Original Article
Serum-tryptase at diagnosis: a novel biomarker improving prognostication in Ph+ CML

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Abstract: Basophilia is an established prognostic variable in Ph-chromosome+ chronic myeloid leukemia (CML). However, in CML, basophils are often immature and thus escape microscopic quantification. We have previously shown that tryptase is produced and secreted by immature CML basophils. In the current study, serum samples of 79 CML patients (chronic phase=CP, n=69; accelerated/blast phase=AP/BP, n=10) treated with BCR/ABL inhibitors, were analyzed for their tryptase content. Serum-tryptase levels at diagnosis were found to correlate with basophil counts and were higher in AP/BP patients (median tryptase: 29.9 ng/mL) compared to patients with CP (11.7 ng/mL; p<0.05). In 20/69 patients with CP, progression occurred. The progression-rate was higher in patients with tryptase >15 ng/mL (31%) compared to those with normal tryptase levels (9%, p<0.05). To validate tryptase as new prognostic variable, we replaced basophils by tryptase levels in the EUTOS score. This modified EUTOS-T score was found to predict progression-free and event-free survival significantly better, with *p* values of 0.000064 and 0.00369, respectively, compared to the original EUTOS score (progression-free survival: p=0.019; event-free survival: p=0.156). In conclusion, our data show that the serum-tryptase level at diagnosis is a powerful prognostic biomarker in CML. Inclusion of tryptase in prognostic CML scores may improve their predictive value.

Keywords: CML, tryptase, survival, scoring system, prognostication

Introduction

The BCR/ABL tyrosine kinase inhibitors (TKI) have substantially improved the prognosis of patients with chronic myeloid leukemia (CML). These drugs produce major cytogenetic (MaCyR) and molecular (MMR) responses in a majority of patients with chronic phase (CP) CML [1-9]. Imatinib remains the golden standard of first-line treatment in CML. However, a number of patients fail to achieve a durable MaCyR during imatinib [10-12]. In these patients, second- or third generation TKI can be administered [12, 13]. Moreover, for young and fit patients stem cell transplantation (SCT) has to be considered. However, SCT is associated with considerable morbidity and mortality and several of the new TKI have major side effects. Therefore, it is of importance to identify 'high risk patients' who may benefit from upfront-use of second-line TKI, SCT or from an early switch from first- to second-line drugs.

Several scoring systems have been developed in order to predict the response to therapy and survival in CML, including the Sokal and Hasford score [14, 15]. Although both scores were established based on data from patients treated with hydroxyurea, busulfan or interferon-alpha, recent studies have shown that these scores are also predictive in patients treated with imatinib [16, 17]. Likewise, the Sokal score is of prognostic value in patients who receive imatinib after interferon-alpha or a 2nd-line TKI after imatinib [18, 19]. The newly developed EUTOS score is the first scoring system that has been established in patients receiving TKI [20]. Basophilia is one of the most significant and well established risk factors in CML and was therefore included in both, the Hasford score
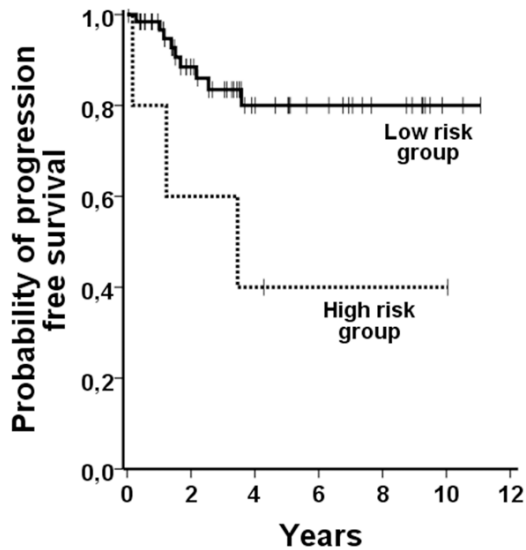
Figure 1. Survival according the EUTOS sore. When EUTOS low and high risk patients were compared with regard to PFS, the differences observed were found to be statistically significant.

and in the EUTOS score [14, 20]. However, the total basophil-compartment of the CML clone often exceeds the morphologically identifiable fraction of basophils which is a critical point because basophilia is employed as key prognostic parameter in the above mentioned scores.

In order to overcome this problem, we screened for potential markers of immature basophils. Tryptase is a serine protease primarily expressed by mast cells and immature basophils. We have previously shown that immature CML basophils express and release tryptase and that elevated serum-tryptase levels are found in a subset of patients with CML at diagnosis [21-23]. However, so far, only little is known about the prognostic value of tryptase in patients with CML. In the present study, we provide evidence that the serum tryptase level is a highly prognostic biomarker in freshly diagnosed patients with CML.

