CCAAT enhancer binding protein alpha (CEBPA) biallelic acute myeloid leukaemia: cooperating lesions, molecular mechanisms and clinical relevance

Anna S. Wilhelmson[1,2,3] and **Bo T. Porse**[1,2,3]

[1]*The Finsen Laboratory, Rigshospitalet, Faculty of Health Sciences, University of Copenhagen,* [2]*Biotech Research and Innovation Centre (BRIC), University of Copenhagen and* [3]*Danish Stem Cell Center (DanStem), Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark*

Summary

Recent advances in sequencing technologies have allowed for the identification of recurrent mutations in acute myeloid leukaemia (AML). The transcription factor CCAAT enhancer binding protein alpha (*CEBPA*) is frequently mutated in AML, and biallelic *CEBPA*-mutant AML was recognised as a separate disease entity in the recent World Health Organization classification. However, CEBPA mutations are co-occurring with other aberrations in AML, and together these lesions form the clonal hierarchy that comprises the leukaemia in the patient. Here, we aim to review the current understanding of co-occurring mutations in *CEBPA*-mutated AML and their implications for disease biology and clinical outcome. We will put emphasis on patterns of cooperation, how these lesions cooperate with CEBPA mutations and the underlying potential molecular mechanisms. Finally, we will relate this to patient outcome and future options for personalised medicine.

Keywords: molecular haematology, co-occurring mutations, disease modelling, CEBPA biallelic acute myeloid leukaemia.

Acute myeloid leukaemia (AML) is a heterogeneous group of aggressive haematological cancers that displays extensive variation in their clinical courses and in response to therapy. AML is characterised by an expansion of immature myeloid precursors at the expense of normal haematopoiesis, eventually leading to bone marrow failure. It is initiated by the step-wise accumulation of genetic alterations affecting proliferation and/or differentiation in haematopoietic stem or progenitor cells. Sequencing efforts in AML, e.g. by The Cancer Genome Atlas (TCGA) and BEAT AML studies (Cancer Genome Atlas Research Network *et al.*, 2013; Tyner *et al.*, 2018), have identified an extensive catalogue of somatic mutations where the recurrent mutations show a high degree of overlap, while mutations with a low frequency are highly divergent. This suggests that the recurrent mutations are the 'true' oncogenic driver mutations causing the disease, whereas low-frequency mutations are likely passenger mutations that do not affect AML biology.

Recent work has shown that AML development constitutes a continuous evolutionary process, where genetic changes are acquired at distinct stages during the course of the disease. Clonal haematopoiesis of indeterminate potential (CHIP) and age-dependent clonal haematopoiesis (ARCH) describe the expansion of haematopoietic clones in healthy individuals and frequently occur in the elderly (Genovese *et al.*, 2014; Jaiswal *et al.*, 2014). Expanded clones harbour lesions in genes that are frequently mutated in haematological malignancies and are particularly enriched for mutations in epigenetic regulators. These lesions have been reported to increase the self-renewal potential of haematopoietic stem cells (HSCs) and constitute the first step along the disease trajectory (Moran-Crusio *et al.*, 2011; Jeong *et al.*, 2018). Whereas the vast majority of CHIP/ARCH individuals do not progress to haematological malignancies, clonal haematopoiesis has been reported to be associated with a tenfold increased risk of developing these diseases, and it therefore constitutes a pre-leukaemic condition (Jaiswal *et al.*, 2014). Additional mutations may drive progression toward cytopaenia, such a clonal cytopaenia of undetermined significance (CCUS), which may eventually develop into myelodysplastic syndrome (MDS) or full-blown AML. Finally, several clones coexist in the patient, which are under continuous selective pressure, not only from treatment approaches, but potentially also from factors influencing their environment, such as infections.

Co-occurring mutations in AML

Co-mutation and mutual exclusivity of recurrent mutations can reveal patterns of mutational co-segregation, and therefore suggest a potential biological cooperation between

Correspondence: Bo Porse, Finsen Laboratory/Rigshospitalet, University of Copenhagen, Ole Maaløes Vej 5, 2200 Copenhagen N, Denmark.
E-mail: bo.porse@finsenlab.dk

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certain mutated genes (Tyner *et al.*, 2018). One hypothesis of leukaemogenesis is the so-called 'two-hit' model where mutations conferring a proliferation/survival benefit cooperate with mutations blocking differentiation (Dash & Gilliland, 2001). However, cooperation may also lie in the selective importance of individual mutations at distinct stages of disease progression, e.g. mutations in epigenetic regulators during clonal haematopoiesis and growth receptors during later disease stages (Shlush *et al.*, 2014; Abelson *et al.*, 2018).

The combination of mutations in AML can be classified as: 'co-mutated', i.e. found together more frequently than would be expected by each gene's individual frequency; 'neutral', i.e. found together in similar frequency as would be expected by each gene's individual frequency; and 'mutually exclusive', i.e. found together less frequently than would be expected by each gene's individual frequency (Papaemmanuil *et al.*, 2016). Co-mutated lesions are usually non-redundant in function and/or cooperate to induce leukaemogenesis, whereas mutually exclusive lesions are either redundant in function or have opposing functions. Mutually exclusive lesions might also arise due to synthetic lethality, if the combination of lesions leads to cell death, whereas the single lesions alone do not.

The fact that two (or more) mutations are found in the same patient does not necessarily mean that they co-occur in the cells, and mutational co-occurrence data of leukaemic cohorts need to be interpreted with caution. Due to clonal heterogeneity, several AML clones harbouring different mutations may exist in a patient, and sequencing of bulk AML cells may reveal co-occurrence of mutations that exist in different clones. That said, approaches using variant allele frequency have been used to establish mutational co-occurrence in individual cells, although these should be interpreted with some caution for the reasons mentioned above. Future efforts into sequencing AML at the single cell level are needed to fully resolve this issue and to firmly establish clonal hierarchies within individual leukaemias.

Oncogenic collaboration is not restricted to mutated genes, but may also involve genes that are subject to transcriptional deregulation, potentially driven by epigenetic changes. In addition, a genetic lesion may even 'collaborate' with a gene that is not subject to transcriptional changes. The latter has been framed non-oncogenic addiction, where a mutation in gene A induces a cellular state in which the cell is dependent on gene B for its survival (Solimini *et al.*, 2007; Luo *et al.*, 2009). Examples of this class include genes in various stress pathways.

