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Prospective evaluation of pro/anti-inflammatory cytokines during TKI treatment in chronic myeloid leukemia patients

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ABSTRACT

Chronic myeloid leukemia (CML) treatment with tyrosine kinase inhibitors (TKIs) has been associated to an increased risk of Arterial Occlusive Events (AOEs), mainly with nilotinib, but the mechanisms underlying these events have been not clarified yet.

Previously, we confirmed in our retrospective cross-sectional study a higher cardiovascular (CV) risk in nilotinib treated patients, particularly if harboring the unfavorable OLR1 polymorphism and we found a nilotinib-associated pro-inflammatory effect.

We started a multicenter prospective study of tyrosine Kinase Inhibitors induced pro-AtherothROmbotic (KIARO) status in CML patients to furtherly investigate a possible pro-atherothrombotic nilotinib-induced status in a cohort of chronic phase (CP)-CML patients treated with first-line imatinib, nilotinib and dasatinib. In particular, our intents were: to assess any changes in the inflammation status during TKI treatment by measuring pro/anti-inflammatory cytokines (IL-6, IL-10, TNFα and ox-LDL) plasma levels; 2) to record AOEs after applying CV SCORE and evaluate its predictive role; 3) to correlate AOEs with altered inflammation status.

A total of 186 CP-CML patients were enrolled in this study of which 89/186 (48%) were treated with imatinib, 59/186 (32%) with nilotinib and 38/186 (20%) with dasatinib. Results from biochemical analyses performed by enzyme-linked immunosorbent assay (ELISA) test showed higher IL-10 levels at 6 and 12 months in imatinib ($p=0.012$ and $p=0.009$, respectively) and dasatinib ($p=0.032$ and $p=0.014$, respectively) cohorts compared to nilotinib, while ox-LDL levels increased at 12 months in the nilotinib cohort ($p=0.041$) in contrast to imatinib and dasatinib. Consequently, IL-6/IL-10 and TNFα/IL-10 ratios were higher in nilotinib cohort compared to imatinib ($p=0.042$, $p=0.044$ at 6 months; $p=0.040$, $p=0.041$ at 12 months) and dasatinib ($p=0.049$, $p=0.040$ at 6 months; $p=0.041$, $p=0.044$ at 12 months), suggesting a TKI-driven pro-inflammatory status in nilotinib treated patients. We recorded an AOE only in 5% of patients and, due to the small number of events detected, it was not possible to establish a correlation between AOEs and pro/anti-inflammatory cytokine levels.

Although we applied the SCORE chart in all CML patients enrolled to better identify patients with high risk to experience AOEs, this parameter was not predictive for our cohort. This result may be explained by the strategic choice of TKI at diagnosis, as documented also by the lower median age of the nilotinib treated patients compared to the other two TKI subgroups and by the higher number of traditional CV risk factors in imatinib cohort respect to nilotinib and dasatinib.

Considering that our results showed a pro-inflammatory status in nilotinib subgroup during the first year of treatment and that AOEs occurred after a median treatment duration of 19,1 months, we believe that a further evaluation of pro/anti-inflammatory cytokines at longer treatment follow-up should be performed to better investigate the correlation between pro-atherothrombotic status and AOEs.

In conclusion, we suggest a careful selection of the TKI treatment, according to the presence of baseline CV risk factors and/or previous CV history, in order to offer the best and safe long-term TKI treatment for CML patients, considering as ultimate goal the possibility of reaching a safe TFR and reducing AOEs associated comorbidities.

INTRODUCTION

Chronic myeloid leukemia: epidemiology and clinical presentation

Chronic myeloid leukemia (CML) is a hematologic malignancy characterized by the clonal proliferation of myeloid progenitor cells. These precursors are characterized by the presence of a specific chromosomal abnormality, the Philadelphia (Ph) chromosome, which is the result of a reciprocal translocation between chromosomes 9 and 22 t(9;22)(q34;q11). This translocation leads to the expression of the BCR-ABL1 gene, generated by the fusion of the breakpoint cluster region gene (BCR) and the Abelson gene (ABL1). The constitutive tyrosine kinase activity of the BCR-ABL1 oncogenic protein is responsible for the activation of the downstream signaling pathways and consequently for the uncontrolled proliferation of the leukemic stem cells (LSCs) [1].

CML accounts for about 15% of all adult leukemias, with an incidence of 1-2 cases per 1000000 individuals and the median age of onset in western countries is approximately 57 years [2,3]. The natural course of this disease consists of three phases: chronic phase (CP), accelerated phase (AP), and blast phase (BP) or blast crisis (BC). The median duration of the CP is of 3 to 5 years then, without any therapeutic intervention, patients progress to AP which in general lasts for about 4 to 6 months before evolving into BP, which can be fatal in 3 to 6 months. CP-CML is generally asymptomatic but, when they appear, the most common symptoms are fatigue, weight loss and malaise, which are related to splenomegaly and anemia. During the CP, LSCs proliferate but still maintain their differentiation potential, which is progressively lost in the AP and BP. The transition to AP is confirmed by the presence of at least one of these hematologic features: $\geq$ 15% blasts, $\geq$ 30% blasts plus promyelocytes, $\geq$ 20% basophils, $\leq$ 100 x 10^9/L platelets unrelated to therapy. Moreover, during this phase additional chromosomal abnormalities, such as Ph chromosome duplication, trisomy 8 or isochromosome 17, can be acquired, causing the progression to BP. The presence of at least 30% of blasts into peripheral blood or bone marrow or the presence of extramedullary blastic foci are the criteria defining BP-CML [4,5].

The Philadelphia chromosome

The Ph chromosome, as it was designated by Nowell in 1962, was the first chromosomal abnormality to be associated to a human leukemia [6]. In the following years, further studies clarified that Ph chromosome is the result of a reciprocal translocation involving the long arms of chromosomes 9 and 22 [7]. This t(9;22)(q34;q11) translocation causes the juxtaposition of the breakpoint cluster region (BCR) gene and Abelson (ABL1) proto-oncogene, generating the BCR-ABL1 fusion protein [8]. The oncogenic potential of BCR-ABL1 protein, due to its constitutive tyrosine kinase activity, was then demonstrated in vivo, since it was able to induce a CML-like phenotype in mice [9].

In particular, the analysis of the genomic breakpoints revealed that their location on the BCR gene is variable, in fact three different BCRs have been described: major (M-BCR), minor (m-BCR) and micro (µ-BCR). Regarding ABL1 gene, exons 1b and 2 are usually involved in the recombination. The two most common breakpoints in M-BCR, which is present in 95% of the cases, involve BCR exons e13/14 and ABL1 exon a2, generating the e13a2 and e14a2 transcripts, also known as b2a2 and b3a2, respectively. They both determine the expression of a 210-kDa BCR-ABL1 protein (p210).

