## Heterogenous mutation spectrum and deregulated cellular pathways in aberrant plasma cells underline molecular pathology of light-chain amyloidosis

Light-chain (AL) amyloidosis (ALA) is a rare but fatal monoclonal gammopathy (MG) causing organ and tissue damage resulting from the deposition of misfolded immunoglobulin free light chains in the form of amyloid fibrils.[1] In some cases, ALA coexists with multiple myeloma (MM) (ALA+MM), which is the second most common blood cancer and is caused by the proliferation of clonal plasma cells (PC).[2] Due to insufficient knowledge of ALA and ALA+MM biology, therapeutic options have mirrored treatment regimens of MM, which focus on the elimination of clonal PC.[3,4] We investigated the mutation and gene expression profiles in clonal aberrant PC (aPC) in order to better understand ALA and ALA+MM etiology and to clarify the molecular differences between individual MG diagnoses.

In order to address this, we analyzed 14 newly diagnosed (untreated) histologically proven ALA samples, 11 ALA+MM samples and 37 MM samples. All ALA+MM and MM samples manifested at least one myeloma-defining event. We isolated DNA and/or RNA from clonal bone marrow PC sorted using CD45-PB, CD38-FITC, CD19-PECy7 and CD56-PE fluorescent antibodies. Samples of frozen aPC from 12 ALA, 10 ALA+MM and 29 MM were subjected to whole genome amplification (WGA) using REPLI-g Mini Kit (Qiagen) employing multiple displacement amplification. Amplified DNA and DNA from peripheral blood (for exclusion of germline variants) served for exome library preparation and sequencing (median coverage 56x, *Online Supplementary Table S3*). RNA was transcribed from six ALA, four ALA+MM and eight MM newly diagnosed patients and hybridized on GeneChip Human Gene 1.0 ST Array. Detailed methods and patient's characteristics are presented in the *Online Supplementary Appendix* and the *Online Supplementary Tables S1, S8*. Four ALA and three ALA+MM samples were used for both the exome and the transcriptome analysis in parallel.

We evaluated only single-nucleotide variants (SNV) due to the potential bias in indels and copy number variations (CNV) introduced by the WGA method.[5] Median numbers of non-synonymous exonic SNV that passed "effect" filters for ALA, ALA+MM, MM cohorts were 16.5, 20.5 and 23, respectively, and the mutation burden was similar across all three cohorts, median 1.36 SNV per megabase (Mb) (range: 0.28-5.86) (Figure 1A, *Online Supplementary Table S2*). The mutation burden did not significantly correlate with age in ALA or MM. For ALA+MM the test was not performed due to the lack of age information of some patients. The intra-sample analysis of clonality was performed in samples exceeding 30 unfiltred non-synonymous SNV and coverage ≥10X in copy number neutral regions. The results were available for 10 ALA, nine ALA+MM and 29 MM patients. Of those, only one clone was observed in one ALA, two ALA+MM, and three MM samples (*Online Supplementary Table S1*). MM samples in our analysis were composed of a higher number of subclones when compared to ALA and ALA+MM, though the difference was not statistically significant (Figure 1B). The median number of subclones for ALA and ALA+MM remained four subclones per sample, while the median for MM was five subclones per sample.

The total pool of mutated genes consisted of 209 genes for ALA, 191 for ALA+MM and 682 for MM (*Online Supplementary Tables S4-S6*). An overlap of gene sets among diagnoses is provided in Figure 1C. Heterogeneity of mutated gene profiles could be observed among all studied cohorts. Pairwise comparisons showed that only six mutated genes (*FAT3, MUC3A, MUC6, PABPC3, RYR3, ZDHHC11*) were present in at least one sample in all three diagnoses. These genes code for large proteins and possess a medium or high gene damage according to the gene damage index score, which points to their polymorphic nature in the normal population. Thus, variants in those genes are unlikely to cause disease,[6] however, the role of some those genes in MM, e.g., *FAT3*, is still debated.[7]

We identified more genes shared between ALA+MM *versus* MM (25) than in ALA *versus* MM (14) or ALA *versus* ALA+MM.[6] This suggests a slightly more similar mutation profile between ALA+MM and MM. From the list of all 209 ALA mutated genes, only 26 genes were shared with a previous sequencing project of Boyle *et al.* 2018[8] and four mutated genes were in common with the original study by Paiva *et al.* 2016[9] (*Online Supplementary Table S7*). Such low gene set overlaps are in line with the assumed mutational heterogeneity in ALA.

