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(EP726) DUAL EZH2 AND BCL6 INHIBITION TARGETS CML STEM CELLS VIA A GENE NETWORK CO-REGULATED WITH C-MYC

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Background: Chronic myeloid leukaemia (CML) is a rare clonal haematopoietic stem cell disorder, driven by the constitutively active tyrosine kinase BCR-ABL1. Inhibition of BCR-ABL1 with tyrosine kinase inhibitors (TKIs) has transformed the treatment of CML. However, low cure rate, TKI resistance and progression to blast phase continue to remain major clinical issues linked to the persistence of TKI-resistant leukaemic stem cells (LSCs) for which new therapeutic approaches are required. Evidence suggests that the Polycomb Repressive Complex 2 (PRC2), catalysed by EZH2, and BCL6 are both required for LSC survival and contribute to an anti-apoptotic phenotype by regulating the expression of apoptotic genes in LSC. Here we examined the molecular interplay between these two key survival factors and examined the consequences on LSC survival of targeting both simultaneously.

Aims: To understand the molecular relationship between BCL6 and EZH2 and provide a therapeutic rationale for the dual targeting of both factors in CML.

Methods: The phenotypic consequences of dual targeting BCL6 and EZH2 *in vitro* were carried out in JURL-MK1 cells using genetic knockdowns (shRNAs) and by using FX1 and GSK343 (inhibitors of BCL6 and EZH2 respectively) (n=3 in all cases). The phenotypic effects of dual targeting were also assessed in primary CML $CD34^+$ cells (n=3) using the two inhibitors. Phenotypic assays included measuring cell counts, apoptosis levels, and colony forming cell (CFC) and long-term colony-initiating cell (LTC-IC) outputs. The genome-wide distribution of BCL6 and EZH2/H3K27me3 targets in CML cells was

determined by ChIP-seq and RNA-seq. Network analysis was performed in the R programming language.

Results:

Genetic knockdown of BCL6 or EZH2 in JURL-MK1 cells resulted in significant decreases in cell expansion ($p<0.05$) and increases in apoptosis relative to scrambled controls ($p<0.05$). Dual targeting enhanced these effects ($p<0.01$). When CML $CD34^+$ cells were treated with FX1 +/- GSK343, the combination resulted in the greatest decreases in CFC outputs and showed a clear therapeutic window versus non-CML $CD34^+$ cells ($p<0.05$). Furthermore, the combination resulted in decreased LT-CIC output when compared to vehicle ($p<0.05$) which far exceeded the effects observed when FX1 or GSK343 were used as single agents – suggesting synergy. Experiments examining the *in vivo* effects of BCL6 and EZH2 inhibitors alone and in combination in mouse models are on-going.

The parallel approaches of ChIP-seq and RNA-seq in JURL-MK1 and CML $CD34^+$ cells provide mechanistic insights into how dual BCL6 and EZH2 targeting mediates this synergy. Surprisingly, BCL6 and EZH2 shared few gene targets globally. However, inhibition of either factor singly or in combination in CML cells resulted in a striking mRNA signature comprising several hundred genes, which had similarities to molecular signatures in public databases enriched for c-MYC targets. By integrating our global datasets with public ones, we constructed a network map depicting a co-regulated network downstream of EZH2 and BCL6 that converges on c-MYC, thus defining novel relationships between three known survival factors in CML for the first time.

Summary/Conclusion: We provide evidence that dual targeting of EZH2 and BCL6 may represent a novel therapeutic strategy for TKI non-responding CML patients. Furthermore, our systems biology approaches have identified a co-regulated network that explores the roles of these factors in CML, which may contain additional drug targets that warrant further investigation.

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(EP727) INVESTIGATING THE IMMUNOLOGICAL INTERACTIONS AFTER TKI THERAPY CESSATION IN CML PATIENTS: PREDICTIONS AND RISK ASSESSMENT USING MATHEMATICAL MODELS

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Background: Chronic myeloid leukaemia (CML) is a serious myeloproliferative disorder. The characteristic *BCR-ABL1*-fusion oncogene is the driving force for tumour growth, while the particular *BCR-ABL1* transcript is used as a marker for diagnosis and monitoring. The introduction of tyrosine kinase inhibitors (TKIs) marks a substantial progress in the therapy of CML. Current research activities focus on the discontinuation of TKIs for patients that achieve at least a major molecular remission (MR3, i.e. 3 log reduction of BCR-ABL1/ABL1 ratios). Several studies that define recurrence as loss of MR3 report CML recurrence rates of about 50%. Although the duration of TKI treatment and of deep molecular remission are associated with a higher probability of treatment free remission (TFR), it is still not possible to prospectively predict recurrence. It has been speculated that the immune system (IS) plays a major role in the control of residual disease levels after stopping TKI, and thus influences the individual recurrence behaviour. We have previously shown that mathematical models of CML can correctly describe patient time courses after TKI stop, if an immunological component is explicitly considered. In line with this, many questions regarding the occurrence of late relapse cases or the optimal treatment regimen before TKI cessation can be addressed using such modelling approaches. However, the correct parameter choice for those mathematical models is limited by the rarity of complete patient time courses with both initial response data *and BCR-ABL1* monitoring after TKI cessation. This calls for innovative approaches to substantiate the basis of these data.

Aims: Model fitting to time course data of CML patients from the DESTINY trial and generation of a virtual trial cohort to address therapy optimization and recurrence behaviour in the context of a larger cohort.

Methods: Unlike most other TKI stop studies, the DESTINY trial (ClinicalTrials.gov NCT 01804985) includes a TKI de-escalation period to half the standard dose for 12 months prior to complete treatment cessation. We applied an established mathematical model (Hahnel et al., Cancer Research

2020) and obtain individual fits for patient time courses from the DESTINY trial. Subsequently, we used permutation methods to sample from the obtained parameter values for each individual patient and thereby generate a larger cohort of virtual patients.

Results: By adopting selection criteria similar to Hahnel et al., we included 74 out of 174 patients from the DESTINY trial in our analysis. We used an advanced optimization algorithm to fit the model to the patient data, explicitly accounting for the half-dose reduction period. Overall, the method was sufficient to correctly describe the general response behaviour, while the recurrence behaviour could only be predicted correctly in about 73% of the patients. Permutating the parameters, we created a larger virtual trial cohort (>1000), that mimics the overall response dynamics of the sampled cohort and allows further comparisons with respect to timing and conditions for CML recurrence after TKI stop.

Summary/Conclusion: Based in the established virtual trial cohort, we are able to further analyse late relapse cases, and to speculate to what extent gradual TKI dose reduction strategies can further promote sustainable TFR.