If the breakpoint occurs in the m-BCR, the e1a2 transcript is obtained, encoding a 190-kDa BCR-ABL1 protein (p190), generally typical of acute lymphoblastic leukemia (ALL). Instead, the µ-BCR is involved in the production of the e19a2 transcript, encoding a 230-kDa BCR-ABL1 protein (p230), associated to chronic neutrophilic leukemia (CNL) (**Figure 1**) [10,11].

Looking at the protein level, BCR-ABL1 constitutive tyrosine kinase activity is caused by the deletion of the inhibitory ABL SRC homology domain 3 (SH3) and by the oligomerization of the BCR coiled-coil domain (CC). Therefore, the protein persists in its phosphorylated status, leading to the activation of the downstream signaling pathways: Jak/STAT and RAS pathways, involved in growth factor independent proliferation and PI3K/Akt pathway, which causes BCL-2 overexpression and LSCs resistance to apoptosis. Moreover, the BCR-ABL1 cytosolic localization is responsible for an altered cell adhesion and the consequent early circulation of precursors [12].

This pathogenetic mechanism explains the key role of BCR-ABL1 protein in leukemic transformation and its eligibility as a target for CML treatment.

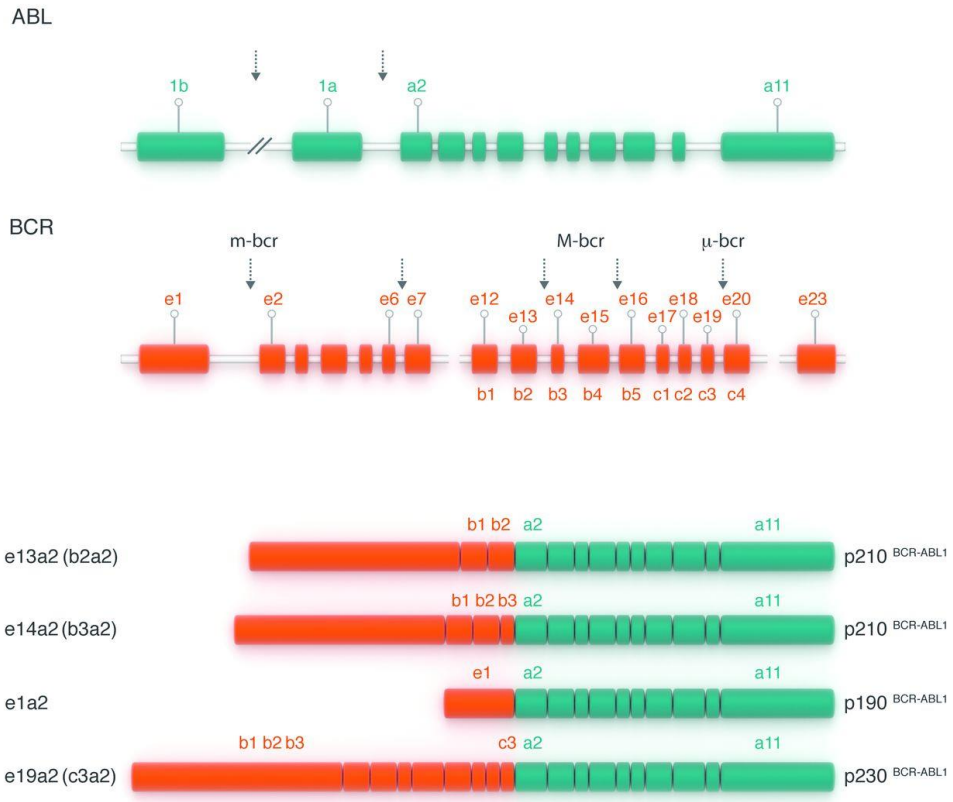

Figure 1. BCR-ABL genomic breakpoints (Mughal TI et al., Haematologica 2016).

Diagnosis, monitoring of CML and prognostic stratification

CML diagnosis generally occurs during CP through a routine blood test, where leukocytosis is documented, and is then confirmed with the detection in peripheral blood (PB) or bone marrow (BM) of Ph chromosome or BCR-ABL1 transcripts by fluorescence in situ hybridization (FISH) and reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. FISH allows the detection of BCR-ABL1+ interphase nuclei (I-FISH), while qualitative RT-PCR is used to detect the different BCR-ABL1 transcripts. Moreover, quantitative RT-PCR (RQ-PCR) is highly sensitive in monitoring minimal residual disease (MRD) [13,14].

Indeed, different levels of response are possible: hematologic, cytogenetic and molecular. Hematologic response (HR) corresponds to the normalization of the blood parameters and constitutes the first level of response. Cytogenetic response (CyR) is based on the detection of Ph+ metaphases by chromosome binding analysis (CBA) and it can be classified into none

(95%), minimal (95-66%), minor (65-36%), partial (35-1%) and complete (CCyR, 0%) [15]. Molecular response (MR), assessed through RQ-PCR, is defined, according to the International Scale (IS), as the ratio of BCR-ABL1 transcripts to ABL1 transcripts. In particular, MR is expressed as the BCR-ABL1^{IS} % on a log scale: major molecular response (MMR) or MR^{3} corresponds to BCR-ABL1^{IS} ≤0.1%, while complete molecular response (CMR) can be MR^{4} if BCR-ABL1^{IS} is ≤0.01% (≥4-log reduction from the standardized baseline), MR$^{4.5}$ if BCR-ABL1^{IS} is ≤0.0032% (≥4.5-log reduction) and MR^{5} if BCR-ABL1^{IS} is ≤0.001% (≥5-log reduction) (**Figure 2**) [16].