Finally, oncogenic cooperation is frequently discussed in terms of response to therapy. However, mutations are generally not under a selective pressure with regards to response to therapy during their evolution in the primary tumour. One exception is tumours that develop secondary to other cancers. Thus, differences in response to therapy may not necessarily reflect oncogenic cooperation.

CEBPA biallelic AML

The gene encoding the transcription factor, CCAAT enhancer binding protein alpha (CEBPA), is biallelically mutated (CEBPAbi) in 2–15% (average 5%) of *de novo* AML patients, with a higher incidence in Asian (6–15%; average 12%) compared to Caucasian (2–6%; average 4%) populations (Table I). CEBPAbi AML has been classified as a novel AML entity in the 2017 revised World Health Organization (WHO) classification of tumours of haematopoietic and lymphoid tissues, and is a favourable prognostic marker in AML, with increased overall survival (OS) and event-free survival (EFS) of patients with CEBPAbi compared to *CEBPA*-wild-type (CEBPAwt) or monoallelic *CEBPA*-mutated patients. When we mention mutations in *CEBPA* in this review, we will be referring to mutations in the context of CEBPAbi AML.

The functions of CEBPA in normal and malignant haematopoiesis have been extensively reviewed previously (Ohlsson *et al.*, 2016; Avellino & Delwel, 2017; Pulikkan *et al.*, 2017). Briefly, CEBPA is a key myeloid transcription factor that is both important for HSC self-renewal and for driving transcriptional programmes during myeloid differentiation (Hasemann *et al.*, 2014; Avellino *et al.*, 2016; Pundhir *et al.*, 2018). CEBPA comes in two forms, the full-length 42 kDa (p42) and the N-terminally truncated 30 kDa isoform (p30). In normal cells, p42 is dominant, but stop and frameshift mutations within the 5′-end of the one-exon *CEBPA* gene, sustain p30 expression at the expense of p42. These N-terminal lesions are frequently combined with C-terminal mutations (although biallelic N-terminal combinations also occur), which either block CEBPA dimerisation with itself and other CEBP family members or block DNA binding. In either event, biallelic *CEBPA* mutations converge at the expression of CEBPA-p30 homodimers as the only form of functional CEBPA in the mutated cells.

Importantly, CEBPAbi AML has successfully been modelled in the mouse with either a mimic of the human N-terminal CEBPA mutant in both alleles (Lp30 or L/L), C-terminal CEBPA mutant in both alleles (K/K) or a combination of C- and N-terminal mutations (K/L) (Kirstetter *et al.*, 2008; Bereshchenko *et al.*, 2009). These models have demonstrated that the sole expression of CEBPA-p30 leads to a block in myeloid differentiation, promotes transcriptional deregulation and is associated with cell cycle defects (Kirstetter *et al.*, 2008). Despite these insights, we still have an incomplete understanding of the mechanistic basis for CEBPA-p30-induced AML, specifically in terms of the functional interactions between *CEBPA* lesions and other genetic and epigenetic aberrations found in human AML.

Table I. Data of co-occurrence of mutations in CEBPA biallelic AML reported in this review come from the listed studies, which were identified during a literature survey for reported CEBPAbi AML cases. Cited references: (Dufour *et al.*, 2010; Chou *et al.*, 2011; Metzeler *et al.*, 2011; Taskesen *et al.*, 2011; Greif *et al.*, 2012; Cancer Genome Atlas Research *et al.*, 2013; Green *et al.*, 2013; Grossmann *et al.*, 2013; Fasan *et al.*, 2014; Kihara *et al.*, 2014; Krauth *et al.*, 2015; Ahn *et al.*, 2016; Lavallee *et al.*, 2016; Metzeler *et al.*, 2016; Papaemmanuil *et al.*, 2016; Theis *et al.*, 2016; Wakita *et al.*, 2016; Wang *et al.*, 2016; Lin *et al.*, 2017; Mannelli *et al.*, 2017; Rose *et al.*, 2017; Konstandin *et al.*, 2018; Su *et al.*, 2018; Tien *et al.*, 2018; Zhang *et al.*, 2019a; Zhang *et al.*, 2019b).

Publication Author	Year	All cases, *n*	Age, years, median (range)	Proportion *de novo* AML, %	Proportion CN-AML, %	CEBPAbi cases, *n* (%)
Ahn	2016	404	52 (15–84)	N.A.	100	51 (13)
Chou	2011	486	52 (15–90)	100	45	45 (9)
Cancer Genome Atlas Research	2013	200	55 ± 16*	100	47	6 (3)
Dufour	2010	467	61 (17–85)	71	100	20 (4)
Fasan	2014	2296	68 (15–100)	89	76	104 (5)
Green	2012	1427	N.A.	N.A.	N.A.	55 (4)
Greif	2012	160	N.A.	N.A.	100	33 (21)
Grossmann	2013	95	58 (15–87)	98		95 (–)
Kihara	2014	197	N.A. (15–64)	100	37	19 (10)
Konstandin	2018	48	57 (20–84)	N.A.	100	48 (–)
Krauth	2015	3157	67 (17–100)	85	62	110 (3)
Lavallée	2016	415	58 (17–87)	94	32	14 (3)
Lin	2017	112	43 (11–79)	N.A.	N.A.	7 (6)
Mannelli	2017	251	57 (16–81)	91	47	16 (6)
Metzeler	2011	220	N.A. (60–83)	N.A.	100	11 (5)
Metzeler	2016	664	57 (18–86)	86	N.A.	27 (4)
Papaemmanuil	2016	1540	† (18–84)	91	N.A.	66 (4)
Rose	2017	4373	67 (18–100)	100	54	136 (3)
Su	2018	553	N.A.	100	N.A.	81 (15)
Taskensen	2011	1182	48 (16–60)	N.A.	100	91 (8)
Theis	2016	113	N.A. (20–76)	96	N.A.	113 (–)
Tien	2018	693	55 (15–94)	100	N.A.	65 (9)
Wakita	2016	184	N.A. (17–86)	100	N.A.	16 (9)
Wang	2016	95	45 (12–88)	N.A.	N.A.	13 (14)
Zhang	2019a	259	23 (2–68)	N.A.	N.A.	26 (10)
Zhang	2019b	609	23 (1–75)	N.A.	N.A.	76 (12)

De novo AML refers to patients with no prior history of myeloid diseases or exposure to leukaemogenic agents, i.e. excluding secondary and therapy-related AML.

