## Structural aberrations are associated with poor survival in patients with clonal cytopenia of undetermined significance

Many patients referred to the hematological department with unexplained cytopenia are diagnosed with idiopathic cytopenia of undetermined significance (ICUS) when diagnostic bone marrow (BM) morphological findings or cytogenetic abnormalities defining myelodysplastic syndromes (MDS) are absent.[1] Approximately half of ICUS patients harbor MDS-related somatic mutations, a condition known as clonal cytopenia of undetermined significance (CCUS), associated with a >13-fold higher risk of progression to MDS or acute myeloid leukemia (AML) than ICUS patients without detectable mutations (i.e., ICUS non-clonal).[1,2] Still, the natural history of ICUS varies considerably; thus, additional prognostic markers remain to be identified.

Single nucleotide polymorphism-based array analysis (SNP-A) in patients with myeloid malignancies allows the identification of chromosomal aberrations beyond the resolution of metaphase cytogenetics (≤5 Mb), i.e., focal copy number aberrations (CNA) and copy-neutral loss of heterozygosity (CNLOH) (*Online Supplementary Figure S1*), that correlate with clinical features and outcome (reviewed by Kanagal-Shamanna *et al.*,[3] Xu *et al.*[4] and Ronaghy *et al.*[5]).

In this study, we investigated whether CNA and CNLOH were also present in ICUS patients and, if so, whether they had prognostic impact.

Patients (n=153) diagnosed with ICUS after routine work-up in Denmark between 2009-2017 were included upon first visit to the Department of Hematology and followed prospectively until death or study cut-off date (*Online Supplementary Methods*).

The study was approved by the Danish Science Ethics Committee and conducted in accordance with the

**Table 1.** Structural aberrations, including relevant genes affected, in the cohort of 153 patients with idiopathic cytopenia that are also recurrently detected in myeloid malignancies.

| A<br>Chromosomal Region | Abnormality type | Relevant gene(s) (if known)* | No. and disorder of cases | Relation* |
|---|---|---|---|---|
| 1p34 | CNLOH | *MPL* | 1 (CCUS) | MDS, MDS/MPN, MPN, AML |
| 1q | CNLOH | | 1 (ICUS non-clonal) | MDS, MPN |
| 2p23 | Deletion | *DNMT3A* | 1 (ICUS non-clonal) | MDS, AML |
| 4q12-q24 | CNLOH, Deletion | *TET2, PDGFRA, FIP1L1* | 2, CNLOH (CCUS); 1, Deletion (CCUS) | MDS, MDS/MPN, MPN, AML |
| 7q22 | Deletion | *CUX1* | 1 (CCUS) | MDS, MDS/MPN, MPN, AML |
| 11q13-q23 | CNLOH | *CBL, CCND1* | 1 (CCUS) | MDS, MDS/MPN, MPN, AML |
| 19p13 | CNLOH | *DNMT1, PRDX2* | 1 (ICUS non-clonal) | MDS |
| 20q | CNLOH | | 1 (CCUS) | MDS |

| B<br>Gene | Chr | Start, bp | End, bp | OMIM | Abnormality | Patient ID† | Disorder of patient | Relation |
|---|---|---|---|---|---|---|---|---|
| *TET2*‡ | 4 | 106,067,032 | 106,200,973 | 612839 | Deletion | 36 | CCUS | MDS, MPN, AML |
| | | | | | CNLOH | 22, 34 | CCUS | |
| *CUX1* | 7 | 101,458,959 | 101,927,250 | 116896 | Deletion | 34 | CCUS | MDS, MPN, AML |
| *DNMT3A*‡ | 2 | 25,455,845 | 25,565,459 | 602769 | Deletion | 6 | ICUS non-clonal | MDS, MPN, AML |
| *IDH2*‡ | 15 | 90,626,277 | 90,645,736 | 147650 | CNLOH§ | 11 | CCUS | MDS, MPN, AML |
| *CALR* | 19 | 13,049,392 | 13,055,304 | 109091 | CNLOH | 13 | ICUS non-clonal | MPN |
| *GNAS* | 20 | 57,414,773 | 57,486,250 | 139320 | CNLOH | 1 | CCUS | MDS, AML |
| *CBL*‡ | 11 | 119,076,752 | 119,178,859 | 165360 | CNLOH | 1 | | MDS, MPN, AML |
| *MLL* | 11 | 118,307,205 | 118,397,539 | 159555 | CNLOH | 1 | | AML |
| *SF1* | 11 | 64,532,076 | 64,546,316 | 601516 | CNLOH | 1 | | MDS, AML |
| *GNB1* | 1 | 1,716,725 | 1,822,526 | 139380 | CNLOH | 14 | CCUS | MDS, AML |
| MPL | 1 | 43,803,475 | 43,820,135 | 159530 | CNLOH | 14 | | MDS, MPN, AML |
| FBXW7 | 4 | 153,242,410 | 153,457,253 | 606278 | CNLOH | 22, 34 | CCUS | AML |
| KIT | 4 | 55,524,085 | 55,606,881 | 164920 | CNLOH | 34 | CCUS | MDS, MPN, AML |
| -Y | | | | | | [10 patients] | ICUS non-clonal/CCUS | MDS, MPN, AML |

