Surface IgM expression and function associate with clinical behavior, genetic abnormalities and DNA methylation in CLL

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Key point 1: High surface IgM level is a potential key factor associated with poorer clinical outcome in CLL

Key point 2: Genetic and epigenetic features influence IgM levels and function in CLL.

ABSTRACT

Chronic lymphocytic leukemia (CLL) with unmutated (U-CLL) or mutated (M-CLL) *IGHV* displays different states of anergy, indicated by reduced surface Immunoglobulin M (sIgM) levels and signaling, consequent to chronic (super)antigen exposure. The subsets also differ in the incidence of high-risk genetic aberrations and in DNA methylation profile, preserved from the maturational status of the original cell. We focused on sIgM expression and function, measured as intracellular Ca^{2+} mobilization following stimulation, and probed correlations with clinical outcome. The relationship with genetic features and maturation status defined by DNA methylation of an 18-gene panel signature was then investigated. SIgM levels/signaling were higher and less variable in U-CLL than in M-CLL and correlated with disease progression between and within U-CLL and M-CLL. In U-CLL, increased levels/signaling associated with +12, del(17p) or NOTCH1 mutations. In M-CLL, there were fewer genetic lesions, while the methylation maturation status, generally higher than in U-CLL, varied and was increased in cases with lower sIgM levels/signaling. These features revealed heterogeneity in M-CLL and U-CLL with clear clinical correlations. Multivariate analyses with phenotype, genetic lesions, or DNA methylation maturation status identified high sIgM levels as a new potential independent factor for disease progression. Multiple influences on sIgM include the cell of origin, the clonal history of antigen encounter *in vivo* and genetic damage. This simple marker compiles these different factors into an indicator worthy of further investigations for prediction of clinical behavior, particularly within the heterogeneous M-CLL subset.

INTRODUCTION

The B-cell receptor (BCR) is the essential functional unit for most normal and neoplastic B-cells.[1, 2] In chronic lymphocytic leukemia (CLL), it is key to survival and proliferation, and is now a therapeutic target of very effective inhibitors of BCR-associated kinases, including SYK, BTK, or phosphoinositide 3'-kinase isoform p110δ (PI3Kδ).[1-6]

The molecular characteristics of the tumor sIg indicate that CLL consists of two major subsets. The one with unmutated (U) Ig gene heavy-chain variable regions (*IGHV*) has arisen from pre-germinal center $CD5^+$ B-cells and the subset with mutated (M) *IGHV* has arisen from post-follicular $CD5^+$ B cells which have undergone somatic hypermutation.[7-9] The nature of the B-cell of origin clearly influences tumor behavior, with U-CLL having a worse prognosis than M-CLL.[10, 11]

The functional characteristics of the tumor sIgM indicate that circulating CLL cells from both subsets are characterized by a degree of anergy. This is defined by variably reduced sIgM, but not sIgD, levels and signaling capacity consequent to chronic (super)antigenic exposure.[1, 2, 12] Analysis of a small cohort of patients indicated that the two subsets differ in mean sIgM levels/signaling capacity, being higher in U-CLL cells than in the more anergized M-CLL.[13-18] The outcome may be that U-CLL cases have a larger proliferative component than M-CLL, explaining more rapid tumor progression in U-CLL.[1, 2, 15] Consistently, U-CLL appear to respond more profoundly to BCR-associated kinase inhibitors (BI) than M-CLL,[19] while duration of the lymphocytosis in the circulation appears more prolonged in M-CLL than U-CLL.[20] However sIgM expression and function is heterogeneous between patients,[13] and the clinical meaning of this heterogeneity has not yet been examined.

DNA methylation is emerging as another key to variable CLL behavior.[21-26] Genome-wide DNA methylation studies have uncovered two major methylation subtypes along with a third intermediate group.[23, 25, 27] These groups largely represent the degree of epigenetic programming experienced by the B-cell of origin in CLL, and are termed low, intermediate and high-programmed CLLs (LP-, IP- and HP-CLLs, respectively).[23, 27] Maturation of DNA methylation patterns is generally concordant with the degree of *IGHV* mutation, with LP- and HP-CLLs mostly composed of U- and M-CLLs, respectively.[25, 27] Variability exists particularly within M-CLL,[25] potentially reflecting a range of maturation of the cell of origin.[27] A 'methylation maturation score' using a panel of selected gene regions has been shown to efficiently represent overall maturation,[27] further dividing M-CLL into at least two subcategories (IP- and HP-CLL) with different clinical behavior.[23, 25, 27]

Genetic alterations also influence outcome, response to (immuno)chemotherapy and clonal evolution.[28-31] Particularly those associated with poorer outcome are markedly enriched in U-CLL.[32-35] Trisomy 12 (+12), 11q(*ATM*) deletion (del(11q)) and 17p(*TP53*) deletion (del(17p)) stratify CLL patients into separate prognostic categories with different survivals,[33] and integration with mutations including *NOTCH1* ΔCT_7544-7545

(*NOTCH1*$_{\Delta CT}$) or those of *SF3B1* help further refine prognosis.[33] Also, although BIs seem dramatically effective in all genetic categories including del(17p),[36] supporting a dominant role of BCR signaling over genetics for clinical efficacy,[37] BI-induced tumor lymphocytosis appears shorter in +12,[20, 38] opening the question if there is any link between specific genetic lesions and BCR characteristics.

In this study, we investigated the links between anergy, deduced global DNA methylation and genetics in CLL. We confirmed that increased sIgM associated with more rapid progression and inferior survival, and revealed heterogeneity in M-CLL. We also showed a correlation with a more aggressive genetic profile particularly in U-CLL. Within M-CLL, a strong inverse correlation was found between IgM and DNA methylation maturation, indicating that profound anergy is associated with a more mature profile.

MATERIALS AND METHODS

CLL patients and samples

The study included samples from a series of 270 consecutive patients with previously untreated sIgM$^+$/D$^+$ CLL recruited in the LPD study at time of initial evaluation at the Department of Hematology of the Southampton University Hospital Trust from January 2001 to May 2015. Diagnosis of CLL was according to the iWCLL2008/NCI criteria and confirmed by a flow cytometry 'Matutes score' >3 in all cases.[39, 40] For clinical association studies, 235 CLL patients with full IgM/D levels/signaling analysis and clinical history with a minimum follow up of 12 months were studied **(Supplementary Table S1)**. Median follow-up of patients that were alive was 99 months. No patients were lost at follow-up. The LPD study was approved by the Institutional Review Boards at the University of Southampton (228/02/t). All patients provided informed consent prior to inclusion in the study.

Phenotypic, signaling capacity and immunogenetic studies.

