Pure erythroid leukemia is characterized by biallelic *TP53* inactivation and abnormal p53 expression patterns in *de novo* and secondary cases

Pure erythroid leukemia (PEL) is a rare type of acute myeloid leukemia (AML) characterized by a neoplastic proliferation of immature erythroblasts associated with a complex karyotype and a poor prognosis.[1-3] PEL can arise *de novo*, but more frequently occurs as a therapy-related neoplasm or transformation from myelodysplastic syndromes (MDS).[4,5] To date, the potential differences between *de novo* and secondary (therapy-related or MDS-derived) PEL have not been well explored. Recent studies have shown that *TP53* mutations are common in PEL[1,5] and that p53 overexpression is also frequent.[6] Strong p53 expression shown by immunohistochemistry has become an important clue in the initial workup of PEL. However, we have observed some PEL cases lacking p53 expression despite the presence of *TP53* mutations. We conducted the current study to investigate *TP53* mutation characteristics and p53 protein expression in PEL and to examine whether secondary PEL cases differ from *de novo* disease. Another aim of this study was to address PEL as an entity that should be histopathologically and genetically defined, *versus* classifying these cases following the current classification scheme in which a history of prior chemoradiation therapy or MDS is relatively more emphasized.

We collected 22 cases of PEL, defined by a predominant proliferation of neoplastic erythroblasts that formed sheets in bone marrow, of which 30% or more were pronormoblasts. The clinical characteristics of our patients are summarized in Table 1. There were 14 men and eight women with a median age of 69 years (range, 37-81). Eleven (50%) patients had a history of chemotherapy for other malignancies (therapy-related), five (23%) had MDS, one (4%) had primary myelofibrosis (PMF), and five (23%) occurred *de novo*. Among 21 patients with treatment information available, five (24%) did not receive any therapy due to poor performance status or poor response to prior treatments for MDS. The remaining 16 patients received treatments after the diagnosis of PEL (Table 1). Four (25%) patients achieved a complete response with incomplete hematologic recovery; the response in three patients (#3, 12, 20) was transient and one patient (#22) remained in complete response with incomplete hematologic recovery at last follow-up, 4.7 months after diagnosis. None of the patients was eligible for stem cell transplantation. Twenty-one patients had clinical follow-up: 20 had died at last follow-up and one patient (#22) was alive. The median survival time for this entire cohort was 2.8 months (range, 0.2-7.3); 2.3 months (range, 0.2-7.3) for therapy-related, 2.6 months (range, 0.4-4.9) for patients with a history of MDS, and 3.9 months (range, 2.2-5.5) for *de novo* PEL.

Targeted next generation sequencing (NGS) with panels composed of genes commonly mutated in myeloid neoplasms was performed on bone marrow samples from 20 patients (19 using an 81-gene panel and 1 using a 28-gene panel) at the time of PEL diagnosis as previously described.[7] One case was tested for *TP53* mutation using Sanger sequencing. In total, 21 cases were tested and all patients had *TP53* mutation(s) (Table 2). A total of 25 TP53 mutations were detected: 18 patients had one *TP53* mutation, two patients (#15 and 17) had two mutations, and one (#5) patient had three mutations. Twenty-two (88%) mutations occurred in the DNA binding domain (exons 5-8), including 12 in exon 5, one in exon 6, four in exon 7, and five in exon 8. The remaining three mutations occurred in exon 4, exon 10, and a splice site, respectively. The types of *TP53* mutations included 19 (76%) missense, 1 (4%) nonsense, 1 (4%) splice site, and 4 (16%) small deletion. Among the four cases with small deletion mutations, three caused frameshift. The median variant allele frequency (VAF) of *TP53* mutations was 35% (range, 1-92.3%). One patient (#13) was not assessed for *TP53* mutation, but immunohistochemistry showed strong and diffuse p53 expression, suggestive of *TP53* mutations. The detailed *TP53* mutational profiles are summarized in Table 2. Among the 16 patients who received treatment, 11 (#3, 4, 7, 8, 11, 15, 17, 18, 20-22) had repeat *TP53* mutation analysis by NGS after treatment and all showed persistent *TP53* mutations. Among the 20 cases tested by NGS, additional gene mutations were detected in nine (45%) patients (*Online Supplementary Table S1*), including *DNMT3A* (n=3; VAF 10.3-29.3%), *NRAS* (n=2; VAF 5% and 26.6%), *TET2* (n=1; VAF <3%), *FLT3* (n=1; VAF 1.7%), *PRPF40B* (n=1; VAF 40.8%), *KMT2A* (n=1; VAF 15.2%) and *GATA2* (n=1; VAF 1.8%). Patient #22 had a history of PMF that was positive for *JAK2* V617F (VAF 32%) and negative for *TP53*. At the time of progression to PEL, *JAK2* V617F was detected with a VAF of 1%, and *TP53* mutation was acquired (VAF 80.6%).