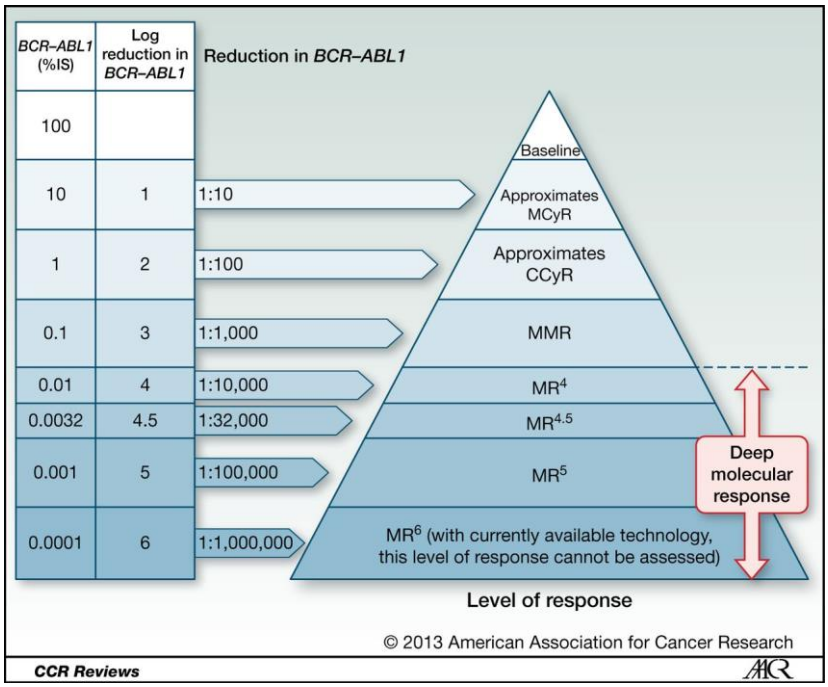

Figure 2. Molecular response levels according to the International Scale (IS) (Mahon FX at al., Clin Cancer Res. 2014).

Regarding prognostic stratification of CML patients at diagnosis, three different systems are used: the Sokal, the Euro and the EUTOS (European Treatment and Outcome Study for CML). All these scores calculate CML patients' survival risk on their baseline clinical and hematological data. In particular, the Sokal and Euro scores divide patients into three risk groups based on their overall survival (OS) during chemotherapy and interferon alpha (IFNα) treatment, respectively. The EUTOS score calculates instead OS on the possibility to achieve CCyR at 18 months in imatinib treated patients. Recently, the ELTS (EUTOS Long Term

Survival) score has been implied, which instead considers CML-related deaths during tyrosine kinase inhibitor (TKI) therapy as endpoint [17-20].

CML therapy before TKIs

Until the last century, CML treatment was predominantly based on the use of cytotoxic agents such as hydroxyurea (HU) and busulfan (BUS), referred to as conventional chemotherapy. These drugs were able to induce HR and reduce CML associated symptoms, providing a better quality of life but with a slight improvement of survival. IFNα demonstrated to be superior to conventional chemotherapy in prolonging survival and delaying disease progression but showed toxicities. Allogeneic bone marrow transplant (alloBMT) constituted instead a curative option, mainly addressed to younger patients with a suitable donor, although carrying some risks of mortality and side effects [21]. The introduction of specific TKIs has completely revolutionized CML treatment, making life expectancy of CML patients comparable to that of the general population [22].

Currently, the four TKIs implied for the frontline treatment of CML are imatinib, nilotinib, dasatinib and bosutinib, while ponatinib is only used in resistant/refractory cases (**Figure 3**). The efficacy of this therapeutic approach has made possible the maintenance of a stable long-term deep response. Indeed, TKI discontinuation is a strategy recently used in the clinical practice with the final goal of the treatment-free remission (TFR) [23,24].

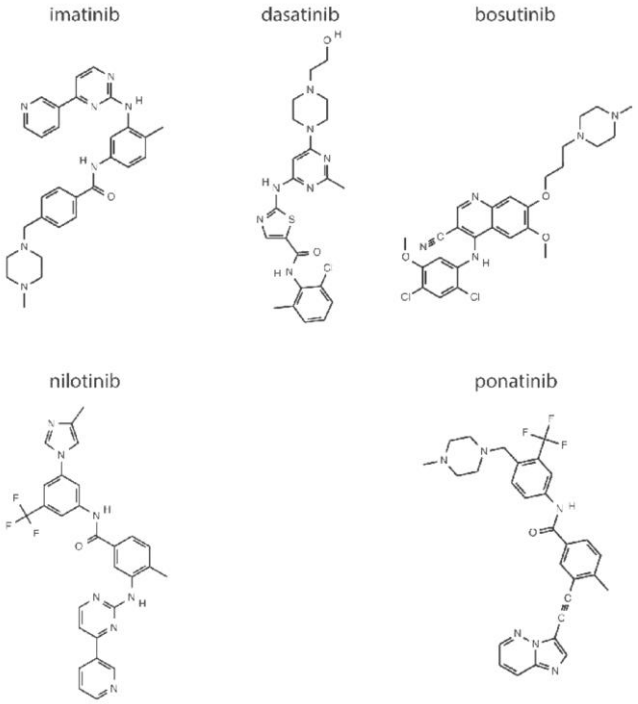

Figure 3. Tyrosine kinase inhibitors (Mughal TI et al., Haematologica 2016).

First generation TKI: Imatinib

Imatinib mesylate (STI571, Gleevec®) was the first TKI to be approved by the Food and Drug Administration (FDA) as first-line treatment for CML. This drug is a derivative of the 2-phenylaminopyridine and is metabolized by CYP3A4. Its mechanism of action is based on the selective inhibition of the ATP-binding site of the BCR-ABL1 protein, blocking the phosphorylation of the proteins involved in the signal transduction and consequently preventing cell proliferation. Although selective for BCR-ABL1, imatinib also inhibits platelet-derived growth factor receptor (PDGFR) and C-KIT tyrosine kinase. The International Randomized Study of Interferon and STI571 (IRIS) compared imatinib 400mg/daily effectiveness to IFN-α plus low dose cytarabine on 1106 CML-CP patients. Interestingly, CCyR rates at 18 months were 76% for imatinib treated patients vs 14% of IFN-α plus cytarabine and progression free survival (PFS) rates at 12 months were 98% and 93%, respectively. The 8-year follow-up of this study showed 81% event free survival (EFS), 92% PFS and 85% OS rates in imatinib treated cohort, confirming a long-term maintained response. Despite the great success of imatinib, about 20% of patients did not achieve CCyR while others underwent side effects or intolerance, developing a long-term drug resistance [25,26]. Imatinib resistance can arise due to the occurrence of point mutations affecting the ATP binding site of the BCR-ABL1 protein and one of the most common mutations is the T315I, which causes a steric hindrance preventing the binding of the drug to the target site. Other mechanisms of resistance are based on the BCR-ABL gene amplification, the overexpression of multidrug-resistant (MDR) glycoprotein and the acquisition of further chromosomal abnormalities [27,28].

To overcome the problem of imatinib resistance, initially the effects of dose escalation were tested in the phase III Tyrosine Kinase Inhibitor Optimization and Selectivity (TOPS) study, where 800mg and 400mg daily doses of imatinib were compared in CP-CML patients. No statistically significant differences in the rates of CCyR and MMR were detected between the two cohorts, although patients treated with 800mg achieved responses in a shorter time. Side effects were more frequent at higher doses but no new imatinib associated adverse events were recorded [29].

