recombination Events in Acute Lymphoblastic Leukemia

Abstract

IKZF1 deletion (ΔIKZF1) is an important predictor of relapse in both childhood and adult B-cell precursor acute lymphoblastic leukemia (B-ALL). Previously, we revealed that COBL is a hotspot for breakpoints in leukemia and could promote IKZF1 deletions. Through an international collaboration, we provide a detailed genetic and clinical picture of B-ALL with COBL rearrangements (COBL-r). Patients with B-ALL and IKZF1 deletion (n = 133) were included. IKZF1 1-8 were associated with large alterations within chromosome 7: monosomy 7 (18%).
isochromosome 7q (10%), 7p loss (19%), and interstitial deletions (53%). The latter included COBL-r, which were found in 12% of the IKZF1 1-8 cohort. Patients with COBL-r are mostly classified as intermediate cytogenetic risk and frequently harbor ETV6, PAX5, CDKN2A/B deletions. Overall, 56% of breakpoints were located within COBL intron 5. Cryptic recombination signal sequence motifs were broadly distributed within the sequence of COBL, and no enrichment for the breakpoint cluster region was found. In summary, a diverse spectrum of alterations characterizes ΔIKZF1 and they also include deletion breakpoints within COBL. We confirmed that COBL is a hotspot associated with ΔIKZF1, but these rearrangements are not driven by RAG-mediated recombination.

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**Introduction**

B-cell precursor acute lymphoblastic leukemia (B-ALL) comprises multiple subtypes defined by structural and numerical chromosomal alterations. These initiating lesions include aneuploidy and chromosomal rearrangements, leading to expression of ectopic fusion proteins and/or to deregulation of gene expression. During the evolution of B-ALL, a series of secondary genomic alterations, such as DNA copy number alterations (CNAs) and sequence mutations, usually emerges [1]. These secondary events commonly involve deletions of genes that encode regulators of cell-cycle (CDKN2A/B) and/or to deregulation of gene expression. During the evolution of B-ALL, multiple subtypes defined by structural and numerical chromosomal alterations. These initiating lesions include aneuploidy and chromosomal rearrangements, leading to expression of ectopic fusion proteins and/or to deregulation of gene expression. During the evolution of B-ALL, a series of secondary genomic alterations, such as DNA copy number alterations (CNAs) and sequence mutations, usually emerges [1]. These secondary events commonly involve deletions of genes that encode regulators of cell-cycle (CDKN2A/B) and/or to deregulation of gene expression. During the evolution of B-ALL, a series of secondary genomic alterations, such as DNA copy number alterations (CNAs) and sequence mutations, usually emerges [1]. These secondary events commonly involve deletions of genes that encode regulators of cell-cycle (CDKN2A/B) and/or to deregulation of gene expression. During the evolution of B-ALL, a series of secondary genomic alterations, such as DNA copy number alterations (CNAs) and sequence mutations, usually emerges [1]. These secondary events commonly involve deletions of genes that encode regulators of cell-cycle (CDKN2A/B) and/or to deregulation of gene expression. During the evolution of B-ALL, a series of secondary genomic alterations, such as DNA copy number alterations (CNAs) and sequence mutations, usually emerges [1]. These secondary events commonly involve deletions of genes that encode regulators of cell-cycle (CDKN2A/B) and/or to deregulation of gene expression. During the evolution of B-ALL, a series of secondary genomic alterations, such as DNA copy number alterations (CNAs) and sequence mutations, usually emerges [1]. These secondary events commonly involve deletions of genes that encode regulators of cell-cycle (CDKN2A/B) and/or to deregulation of gene expression. During the evolution of B-ALL, a series of secondary genomic alterations, such as DNA copy number alterations (CNAs) and sequence mutations, usually emerges [1]. These secondary events commonly involve deletions of genes that encode regulators of cell-cycle (CDKN2A/B) and/or to deregulation of gene expression. 

**Material and Methods**

**Patients**

This study included 146 patients diagnosed with B-ALL and IKZF1 deletions. This patient cohort comprised two independent groups. The first one included 133 patients with 1) IKZF1 complete deletion, Δ1-8 (n = 104); or 2) intragenic deletion of IKZF1 including exon 8, Δn-8 (n = 29). The second group referred to 13 patients already diagnosed with IKZF1 deletions and COBL-r at collaborating centers [8–10]. Patient samples and information were obtained through the cooperative efforts of nine centers worldwide, and the project was conducted in accordance with the declaration of Helsinki.

