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Chapter 6

CXCL13 levels are elevated in patients with Waldenström's Macroglobulinemia, and are predictive of major response to ibrutinib.

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Waldenstrom macroglobulinemia (WM) is characterized by bone marrow (BM) infiltration of monoclonal Immunoglobulin M (IgM) secreting lymphoplasmacytic lymphoma (LPL), and typically presents with anemia. *MYD88* and *CXCR4* activating somatic mutations (*CXCR4*^{MUT}) are common in WM, and found in 90-95% and 35-40% of WM patients, respectively.¹⁻³ Activating mutations in *MYD88* support tumor growth via nuclear factor kappa-light-chain enhancer-of-activated B-cells (NF-κB) that is triggered by IL-1 receptor-associated kinases (IRAK4/IRAK1) and Bruton's tyrosine kinase (BTK).⁴ A distinct transcriptome signature based on *MYD88* and *CXCR4* mutation status has been observed⁵.

Ibrutinib is a BTK inhibitor that is approved for the treatment of WM. Ibrutinib is highly active in WM patients, and responses are impacted by *MYD88* and *CXCR4* mutation status. Patients with *MYD88*^{WT} lack major responses, while those with mutated *MYD88* show decreased response rates, and delayed time to response if carrying a *CXCR4* mutation.^{2,6} Importantly, rapid improvements in hemoglobin levels were observed even in patients with modest or no changes in BM tumor infiltration, suggesting a contributing mechanism in addition to tumor debulking.⁶ Anemia in some WM patients may be related to elevated hepcidin levels produced by LPL cells.⁷ However, the effect of ibrutinib on hepcidin remains unknown. Serum cytokines are important in WM biology and can be produced either by the malignant cells, the surrounding microenvironmental cells, as well as by cells of the immune system.⁸ The anti-tumor effect of ibrutinib may impact all of these compartments, including cytokines that may support growth and survival of tumor cells, and contribute to the morbidity in WM, including anemia.^{9,10} As such, we aimed to characterize the serum cytokine profile in WM patients based on *MYD88* and *CXCR4* mutation status, and to characterize serum cytokine and hepcidin changes in response to ibrutinib therapy.

We first analyzed off-therapy samples of 86 WM patients: 52 previously untreated, and 34 previously treated patients, including 3 mutational subgroups: *MYD88*^{L265P}/*CXCR4*^{WT} (n=45), *MYD88*^{L265P}/*CXCR4*^{MUT} (n=32) and *MYD88*^{WT}/*CXCR4*^{WT} (n=9), including 12 patients with frameshift and 20 patients with nonsense *CXCR4* mutations. Samples from 20 age and gender matched healthy donors (HD) were used for comparison. We then analyzed serial samples of 29 relapsed and/or refractory patients who received ibrutinib therapy on a prospective clinical trial, and for whom baseline and post-treatment serum samples taken one year after initiation of therapy were available.⁶ These included 3 mutational subgroups: *MYD88*^{L265P}/*CXCR4*^{WT} (n=19), *MYD88*^{L265P}/*CXCR4*^{MUT} (n=9) and *MYD88*^{WT}/*CXCR4*^{WT} (n=1). All consecutive patients participating in this trial at the DFCI were included, apart from 10 patients who were missing samples, and 4 patients who went off trial within the first year (see supplementary data). A total of 24 cytokines were analyzed including soluble CD27 as a known biomarker in WM (details in supplementary data). Hepcidin was measured as previously described.¹¹ Iron-related parameters (iron, ferritin, transferrin) were tested at the St. Antonius Hospital, using the Cobas 6000 analyzer. Statistical analy-

sis was performed with R software (R Core Team (2015) R: A language and environment for statistical computing). As previously described, allele-specific PCR was used to detect MYD88L265P and CXCR4 mutations.⁶ Additional Sanger sequencing was performed for CXCR4. Clinical response to ibrutinib at one year was determined using consensus-based response criteria, wherein major response includes complete, very good partial or partial responses and non-major response includes minor response, stable disease or progressive disease.¹² Clinical parameters and cytokine levels were analyzed following log 2 transformation. Correlation was tested using Spearman's rho and when appropriate Holm-Bonferroni multiple-hypothesis correction applied. Adjusted p-values <0.05 were deemed significant. The DF/HCC Institutional Review Board approved the study.