Patients and methods

Patients

Seventy-nine patients with Ph+ CML seen at our institution who received first line treatment with TKI were examined retrospectively. Sixty-nine had CML-CP, 9 accelerated phase (AP) CML and one blast phase (BP) CML. Seventy-

six patients received imatinib (400 mg/day, n=74 or 600 mg/day, n=2), two CP-patients received dasatinib (100 mg/day) and one nilotinib (600 mg/day). Parameters recorded at diagnosis included complete blood counts (CBC) and differential counts, bone marrow (bm) morphology and histology, karyotyping according to standard techniques [24], BCR-ABL transcript levels, and serum-tryptase levels. The patients' characteristics are shown in Supplemental Table S1. In all patients, the Hasford score, Sokal score, and EUTOS score, were calculated. Follow-up investigations were based on the recommendations of the European-Leukemia-Net (ELN) [25, 26]. If necessary, FISH and/or BCR/ABL mutation analyses were performed. All patients gave written informed consent before blood donation and bm puncture. The study was approved by the Local Ethics Committee of the Medical University of Vienna.

Tryptase measurements

Serum-tryptase levels were measured by a commercial fluoroenzyme-immunoassay (FIA, Thermo Fisher Scientific, Uppsala, Sweden) as described [21-23]. In all CML patients, serum tryptase levels were measured prior to therapy.

qPCR of BCR/ABL and tryptase mRNA levels in peripheral blood (pb) cells

BCR-ABL transcripts were quantified by real-time qPCR according to standard methods using the Ipsogen BCR-ABL Mbcr Kit (Quiagen, Hilden, Germany) and the LightCycler 2.0-System (Roche, Mannheim, Germany) [27]. BCR/ABL levels were expressed as percent of ABL after adjusting PCR data according to the international scale (IS) [28]. In a subgroup of our CML patients (CP, n=36; AP, n=7), tryptase mRNA levels were determined. Tryptase mRNA expression was normalized to ABL copy numbers. Detailed information is provided in the Supplemental materials.

Statistical analyses

Differences between patients' groups according to various risk-factors and scores were calculated by Kruskal-Wallis or Mann-U-test. To weigh different overlapping prognostic variables, a canonical analysis of the relationship
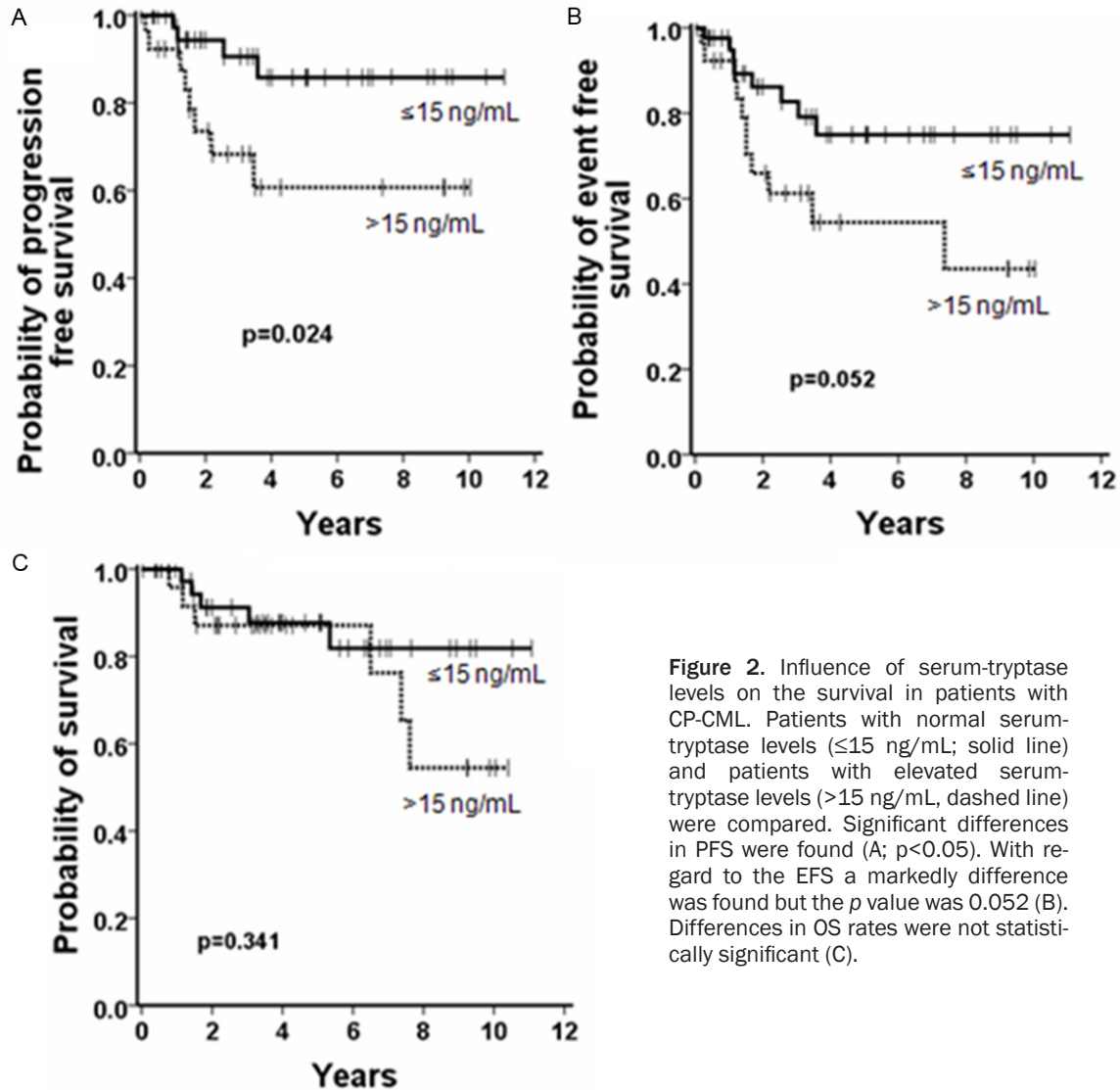
Figure 2. Influence of serum-tryptase levels on the survival in patients with CP-CML. Patients with normal serum-tryptase levels (≤15 ng/mL; solid line) and patients with elevated serum-tryptase levels (>15 ng/mL, dashed line) were compared. Significant differences in PFS were found (A; p<0.05). With regard to the EFS a markedly difference was found but the *p* value was 0.052 (B). Differences in OS rates were not statistically significant (C).