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(EP728) CHARACTERIZATION OF PHENOTYPIC AND GENOTYPIC MARKERS AS PREDICTORS OF RELAPSE DURING TREATMENT-FREE REMISSION IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Background: Chronic myeloid leukemia (CML) is caused by spontaneous generation of a mutated tyrosine kinase with constitutive activation (BCR-ABL). After several years of treatment with tyrosine kinase inhibitors (TKIs), patients with sustained, deep molecular response (DMR) may interrupt treatment but approximately 50% experience relapse at some point after withdrawal. In addition to their direct effect on the BCR-ABL+ cancerous clone, TKIs are also immunomodulatory drugs that induce a potent antileukemic, cytotoxic response during treatment. It is unknown why this response, based on Natural Killer (NK) and CD8+ T cells, is only conserved in some patients after discontinuation, whereas others relapse and have to restart treatment.

Aims: To analyze phenotypic and genotypic markers that may be used as predictors of relapse in patients with CML during treatment-free remission (TFR).

Methods: We recruited 93 samples for analysis divided in 5 groups. "On-TKI": 45 patients with CML on treatment with TKIs for at least 9 months (imatinib (11), nilotinib (9), dasatinib (20), bosutinib (5)); "Off-TKI": 17 patients on successful TFR for at least 7 months; "Relapse": 7 patients who relapsed during TFR: 3 samples prior to TKI reintroduction and 4 samples who had already restarted TKIs; "New diagnosis": 4 patients still untreated with recent CML diagnosis; and 20 healthy donors as basal controls. PBMCs were subjected to phenotypic analysis by flow cytometry. Genotyping of HLA-E and KIR genes was performed using real-time qPCR.

Results: 1) Patients' results are shown in Table 1. 2) Treatment with TKIs induced an increase of 8.6±1.2% ((p<0.001) in CD56+ Natural Killer (NK) cells regarding healthy controls. This increase was sustained in patients "Off TKI" during successful TFR but it was reduced 9.5±1.4% in patients "Off TKI" on relapse. This cell population was not recovered in these patients even after restarting TKI treatment. 3) A population of NK cells with cytotoxic phenotype CD3-CD56+CD16+ was increased 8.1±2.8% in "Off TKI" patients during TFR, regarding patients "On TKI". This population was reduced 12±3.8% in patients "Off TKI" who relapsed but increased 18.8±6.7% once treatment was restarted. 4) Populations of cytotoxic cells CD8+TCRgd+ and CD8-TCRgd+ were

respectively increased 19±3.9% ($p<0.0001$) and 6.1±1.0% ($p<0.05$) in “Off TKI” patients regarding healthy controls but they were reduced 17.95±1.3% and 7.8±0.1%, respectively, in “Off TKI” patients who relapsed. 5) Analysis of HLA-E alleles showed that 60% of patients “Off TKI” were heterozygous for HLA-E, whereas only 15.4% of patients on relapse were heterozygous, being predominant (61.5%) homozygous HLA-E*0103/0103 genotype in this group. 7) KIR genes encoding for inhibitory molecules KIR2DL2 and KIR2DL5 and activating molecules KIR2DS2 and KIR2DS3 were present in >71% of patients who relapsed after treatment interruption, regarding <50% in “Off TKI”. Consequently, 86% of patients who relapsed showed KIR haplotypes BX.

Table 1. Phenotypic analysis in different groups of patients with CML. Leukocytes counts displayed in %.

	CD56+	CD3-CD56+CD16+	CD8+TCRγδ+	CD8-TCRγδ+	CD86+
Healthy donors	10,4±0,6	11±1,1	5,5±0,7	5,7±0,9	5,3±0,5
On TKI	19±1,4	12,9±1,7	11,8±1,2	6,5±0,9	13,4±1,2
Off TKI	13,8±1,7	19,1±3,9	24,5±4,6	11,8±1,9	10,9±1,2
Relapse	4,3±0,3	7,1±0,1	6,5±3,3	4,0±,2,0	20,1±0,2
On TKI after relapse	2,9±1,1	25,9±6,8	9,7±5,0	4,3±2,1	1,7±0,2
New CML diagnosis	6±04	2,4±0,5	4,1±1,3	3,3±1,0	14,2±6,5

Summary/Conclusion: We identified several biomarkers as potential predictors of relapse in CML patients during TFR: CD56+<4%; CD3-CD56+CD16+<7%; CD8+TCRgd+<7%; CD8-TCRgd+<4%; CD86+>20%; homozygosis for HLA-E*0103; and KIR haplotypes BX. These biomarkers need to be validated in a larger, longitudinal cohort of patients.

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(EP729) SETD2/KDM4A-MEDIATED H3K36ME3 LOSS IN CHRONIC MYELOID LEUKEMIA PATIENTS IN BLAST CRISIS CAN BE THERAPEUTICALLY TARGETED

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Background:

Genomic instability is a hallmark of chronic myeloid leukemia in chronic phase (CML-CP) resulting in BCR-ABL1 mutations inducing resistance to tyrosine kinase inhibitors (TKIs) and/or additional chromosomal abnormalities leading to disease relapse and/or progression. The histone methyltransferase SETD2 is responsible for tri-methylation of lysine 36 on histone H3 (H3K36me3), that is implicated in regulation of transcription, DNA mismatch repair, homologous recombination and alternative splicing. KDM4A de-methylates H3K36Me3.

Aims:

We aimed to investigate mechanisms involved in SETD2 loss of function and to asses if SETD2/H3K36me3 loss may be considered a promising druggable lesion.

Methods:

Protein expression and activation was assessed by Western Blotting. Apoptotic cell death was quantified by annexinV/propidium iodide staining. Drug cytotoxicity was evaluated by clonogenic assays.

Results:

In CML, SETD2 reduction was detected in 86% of patients (pts) with blast crisis (BC) as compared to a pool of healthy donors and to chronic phase (CP) pts at diagnosis. This phenomenon is due to translation of a wild-type protein followed by its rapid proteasomal degradation. Proteasome inhibition restored SETD2 expression that was found to interact with Aurora Kinase A (AKA), frequently overexpressed in CML. AKA phosphorylates SETD2, and its inhibition rescued SETD2 expression and activity. We performed clonogenic assays in LAMA84 ($SETD2^{high}$/$H3K36Me3^{high}$), K562 ($SETD2^{low}$/$H3K36Me3^{loss}$) and KCL22 ($SETD2^{loss}$/$H3K36Me3^{loss}$) cells. The extent of reduction of clonogenic growth after proteasomal and AKA inhibition was found to be inversely correlated to SETD2 residual expression. In the aforementioned cell lines, proteasomal inhibition by bortezomib, carfilzomib and ixazomib and AKA de-phosphorylation by Danusertib caused a time-dependent increase of annexin-V-positive cells by activating the mitochondrial apoptotic pathway as reflected by an increase in Bax expression and induction of the cleavage of caspase-3,-9 and PARP.

Reduced SETD2/H3K36Me3 levels, in association with AKA hyper-activation, were also detected when the CD34+ cell fraction of 20 CML-CP pts was compared to the total mononuclear cell fraction or to the CD34+ compartment obtained from a pool of healthy donors.