For all 86 WM patients, the median age was 63 (range 31-94 years); median BM infiltration was 58% (range 3-95%); serum IgM level was 3,632 (range 134-7,400 mg/dL); and 64/86 (74%) patients were male. The median age of the HD was 57 (38-80 years), and 15/20 (75%) were male. Compared to HD, CXCL10 (+1.7 fold; $p=0.004$), IL10 (+13.9; $p<0.001$), IL2RA (+4.3 fold; $p<0.001$), CXCL13 (+33.8 fold; $p<0.001$) and sCD27 (+174.1 fold; $p<0.001$) were significantly higher in WM patients, regardless of prior therapy status (**Table 1; Figure 1A**). In WM patients, serum levels of CXCL13, IL2RA and sCD27 strongly correlated with hemoglobin levels with correlation coefficients of -0.55, -0.55, and -0.47, respectively ($p<0.001$ for all comparisons) (**Figure 1B**). Serum CXCL13 ($\rho=0.56$; $p<0.001$) and sCD27 ($\rho=0.46$; $p<0.001$) levels correlated with BM infiltration (**Figure 1C**), whereas no cytokine significantly correlated with serum IgM levels.

We then looked at cytokine differences between mutational subgroups in the WM patients. Although IL6 levels were not significantly different between all WM patients and HDs, significantly higher IL6 levels were observed among *MYD88*^{L265P}*CXCR4*^{WT} patients compared to HD (+1.8 fold; $p=0.001$). Moreover, among *MYD88*^{L265P} mutated patients, IL2RA (+2.2 fold; $p=0.025$), IL1RA (+1.5 fold; $p=0.003$), CXCL10 (+1.5 fold; $p=0.026$) and sCD27 (+1.7 fold; $p=0.016$) were higher in *CXCR4*^{WT} versus *CXCR4*^{MUT} patients. IL1RA ($p=0.011$) and CXCL13 ($p=0.016$) were also lower among *CXCR4* frameshift but not *CXCR4* nonsense mutated patients versus those who were *CXCR4*^{WT}. We found no differences in cytokines between those patients with symptomatic ($n=28$) and asymptomatic ($n=24$) disease in the previously untreated group. All previously treated patients had symptomatic disease.

We next analyzed the samples of 29 previously treated, symptomatic WM patients who participated in a prospective clinical trial with single agent ibrutinib⁶. Twelve of 24 cytokines showed a significant change following one year on ibrutinib (**Figure 2A**). Among these cytokines, TNF- α (-1.6 fold), IL2RA (-2.2 fold) and CXCL13 (-38.2 fold) showed the most significant decrease ($p<0.001$ for all). Of the cytokines evaluated, only baseline CXCL13 was associated with attainment of a major response after one year on ibrutinib: at baseline, CXCL13 was 451.6 fold higher in patients who achieved major responses versus those without a major response ($p=0.049$; **Figure 2B**). Following treatment at

Table 1: Summary of Cytokine Findings in WM patients (all, untreated and previously treated) versus healthy donors

