REVIEW ARTICLE

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Emerging epigenetic therapeutics for myeloid leukemia: modulating demethylase activity with ascorbate

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ABSTRACT

The past decade has seen a proliferation of drugs that target epigenetic pathways. Many of these drugs were developed to treat acute myeloid leukemia, a condition in which dysregulation of the epigenetic landscape is well established. While these drugs have shown promise, critical issues persist. Specifically, patients with the same mutations respond quite differently to treatment. This is true even with highly specific drugs that are designed to target the underlying oncogenic driver mutations. Furthermore, patients who do respond may eventually develop resistance. There is now evidence that epigenetic heterogeneity contributes, in part, to these issues. Cancer cells also have a remarkable capacity to 'rewire' themselves at the epigenetic level in response to drug treatment, and thereby maintain expression of key oncogenes. This epigenetic plasticity is a promising new target for drug development. It is therefore important to consider combination therapy in cases in which both driver mutations and epigenetic plasticity are targeted. Using ascorbate as an example of an emerging epigenetic therapeutic, we review the evidence for its potential use in both of these modes. We provide an overview of 2-oxoglutarate dependent dioxygenases with DNA, histone and RNA demethylase activity, focusing on those which require ascorbate as a cofactor. We also evaluate their role in the development and maintenance of acute myeloid leukemia. Using this information, we highlight situations in which the use of ascorbate to restore 2-oxoglutarate dependent dioxygenase activity could prove beneficial, in contrast to contexts in which targeted inhibition of specific enzymes might be preferred. Finally, we discuss how these insights could be incorporated into the rational design of future clinical trials.

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Introduction

Acute myeloid leukemia (AML) is a cancer that arises from the stem and progenitor cells of the hematopoietic system. These abnormally differentiated, clonal cells infiltrate the bone marrow, blood and other tissues. Unless treatment is initiated early, the rapid onset of this proliferative disease is fatal. Over the past 50 years there have been remarkable improvements in the treatment of AML. Initially incurable, the 5-year survival for young patients (<60 years old) is now greater than 50%.[1] This progress was initiated in the 1970s when the effectiveness of cytarabine and anthracyclines was discovered. Subsequently, various regimens involving combinations of the two drugs were studied to find the optimum dose, with bone marrow transplants introduced as an option in 1977. Prognosis has been even better for acute promyelocytic leukemia, a subset of AML in which the use of all-*trans* retinoic acid (ATRA) and arsenic trioxide has increased 5-year survival to 90%. However, for older patients, for whom intensive chemotherapy is not an option, outcomes remain bleak with a median survival of 5-11 months.[2] Clearly, more effective treatments for AML are needed, with important clinical questions remaining, including why is there a variable response to drug therapy, and why do some patients develop resistance to therapy? When considering new treatments for AML, it is important to first evaluate our current understanding of the contributing

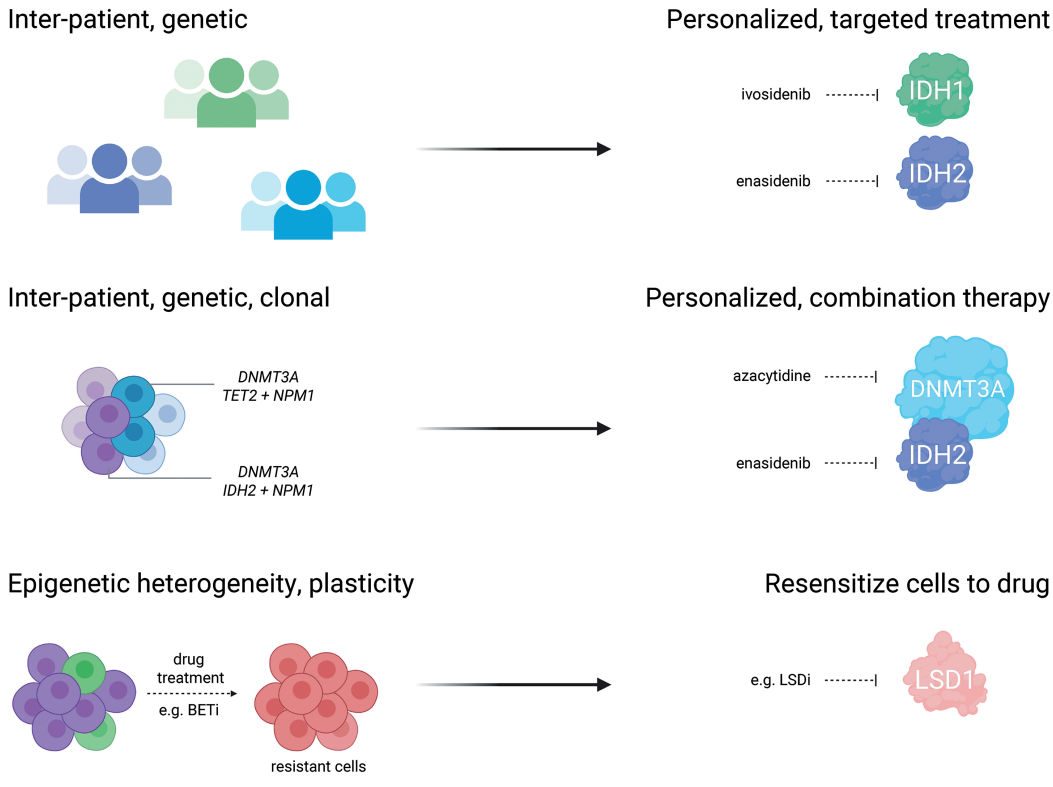

Figure 1. Levels of heterogeneity in acute myeloid leukemia and potential treatment strategies to address them. See text for further details.

variables. This will help to frame current and emerging treatment strategies for AML (Figure 1), and provide a framework for considering ascorbate as an epigenetic therapeutic.