of these parameters was applied. Progression was defined as hematologic, cytogenetic (reappearance of BCR/ABL) or molecular (>1 log increase after major molecular response) relapse, or appearance of additional chromosomal abnormalities in BCR/ABL-positive or BCR/ABL-negative cells according to ELN guidelines [26]. Detailed information is provided in the Supplemental materials.

Establishment of the EUTOS-T score

Based on the correlation between basophils and tryptase and the superior prognostic value of tryptase levels, we established a new EUTOS score system in which basophil counts were replaced by tryptase levels. Like in the EUTOS score, spleen size was measured in centime-

ters below the costal margin. Spleen size and tryptase were weighed. According to this calculation the formula of the EUTOS Score was adapted as follows: serum-tryptase (ng/ml) + 5* spleen size. The cutoff for this EUTOS-T score was determined by the relationship between the score and the zero residuals [29]. A score of 80 points or less indicated low risk and more than 80 points high risk disease.

Results

Survival in TKI-treated patients with CML

The median follow up period was 3.5 years (range 0.04 to 12.76 years). OS in all patients was 67% at 5 years, the PFS reached a plateau at 75% after 3.6 years. In CML-CP patients, the
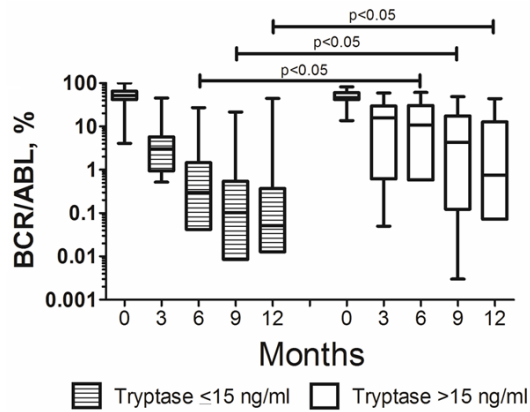
Figure 3. Follow up of bcr/abl levels in patients with normal and elevated serum tryptase levels. The bcr/abl levels decresed markedly in both, patients with normal (filled boxes) and elevated serum tryptase levels (empty boxes). However, The differences between the two groups with regard to transcript levels of bcr/abl observed at 6, 9 and 12 months were found to be statistically significant (p<0.05).

OS was 88% after 5 years and the PFS reached a plateau at 75% after 3.6 years. When applying the EUTOS score in our CML-CP patients, significant differences among the two groups were found for PFS (p=0.019; **Figure 1**), but not for OS (p>0.05). We also confirmed the prognostic value of the Sokal score (PFS p=0.042; OS p=0.012) and the Hasford score (PFS, p=0.042; OS, p=0.050).