Finally, it has already been shown that alterations of epigenetic regulators such as the KDM4 family members control tumor cell proliferation in a variety of cancers including acute myeloid leukemia and that may be used as putative therapeutic targets in a SETD2 mutated context. Starting from these evidences we checked K562 ($SETD2^{low}$/$H3K36Me3^{loss}$), LAMA84 ($SETD2^{high}$/$H3K36Me3^{high}$) and KCL22 ($SETD2^{loss}$/$H3K36Me3^{loss}$) cell lines for KDM4A expression to confirm if KDM signaling and its inhibition may be considered a molecular therapeutic modality in CML. We observed that in K562 cells KDM4A is overexpressed and able to de-methylate H3K36me3 by directly interacting with histone H3 and inducing the loss of H3/SETD2 binding. Further experiments will be performed to confirm this mechanism in

BC CML pts showing residual SEDT2 activity to test if KDM4A may be considered a druggable target.

Summary/Conclusion:

Phosphorylation by AKA contributes to SETD2 non-genomic loss of function in BC CML and in CD34+ leukemic progenitors. Restoring physiological H3K36Me3 may help to improve the outcome of BC pts and might be an attractive opportunity to interfere with persistence of leukemic progenitors. Supported by AIRC (project 23001), AIL and Italian Ministry of Health, project GR-2016-02364880.

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(EP730) DASATINIB INDUCES ENDOTHELIAL TO MESENCHYMAL TRANSITION IN HUMAN VASCULAR ENDOTHELIAL CELLS

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Background:

Vascular adverse events (VAEs) have become a serious clinical problem in CML patients who receive some of the tyrosine kinase inhibitors (TKIs). Previous studies found that endothelial to mesenchymal transition (EndMT) could contribute to various cardiovascular diseases.

Aims:

To investigate the influence of TKIs on the EndMT in human vascular endothelial cells (VECs).

Methods:

EA.hy926 human VECs were cultured with clinically relevant concentrations of dasatinib 0.2 μM, ponatinib 0.1 μM, imatinib 4.0 μM, bosutinib 0.5 μM, and nilotinib 3.0 μM. The morphological changes of cells were observed under microscope. Using RT-qPCR, mRNA expression levels of mesenchymal markers *vimentin, FSP1, α-SMA, fibronectin* and inducer of EndMT *TGF-β* were evaluated. The protein expressions of endothelial markers VE-cadherin and CD31, mesenchymal marker vimentin were detected by western blotting and immunofluorescence staining. ERK1/2 and AKT phosphorylation levels were detected by western blot analysis. Using murine TGF-β ELISA kit, serum levels of TGF-β in mice (n = 3 mice at each time point) were measured at 6, 24 and 48 h following intravenous dasatinib (10 mg/kg) or control (DMSO) diluent administration.

Results:

Exposure of VECs to dasatinib, but not to other TKIs, caused mesenchymal transformation as assessed by morphological examination; VECs dramatically changed their morphology from cobblestone-like shape to a spindle-like shape with fewer cell-cell contact after exposure to dasatinib for 24-48 hours. This change was accompanied by a significant increase in the mRNA expression levels of mesenchymal markers including *vimentin, FSP1, α-SMA* and *fibronectin* ($p<0.001$, $p<0.05$, $p<0.05$, $p<0.01$, respectively). In addition, protein expression level of vimentin showed significant increase ($p<0.01$), whereas endothelial markers VE-cadherin and CD31 protein levels were significantly decreased ($p<0.01$). The relative mRNA expression level of a key regulator and inducer of the EndMT, *TGF-β* was

significantly ($p<0.001$) upregulated in dasatinib treated cells. Furthermore, serum levels of murine TGF-β1 were significantly increased ($p<0.01$) at 6 h and 24 h after intravenous administration of dasatinib and dropped significantly ($p<0.01$) after 48 h of dosing. Dasatinib-induced EndMT in VECs were associated with activation of smad-independent TGF-β signaling pathways, including ERK1/2 and AKT. Recombinant Human TGF-β increased the dasatinib-induced an increase in the ERK1/2 phosphorylation level by two-fold; whereas the neutralization antibody to TGF-β attenuated the dasatinib-induced increase in the ERK1/2 phosphorylation level. Of note, an inhibitor of ERK (PD98059) attenuated the expression of mesenchymal marker vimentin. Other investigators previously reported that bosutinib had protective effects against VAEs. Consistent with their observations, co-treatment of VECs with both dasatinib and bosutinib, interestingly, resulted in significantly reduced the spindle shape formation, phosphorylation of ERK as well as reduced expression of mesenchymal markers observed with dasatinib.

Summary/Conclusion:

Our findings showed that exposure of VECs to dasatinib induced expression of TGF-β and upregulated the expression of mesenchymal markers via, at least in part, ERK signaling pathway. Inhibitors of TGF-β and ERK attenuated the dasatinib-induced mesenchymal markers. Co-treatment of dasatinib and bosutinib efficiently protected the morphological and molecular properties of ECs. Taken together, dasatinib and bosutinib play distinct roles in VECs.

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(EP731) THE CRISPR/CAS9 SYSTEM EFFICIENTLY REVERTS THE TUMORIGENIC ABILITY OF BCR/ABL AND RESTORING THE NORMAL HAEMATOPOIESIS IN A MOUSE MODEL OF CHRONIC MYELOGENOUS LEUKAEMIA.

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Background: Chronic myelogenous leukaemia(CML) is a haematological neoplasia driven by translocation t(9;22) in the haemopoietic stem cell(HSC) compartment, generating the fusion oncoprotein BCR-ABL with a constitutive tyrosine kinase(TK) activity. The specific TK inhibition(TKI) is the base for current treatments. However, TKI resistances are observed in up to 25% of patients so, new therapeutic approaches for these patients are needed. The recently developed CRISPR system could be a definitive therapeutic option for these patients. The generation of human-mice model of bone marrow transplantation would allow to explore the potential of this technology to correct genetic disorders in hematological diseases

Aims: To study the ability of CRISPR technology for disrupting the BCR/ABL oncogene sequence in CML stem cells and for restoring the normal haematopoiesis

Methods: For the mouse stem cell transplantation a CML mouse model expressing the human BCR/ABL was used as a donor of CML leukemic stem cells(LSCs). In parallel, human CD34+ cells(hLSCs) from new diagnosed CML patients were also used to perform a bone marrow transplantation improving a xenograft mouse model. Human and mouse LSCs were electroporated with the CRISPR components, including two sgRNAs targeting the TK domain of BCR/ABL and were transplanted into the immunodeficient NSG mice. CRISPR genome edition was determined by PCR and Sanger sequencing. BCR/ABL oncogene expression was evaluated by qPCR to ensure total mRNA abolition. Finally, protein expression was measure by immunofluorescence of LSC. To study the ability of edited LSCs for restoring the normal hematopoiesis, peripheral blood cell populations were studied 2 and 4 months after transplantation, by blood smears and flow cytometry. Human-mouse transplant host were euthanized 6 months after transplantation, and BCR/ABL expression of hCD45+ cell population from bone marrow was analyzed by

qPCR. Myeloid and lymphoid populations were measure by flow cytometry to analyze the hematopoiesis derived from the graft of human LSCs