	WM Median Fold Change Relative to Healthy Donors					
	All (N=86)	p-value	Untreated (N=52)	p-value	Treated (N=34)	p-value
<i>TNF</i>	-1,03	NS	-1,27	NS	1,45	NS
<i>IL6</i>	1,38	NS	1,19	NS	1,85	0,0007
<i>IFN</i>	-3,46	NS	-2,78	NS	-4,10	NS
<i>CXCL10</i>	1,66	0,0038	1,47	NS	2,07	0,0001
<i>IL10</i>	13,95	0,0005	11,25	0,0041	16,29	0,0007
<i>CCL2</i>	-1,11	NS	-1,19	NS	-1,06	NS
<i>IL8</i>	1,04	NS	-1,05	NS	1,30	NS
<i>IL1b</i>	-1,77	NS	-2,06	NS	-1,36	NS
<i>IL7</i>	1,00	NS	-1,12	NS	1,09	NS
<i>IL1RA</i>	1,21	NS	1,10	NS	1,28	NS
<i>CCL3</i>	-1,04	NS	-1,27	0,0033	1,12	NS
<i>CCL4</i>	1,01	NS	-1,12	NS	1,10	NS
<i>IL4</i>	-1,27	NS	-1,12	NS	-1,57	NS
<i>IL2</i>	-1,42	NS	-1,32	NS	-1,84	NS
<i>GMCSF</i>	-1,64	NS	-1,52	NS	-2,10	NS
<i>IL2RA</i>	4,28	0,0006	3,45	0,0057	4,66	0,0003
<i>CXCL9</i>	-1,69	NS	-1,94	NS	-1,52	NS
<i>IL12</i>	1,02	NS	1,12	NS	-1,34	NS
<i>IL15</i>	-1,03	NS	-1,17	NS	1,13	NS
<i>IL13</i>	-1,99	NS	-2,31	NS	-1,65	NS
<i>CCL11</i>	-1,09	NS	-1,09	NS	-1,08	NS
<i>CXCL12</i>	-1,09	NS	-1,11	NS	1,00	NS
<i>CXCL13</i>	33,77	<0.0001	27,76	<0.0001	163,60	<0.0001
<i>CD27</i>	174,07	<0.0001	139,01	<0.0001	203,41	<0.0001

one year, a significantly greater decrease in CXCL13 levels was observed in patients who attained a major response (-306.9 fold) versus those who did not (-2.3 fold) ($p=0.019$; **Figure 2C**). These findings were also true using logistic regression to account for age, gender, *MYD88* and *CXCR4* mutational status (including the distinction between *CXCR4* frameshift and nonsense-mutations), and baseline BM involvement ($p=0.035$ for baseline CXCL13, $p=0.005$ for change in CXCL13). CXCL13 levels did not differ based on *CXCR4* mutational status ($p=0.199$). Changes in CXCL13 correlated with changes in hemoglobin ($\rho=-0.64$; $p=0.005$; **Figure 2D**) and serum IgM levels ($\rho=0.68$; $p=0.002$; **Figure 2E**).