Peripheral blood mononuclear cells (PBMC) were prepared and stored in liquid nitrogen. Prior to each assay, cells were thawed, washed and allowed to recover in complete RPMI1640 medium (supplemented with 10% fetal calf serum, 2 mM glutamine, and 1mM sodium pyruvate), for 1 hour at 37°C.[41] Phenotypic, signaling capacity and immunogenetic studies were performed using established internal standard operating procedures.[13, 42]

For phenotypic analyses, sIgM and sIgD levels were determined on the CD19$^+$/CD5$^+$ CLL cells using soluble rabbit F(ab')$_2$ phycoerythrin(PE)-conjugated anti-human IgM or fluorescein isothiocyanate(FITC)-conjugated anti-human IgD or control polyclonal antibodies (DAKO, Ely, UK) and Peridinin-chlorophyll-proteins-Cyanine5.5(PerCP-Cy5.5)-conjugated anti-CD5 and allophycocyanin(APC)-conjugated anti-CD19 monoclonal antibodies (Biolegend, London, UK). Surface staining was carried out on 1x10^6 PBMC in 100µl FACS buffer on ice for 30 minutes in all cases. Cells were washed in 2 ml FACS

buffer and resuspended in 300µl FACS buffer and total 10000 events were acquired before analysis. Expression of CD38, ZAP70 and CD49d was analyzed as reported.[43] Cutoff points for CD38, ZAP70 and CD49d positivity were 30%, 20% and 30%, respectively. Mean fluorescence intensity (MFI) was calculated as [Test antibody GeoMean-Control antibody GeoMean] for all markers included in the study.

Signaling capacity was measured as percentage intracellular Ca^{2+} [iCa^{2+}] mobilization following stimulation.[13] Briefly, 10^7 PBMC/mL were incubated with 4 µM Fluo3-AM (Invitrogen, Paisley, United Kingdom) and 0.02% (vol/vol) Pluronic F-127 (Sigma, Poole, UK) for 30 minutes at 37°C. Cells were then washed and resuspended at 5×10^6 cells/mL at room temperature, warmed to 37°C for 5 minutes prior to acquisition for 35 seconds to obtain the background fluorescence (unstimulated cells). Following addition of 20 µg/mL goat $F(ab')_2$ anti–human IgM or IgD (Southern Biotechnology, Cambridge, UK) or control antibodies. Data were acquired for 10 minutes. Maximum Calcium release was observed within the first 2 minutes in all circumstances. Treatment with 1 µM ionomycin (Sigma) was used to confirm viability of samples and exclude negative artefacts. Percent iCa^{2+} mobilization was calculated as [Peak(all events)-Mean Y(unstimulated cells)/%$CD19^+$cells]x100, where %$CD19^+$cells was the percentage of $CD19^+$ cells in the live lymphocyte gate of the test sample.

A FACScalibur flow cytometer (Becton Dickinson, Oxford, UK) was used for acquisition in all circumstances. Analysis of all phenotypic and signaling profiles was performed and uniformly reviewed by two independent researchers (I.T. and I.H.) using FlowJo software v9.5.2 (Tree Star, Ashland, Oregon).

The full IGHV-IGHD-IGHJ-Cµ Constant region rearrangements were amplified from complementary DNA in all circumstances and directly sequenced bi-directionally using our primers from leader to constant IGHM region.[13, 43, 44] Sequences were aligned to ImMunoGeneTics directories, and considered mutated if homology to the corresponding germ line gene was <98%. Tumors, using IGHG or IGHA rearrangements (which required amplification with a primer specific to Cγ or Cα constant region) and/or expressing IgG or IgA on the CLL cells by flow cytometry, were excluded from the study.

Genetic studies

Interphase fluorescence-in-situ-hybridization (FISH) was performed at the Wessex Regional Genetics Laboratory in Salisbury, using the probes (Vysis) LSI13 and LSID13S319 for del13q14 (del13q), CEP12 for chromosome 12 aneuploidy (+12), LSIp53 for del17p13 (**del(17p)**); and LSIATM for del11q22-q23 (**del(11q)**). *NOTCH1*$_{\Delta CT}$ mutation was sought by ARMS PCR and Sanger sequencing.[45] In the CLL harboring the *NOTCH1*$_{\Delta CT}$, mutated allele frequency was determined by digital droplet PCR (ddPCR).[46] Briefly, 50 ng DNA and 70 µl oil were loaded into a cartridge (Bio-Rad, Milan, Italy) to form 20,000 monodispersed 1 nl surfactant-stabilized droplets per sample. Droplets were

transferred into a 96-well PCR plate (40 μl/well). Droplet PCR amplification was performed using the primePCR ddPCR_mutation_assay_*NOTCH1*_p.P2514fs*4 human kits for wildtype or mutant c.7541_7542delCT (Bio-Rad) and a Veriti DX thermal cycler (Applied Biosystems). Fluorescence of amplified products were read using a QX200 droplet reader (Bio-Rad) and analyzed with a QuantaSoft software (Bio-Rad). Frequency of $NOTCH1_{\Delta CT}$ allele in the tumor population was calculated as *[(*ΔCT droplets/total droplets)*(% CLL cells per sample)]. *SF3B1* mutations were sought by HRM-PCR and confirmed by Sanger sequencing, as previously described.[47]

DNA Methylation studies

Genomic DNA isolated CLL samples was bisulfite-converted using the EZ-DNA Methylation Kit (Zymo Research). Targeted DNA methylation analysis was performed using MassARRAY (Agena Biosciences) by PCR amplification of 18 genomic regions. Briefly, regions were selected from previous genome-wide analyses using Infinium 450K array data that were differentially methylated between CLL samples and, when combined, retained high discriminatory power to separate the three CLL DNA methylation subtypes.[27] To determine LP-, IP- and HP-CLL DNA methylation subtypes, CpG methylation values were averaged per genomic region and then used to subgroup patients by consensus clustering.[27] Reduction of amplicon methylation data to a singular value representing the methylation maturation score (MMS) per sample was calculated by subtracting from 1 the methylation value (range 0-1.0) for amplicons associated with hypomethylation programming and then calculating the mean methylation for all 18 amplicons.

Statistical analyses and clinical association studies

Supplementary Table S1 summarizes the clinical variables recorded at presentation. The clinical variables recorded at follow-up were date of progression requiring treatment for the first time and date of lymphocyte doubling from diagnosis according to the IWCLL2008/NCI guidelines,[40] and date of death. Time to progression requiring first treatment (TTFT), time to lymphocyte doubling (LDT) and overall survival (OS) were measured from date of CLL diagnosis to date of progressive and/or symptomatic disease requiring treatment according to iWCLL2008/NCI guidelines (TTFT),[40] to date of lymphocyte doubling (LDT), or to date of death or last follow-up (OS). TTFT was used as primary endpoint, while OS was used as a secondary endpoint to avoid the chemotherapy and kinase inhibitors as confounders. We used our previous cut-offs of 50 and 5% to distinguish patients with high/low sIgM/D (MFI) or signaling capacity (iCa^{2+} mobilization %), respectively.[13] These cut-offs corresponded to the best cut-offs by ROC and Youden's T-tests when treatment was used as a state variable.

Categorical variables were compared by χ^2 test or Fisher's exact test when appropriate. Continuous variables were compared by Mann-Whitney non-parametric test for 2 or k independent samples. All statistical tests were two-sided. Statistical significance was

defined as p value <0.05. Survival analysis was done by Kaplan-Meier method using log-rank statistics. Multivariate analysis was done by Cox proportional hazard regression. A variable was entered into the model if the probability was ≤0.05. Statystical analyses were performed with the Statistical Package for the Social Sciences (SPSS) software v.22.0 (Chicago, IL) and Graphpad Prism 6 software (La Jolla, CA).

RESULTS

U-CLL and M-CLL have different levels of signal-responsive sIgM

Surface IgM levels and signaling capacity were investigated in 270 CLL patients. Levels of sIgM were broadly variable (range 3-918, median 50, coefficient of variation (CV) 144%), as was sIgM signaling capacity (range 0-100, median 25, CV 87%). A significant correlation was present between sIgM levels and signaling capacity (r=0.55, p<.0001, 95% CI 0.44-0.64).