Twenty cases underwent conventional karyotyping at the time of PEL diagnosis and all (100%) had complex karyotypes (*Online Supplementary Table S1*). Among 19 cases with karyotype data available, 12 (63%) had -5/5q-, 12

Table 1. Clinical characteristics of pure erythroid leukemia.

Case #	Sex	Age years	F/U months	Treatment	Response	Status at F/U	History
1	M	77	2.8	None	N/A	Dead	Therapy-related (B-ALL and DLBCL)
2	M	66	0.2	None	N/A	Dead	Therapy-related (PCN)
3	F	68	7.3	Decitabine + Venotoclax, 4 cycles Azacitidine + Hu5F9-G4, 1 cycle	Transient CRi, 2.1 months	Dead	Therapy-related (ovarian cancer)
4	M	55	1.4	CLIA + Venetoclax, 1 cycle	No	Dead	Therapy-related (DLBCL)
5	F	70	1.7	Decitabine + Venetoclax, 1 cycle	No	Dead	Therapy-related (PCN)
6	M	48	2.3	Fludarabine + AraC + Idarubicin, 1 cycle	No	Dead	Therapy-related (AML)
7	F	54	4.8	Cytarabine + Daunorubicine, 1 cycle Decitabine + Venetoclax, 2 cycles	No	Dead	Therapy-related (breast cancer)
8	M	66	6.3	Azacitidine, 4 cycles	No	Dead	Therapy-related (PCN)
9	F	81	0.8	None	N/A	Dead	Therapy-related (DLBCL)
10	M	69	2.1	Low dose Cytarabine + Venetoclax, 1 cycle	No	Dead	Therapy-related (lung cancer)
11	M	56	4.4	ASTX660 + ASTX727, 1 cycle	No	Dead	Therapy-related (PCN)
12	M	76	4.9	Sapacitabine, 3 cycles	Transient CRi, 2 months	Dead	MDS
13	F	37	0.4	None	N/A	Dead	MDS
14	M	78	2.6	Low dose Cytarabine + Venetoclax, 2 cycles	No	Dead	MDS
15	M	79	3.6	FF1101 (BET inhibitor), 2 cycles	No	Dead	MDS
16	M	60	1.3	None	N/A	Dead	MDS
17	F	78	2.2	Azacitidine + Nivolumab, 2 cycles	No	Dead	De novo
18	M	59	5.5	Azacitidine, 3 cycles FIA + Venetoclax, 1 cycle	No	Dead	De novo
19	F	78	N/A	N/K	N/K	N/K	De novo
20	M	65	5.0	Azacitidine + Venetoclax, 2 cycles	Transient CRi, 2.6 months	Dead	De novo
21	M	72	2.8	Decitabine + Venetoclax, 1 cycle	No	Dead	De novo
22	F	73	4.7	Azacitadine + Venclexta + Magrolimab, 3 cycles	CRi at the last F/U, 3 months	Alive	PMF

AML: acute myeloid leukemia; B-ALL: B-acute lymphoblastic leukemia; BET: bromodomain and extra-terminal; CLIA: cladribine, idarubicin, and cytarabine; CRi: complete response with incomplete hematologic recovery; DLBCL: diffuse large B-cell lymphoma; FIA: fludarabine, idarubicin, cytarabine; F/U: follow up; MDS: myelodysplastic syndrome; N/A: not applicable; N/K: not known; PCN: plasma cell neoplasm; PMF: primary myelofibrosis.

(63%) had -7/7q-, and nine (47%) had concomitant -5/5q- and -7/7q-. The status of 17p/*TP53* was assessed by conventional karyotyping and/or fluorescence *in situ* hybridization (FISH) in 17 cases: deletion of 17p and/or *TP53* was detected in 13 (76%) cases (Table 2). The remaining four patients were negative but three (cases # 5, 15, and 17, Table 2) had more than one *TP53* mutation by NGS, raising the possibility that both alleles were affected by *TP53* mutations. In one patient (#12), the status of 17p/*TP53* was unknown, but the VAF of *TP53* mutation was 92.3%, consistent with the loss of wild-type *TP53*.

