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Plasma proteomics in CML patients before and after initiation of tyrosine kinase inhibitor therapy reveals induced Th1 immunity and loss of angiogenic stimuli

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\section*{A B S T R A C T}

\textbf{Background and aims:} The simultaneous measurement of many proteins is now possible using multiplex assays. In this pilot study we investigated a total of 124 proteins in plasma from chronic myeloid leukemia (CML) patients with the purpose of identifying proteins that are differentially expressed at diagnosis and after tyrosine kinase inhibitor (TKI) treatment initiation.

\textbf{Methods:} Samples were taken from 14 CML patients at diagnosis and after three months of TKI treatment (imatinib or dasatinib). Samples were analyzed by Mesoscale Discovery, Myriad RBM MAP technology and Olink Proseek.

\textbf{Results:} Multiple plasma proteins were differentially expressed before and after initiation of TKI therapy. Protein patterns demonstrated a possible shift towards Th1-immunity and reduced angiogenic stimuli. Further, some plasma proteins were identified that can be of potential interest to study further for biologic, prognostic or therapeutic significance such as E-selectin, uPAR, growth hormone and carbonic anhydrase IX.

\textbf{Conclusions:} Plasma proteomics seems feasible and useful in CML patients, both for studying patterns of protein expression and for identifying single proteins differentially expressed before and after treatment. Plasma proteomics may be useful to map disease activity and biological processes. Hence, plasma proteomics can be used to understand drug mechanisms and treatment responses in CML.

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1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by the constitutively active tyrosine kinase BCR-ABL1 [1]. Today, standard treatment with a tyrosine kinase inhibitor (TKI), conveys excellent long term-prognosis [2,3] but there are still problematic issues in a smaller proportion of CML patients related to TKI resistance and long-term treatment side effects and costs. Studies reporting on long-term remissions after stopping TKI treatment are beginning to emerge [4–6], but in clinical practice TKI treatment is still considered life-long and associated with adverse side effects such as fatigue [7] and cardiovascular events [8]. Much is still unknown about why patients respond differently to TKI treatment and why some patients are able to stop TKI treatment without disease relapse while others relapse quickly.
2. Materials and methods

2.1. Patient samples and clinical parameters

Frozen citrate plasma samples were taken from chronic phase CML patients at diagnosis (baseline) and after three months of treatment with imatinib (n = 9) or dasatinib (n = 5). All samples were obtained from Helsinki University Central Hospital and the patients were included in the clinical study NordCML006 [13] randomizing patients in chronic phase CML to treatment with either imatinib or dasatinib as first line treatment. Patients and study results are described in detail elsewhere [13]. Clinical characteristics (Table 1) were collected from the study case report forms to correlate them to plasma proteins. Sokal scores were calculated as previously described [14]. Treatment responses were assessed by qRT-PCR for BCR-ABL1 and major molecular response (MMR) was defined as BCR-ABL1 \( \leq 0.1\% \). Data on leukemic stem cells were obtained from a spin-off project to the clinical study [11]. The study was conducted in accordance with the Helsinki Declaration and was approved by the Regional Research Ethics Committee. All patients gave their written informed consent.

2.2. Multiplex analyses

All samples were analyzed on three different multiplex platforms: Mesoscale Discovery, Myriad RBM MAP technology and Olink Proseek. Methods for the Mesoscale and Myriad RBM kits have been previously described [15] and will therefore only be commented briefly.

2.3. Human proinflammatory 9-plex ultra-sensitive kit by Mesoscale

Analysis of the Human proinflammatory 9-plex Ultra-Sensitive kit (Mesoscale Discovery, Rockville, MD, USA) was performed according to the instructions from the manufacturer and samples were run in duplicates. The Lower Limit Of Detection (LLOD) was defined as 2.5 standard deviations above assay background and values below the LLOD were reported as “below detection range”.