(A) List of copy number aberrations (CNA) and copy neutral loss of heterozygosity (CNLOH) in our study cohort of patients with idiopathic cytopenia of undetermined significance (ICUS), that are also recurrently detected in patients with myeloid malignancies. These include ten CNA and CNLOH in eight chromosomal regions identified in eight patients (two patients had more than one). The cases listed in this table are all included in (B) except for chromosome 1q abnormality, as the gene(s) at this location that is relevant for myeloid malignancies is unknown. (B) List of genes recurrently affected in myelodysplastic syndromes, myeloproliferative neoplasms and/or acute myeloid leukemia that were involved in CNA and/or CNLOH in the ICUS patients. The cases listed in this table are all included in (A), except for the one marked by a section sign (§) as CNLOH(15q) is not reported as a recurrent lesion in myeloid malignancies. *From reviews by Kanagal-Shamanna *et al.*[3] and Xu *et al.*[4] †Patient ID in accordance with *Online Supplementary Tables S2, S3*. ‡Genes included in the 20-gene panel and sequenced in the study. §Not included in (A). CNLOH: copy-neutral loss of heterozygosity; CCUS: clonal cytopenia of undetermined significance; ICUS: idiopathic cytopenia of unknown significance; MDS: myelodysplastic syndromes; MPN: myeloproliferative neoplasms; AML: acute myeloid leukemia; Chr: chromosome; bp: base pairs; OMIM: online Mendelian inheritance in man.

Helsinki Declaration. All patients provided written informed consent.

Targeted next-generation sequencing of diagnostic samples was performed using a custom-designed multiplex Ion Ampliseq panel (Thermo Fischer Scientific, Waltham, MA, USA) including 20 genes recurrently mutated in MDS (*Online Supplementary Appemdix*). SNP-A was performed on diagnostic samples using the Infinium CytoSNP-850K v1.1 BeadChip (Illumina, San Diego, CA, USA). Illumina intensity files (.idat files) were analyzed visually with the GenomeStudio software version 2011.1 (Illumina) (*Online Supplementary Appemdix*).

Patient characteristics at baseline are presented in the *Online Supplementary Table S1* and the *Online*