We performed p53 immunohistochemistry on 21 cases

and correlated the results with *TP53* mutation types (Table 2). Sixteen (76%) cases of PEL were strongly and uniformly positive for p53; 15 had missense mutations and one had a deletion mutation but no frameshift (#3). In the remaining five (24%) cases, p53 expression was completely absent in the neoplastic cells (null pattern). In cases negative for p53 expression, three (#6, 16, 21) had *TP53* frameshift mutations, one (#18) had a nonsense mutation, and one (#1) had a splice site mutation. Representative cases of PEL with p53 overexpression and completely absence of p53 expression are shown in Figure 1A and B. In this study, two patients (cases #3 and 14) had a single *TP53* mutation with a VAF less than 15%. In both cases, erythroblasts formed sheets in the core biopsy and were diffusely and strongly positive for p53 by immunohistochemistry. These findings suggest that most of the erythroblasts had mutated *TP53* and the low VAF of *TP53* mutation may be due to hemodiluted specimen submitted for molecular analysis. However, we also cannot exclude the possibility that only a subclone of leukemic cells had *TP53* mutation.

These data demonstrate that PEL is characterized by biallelic *TP53* alterations, frequently present as a mutation in one allele and deletion in another allele. In cases with no *TP53* deletion, two or more mutations were often detected. Mutations frequently seen in other myeloid neoplasms are less common in PEL, indicating that biallelic loss of *TP53* function is a feature of PEL and may play a

Table 2. *TP53* mutational profiles and p53 protein expression in pure erythroid leukemia.

Case #	Monosomy 17 or *TP53* Deletion (karyotype or FISH)*	Number of *TP53* Mutation	Biallelic *TP53* Inactivation	*TP53* Mutation (Ref: NM_000546.5)	Type of Mutation	VAF %	Exon(s)	IHC-p53
1	yes	1	yes	c.673-2A>T	splice site	74.9	splice site	negative
2	yes	1	yes	c.405C>G p.C135W	missense	42.1	5	positive
3	yes	1	yes	c.534_536del p.H179del	deletion, no frameshift	11.9	5	positive
4	yes	1	yes	c.818G>A p.R273H	missense	59.6	8	positive
5	no	3	likely yes	c.715A>G p.N239D c.401T>G p.F134C c.329G>T p.R110L	missense missense missense	1 29.3 32	4,5,7	positive
6	N/K	1	N/K	c.501del p.Q167fs	deletion, frameshift	62.6	5	negative
7	N/K	1	N/K	c.524G>A p.R175H	missense	37.2	5	positive
8	yes	1	yes	c.377A>C p.Y126S	missense	23.0	5	positive
9	N/K	1	N/K	c.715A>G p.N239D	missense	42.6	7	positive
10	yes	1	yes	c.818G>C p.R273P	missense	27.2	8	positive
11	yes	1	yes	c.488A>G p.Y163C	missense	48.0	5	positive
12	N/K	1	likely yes	c.797G>A p.G266E	missense	92.3	8	positive
13	N/K	N/D	N/K	N/D	N/D	N/D	N/D	positive
14	yes	1	yes	c.745A>G p.R249G	missense	8.4	7	positive
15	no	2	likely yes	c.434T>G p.L145R c.1010G>C p.R337P	missense missense	20.4 18.5	5,10	N/D
16	yes	1	yes	c.455del p.P152fs	deletion, frameshift	70.1%	5	negative
17	no	2	likely yes	c.844C>T p.R282W c.734G>T p.G245V	missense missense	35.1 14.4	7,8	positive
18	yes	1	yes	c.493C>T p.Q165*	nonsense	39.1	5	negative
19	no	1	probably no	c.476C>T p.A159V	missense	16.0	5	positive
20	yes	1	yes	c.590T>G p.V197G	missense	15.1	6	positive
21	yes	1	yes	c.558del p.D186fs	deletion, frameshift	47.1	5	negative
22	yes	1	yes	c.824G>T p.C275F	missense	80.6	8	positive

IHC: immunohistochemistry; N/D: not done; N/K: not known; VAF: variant allele frequency; FISH: fluorescence *in situ* hybridization. *Detailed karyotype and FISH findings are listed in the *Online Supplementary Table S1*.

critical role in the development of PEL. Of note, biallelic *TP53* alteration is not specific to PEL and can be seen in other myeloid neoplasms, such as AML[8] and therapy-related MDS.[9] Thus, *TP53* mutations alone may not be sufficient to block the differentiation of erythroid lineage and drive pronormoblast proliferation, a pathognomonic feature of PEL. Alterations of other genes (not covered in our mutation panels) or pathways involved in erythroid differentiation likely also play a role in PEL development.