Levels were less variable and significantly higher (p<.0001, **Figure 1A**) in U-CLL (range 8-781, median 72) than in M-CLL (range 3-918, median 39). Signaling capacity was also variable and significantly different between the two subsets (ranges 8-99, median 43 in U-CLL; 0-100, median 17 in M-CLL, p<.0001, **Figure 1B**). These data validated previous findings in a separate smaller cohort of patients.[13] Of interest, the subset of CLL using *IGHV3-21* (n=9), which typically have an aggressive course,[48-50] also had significantly higher IgM levels and signaling capacity than non-*IGHV3-21* M-CLL, irrespective of *IGHV3-21* mutational status **(Supplementary Figure S1)**. In M-CLL, there was a significant group of cases with no detectable ability to signal (<5% Ca^{2+} flux) and these correlated with a very low expression indicating a deeply anergic group, not found in U-CLL. In each subset a significant correlation between levels and signaling was maintained, although this was more evident in the M-CLL subset (r=0.38 p=0.0012 in U-CLL, r=0.52 p=0.0001 in M-CLL).

IgD levels (range 2-471, median 33, CV 114%) and signaling capacity (range 1-100, median 52, CV 58%) were less variable and no significant differences were observed between U-CLL and M-CLL **(Supplementary Figure S2A)**, nor the variations showed robust correlations with IgM levels.

Levels of functional sIgM predict progression of CLL

TTFT was used as a primary indicator of natural progression to investigate the role of sIgM in 235 CLL patients **(Supplementary Table S1)**. Both high sIgM expression and high sIgM signaling associated with significantly more rapid progression **(Figure 2A-B)**. *IGHV* status is an independent prognostic factor of progression and this previously unpublished cohort at the CSU confirmed its relevance **(Supplementary Figure S3)**. To understand potential individual relevance of BCR associated characteristics and explore significance of IgM

variability in each U-CLL and M-CLL subset, we initially performed a multivariate Cox regression adjusted for *IGHV* status, sIgM levels and sIgM signaling. This revealed that sIgM levels and signaling predicted progression of CLL in a fashion independent from *IGHV* status **(Table 1)**. Hence, we analyzed sIgM within U-CLL or M-CLL separately **(Figure 2C-F)**. High levels associated with more aggressive behavior within U-CLL or M-CLL **(Figures 2C and 2E).** High and low signaling also separated U-CLL and M-CLL in two categories with different outcome (**Figure 2D and 2F**). The differences were most evident within M-CLL, .of which the subset with high IgM levels/signaling appeared to have a progression apparently as rapid as the U-CLL with low IgM levels/signaling (Supplementary Figure S4). This overlap also highlights the fact that, although the cell of origin has a major influence on tumor behavior, other influences on the BCR can, in a minority of cases, lead to a convergent clinical outcome.

When sIgD was analyzed, no significant different survivals could be documented between CLL with high vs low levels/signaling **(Supplementary Figure S2B)**.

sIgM levels and signaling associate with poor-risk genetic lesions, which are enriched in U-CLL

We then investigated links between IgM features and specific FISH lesions (isolated del13q, +12, del(11q), del(17p), n=189), or mutations of *NOTCH1* (n=220) or *SF3B1* (n=189). U-CLL were enriched for poor-risk genetic lesions compared to M-CLL **(Supplementary Table S1 and Supplementary Figure S5),** as expected.[33]

Analysis of FISH lesions revealed that CLL subsets harboring +12 or del(17p) had significantly higher sIgM levels and signaling capacity than Del13q **(Figure 3A-B).** The associations held even when U-CLL or M-CLL were analyzed separately **(Figure 3C-D)**.

NOTCH1$_{\Delta CT}$ was identified in 13/220 patients. Eleven-of-thirteen CLL with *NOTCH1*$_{\Delta CT}$ were U-CLL. We interrogated the U-CLL cohort for associations of *NOTCH1*$_{\Delta CT}$ status with IgM levels/signaling. Although only mean sIgM level were higher in *NOTCH1*$_{\Delta CT}$ U-CLL**,** *NOTCH1*$_{\Delta CT}$ U-CLL appeared to cluster in two separate groups with high and low IgM levels or signaling capacity **(Figure 4A)**. Subsequent analysis by ddPCR revealed variable *NOTCH1*$_{\Delta CT}$ allele frequency (range 11.4-90.8), and the variability strongly correlated with IgM levels and signaling capacity **(Figure 4B)**.

SF3B1 mutations were found in 12/189 CLL (5/62 U-CLL and 7/127 M-CLL). In U-CLL, no differential IgM levels/signaling were seen, possibly due to significant enrichment with other genetic lesions in U-CLL **(Supplementary Table S1 and Figure S5)**. Conversely in M-CLL, the samples with *SF3B1* mutations (n=7) had higher sIgM levels/signaling if compared to those with isolated del13q or no +12/ *NOTCH1*$_{\Delta CT}$/del(17p) (n=87) (**Figure 4C**).

DNA methylation maturation status inversely associates with IgM levels/signaling capacity in M-CLL

The DNA MMS, which represents the degree of CLL epigenetic maturity, was analyzed in 66 CLL samples, which were selected based on availability of material and tumor cell purity >85%, as previously described.[27] The immunogenetic characteristics, IgM/D level and signaling capacity and genetic characteristics of the individual samples are described in **Supplementary Table S2**. Correlations between MMS and IgM were sought in the 57 CLL with full FISH/genetic details available from this selection **(Supplementary Table S2)**. U-CLL had a lower MMS (range 0.12-0.54, median 0.20) than M-CLL (MMS 0.48-0.85, median 0.68, **Figure 5A**) as expected from previous findings.[27] A lower MMS was found in CLL with high sIgM levels or signaling capacity than in CLL with low IgM levels or signaling capacity, respectively **(Figure 5A)**. The cases with an intermediate MMS (0.5±0.1) were enriched in CLL using *IGHV3-21*, as expected.[23, 25] *IGHV3-21*$^{+}$ CLL were all strong signalers and explained 50% (2/4) of the exceptions with higher MMS in U-CLL. No significant correlations between IgM levels or signaling and methylation maturation were found if U-CLL only were analyzed **(Figure 5B)**. Analysis of M-CLL revealed a strikingly high correlation between maturation of methylation and reduction of sIgM levels ($r=0.67$, $p<0.001$) or signaling capacity ($r=0.71$, $p<0.001$), and the great majority of these M-CLL carried no genetic lesions (**Figure 5B**).

Level of sIgM may be an independent prognostic factor of TTFT in CLL

High sIgM levels associated not only with more rapid TTFT, but also with advanced stage, with ZAP-70≥20%, CD38≥30%, CD49d≥30% and with shorter LDT and shorter OS **(Supplementary Figures S6-S7)**. TTFT was used as endpoint to determine the potential role of sIgM as a clinical prognostic parameter against either known phenotypic, or genetic or methylation risk categories verified by univariate analysis in this cohort **(Supplementary Table S3)**.

When a multivariate analysis adjusted for high sIgM levels and phenotypic markers CD38≥30%, ZAP-70≥20%, high sIgM levels scored as the strongest prognostic parameter of short TTFT **(Table 2**). A multivariate analysis adjusted for high sIgM levels, U-IGHV status and genetic factors (+12/no FISH lesions, del(11q), and del(17p)/*NOTCH1*$_{\Delta CT}$), demonstrated that high IgM levels was also a powerful prognostic factor of TTFT independent from genetics **(Table 3)**.