2.4. Multi-analyte profiling (MAP) technology by Myriad RBM

Tubes with frozen patient samples were shipped to Myriad RBM (Salt Lake City, UT, USA). Analysis of the custom human MAP containing 44 different plasma proteins was run according to the laboratory’s standard operating procedures. Samples were run in singlets. The Least Detectable Dose (LDD) was defined as the mean +3 standard deviations of 20 blank readings and the Lower Limit of Quantitation (LLOQ) was defined as the concentration of an analyte at which the coefficient of variation of replicate standard samples was \( \geq 30\% \) and values below this limit were reported as <LLOQ.

2.5. Olink Proseek multiplex oncology 1

The Olink Proseek multiplex Oncology 1 comprises 92 cancer-related analytes. Frozen aliquots of patient samples were sent to Olink Bioscience in Uppsala, Sweden, and analyzed there according to the laboratory’s standard operating procedure. Samples were run in duplicates. The Limit Of Detection (LOD) was defined as 3 standard deviations above background and values below this limit were reported as <LOD. At the time of analysis it was not recommended to determine absolute concentrations from the Olink kit and values are thus reported in relative units referred to as normalized protein expression (NIPX).

2.6. Data analysis

The focus of this study was to investigate treatment-related changes in the levels of different analytes before and after treatment in an explorative purpose and not to determine and compare exact concentrations. We therefore included extrapolated data in the analyses. Since non parametric tests were used to assess differences, values were set to 0 if they were below detection range in the MSD kit, below the LLOQ in the Mesoscale kit, and below the LOD in the Olink kit. For duplicate samples in the MSD and Olink kits with one value above the LOD/LLOD and one value below, the value below was set to 0 and the mean value was calculated in analogy with the other duplicate samples. Analytes that were detectable in fewer than three samples (approximately 20%) before and after treatment were excluded from further analysis.

2.7. Statistical analysis

Statistical analyses were done in Graph pad prism and SPSS. Since Gaussian distribution could not be assumed, non-parametric tests were used. Wilcoxon matched-pairs signed rank test was used to assess differences for each analyte before and after treatment. To assess correlations, the Spearman \( r \) was used. Independent samples Mann-Whitney U test was used to assess differences in the levels of analytes according to treatment response and TKI type. P-values of \( <0.05 \) were considered statistically significant. No corrections were made for multiple testing since this was an explorative study.

3. Results

3.1. Platform output

On the MSD platform, nine analytes were run. With extrapolated values included, only one (11%) analyte did not have measurable concentrations as defined above. For five (56%) of the analytes extrapolated data were included. Forty-four proteins were analyzed with the Myriad platform and 21 (48%) of these had measurable concentrations as defined above. Extrapolated data were included for eighteen (41%) of the analytes. Using the Olink platform, 92 analytes were run and 82 (89%) of these had measurable values before and after treatment. For sixteen (17%) analytes one or more extrapolated values were included in the analyses. Several analytes showed statistically significant differences before and after three months of TKI treatment and are summarized in Table 2. Several analytes were included in more than one kit (CD40 ligand, E-selectin, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN\( \gamma \)), interleukin (IL) 12p70, IL1 receptor (\( \kappa \)) antagonist (IL1R\( \kappa \)), IL2, IL4, IL6, IL6 R alpha (IL6R\( \alpha \)), IL7, IL8, IL10, monocyte chemotactic protein (MCP)-1, matrix metalloproteinase 3 (MMP3), stem cell factor (SCF), tumor necrosis factor
alpha (TNFα) and vascular endothelial growth factor A (VEGF-A)). In most cases we found similar trends concerning the direction of change post treatment (i.e. increase/decrease/unmeasurable). For a few analytes, however, results from different kits were contra-
dicting (IFNγ, IL6, IL8, IL10, and MMP3). Concentrations of these analytes were generally low in plasma and results were close to the platforms’ limits of detection.

Some categories of proteins were evaluated in more detail: immunological cytokines, chemokines and their receptors, angiogenesis factors, hematopoietic growth factors and other analytes significantly altered by treatment. Plasma proteins were correlated to Sokal score, white blood cell counts (WBC) at baseline, the level of Philadelphia (Ph+) positive stem and progenitor cells at baseline and to treatment response in terms of major molecular response (MMR) at 12 months.