Genetic heterogeneity in acute myeloid leukemia

As evidenced by the variable response to chemotherapy, AML is a heterogeneous disease. As the technologies available to investigate AML have developed, our understanding of where this heterogeneity lies has expanded. Morphological and cytogenetic heterogeneity have been studied for over 30 years and formed the basis for the French-Amercian-British classification system.[3] As genetic sequencing technologies improved, frequently mutated genes were added to classification and prognostic prediction by the World Health Organization[4] and European LeukemiaNet.[5] To date, next-generation sequencing technologies have shed the greatest light on heterogeneity in AML. In 2016, a landmark study that sequenced DNA samples from more than 1,540 AML patients found recurrent mutations in over 100 genes.[6] Combining this information with cytogenetic and clinical data has shown that AML comprises at least 11 different subgroups with prognostic relevance.[6,7] Therefore, substantial heterogeneity in molecular pathology underpins the apparently homogenous phenotype of patients presenting in the clinic. Single-cell sequencing has added another layer of complexity to this scenario: multiple AML clones may exist in the same patient with their relative proportions changing over time with treatment and relapse,[8,9] with different co-occurring mutations contributing to different clinical outcomes.[6,7]

A further insight emerging from large studies using next-generation sequencing to investigate AML is that the mutational burden is low in AML compared to other cancers, and that proteins involved in epigenetic processes are early drivers of the cancer phenotype.[6,10] This information is of particular interest because the reversibility of epigenetic modifications makes these processes, and the proteins that mediate them, attractive drug targets.[11-13] These data have also highlighted the potential for precision medicine, where treatments can be matched to the patient on a case-by-case basis. In the past 5 years, selective inhibitors for mutations in *FLT3*,[14] *IDH1*[15] and *IDH2*[16] have gained approval from the Food and Drug Administration. There are also a growing number of clinical trials for drugs targeting epigenetic writers (DOT1L, PRMT5), readers (BRD2/3/4) and erasers (HDAC, LSD1).[13] However, the major difficulties with targeted therapy to date, epigenetic or otherwise, are familiar adversaries: variable response to the drug, and drug resistance. At first glance this seems to be a repetition of the same issues experienced with chemotherapy. However, the nuance here is that patients with the same mutations respond quite differently to treatment. This is reported even with highly specific drugs that are designed to target the common oncogenic driver mutation.[15,17] By uncovering deeper layers of heterogeneity in AML, this obstacle has provided an indication as to how it may be overcome.

Epigenetic heterogeneity, plasticity and drug resistance

We have highlighted two reasons why patients respond quite differently to treatment; inter-patient heterogeneity (genetic) and intra-patient heterogeneity (genetic/clonal). However, these factors alone do not explain why some patients eventually develop resistance to a targeted drug.

The most commonly proffered reason is that the development of new mutations confers a survival advantage.[15,18] This can be due the founding clone in the primary tumor gaining mutations and evolving into the relapse clone, or a subclone of the founding clone surviving initial therapy, gaining additional mutations and expanding at relapse.[19] However, there is growing evidence that non-genetic resistance also plays a role.[20] Two key variables that contribute towards this outcome are epigenetic heterogeneity and epigenetic plasticity.

Epigenetic heterogeneity refers to the different epigenetic states across a population of genetically identical cells. This is a well-studied phenomenon in embryology, whereby multicellular organisms generate a vast array of cell phenotypes from a single genome. What is remarkable about normal development is the capacity of cells and organisms to produce consistent phenotypic outcomes despite being challenged by variable conditions.[21,22] Waddington coined the term "canalization" to refer to this capacity of cells and used it interchangeably with the word "buffering".[23] Cancers in general and AML in particular also display buffering by producing a consistent phenotype. However, this apparent homogeneity can be unmasked by chemotherapy or targeted treatment that provides a large selection pressure. Epigenetic heterogeneity across a population of genetically identical cells means that some will be in a transcriptional state that confers resistance. Depending on the size of the fraction with resistance, this could manifest as an initial response to treatment, followed by relapse once the resistant clone has had time to regenerate the disease. Importantly, epigenetic heterogeneity has been correlated with poorer outcomes and a shorter time to relapse in AML.[24]

A closely related concept is epigenetic plasticity, whereby a cell can alter its epigenetic state in a heritable manner in response to stimuli. Those cells that are able to explore the epigenetic landscape more extensively will be more likely to discover resistant states. How epigenetic heterogeneity and plasticity enable non-genetic resistance is of great interest and we recommend the review by Waddington for further discussion.[23] Of interest for this review is that: (i) both genetic and non-genetic evolution contribute to resistance in AML[24-26] and (ii) epigenetic plasticity is a new target for therapy in AML.[27]

Ascorbate as an emerging epigenetic therapeutic

Our increased understanding of heterogeneity in AML has opened up new strategies for therapy (Figure 1). Drugs that inhibit or activate epigenetic proteins can be used to target either oncogenic driver mutations, or epigenetic plasticity. With this in mind, we review the current evidence on whether ascorbate can be used as an epigenetic therapeutic in AML. Ascorbate is synthesized from glucose in the liver or kidneys of most animals. However, humans have acquired mutations in L-gulonolactone oxidase, the terminal synthetic enzyme, and must therefore obtain ascorbate from their diet.[28] Ascorbate has an essential role in human physiology through its capacity to act as an electron donor and cofactor for a wide range of enzymes including many of the 2-oxoglutarate dependent dioxygenases (OGDD).[29,30] This is a large family of enzymes that includes the first enzymes known to utilize ascorbate as a cofactor, the collagen hydroxylases.[31] The OGDD utilize non-heme iron (Fe^{2+}), 2-oxoglutarate and oxygen to catalyze a wide range of hydroxylation reactions. Many enzymes function as DNA, histone and RNA demethylases and have been shown to require ascorbate to maintain optimal activity (Table 1). This biochemistry underpins ascorbate's link to epigenetics and has received increasing attention in multiple contexts including cancer.[32,33] The findings are particularly relevant for AML because many of these demethylase enzymes are involved in the development and maintenance of AML (Table 1). Of the 20 OGDD enzymes with demethylase activity listed in Table 1, 17 play a role in the development or maintenance of AML, 15 require ascorbate as a cofactor, and 12 fall into both categories (see Table 1 for details).