Serum-tryptase levels in various phases of CML

The median serum-tryptase level in all patients was 12.5 ng/mL (range: 1.4-67.7 ng/mL). In 33 patients (41.8%), elevated serum-tryptase levels (i.e. >15 ng/mL) were detected, and 46 (58.2%) patients presented with normal enzyme levels. Significantly higher median tryptase levels were recorded in patients with advanced CML (CML-AP/CML-BP: 29.9 ng/mL; range 8.1-67.7) compared to patients with CML-CP (11.7 ng/mL, range 1.4-65.5; p<0.05; Supplemental Figure 1A). Serum-tryptase levels >15 ng/mL were found in 38% of CP-patients and in 70% of advanced CML patients. Significant differences in tryptase levels were also found when comparing prognostic risk groups of the Sokal (low, 6.3 ng/mL; intermediate, 10.7 ng/mL; high, 24.7 ng/mL), Hasford (low, 8.5 ng/mL; intermediate, 14.7 ng/mL; high 20.2 ng/mL), and EUTOS Score (low, 10.3 ng/mL; high, 33.2 ng/mL; p<0.05; Supplemental Figure 1B-D).

Identification of serum-tryptase as independent prognostic variable in CML

Twenty-six of our CML-CP patients (38%) had elevated serum-typtase levels, and 43 (62%) normal enzyme levels. In the cohort with high-tryptase, 30.8% (8/26) of the patients had a disease progression compared to 9.3% (4/43) in the normal serum-tryptase group (p<0.05). The 5-year PFS was 86% and 61% in the low and high tryptase group, respectively (**Figure 2A**). Marked differences were also seen when calculating EFS. Here, an event occurred in 42.3% (11/26) of patients with elevated serum-tryptase and 18.6% (8/43) of patients with normal tryptase. The 5-year EFS rates were 75% and 54% in the low and high tryptase group, respectively (**Figure 2B**; p=0.052). No significant differences in OS were found (p>0.05). Of the 11 patients (median age 75 years at diagnosis) who died during the observational period, six patients were in the high-tryptase group and five in the low-tryptase group. The 5-year OS rates were 87% for both the tryptase ≤15 ng/mL and the tryptase >15 ng/mL group (**Figure 2C**).

Comparison of tryptase with other prognostic variables

We found a positive correlation between serum-tryptase levels and other prognostic variables including basophils, peripheral blast cell counts, and bm blasts in CML-CP. Canonical analysis of the relationship of these parameters and PFS showed that tryptase had the highest weight. Multivariate analysis, including other pb-parameters i.e. white blood cell counts, platelet counts, percentage of eosinophils, and hemoglobin together with tryptase showed, that tryptase was an independent prognostic variable with regard to PFS.