3.2. Immunological cytokines, chemokines and their receptors

Significantly altered analytes are shown in the overview waterfall plot in FIG. 1A that demonstrates the mean fold change (+SD) of all patients. We divided the markers in pro- and anti-tumorigenic as shown in FIG. 1A, in which pro-tumorigenic markers also are Th2-promoting molecules and the anti-tumorigenic markers are Th1-promoting molecules. Most patients had upregulation of one or more Th1 markers and simultaneous downregulation of one or more Th2 markers. Except for IL8 which was increased (p = 0.0105 and 0.0067, Mesoscale and Olink, respectively), all significantly altered pro-tumorigenic analytes decreased post treatment initiation: myeloperoxidase (MPO) (p = 0.0001), IL10 (p = 0.027), C-C motif ligand (CCL4) (p = 0.0203), myeloid progenitor inhibitory factor (MIPF1) (p = 0.0084), IL6R (p = 0.0012), IL2R alpha (p = 0.0031), transforming growth factor beta1 (TGFβ1) (p = 0.0166) and MHC class 1 polypeptide-related sequence A (MICA) (p = 0.0122). The level of IL8 was significantly higher pre-treatment in patients responding well to treatment (MMR at 3, 9 and 12 months; p = 0.038, 0.008 and 0.014, respectively), the level of IL6R was higher pre-treatment in patients not reaching MMR by 3 months (p = 0.016) and both CCL24 and MICA were higher pre-treatment in patients not reaching MMR by 9 months (p = 0.026 and 0.026, respectively). The level of TGFβ1 at baseline was correlated to the percentage of Ph+ stem and progenitor cells at baseline (Spearman r = 0.62; p = 0.0212; and r = 0.54; p = 0.0491) (FIG. 1B). The principal Th2 cytokine IL4 was analyzed with both Olink and Myriad but had no measurable concentrations in either kit. Among the Th1 promoting markers in our material, some analytes had low or unmeasurable values in all kits where they were included (IFNγ, IL2, TNF beta), probably reflecting low concentrations in plasma since they normally act locally in a cell to cell manner. For IFNγ, which is considered the “hallmark cytokine” of Th1 and were included in all kits, results were uncertain with unmeasurable concentrations in >50% of samples on all platforms. However, in the ultrasensitive Mesoscale kit, a significant increase was seen post treatment (p = 0.0273). Increases were also seen in IL12 (p = 0.0040) and C-X-C motif ligand (CXCL9) monokine induced by gamma interferon (MIG) (p = 0.0007) whereas were decreased in CD40 (p = 0.0308), TNF receptor super family member (TNFRSF) 14 (p = 0.0001), TNFR 1 and 2 (p = 0.0067 and 0.0040, respectively), IL1Ra (p = 0.0006), macrophage inflammatory protein 1beta (MIP1β) (p = 0.0039) and monocyte chemotactic protein 4 (MCP-4) (p = 0.0004). Pre-treatment levels of CD40 were positively correlated to Sokal score (Spearman r = 0.76; p = 0.002) (FIG. 1C) and CXCL9 at baseline was negatively correlated to baseline WBC (Spearman r = −0.55; p = 0.0438). No correlations were seen between other immunological cytokines and Sokal score, WBC, Ph+ stem or progenitor cells or treatment response.

3.3. Angiogenic factors

Statistically significant decreases post treatment were seen for several angiogenic factors (FIG. 2): CD31/platelet endothelial cell adhesion molecule (PECAM)-1 (p = 0.0067), E-selectin (p = 0.0006), hepatocyte growth factor (HGF) (p = 0.0001), placenta growth factor (PIGF) (p = 0.0085), transforming growth factor (TGF) α (p = 0.0052) and VEGF-A (p = 0.0245). VEGF-D on the other hand, was significantly increased post treatment (p = 0.0012). No significant correlations were found between angiogenic factors and clinical parameters (Sokal score, white blood cell count), leukemic stem or progenitor cells at baseline or treatment response.