Our datasets were not large enough to perform analysis of significantly mutated genes. Within the ALA, ALA+MM and MM cohort, genes mutated in more than one patient represented only 2, 6 and 47 genes (1%, 3% and 6.9%), respectively (Figure 1C). Such a marked heterogeneity was also detected in previous ALA exome studies.[8,9] However, Boyle's work reported that 16% of the genes in ALA were mutated more than once.[8] The observed difference can be explained by the different approach for the separation of target populations of cells and by different variant calling algorithms.

We performed comparison of all mutated genes with the 63 known MM drivers obtained from Walker *et al.* 2018.[10] The results revealed that the average number of drivers per patient was lower in ALA and ALA+MM *versus* MM, even though the differences were not significant (Figure 1D). The total number of drivers present in the entire set of samples (ALA, ALA+MM and MM) was 28 (Figure 1E, *Online Supplementary Table S2*). The only shared mutated drivers among the diagnoses were *NRAS* found in ALA+MM and MM, and *DIS3* present in ALA and MM. Interestingly, ALA and ALA+MM did not possess any SNV in the same driver gene (Figure 1E). Previously identified ALA drivers *DIS3* and *EP300* overlap with our ALA dataset and *NRAS* and *TRAF3* overlap with our ALA+MM dataset[8] (*Online Supplementary Table S7*). The most frequent functional driver categories were epigenetic regulators in ALA, NF-κB pathway in ALA+MM and MEK/ERK pathway in MM (Figure 1E). Interestingly, the NF-κB pathway was previously suggested to be one of the main affected pathways for ALA.[8]

Surprisingly, the differences at the mutational level did not manifest at the gene expression level. Our analysis did not reveal any differentially expressed genes between ALA and ALA+MM despite using several thresholds of significance. The expression analysis yielded 783 deregulated genes (837 probe sets) on the level of 0.05 P-value and fold change above 2 or below 0.5 in ALA or ALA+MM compared to MM (*Online Supplementary Table S9-S10*).

Genes uniquely upregulated in ALA fall into the regulation of B-cell activation, phagocytosis or regulation of protein localization pathway gene ontology (GO) terms (Figure 2A, *Online Supplementary Table S11*), while genes that were exclusively downregulated in ALA belonged to the mitochondrial translation and ribosome biogenesis
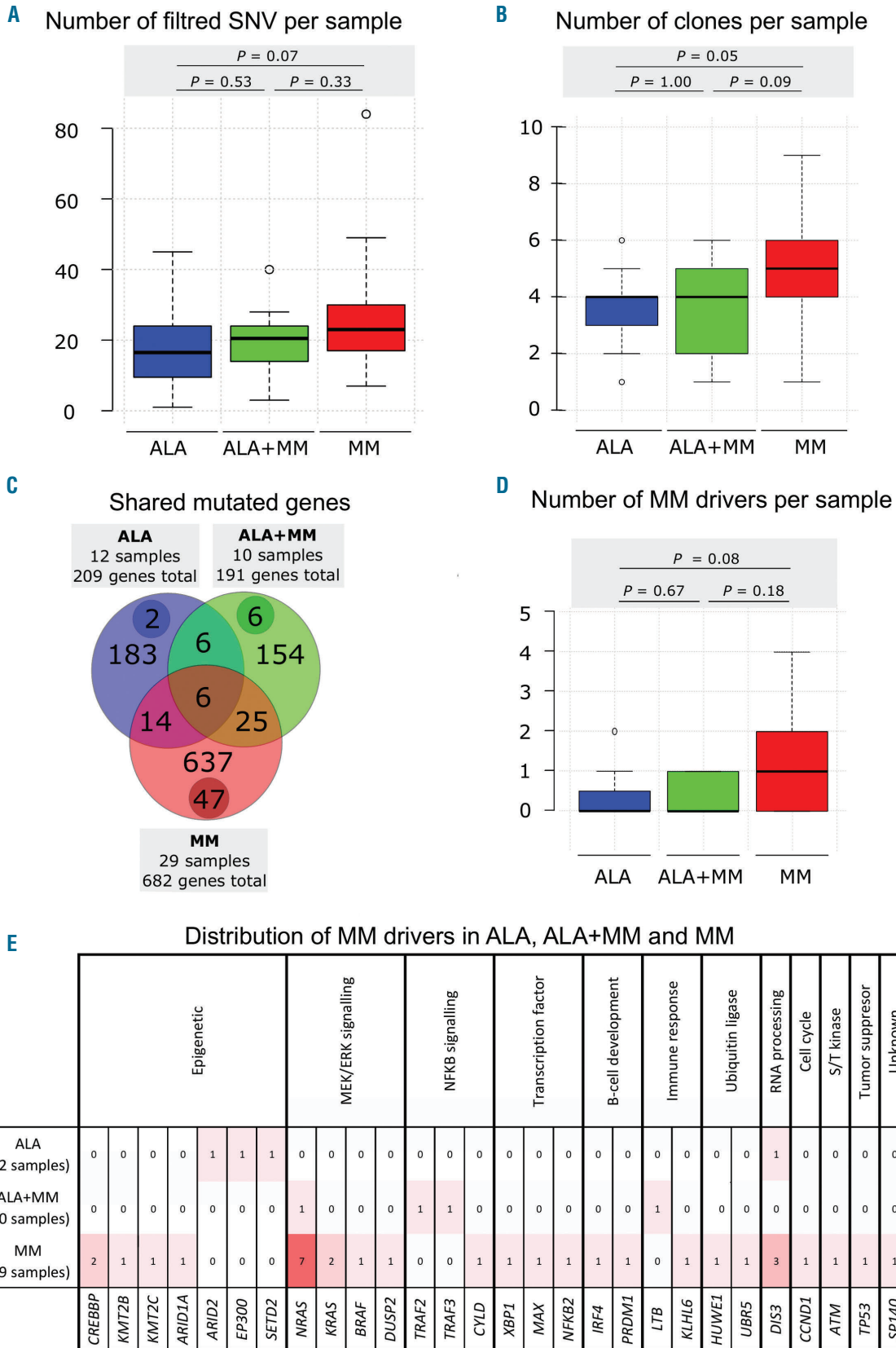
**Figure 1. Profiling of somatic variants.** (A) Distribution of numbers of filtered single nucleotide variants (SNV) per sample in light-chain amyloidosis (ALA), ALA plus multiple myeloma (ALA+MM) and MM. (B) Distribution of subclones in ALA, ALA+MM and MM. (C) Overlap of sets of mutated genes in ALA, ALA+MM and MM (large circles); numbers of genes mutated in more than two samples within ALA, ALA+MM and MM (small circles). (D) Distribution of MM drivers per sample in ALA, ALA+MM and MM. (E) Heat-map of occurrence of mutated drives in each cohort. Cohort sizes are given in brackets. The assignment of drivers into functional categories was performed according to Walker *et al.*[8]; ST kinase: serine/threonin kinase.