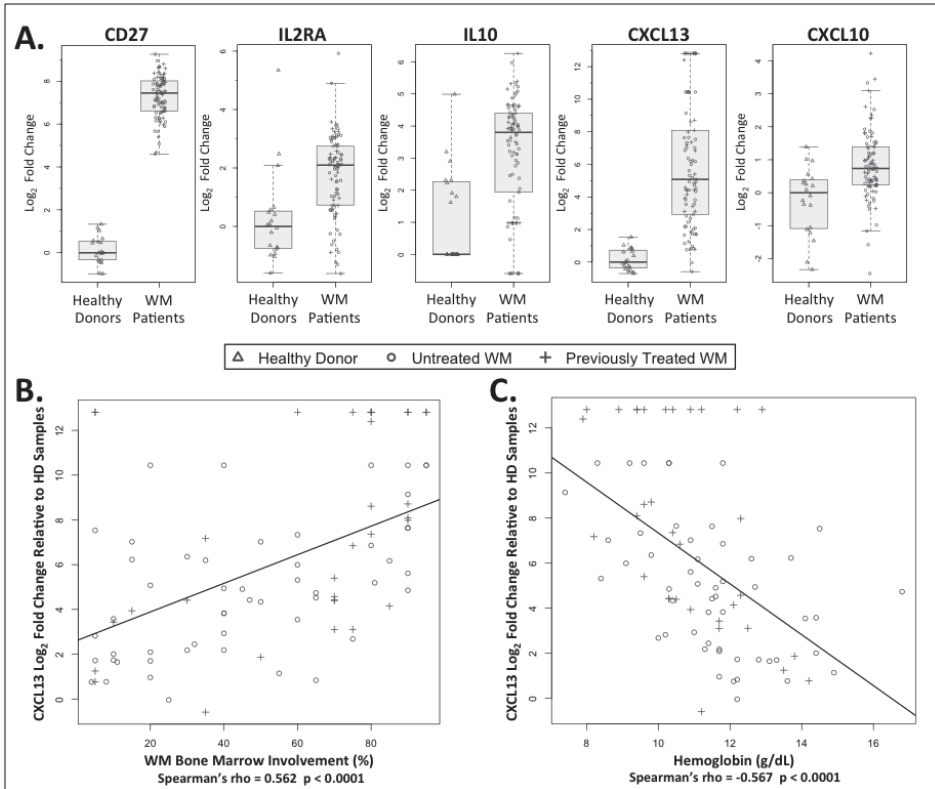
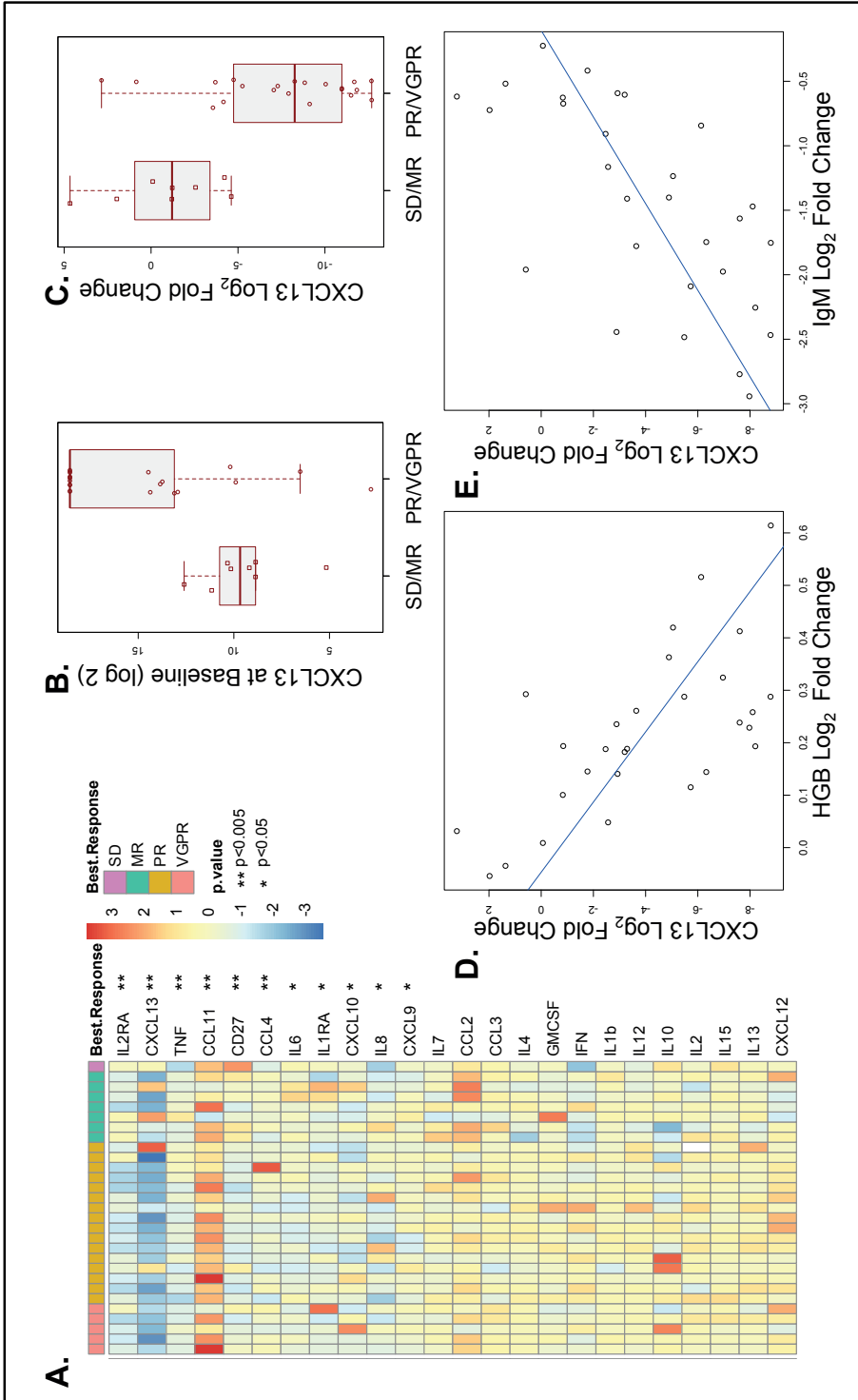