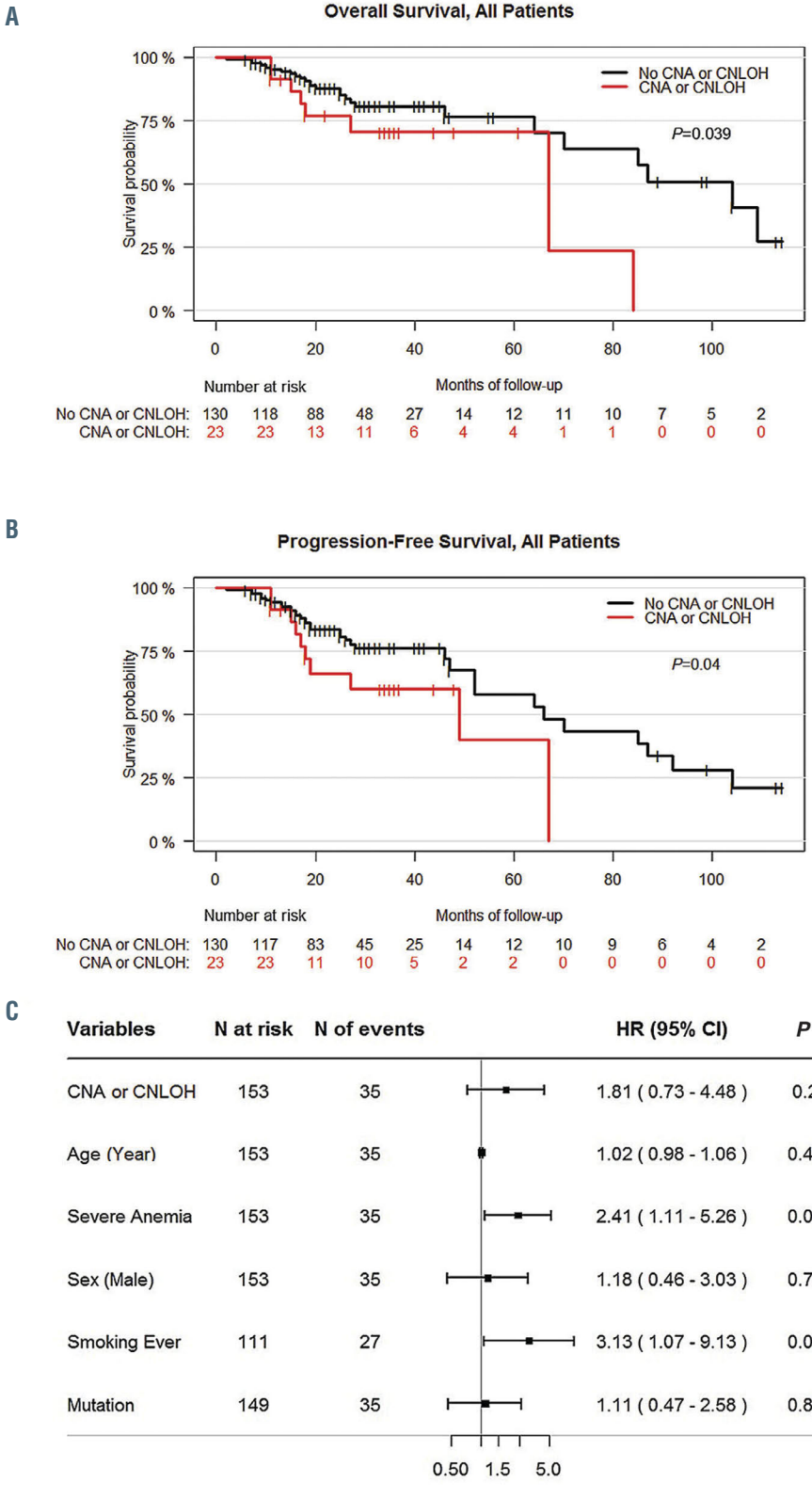


**Figure 1. Single nucleotide polymorphism-based array analysis-detected structural aberrations demarcate survival in patients with idiopathic cytopenia of undetermined significance but are not an independent adverse prognostic factor.** Kaplan-Meier estimates of (A) overall survival and (B) progression-free survival of the group of idiopathic cytopenia of undetermined significance (ICUS) patients with copy number aberrations (CNA) or copy neutral loss of heterozygosity (CNLOH) (excluding loss of the Y chromosome; red curve) and the group of ICUS patients without CNA or CNLOH (black curve). (C) Forest plot of hazard ratios (HR) including 95% Confidence intervals (CI) for all-cause mortality in multivariable analysis in ICUS patients (n=109 with complete data). Overall survival was measured from first bone marrow investigation (=inclusion) to death from any cause. Progression-free survival was measured from first bone marrow investigation to progression to a myeloid cancer or death from any cause. Annotated *P*-values are from two-sided log-rank tests. Severe anemia was defined as hemoglobin <100 g/L. Mutations were identified by targeted next generation sequencing using a 20-gene panel with a lower limit of detection at 2%.

*Supplementary Figure S2* illustrates the workflow. Median age was 69 years (range, 17-94 years) and two-thirds were male. Sixty-four patients (42%) had ≥1 mutation(s) in MDS-related genes with the most commonly affected genes being *TET2, DNMT3A* and *SRSF2* (*Online Supplementary Table S2*).

SNP-A identified a total of 25 structural aberrations (excluding loss of the Y chromosome [LOY]); 12 deletions, eight CNLOH and five gains, in 23 of 153 patients (15%) (*Online Supplementary Table S3, Online Supplementary Figure S3*).