**A** Upregulated genes
- comparison with MM

response to endoplasmic
reticulum stress[4]

ALA        ALA+MM

regulation of B-cell activation[1]
phagocytosis, engulfment[2]
humoral immune response
mediated by circulating Ig[3]

217    97    52

not significant

**B** Downregulated genes
- comparison with MM

mitochondrial RNA metabolism[7]

ALA        ALA+MM

mitochondrial translation[5]
ribosome biogenesis[6]
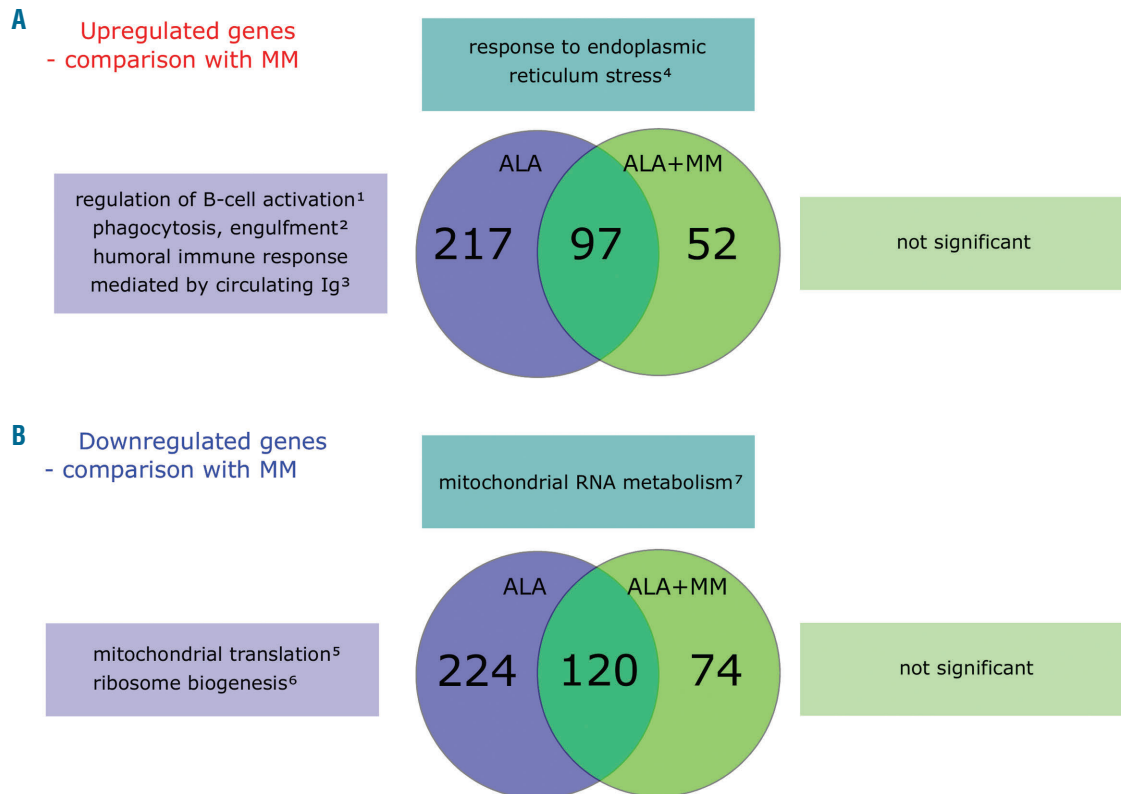
224    120    74