**Detection of IKZF1 Deletions and CNAs**

IKZF1 deletions and CNAs in EBF1, JAK2, CDKN2A, CDKN2B, PAX5, ETV6, BTF1, RBL1, and the pseudoautosomal region 1-PAIR (SHOX, CRLF2, C2F2RA, IL3Rα, and P2RY8) were determined by either multiplex ligation-dependent probe amplification (MLPA), or single nucleotide polymorphism (SNP) array analyses. The SALSA MLPA P335-(A3-B2) and/or SALSA MLPA P202-B1 (MRC Holland) were used for MLPA experiments, and the data analyses were performed using Coffalyser software. The CytoScan HD Array (Affymetrix) assessed the occurrence of CNAs in the Brazilian (partially), British, and French cohorts. Data were analyzed with Chromosome Analysis Suite software, version 3.2 (Applied Biosystems), and the GRCh38/hg38 build of the Human Genome Assembly.

**Characterization of COBL Breakpoints**

We developed customized MLPA assays for the screening of CNAs within chromosome 7. The probes’ design and assay conditions have been previously described [9]. To validate our SNP array and MLPA findings, we performed either multiplexed long-distance PCR (M-PCR) or long-distance inverse (LDI)-PCR. These PCR approaches allowed us to confirm COBL-r and to determine the breakpoints at nucleotide level.

**Patient Data and Survival Analyses**

For the comparison of laboratory and clinical data between patients with or without COBL-r, we used the Pearson χ² test. Overall survival (OS) was defined as the time in months from the date of diagnosis to death or to the last follow-up assessment for patients alive. Kaplan-Meier method was used to estimate OS rates of patients according to COBL status with differences compared by the log-rank test. Statistical analyses were performed using R software, version 3.5.2, and P values < .05 were considered statistically significant.

**Analysis of Activation-Induced Deaminase (AID) or RAG Recognition Signal Sequences in Recombined COBL Alleles**

An agnostic search for motifs located within fragments spanning 50 bp from the breakpoint junctions of COBL-r was performed using MEME. The limit of output motifs was set to 5, and width ranged from 2 to 15 bp. The FIMO (Find Individual Motif Occurrences) tool was used for the analysis of WGCW and CG sequences, and the recombination signal sequence (RSS) consensus sequence was used to search for cryptic RSS sequences [11,12].
Results
Most of the deletions encompassing \(\text{IKZF1}\) exon 8 were classified as whole-gene deletions (\(\Delta 1-8: 78\%\) vs. \(\Delta n-8: 22\%\)). The CNAs found in the cohort were associated with monosomy 7 (14\%; \(n = 16\)), isochromosome 7q, i(7q); 7p loss (15\%; \(n = 17\)), and interstitial deletions within 7p (66\%; \(n = 76\)) (Figure 1A). Among the \(\Delta n-8\) group, most of the alterations (93\%) were \(\text{IKZF1}\) deletions detectable by the M-PCR, i.e., \(\Delta 2-8\) or \(\Delta 4-8\) (Figure 1B). Alternatively, 47\% of the \(\Delta 1-8\) group presented with aberrations involving loss of the whole 7p, such as monosomy 7 (18\%; \(n = 19\)), isochromosome 7q (10\%; \(n = 10\)), and 7p loss (19\%; \(n = 20\)). The remaining 55 patients (53\%) harbored interstitial deletions; \(\text{COBL}\)-r were found in 12\% of the \(\Delta 1-8\) cohort (Figure 1C).