We also measured tryptase mRNA levels in circulating leukocytes of 43 patients (CP, n=36; AP, n=7). In all CML patients tested pb-cells expressed tryptase mRNA. As expected, tryptase mRNA levels varied from patient to patient. As expected, tryptase mRNA levels correlated with serum-tryptase levels as assessed by Pearson correlation (r=0.56; p<0.001) (Supplemental Figure 2).
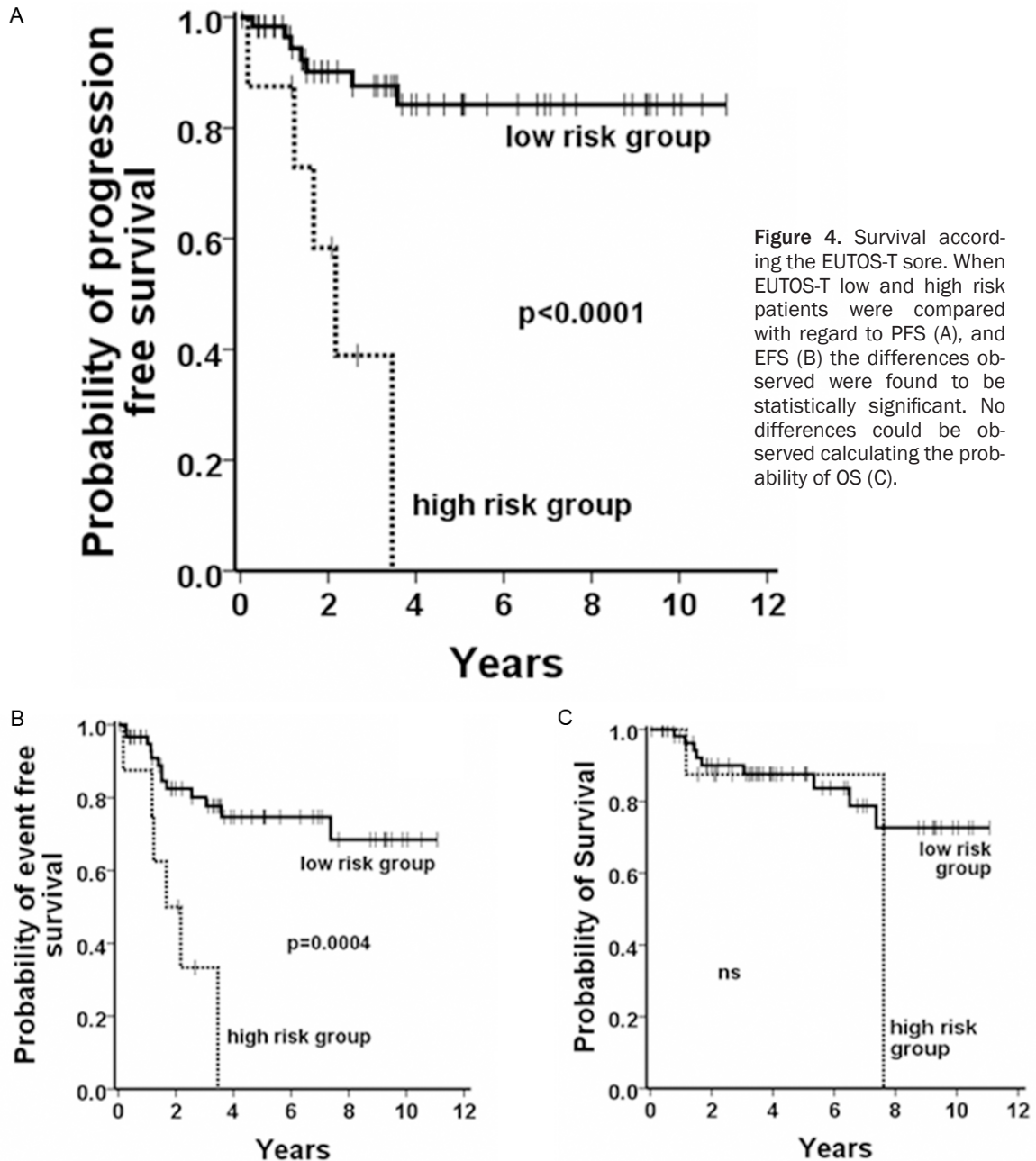
A

Figure 4. Survival according the EUTOS-T sore. When EUTOS-T low and high risk patients were compared with regard to PFS (A), and EFS (B) the differences observed were found to be statistically significant. No differences could be observed calculating the probability of OS (C).

B

C

Transcript levels of BCR/ABL were also measured in the follow up and compared to tryptase levels. In both groups of patients (those with elevated and those with normal tryptase), a marked decrease in BCR/ABL was observed over time. However, in the group of patients with tryptase levels ≤15 ng/ml, the decrease of BCR/ABL was faster during the first year when compared to patients with serum-tryptase levels >15 ng/ml. These differences in the BCR/ABL transcript levels in the two groups of

patients were found to be statistically significant at 6, 9 and 12 months (**Figure 3**).

A proposed score including tryptase as prognostic variable: EUTOS-T

To evaluate whether the prognostic value of the EUTOS score in CML-CP would increase by including tryptase, we replaced the percentage of basophils by serum-tryptase levels. Using this modified EUTOS-T score, 61 patients with

CML in CP (88.4%) were classified as low risk and 8 patients (11.6%) were considered high risk. Significant differences were found between the two risk groups in both, the PFS and EFS (p<0.0001; **Figure 4**). The 5-year PFS rates were 84% and 0% for the low and high risk group, respectively. Of all patients, 11.5% (8/61) of the low risk group and 62.5% (5/8) of the high risk group progressed. EFS rates at 5 years were 75% and 0% in low and high risk patients, respectively. Of all patients with 21.3% (13/61) in the low risk group developed an event and 75% (6/8) in the high risk group. No significant difference was found between these two groups with regard to OS.

Discussion

The BCR/ABL TKI have significantly improved the survival in patients with CML. The rate of complete cytogenetic response increased from 30% (with interferon-alpha) to >70% with imatinib [1-8, 30, 31]. However, still several patients are unresponsive to imatinib or relapse. Recently, second- and third-generation TKI have been developed and have shown beneficial results in untreated and imatinib-resistant CML [6, 7]. However, due to their side effects, these novel TKI cannot be offered to all patients. Currently available guidelines suggest that these drugs should be reserved for those who are at high risk for disease-progression [10-12]. For patients who are resistant to novel TKI or present with BP, hematopoietic SCT remains a treatment option [32, 33]. Whereas in advanced CML it is straightforward to offer SCT, the treatment algorithm in CP is more complex. Although several scores have been established in the past, it is difficult to estimate the exact risk of progression during TKI therapy.