3.4. Hematopoietic growth factors

Among the hematopoietic growth factors, significant changes after TKI treatment were seen in Fms-like tyrosine kinase 3 ligand (Flt3L), SCF and thrombopoietin (TPO) (FIG. 3). Flt3L decreased after treatment (p = 0.0166). SCF was analyzed with both Olink and Myriad and an increase was detected with both kits but was only significant with the former (p = 0.0107). TPO was significantly increased after treatment initiation (p = 0.0295). Flt3L levels were higher at baseline in patients responding optimally to treatment (MMR at 12 months, p = 0.009) but no other correlations were found between hematopoietic growth factors and clinical parameters or leukemic stem or progenitor cells at baseline or treatment response.

3.5. Other analytes significantly altered by treatment

Increases after treatment initiation were noted for carbonic anhydrase IX (CAIX) (p = 0.0005) (FIG. 4A), folate receptor alpha (p = 0.0009) (FIG. 4B), kallikrein 11 (p = 0.0107) (FIG. 4C) and Erb B4 (p = 0.0052) (FIG. 4D). Decreases were seen in the levels of growth hormone (p = 0.0166) (FIG. 4E), tetratrate-resistant acid phosphatase (TRACP) (p = 0.0295) (FIG. 4F), urokinase plasminogen activator surface receptor (uPAR) (p = 0.0004) (FIG. 4G), cathepsin D (p = 0.0016) (FIG. 4H) and Cystatin B (p = 0.0107) (FIG. 4I). No significant correlations were found between these analytes and clinical parameters (Sokal score, white blood cell count) or leukemic stem or progenitor cells at baseline.

3.6. Dasatinib versus imatinib

Since dasatinib is a multi kinase inhibitor the effect may be different from imatinib even if they in general have a similar mechanisms of action. Therefore, a statistical evaluation was done dividing the cohort into dasatinib (n = 5) and imatinib (n = 9). Most analytes behaved similarly in patients treated with either TKI but caution needs to be considered for the small cohort sizes. However, IFNγ gamma was significantly higher post treatment initiation in dasatinib treated patients (p = 0.042) and when comparing mean fold changes between treatment arms, a larger decrease in pro-tumorigenic analytes IL10 (p = 0.002), IL6R (p = 0.012) and MIPF1 (p = 0.029) was noted in dasatinib treated patients. Further, for some anti-tumorigenic analytes (CD40, p = 0.007; TNFR, p = 0.007 and TNFRIL, p = 0.004), the decrease was more pronounced in dasatinib treated patients.

4. Discussion

Proteomics is an area of growing interest and to our knowledge, the usefulness of plasma proteomics in CML patients has not been previously studied. In this pilot study, we analyzed a total of 124 different proteins in plasma from 14 CML patients before and three months after initiation of TKI treatment using three different multiplex platforms. The time point was chosen because at earlier time points, the disease burden in peripheral blood is often still large and might make it more difficult to discriminate between TKI responders and non-responders. Also, the
treatment response at three months has been increasingly recognized as prognostically important for long-term survival [16]. Many protein markers were significantly altered after three months of TKI treatment. Results for markers present in low concentration in plasma were sometimes contradicting between platforms, which may reflect the specificity and sensitivity variation of the platforms used. It also highlights possible difficulties of analyzing multiple markers of different concentration ranges in a single sample at one dilution. Nevertheless, we studied patterns of protein expression of immunological cytokines and chemokines, angiogenic factors and hematopoietic growth factors. Further, we identified a number of plasma proteins that could be of potential interest to study further.