CN-AML, cytogenetically normal AML; N.A., data not available.

*Mean ± SD.

†Three included studies: HD98A (n = 627) median age 47 (18–65) years, HD98B (n = 173) median age 66 (58–84) years, and 07/04 (n = 740) median age 49 (18–61) years.

Co-occurrence of mutations in CEBPA biallelic AML

In CEBPAbi AML, *CEBPA* lesions co-occur with mutations in numerous genes with diverse cellular functions. As for other AML subtypes, the recurrently mutated genes belong to epigenetic regulators, transcription factors, cell-signalling factors, splicing factors, members of the cohesin complex and tumour suppressors. In contrast, *CEBPA* lesions do not appear to co-occur with classical aberrations such as inversions [e.g. inv(16), inv(3)] and translocations [e.g. t(15;17), t(8;21), t(6;9) and mixed-lineage leukaemia (MLL)-fusions]. Specifically, in the large study by Papaemmanuil *et al.* only two cases out of 66 CEBPAbi AML did co-occur with the 280 reported cases of AML with classical cytogenetic aberrations (Papaemmanuil *et al.*, 2016). Thus, for the purpose of the present review, we therefore focused on mutations in protein-coding genes. In order to obtain more quantitative data for the co-occurrence of mutations in CEBPAbi AML, we surveyed the literature and identified 26 studies with reported CEBPAbi AML cases (Table I), including four large next-generation sequencing (NGS)-based studies, with one representing cytogenetically normal (CN-)AML, and three *de novo* AML (Papaemmanuil *et al.*, 2016; Konstandin *et al.*, 2018; Su *et al.*, 2018; Zhang *et al.*, 2019b) (Fig 1). From this, it is apparent that mutational burden differs between studies which, in turn, might be due to choice of methodology, i.e. panel vs. exome sequencing, and/or differences in patient

cohorts with respect to both the type of AML and population characteristics. In the reviewed literature, data regarding clinical outcomes, i.e. survival and relapse frequency, are sparse; however, when available, the impact of co-occurring mutations on clinical outcome have been mentioned.

Epigenetic regulators

Epigenetic dysregulation is central to AML development and biology, and include aberrant DNA methylation, histone methylation and histone acetylation (Gallipoli *et al.*, 2015). Hence co-occurrence of mutations in genes coding for epigenetic factors and CEBPAbi mutations is frequent.

DNA methylation

The ten–eleven translocation (TET) family of proteins catalyse the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), thus leading to DNA demethylation. Co-occurrence of *TET2* mutations in CEBPA-mutated AML is prevalent with up to 40% of the CEBPAbi cases being reported to have mutated *TET2* (Table I). Konstandin *et al.* (2018) reported *TET2* mutations and *CEBPA* to be co-mutated, i.e. found together more frequently than would be expected by each gene's individual frequency. When analysing clinical outcomes in the context of CEBPAbi AML, *TET2*-mutated cases had shorter OS and EFS than *TET2*wt cases (Grossmann *et al.*, 2013; Konstandin *et al.*, 2018).

Moreover, the isocitrate dehydrogenase 1 and 2 (IDH1/2) enzymes, which catalyse the oxidative decarboxylation of isocitrate to α-ketoglutarate, are both recurrently mutated in CEBPAbi AML. The leukaemogenic effect of mutant IDH1/2 is thought to be the result of inhibition of the TET enzymatic function by aberrant production of the oncometabolite 2-hydroxyglutarate (2-HG). These gain-of-function mutations are co-occurring with CEBPAbi in around 10% of the cases (Table I). *IDH1/2* and *CEBPA* mutations have been reported to be mutually exclusive, i.e. found together less frequently than would be expected by each gene's individual frequency (Fasan *et al.*, 2014; Konstandin *et al.*, 2018). Additionally, *IDH1/2* mutations have been shown not to have an impact on OS or EFS (Grossmann *et al.*, 2013).

Mutations in the *WT1* gene encoding the transcription factor and tumour suppressor gene Wilms tumour 1 (WT1; discussed here due to its impact on TET2 function) co-occur with *CEBPA* lesions in up to 30% of the CEBPAbi cases (Table I). WT1 has been shown to recruit TET2 to its target genes and *WT1* mutations result in loss of function of TET2, and, in line with this, mutations in *TET2*, *IDH1/2* and *WT1* are reported as mutually exclusive in AML (Rampal *et al.*, 2014; Wang *et al.*, 2015). *WT1* and *CEBPA* are significantly co-mutated in CEBPAbi AML, but effects on EFS are divergent, with Grossmann *et al.* and Zhang *et al.* reporting no significant impact, while Su *et al.* reported shorter EFS (Grossmann *et al.*, 2013; Fasan *et al.*, 2014; Krauth *et al.*, 2015; Lavallee *et al.*, 2016; Papaemmanuil *et al.*, 2016; Su *et al.*, 2018; Zhang *et al.*, 2019b). Nonetheless, *WT1* mutations had no impact on OS in any of these studies (Grossmann *et al.*, 2013; Su *et al.*, 2018; Zhang *et al.*, 2019b).

Finally, DNA methyltransferase 3 alpha (DNMT3A), an enzyme responsible for *de novo* DNA methylation, is also prevalently mutated in CEBPAbi AML cases, where up to 15% carry *DNMT3A* mutations (Table I). Further, *DNMT3A* and CEBPAbi are classified as mutually exclusive (Ahn *et al.*, 2016; Metzeler *et al.*, 2016; Papaemmanuil *et al.*, 2016; Konstandin *et al.*, 2018). *DNMT3A* mutations have no impact on OS or EFS in the context of CEBPAbi AML (Grossmann *et al.*, 2013).