We identified \(\text{COBL}\)-r in 25 B-ALL cases with \(\text{IKZF1}\) deletions (Figure 1D). \(\text{COBL}\)-r were detected by MLPA screening in 133 patients with B-ALL and \(\text{IKZF1}\) deletions (\(n = 12\)) or SNP array/NGS investigation performed at collaborating centers (\(n = 13\)), as described in the methodology. The loss of genes located within 9p locus — \(\text{PAX5}\) (\(n = 11\)), \(\text{CDKN2A}\) (\(n = 10\)), and \(\text{CDKN2B}\) (\(n = 7\)) — and \(\text{ETV6}\) deletions (\(n = 10\)) were the most recurrent additional alterations among these patients (Figure 1E). Demographic and laboratory data for the 25 patients with \(\text{COBL}\)-r showed they included 17 males and 8 females who were mainly children and adolescents (\(n = 21\)), with a median age at diagnosis of 5.5 years (range 1-59 years) and a median white blood cell count of 7.5 × 10⁹/l (range 1.5-459.6 × 10⁹/l) (Table 1). The patients were treated on diverse therapy protocols (Table 2). Seven patients relapsed within a median of 5.5 years (range 1.4-16.3 years), and six of them experienced isolated disease recurrence in the bone marrow (\(n = 5\)) or testes (\(n = 1\)). One patient relapsed at both sites: bone marrow and central nervous system. The median follow-up was 5.1 years (range 0.3-16.3 years), and the five deaths (mainly in patients with early relapse) occurred at a median 4.2 years (range 0.3-5.5 years) following diagnosis. In addition, the comparison between patients...
Table 1. Demographic and Laboratory Characteristics of B-ALL Cases with COBL Rearrangements.*

| Patient | Age (Years) | Gender | WBC (×10⁹/l) | Blasts at BM | 5' Breakpoint | 3' Breakpoint | Detection Method | Karyotype |
|---------|-------------|--------|--------------|-------------|--------------|--------------|----------------|-----------|
| P004    | 1           | F      | 96.000       | 95%         | ELMO1 intron 14 | Upstream COBL | SNP array       | 46XX, +2(13)(p13;q12), +del(4)(q22), +12(12)(q11), del(11)(q13), del(12)(q12), +14(q10), +15(q22), +16(q22), +18, +mar1, +mar2 |
| P034    | 1           | M      | 3.400        | 98%         | RAD500 intron 5 | COBL intron 5 | LDI-PCR         | NA        |
| P086    | 1           | M      | 5.000        | 95%         | 7pter          | COBL intron 7 | MLPA/LDI-PCR    | NA        |
| P120    | 1           | F      | 459.600      | 80%         | 7pter          | COBL intron 5 | SNP array/M-PCR | NA        |
| P056    | 2           | F      | 1.600        | 98%         | STAT1 intron 1 | COBL intron 5 | LDI-PCR         | 46XX     |
| P122    | 2           | M      | 13.400       | 97%         | 7pter          | COBL intron 7 | MLPA           | NA        |
| P145    | 2           | F      | 92.000       | 98%         | TCRG2C intron 1 | COBL intron 13 | SNP array       | 47XX, del(7)(p11), +10, +add(12)(p13), del(12)(p12) [4] |
| P022    | 4           | F      | 7.500        | 85%         | 7pter          | COBL 2-5      | MLPA           | 46XX     |
| P005    | 16          | M      | 10.000       | 95%         | IKZF1 intron 3 | COBL intron 5 | SNP array/M-PCR | 46XY     |
| P074    | 5           | M      | 16.400       | 78%         | 7pter          | COBL intron 5 | LDI-PCR         | NA        |
| P079    | 5           | F      | 5.700        | 88%         | 7pter         | COBL 8-13     | SNP array/M-PCR | NA        |
| P121    | 5           | F      | 7.470        | 7%          | 7pter          | COBL intron 5 | SNP array       | NA        |
| P144    | 5           | M      | 31.000       | 7pter       | 7pter         | COBL intron 5 | SNP array       | 47XX, +2(13)(p13), +del(4)(q22), del(11)(q13), del(12)(q12), +14(q10), +15(q22), +16(q22), +18, +mar1, +mar2 |

* Patient had COBL rearrangement and 7p12-3;GRB10 intron 5 deletion.