Targeting driver mutations: restoring tumor-suppressor activity

The use of ascorbate as a drug differs from the typical scenario in which small molecules are designed to inhibit catalytic activity or interfere with protein-protein interactions. The proposed mechanism of action is that ascorbate could promote enzyme activity in cases in which decreased activity contributes to the AML phenotype. This requires residual functional enzyme to be present. We therefore propose two further criteria to select likely molecular contexts in which ascorbate might function to target oncogenic drivers. Firstly, evidence that the target demethylase functions as a tumor-suppressor in AML and secondly, that loss-of-function mutations are heterozygous to ensure there is residual functional enzyme. Using the information collated in Table 1 along with previously published next-generation sequencing data from 878 AML patients accessed through cBioPortal,[34] we found three demethylases that fit these criteria: *TET2*, *KDM3B* and *KDM6A* (Figure 2). The rest of the demethylases from Table 1 are either oncogenes in AML, are tumor suppressor genes but have increased or mixed gene expression profiles, or there is no current evidence that ascorbate acts as a cofactor. *TET2*, *KDM3B* and *KDM6A* are discussed here first, with the more diverse group of enzymes considered in the subsequent sections.

Context 1. Heterozygous mutations in *TET2*

Mutations in *TET2* arise in approximately 10% of cases of AML.[35-37] Similar to other mutations that affect epigenetic regulation in AML, *TET2* mutations arise early in the development of hematopoietic malignancies.[6] Mutations in *TET2* and other epigenetic regulators are commonly found in otherwise healthy individuals with clonal hematopoiesis, who are at risk of subsequently developing blood cancers such as AML.[38-40] Mutations in *TET2* increase hematopoietic stem cell (HSC) self-renewal and the expansion of myeloid lineage cells.[41] The acquisition of subsequent mutations can lead to AML.[42,43] These findings support a double-hit model in which premalignant HSC with *TET2* mutations subsequently undergo further mutations leading to the development of cancer.[44]

TET2 has a fundamental role in hematopoiesis, and enables the appropriate differentiation of HSC.[41] The TET enzymes are involved in active demethylation of DNA *via* oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxylcytosine.

Table 1. Iron and 2-oxoglutarate-dependent epigenetic enzymes, their dependency on ascorbate and link to myeloid malignancies.

Gene	Synonyms	Protein	Substrate	Effect of ascorbate[a]	Role in myeloid malignancies[b]
TET1	CXXC6, KIAA1676, LCX	Methylcytosine dioxygenase TET1	5mC, 5hmC and 5fC	Increased activity *in vitro*[51,91] and in cell culture[50,51,53,92–94]	Oncogene in AML[95] and MLL-rearranged leukemia[96]. Reduced expression in AML[97] Increased expression in MLL-rearranged leukemia[96]
TET2	KIAA1546, Nbla00191	Methylcytosine dioxygenase TET2	5mC, 5hmC and 5fC	Increased activity *in vitro,*[91] in cell culture,[50,51,53,61,92] mouse models[41,52] and clinical setting[89]	Tumor suppressor in AML, MDS, CMML[35,36,41,52,97]
TET3	KIAA0401	Methylcytosine dioxygenase TET3	5mC, 5hmC and 5fC	Increased activity *in vitro*[91] in cell culture[53,92] and a mouse model[41]	Increased expression in AML - high expression of TET3 may be a positive prognostic marker.[97] Possible tumor suppressor in AML[98]
KDM2A	CXXC8, FBL11, FBL7, FBXL11, JHDM1A, KIAA1004	Lysine-specific demethylase 2A	H3K36me1/me2	Increased activity in cell culture[73,74]	Tumor suppressor in MLL-rearranged leukemia - Over expression induces differentiation of MLL-AF10 transformed cells.[72]
KDM2B	CXXC2, FBL10, FBXL10, JHDM1B, NDY1, PCCX2	Lysine-specific demethylase 2B	H3K36me1/me2 H3K4me3	Increased activity in cell culture[73,74]	Oncogene, required for AML cellular proliferation. Highly expressed in AML.[99]
KDM3A	JHDM2A, JMJD1, JMJD1A, KIAA0742, TSGA	Lysine-specific demethylase 3A	H3K9me2/me1	Increased activity *in vitro*[65] and in cell culture[66]	Unknown[b]
KDM3B	C5orf7, JHDM2B, JMJD1B, KIAA1082	Lysine-specific demethylase 3B	H3K9me2/me1	Increased activity in cell culture[66]	Tumor suppressor, transcriptionally regulates HOXA1 through retinoic acid response elements in acute myeloid leukemia.[63] Also commonly deleted, or underexpressed in AML/MDS.[100,101]
KDM3C	JHDM2C, JMJD1C, KIAA1380, TRIP8	Lysine-specific demethylase 3C	H3K9me2/me1	Unknown[a]	Required for the survival and proliferation of some AML cell lines.[102]
KDM4A	JHDM3A, JMJD2, JMJD2A, KIAA0677	Lysine-specific demethylase 4A	H3K9me3/me2 H3K36me3/me2 H1.4K26me3/me2	Increased activity *in vitro*[103] and in cell culture[104]	Required for MLL-AF9 translocated AML.[105]
KDM4B	JHDM3B, JMJD2B, KIAA0876	Lysine-specific demethylase 4B	H3K9me3/me2 H3K36me3/me2 H1.4K26me3/me2	Increased expression and activity in cell culture[104,106]	Required for MLL-AF9 translocated AML.[105]
KDM4C	GASC1, JHDM3C, JMJD2C, KIAA0780	Lysine-specific demethylase 4C	H3K9me3/me2 H3K36me3/me2 H1.4K26me3/me2	Increased activity in cell culture[104]	Required for MLL-AF9 translocated AML.[105]
KDM5A	JARID1A, RBBP2, RBP2	Lysine-specific demethylase 5A	H3K4me3/me2/me1	Increased expression in cell culture[107]	Potentially oncogenic in AML.[78] NUP98/JARID1A gene fusion linked to pediatric acute megakaryoblastic leukemia.[108]
KDM5B	JARID1B, PLU1, RBBP2H1	Lysine-specific demethylase 5B	H3K4me3/me2/me1	Decreased expression in cell culture[109]	Potential tumor suppressor - MLL-AML.[77] Potentially oncogenic – CML,[110] AML[78,79]
KDM5C	DXS1272E, JARID1C, SMCX, XE169	Lysine-specific demethylase 5C	H3K4me3/me2/me1	Unknown[a]	Associated with chemoresistance - pediatric AML[111]
KDM5D	HY, HYA, JARID1D, KIAA0234, SMCY	Lysine-specific demethylase 5D	H3K4me3/me2/me1	Increased activity *in vitro*[112]	Unknown[b]
KDM6A	UTX	Lysine-specific demethylase 6A	H3K27me3/me2	Increased activity *in vitro*[71] in cell culture[70]	Tumor suppressor. Low expression correlated with adverse outcome in male AML patients. Mutations in KDM6A associated with cytarabine resistance.[68] Homozygous loss