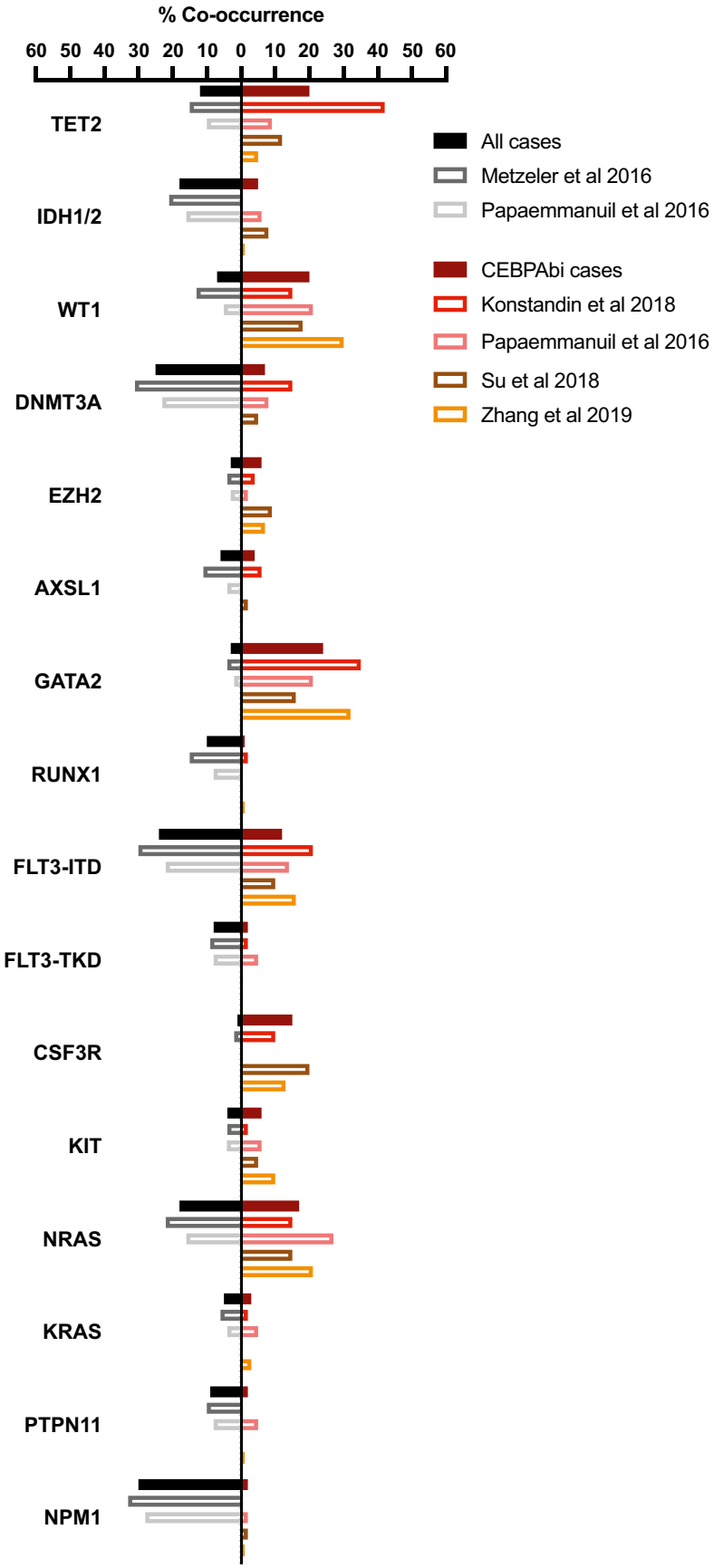
Collectively, genetic lesions predicted to either reduce (*DNMT3A*) or enhance (*TET2*, *IDH1/2*, *WT1*) DNA methylation are frequent in CEBPAbi AML. However, co-mutation seems to be restricted to *TET2* and *WT1*. Concordantly, *TET2*- and *WT1*-mutated AML shared nearly the same methylation profile, where the affected sites constitute a subset of those affected in *IDH1/2*-mutant AML (Rampal *et al.*, 2014). Whereas the functional consequences of this remain unknown, future experiments in mice promise to uncover the mechanistic basis for how *TET2* and *WT1* mutations collaborate with *CEBPA* lesions in the context of CEBPAbi AML.

Histone modification

The lysine methyltransferase 2 family (KMT2) mediates transcriptional activation, and mutations in the genes encoding KMT2A (*KMT2A/MLL1*), KMT2C (*KMT2C/MLL2*) and KMT2D (*KMT2D/MLL4*) have been reported to co-occur with *CEBPA* lesions in a few cases (Table I). *KMT2A* partial tandem duplication (*KMT2A-PTD*) and *CEBPA* lesions have been reported to be mutually exclusive, and several large studies have found no overlap between the two aberrations (Dufour *et al.*, 2010; Greif *et al.*, 2012; Grossmann *et al.*, 2013; Fasan *et al.*, 2014; Metzeler *et al.*, 2016; Su *et al.*, 2018).

KMT2A is part of a large protein complex involved in transcriptional activation, and although mutations in

Fig 1. Data on mutational co-occurrence from four large next-generation sequencing (NGS) based studies with Konstandin et al. (2018) (n = 48) representing cytogenetically normal (CN-)AML and Papaemmanuil et al. (2016) (n = 66), Su et al. (2018) (n = 81) and Zhang et al. (2019b) (n = 76) representing *de novo* AML. For comparison, data from the same cohorts representing all cases have been added when available; Metzeler et al., (2016) (n = 664) CN-AML cases and Papaemmanuil et al. (2016) (n = 1540) *de novo* AML cases. Data from the individual studies are represented by non-filled bars. Solid bars represent a calculated average from the studies included (percent of cases with co-occurring mutations in all samples (left) or in CEBPAbi cases (right).



KMT2 members are infrequent in CEBPAbi AML, the KMT2A complex was recently identified as a potential druggable target in this AML subtype (Liedtke & Cleary, 2009; Schmidt *et al.*, 2019). Specifically, the authors demonstrated that CEBPA-p30 and KMT2A interacted physically on chromatin. Deletion of KMT2A or mutations within functionally important KMT2A domains, such as the WD repeat domain 5 (WDR5)-interacting domain or the Menin-binding-motif, all showed anti-proliferative effects, whereas targeting the catalytic SET domain had no impact. Moreover, a small-molecule inhibitor, which disrupts the Menin-KMT2A interaction, displayed high activity in *CEBPA*-mutated cell lines. This, along with an earlier report demonstrating that a small-molecule inhibitor targeting the WDR5-KMT2A interaction also affected proliferation of *CEBPA*-mutant cell lines, points to the KMT2A complex being a potential target in CEBPAbi AML (Grebien *et al.*, 2015).