Figure 1: Serum cytokine levels in WM patients and healthy donors

Serum levels for the five cytokines that were different between 86 (52 untreated and 34 previously treated) WM patients and 20 healthy donors. Data represents log₂-transformed fold change values relative to the median of the healthy controls. All p-values were adjusted using Holm-Bonferroni correction for multiple hypothesis testing. A) All five cytokines were increased in WM patients with median levels of CD27 174.1 fold ($p < 0.001$), IL2RA 4.3 fold ($p < 0.001$), IL10 13.9 fold ($p < 0.001$), CXCL13 33.8 fold ($p < 0.001$) and CXCL10 1.7 fold ($p = 0.004$) increased over median healthy donor concentrations. B) Log₂-transformed CXCL13 levels positively correlated with percentage of WM bone marrow involvement (Spearman's rho=0.562; $p < 0.001$). C) Hemoglobin levels negatively correlated with CXCL13 levels (Spearman's rho=0.567; $p < 0.001$).

Figure 2: CXCL13 response to ibrutinib therapy →

Serum cytokine levels were assessed for 29 WM patient samples prior to the start of ibrutinib therapy and again after 1 year of therapy with ibrutinib. A) A row scaled heat map sorted by clinical response to ibrutinib at 1 year and statistical significance of the cytokine change demonstrating decreasing CXCL13 and increasing CCL11 levels following therapy. B) Baseline levels of CXCL13 were higher in patients who ultimately achieved a major response following one year of ibrutinib therapy ($p = 0.049$). C) Changes in CXCL13 levels at one year were also greater in those patients obtaining a major response versus patients who did not achieve a major response ($p = 0.019$). D) Changes in CXCL13 levels correlated with hemoglobin levels (Spearman's rho=-0.64; $p = 0.005$) and serum IgM response to ibrutinib (rho=0.68; $p = 0.002$)



We also studied changes in hepcidin levels to clarify whether improvements in anemia following ibrutinib were related to this protein. At baseline, hepcidin levels correlated with hemoglobin levels ($\rho = -0.39$, $p = 0.02$), but not with serum IgM, BM infiltration, or other cytokines evaluated. After one-year of ibrutinib, median hepcidin levels decreased from 67 (range 17.5-527.2 ng/ml) to 49 (range 8.9-225 ng/ml; $p = 0.013$); however there were no significant differences in decrease between patients with or without a major response, nor did they correlate with changes in hemoglobin, serum IgM or BM tumor involvement (data not shown).

The differences in cytokine levels that we observed between HD and WM patients were generally consistent with previously findings in WM,⁸ although these studies did not account for *MYD88* and *CXCR4* mutation status. Several cytokines were found to be lower in *MYD88*^{L265P}*CXCR4*^{MUT} versus *MYD88*^{L265P}*CXCR4*^{WT} patients, which may relate to the suppression of *MYD88*^{L265P} induced inflammatory pathways in patients with *MYD88*^{L265P}*CXCR4*^{MUT} status as found in a recent transcriptome study⁵.