Median sizes of the aberrations were deletions: 248 Kb (range, 131.6-2,867.5 Kb), CNLOH: 82.9 Mb (range, 11.6-137.1 Mb) and gains: 1.3 Mb (range, 0.6-2.6 Mb) ranging from minor genomic segments to entire chromosomes.

Mutations in MDS-related genes were present in 12 of 23 patients (52%) with CNA/CNLOH (*Online Supplementary Table S3*). Thus, a marker of clonal hematopoiesis was identified in 11 of 85 ICUS patients (13%) in whom no abnormalities were detected by conventional cytogenetics or targeted sequencing (*Online Supplementary Figure S4*).

The CNA/CNLOH identified in the ICUS patients were largely overlapping with recurrent CNA/CNLOH of known or likely clinical significance in patients with myeloid malignancies (10 of 25 CNA/CNLOH; 40%) (Table 1A).[3,4,6] Correspondingly, many genes frequently mutated in MDS, myeloproliferative neoplasms and/or AML were located within the sites of CNA/CNLOH (Table 1B; *Online Supplementary Figure S5*).

Mean corpuscular volume (median, 97 *vs.* 90 fL; *P*=0.014) and ferritin level (median, 260 *vs.* 173 μg/L;

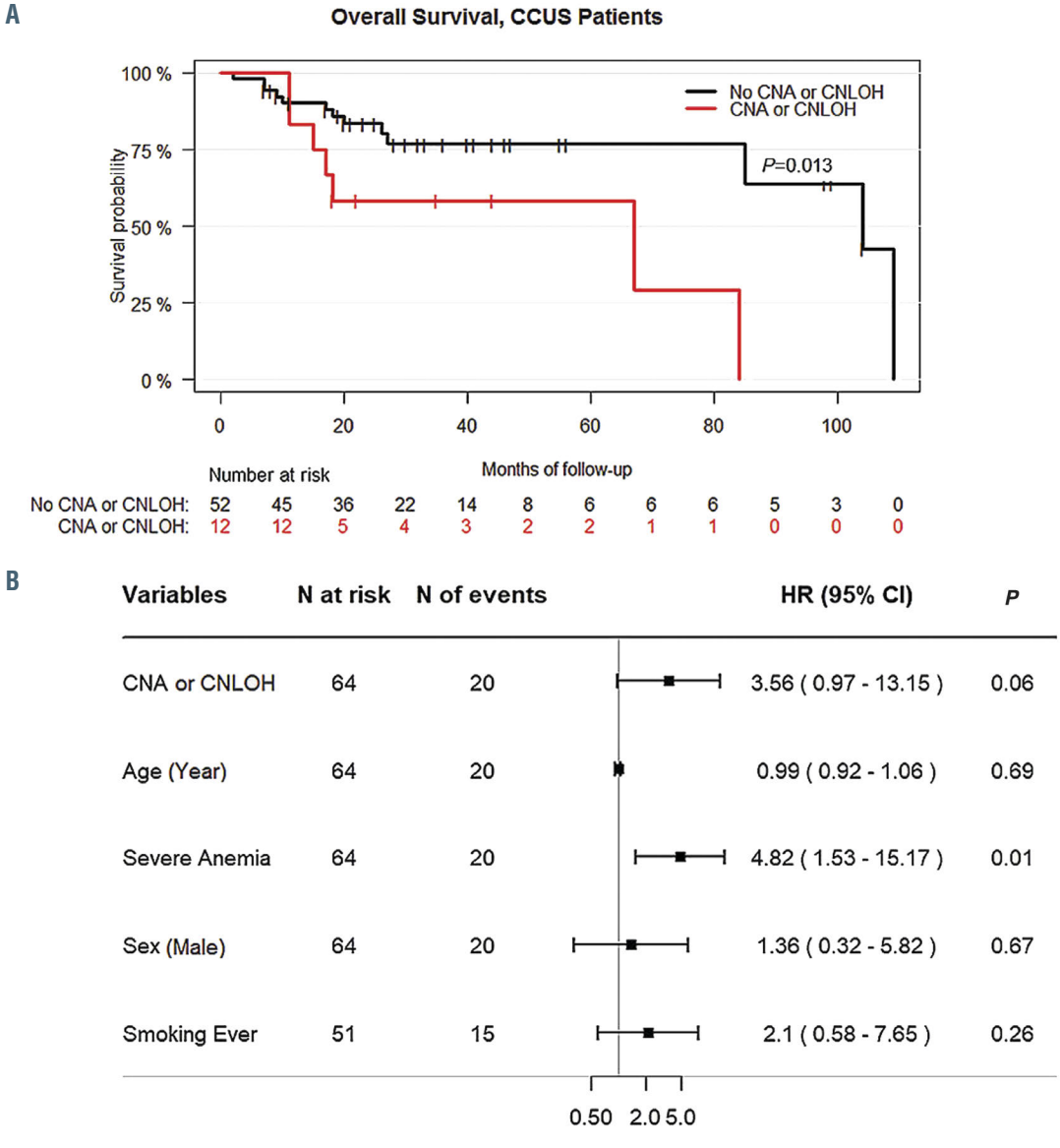


**Figure 2. In patienst with clonal cytopenia of undetermined significance, the presence of additional structural aberrations is associated with an increased hazard of all-cause mortality in univariable and multivariable analysis.** (A) Kaplan-Meier estimates of overall survival of the group of clonal cytopenia of undetermined significance (CCUS) patients with copy number aberrations (CNA) or copy neutral loss of heterozygosity (CNLOH) (excluding loss of the Y chromosome; red curve) and the group of CCUS patients without CNA or CNLOH (black curve). (B) Forest plot of hazard ratios (HR) including 95% Confidence Intervals (CI) for all-cause mortality in multivariable analysis in CCUS patients (n=51 with complete data). Overall survival was measured from first bone marrow investigation (=inclusion) to death from any cause. Patients alive at the last date of follow-up were censored at that time. Annotated *P*-values are from two-sided log-rank tests. Severe anemia was defined as hemoglobin <100 g/L.