Several genes involved in epigenetic repression via the polycomb repressive complexes (PRC) 1 and 2 have been found to be recurrently mutated in CEBPAbi AML cases. Enhancer of zeste homologue 2 (EZH2) is a histone methyltransferase responsible for depositing the trimethyl mark on lysine 27 of histone 3 (H3K27me3), and, as a part of PRC2, the enzyme mediates transcriptional repression. *EZH2* is mutated in 6% of CEBPAbi AML cases (Table I). Likewise, mutations in additional sex combs like-1 (*ASXL1*), encoding a protein which is also part of the PRC2 complex as well as the polycomb repressive deubiquitinase complex, are found to co-occur with *CEBPA* lesions in 4% of the cases (Table I). Further, the genes encoding the BCL6 corepressor (BCOR) and its homologue, BCOR-like1 (BCORL1), are both recurrently mutated in AML (Tiacci *et al.*, 2012). BCOR is part of the PRC1.1 complex, which also mediates transcriptional repression. *BCOR* and *BCORL1* mutations have been reported to co-occur with *CEBPA* lesions in two CEBPAbi cases each (Table I). Collectively, these finding suggest that lesions in PRC1/2 complexes collaborate with *CEBPA* mutations in the context of CEBPAbi AML.

Finally, mutations in the genes encoding histone acetyl transferases have also been reported in CEBPAbi AML, including the CREB binding protein (CREBBP or CBP) and E1A binding protein p300 (EP300) (Lavallee *et al.*, 2016; Papaemmanuil *et al.*, 2016). Specifically, *EP300* lesions were found to be significantly co-mutated with *CEBPA*, as it appeared in four cases (6%) of CEBPAbi AML cases compared to an overall frequency of *EP300* mutations of 1·5% in AML (Papaemmanuil *et al.*, 2016).

Collectively, mutations in genes affecting histone modifiers are co-occurring in CEBPAbi AML, and some of these likely have functional ramifications, although the mechanistic details are lacking. In particular, the KMT2A complex appears to be of functional importance, and it constitutes a potential therapeutic target in this AML subtype.

Transcription factors

Several transcription factors, including important haematopoietic linage-specific transcription regulators, are recurrently mutated in AML, and therefore also in CEBPAbi AML.

GATA binding protein 2 (GATA2), an important transcription factor for the haematopoietic lineage, is abundantly and heterozygously (when reported) mutated in 15–35% of CEBPAbi AML cases, and indeed *GATA2* and *CEBPA* are significantly co-mutated (Table I). As for clinical outcome, *GATA2*-mutated CEBPAbi AML cases have been shown to have longer OS and EFS compared to *GATA2*wt CEBPAbi cases (Greif *et al.*, 2012; Fasan *et al.*, 2013; Grossmann *et al.*, 2013; Hou *et al.*, 2015; Tien *et al.*, 2018). As for a specific functional interaction between *GATA2* and *CEBPA* lesions, mutations within the N-terminal zinc finger (ZF1) of GATA2 reduced CEBPA-p30-dependent transcriptional activation in a reporter assay (Greif *et al.*, 2012). This finding raises the possibility that *GATA2 ZF1* mutations may collaborate with *CEBPA* lesions in order to deregulate the expression of downstream target genes. Interestingly, CRISPR/Cas9-mediated deletion of *GATA2* leads to reduced proliferative capacity and induction of myeloid differentiation in CEBPA-p30 expressing cells (Schmidt *et al.*, 2019). This finding suggests that, whereas the heterozygous mutations of *GATA2* found in patients with CEBPAbi lesions promote leukaemogenesis, the complete loss of GATA2 is incompatible with leukaemic growth. We hypothesise that this is due to the importance of GATA2 in maintaining self-renewal, a property of GATA2 clearly established in HSCs (Lim *et al.*, 2012).

Runt-related transcription factor 1 (RUNX1) is important for differentiation of haematopoietic cells. *RUNX1* mutations have been reported to co-occur with *CEBPA* lesions in AML at low frequencies, and in some studies as not co-occurring at all, despite the fact that *RUNX1* lesions are found in 9% of overall AML cases (Table I). Thus, *RUNX1* and *CEBPA* lesions are mutually exclusive. However, this does not necessarily apply to translocations involving the *RUNX1* locus such as *RUNX1-ETO* and *RUNX1-ETV6* (Fasan *et al.*, 2014; Papaemmanuil *et al.*, 2016).

Experimentally, *RUNX1* has been reported to control the expression of *CEBPA,* which could potentially render *CEBPA* lesions irrelevant in a *RUNX1*-mutated context (Guo *et al.*, 2012). Indeed, the expression of *CEBPA* is reduced in *RUNX1*-mutated AML (Grossmann *et al.*, 2012).

Finally, work in mice has shown that the SRY-box transcription factor 4 (SOX4), which is repressed by wt CEBPA, is increased in the leukaemic stem cell compartment in a mouse model of *CEBPA*-mutant AML and that its repression restored myeloid differentiation (Zhang *et al.*, 2013). These findings suggest that mutated CEBPA loses its normal ability to repress *SOX4* expression and that targeting SOX4 in the

context of CEBPAbi AML may constitute a potential treatment option.

Collectively, *CEBPA* is co-mutated with *GATA2* and mutually exclusive to mutations in *RUNX1*, thus highlighting the differential impact that transcription factor mutations has on AML biology.

Cell-signalling factors

Oncogenic signalling in the receptor tyrosine kinase (RTK)/RAS-signalling pathway is frequently activated in cancer, and AML is no exception. Thus, mutations in genes encoding proteins involved in this pathway are found in more than half of all AML cases, and these are often gain-of-function mutations (Papaemmanuil *et al.*, 2016).

Lesions in the gene encoding the FMS-like tyrosine kinase 3 (FLT3) occur in approximately 30% of all AML cases, where the internal tandem duplication (ITD) is most frequent, followed by mutations in the tyrosine kinase domain (*FLT3-TKD*). *FLT3-ITD* and *FLT3-TKD* co-occur with *CEBPA* aberrations in around 15% and 2% of cases, respectively (Table I). Moreover, both *FLT3-ITD* and *FLT3-TKD* are considered mutually exclusive with *CEBPA* lesions, but for *FLT3-TKD*, this negative association was only reported in two studies (Dufour *et al.*, 2010; Taskesen *et al.*, 2011; Greif *et al.*, 2012; Fasan *et al.*, 2014; Kihara *et al.*, 2014; Ahn *et al.*, 2016; Papaemmanuil *et al.*, 2016; Konstandin *et al.*, 2018). *FLT3-ITD* has no impact on OS or EFS (Grossmann *et al.*, 2013; Zhang *et al.*, 2019b). However, OS is significantly shorter in *FLT3-ITD* than in FLT3-wt cases if only CN-CEBPAbi AML cases are considered (Zhang *et al.*, 2019b).