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					of UTX in mice associated with AML[113]
KDM6B	JMJD3, KIAA0346	Lysine-specific demethylase 6B	H3K27me3/me2	Increased activity in cell culture[70,94]	Potential oncogene in AML – involved in the regulation of HSC self-renewal, knockout impairs self-renewal.[114] Overexpressed in MDS CD34+ cells[115]
KDM7A	JHDM1D, KDM7, KIAA1718	Lysine-specific demethylase 7A	H3K9me2/me1 H3K27me2/me1 H4K20me1	Increased activity *in vitro*[116]	Unknown[b]
KDM7B	PHF8, KIAA1111, ZNF422, JHDM1F	Histone lysine demethylase PHF8	H3K9me2/me1 H3K27me2 H4K20Me1	Unknown[a]	Regulates ATRA response in APL. Downregulated in ATRA-resistant APL cells[117]
FTO	KIAA1752	Alpha-ketoglutarate-dependent dioxygenase FTO	N^6-mA mRNA	Increased activity *in vitro*[118]	Oncogene in AML. Inhibition of ATRA-induced AML cell differentiation[119]

[a]'Unknown', we did not find evidence linking these enzymes to dependence on ascorbate as well as KDM4D, KDM4E, KDM6C, KDM7C, ALKBH1, ALKBH5, RIOX1, RIOX2, JMJD6, and JMJD8. *in vitro*, in which activity was tested using purified protein or cell lysate. [b]'Unknown', we did not find evidence linking these enzymes to the development or maintenance of myeloid leukemia.

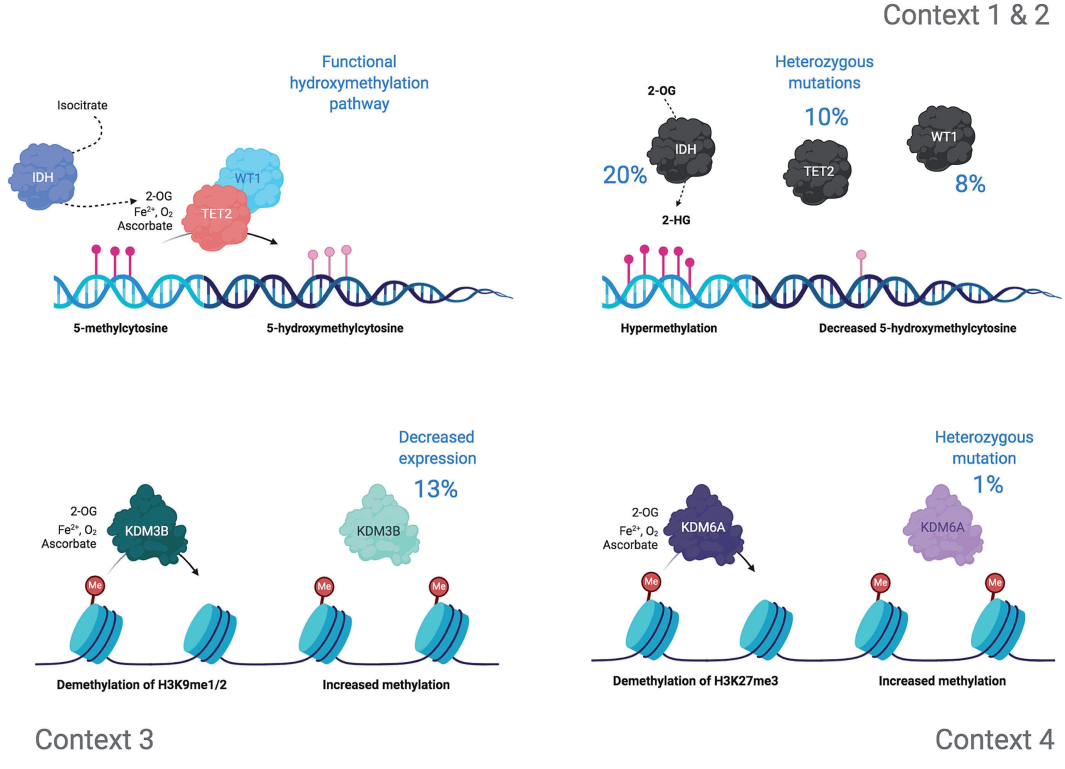

Figure 2. Ascorbate-dependent demethylases that function as tumor suppressors in acute myeloid leukemia. *Top left panel.* Relevant components of the DNA hydroxymethylation and active demethylation pathway are depicted. *Top right panel.* Heterozygous mutations seen in *de novo* acute myeloid leukemia (AML) that result in decreased TET2 activity. These mutations are mutually exclusive but collectively are found to be mutated in 30-50% of AML patients.[6,59,60,122] *Bottom left panel.* Decreased *KDM3B* expression is seen in 13% of AML patients at first presentation. *Bottom right panel.* Heterozygous mutations in *KDM3B* are only seen in 1% of patients at first presentation. These data are based on next-generation sequencing of 878 AML patients accessed through cBioPortal[34] (DNAseq n=878; RNAseq n=165). 2-OG, 2-oxoglutarate; 2-HG, 2-hydroxyglutarate.

These molecules are intermediates in the pathway of DNA demethylation, and 5hmC may be an epigenetic mark with its own reader.[45-48] Loss-of-function mutations in *TET2* cause a significant reduction in 5hmC levels, highlighting the importance of TET2 in hydroxymethylation and epigenetic regulation more broadly.[49]

TET2, as a member of the family of OGDD, requires Fe^{2+}, oxygen and 2-oxoglutarate for activity. It is now well-established that ascorbate also increases TET enzyme activity and leads to an increase in 5hmC in multiple cell culture and animal models.[33,37,50,51] While still an area of active research, ascorbate has been shown to act as an electron donor to reduce inactive Fe^{3+} back to its catalytically active Fe^{2+} state, thereby increasing the Fe^{2+} necessary for TET activity.[32,33,50] Furthermore, Yin *et al.* used fluorescence quenching to demonstrate that ascorbate uniquely interacts with the C-terminal catalytic domain of TET2, with other strong reducing chemicals not having

a similar effect.[50] This suggests something specific about the interaction between ascorbate and the TET enzymes, although more structural evidence is required.[50]