Basophilia is one of the most powerful predictive markers in CML [14, 20]. However, the identification and quantification of immature basophils may be difficult, since the cells often exhibit little or no granulation. Tryptase, a serine protease expressed by mast cells and immature basophils has been suggested as potential marker of the total basophil burden in CML. In contrast to mature basophils, immature (CML) basophils are able to synthesize and to release substantial amounts of tryptase [21]. Indeed, elevated tryptase levels were found in CML [22] which was confirmed in the current study. Moreover, pb leukocytes in CML expressed substantial amounts of tryptase mRNA. Other blood leukocytes do not express tryptase under physiologic conditions [34-35]. However, immature myeloid cells can produce substantial amounts of tryptase and the enzyme is elevated in patients with acute myeloid leukemia, myelodysplastic syndromes and chronic eosinophilic leukemia [22, 35-38].

Elevated serum-tryptase levels were detected more frequently in patients with advanced phase CML compared to patients with CP CML. The fact, that an elevated tryptase level is of prognostic significance was confirmed by the observation that enzyme levels were significantly higher in high risk patients than in low risk patients in prognostic scoring systems, including the Sokal, Hasford, and EUTOS score. Moreover, CP patients with elevated tryptase levels had a significantly higher progression- and event rate compared to patients with normal tryptase levels. Finally, the decrease in the BCR/ABL, one of key markers indicating responses in CML patients in the follow up, was faster and deeper in the group of patients with normal serum tryptase levels compared to those with elevated enzyme levels. An interesting finding was that tryptase was not of predictive value for OS. Nevertheless, a `late drop` in the survival curve in the high tryptase group and a higher mortality-rate in this group was observed. In this regard, it is noteworthy that several high risk patients switched from one TKI to another and that this may explain the discrepancy in the prognostic value of tryptase regarding PFS and OS. In line with this hypothesis, the majority of our CML patients who progressed and then switched the TKI is still alive. In order to detect an influence of an elevated tryptase level on survival, a longer observation period may be necessary. Overall, tryptase at diagnosis seems to be a strong prognostic parameter predicting disease progression in CML and could thus be of importance in the decision to treat patients with CP CML.

Although we found a correlation between the percentage of pb basophil counts and serum tryptase levels, there are patients with high tryptase levels and a rather low basophil count and vice versa. This difference may be explained by the fact that tryptase is primarily expressed in immature basophils, which are often difficult to identify due to their hypo-granulated (or even

non-granulated) cytoplasm. Therefore, the total basophil burden can be underestimated in such patients. On the other hand, the percentage of basophils in the pb may not always reflect the total basophil body burden. Tryptase measurement may thus be a preferable marker in CML.

We also analyzed the prognostic value of tryptase concerning PFS and EFS. So far, the only established scoring system developed for patients receiving TKI is the EUTOS score. The PFS of our patients differed significantly between the high and low risk group according to the EUTOS score which is in line with other publications [19, 39]. In an attempt to improve the prognostication by EUTOS, we replaced the percentage of basophils with serum tryptase level. Indeed, with a cut off of 80 ng/ml, this modified EUTOS-T score was able to distinguish significantly between low and high risk patients regarding EFS and PFS.

In conclusion, tryptase is a new robust prognostic marker in CML. Elevated serum-tryptase levels are of prognostic significance for PFS and EFS. Moreover, when used to replace the percentage of basophils in the EUTOS score, tryptase appears to improve this scoring system, most probably by better reflecting the total basophil burden (mature+immature basophils) in these patients.

Acknowledgements

Disclosure of conflict of interest

WRS received honoraria from Novartis, TP, GH, SH, CS, CM, and MK have nothing to disclose. PV is a consultant of Novartis, received research support from Novartis and received honoraria from Novartis, BMS, Pfizer and Ariad.