The importance of the immune system in tumor development has been shown for various cancer forms [17] and many cytokines and chemokines are well known components of pro- and anti-tumor immunity [18]. Previous studies have pointed at a suppression of Th1-type immunity in untreated CML patients and a restoration of Th1 immunity after treatment [19–22]. In our material, a general pattern was noted in which a decrease of pro-tumorigenic analytes was seen (VEGF, TGFβ, IL10, CD31, MICA) while some analytes known to be of importance to Th1 immune responses and anti-cancer immunity were increased after TKI initiation (IL12, CXCL9/MIG, IFNγ). This likely reflects a restoration of normal immune functions after treatment initiation since TKI treatment reduces the number of tumor cells as well as myeloid-

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**Fig. 1.** Immunological cytokines, chemokines and their receptor.

(A) Waterfall plot demonstrating mean fold changes (+SD) in significantly altered cytokines and chemokines related to tumor immunity. The dashed line separates cytokines and receptors considered as pro-tumorigenic/Th2 promoting from those considered as anti-tumorigenic/Th1 promoting. Results for IL8, IL10 and IFN gamma are from the MSD platform; CD40, MIP1 beta, MCP4, IL6R and MIPF1 from the Myriad platform and all other analytes from the Olink platform. (B) Correlation between TGFβ1 and the percentage of Ph+ leukemic stem cells (LSC) at baseline using Spearman r. Data on leukemic stem cells were obtained from a previously published study where the CD34+ cell fraction was first sorted from bone marrow mononuclear cells by magnetic beads and then subdivided into stem (CD34+CD38-) and progenitor (CD34+CD38+) cell fractions where the former was considered as the LSC [11]. (C) Correlation between pre-treatment levels of CD40 and Sokal score using Spearman r. NPX = normalized protein expression.
derived suppressor cells (MDSC) [22]. Contradicting results were seen for the T-cell co-stimulatory molecule CD40 [23] and pro-inflammatory cytokine IL8. CD40 was decreased after treatment initiation in our samples. We have previously shown that CD40 expression is significantly decreased on myeloid cells in CML patients compared to healthy controls and that the expression increase on the cell surface of myeloid cells after TKI therapy initiation [22]. Hence, the decrease in CD40 plasma levels shown herein might reflect a reduced shedding of CD40 from the cell membrane of antigen presenting cells. IL8, which is known to have tumor promoting functions in solid malignancies, was increased after TKI treatment and a higher baseline level was correlated to a good treatment response. Hayashi et al. [24] previously described elevated levels of IL8 in CML patients treated with imatinib and dasatinib compared to healthy controls. They also found a correlation between IL8 levels and NK-cell reactivity. NK-cell function has previously been linked to disease control in CML [10,25,26].

Several studies indicate a role for angiogenesis in hematological malignancies. VEGF-A is overexpressed in both AML and CML [27,28], and increased bone marrow vascularity has been found in CML [27]. PIGF and HGF have been shown to be elevated in CML patients and reflect disease burden [29–31]. Expression of VEGF-D has been described in myeloid progenitors and in early blast cells of the granulocytic line in CML patients but also in reactive lymphoid tissue [32]. In AML, higher levels of VEGF-D were associated with a higher survival probability after chemotherapy [33]. We noted

Fig. 2. Angiogenic factors.
Differences before and after three months of TKI treatment were assessed with the Wilcoxon signed ranks test. (A) PECAM 1, (B) HGF, (C) PIGF, (D) TGFα, (E) VEGF-D, (F) E-selectin. Long horizontal bars represent median values; short bars represent max and min. NPX = normalized protein expression.
Fig. 3. Hematopoietic growth factors.
Differences before and after three months of TKI treatment were assessed with the Wilcoxon signed ranks test. (A) Flt3 ligand, (B) SCF, (C) THPO. Long horizontal bars represent median values; short bars represent max and min. NPX = normalized protein expression.

Fig. 4. Analytes significantly altered post treatment initiation.
Differences before and after three months of TKI treatment were assessed with the Wilcoxon signed ranks test. (A) CAIX, (B) FR alpha, (C) Kallikrein11, (D) ErbB4, (E) TRACP, (G) uPAR, (H) CathepsinD, (I) CystatinB. Long horizontal bars represent median values; short bars represent max and min. NPX = normalized protein expression.

a general decrease of angiogenic factors after treatment that could reflect a normalization of bone marrow angiogenesis after TKI treatment. The increase in VEGF-D levels that we found could possibly be connected to an immune activation and restoration of immune functions as previously described.