A number of prominent studies have explored the specific relationship between TET activity, ascorbate and the development of hematologic malignancies. Agathocleous *et al.* used *Gulo*$^{-/-}$ mice (which lack the ability to synthesize ascorbate) and showed that ascorbate deprivation resulted in a higher level of HSC relative to body mass and significantly reduced 5hmC levels in HSC, findings that are consistent with reduced TET activity.[52] The authors then transplanted *Flt3*^{ITD}/*Tet2*$^{\Delta/+}$ AML cells into both *Gulo*$^{-/-}$ and control mice which resulted in accelerated development of leukemia in the *Gulo*$^{-/-}$ mice compared with the controls. Administration of ascorbate to the *Flt3*^{ITD}/*Tet2*$^{\Delta/+}$/*Gulo*$^{-/-}$ mice suppressed leukemia development and prolonged survival. These results suggest that ascorbate could be beneficial in the context of leukemia with *TET2* mutations. Interestingly, HSC also exhibited a 14-fold increased level of the ascorbate transporter Slc23a2 and a 6-fold higher concentration of ascorbate compared to the levels in mature immune cells, further indicating the importance of intracellular ascorbate availability to support cellular differentiation.[52] In another study, Cimmino *et al.* used RNA interference to induce and reverse the knockdown of *Tet2* in mice.[41] *Tet2* knockdown caused aberrant self-renewal of HSC, which was reversed upon the restoration of TET2 activity. The restoration of TET2 activity promoted myeloid differentiation, cell death and DNA demethylation. Additionally, ascorbate administration was shown to pharmacologically mimic the effect of *Tet2* restoration.[41] An important corollary of these findings is that ascorbate depletion mimics a loss of TET2. Indeed, ascorbate deficiency was able to cooperate with *Flt3*^{ITD} to promote leukemogenesis in a manner similar to *Tet2* loss.[52] A number of studies have found that patients with hematologic cancers are ascorbate deficient,[53-55] and these patients are likely to have decreased TET2 activity even in the absence of *TET2* mutations.

Collectively, these results indicate that the restoration of TET2 activity *via* ascorbate supplementation could provide an avenue for reversing disease progression in AML cases linked to heterozygous loss-of-function mutations in *TET2*. However, it is also important to address the issue of specificity. To what extent are the effects of ascorbate seen in the above studies mediated by mechanisms other than increased TET2 activity? Both Agathocleous *et al.* and Cimmino *et al*. found that ascorbate also had a beneficial effect when both copies of TET2 were abrogated.[41,52] It is possible that ascorbate could be increasing the activity of other OGDD including histone demethylases. Agathocleous *et al.* did not find any evidence of increased histone methylation. Furthermore, there were no significant changes in the expression levels of *Tet1-3*, loss of bone collagen, depletion in carnitine or increased reactive oxygen species. Interestingly, Cimmino *et al.* found that knocking down *Tet3* in addition to *Tet2* resulted in a loss of the beneficial effect of ascorbate on both colony-forming potential as well as 5hmC. These results suggest that Tet3 was responsible for the residual effect of ascorbate in the absence of Tet2. Another mechanism proposed in the literature is that ascorbate can act as a pro-oxidant when used at high concentrations.[28] Specifically, adding millimolar amounts of ascorbate to cell culture media can generate extracellular hydrogen peroxide *via* redox cycling, leading to increased cell death. Alternatively, cells can take up dehydroascorbate, an oxidation product of ascorbate, *via* GLUT1 which could lead to oxidative stress as the cells reduce it back to ascorbate. Agathocleous *et al.* did not investigate this possibility because they were not using supraphysiological concentrations of ascorbate. Cimmino *et al.* used catalase as a control and showed that this had no effect on ascorbate blocking the aberrant replating capacity of *Tet2*$^{\Delta/+}$ AML cells. Furthermore, using dichlorofluorescein fluorescence, they were not able to detect generation of intracellular reactive oxygen species, even though this method can detect as little as 50 μM hydrogen peroxide. Given these results and the low concentrations of ascorbate used, it is unlikely that the effects seen were mediated by the production of hydrogen peroxide.

Context 2. Mutations in *IDH1*, *IDH2* and *WT1* also decrease TET2 activity

In addition to heterozygous *TET2* mutations, decreased TET2 activity can result from mutations in *IDH1*, *IDH2*, and *WT1*, particularly in the context of AML (Figure 2).[56-58] Mutant isocitrate dehydrogenase (IDH) enzymes generate the oncometabolite 2-hydroxyglutarate, which acts as a competitor of TET2, in competition with its physiological substrate, 2-oxoglutarate.[56] Wilms tumor protein 1 (WT1) is a transcription factor that recruits TET2 to DNA, enabling promoter demethylation.[59] In both contexts, hypermethylation and decreased hydroxymethylation provide evidence of decreased TET2 activity. Data from cBioPortal and Wang *et al*.[59] clearly show that mutations in the IDH1/2-TET2-WT1 pathway are mutually exclusive. Furthermore, they are collectively present in 30–50% of AML cases.[6,59,60] Together, they constitute a distinct subtype of AML characterized by dysregulated DNA (hydroxy)methylation (Figure 2).

Ascorbate administration to mouse cells with *IDH1* mutations has also been shown to reduce the rate of cellular proliferation and increase the expression of genes associated with hematopoietic differentiation. In this experiment, *TET2* knockdown by short interfering RNA led to lower 5hmC increases in ascorbate-treated *IDH1*-mutant cells, a finding consistent with a TET2-dependent mechanism.[61] Importantly, this study excluded the generation of hydrogen peroxide by using 2-phosphoascorbate. This analogue of ascorbate does not redox cycle and therefore does not generate hydrogen peroxide but is converted to ascorbate during transport across the cell membrane.