Results: In CML-LSCs from human and mice the CRISPR system induced a deletion in the BCR/ABL sequence triggering lower expression of both in RNA and protein level. Of note, 6 months after transplantation, the presence of the deletion in mature lymphoid and myeloid cells, exhibiting the totipotent ability of edited LSC, to contribute to all hematopoietic lineages in the host mice. Peripheral blood of mice transplanted with mouse un-edited LSCs(controls), showed a high percentage of myeloid cells, like those found in leukemic donor mice. However, in transplanted mice with edited cells, myeloid population was significantly reduced, reaching the levels observed in wild type mice. Moreover, the lymphoid population in transplanted mice with CML-edited cells were higher than in controls, reaching the level observed in wild type mice. Accordingly, in the bone marrow of mice transplanted with human un-edited LSC an inverse correlation between high CD117+ and low CD19+ cell populations was observed. In addition, in human edited-LSC transplants we found low CD117+ cell population accompanied with high CD19+ cell population, in agreement with observed in normal hematopoiesis derived from healthy human stem cells transplanted into NSG mouse, suggesting the recovery of normal hematopoiesis

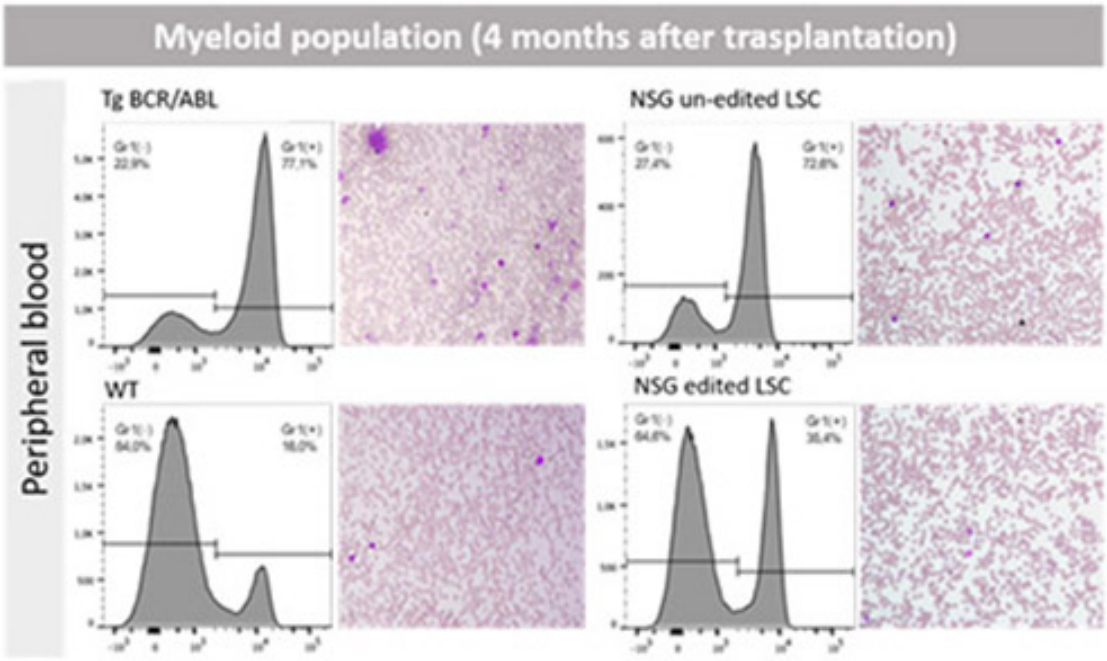

Figure 1. Analysis of peripheral blood 4 months after transplantation into NSG mice. The myeloid cells Gr1+ were quantified by Flow cytometry, and visualized by blood smears.

Summary/Conclusion: CRISPR technology allows to induce BCR/ABL null mutations in the CML-LSCs, disrupting the myeloid bias and restoring the normal hematopoiesis. These results are a proof-of-principle of the therapeutic potential of CRSPR genome edition tool in haematological diseases.

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(EP732) SNPS IN ABC GENES CORRELATES WITH MOLECULAR RESPONSE IN CML PATIENTS TREATED WITH NILOTINIB

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Background: Targeted therapy with tyrosine kinase inhibitors (TKIs) have dramatically changed the treatment of CML. However, a significant proportion of patients still fail to maintain a major molecular response (MMR, MR3) over time. The over expression of MDR-ABC transporters, such as ABCB1, ABCG2 and ABCC2 represents a potential mechanisms of primary resistance. Single nucleotide polymorphisms in these genes may thus influence molecular response in CML patients.

Aims: to determine the effect of 7 SNPs in 4 ABC genes on the achievement and/or loss of molecular response in 90 CML patients treated with nilotinib at 6 Italian sites.

Methods: the following SNPs were examined: ABCC1 5463T>A (rs212090), ABCC2 3972C>T (rs3740066), ABCC2 rs4148386, ABCC2 1549G>A (rs1885301), ABCG2 34G>A (rs2231137), ABCG2 rs2231142, ABCB1 3435C>T (rs1045642). The alleles frequencies and their association with response to nilotinib were obtained for the dominant, codominat and recessive models. Hardy-Weinberg equilibrium was verified for all examined SNPs. Differences in genotype and allele's distributions among CML patients, together with the associations between genotypes and response, resistance or loss of response to nilotinib were assessed by Kaplan-Meier method and Cox Regression analysis.

Results: The characteristics of patients are listed in table 1. Evaluation of molecular response was conducted in accordance to 2013 ELN criteria. Overall, 92% achieved MR3 during treatment. 15% of these patients subsequently lost MR3 during treatment, after a median time of 14 months

(range 4-78). 74% of patients receiving nilotinib achieved a DMR (deep molecular response defined as MR≥4). ABCC2 rs374066, ABCG2 rs2231137 and ABCB1 rs1045642 influenced the probability of achieving MR3 or the time to MR3. Patients with ABCC2 3972C/C or 3972C/T genotype and ABCB1 3435T/T genotype had a higher probability to reach MR3 in a shorter time (p=0.02, p=0.004, p=0.01 respectively). On the contrary, patients with ABCG2 34G/G had lower probability to achieve a molecular response (p=0.005). Regarding MR4, ABCC2 3972 C/C positively affected the achievement of MR4 together with ABCB1 3435C/C and T/T (p=0.02, p=0.007, p=0.003, respectively). Concerning MR4.5 achievement, we were not able to find any statistically significant effect from any of the studied SNPs. We further investigated if there was an association between the different distribution of genotypes or haplotypes of the studied SNPs and loss of molecular response to Nilotinib. Our results showed that ABCG2 rs2231142 GG variant was associated with a decreased risk of MR3 loss (p=0.02). No significant association was found between other studied SNPs and loss of MR3, MR4 and MR 4.5.