As mutational analysis often takes time, checking p53 expression status by immunohistochemistry has been used as a surrogate to predict the presence of *TP53* mutations.[6,10,11] One caveat is that *TP53* mutations do not always correlate with p53 overexpression. In the current study, approximately one quarter of PEL cases showed a null pattern by immunohistochemistry. In these cases, *TP53* mutations were either frameshift, nonsense, or involved a splice site. Of note, the null pattern of p53 expression can usually be distinguished from the "negative" wild-type pattern which often shows variable p53 expression in a subset of cells and the staining intensity ranges from weak to moderate (Figure 1C). In some cases, however, as-

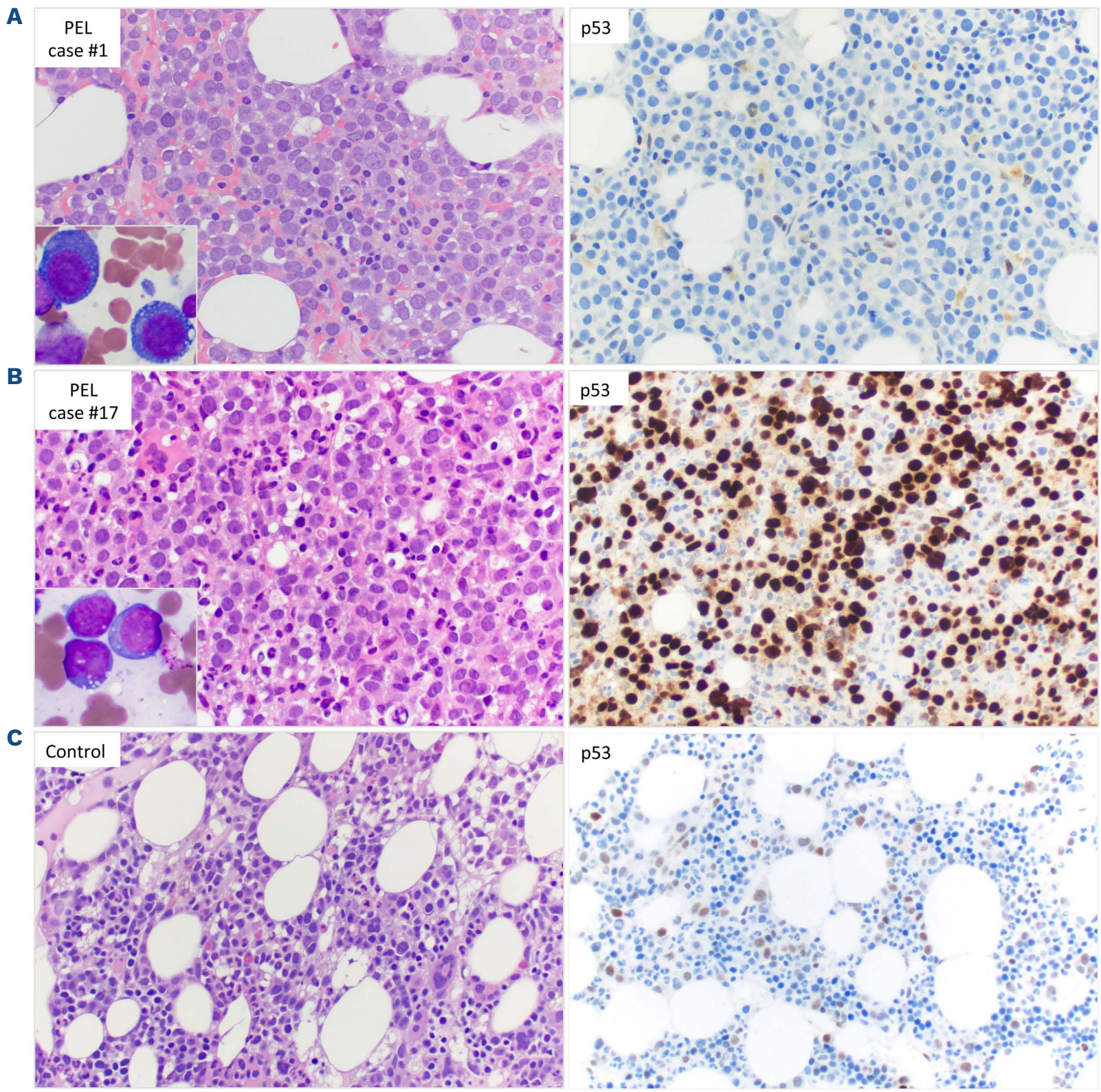

Figure 1. The expression pattern of p53 by immunohistochemistry in pure erythroid leukemia. Immunohistochemistry shows two patterns of p53 expression: complete absence of p53 expression (case #1, upper panel) and uniform and strong overexpression (case #17, middle panel). Of note, in the case with absence of p53 expression in tumor cells (case #1, upper panel), there were scattered reactive cells in the background variably positive for p53, serving as positive controls. A normal bone marrow and its p53 expression by immunohistochemistry is illustrated in the lower panel, in which p53 is variably expressed in a subset of cells with weak to moderate intensity.

sessment of p53 using immunohistochemistry can be challenging, especially in cases where PEL is mixed with residual normal hematopoietic cells in the background which have a wild-type p53 staining pattern.

Lastly, we suggest that the category of PEL should be preserved, despite the fact that some cases also can be classified as therapy-related AML/MDS or AML with myelodysplasia-related changes (AML-MRC) using the current World Health Organization (WHO) criteria. We believe classifying these cases as something other than PEL does not fully capture the distinctive features of this disease. The rationale for this proposal includes: i) PEL cases, irrespective of their origin (*de novo* or secondary), share similar clinicopathological features including poor response to treatment, dismal prognosis, complex karyotype, and biallelic *TP53* alterations. By contrast, the WHO-defined categories of therapy-related AML/MDS or AML-MRC are highly heterogeneous at the molecular level, and are associated with highly variable prognoses for different patient subsets.