In one clinical study, a patient with AML experienced a full clinical remission for 2.5 years following treatment with intravenous ascorbate.[62] The patient had mutations in a number of genes including *DNMT3a*, *NPM1*, *TET2* and *WT1*. The *TET2* and *WT1* mutations were present in separate subclones, both mutations were heterozygous, and both involved truncation of the resultant protein leading to a loss of activity.[62] Interestingly, the *WT1* clone did not emerge at relapse, suggesting that it may have been more sensitive to treatment. Together with the evidence in Context 1, these findings support the hypothesis that the clinical outcome of patients suffering from AML involving reduced TET2 activity might be improved by treatment with ascorbate.[28]

Context 3. Decreased *KDM3B* expression

An unexplored context for the potential benefit of ascorbate in AML is the altered expression of KDM3B. Decreased expression of this demethylase occurs in 13% of AML cases (Figure 2). This was determined by an *in silico* analysis of cancer patients' data from the COSMIC database.[63] This study showed that, in contrast to other cancers, decreased KDM3B expression is a feature of AML. On the other hand, *KDM3B* acts as an oncogene in acute lymphoblastic leukemia,[64] which highlights the importance of determining disease- and subtype-specific roles for each demethylase. Although homozygous deletions of *KDM3B* are found in AML, these only account for 1% of AML cases, suggesting that most cases with decreased expression could also express some functional enzyme. KDM3B (originally known as JHDM2A) specifically demethylates mono- and di-methyl lysine at position 9 on H3 (H3K9).[65] Knockdown of this demethylase results in a build-up of H3K9me2 at specific promotors, and decreased expression of genes at these locations. Importantly, KDM3B is also a known tumor-suppressor in AML, specifically in cell lines with *MLL-AF6/9* or *PML-RARA* translocations.[63] Overexpression of KDM3B in these cell lines resulted in repressed colony formation, suggesting that restoration of KDM3B activity is beneficial in specific AML contexts.[63]

Although pharmacological restoration of KDM3B has not been investigated in the context of AML, there is clear evidence that ascorbate activates KDM3B in embryonic stem cells.[66] It was shown that ascorbate induced widespread demethylation of H3K9me2 in a KDM3A- and KDM3B-dependent manner. Given that H3K9 methylation is a repressive mark with regards to transcription, it is possible that demethylation due to KDM3B activation in the AML context reverses repression required for maintenance of AML. This hypothesis is supported by evidence that came from a screen of over 200,000 small molecules in which Xu *et al.* found that KA-7 was capable of upregulating KDM3B activity.[67] KA-7 repressed proliferation and colony-forming assays in *MLL*-rearranged acute leukemia with a concomitant increase in KDM3B activity (demonstrated by increased demethylation of H3K9). These data are proof-of-principle that increasing the activity of KDM3B may be beneficial for AML patients with decreased *KDM3B* expression. Whether this increase in activity could be achieved with ascorbate should be explored in the preclinical setting as heterozygous mutations of *TET2* have been tested.

Context 4. Heterozygous *KDM6A* mutations, and decreased expression at relapse

Of the contexts discussed in this section, heterozygous *KDM6A* mutations constitute the smallest percentage of cases of *de novo* AML. Along with the seminal paper by Papaemmanuil *et al.*,[6] COSMIC and cBioportal data[34] show that only 1% of patients with *de novo* AML have a heterozygous loss-of-function mutation (Figure 2). However, *KDM6A* appears to be a tumor suppressor; low *KDM6A* expression is associated with poor survival outcomes and when *KDM6A* mutations arise at relapse they confer cytarabine resistance.[68] Although cells used in follow-up cell culture experiments were either *KDM6A*-null or *KDM6A*-replete, AML patients are more likely to have heterozygous mutations than homozygous deletions.[69] Therefore, there is likely to be functional KDM6A present, with the potential for ascorbate to increase residual enzyme activity. Interestingly, the dependence of KDM6A on ascorbate has been demonstrated both using the purified protein as well as in a hematopoietic stem cell model.[70,71] Ascorbate was required for KDM6A-mediated demethylation of H3K27, which is a repressive chromatin mark. KDM6A occupies the promotors of *HOX* gene clusters, and demethylation of H3K27 is critical for expression of some of the *HOX* genes. Zhang *et al.* found that ascorbate was critical for the differentiation of hemogenic-endothelial cells into hematopoietic cells in a KDM6A-dependent manner. Collectively this evidence suggests that ascorbate may be beneficial for a small percentage of patients (~1%) at presentation, and potentially a much larger proportion of patients at relapse (see Context 7). As with *KDM3A*, this hypothesis requires further investigation at the preclinical level.

Context-dependent roles for epigenetic 2-oxoglutarate-dependent dioxygenases in acute myeloid leukemia

Thus far, we have considered contexts in which activation of tumor suppressor activity might be beneficial. However, our analysis of the information available has highlighted some other contexts with greater complexity. The remaining demethylases in Table 1 are either oncogenes in AML, are tumor suppressor genes with increased or mixed gene expression profiles, or there is no current evidence that ascorbate acts as a cofactor. Important contexts to consider are those in which the demethylase expression is heterogeneous across the patient group or those in which ascorbate-dependent demethylases are actually required for the maintenance of AML, i.e., they function as oncogenes (Figure 3). While an in-depth consideration is outside the remit of this review, it is important to be aware of contexts in which inhibition of the respective demethylase might be recommended on a mechanistic basis.

Context 5. Heterogeneous expression across patients with acute myeloid leukemia

The demethylases *KDM2A*, *KDM2B*, *KDM4C*, *KDM5A*, *KDM6B*, *KDM7A*, and *KDM7B*, are neither consistently upregulated nor downregulated across AML patients. Of these, *KDM2A* is a tumor-suppressor in the context of *MLL*-rearranged leukemia, in which demethylation of H3K36 leads to inactivation of genes required for maintenance of leukemia.[72] This enzyme requires ascorbate for optimal activity;[73,74] however, this finding needs to be validated in the setting of leukemia before this can be considered as a context for treatment with ascorbate.