For clinical correlations with DNA methylation status, analyses were done against the 3 discrete LP-, IP- and HP-CLL categories.[27] All LP-CLL patients grouped into U-CLL (19/23 U-CLL) and were IgM signalers, while M-CLL were either IP-CLL (14/43 M-CLL) or HP-CLL (29/43 M-CLL) with high or low IgM levels/signaling, consistent with the previously described broader methylation heterogeneity in M-CLL than U-CLL **(Supplementary Figure S8)**.[25, 27] Hence we focused on M-CLL with a multivariate analysis adjusted for IgM levels (high vs low) and methylation status (IP vs. HP) to understand relative relevance of

these two parameters specifically in M-CLL. The analysis revealed a similarly independent value of high IgM and IP-CLL status in predicting disease progression, despite the low numbers entered (n=43, **Supplementary Figure S8**).

DISCUSSION

The overall data add new support to the critical role of anergy in CLL. We now observe and report that it is the degree of anergy operating on sIgM, but not on sIgD, which appears to associate most strongly with slower disease progression in patients. This observation reveals intra-subset variability which adds more prognostic information.

Evidence for interaction of CLL cells with putative (super)antigen *in vivo* is provided by the downregulation of sIgM expression in the tumor cells, that can be reversed *in vitro* and during circulation following engagement in tissue sites.[13, 51] In healthy individuals, the natural levels of sIgM in B-cells are higher than in CLL,[52, 53] and in memory B cells are higher than in naive B-cells.[54] This is not reflected in the leukemic counterparts, M-CLL and U-CLL. The reason for this is that expression levels in CLL are modulated by events *in vivo*. The major perturbation is antigen-induced anergy which operates in both subsets but appears more profound in M-CLL.[1] This is the likely determinant of the differential disease behavior between subsets. However, in both subsets the degree of anergy is variable, being especially broad in M-CLL. Dissecting this variability within M-CLL now reveals that the factor which most associates with disease progression is the level of sIgM.

An additional influence on disease progression is genetic damage. Here we learn that sIgM levels/signaling are different in different genetic categories in CLL, suggesting influences also by intrinsic tumor-related factors. This is particularly evident in U-CLL where genetic lesions with inferior prognosis including +12, del(17p) and/or *NOTCH1*$_{\Delta CT}$ associate with higher IgM. CLL cells with loss of *TP53* and *NOTCH1*$_{\Delta CT}$ mutation have a survival and proliferation advantage over those without these lesions.[55] The +12 translocation has also been associated with up-regulation of levels and signaling of integrins including CD49d and CD38.[56] Our data show an association between IgM with CD49d or CD38 levels in both U-CLL and M-CLL.

Global DNA methylation status of CLL cells is closely related to the B-cell-of-origin and is remarkably preserved post-transformation during disease course.[25, 57, 58] The degree of DNA methylation maturity in CLL (as defined by parallel changes occurring in normal B cell development) can be used to conveniently divide CLL into three subgroups across U-CLL and M-CLL namely LP-, IP- and HP-CLL.[25, 27] The highest degree of DNA methylation variability is seen amongst M-CLLs, composed mainly of the HP-CLL methylation subgroup but also includes many IP-CLLs, as highlighted by the methylation maturation score. Analysis of the corresponding sIgM levels now reveals a remarkable inverse relationship between sIgM expression/function and methylation maturation in M-CLL. It appears that cells derived from more mature B cells within the spectrum of M-CLL may be

more susceptible to induction of anergy. This could be intrinsic to the normal counterpart of memory-like B cells. Alternatively, it could be due to the nature of the autoantigens.[1]

An association with DNA methylation maturity and increasingly favorable outcome has been reported and is confirmed here by a significant increase of TTFT in HP- compared to IP-CLLs **(Supplementary Figure S8)**.[23, 25, 27] We now show that both the reduced sIgM and the higher methylation maturation score within M-CLL associated with a slower progression of disease. These results add that not only do IP-CLLs differ from HP-CLLs regarding their correlation with prognostic markers and clinical outcome, but rather simply with sIgM levels and signaling capacity.

Our study highlights the potentially relevant clinical role of IgM levels in CLL progression. In this cohort, 55% patients required chemoimmunotherapy, and 16% of them (of which 75% U-CLL) were treated with inhibitors of BCR pathway-associated kinases that prolonged patients' survivals.[6] Hence we chose TTFT as the primary endpoint of disease progression. Our analysis of IgM levels in separate multivariate analyses with known phenotypic, genetic or methylation prognostic markers of progression revealed the independent role of sIgM levels. Our data highlight the potential utility of IgM to identify those CLL with more aggressive behavior, although their routine use in a clinical setting will require standardization and validation in independent cohorts.

In conclusion, we emphasize the importance of sIgM as an indicator of tumor cell origin and behavior. SIgM levels and function appear to reflect the critical factors operating on CLL in vivo, including genetic damage. Not only does it include features of the cell of origin but it is a meaningful marker of interaction with antigen. This simple marker could now be worthy of further investigations to verify its role to assist prediction of tumor behavior between and within the major subsets, particularly within the heterogeneous M-CLL subset.

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AUTHORSHIP CONTRIBUTION

AD'A, SD, IT, IH performed research and analyzed data. LC, ML, MR-Z and JS contributed to genetic analyses. PWJ contributed to identification and provision of well characterized biological samples. CP contributed to methylation studies. AJS and GP contributed to analysis and interpretation of data. CCO performed, analyzed and interpreted methylation studies and co-wrote the manuscript. FF designed the study, analyzed and interpreted data. FKS and FF wrote the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. Surface IgM expression and signal capacity in CLL subgroups. sIgM expression (MFI) and sIgM signaling capacity (iCa^{2+} %) were analyzed by flow cytometry in 270 patients with CLL. The patient cohort was divided by *IGHV* mutational status to assess (A) sIgM expression and (B) anti-IgM signaling capacity. Horizontal lines indicate mean values. The statistical significance of difference was analyzed using the Mann-Whitney test.

Figure 2. The significance of sIgM levels and signaling for TTFT in CLL. The previous cut-offs of 50 and 5% were used to distinguish patients with high or low sIgM (MFI) or signaling capacity, respectively.[13] These cut-offs corresponded to the best cut-offs by ROC and Youden's T-tests when treatment was used as a state variable. (A) Patients with sIgM expression above (IgM MFI high, dotted line) or below (IgM MFI low, continuous line) the MFI cut-off of 50 were investigated for time to progression from diagnosis to requirement of treatment for the first time (TTFT). (B) Patients with sIgM signaling capacity above (IgM signaler, dotted line) or below (IgM non-signaler, continuous line) the cut-off of 5% were investigated for progression requiring treatment. Association between sIgM levels (C, E) and signaling capacity (D, F) with TTFT was also investigated in U-CLL and M-CLL, respectively. Survival analysis was performed by Kaplan-Meier algorithm.

Figure 3. sIgM levels or signaling and FISH lesions in CLL. CLL samples were investigated for sIgM expression (MFI) and signaling capacity (iCa^{2+} %) by flow cytometry. The CLL patient cohort were divided by FISH lesions according to D□hner hierarchical model. Association between (A) IgM levels, or (B) signaling capacity with FISH lesions was investigated. Association between sIgM levels (C, E) and signaling capacity (D, F) with FISH lesions was also investigated in the U-CLL and M-CLL patient cohort, respectively. Horizontal bars indicate mean values. Statistical analysis were done by comparing isolated del13q versus each other individual FISH category using the Mann-Whitney test (2-tailed, 95% CI). P-values are represented only for statistically significant differences.