Table.1

CLINICAL CHARACTERISTIC	N	(%)
Age at diagnosis, years median (range)	48 (18-74)	
Age at Nilotinib, years median (range)	50 (18-74)	
Sex		
Male	52	58
Female	38	42
Sokal score		
Low	30	33
Intermediate	33	37
High	25	28
nv	2	2
EURO/Hasford		
Low	50	56
Intermediate	31	34
High	6	7
nv	3	3
EUTOS		
Low	83	92
High	1	1
nv	6	7
Nilotinb treatment		
I ine	46	51
II Line	44	49

Conclusions/summary: This study correlates SNPS in the ABC genes with molecular response with Nilotinib. Interestingly, patients with ABCG2 34G/G had a lower probability of achieving a sustained molecular response, potentially limiting the number of patients with these SNP evaluable for treatment-free remission. Further studies in lager series are warranted to confirm our preliminary experience.

CML AdvocatesNetwork

(EP733) BONE MARROW NICHE REPROGRAMMING OF LEUKAEMIA STEM CELLS IN CHRONIC MYELOID LEUKAEMIA

Forde, Eóghan / Wheadon, Helen / Copland, Mhairi / Dzierzak, Elaine

Background:

Eradicating leukaemic stem cells (LSCs) and curing stem cell-derived cancers is hugely challenging. This is exemplified in chronic myeloid leukaemia (CML), where the t(9;22)(q34;q11) chromosomal translocation results in an abnormal chimeric BCR-ABL1 tyrosine kinase and LSC transformation. Tyrosine kinase inhibitors (TKI) targeting the BCR-ABL1 protein are considered an exemplar of precision medicine in CML, but they do not cure the vast majority of patients. Unmet clinical needs arise from TKI resistance and the persistence of LSCs sequestered in protective niches within the bone marrow microenvironment. More recently the role of BM mesenchymal stromal cells (BM-MSC) and their contribution to malignant selection and non-cycling of adherent cells has been associated with the limited effectiveness of TKI in targeting *BCR-ABL1*$^{+}$ cellular proliferation and apoptosis.

Aims:

In our study, we set out to identify genes downstream of established cytokine autocrine loops which drive the evolution of CML-LSC persistence in a *BCR-ABL1* independent manner.

Methods: We analysed publicly available CML RNAseq data obtained from Giustacchini, *et al*. (GSE76312) and Agarwal, *et al*. (GSE124125). Cell culture of human stromal, myeloid and lymphoid CML cell lines alongside primary CD34+ enriched normal donor and CML samples was performed. Following informed consent, samples were collected in accordance with the Declaration of Helsinki, and approval of the Greater Glasgow and Clyde National Health Service. CML cell lines and primary CD34+ haemopoietic cells were either cultured on plastic or co-cultured on confluent BM-MSC for up to 72 hours. Real-time PCR using Taqman or Fluidigm platforms was used to verify the published RNA sequencing data and Western Blot analysis to confirm protein expression.

Results:

Collectively, our further analyses inferred the expected widespread disturbances of many haemopoietic networks. We identified gene sets enriched in CML LSCs downstream of previously described axes; these included established IL-6, TGF-β-BMP, TNF-α as well as PI3K-BMP associated NF-κB- expression pathways. In further analyses, significantly enriched genes identified from both murine and human CML LSCs were mined

for overlapping genes of novel interest. Interestingly, compared to normal haemopoietic cells, and the highly proliferative LSCs that have been previously shown to disappear upon TKI therapy, LSCs from quiescent BCR-ABL+ populations, K562 cells and independent murine CML datasets showed enrichment for genes encoding the Pituitary tumour-transforming gene 1 (*PTTG1*) and its associated interacting protein (*PTTG1IP*). This was also seen to be further elevated during experiments using co-cultures with BM-MSCs. Several genes, including c-*Myc*, *p53*, *FGF2*, *p21*, prolactin and *MMP2*, are transcriptional targets of PTTG1. These targets of PTTG1 are currently being assessed and validated *in vitro*.

Summary/Conclusion:

Re-analysis of existing transcriptomic datasets provided insights into pathways that are potentially involved in promoting survival of a subset of quiescent BCR-ABL+ LSCs via a BCR-ABL-independent mechanism. Experiments are ongoing to investigate how such an axis may promote the BM-MSC niche to harbour and selectively maintain LSC persistence.

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(EP734) DIGITAL PCR FOR THE MEASUREMENT OF BCR- ABL1 IN CML: A NEW DAWN?

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Background: Sequential measurement of BCR-ABL1 mRNA levels by reverse transcription quantitative PCR (RT-qPCR) is embedded in treatment pathways and clinical trials in Chronic Myeloid Leukaemia (CML). It has played an important role in the remarkable improvement in patient survival seen in CML. Standardisation efforts have reduced inter- and intra-laboratory variation in BCR-ABL1 measurement; however, these remain an issue for laboratories. Reverse transcriptase droplet digital PCR (RT-ddPCR) may overcome these issues, but a definitive, large-scale multi-centre study comparing RT-qPCR and RT-ddPCR has yet to be published.

Aims: To establish if RT-ddPCR is a viable alternative to RT-qPCR, in an international interlaboratory study.

Methods: Ten randomised, cell line based BCR-ABL1 positive samples (labelled A-J) with BCR-ABL1 levels ranging from $MR^{1.0}$-$MR^{5.0}$ at half log intervals were dispatched to 26 participants. Sample A-G represented BCR-ABL1 levels between $MR^{1.0}$ and $MR^{4.0}$, important to detect within standard treatment protocols. Samples (H-J) represented 'deep response' levels ($MR^{4.5}$, $MR^{4.7}$ and $MR^{5.0}$), important to detect in treatment cessation trials. All participants were asked to extract RNA and perform reverse transcription using their standard in house protocol and test the samples with the QXDX BCR-ABL %IS kit, using either the QX200 Auto or Manual DG system. Where participants were currently reporting BCR-ABL1 quantification results using RT-qPCR,

all study samples were run in parallel using local methodology for comparison to the RT-ddPCR results (n=11). International Scale results were analysed for this study.

Results: Digital PCR results were returned by 23 participants; 11 of these participants returned both RT-qPCR and RT-ddPCR allowing direct comparison which will provide the focus of this abstract. When results were compared for the 11 participants who performed both RT-ddPCR and RT-qPCR, all participants detected BCR-ABL1 in all samples down to $MR^{4.0}$, using both techniques. For deep response samples, RT-ddPCR showed a detection rate of 90.9% at $MR^{4.5}$, 81.2% at $MR^{4.7}$ and $MR^{5.0}$ compared to 90.9% at $MR^{4.5}$, 90.9% at $MR^{4.7}$ and 72.7% at $MR^{5.0}$ for RT-qPCR. Interlaboratory CV was lower across all samples for RT-ddPCR compared to RT-qPCR: average CV% for RT-ddPCR was 23.6% cf. 44.4% for RT-qPCR down to MR4.0; and 55.6% and 87.8% respectively for deep response samples. Statistical significance ($P<0.05$) could be demonstrated in 7/10 samples. Nine out of 11 participants RT-ddPCR results showed better linearity from $MR^{1.0}$ to $MR^{4.0}$ (average R2=0.9991) compared to RT-qPCR (average R2=0.9751). Six of these participants showed a statistically significant difference ($P<0.05$).