[12,13] We believe that the distinctive clinicopathologic and molecular features of PEL may be obscured when these neoplasms are placed in the therapy-related AML/MDS or AML-MRC WHO categories; ii) the survival of PEL patients with a history of receiving cytotoxic therapy or MDS is similar to *de novo* PEL patients but is worse than patients with therapy-related AML[12] and AML-MRC,[13] respectively; iii) all PEL cases, whether they are *de novo* or secondary, share distinctive morphologic features with prominent pronormoblast proliferation. Pronormoblasts have been shown to play an important role in treatment resistance and increased pronormoblasts have contributed to a poorer prognosis in AML patients.[14,15] By keeping secondary PEL cases in the category of PEL, these cases can be studied together to explore therapeutic strategies targeting the neoplastic pronormoblasts. Of note, the number of *de novo* PEL cases in our study is relatively small and future studies to include more cases will be valuable.

In summary, we show that PEL is characterized by biallelic *TP53* loss-of-function, a complex karyotype, poor response to AML or MDS directed therapy, and a very dismal prognosis. These unique features are the same for *de novo* or secondary cases of PEL, and therefore we advocate for keeping them under the entity of PEL to facilitate further studies and drug discovery. Immunohistochemistry for p53 can be used as a preliminary screening tool to assess *TP53*; strong p53 expression correlates with missense mutations of *TP53* and a null p53 pattern is often associated with frameshift, nonsense, or *TP53* mutations involving splice sites.

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Contributions

HF had substantial contributions to the design of the work, drafting the work, revising it, and the acquisition, analysis, interpretation of data for the work. WW had substantial contributions to the conception and design of the work, revising it critically for important intellectual content, and analysis, interpretation of data for the work. SAW and LJM had substantial contributions to the conception and design of the work and revising it critically for important intellectual content. JDK, SE, DHK, MT, ZT, SL, ZH, FZJ, KPP, TJM, and TK had contributions to the acquisition, analysis of data for the work and revising it critically for important intellectual content. All authors had final approval of the version to be published and an agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data-sharing statement

All data are available for sharing upon request to the corresponding author.

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