*P*=0.043) were significantly higher in ICUS patients with CNA/CNLOH, and LOY was more preponderant (29% vs. 8% of males; *P*=0.028) than in ICUS patients without CNA/CNLOH (*Online Supplementary Table S4*). Notably, there was no significant correlation between the presence of CNA/CNLOH and the mutational profile.

Of utmost importance for the clinical relevance is whether the presence of chromosomal lesions translates to more precise prognostication in ICUS patients. Median follow-up time was 25 months (range, 2-114 months). No patients were lost to follow-up, the only censoring was administrative at the study cut-off date. Twenty patients had a follow-up BM investigation performed for suspected MDS. A total of 15 of 153 patients (10%) progressed to myeloid malignancy (MDS, n=12; AML, n=2; chronic myelomonocytic leukemia, n=1), and 35 died (23%) of any cause. All patients but one who progressed carried somatic mutation(s).

Survival of ICUS patients with CNA/CNLOH was significantly shorter than of patients without CNA/CNLOH for overall survival (OS) (median, 67 *vs.* 104 months; *P*=0.039; hazard ratio [HR]=2.26; 95% Confidence Interval [CI]: 1.04-4.93), and progression-free survival (PFS) (median, 49 *vs.* 66 months; *P*=0.04; HR=2.07; 95%CI: 1.04-4.14) (Figure 1A and B; *Online Supplementary Table S5*). In multivariable analysis adjusting for age, sex, severe anemia, mutational status and smoking, CNA/CNLOH were not associated with adverse outcomes in ICUS patients (Figure 1C; *Online Supplementary Figure S6; Online Supplementary Table S5*).

The presence of somatic mutation(s) was significantly associated with inferior PFS (HR=2.40; 95%CI: 1.30-4.43; *P*=0.004), but not OS (HR=1.60; 95%CI: 0.82-3.16; *P*=0.2) in univariable analysis. Somatic mutation(s) was a borderline significant independent factor for inferior PFS (adjusted HR=2.03; 95%CI: 0.97-4.26; *P*=0.06) (*Online Supplementary Figure S6*).