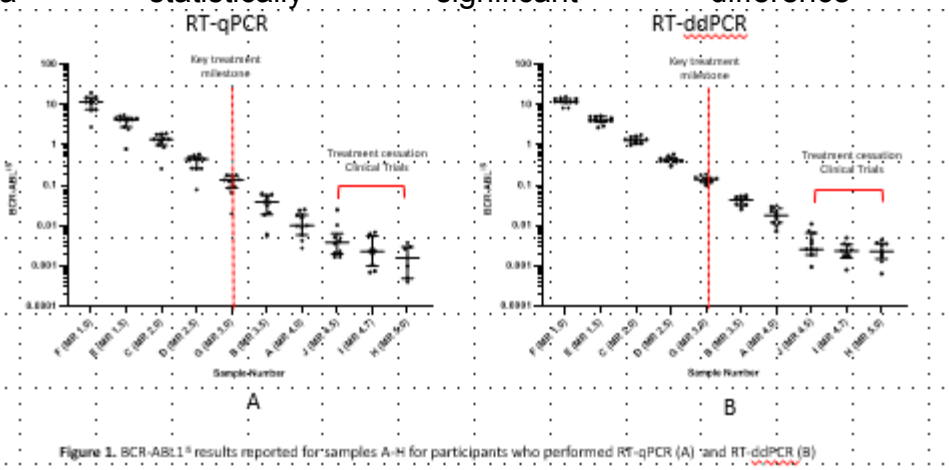

Figure 1. BCR-ABL1[S] results reported for samples A-H for participants who performed RT-qPCR (A) and RT-ddPCR (B)

Summary/Conclusion: Digital PCR is a viable alternative to traditional RT-qPCR offering decreased inter-laboratory variation and better assay linearity when directly compared to RT-qPCR. No difference in performance was seen in this study in the ability to detect BCR-ABL1 in 'deep response' samples required by treatment cessation studies; however, stochastic sampling error limited the possibility of reliable detection of BCR-ABL1 when so few copies are present given the standard sample input into the assay. Further appropriately powered studies will be needed to determine performance at this level. The ability to detect deep responses with ddPCR, matched with an improved linearity and reduced CVs, compared to qPCR, is of benefit when comparing clinical results across different laboratories, improving individual patient management and the results of clinical trials.

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(EP735) DIGITAL DROPLET PCR IS A FAST AND EFFECTIVE TOOL FOR DETECTING T315I MUTATION IN CHRONIC MYELOID LEUKEMIA.

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Background: After introduction of the Tyrosine Kinase Inhibitors (TKIs) into the clinical practice, the outcome of patients affected by Chronic Myeloid Leukemia (CML) is dramatically improved. Nevertheless, about 30% of patients must change TKI for intolerance or resistance. The real mechanisms of resistance are still not fully elucidated, but point mutations in the ABL1 gene are responsible for at least 10% of failures. Among these mutations, the T315I seems to be the worst one, causing resistance to all TKIs except Ponatinib. Thus, the rapid identification of T315I could be a relevant goal

Aims: To assess feasibility and sensitivity of digital droplet PCR (ddPCR) assays for T315I, both on cDNA and gDNA and to compare these results with those obtained by Sanger and/or NGS. Mutational status was assessed by the FAM/HEX mutation detection ddPCR assay ABL1 p.T315I c.944 C>T (Biorad).

Methods: In the context of the “Campus CML” working group, 44 samples from 6 Italian Centers were centralized in the Molecular lab of Hematology of Pisa that performed ddPCR. In order to compare ddPCR results with Sanger and NGS, only cases already detected as T315I-mutated by one of these techniques have been analyzed. In 15 cases ddPCR was compared to both Sanger and NGS; in the remaining samples, only to Sanger or NGS. Two events in the FAM channel were considered as the minimum cut off for defining a sample as mutated.

Results:

1) feasibility: in 42/44 cases the ddPCR was successful: in one case also the BCR-ABL1 QT-PCR failed. **2) sensitivity**: the minimum mutational burden detected was 0.02%, so confirming the value of $1x10^{-3}$ stated by producer. **3) d*dPCR* vs Sanger**: 25 samples were concordant; 5 cases resulted mutated by ddPCR but not by Sanger (all mutated by NGS), whereas no samples were wild-type by ddPCR but mutated by Sanger. This comparison showed that ddPCR recovered 16% of mutated cases in respect of Sanger. 4) d**dPCR vs NGS**: 19 samples were concordant; 2 cases, mutated by NGS, resulted wild-type by ddPCR; on the other hand, other 2 cases wild-type by NGS were mutated by ddPCR. The VAF of these cases was 0.43% and 0.39%, values under the sensitivity limit of NGS. Out of the 2 failing cases in ddPCR, one resulted mutated on gDNA but not on cDNA. This comparison showed that ddPCR and NGS are superimposable. **5) cDNA vs gDNA:** in 16 samples ddPCR was performed both on cDNA and on gDNA: we observed that 2 cases resulted T315I-mutated only on gDNA. The VAF of these cases was 0.01% and 0.05%, respectively. One of these patients became wild-type on both nucleic acids after Ponatinib. In the other case the test was performed on DNA extracted from liquor during a SNC CML localization; after intratecal chemotherapy, the test became negative.

Summary/Conclusion:

ddPCR is a promising molecular technique that allows a quantitation of gene expression or detection of mutations without a reference curve, with costs comparable with those of the “classic” QT-PCR. With this work we demonstrated that ddPCR represents a valid tool for assessing in few hours the presence of T315I mutation, either on cDNA or on gDNA. This aspect could be clinically relevant, because many failing patients are not mutated on cDNA. This observation could mirror the persistence of the CML leukemic stem cell in the bone marrow niche. Our study shows that ddPCR is comparable to NGS and slightly superior to Sanger.

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(EP736) NOVEL MULTIPLEX DROPLET DIGITAL PCR ASSAYS TO MONITOR MINIMAL RESIDUAL DISEASE IN CHRONIC MYELOID LEUKEMIA PATIENTS SHOWING ATYPICAL BCR-ABL1 TRANSCRIPTS

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Background:

Chronic myeloid leukemia (CML) is characterized by a BCR–ABL1 fusion protein, originating from the translocation between chromosome 9 and 22 (t(9;22)), which is also the molecular marker for the evaluation of minimal residual disease (MRD). Although 98% of patients shows conventional translocations (e13a2 and e14a2), 2% of them has breakpoints located in unusual regions giving rise to atypical transcripts, such as e13a3, e14a3 and e19a2. Quantitative Real-Time PCR (RT-PCR) is the standardized method for molecular response evaluation, but no assays have been set to quantify these rare transcripts. Currently, MRD monitoring is performed almost exclusively by non-quantitative NESTED PCR. These limitations make impossible the recognition of a major molecular remission achievement, which is essential for prognostic purposes to avert the risk of progression and to decide on therapy.