This is the first report on inflammatory cytokine changes following ibrutinib therapy in WM, and our findings paralleled changes observed in CLL patients on ibrutinib therapy for CXCL13, IL8, CXCL10, CCL4, CCL11, IL1RA, and TNF- α .⁹ These changes may be related to on-target tumor effects leading to decreased cytokine production by LPL cells and/or the impact of ibrutinib on microenvironmental cells such as T-cells and macrophages. Although hepcidin levels showed a modest decrease, changes in hepcidin or BM infiltration did not account for the robust improvements in hemoglobin levels observed in ibrutinib-treated WM patients.

An important finding was the behavior of CXCL13 as a robust predictive marker of major response to ibrutinib in WM. A high CXCL13 at baseline was a strong predictor of achieving a PR or better after 1 year, and major responses were associated with a deep suppression of CXCL13 levels. To our knowledge there are no published data on the qualities of CXCL13 as a predictive marker of response to ibrutinib in CLL or other B-cell malignancies. CXCL13 is a chemokine that is expressed in lymphoid organs by follicular dendritic cells and macrophages, and is produced by LPL cells.⁵ CXCL13 attracts mast cells to the microenvironment in angioimmunoblastic T-cell lymphoma.¹³ Excessive BM mast cells are a known disease characteristic of the WM BM niche, and may provide support for WM cell growth and survival through CD40L and sCD27-CD70 interaction.¹⁴ In CLL, ibrutinib has been shown to strongly inhibit CXCL13 mediated adhesion of CLL cells to the BM and lymph node microenvironment, including interaction with macrophages.^{9,15} Our data suggest a role for CXCL13 in WM tumor biology and sensitivity to ibrutinib therapy, and warrant further investigation as a biomarker for ibrutinib therapy, as well as a potential therapeutic target.

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Author contributions: ST, ZH, JV, NT, MJK, CP and GY designed the study. JV, ZH, PB, NT, TG, KM, LX performed the research. JV, ZH performed data analysis. JV, ZH, ST, SP, MJK wrote the manuscript. ZH, ST, SP, MJK supervised the study.

Supplementary methods for cytokine testing

The following 24 cytokines were analyzed including soluble CD27 as a known biomarker in WM: TNF- α , IL6, IFNG, CXCL10, IL10, CCL2, IL8, IL1b, IL7, IL1RA, CCL3, CCL4, IL4, IL2, GMCSF, IL2RA, CXCL9, IL12, IL15, IL13, CCL11, CXCL12, CXCL13, sCD27.

All samples were kept at -80°C until analysis, and evaluated in duplicate.

We used magnetic multiplex enzyme-linked immunosorbent assays (R&D Systems Inc., Minneapolis, MN).

Luminex XMAP Technology MAGPIX System version 4.2 was used for reading plates. xPONENT software was used to analyze data.

Supplementary data on four patients that went off study within the first year of the trial.

Case Number	CXCR4 status	TIME ON IBR (Months)	On/off IBR at the time of sampling	CXCL13 level (pg/ml)	Clinical status
1	CXWT	baseline	off	1150	baseline
1	CXWT	1	on	708	Partial response
1	CXWT	9	on	4590	Progressive disease; off study
2	CXWT	baseline	off	129	baseline
2	CXWT	1	on	134	Stable Disease
2	CXWT	4	on	176	Stable disease; Off study due to progressive complications of systemic AL amyloidosis
3	CXMUT (NS)	baseline	off	389344	baseline
3	CXMUT (NS)	7	on	5026	Partial response
3	CXMUT (NS)	9	off	389344	Off study due to toxicities, then progressive disease shortly after that (last sample was taken after stopping the drug)
4	CXMUT (NS)	baseline	off	198	baseline
4	CXMUT (NS)	1	on	59	Minor response
4	CXMUT (NS)	4	on	62	Minor response Off study due to toxicities

CXWT= CXCR4 wildtype

CXMUT=CXCR4 mutated

NS= nonsense mutation

All 4 cases carried the MYD88L265P mutation

IBR- ibrutinib

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