Figure 4. sIgM levels or signaling and NOTCH1 or SF3B1 mutations in CLL. Associations or correlations of $NOTCH1_{\Delta CT}$ with sIgM levels or signaling capacity were sought following identification of $NOTCH1_{\Delta CT}$ by ARMs PCR and Sanger sequencing or following determination of $NOTCH1_{\Delta CT}$ allele frequency by digital PCR in the tumor population. (A) Associations $NOTCH1_{\Delta CT}$ with sIgM levels or signaling capacity. (B) Correlation analyses between $NOTCH1_{\Delta CT}$ allele frequency and sIgM expression and signaling was assessed by digital PCR in those cases that scored $NOTCH1_{\Delta CT}$ mutants by ARMS-PCR/sequencing. (C) *SF3B1* mutational status was determined by HRM-PCR and confirmed by Sanger sequencing. Cases with and without *SF3B1* mutation were investigated for association with sIgM expression and sIgM-mediated signaling responses. Horizontal bars indicate mean values. Statistical analyses for associations were performed using the Mann-Whitney test (2 tailed, 95% CI). Statistical analyses for correlations were performed using the Spearman Rank correlation test.

Figure 5. Surface IgM levels or signaling capacity and DNA methylation maturation status in CLL. DNA methylation profiling of 18 selected regions was determined by MassARRAY in 57 CLL samples and a methylation maturation score was calculated. (A) The association between methylation maturation score and *IGHV* status (U-CLL vs. M-CLL), sIgM level status (sIgM MFI high vs sIgM MFI low) and signaling capacity (sIgM high-signaler vs low-signaler) are represented. Horizontal bars indicate mean values. Statistical analysis was performed using the Mann-Whitney test (2 tailed, 95% CI). (B) Correlation analysis between methylation methylation score and sIgM levels and signaling capacity in U-CLL and in M-CLL. Statistical analyses were performed using the Spearman Rank correlation test. In all panels, empty symbols represent CLL with isolated del13q or negative FISH, black filled symbols represent CLL with either +12 or del(11q) or del(17p) or *NOTCH1*$_{\Delta CT}$ or SF3B1 mutation. Square symbols represent *IGHV3-21*$^{+}$ CLL. MMS: methylation maturation score. NS: not significant.

Table 1. Cox multivariate analysis of BCR- associated parameters for time to progression requiring first treatment (TTFT)

	SE	p-value	HR (95% CI)
IgM MFI>50	.215	.000	2.586 (1.698-3.938)
IgM iCa^{2+}≥5%	.292	.045	1.796 (1.013-3.181)
***IGHV*≥98%**	.202	.000	2.476 (1.668-3.675)

Number of cases entered in the multivariate analysis=206; Number of events=112, SE: standard error. HR: hazard ratio. CI: confidence interval.

Table 2. Cox multivariate analysis of high IgM and phenotypic prognostic parameters associated with time to progression requiring first treatment (TTFT)

	SE	p-value	HR (95% CI)
IgM MFI>50	.211	**.000**	2.501 (1.654-3.782)
CD38≥30%	.205	**.011**	1.682 (1.127-2.513)
ZAP-70≥20%	.192	**.003**	1.781 (1.223-2.594)

Number of cases entered in the multivariate analysis=222; Number of events=124. SE: standard error. HR: hazard ratio. CI: confidence interval.

Table 3. Cox multivariate analysis of high IgM and molecular prognostic parameters associated with time to progression requiring first treatment (TTFT)

	SE	p-value.	HR (95% CI)
IgM MFI>50	.223	.015	1.718 (1.109-2.662)
***IGHV*≥98%**	.227	.004	1.917 (1.228-2.993)
+12/FISH neg	.223	.001	2.099 (1.355-3.251)
Del(11q)	.330	.048	1.918 (1.005-3.663)
Del(17p)/*NOTCH1*$_{\Delta CT}$*	.306	.493	1.233 (.678-2.245)

Number of cases entered in the multivariate analysis=184; Number of events=108. *Since no difference in TTFT was observed between patients with *NOTCH1*$_{\Delta CT}$ and del(17p), these lesions were pooled together for multivariate survival analyses (also refer to **Supplementary Table S3**). SE: standard error. HR: hazard ratio. CI: confidence interval.

Figure 1

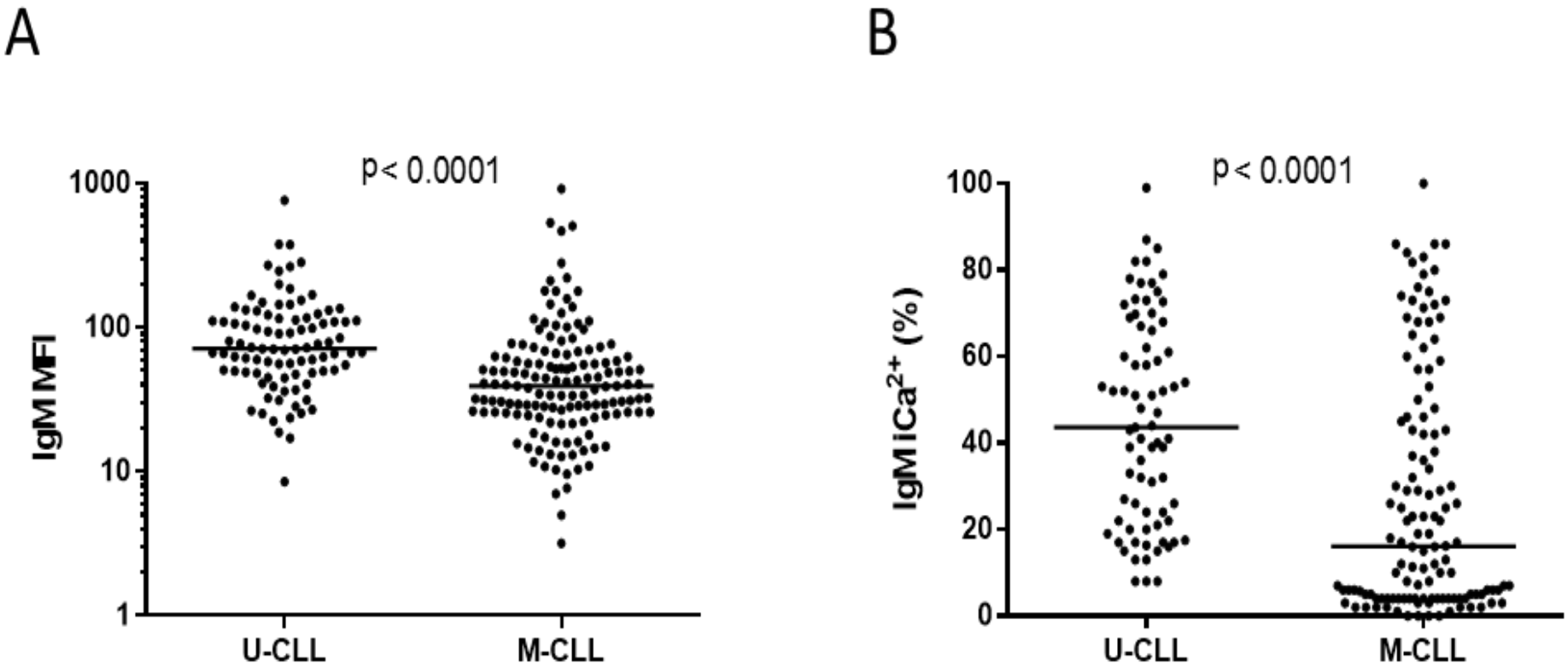
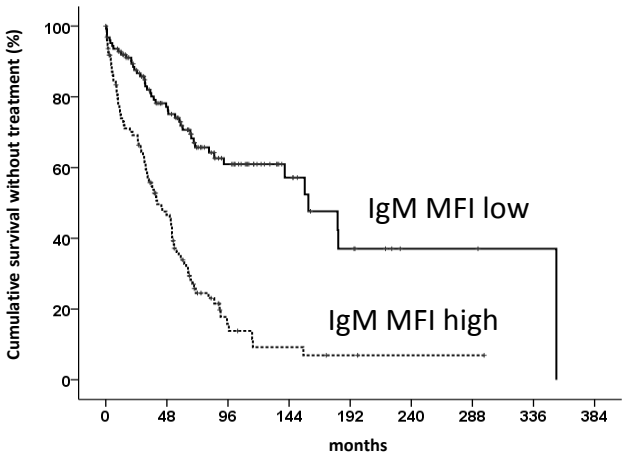