Some of the analytes in our material have been connected to the function of leukemic stem cells. In a murine study, BCR/ABL positive progenitors where shown to be more dependent on selectins for homing and engraftment than normal stem cells [34] and especially E-selectin was found to be critical to leukemic stem cell engraftment. In this small material we found that E-selectin decreased after treatment initiation but was not correlated to Ph+ stem- or progenitor cells. However, we found that TGF beta, which has been connected to the maintenance of leukemia initiating stem cells [35], was correlated to the leukemic stem cell burden at baseline.

In regard to hematopoietic factors, the level of Flt3L was decreased while an elevated level of SCF was noted post treatment initiation. Flt3L interacts with Flt3 that is expressed in primitive hematopoietic progenitors (CD34+) and signaling through Flt3 is important in early hematopoiesis [36]. The decrease that we found
in Flt3L could perhaps be linked to the depression of hematopoiesis often seen upon TKI treatment. However, higher baseline levels of Flt3L were correlated to good treatment responses. Flt3L signaling is also known to have potent effects on development of the immune system and knock-out mice lacking Flt3L have reduced numbers of myeloid and lymphoid progenitors in bone marrow as well as a marked deficiency of DCs and NK-cells in lymphoid tissue [36]. Perhaps higher measurable Flt3L levels is a marker for maintained immune functions conveying better disease control. SCF binds to a receptor tyrosine kinase called c-kit and hematopoietic stem cells at all stages of differentiation express c-kit on their surface [37]. In a study on patients with gastrointestinal stromal tumors treated with imatinib [38], levels of SCF increased after 1 and 6 months of treatment. The proposed explanation was a compensatory mechanism to maintain hematopoiesis and other c-kit-related functions and our finding of elevated SCF levels post treatment initiation might support this thesis.

Among the plasma proteins not sorted into any category of analytes, CAIX, GH and uPAR were considered to be of potential interest. CAIX has been identified as a tumor-associated antigen in AML, and a high expression of CAIX was associated with a favorable outcome [39]. We found that CAIX increased after three months of TKI treatment. CAIX in CML has not been extensively studied and could be of potential interest to further evaluate. GH levels were decreased after treatment initiation. Imatinib has been shown to cause growth impairment in pre-pubertal children treated for CML [40] and in a study of 17 imatinib treated CML patients, 70% had severe GH-deficiency [41]. Our findings can thus be in line with other studies but the clinical significance in the adult population is unclear. However, studies have shown that adults with GH deficiency are both physically and psychologically less healthy than their age matched peers [42]. The level of uPAR was decreased after treatment initiation. uPAR is expressed on myeloid precursors but not on CD34+ hematopoietic stem or progenitor cells [43]. In CML, uPAR gene expression is higher in blast crisis than in chronic phase [44]. In a study by Mustjoki et al., soluble uPAR (suPAR) was found to be elevated in AML and decreased during chemotherapy, suggesting that suPAR was produced by tumor cells [45]. uPAR could be of potential interest to further study for prognostic significance in CML.

In conclusion, multiple plasma proteins were differentially expressed before and after initiation of TKI therapy and some patterns of protein expression were in agreement with previous findings, supporting the accuracy of the multiplex assays. Some differences were found according to treatment responses, but our cohort was too small to enable identification of TKI resistance profiles. This would have to be addressed in larger patient cohorts. We identified some plasma proteins that could be of potential interest to study further. E-selectin should be evaluated for its biological and prognostic significance. CAIX has to our knowledge not been extensively studied in CML and may have prognostic and perhaps also therapeutic value. uPAR might be of biological and/or prognostic significance. Finally, GH could be of interest to study further for a possible connection between TKI-induced GH deficiency and fatigue as a TKI related side effect. Thus, simultaneous measurement of many proteins in experimental samples using multiplex assays seems feasible and useful in CML patients.

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