Context 6. Inhibiting oncogenic demethylase activity

The demethylases *TET1*, *KDM3C*, *KDM4A*, *KDM4B*, *KDM5B*, *KDM5C* and *FTO*, are individually mutated in <1% of cases, whereas they each display increased expression in 5-7% of AML cases (Figure 3). There is evidence that *TET1*, *KDM3C*, *KDM4A*, *KDM4B*, *KDM5B* and *FTO* function as oncogenes in some AML contexts (see Table 1 for details). It is therefore interesting to consider that the inhibition of demethylases may provide clinical benefit. This possibility has been considered since the initial discovery of histone demethylases, and we sug-

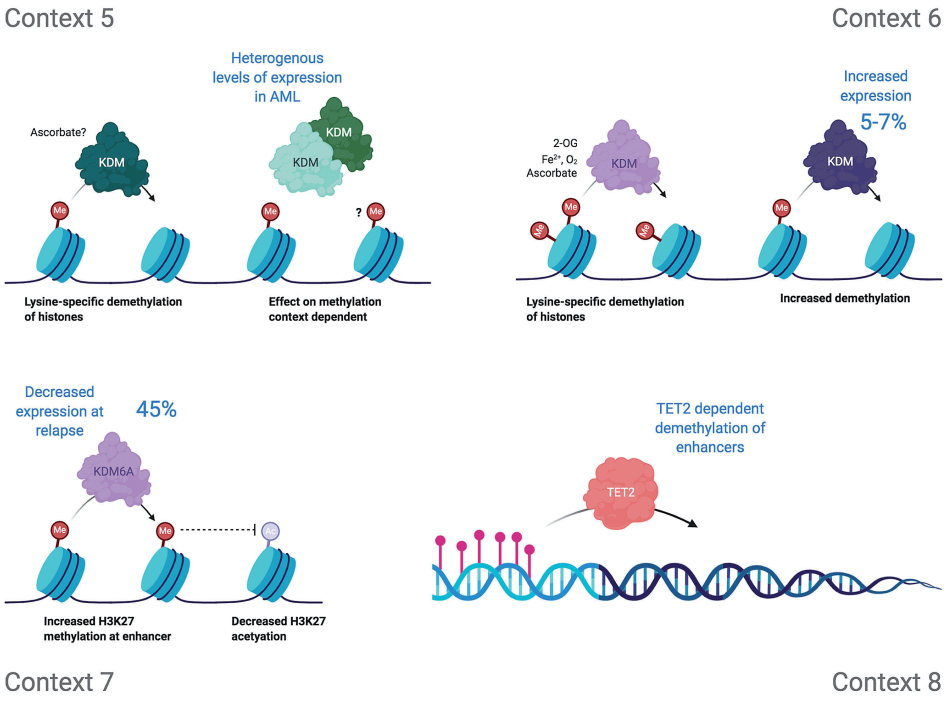

Figure 3. Context-dependent roles for demethylases in acute myeloid leukemia. *Top left panel.* Heterogeneous expression of histone KDM in acute myeloid leukemia (AML) including some for which there is no clear evidence that ascorbate is required for optimal activity. *Top right panel.* A number of histone KDM are oncogenes and have upregulated expression in 5-7% of AML patients. Targeted inhibition of these enzymes might be beneficial. *Bottom left panel.* Decreased *KDM6A* expression is seen in 45% of AML patients at relapse and confers resistance to cytarabine.[69] *Bottom right panel.* TET2 can demethylate enhancers and thereby play a role in epigenetic plasticity. Data are based on studies of 878 AML patients and were accessed through cBioPortal[34] (DNAseq n= 878; RNAseq n= 165). 2-OG, 2-oxoglutarate.

gest the review by Thinnes *et al.* for further reading on this subject.[75] It is interesting to note that histone deacetylase inhibitors are being investigated in clinical trials,[13] whereas histone demethylase inhibitors have lagged behind.[76] This is likely due to the multiple roles that histone demethylases play in AML disease progression. For example, there are conflicting reports in the literature as to whether *KDM5B* inhibition suppresses or promotes leukemogenesis.[77-79] Clearly, further investigation is required to elucidate the specific contexts in which inhibition of these demethylases might have clinical benefit.

Targeting epigenetic plasticity

Cancer stem cells can evade therapy by exploring the epigenetic landscape and finding transcriptional states that confer resistance.[20,27,80] This is a form of Lamarckian induction that is enabled by epigenetic plasticity. For example, drug treatment may result in enhancer switching[81] to maintain the expression of key survival genes.[27] While targeting this capacity of cancer cells is a relatively new concept, there is some evidence that *KDM6A* and *TET2* may enable plasticity in some contexts.

Context 7. Increasing KDM6A activity to overcome drug resistance

Epigenetic proteins that mediate plasticity have been identified in different models of drug resistance in AML. Using overexpressed MLL-AF9 fusion protein to generate cell culture and mouse models of AML, BET inhibitor therapy was employed to create a robust model of drug resistance.[25] Using this model, targeting the histone demethylase Lsd1(Kdm1a) overcame non-genetic, acquired resistance and re-sensitized the cells to BET inhibition.[27] Interestingly, the CRISPR screen that identified *Lsd1* as a target also showed that targeting *Utx* (*Kdm6a*) and *Mll4* prevented differentiation of the resistant population. *Lsd1* and *Mll4* counter each other *via* demethylation/methylation of H3K4 at enhancers, suggesting that enhancer switching was mediating the resistant phenotype.[27] Consistent with the emergence of new enhancers, there were also increased H3K27 acetylation and related markers at regions of increased chromatin accessibility. Importantly, KDM6A demethylates H3K27 in an ascorbate-dependent manner,[70,71] which must occur prior to H3K27 acetylation.[82] Given that knocking down *Kdm6a* prevented differentiation and resensitization, it is possible that ascorbate could increase Kdm6a activity and thereby augment the effect of Lsd1 inhibition in restoring sensitivity to BET inhibition. Further evidence in support of this hypothesis comes from clinical studies: when *KDM6A* mutations arise at relapse they confer cytarabine resistance.[68] A follow-up study found that 45% of relapsed patients have lower *KDM6A* expression at relapse than at initial presentation.[69] The same study found that *KDM6A* null cells were more resistant to cytarabine and daunorubicin, with re-expression of *KDM6A* restoring sensitivity to cytarabine. Determining whether treatment with ascorbate phenocopies *KDM6A* overexpression is a critical next step. Given that there are relatively few options for relapsed AML patients, investigating this possibility is rel-

Table 2. Clinical trials involving treatment with ascorbate for acute myeloid leukemia and myelodysplastic syndromes.