FLT3-ITD results in the constitutive activation of FLT3, but this is not sufficient to promote AML development in mice. Still, in the context of the mouse model of CEBPAbi AML (K/L), *FLT3-ITD* accelerates leukaemic development, and this is associated with an increase in granulocyte-monocytic progenitors (GMPs) during the pre-leukaemic phase (Reckzeh *et al.*, 2012). Moreover, gene expression analysis of leukaemic GMPs revealed that *FLT3-ITD* was associated with a more immature phenotype and increased self-renewal. The mutual exclusiveness of *FLT3* and *CEBPA* lesions in human AML, and the observed acceleration of *CEBPA*-mutant AML in the context of the murine model, appears conflicting at a first glance. Nonetheless, *FLT3* activating mutations have been shown to selectively impede the function of CEBPA-p42, but not CEBPA-p30, via phosphorylation of a site (S21) present only in CEBPA-p42 (Radomska *et al.*, 2006; Radomska *et al.*, 2012). Conversely, CEBPA-p30 over-expression has been shown to increase *FLT3* expression (Alachkar *et al.*, 2015). These reciprocal interactions between FLT3 and CEBPA are likely to reduce the selective pressure for lesions in both genes during leukaemic initiation, which could explain their mutual exclusivity. Yet, when the two lesions are combined in the context of the mouse model, their combination may accelerate leukaemic growth during the expansion phase.

Mutations in the genes encoding the cytokine receptors colony stimulating factor 3 receptor (CSF3R; Cluster of Differentiation 114 [CD114] or granulocyte colony stimulating factor receptor [G-CSF-R]) and KIT (CD117 or stem cell factor receptor [SCF-R]) are co-occurring with *CEBPA* lesions in 10–20% and 2–10% of CEBPAbi AML cases, respectively (Table I). *CSF3R* and *CEBPA* are co-mutated; however, the clinical significance remains controversial (Lavallee *et al.*, 2016; Konstandin *et al.*, 2018; Zhang *et al.*, 2018). Whereas Su *et al.* reported both EFS and OS to be shorter in *CSF3R*-mutated cases of CEBPAbi AML, similar effects were not identified in another study (Su *et al.*, 2019; Zhang *et al.*, 2019b). Likewise, KIT mutations have been shown to affect EFS adversely when restricting the analysis to CN-AML, while OS and EFS are not changed when comparing to all CEBPAbi AML cases (Zhang *et al.*, 2019b).

CSF3R predominantly signals through the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway and, interestingly, CEBPAbi AML has been reported to be more sensitive to JAK inhibitor treatment *ex vivo* than CEBPAwt AML, with *CSF3R*-mutated CEBPAbi AML samples being particularly sensitive to JAK-inhibition (Lavallee *et al.*, 2016).

Additionally, mutations in JAK2, a non-receptor tyrosine kinase, have been reported to co-occur with *CEBPA* aberrations in six cases, and JAK3 in seven cases of CEBPAbi AML (Kihara *et al.*, 2014; Lin *et al.*, 2017; Konstandin *et al.*, 2018; Su *et al.*, 2018; Zhang *et al.*, 2019b).

Mutations in *NRAS* (20%) and *KRAS* (5%) are commonly found in AML and CEBPAbi AML cases mimic the overall mutation frequencies in AML with NRAS and KRAS aberrations co-occurring with lesions in *CEBPA* in 10–30% and 2–5% of cases, respectively (Table I). One study has reported *NRAS* and *CEBPA* as significantly co-mutated, but *NRAS* mutations have no impact on OS or EFS (Grossmann *et al.*, 2013; Papaemmanuil *et al.*, 2016; Zhang *et al.*, 2019b).

Last among members of the RTK/RAS-signalling pathway, the gene encoding the enzyme tyrosine-protein phosphatase non-receptor type 11 (PTPN11) is mutated in 10% of AML cases. *PTPN11* mutations have been reported in CEBPAbi AML at variable frequencies from 0% to 5%, but *PTPN11* and *CEBPA* lesions have not been reported to be mutually exclusive (Table I).

Finally, Zhang *et al.* investigated the significance of mutations in RTK/RAS pathway genes (i.e. *NRAS*, *KRAS* and *PTPN11* mutated) in CEBPAbi AML, and found no significant effects on OS or EFS as compared to non-mutated CEBPAbi cases, neither when comparing all *de novo* cases nor the CN-AML subgroup. Whereas mutations in tyrosine kinase genes (i.e. *FLT3-ITD*, *CSF3R*, *KIT* and *JAK3* mutated) resulted in significantly shorter OS and EFS in CEBPAbi CN-AML as compared to non-mutated cases; however, this difference was no longer seen when comparing all CEBPAbi AML (Zhang *et al.*, 2019b).

In summary, whereas mutations in the RTK/RAS pathway are frequent in CEBPAbi AML, they do not seem to cooperate extensively with lesions in *CEBPA*.

Splicing factors

Splicing factors are recurrently mutated in AML, with lesions found in around 10% of patients with AML, including the genes encoding the serine and arginine rich splicing factor 2 (SRSF2), the splicing factor 3b subunit 1 (SF3B1) and the U2 small nuclear RNA auxiliary factor 1 (U2AF1) (Zhou & Chng, 2017).

In CEBPAbi AML, a few cases of co-occurring mutations in splicing-factor genes have been reported; *SRSF2* was found to co-occur with *CEBPA* in 10 cases of CEBPAbi AML, *SF3B1* in one case and *U2AF1* in two cases (Table I). In addition, the zinc finger CCCH-type, RNA binding motif and serine/arginine rich 2 gene (*ZRSR2*), which encodes a protein that is associated with the U2 small nuclear ribonucleoprotein (snRNP) complex, was mutated in one case (Lavallee *et al.*, 2016). Hence, splicing-factor mutations are not common in CEBPAbi, and, moreover, *SRSF2* and *CEBPA* mutations have been shown to be mutually exclusive (Papaemmanuil *et al.*, 2016).