When we analyzed CCUS patients (n=64) as a subgroup the adverse effect of CNA/CNLOH on OS was more pronounced than in the entire study population of ICUS non-clonal and CCUS patients. After a median follow-up of 24 months (range, 2-109 months), median OS was 67 months in the group of CCUS patients with CNA/CNLOH compared with 104 months in the group of CCUS patients without CNA/CNLOH (*P*=0.013) (Figure 2A). The corresponding HR was 3.22 (95%CI: 1.22-8.51) for all-cause mortality in CCUS patients with CNA/CNLOH. Notably, also in multivariable analysis the presence of CNA/CNLOH in CCUS patients conferred a more than three times higher hazard of death, which was borderline significant (adjusted HR=3.56; 95%CI: 0.97-13.15; *P*=0.056) (Figure 2B). Interestingly, this increased mortality hazard was not driven by progression to overt myeloid malignancy as an association with PFS was less evident (*Online Supplementary Figure S7*).

On the other hand, in a separate analysis of the patients with non-clonal ICUS, the presence of CNA/CNLOH was not associated with shorter survival (*Online Supplementary Figure S8*).

CCUS patients with CNA/CNLOH had a significantly higher variant allele frequency of somatic mutations than CCUS patients without CNA/CNLOH (median 36% *vs.* 24%; *P*=0.039) and were more likely to have LOY (*P*=0.035) and macrocytosis (*P*=0.022) (*Online Supplementary Table S6*). There was no significant difference between the two CCUS groups with respect to age, sex, adverse mutations, number of mutations, smoking or hematological parameters.

Multiple studies have demonstrated worse survival of patients with myeloid malignancies harboring cryptic chromosomal lesions compared with patients without cryptic lesions.[3,4] Akin to our findings, the impact of CNA/CNLOH was generally more pronounced on OS than PFS, and SNP-A improved prognostic stratification in primarily lower-risk MDS patients including patients with a normal karyotype.[7,8]

To our knowledge, only three smaller previous studies have reported on CNA/CNLOH in patients with ICUS/pre-MDS with frequencies at 15-32%.[9–11] However, their study designs did not enable distinction between ICUS non-clonal and CCUS or correlation to clinical outcomes.

SNP-A in large patient cohorts from genome-wide association studies including healthy controls showed that the frequency of mosaic CNA/CNLOH in peripheral blood increases to approximately 2-3% for age >75 years.[12] Even when only considering clonal mosaicism (*Online Supplementary Table S3*), the frequency of autosomal mosaic CNA/CNLOH was 2-3-fold higher in our study population. This suggests that the loss of chromosomal integrity found by SNP-A in the ICUS patients was related to their disorder, rather than their advanced age.

Our study has certain limitations. Firstly, follow-up was relatively short given the cohort size and the life expectancy of ICUS/CCUS patients. This may have influenced the lack of statistical significance in multivariable analysis. Obviously, our findings need validation in an independent cohort.

Secondly, DNA from BM was not available for SNP-A in all patients, hence, granulocytes from peripheral blood were the source of DNA in these cases (*Online Supplementary Methods*). We considered this feasible as previous studies have shown a high concordance (95%) for SNP-A karyotype between peripheral blood and BM as also seen for somatic mutations.[8,13]

Thirdly, germline DNA was not available as matched DNA reference to allow definitive distinction between acquired and constitutional aberrations. Some aberrations, especially small CNA, appeared to be fully clonal (i.e., not mosaic) (*Online Supplementary Table S3*) and therefore could be germline variants potentially predisposing to disease development. However, extensive MDS-associated aberrations and LOY were also present in a fully clonal state, as observed previously.[14] Furthermore, it has been demonstrated that large (≥25 Mb) and/or telomeric CNLOH do not require verification as they do not occur in non–clonal control DNA.[7,15]

Finally, due to the scarcity of surplus sample material we were unable to proceed with sequencing of myeloid malignancy-associated genes that were not included in our 20-gene panel and were found to be affected by deletion or CNLOH in a subset of patients (Table 1B). Sequencing of these genes was compelling as regions of acquired CNLOH may pinpoint homozygous loss of tumor suppressor genes or oncogenes with homozygosity of mutations.[3,15]

Besides these limitations, our data document that additional structural aberrations detected by SNP-A may influence the variability in the clinical course among CCUS patients and distinguish patients with a markedly worse OS. By contrast, in the group of ICUS non-clonal patients, CNA/CNLOH had no impact on survival. Newer technologies such as whole-genome sequencing, capable of simultaneously detecting mutations and CNA, are increasingly being used in the diagnostic setting. We believe our study emphasizes the importance of the compound analysis of mutations and structural aberrations in CCUS patients.

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