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Supplementary data

Materials and methods

qPCR of BCR/ABL and tryptase mRNA levels in pb-cells

BCR-ABL transcripts were quantified by Real-time PCR according to a published protocol, using the Ipsogen BCR-ABL Mbcr Kit (Quiagen, Hilden, Germany) and the LightCycler 2.0-System (Roche, Mannheim, Germany) [43]. BCR/ABL levels were expressed as percent of ABL after adjusting PCR data according to the international scale (IS) [44]. In a subgroup of our CML patients (CP, n=36; AP, n=7) tryptase mRNA levels were determined. In brief, tryptase and ABL mRNA levels were quantified on a CFX96 Touch Real-Time PCR Detection System (Biorad, Hercules, CA) using iTaq SYBR Green Supermix with ROX (Biorad) and the following primers: huTPS_fwd 5'-CGG GAA CAC CCG GAG GGA CT-3', huTPS_rev 5'-GCC TGC AGC CAG GTG CCA TT-3', huABL_fwd 5'-TGT ATG ATT TTG TGG CCA GTG GAG-3', and huABL_rev 5'-GCC TAA GAC CCG GAG CTT TTC A-3'. Absolute copy numbers were determined from a standard curve prepared with plasmid standards. Tryptase mRNA expression was normalized to ABL copy numbers.

Statistical analysis

Differences in tryptase levels between patients' groups according to various risk-stratifications, were calculated by Kruskal Wallis or Mann U test. The correlation between tryptase levels and basophils, peripheral blast cells and white blood counts was analyzed by linear regression. To weigh different overlapping prognostic variables, a canonical analysis of the relationship of these parameters was applied. Tryptase mRNA and serum-tryptase levels were correlated by Spearman correlation. Overall survival (OS) was defined as the time from diagnosis to death and progression free survival (PFS) as the time from TKI start to loss of response, event free survival (EFS) as time from TKI start to progression or death. Progression was defined as hematologic, cytogenetic (reappearance of bcr/abl) or molecular (>1 log increase after major molecular response) relapse, or appearance of additional chromosomal abnormalities in BCR/ABL-positive or BCR/ABL-negative cells according to ELN guidelines. The prognostic value of variables was analyzed by Cox regression, differences in survival between the patients' cohorts were calculated log rank test. A *p*-value <0.05 indicated statistical significance.

Table S1. Patients' Characteristics

	All patients (n=79)	Chronic Phase (n=69)	Advanced phase (AP=9; BP=1)
Age; median (range)	54 (21-86)	55 (21-84)	53.5 (27-86)
Female/male ratio	1/1.63	1/1.6	1/2.33
WBC (G/L)	94 (14.5-365.44)	79.9 (14.5-365.4)	178.6 (22.7-337.3)
Hemoglobin (g/dL)	11.9 (7.7-15.7)	12.2 (7.7-15.7)	10.5 (9.0-13.9)
Platelets (G/L)	373 (71-1375)	351 (71-1375)	390 (322-958)
Basophils (%)	4 (0-30)	4 (0-14)	14 (7-30)
Eosinophils (%)	3 (0-16)	2 (0-16)	6 (2-13)
Bm blasts (%)	2 (0-6)	1 (0-6)	3.5 (2-10)
Sokal Score (%; low/ intermediate/high)	38/34/28	42/36/22	10/20/70
Hasford Score (%; low/intermediate/high)	38/46/16	42/48/10	10/30/60
EUTOS Score (%; low/high)	85/15	94/6	30/70