Functional approaches have also been undertaken to study the importance of de-regulation of splicing in the context of CEBPAbi AML. Using an *in vivo* short-hairpin RNA (shRNA) screen, our laboratory recently reported the identification of the non-mutated splicing regulator, RNA binding motif protein 25 (RBM25), as a novel tumour suppressor in CEBPAbi AML (Ge *et al.*, 2019). Specifically, we found that *RBM25* downregulation accelerates leukaemogenesis and shortens survival of mice transplanted with *CEBPA*-mutant AML. The effects of RBM25 were not restricted to CEBPAbi AML, and, mechanistically, we demonstrated that RBM25 controls splicing of the genes encoding the apoptotic regulator B-cell lymphoma-extra large (BCL-X) and bridging integrator 1 (BIN1), an inhibitor of the universal oncogene MYC. Thus, decreased *RBM25* levels inhibited apoptosis and promoted the expression of MYC target genes, thereby driving oncogenic expansion. In accordance, patients with AML with low *RBM25* expression displayed inferior outcomes.

In summary, whereas mutations in splicing factors do not co-occur frequently with *CEBPA* lesions, transcriptional deregulation of splicing factors may constitute an overlooked driver of AML development.

Cohesins, nucleophosmin 1 (NPM1) and tumour protein p53 (TP53)

The cohesin complex plays multiple roles in the context of chromatin such as promoting chromosome segregation during mitosis, maintaining chromatin boundaries, as well as facilitating the looping between different gene regulatory regions. Members of the cohesin complex are often mutated in AML, and the genes encoding stromal antigen 2 (STAG2) and RAD21 are found to be mutated in 6% and 2–9% of CEBPAbi AML cases, respectively (Table I). Similarly, the cohesin-complex components structural maintenance of chromosomes 1A and 3 (SMC1A/SMC3) have been reported to be mutated in CEBPAbi AML, albeit few cases have been identified (Lavallee *et al.*, 2016; Konstandin *et al.*, 2018). Correspondingly, the TCGA research network reported cohesion mutations in one of 16 CEBPAbi cases (Cancer Genome Atlas Research Network *et al.*, 2013). As of yet, no experimental studies have addressed the functional importance of cohesion complex lesions in the context of CEBPAbi AML.

Despite being one of the most frequent molecular abnormalities in AML, nucleophosmin 1 (*NPM1*) gene mutations are rarely found in CEBPAbi AML cases. Several studies have reported either no or only a modest overlap been *NPM1* and *CEBPA* in the range of 2–3% of CEBPAbi cases (Table I). Hence, *NPM1* and *CEBPA* mutations are mutually exclusive, with several studies reporting a negative association (Verhaak *et al.*, 2005; Dufour *et al.*, 2010; Taskesen *et al.*, 2011; Fasan *et al.*, 2014; Kihara *et al.*, 2014; Ahn *et al.*, 2016; Metzeler *et al.*, 2016; Papaemmanuil *et al.*, 2016; Konstandin *et al.*, 2018).

In a recent study, Gu *et al.* (2018) have elucidated the mechanism for the leukaemogenic effects of mutated *NPM1*, and these results might give clues as to why these mutations are mutually exclusive with *CEBPA* lesions. Mutant NPM1 aberrantly accumulates in the cytoplasm and also promotes the nuclear export of the myeloid transcription factor PU.1. The loss of nuclear PU.1 leads to a change in the repertoire of interaction partners for CEBPA and RUNX1, from coactivators to corepressors; hence, resulting in the shift towards repression of myeloid differentiation genes. Taken together, this suggests at least partly redundant functions for mutant NPM1 and *CEBPA* lesions in AML, i.e. a block in myeloid differentiation that may explain their mutual exclusivity in AML.

Finally, tumour protein p53 (TP53) is a tumour suppressor that is mutated in 6% of *de novo* AML cases, and *TP53* mutations co-occurred with *CEBPA* lesions in six CEBPAbi cases (Table I).

The most commonly co-mutated and mutually exclusive genes and their effect on OS/EFS are summarised in Table II.

Additional insights from experimental work

Mechanism-based studies have been instrumental in providing novel insights into AML biology, and a number of these have been highlighted throughout the present review. It is clear from this work that myeloid progenitors are subject to a strong selective pressure for genetic or transcriptional changes that increase the ratio of CEBPA-p30 to CEBPA-p42. Apart from mutations and activation of FLT3, the p30/p42 ratio may also change by the expression of tribbles pseudokinase 2 (TRIB2). *TRIB2* is highly expressed in a subgroup of patients with AML who display features of CEBPAbi

Table II. Summary of the recurrently co-mutated or mutually exclusive co-occurring mutations in CEBPAbi cases and their effects on clinical outcomes.

Mutation	Frequency in CEBPAbi, % (min–max)	Classification	Effect on OS	Effect on EFS
GATA2	24 (14–39)	Co-mutated	↑	↑
WT1	20 (14–30)	Co-mutated	↔	↓/↔
TET2	20 (5–42)	Co-mutated	↓	↓
CSF3R	15 (10–20)	Co-mutated	↓/↔	↓/↔
FLT3	14 (3–23)	Mutually exclusive	↓/↔	↔
DNMT3A	7 (0–15)	Mutually exclusive	↔	↔
IDH1/2	5 (0–14)	Mutually exclusive	↔	↔
NPM1	2 (0–4)	Mutually exclusive	N.A.	N.A.
RUNX1	1 (0–6)	Mutually exclusive	N.A.	N.A.

↑, increased; ↔, unaltered; and ↓, decreased. N.A., data not available; OS, overall survival; and EFS, event free survival.

AML, but without mutations in CEBPA (Keeshan *et al.*, 2006). Recent work has shown that TRIB2 facilitates the specific degradation of the CEBPA-p42 isoform via ubiquitination of lysine 48, a residue not present in CEBPA-p30, and consequently that TRIB2-mediated leukaemogenesis is dependent on the presence of the CEBPA-p42 isoform (O'Connor *et al.*, 2016). At present, the underlying driver responsible for the transcriptional upregulation of *TRIB2* is not known, but inhibition of the TRIB2-mediated degradation of CEBPA may constitute a novel therapeutic option in CEBPAbi AML.