A second and a third generation of TKIs with a greater efficacy and a broader spectrum of activity were developed and included nilotinib, dasatinib, bosutinib and ponatinib.

Second and third generation TKIs

Nilotinib (AMN107, Tasigna®) is a second generation TKI with an analog structure to imatinib but showing a 50-fold higher activity and a greater efficacy on resistant cases, except for T315I. Indeed, nilotinib has a higher affinity for BCR-ABL1 inactive conformation and its potency has been demonstrated both in vitro and in vivo. As imatinib, also nilotinib effect is extended to PDGFR and C-KIT tyrosine kinases [30].

Nilotinib showed promising results in the phase 3 randomized, multicenter and open-label ENESTnd (Evaluating nilotinib Efficacy and Safety in Clinical Trials – Newly Diagnosed Patients) trial, where its efficacy and safety were compared to those of imatinib at different doses: patients treated with nilotinib 300 mg or 400 mg twice a day and patients treated with imatinib standard therapy of 400mg/day. The established primary end point was the rate of MMR at 12 months, which in both nilotinib groups was nearly twice than in imatinib group (44% and 43% vs 22%). CCyR rate at 12 months, considered as the secondary end point, was 80% and 78% vs 65% respectively in the three cohorts of patients. Moreover, nilotinib treated patients had <1% progression rate from AP to BP after 18 months of therapy and these results were confirmed at 24 months follow-up (**Figure 4**) [31,32].

Currently, nilotinib is used as frontline treatment in CML or as a second-line in case of intolerance or resistance to imatinib.

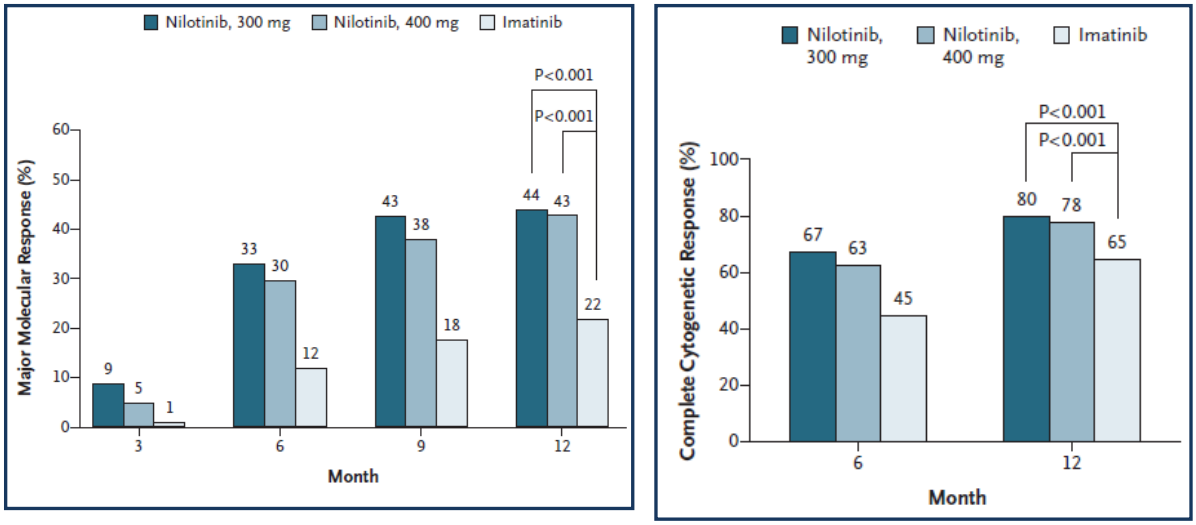

Figure 4. Rates of MMR and CCyR of treatment in ENESTnd trial (Ref 31).

Dasatinib (BMS-354825, Sprycel®) is a second generation TKI 325-fold more potent than imatinib in vitro and, additionally, able to inhibit several imatinib-resistant BCR-ABL1 mutants, except for the T315I. Moreover, this TKI has a broader spectrum of activity, in fact it is active also against the Src kinases family, involved in the cell signaling pathway [33,34].
The Dasatinib Versus Imatinib Study in Treatment-Naïve Chronic Myeloid Leukemia Patients (DASISION) phase 3 randomized trial compared imatinib 400mg/daily to dasatinib 100mg/daily in newly diagnosed CP-CML patients. At 12 months follow-up, CCyR rate was higher with dasatinib than with imatinib (77% vs. 66%, P=0.007) as the rate of MMR (46% vs. 28%, P<0.0001) [35]. The 5-year follow-up of this study revealed that dasatinib induced deeper responses in shorter times respect to imatinib but PFS and OS were comparable (85% vs 86% and 91% vs 90%, respectively) [36].

Bosutinib (SKI-606, Bosulif®) belongs to the second generation TKIs and, as dasatinib, is a dual Src/ABL inhibitor. It was initially approved only for second line treatment in case of intolerance/resistance but, after the results obtained in the Bosutinib Trial in First-Line Chronic Myelogenous Leukemia Treatment (BFORE), it was introduced also as first-line therapy. In this phase 3 trial, 536 CML patients were treated with 500mg/daily of imatinib or bosutinib and CCyR and MMR rates at 12 months were significantly higher in bosutinib compared to imatinib (77.2% vs 66.4% and 47.2% vs 36.9%, respectively) [37].

Ponatinib (AP24534, Iclusig®) is instead a third generation TKI used as second-line treatment in case of resistant/refractory CML. It is more potent than all the other TKIs and is the only one effective against BCR-ABL1 T315I mutant which characterizes up to 20% of CML patients. In the phase 2 of the Ponatinib Ph+ ALL and CML Evaluation (PACE) trial the responses obtained were durable and independent from the BCR-ABL1 mutation status, although 12% discontinued due to adverse events.
Indeed, ponatinib was first approved in the United States but, after evidences of cardiovascular (CV) toxicity, its use was restricted only to the cases of intolerance/resistance to first-line treatment and in particular to T315I patients [38-40].

Arterial occlusive events during TKI treatment

The introduction of TKIs in the clinical practice for the treatment of CML has notably improved patients' life expectancy, making CML a curable disease. Therefore, the safety and toxicity of this drugs constitute a matter of concern since the occurrence of adverse events (AEs) can lead to dose reduction or therapy discontinuation. TKIs' related AEs can be classified into hematological and non-hematological: hematological AEs generally include anemia, thrombocytopenia and neutropenia, while among non-hematological AEs the attention has been particularly focused on Arterial Occlusive Events (AOEs) [41]. Imatinib is generally well tolerated and induces mild to moderate AEs [42] while AOEs have been mostly associated to the treatment with second or third generation TKIs [43-45].