A
p< 0.0001
1000
100
10
1
IgM MFI
U-CLL
M-CLL
B
p < 0.0001
100
80
60
40
20
0
IgM ICa2+ (%)
U-CLL
M-CLL

Figure 2

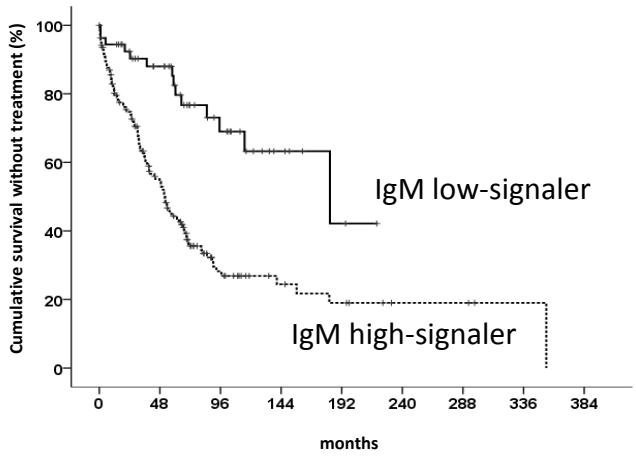
A

All CLL



sIgM MFI	Median months	95% CI	Events	P-value
Low	159	115-202	45/126	<0.001
High	40	26-55	84/109	

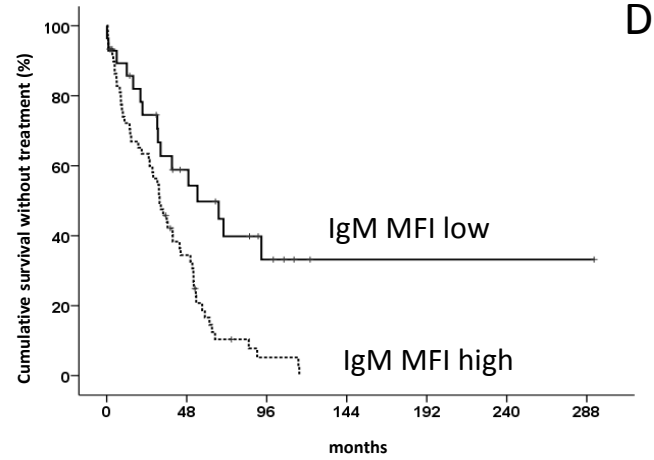
B



sIgM iCa^{2+}	Median months	95% CI	Events	P-value
Low-signaler	182	70-280	14/54	<0.001
High-signaler	52	42-62	99/154	

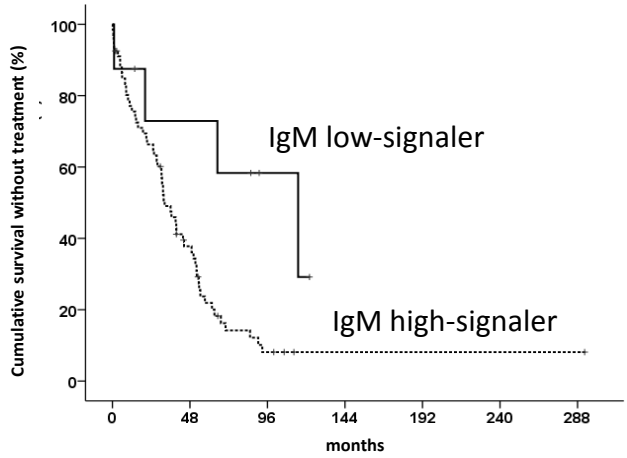
C

U-CLL



sIgM MFI	Median months	95% CI	Events	P-value
Low	55	15-93	16/28	0.001
High	32	23-40	53/60	

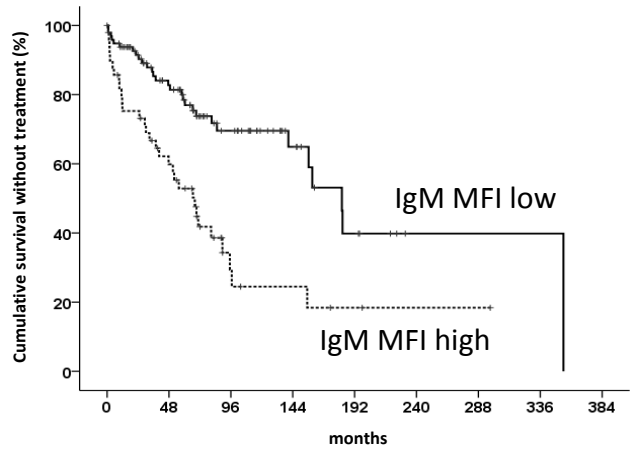
D



sIgM iCa^{2+}	Median months	95% CI	Events	P-value
Low-signaler	115	39-191	4/8	0.025
High-signaler	32	23-42	56/67	

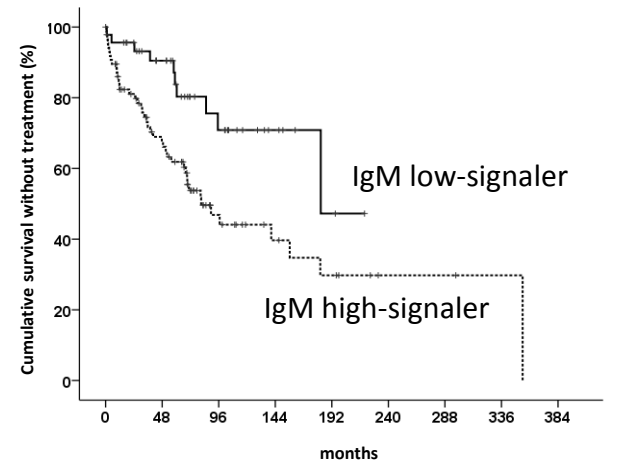
E

M-CLL



sIgM MFI	Median months	95% CI	Events	P-value
Low	182	154-210	29/98	<0.001
High	68	44-92	31/49	