Table 2. Clinical Characteristics and Outcome of B-ALL Cases with COBL Rearrangements

| Patient | Clinical Trial | CNS Disease | MRD Day 33 | MRD Day 78 | PR | CMR Day 33 | Relapse | Outcome | Follow-Up (Months) |
|---------|----------------|-------------|------------|------------|----|------------|---------|---------|-------------------|
| P147    | AALL20232_J    | NA          | NA         | NA         | NA | NA         | NA      | NA      | NA                |
| P068    | AALL0331/UKALLR3 | No         | Negative   | Negative   | NA | Yes        | BM      | Dead    | 45                |
| P034    | AIEOP-BFM ALL2000 | No        | Negative   | Negative   | NA | Yes        | BM      | Dead    | 45                |
| P022    | AIEOP-BFM ALL2000 | No        | Negative   | Negative   | NA | Yes        | BM      | Dead    | 45                |
| P114    | AIEOP-BFM ALL2000 | No        | NA         | NA         | NA | Yes        | BM      | Dead    | 45                |
| P120    | AIEOP-BFM ALL2000 | No        | Positive   | Positive   | NA | Yes        | BM      | Dead    | 45                |
| P104    | ALL 1C 2009    | Yes        | Negative   | Negative   | NA | Yes        | BM      | Dead    | 45                |
| P145    | UKALL2003      | No         | Negative   | Negative   | NA | Yes        | BM      | Dead    | 45                |
| P146    | UKALL2003      | No         | Negative   | NA         | NA | Yes        | BM, CNS | Dead    | 45                |
| P144    | UKALL97        | No         | NA         | NA         | NA | Yes        | NA      | No      | 1st CR 26         |
| P143    | UKALL97        | No         | NA         | NA         | NA | Yes        | BM      | Dead    | 45                |
| P003    | CAALLF01       | No         | Negative   | NA         | NA | Yes        | NA      | No      | 1st CR 167       |
| P079    | COALL 05-92    | No         | NA         | NA         | NA | Yes        | BM      | Dead    | 45                |
| P008    | COALL 06-97    | No         | Negative   | NA         | NA | Yes        | NA      | Testes  | 2nd CR 195       |
| P074    | COALL 06-97    | No         | Negative   | NA         | NA | Yes        | BM      | Dead    | 45                |
| P069    | COALL 07-03    | No         | NA         | NA         | NA | Yes        | NA      | No      | 1st CR 123       |
| P004    | EORTC 58081    | No         | Negative   | Negative   | NA | Yes        | NA      | No      | 1st CR 49        |
| P090    | EsPhALL        | No         | Negative   | Positive   | NA | Yes        | NA      | Dead    | 45                |
| P006    | FRALEL 93      | No         | Positive   | Positive   | NA | Yes        | NA      | Relapse | 219               |
| P002    | FRALEL 93      | No         | Positive   | Negative   | NA | Yes        | NA      | No      | 1st CR 15        |
| P112    | GBTLIALL99     | No         | NA         | NA         | NA | Yes        | NA      | Alive   | NA                |
| P121    | GBTLIALL99     | No         | Negative   | Negative   | NA | Yes        | NA      | Alive   | NA                |
| P005    | GAAAPH         | No         | Positive   | Positive   | NA | Yes        | NA      | No      | Alive 27         |
| P056    | NA             | No         | NA         | NA         | NA | Yes        | BM      | Lost follow-up | 17                |
| P001    | NA             | F          | 50%        | 88%        | NA | Yes        | NA      | No      | SCL, alive 9      |

BM, bone marrow; CNS, central nervous system; CMR, complete morphological remission; MRD, minimal residual disease; NA, not available; PR, prednisone response; SCT, stem-cell transplantation; WBC, white blood cell count. MRD-negative status was defined as <0.01% leukemic cells in bone marrow and peripheral blood.
with COBL-r vs. COBL wild-type revealed that both groups presented similar laboratory and clinical characteristics (Table 3). Among the B-ALL cytogenetic abnormalities, TCF3-PBX1 and ETV6-RUNX1 were exclusively found in patients without or with COBL-r, respectively (Figure 1F). Although we did not observe any significant difference in the frequency of additional gene deletions when comparing patients with vs. without COBL-r, it is worthy of note that CDKN2A, ETV6, and PAX5 deletions were more frequent in patients with COBL-r (Figure 1G). Follow-up data were available for 111 B-ALL patients with (n = 20) or without COBL-r (n = 91). The OS of patients with COBL-r was similar to those with IKZF1 deletion only (hazard ratio, 1.278; 95% CI, 0.35-4.68; P = .646) (Figure 1H).