Indeed, TKIs have been defined as the main anticancer drug involved in the development of peripheral arterial occlusive disease (PAOD) [46]. Several retrospective studies reported the occurrence of AOEs during TKIs treatment in CML patients such as PAOD, QT interval prolongation, coronary artery disease (CAD) and other vascular complications. In particular, nilotinib showed a higher CV toxicity and PAOD rates compared to the other TKIs, especially at higher doses [47-49]. Moreover, the frequency of these events increased with long-term treatment, as showed by the 5-year follow-up of the ENESTnd trial, in which 10% of nilotinib treated patients experienced AOEs compared to 2,5% of patients treated with imatinib [50].

As reported in the 5-year follow-up of the phase 2 PACE trial, also ponatinib showed a significant CV toxicity and was associated to AOEs in 31% of CP-CML patients [40]. Additionally, some real-life studies confirmed the increased risk of AOEs in ponatinib treated patients, especially at a longer follow-up, with a previous nilotinib exposure considered as a risk factor [44,51].

The correlation between TKIs treatment and these events has not been completely understood yet, the hypothesis is that of an off-target effect of these drugs. In particular, ponatinib and nilotinib inhibit several molecules involved in the regulation of endothelial cells and in atherosclerosis, such as the vascular endothelial growth factor (VEGF) receptor KDR and discoidin domain receptor 1 (DDR1), respectively [52]. Moreover, nilotinib-induced upregulation of pro-atherogenic adhesion-proteins such as ICAM-1, E-selectin and VCAM-1 was also demonstrated in vitro and in nilotinib treated mice [53]. Recently, metabolomic analyses have been implied to better understand the mechanisms underlying these side effects, revealing a different metabolic profile in CML patients who experienced AOEs compared to the others [54].

In our retrospective cross-sectional study conducted on 110 CP-CML patients treated with nilotinib and imatinib, we investigated the correlation between atherothrombotic events and the presence of traditional CV risk factors and the lectin-like ox-LDL receptor-1 (OLR-1) gene polymorphism, encoding LOX-1 receptor. Moreover, we performed the biochemical analysis of some molecules involved in the atherothrombotic process. Results evidenced the possibility of a nilotinib-induced pro-atherothrombotic effect, due to the association of the detrimental IVS4-14 G/G LOX-1 polymorphism, causing lipid-peroxidation, and a pro-inflammatory state given by the lower anti-inflammatory interleukin-10 (IL-10) cytokine levels [55].

As reported in several studies, the presence of traditional CV risk factors such as diabetes, hypercholesterolemia, hypertension and smoking can contribute to the occurrence of AOEs [52]. The combination of these factors can lead to the development of an inflammatory and pro-atherogenic status in which are involved molecules such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL6) and oxidized low-density lipoprotein (ox-LDL) [56]. Additionally, some authors investigated the mechanisms generating PAOD by measuring standard ankle brachial index (ABI) in nilotinib treated CML patients [49].

A systemic coronary risk evaluation (SCORE) chart, based on gender, age, systolic blood pressure, total cholesterol levels and smoking habit, has been applied to CML patients treated in frontline or second-line with nilotinib, in order to evaluate the efficacy of this tool in predicting the risk of atherosclerosis development [57].

Furthermore, a very recent study showed that high cholesterol and low-density lipoprotein (LDL) levels and a baseline high SCORE risk in CML patients are associated to an increased occurrence of AOEs [58].

Therefore, the most recent guidelines for the management of CML patients put a major focus on the AEs, considering their long-term life expectancy, and on the careful selection of the first-line TKI. For instance, the use of second generation TKIs has been recommended in patients at intermediate- or high-risk Sokal and Euro scores and in case of younger patients, who have higher probability of reaching TFR. Regarding AOEs, the current clinical practice considers the baseline evaluation of the CV patients' risk factors and the TKI safety profile. Indeed, imatinib is suggested in older CML patients with preexistent CV comorbidities [59], while in case of ponatinib treatment, a prophylaxis with aspirin has been hypothesized, but this option is still under debate. Moreover, the occurrence of AOEs is assessed and monitored throughout treatment duration.

AIM OF THE STUDY

The introduction of TKIs completely changed CML treatment scenario, bringing a significant improvement in CML patients life expectancy and making this disease curable. Consequently, clinicians' attention has mainly been focused on the long-term efficacy, safety and toxicity of these drugs. Among TKIs-induced AEs, AOEs have become important issues, following to several studies reporting the increased frequency of these events especially during treatment with second and third generation TKIs. In particular, nilotinib was associated to higher incidence of atherothrombotic events and PAOD compared to other TKIs. The mechanisms underlying these AEs have not been clarified yet; some studies showed nilotinib atherogenic role, based on the involvement of molecules regulating vascular endothelial cells and atherosclerotic process. The presence of traditional CV risk factors such as age, sex, diabetes, hypercholesterolemia, hypertension and smoking can contribute to the development of these events. For this reason, the current clinical practice has moved toward considering these factors during TKI choice, in order to reduce the possibility for CML patients to undergo AOEs. Results from our retrospective cross-sectional study suggested a possible nilotinib-induced pro-atherothrombotic status and provided the rationale to prospectively study clinical and biochemical pro-atherothrombotic profiles in CP-CML patients at diagnosis and during front line TKI treatment. To further investigate our initial hypothesis, we started a multicenter prospective study of tyrosine Kinase Inhibitors induced pro-AtherothROmbotic (KIARO) status in CML patients, in which we evaluated a possible pro-atherothrombotic effect behind CV toxicity in first-line imatinib, nilotinib and dasatinib treated CP-CML patients.

Our intents were: 1) to assess any changes in the inflammation status during TKI treatment through measurement of pro/anti-inflammatory cytokines (IL-6, IL-10, TNFα and ox-LDL); 2) to record AOEs after applying CV SCORE and evaluate its predictive role; 3) to correlate AOEs with altered inflammation status. The final goal was to prospectively study TKIs influence on the pro/anti-inflammatory background as one of the possible mechanisms involved in the increased rate of atherothrombotic events observed in CML patients, particularly in those treated with nilotinib.

MATERIALS AND METHODS

Patients population

From September 2013, consecutive CP-CML patients referring to 11 Italian Hematology Units were enrolled in the study at the time of undergoing first-line treatment with imatinib, nilotinib or dasatinib according to physician choice. All patients gave written informed consent according to the local ethical committee policy. At enrollment, the presence of traditional CV risk factors such as hypertension, familiarity, dyslipidemia, diabetes mellitus, cigarette smoking and BMI and other comorbidities was assessed. Sokal score and ABI were calculated for each patient and data regarding additional cytogenetic abnormalities (ACA) were collected. Consequently, patients were stratified according to their SCORE values. During treatment follow-up, the occurrence of AOEs was registered. In particular, the result was considered positive in case of PAOD, acute coronary syndrome (ACS), transient ischemic attack (TIA) or definite ischemic stroke of atherothrombotic origin.