F



sIgM iCa^{2+}	Median months	95% CI	Events	P-value
Low-signaler	183	135-192	10/46	0.005
High-signaler	81	52-110	43/87	

Figure 3

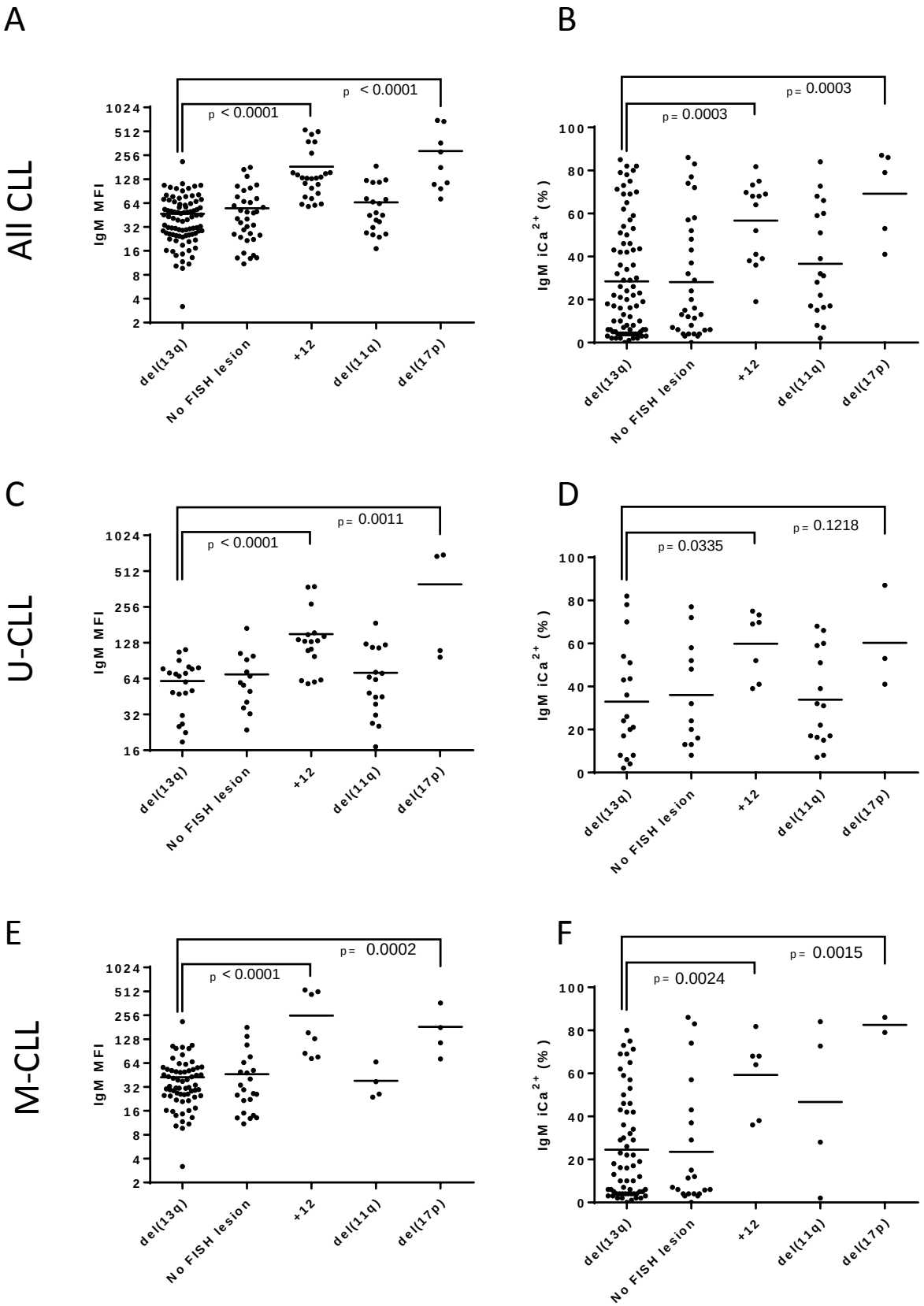

A
All CLL
IgM MFI
p < 0.0001
p < 0.0001
del(13q)
No FISH lesion
+12
del(11q)
del(17p)
B
IgM iCa2+ (%)
p = 0.0003
p = 0.0003
C
U-CLL
p = 0.0011
p < 0.0001
D
p = 0.1218
p = 0.0335
E
M-CLL
p = 0.0002
p < 0.0001
F
p = 0.0015
p = 0.0024