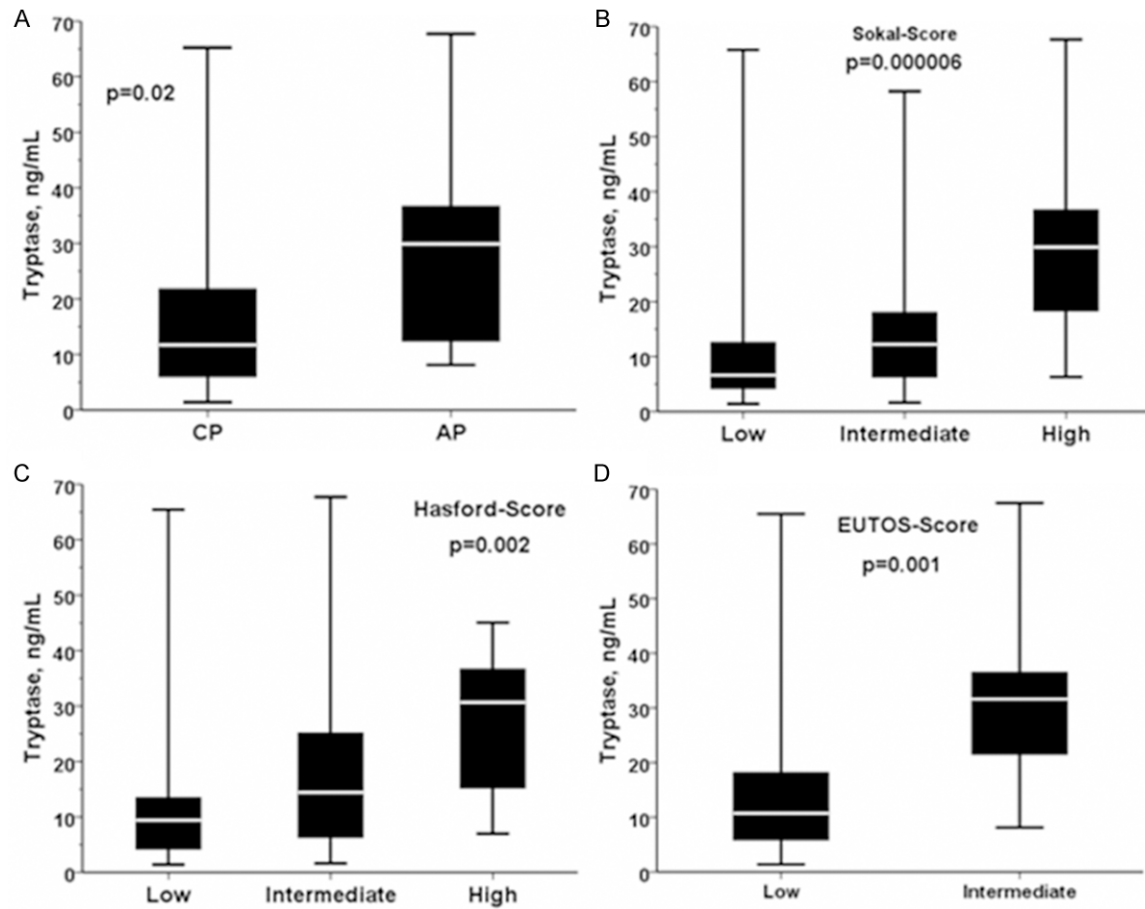WBC, white blood cell count; Bm, bone marrow; AP, accelerated phase; BP, blast phase.

Figure S1. Serum-tryptase levels in chronic and advanced phase CML and in the different risk stratification systems. The box represents the 25-75% percentile of tryptase levels in each group, the horizontal line within the boxes defines the median and the whiskers represent the range. Statistically significant differences between chronic and advanced phase CML were found (p<0.05; A). Tryptase levels between low, intermediate and high risk patients according Sokal (B) and Hasford (C) score were found to differ significantly as well as EUTOS high and low risk patients (D).
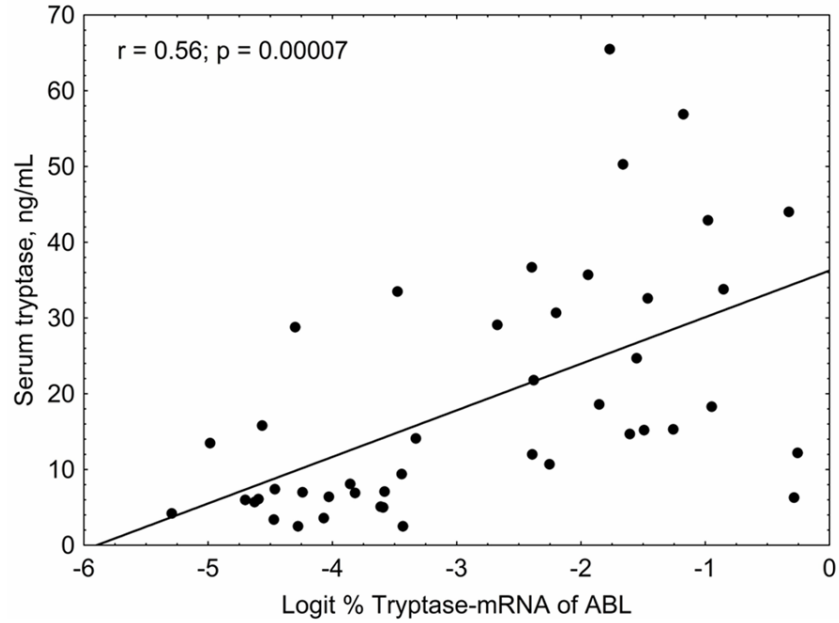
Figure S2. Correlation between tryptase mRNA- and serum tryptase levels. Peripheral blood mononuclear cells of 43 patients with CML (CP, n=36; AP, n=7) were examined for the presence of tryptase mRNA by qPCR as described in the text of the main document. ABL served as a reference gene. In the same patients, we also measured serum tryptase levels by fluoroenzyme-immunoassay. The figure shows tryptase mRNA levels as percent of ABL and tryptase levels in ng/ml. As visible, a significant correlation between tryptase mRNA and serum tryptase levels was obtained (by Pearson correlation).