not significant

**Figure 2. Gene Ontology (GO) analysis of genes differentially expressed in light-chain amyloidosis (ALA) and ALA plus multiple myeloma (ALA+MM) *versus* MM diagnoses and assigned into the category "biological process".** (A) The overlap of upregulated genes in ALA and ALA+MM. The top upregulated pathways are provided for the colored intersections. (B) The overlap of downregulated genes in ALA and ALA+MM. The top downregulated pathways are provided for the colored intersections. [1]$P$=0.000002, [2]$P$=0.000283, [3]$P$=0.00078, [4]$P$=0.000011, [5]$P$=0.001867, [6]$P$=0.04333 and [7]$P$=0.02736.

GO terms (Figure 2B, *Online Supplementary Table S11*). The small number of deregulated genes between MM and ALA+MM only, did not allow for identification of pathways uniquely affected in these entities (Figure 2A-B, *Online Supplementary Table S11*).

Although our dataset was relatively small (18 samples), we detected a specific pattern of gene expression common in ALA and ALA+MM *versus* MM. Interestingly, the expression profiles and pathways specific for ALA/ALA+MM pointed to downregulation of genes involved in mitochondrial RNA metabolism and translation (Figure 2, *Online Supplementary Table S11*). Ribosomal deregulation in ALA was previously indicated by Kryukov *et al.*[11] in 2016, however, our set of ribosomal proteins is involved in the function of mitochondrial rather than cytoplasmic ribosomes. We can speculate that downregulation of mitochondrial translation is a compensatory mechanism for increased stress. Downregulation of mitochondrial RNA to avoid oxidative stress was described by Crawford *et al.*[12] in 1997 and oxidative stress as well as endoplasmtic reticulum stress were found to be elevated in ALA *versus* MM PC.[13]

One of the most important aims and biological questions of our study was to characterize the mutation and subclonal profile of ALA+MM and determine whether it is more similar to ALA or MM. In order to answer this question, we first defined similarities at the level of the somatic variants. More mutated genes were shared between ALA+MM and MM. On the other hand, there were more similarities in the number of subclones between ALA and ALA+MM compared to MM. This observation is supported by a clonality study using cyto-

genetic methods.[14] Results of Bochtler *et al.*[14] demonstrated that all PC dyscrasias containing amyloid deposits share less clones compared to non-ALA counterparts. Furthermore, we compared the gene expression levels to gain additional insights into ALA+MM. Surprisingly, transcription profiles of ALA and ALA+MM were indistinguishable, while MM was a clearly separate entity, but still closer to ALA+MM than to ALA.

Based on these results, we conclude that ALA+MM share a mutation profile which is more similar to MM, but these changes were not manifested on the gene expression level, or on the level of plasma cell infiltration. The typical myeloma symptoms present in ALA+MM may thus be caused by mechanisms other than the global expression profile of aPC.

Our detailed study of ALA diseases represents an important step towards improved understanding of their genetic and transcriptomic background, which is a perquisite for development of optimal treatment strategies in the future.

All sequencing reads mapped on the reference genome are deposited in European Genome-phenome Archive (EGA) with accession code EGAS00001004214. The gene expression data-matrix is available in the *Online Supplementary Table S10*.

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*Contributions: ZC and TS contributed equally to this manuscript , processed nucleic acid samples, designed experiments, prepared the figures and wrote the manuscript; PV, JP, LB, EB, AM and MMo performed bioinformatic and statistical analysis; KG, JF and MZ performed cell sorting and nucleic acid isolation; TJ, LP, MS, SG, WWJ, AWG, AJ, AB, MH, LH, MMi, GM, PR, JD, AJ, JS and AC diagnosed patients, collected samples of peripheral blood and bone marrow and provided patient's clinical data; GM provided the multiple myeloma exome data; FK designed the study and organized the initial sample collection and experiments; RH designed the study and managed the team; MS, MH and TJ designed experiments and wrote the manuscript. All authors have commented on and proofread the manuscript.*

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