Recently studies showed that discontinuing tyrosine kinase inhibitor (TKI) treatment in patients who have achieved a stable deep molecular response could decrease the cost of therapy and together reduce the treatment side effects, increasing CML patients' quality of life. Patients with atypical transcripts are automatically excluded from benefits of this clinical approach because it is not possible to quantify their molecular response.

Droplet digital PCR (ddPCR) has recently emerged as an alternative to RT-PCR to monitor MRD in CML patients. ddPCR, which allows the absolute quantification of target molecules independently by standard curves, seems appealing for the monitoring of molecular response also in patients with BCR-ABL1 atypical transcripts.

Aims: The aim of this study was to develop a method to evaluate molecular response in CML patients characterized by atypical breakpoints, in order to improve the prognostic information that could allow to guide the therapeutic choices and/or the TKI discontinuation.

Methods: We designed two multiplex ddPCR assays able to quantify BCR-ABL1/ABL1 levels in e13a3, a14a3 and e19a2 CML patients. Primers efficiency and limit of detection were evaluated. The assays were validated in a cohort of 11 CML patients (n. 3 e13a3, n. 2 e14a3 and n. 6 e19a2) at diagnosis and during follow up. 65 RNA samples were collected and ddPCR results were compared with NESTED PCR.

Results: Selected primers showed high efficiency (95-102%) and selectivity. Further, results indicated that our assays could quantify a reduction of BCR-ABL1 transcript of up to 10^{-4}. The BCR-ABL1 fusion transcripts were evaluated comparing ddPCR and NESTED PCR in patient's samples taken at diagnosis and during follow up. ddPCR and NESTED PCR identified congruently 10 negative and 40 positive patients, while 11 samples were recognized as positive only in ddPCR (McNemar's test $p<0.01$). Disagreement between techniques could be explained by the inaccuracy found with NESTED PCR when BCR-ABL1/ABL1 level was less than 1.5%. Further, our data identify 3 patients who achieved and maintained a deep molecular response for almost two years. Thanks to the possibility of MRD monitoring, they could represent good candidates for a future treatment free remission approach.

Summary/Conclusion: We described a new high sensible/specific method for molecular monitoring of atypical BCR–ABL1 fusion transcripts in CML patients, useful to better define MRD levels. Our methodology could play the foundation to include also CML patients with atypical breakpoints in TKI discontinuation therapeutic approach.

CML AdvocatesNetwork

(EP737) XPERT ® BCR-ABL ULTRA: A METHOD OF MONITORING CONVERSION FACTOR STABILITY IN DIAGNOSTIC LABORATORIES

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Background: The Xpert® BCR-ABL Ultra system provides a quantitative test for BCR-ABL1 major breakpoint (p210) transcripts and automates the entire test process including RNA isolation, reverse transcription, and fully nested real-time PCR of *BCR-ABL1* target gene and *ABL1* reference gene with results expressed on the international scale (IS). Every new lot of Xpert® BCR-ABL Ultra is calibrated to secondary standards that are produced from WHO IS primary standards so that the test reports results according to the IS.

A previous version of the *BCR-ABL1* Cepheid test generated results that were significantly different from the in-house *BCR-ABL1/ABL1* quantitative PCR (qPCR) values, but they became non-significant when adjusted using the laboratory conversion factor (CF) (Gerrard et al. Leukemia Research 49 (2016) 47–50). These metrics can help decide whether to update a CF value, especially where a significant change in CF might lead to a discontinuity in ongoing patient monitoring.

Aims: An investigation was carried out to determine whether a new version of the *BCR-ABL1* Cepheid test, Xpert BCR-ABL Ultra (Xpert BCR-ABL Ultra (CE-IVD; US IVD; Cepheid, Sunnyvale CA USA)), designed to detect *BCR-ABL1/ABL1* ratios as low as 0.0032%, is equivalent to the in-house qPCR assay and also suitable for CF assessment.

Methods: *BCR-ABL1* ratios were calculated from 88 patients using fresh blood samples analysed with both the Xpert BCR-ABL Ultra and an established real-time quantitative PCR assay (qPCR). Additionally, a control cell lysate panel with known *BCR-ABL1* concentrations was assessed using the Xpert BCR-ABL Ultra.

Results: The control cell lysate panel consisted of 5 samples with different *BCR-ABL1/ABL1* ratios plus a 0% B*CR-ABL1* negative sample. They were assessed in triplicate with the Xpert BCR-ABL Ultra and demonstrated a strong correlation between the predicted and measured values (R^2=0.984) indicating that results are reproducible and accurate. There was no *BCR-ABL1* detected in the 0% IS sample.

All qPCR results were converted to IS and compared to the IS result produced by the Xpert BCR-ABL Ultra and there was a strong correlation between the 2 techniques (r^2=0.954). To assess whether the results were equivalent, a

Bland-Altman plot was produced. The mean ratio between the two techniques was 1.018, meaning that the two techniques gave highly similar results.

Summary/Conclusion: The Xpert BCR-ABL Ultra test provides IS *BCR-ABL1* accurate and reproducible results. The test is therefore suitable to assess a laboratory conversion factor, down to a *BCR-ABL1* ratio of 0.0032% (MR4.5). There is more variability in both assays at the lower BCR-ABL1 ratios and this will continue to be an inherent problem of assessing low BCR-ABL1 transcript numbers regardless of the technique. The Xpert BCR-ABL Ultra system is significantly simpler and less time consuming than sample exchange programmes and allows for more regular assessment of CF, particularly if a laboratory procedure has undergone significant change since the last CF assessment. Alternatively, in low-throughput laboratories, the Xpert BCR-ABL Ultra test could replace in-house qPCR methods thus making assessment of CF obsolete.

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(EP738) IMATINIB IS ABLE TO INTERFERE WITH IMMUNE RESPONSE AND INFLAMMATION IN CHRONIC MYELOID LEUKEMIA PATIENTS: RESULTS FROM THE NANOSTRING APPLICATION.

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Background: Tyrosine kinase inhibitors (TKIs) changed the destiny of patients with chronic myeloid leukemia (CML), but one third of them do not reach or loss the deep molecular response. The mechanisms of resistance are not fully elucidated: perhaps resistant LSC remain hidden in the hypoxic zones of the bone marrow niche where they do not proceed to the RNA and protein synthesis. Moreover, the presence of high levels of PD1 and PD-L1 could reduce the efficacy of the immune system against persistent LSC. TKIs have been described to interfere with immune system, as demonstrated also by the higher rate of TFR success in cases previously treated with interferon.