Figure 4

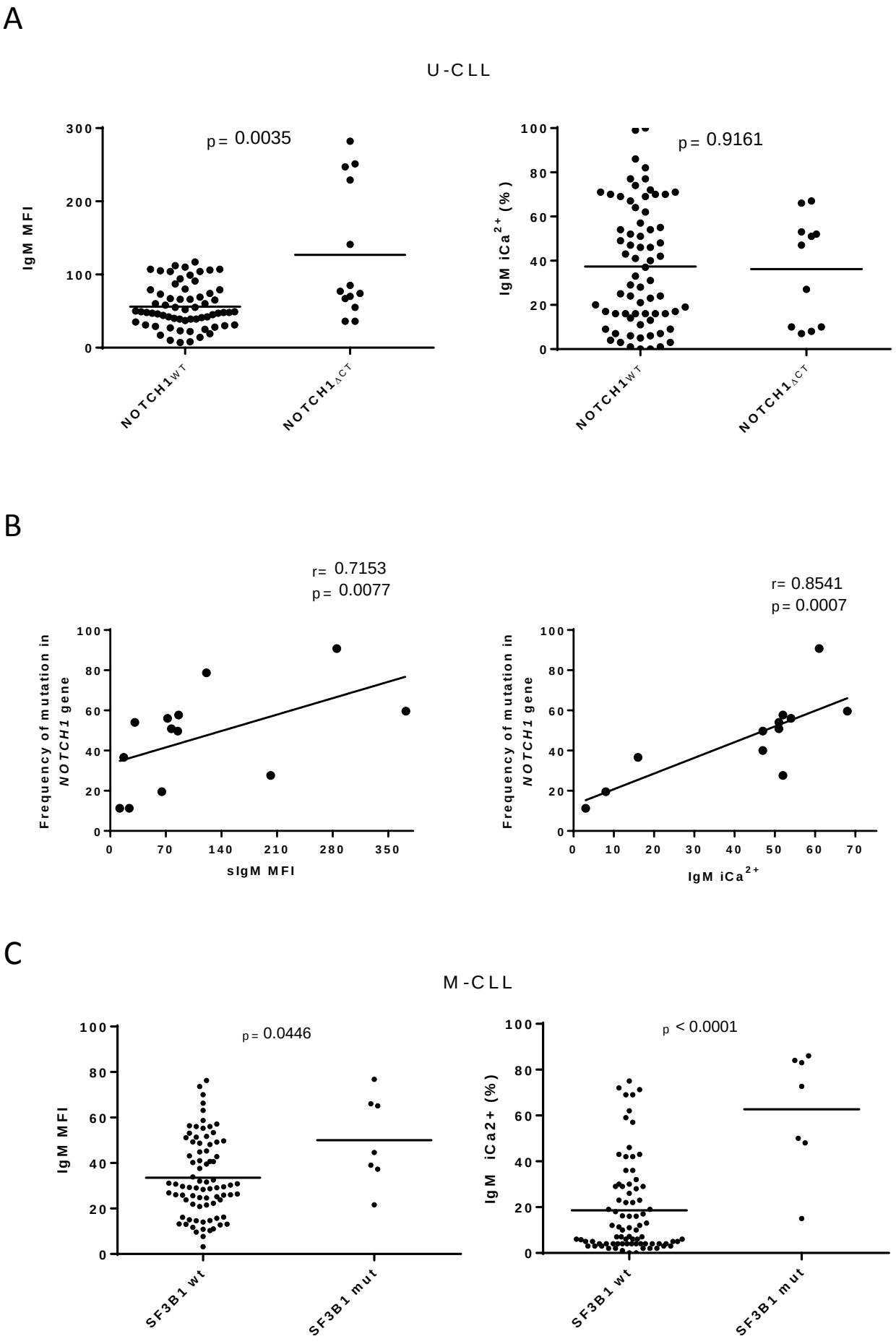
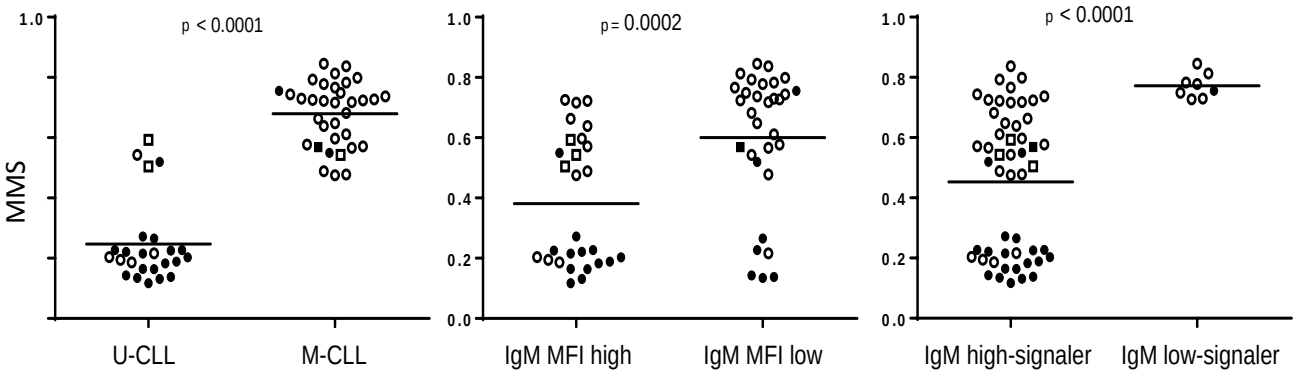
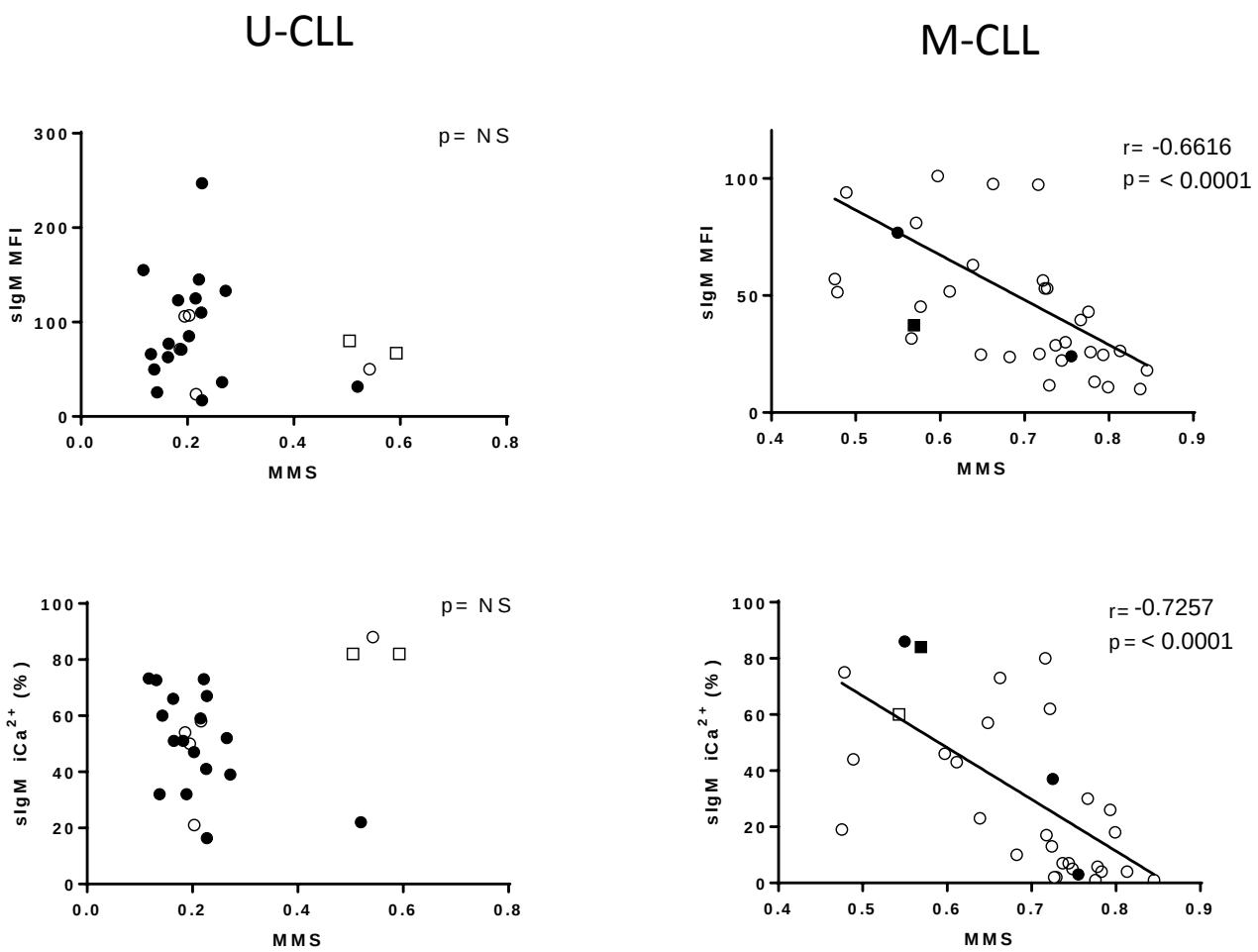

A
U-CLL
IgM MFI
p = 0.0035
NOTCH1WT
NOTCH1ΔCT
IgM iCa2+ (%)
p = 0.9161
NOTCH1WT
NOTCH1ΔCT
B
r= 0.7153
p = 0.0077
Frequency of mutation in NOTCH1 gene
sIgM MFI
r= 0.8541
p = 0.0007
Frequency of mutation in NOTCH1 gene
IgM iCa2+
C
M-CLL
IgM MFI
p = 0.0446
SF3B1 wt
SF3B1 mut
IgM iCa2+ (%)
p < 0.0001
SF3B1 wt
SF3B1 mut

Figure 5

A


MMS
p < 0.0001
p = 0.0002
p < 0.0001
U-CLL
M-CLL
IgM MFI high
IgM MFI low
IgM high-signaler
IgM low-signaler

B


U-CLL
M-CLL
p = NS
r = -0.6616
p = < 0.0001
sIgM MFI
MMS
p = NS
r = -0.7257
p = < 0.0001
sIgM iCa2+ (%)
MMS