Blood samples

All consecutive patients underwent a peripheral blood withdrawal at diagnosis and at the established timepoints of 1, 3, 6 and 12 months of either TKI treatment. Blood samples in citrate were centrifugated in order to isolate plasma samples, which were stored at -80°C locally at each Hematology Unit and then shipped to the Hematology laboratory of Siena to perform biochemical analysis.

IL-10, IL-6, TNFα and ox-LDL analysis

IL-10, IL-6, TNFα and ox-LDL levels of each patient, at diagnosis and at the selected follow-up timepoints, were measured through enzyme-linked immunosorbent assay (ELISA) test. Regarding the first three cytokines, the kit implied was the high sensitivity Quantikine ELISA assays (R&D systems), while for ox-LDL was Immundiagnostik AG ELISA kit. Biochemical analyses were performed according to the manufacturer's instructions, exploiting the principle of the quantitative sandwich enzyme immunoassay technique. Firstly, plasma samples were added to a 96 wells microplate where a monoclonal antibody specific for each cytokine was already pre-coated. Standards provided by the kit, used to create a standard reference curve to compare samples concentrations, were also added to the plate. All samples and standards were tested in duplicate. During the first incubation period, the monoclonal antibodies immobilized on the wells capture the antigens in patients' samples. Any unbound substance was removed through a series of washes and then a polyclonal antibody specific for the cytokine was added to the wells. After an incubation period, a substrate solution and an amplifier solution were applied to obtain a colorimetric reaction.

Different enzymes and substrates are implied for the colorimetric reaction: IL-6 and IL-10 kits provide specific antibodies conjugated with alkaline phosphatase and use NADPH as substrate, TNFα kit exploits biotin-streptavidin affinity while ox-LDL antibody is peroxidase-conjugated and its substrate is tetramethylbenzidine (TMB). Color development is directly proportional to the concentration of the cytokine present into the sample.

The last step consisted in measuring the color intensity, prior stopping the colorimetric reaction with a specific stop solution. All cytokines levels were measured by using the Mindray MR-96A Elisa Reader: the optical density (OD) of each well should be read within 30 minutes from the addition of the stop solution, setting the indicated wavelength and a correction wavelength to remove optical imperfections. Standard curve was created with a software generating a four-parameter logistic (4-PL) curve-fit. Final samples concentrations were calculated as the average of the duplicate readings and were normalized subtracting the average zero standard OD.

Statistical analysis

Multivariate analysis and the Cox proportional-hazards modelling were used to evaluate the putative relations among end-points and both parametric and nonparametric data in the whole cohort of subsequent CP-CML patients in CCyR. For this purpose, the calculated number to detect a significant difference in parametric and not parametric variables evaluated, with 90% power at p=0.01 was 134, if at least 10 events would occur. Therefore, the putative relation for each variable was determined by applying the statistical model, consisting of a formal test for interaction. Moreover, a resampling technique (exact tests in SPSS 2003 module) was employed for the final validation of data together with a discrimination analysis (Hosmer–Lemeshow method), assuming a $p< 0.05$ as indicating a statistical significance.

Additionally, we employed the Mann Whitney U-test and the Wilcoxon test to estimate putative differences in the levels of each single biochemical variable for comparisons between and within groups. The Kendall rank correlation coefficient was used to measure the relationship among measurable variables.

All calculations were performed using the SPSS library version 13 (SPSS Inc. Chicago, IL).

RESULTS

Patients clinical data

Between September 2013 and September 2018, a total of 186 CP-CML patients were enrolled in this study. Of them, 107 (58%) were males and 79 (42%) were females and their median age at diagnosis was 60 years (range 24-90 years). Regarding TKI therapy, 89/186 (48%) were treated in first-line with imatinib, 59/186 (32%) with nilotinib and 38/186 (20%) with dasatinib. Median age at diagnosis was different among the three TKI cohorts: 69 years (range 32,4-90 years) in imatinib, 51 years (range 24,6-88 years) in nilotinib and 56 years (range 32-79 years) in dasatinib. The median follow-up since CML diagnosis was 23,3 months (range 1,1-64,6 months). In particular, patients in imatinib cohort were treated for a median time of 21 months (1,2-62,7 months), those in nilotinib had a median duration treatment of 24 months (1,5-64,6 months), while for those in dasatinib the median treatment duration was of 27 months (3,2-59,8 months). Sokal score was low in 62/186 patients (33%), intermediate in 85/186 patients (46%), and high in 39/186 patients (21%). Additional cytogenetic abnormalities were reported for 16/186 patients (8,6%).

Traditional CV risk factors such as hypertension, familiarity, hypercholesterolemia, diabetes mellitus, cigarette smoking and BMI were evaluated in all patients. Of the total patients' population, 50/186 (27%) (19 imatinib, 23 nilotinib, 8 dasatinib) had no CV risk factors at diagnosis; 55/186 (30%) (22 imatinib, 22 nilotinib, 11 dasatinib) had only one CV risk factor and 47/186 (25%) (27 imatinib, 8 nilotinib, 12 dasatinib) presented two CV risk factors. Furthermore, 19/186 (10%) (11 imatinib, 4 nilotinib, 4 dasatinib) and 14/186 (7,5%) (9 imatinib, 2 nilotinib, 3 dasatinib) respectively reported three and four CV risk factors and only 1/186 (0,5%) had five CV risk factors and was treated with imatinib. The distribution of the different CV risk factors among the three cohorts of patients is summarized in Table 1.

Traditional CV risk factors	IMATINIB (n=89)	NILOTINIB (n=59)	DASATINIB (n=38)
Diabetes mellitus	16/89 (18%)	1/59 (1,7%)	11/38 (29%)
Smoking	15/89 (17%)	8/59 (15,5%)	7/38 (18%)
BMI≥30	11/89 (12%)	4/59 (7%)	4/38 (10,5%)
Hypertension	51/89 (57%)	19/59 (32%)	16/38 (42%)
Hypercholesterolemia	31/89 (35%)	12/59 (20%)	8/38 (21%)
Familiarity	27/89 (30%)	14/59 (28%)	13/38 (34%)

Table 1. Traditional CV risk factors.

Of note, 20 out of 186 (11%) patients underwent an AOE before starting TKI treatment: 17/20 (85%) were treated with imatinib while the remaining 3/20 (15%) belonged to dasatinib cohort; none of them was treated with nilotinib. According to the Sokal score stratification, in the imatinib cohort 3 out of 17 patients had a high Sokal score, 13 had an intermediate score and one had a low score, while 2 out of 3 dasatinib treated patients were classified as high Sokal score and the other as intermediate. Furthermore, we investigated the presence of CV risk factors in these 20 patients who already experienced AOEs and we found that 16/20 (80%) of them had ≥2 CV risk factors.