Aims:

To perform gene expression profile in patients receiving Imatinib at diagnosis and after 6 months of treatment, focusing on immunity and inflammation.

Methods:

the Nanostring nCounter Human Myeloid Innate Immunity V2 panel was employed for simultaneously analyzing RNA levels of 730 genes in 6 patients at diagnosis and in 5 after 6 months of treatment with Imatinib. These genes belong to angiogenesis, adhesion, antigen presentation, complement, pathogens response, lymphocyte activation, extra-cellular matrix, chemokines, interferon, cytokines pathways. Principal Component Analysis was performed in order to also distinguish between sensitive (3) and resistant (2) patients.

Results:

1) immunity cells: overall, by 6 months Imatinib significantly reduced dendritic and mast cells, while increased cytotoxic, exhausted CD8+ and NK lymphocytes. Neutrophils and B cells remained substantially at the same levels measured at diagnosis. Interestingly, in the resistant cases some differences were observed: NK number at diagnosis was lower and after 6

months of Imatinib dendritic and mast cells increased instead of decreasing. **2) down-regulated genes:** 69 genes were significantly down-regulated during treatment ($p<0.05$); these genes are involved in angiogenesis, cell cycle, cell growth, chemokines, antigen presentation, adhesion, extracellular matrix, and T cell immunity. Among them, MMP8 and MMP9, whose low levels have been already shown to be prognostically positive, several MAPK, ANGIOPOIETIN-1, CD70, that is able to activate Wnt pathway and sustain the stemness of acute leukemia cells, several S100 genes, whose de-regulation is able to reduce macrophagic activity, ELL2, a transcriptional factor, and several adhesion molecules, such as CEACAM1, CEACAM8, NECTIN1, and some pro-inflammatory genes (PGLY1, PCARD). Finally, a Th2 immunological response seems to be reduced in favor of the Th1 one. Interestingly, resistant patients presented a gene expression pattern at diagnosis different from that of sensitive cases, with a higher number of gene with low expression; **3) up-regulated genes:** only 16 genes resulted significantly up-regulated. Among them, CD74, FOSL1 and TLR5, that are commonly involved in autoimmune diseases, NFATC2, that mirrors T-cell activation, and IL12, involved in T and NK cells activation, so inducing a good T and NK control of disease but also the activation of some pro-inflammatory aspects.

Summary/Conclusion: Conclusions: even if on a very small number of cases, our study shows that Imatinib impacts on inflammation, immunity and microenvironment, inhibiting cell growth, proliferation, stemness, adhesion to the bone marrow niche but also sustaining some genes involved in autoimmune diseases and supporting NK response. These results could explain some phenomena commonly observed in the clinical practice. Acknowledgments: this study has been performed with the support of University of Pisa PRA 2018 PI Prof. Petrini

CML AdvocatesNetwork

(EP739) DO NOT WASTE THE DATA: A PIPELINE FOR EVALUATION OF BCR-ABL1 KINASE DOMAIN MUTATION USING INDIVIDUAL THRESHOLDS

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Background: Next-generation sequencing (NGS) is technology enabling to detect low-level BCR-ABL1 mutations, thus to early reveal a resistant clone of chronic myeloid leukemia (CML) to tyrosine kinase inhibitors (TKI). For reliable data evaluation, it is important to set up a threshold of sensitivity to distinguish artificial variants from true mutations. In the previous works, it has been shown that the error frequency and distribution depend on the sequence context and that some of the nucleotide changes occur in higher frequencies.

Aims: To count the individual thresholds for mutations detected through the entire kinase domain (KD) of BCR-ABL1 by NGS and to implement the bioinformatic pipeline into the routine laboratory practice.

Methods: RNA samples from leukocytes were isolated from 30 healthy donors and the transcript was subsequently analysed for ABL1 KD nucleotide variants. The NGS libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina) and sequenced on the Miseq platform. Data were processed with the NextGENe software (Softgenetics). From the numbers of each nucleotide on each sequenced position of 30 healthy donors, the values of the limits of blank (LoB), detection (LoD) and quantification (LoQ) were counted.

Results: Sequenced amplicons of ABL1 KD of healthy donors were 1475 bp long and covered the amino acids V33-D504 (ref. seq. NM_005157). Only those nucleotide positions with minimum of 1000 reads both in forward and reverse reads were used and number of reads were normalized to 3000 total reads to count the limits. The LoQ of specific nucleotide change across the amplicon was 30 for the transversions, as for the transitions it was 68 for A/G, 28.5 for C/T, 35 for G/A and 68.5 for T/C. Detection of mutations was improved by calculation of LoQs for each nucleotide position. Then the final thresholds were generated for each nucleotide position from the greater LoQ value - specific nucleotide change versus each nucleotide position. As a result, each position has its own threshold both for transversion and transition. The limits of sensitivity of top 24 resistant mutations were counted (LoQ/3000*100) with the median 1.5% (1.0-3.7%) of mutated BCR-ABL1 from total BCR-ABL1 (Figure

1). In every routine analysis two healthy controls are included and examined using LoDs to avoid possible error spots of the particular analysis. For an automatic processing of the evaluations of sequences of CML patients the inhouse programme called NextDOM was created using Matlab software. NextDOM controls the quality of the data by counting the number of reads of particular positions and excluding positions with inadequate numbers of reads. NextDOM analyses the data after primary processing with NextGENe software and additionally implements the threshold set giving the statistically significant variants as an outcome (p-value = 0.05).

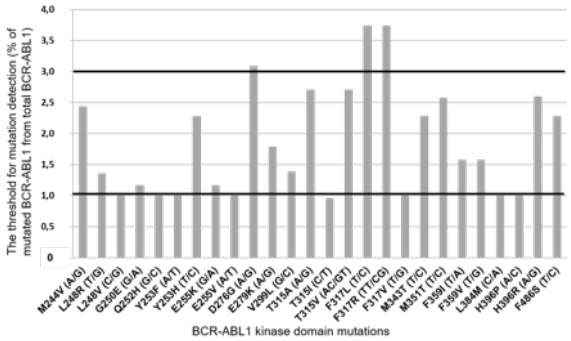

Figure 1. The graph represents the calculated values of individual thresholds for top 24 clinically relevant mutations of BCR-ABL1 kinase domain. The bold lines highlight the most frequently used uniform thresholds levels - 1% and 3%.

Summary/Conclusion: Published studies applied the threshold of sensitivity for BCR-ABL1 mutation detection by NGS to 1%, 3% or 5% uniformly for the whole sequenced region. Here, the innovative approach using sequencing data from samples of healthy donors was applied to calculate the threshold levels for each sequenced position individually. This approach represents an economical work with data, which does not “waste” variants at significant levels - true mutations - that may be filtered out using universal thresholds of 5% or 3%. On the other hand, the universal threshold 1% cannot be applicable for many variants with high probability of false-positive mutation calling.