The breakpoints of the COBL-r were determined at nucleotide level in 11 of the 25 cases, and 56% of breakpoints were located within COBL intron 5 (Figure 2B). To address the possible causes of these breakpoints, we first performed an agnostic motif search. This analysis identified the motif CASWGTGG (E-value = 0.87) within all 22 breakpoint sequences of COBL-r (Figure 2A; Supplementary Table S1). CASWGTGG is similar to the heptamer of the RAG RSS, which is composed of a heptamer (5′-CAGCTG-3′) and a nonamer (5′-ACAAAACC-3′) sequence, interspaced by 12 or 23 random nucleotides associated to RAG-type rearrangements. Since the nonamer was absent in our first analysis, we then investigated the presence of complete motifs associated with the occurrence of rearrangements in leukemia: cryptic RSS sequence (RAG-type fusions), WGCW (AID-type fusions), and CG sequences. WGCW rearrangements in leukemia: cryptic RSS sequence (RAG-type fusions), WGCW (AID-type fusions), and CG sequences. WGCW and CG sequences were not found; however, we identified cryptic RSS in 5 out of 22 breakpoint regions, although none of them were spanning breakpoints within COBL (Supplementary Table S2).

Additionally, we performed a robust analysis for the identification of cryptic RSSs within the whole sequence of COBL (Figure 2, C-D). The results revealed a broad distribution of this motif throughout the gene and no enrichment for the breakpoint cluster located in intron 5 of COBL.

### Discussion

In this study, among the Δ-8 group, the majority of the alterations were IKZF1 deletions which had already been detected by the M-PCR method [8]. Since the remaining Δ-8 samples harbored deletions restricted to IKZF1, either they had DNA fusions outside the breakpoint cluster region of IKZF1 or M-PCR failed to detect them. Among the Δ1-8 group, COBL-r were found in 12% of the patients. This result revealed that COBL-r are more frequent among Δ1-8 but rarely related to Δ-8. Although COBL-r were not detected within the Δ-8 group for the cases included in the current proposal, we found these deletions in patients with COBL-r from a previous study [9]. These patients had IKZF1-COB1 fusions, which involved IKZF1 intron 1 (n = 1) or intron 3 (n = 2) and COBL intron 5.

Confirming the idea that COBL represents a genomic hotspot for IKZF1 deletions in B-ALL [9], we identified COBL-r in 25 B-ALL cases with IKZF1 deletions. Although either good (ETV6-RUNX1, high hyperdiploid) or high-risk (BCR-ABL1) cytogenetic groups have been observed in some of these patients, most of them had the so-called “B-other” subclassification with either normal or other abnormal (BCR-JAK2, IGH-EPOR) cytogenetic profile. In the current genetic risk stratification, many cases with COBL-r would then be classified as intermediate cytogenetic risk. We found that these patients also presented other secondary abnormalities commonly identified in B-ALL, such as PAX5 and CDKN2A/B and ETV6 deletions. This combination is of special interest because these patients could benefit from the newly proposed combined risk stratification strategies, such as IKZF1plus or the UKALL-CNA classifiers [13–15]. Considering that patients with B-ALL share similar laboratory and clinical characteristics regardless of COBL involvement, IKZF1 deletions may play a major role on risk stratification for these patients.

Regarding the breakpoints of the COBL-r, it is remarkable that 56% of them were located within COBL intron 5, although this region represents only 16% (47,770 out of 300,587 bp) of the entire gene. Based on this observation, we formulated two hypotheses to explain the presence of a hotspot for breakpoints within COBL: 1) the production of a truncated COBL protein, encoded by exons 1-5 only, could have a role on leukemogenesis, or 2) there is a breakage mechanism involving COBL intron 5, thus enriching this area for gene rearrangements.

COBL protein has three Wiskott-Aldrich syndrome protein homology 2 domains for actin binding. It shows substantial expression in neurons and muscle cells, although levels are low in blood [16]. COBL functions as an actin nucleator, controlling neuronal morphology and development [17]. Considering that COBL does not play a direct role in lymphoid development, the enrichment of COBL-r in B-ALL is more likely to be related to mechanisms controlling DNA breakage and promotion of genetic fusions.