Mechanism-based studies have also yielded insights into the downstream targets playing functional roles in CEBPAbi AML. Apart from SOX4 mentioned above, our laboratory recently discovered that the ectoenzyme CD73 (5'-nucleotidase ecto [*NT5E*]) is upregulated in human CEBPAbi AML, as well as in the mouse model of *CEBPA*-mutant AML. We demonstrated that this up-regulation was due to the specific binding of the CEBPA-p30 isoform to a normally silenced enhancer of the *NT5E* gene (Jakobsen *et al.*, 2019). Knockdown of *NT5E* delayed AML development in *CEBPA*-mutant AML in mice, both by targeting *NT5E* and its enhancer. CD73 catalyses the conversion of AMP to adenosine, and we find that this sets up a tumour-promoting adenosinergic autocrine signalling loop, which also appears to be operative in human CEBPAbi AML. Interestingly, the combined inhibition of CD73 and the adenosine receptor, A2AR, resulted in increased survival time of mice transplanted with CEBPA-mutated leukaemic cells. Hence, blocking the CD73/A2AR axis represents a potential new druggable target in CEBPAbi AML.

Clonal evolution in CEBPA biallelic AML

Clonal evolution is an important aspect of AML biology and we therefore surveyed the limited number of reports describing paired analyses of diagnostic and relapse CEBPAbi samples (Tiesmeier *et al.*, 2003; Shih *et al.*, 2006; Grossmann *et al.*, 2013; Tawana *et al.*, 2015; Li & Su, 2019). Given the different technologies used in these studies, and the limited sample numbers, it was not possible to discern any patterns of changes in the presence of co-occurring mutations. However, if we focus on the mutations in CEBPA itself an interesting pattern emerges. Specifically, we find that whereas the overwhelming majority of somatic CEBPAbi cases harbour the same *CEBPA* mutations at both diagnosis and relapse, the pattern is markedly different in familial cases. Here relapse is associated with the acquisition of new C-terminal lesions, which in combination with the germline N-terminal mutation, drive the development of a new leukaemic clone. In future studies it will be important to more adequately address the patterns of co-occurrence mutations during clonal evolution in CEBPAbi AML.

Implications for personalised medicine

The recent progress in NGS has given rise to a new challenge in AML treatment, i.e. how to translate the knowledge of the mutational status of a patient into tailor-made therapy targeting the specific aberrations of a given leukaemia. The first steps towards personalised medicine have already been taken, where identification of oncogenic driver mutations and the understanding of how they sustain disease development and maintenance are heavily researched.

During the last couple of years, several targeted therapies against AML carrying specific driver mutations have been approved: *FLT3*-mutated AML (Midostaurin; Rydapt® & Gilteritinib; Xospata®), and *IDH1/2*-mutated AML (IDH1: Ivosidenib; Tibsovo® & IDH2: Enasidenib; Idhifa®) (DiNardo & Perl, 2019). Thus, following the clinical outcomes for patients with CEBPAbi AML carrying *FLT3* and/or *IDH1/2* mutations treated with these new targeted therapies will be interesting.

Hopefully, this is the start of a new era in AML treatment that will improve survival and quality of life for patients with AML, aided by large studies investigating drug sensitivity in conjunction with mutational status (e.g. the BEAT AML study (Tyner *et al.*, 2018)). Albeit finding a druggable target for specific driver mutations might become more challenging if these mutations are in, for example, transcription factors, commonly viewed as non-druggable. Basic research might shed light over new druggable targets in cell or animal models for AML with specific mutational patterns. Recent publications have indicated the CD73/A2AR axis, the KMT2A complex and the CSF3R-JAK/STAT-signalling pathway as potential druggable targets in *CEBPA*-mutated AML. Still, the future therapeutic opportunities for these targets remain to be tested clinically (Grebien *et al.*, 2015; Lavallee *et al.*, 2016; Jakobsen *et al.*, 2019; Schmidt *et al.*, 2019).

The high degree of mutations in druggable tyrosine kinase genes (e.g. *CSF3R*, *KIT* and *JAK2/3*) should suggest that this subgroup of CEBPAbi patients would be responsive to

tyrosine kinase inhibitors (such as Tofacitinib; Xeljanz® or Ruxolitinib; Jakavi®), which are already in clinical use for other diagnoses. As mentioned above, Lavallee *et al.* found CEBPAbi AML to be highly sensitive to JAK inhibitors *ex vivo* (Lavallee *et al.*, 2016). Moreover, it will be interesting to follow how patients with CEBPAbi AML respond to newly approved treatment regimens such as BCL-2 inhibitor (Venetoclax; Venclyxto®) in combination with DNA methyltransferase inhibitor (Azacitidine; Vidaza® and Decitabine; Dacogen®). These treatments might be efficient, especially in *TET2*-mutated CEBPAbi cases and, by extension, in *IDH1/2*- and *WT1*-mutated cases (Duy *et al.*, 2019).

Conclusions

Here we have reviewed the current knowledge of mutational patterns in the context of CEBPAbi AML, excluding classical inversions and translocations that do not co-occur with *CEBPA* lesions. We note that relatively few data sets are available and that these deal mainly with point mutations in protein encoding genes. Thus, co-occurrences involving structural variations affecting gene regulation (e.g. enhancer hijacking etc.) are, to date, essentially unknown and therefore constitute an additional potential layer of biological complexity likely to influence the biology of, and clinical response in, CEBPAbi AML.

While CEBPAbi AML is generally associated with a good prognosis, this may vary widely depending on the co-occurrence of aberrations in other genes. Indeed, *CEBPA* is frequently co-mutated with lesions in *GATA2*, *WT1, TET2* and *CSF3R,* and this is generally associated with a worsened outcome. In contrast, mutations in factors such as *DNMT3A, FLT3, IDH1/2, RUNX1* and, in particular, *NPM1* are mutually exclusive with lesions in *CEBPA*. Mechanistic studies promise to uncover therapeutic vulnerabilities resulting from these functional interactions. In the future, with the introduction of clinical genomics in routine diagnosis, data from large cohorts will allow further stratification based on less frequent mutations, as well as on sex and ethnicity. We believe that this holds great potential for tailoring treatment and thus improving patient outcomes in the near future.

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Conflicts of interest

The authors have no conflicting interests to disclose.

Author contributions

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