IL-10, IL-6, TNFα and ox-LDL

IL-6, TNFα, IL-10 and ox-LDL plasma levels were measured through ELISA test in all CP-CML patients at diagnosis and at 1, 3, 6 and 12 months of TKI treatment. Median values of all cytokine levels both in the whole cohort and in the three TKI subgroups of patients are reported in Tables 2A, 2B, 2C and 2D.

Looking at the cytokines trend, no statistically significant differences were detected in TNFα and IL-6 levels during the first 12 months of treatment in the three arms of treatment (**Figure 5**). On the other hand, data showed that IL-10 levels were comparable among the three TKIs cohorts at baseline but showed a remarkably different evolution during treatment. Indeed, IL-10 levels were higher at 6 and 12 months in imatinib (p=0.012 and p=0.009, respectively) and dasatinib (p=0.032 and p=0.014, respectively) cohorts compared to nilotinib. In addition, ox-LDL levels remained comparable until 6 months of treatment and then increased at 12 months only in the nilotinib cohort (p=0.041) in contrast to imatinib and dasatinib (**Figure 6**). Consequently, looking at the IL-6/IL-10 and TNFα/IL-10 ratios, we found an interesting

difference in nilotinib cohort. Indeed, in this group of patients, IL-6/IL-10 and TNFα/IL-10 ratios were higher compared to imatinib (p=0.042, p=0.044 at 6 months; p=0.040, p=0.041 at 12 months) and dasatinib (p=0.049, p=0.040 at 6 months; p=0.041, p=0.044 at 12 months), suggesting a TKI-driven pro-inflammatory status in nilotinib treated patients (**Figure 7**).

IL-6 [pg/ml]	WHOLE COHORT	IMATINIB (n=89)	NILOTINIB (n=59)	DASATINIB (n=38)
baseline	2,257 (0-44)	2,28 (0-44)	1,66 (0-20,196)	2,57 (0,2-21,9)
1 month	1,339 (0-15,97)	2,339 (0,25-14,633)	1,2085 (0-15,97)	0,83 (0-7,454)
3 months	1,1965 (0-82)	1,392 (0-82)	1,096 (0-4,57)	1,0895 (0,17-15,869)
6 months	1,15 (0-18,27)	1,784 (0,06-15,65)	0,834 (0-18,27)	1,0905 (0,06-6,91)
12 months	1,391 (0-18,08)	1,804 (0,65-9,86)	1,2 (0-18,08)	1,347 (0-14,581)

Table 2A. Median IL-6 levels in the three TKI cohorts.

TNFα [pg/ml]	WHOLE COHORT	IMATINIB (n=89)	NILOTINIB (n=59)	DASATINIB (n=38)
baseline	1,523 (0-18,64)	1,5775 (0,006-18,641)	1,396 (0-9,491)	1,655 (0-17,94)
1 month	0,987 (0-8,06)	1,0395 (0-3,006)	1,003 (0-8,06)	0,93 (0-377)
3 months	1,12 (0-33,81)	1,191 (0-33,818)	1,0345 (0-9,238)	1,1085 (0-3,9)
6 months	0,901 (0-12,83)	0,9845 (0-6)	0,84 (0-12,83)	0,852 (0-3,92)
12 months	1,129 (0-30)	1,157 (0-7,88)	1,1085 (0-30)	1,001 (0-18,26)

Table 2B. Median TNFα levels in the three TKI cohorts.

IL-10 [pg/ml]	WHOLE COHORT	IMATINIB (n=89)	NILOTINIB (n=59)	DASATINIB (n=38)
baseline	0,3245 (0-36,77)	0,4 (0-36,577)	0,33 (0-26,23)	0,2 (0-7,536)
1 month	0,25 (0-39,21)	0,687 (0-17,152)	0,19 (0-39,21)	0,13 (0-5,943)
3 months	0,335 (0-76,89)	0,62 (0-76,895)	0,1995 (0-19,06)	0,06 (0-8,76)
6 months	0,25 (0-31,06)	0,31* (0-20,772)	0,165 (0-31,06)	0,25# (0-9,056)
12 months	0,19 (0-17,5)	0,376** (0-17,49)	0,094 (0-14,281)	0,215## (0-16,62)

*p=0.012; ** p=0.009. # p=0.032; ## p=0.014.

Table 2C. Median IL-10 levels in the three TKI cohorts.

ox-LDL [pg/ml]	WHOLE COHORT	IMATINIB (n=89)	NILOTINIB (n=59)	DASATINIB (n=38)
baseline	136,19 (14,07-3595,85)	109,48 (14,07-3115,3)	153,72 (14,82-3595,85)	101,04 (27,83-2168,9)
1 month	99,42 (16,51-2546,64)	93,01 (16,51-2452,09)	123,82 (37,86-2546,64)	88,42 (17,46-1854,29)
3 months	103,92 (1,27-6911,9)	90,6 (2,72-2486,9)	154,67 (46,84-6911,9)	100,995 (1,27-1039,16)
6 months	93,52 (9,97-2560,9)	84,3 (9,97-2560,9)	164,8 (37,76-1003,6)	70,37 (21,41-517,01)
12 months	90,83 (5,74-2392,46)	82,005 (5,74-1884,65)	171,68* (42,41-2392,46)	70,29 (30,22-993,02)

*p=0.041.

Table 2D. Median ox-LDL levels in the three TKI cohorts.

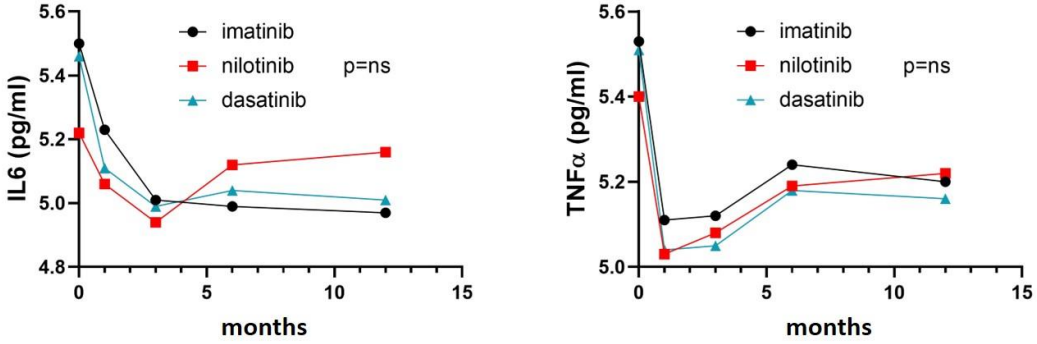

Figure 5. IL-6 and TNFα trends.