Usually, genetic rearrangements in lymphoid malignancies are caused by either AID or the RAG complex. Therefore, we searched for motifs located within the breakpoint junctions of COBL-r, which could potentially provide a rational explanation for the observed

### Table 3. Demographic and Laboratory Data of Patients with B-ALL∗

| Parameter                  | COBL Rearrangement | P Value |
|----------------------------|--------------------|---------|
|                            | No                 | Yes     |         |
|                            | n = 121            | n = 25  |         |
|                            | n (%)              | n (%)   |         |
| Gender                     | Male               | 48 (39.7) | 8 (32.0) | 0.473  |
|                            | Female             | 73 (60.3) | 17 (68.0) |         |
| Age at diagnosis           | <1 year            | 2 (1.7) | 0 (0.0) | 0.701  |
|                            | 1-9 years          | 49 (41.5) | 12 (48.0) |         |
|                            | ≥10 years          | 67 (56.8) | 13 (52.0) |         |
| WBC (×10⁹/μL)              | <50                | 85 (70.2) | 16 (69.6) | 0.948  |
|                            | ≥50                | 36 (29.8) | 7 (30.4) |         |
| NCI risk                   | Standard           | 69 (58.0) | 14 (60.9) | 0.797  |
|                            | High               | 50 (42.0) | 9 (39.1) |         |
| CNS disease                | No                 | 96 (94.1) | 23 (95.8) | 0.741  |
|                            | Yes                | 6 (5.9) | 1 (4.2) |         |
| Prednisone response        | Good               | 66 (91.7) | 11 (78.6) | 0.143  |
|                            | Poor               | 6 (8.3) | 3 (21.4) |         |
| Relapse                    | No                 | 75 (70.8) | 13 (65.0) | 0.607  |
|                            | Yes                | 31 (29.2) | 7 (35.0) |         |
| Outcome                    | Alive              | 94 (77.7) | 20 (83.3) | 0.604  |
|                            | Dead               | 27 (22.3) | 4 (16.7) |         |

WBC, white blood cell count; NCI, National Cancer Institute of US; CNS, central nervous system.

* Pearson χ² calculation.
chromosomal rearrangements. RSSs are recognized by RAG enzymes during V(D)J recombination, and previous studies have located cryptic RSS immediately internal to the breakpoints of intragenic deletions of \textit{IKZF1}. Although the mutual motif within sequences spanning the breakpoints was similar to the heptamer sequence, our results do not support the idea that aberrant RAG-mediated recombination is the mechanism responsible for \textit{IKZF1} and \textit{COBL} codeletions.

Figure 2. Identification of motifs within the breakpoint sequences. (A) An agnostic motif search using MEME identified the sequence CASWGTGG (E-value = 0.87) among 22 breakpoint sequences. (B) The map of 19 deletion breakpoints (red triangles) within \textit{COBL} revealed a hotspot located at intron 5. Three breakpoints were detected within a downstream region of 7p12.1. The sequences highlight two breakpoint clusters located at \textit{COBL} intron 5. The mapped cryptic recombination signal sequences were not statistically significant. The cryptic recombination signal sequences (cRSS) with a spacer of 12-bp (c) and 23-bp (d) were mapped along \textit{COBL} gene. The highest RIC scores represent cRSS (blue dots) associated with RSS functionality. The gray area highlights the \textit{COBL} intron 5 and breakpoint cluster regions.
In summary, our results highlight COBL as a hotspot for interstitial deletions within chromosome 7, especially for deletions including the IKZF1 gene. Most of the COBL-r arose within COBL intron 5, leading to complete deletion of IKZF1; nevertheless, we also observed fusions between both genes. The analysis of the breakpoint sequences revealed a common motif resembling the heptamer of recombination signal sequence, but the analysis of the whole consensus sequence did not provide evidence for RAG-mediated recombination. Lastly, our study demonstrates that patients with IKZF1 deletions are associated with worse outcome regardless of COBL-r.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.02.002.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contribution
Conception and design: B. A. L., C. M., R. M., M. E. Experiments, data analysis, and interpretation: B. A. L., C. M., T. C. B., C. P. P., N. D., M. B., U. S., C. P., N. C. V., R. M., M. E. Provision of samples, data collection, and assembly: B. A. L., M. B. M., N. D., C. J.H., U. S., M. H., M. S. P. O., G. C., R. S., C. N. A., G. T., S. G., S. B. Writing and/or revision of the manuscript: B. A. L., R. M., M. B. M., C. J. H., G. C., R. S., C. N. A., M. E. Final manuscript approval: all authors.

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