Trial number	Conditions	Specific mutations	Drug combinations	Control	Phase	Status
NCT00184054	AML	N/A	Arsenic trioxide + ascorbate (IV)	Single-arm	II	Completed
NCT03397173	MDS, AML	Heterozygous *TET2* mutations required for patient enrolment	Azacitidine + ascorbate	Single-arm	II	Recruiting
NCT00329498	MDS, AML	N/A	Manipulation of ascorbate levels	Single-arm	II	Completed[120]
NCT03999723	MDS, AML, CMML	N/A	Azacitidine + ascorbate	Azacitidine + placebo	II	Recruiting
NCT00671697	MDS, AML	N/A	Decitabine, arsenic trioxide + ascorbate	Single-arm	I	Completed[121]
NCT02877277	MDS, AML	*TET2*, *DNMT3A*, *IDH2* mutations identified	Azacitidine + ascorbate	Azacitidine + placebo	N/A	Completed[89]
Not registered	AML	N/A	Low-dose DCAG + ascorbate (IV)	DCAG[a]	N/A	Completed[86]

AML: acute myeloid leukemia, N/A: not applicable; IV: intravenous; MDS: myelodysplastic syndrome; [a]DCAG: low-dose decitabine prior to aclarubicin and cytarabine.

evant for a large proportion of patients who are resistant to therapy.

Context 8. Heterogeneous roles for TET2 at enhancers

A number of studies have found that TET2 has the capacity to demethylate DNA at enhancers. This has been demonstrated in embryonic stem cells, hematopoietic stem cells, AML and breast cancer.[43,83,84] In the context of AML, loss of TET2 promotes leukemogenesis *via* hypermethylation of enhancers.[43] Therefore, in those cases in which heterozygous *TET2* mutations are part of the natural history of AML, DNA demethylation at key enhancers would be an additional mechanism in favor of ascorbate treatment. However, the specific enhancers that TET2 binds to are cell-specific and likely to be influenced by AML subtype.[83] It is possible that resistance to targeted treatment in *TET2* wild-type AML could arise by TET2-dependent demethylation of enhancers leading to the expression of key survival genes. In this scenario direct inhibition of TET2 with small molecules might be more effective.[85]

Clinical trials and conclusions

By providing an overview of the ascorbate-dependent OGDD with demethylase activity, we have highlighted a range of contexts in which ascorbate could be used as an epigenetic therapeutic in AML (Contexts 1-4 and 7). Undoubtedly, the greatest weight of preclinical data supporting this is in AML with decreased TET2 activity (Contexts 1 and 2). For Contexts 3-8 much pre-clinical work remains to be done to determine whether ascorbate or, conversely, OGDD inhibition may be of clinical benefit. The question remaining is, how can this be translated into clinical benefit?

It is important to note that no single-agent therapy has been effective in AML; combination therapy has been the foundation of all successful treatments. Ascorbate will therefore need to be combined with chemotherapy or other targeted therapies in randomized clinical trials in order to gauge its utility in the clinic. Thus far, only one randomized trial has reported outcomes from such a comparison: ascorbate plus decitabine prior to aclarubicin and cytarabine (A-DCAG, n=39) was compared to DCAG alone (n=34).[86] Zhao *et al.* found that A-DCAG significantly increased the chance of clinical remission after first induction, as well as extending median overall survival by 6 months when compared to DCAG alone in patients over 60 years of age. This result is promising, although the findings need to be validated in a larger, randomized cohort. In addition to lack of randomization, we have noted that clinical trials looking at the benefit of ascorbate (Table 2) do not systematically evaluate the potential proposed in Contexts 1 and 2. Because most trials did not investigate *TET2/IDH/WT1* mutational status, there was no way to infer the underlying mechanism of action. Future clinical trials involving ascorbate will need to stratify response by mutation status if epigenetic mechanisms of action are to be validated in patient cohorts.

Another consideration is the route of ascorbate administration. This is important because the maximum steady-state plasma concentration achievable by oral dosing is approximately 100 μM.[87] Preclinical and clinical data suggest that oral administration may be adequate for the maintenance of TET2 function.[52,88] Specifically, Agathocleous *et al.* found that oral supplementation was sufficient to prevent the onset of leukemia when Gulo$^{-/-}$ mice were transplanted with Tet2$^{\Delta/+}$;Flt3$^{ITD/+}$ leukemic cells. Although they did not measure 5hmC changes with supplementation, they did show that the 5hmC:mC ratio was lower in hematopoietic progenitor cells from Gulo$^{-/-}$ mice transplanted with Tet2$^{\Delta/+}$;Flt3$^{ITD/+}$ leukemic cells than in Gulo$^{+/+}$ mice transplanted with the same cells.[52] Furthermore, Gillberg *et al.* found that oral administration of ascorbate was able to increase the 5hmC:mC ratio in patients undergoing azacytidine treatment for myeloid neoplasms.[89] On the other hand, Cimmino *et al.* used 250 μM ascorbate in cell culture, and intraperitoneal injections in experiments with mice to achieve similar results.[41] Whether or not increasing plasma concentrations beyond the physiological maximum of 100 μM

increases TET2 activity further and provides additional clinical benefit in humans needs to be investigated. Although achieving supraphysiological levels would require administration *via* an intravenous route, this should be distinguished from high-dose intravenous trials in other cancers in which plasma ascorbate concentrations can be manipulated to exceed 10 mM transiently. Interestingly, modeling of ascorbate distribution through tissues suggests that 1 mM ascorbate is sufficient for saturation of tissues distant from the blood vessel supply.[28] Increasing the plasma concentration above this level may not have an additional impact *via* the epigenetic mechanisms that we have described in this paper.

Therefore, for clinicians and hematology researchers looking to investigate the use of ascorbate as an epigenetic therapeutic for AML, we recommend the following: (i) at a minimum, consider mutation, copy number, and gene expression profiling for *TET2*, *IDH1*, *IDH2*, *WT1*, *KDM3B*, and *KDM6A* and (ii) determine an appropriate dosing regimen. As part of this, whether or not supraphysiological concentrations by intravenous administration can increase OGDD activity beyond that achieved by oral supplementation needs to be investigated. In order to do this, levels of ascorbate and 5hmC (or histone methylation) in myeloid cells should be measured. Furthermore, the frequency of dosing and temporal relationship to standard-of-care treatment will need to be established. Where possible, deep sequencing[90] to obtain mutational, copy number and gene expression profiling details should be carried out at diagnosis, remission and relapse to provide information on the sensitivity of subclones to treatment. This will provide ample data to determine whether epigenetic mechanisms of action for ascorbate can be validated in patient cohorts.

Disclosures

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Contributions

ABD, CCSD and MCMV wrote the manuscript and approved the final version.

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