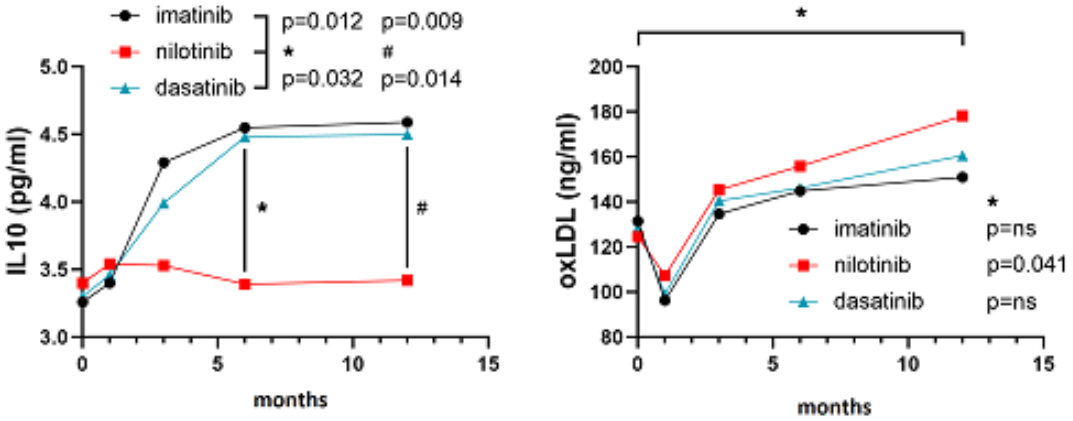

Figure 6. IL-10 and ox-LDL trends.

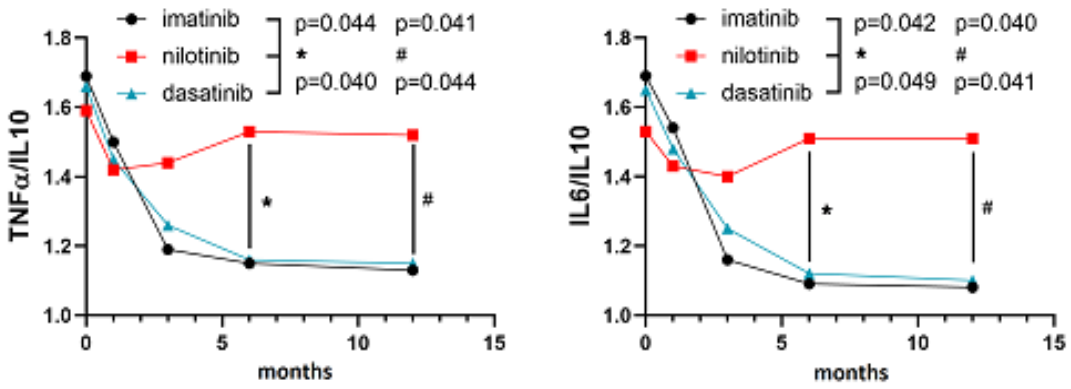

Figure 7. TNFα/IL-10 and IL-6/IL-10 ratios.

Clinical and serological parameters

Clinical and serological parameters such as total cholesterol levels, high density lipoprotein (HDL) and LDL levels, triglycerides, systolic blood pressure (SBP), diastolic blood pressure (DBP) and glycemia were measured at baseline and at 1, 3, 6, 12 months of TKI treatment. Median values of all these variables are reported in Tables 3A and 3B. Regarding total cholesterol, HDL and tryglicerides levels, no statistically significant differences were appreciated. On the other hand, we observed increased levels of LDL ($p<0.05$) only in the nilotinib cohort at 3 and 12 months of treatment, regardless of the concomitant use of CV medications, compared to imatinib and dasatinib cohorts. SBP, DBP and glycemia values did not undergo significant changes in all the timepoints of treatment both in the whole cohort and among the three subcohorts. Additionally, no significant data were obtained from ABI values, being calculated only in a minority of CML patients enrolled.

Total cholesterol [mg/dl]	**WHOLE COHORT**	**IMA (n=89)**	**NILO (n=59)**	**DASA (n=38)**	**HDL [mg/dl]**	**WHOLE COHORT**	**IMA (n=89)**	**NILO (n=59)**	**DASA (n=38)**
baseline	172 (78-287)	174 (83-249)	175 (100-233)	147 (92-287)	**baseline**	41 (17-105)	40 (25-95)	44 (17-105)	37 (18-76)
+1 month	171 (79-271)	151 (79-224)	193 (96-271)	183 (116-238)	**+1 month**	54 (28-93)	52 (32-85)	57 (33-90)	53 (28-93)
+3 months	181 (84-283)	167 (84-259)	210 (122-283)	185 (117-265)	**+3 months**	56 (23-124)	55 (23-89)	59 (37-106)	51 (32-124)
+6 months	180 (80-312)	159 (80-245)	212 (134-312)	182 (140-273)	**+6 months**	54 (19-139)	50 (19-81)	59 (37-97)	47 (31-139)
+12 months	181 (85-308)	156 (90-245)	198 (156-308)	187 (85-267)	**+12 months**	52 (23-102)	48 (23-92)	59 (32-99)	47 (27-102)
LDL [mg/dl]	**WHOLE COHORT**	**IMA (n=89)**	**NILO (n=59)**	**DASA (n=38)**	**Triglycerides [mg/dl]**	**WHOLE COHORT**	**IMA (n=89)**	**NILO (n=59)**	**DASA (n=38)**
baseline	100 (26-205)	101 (26,2-163,6)	102 (39,4-155,8)	80 (26,4-205)	**baseline**	146 (51-477)	143 (61-477)	149 (49-299)	172 (71-418)
+1 month	95 (21-190)	77 (21,2-151,2)	119 (29-190,4)	105 (39,2-166,2)	**+1 month**	88 (35-345)	87 (44-319)	79 (44-164)	106 (41-345)
+3 months	104,2 (20,2-197,6)	90 (20,4-160,4)	130* (37,4-197,6)	107 (44-197,6)	**+3 months**	105 (34-260)	104 (37-246)	99 (34-200)	141 (45-260)
+6 months	102 (21-218)	87 (17-166,6)	132 (70,6-218)	101 (34,8-190,6)	**+6 months**	113 (49-280)	117 (49-280)	95 (49-243)	139 (59-272)
+12 months	106,8 (21-207,2)	83 (21,4-165)	120* (74,4-207,2)	113 (26-181)	**+12 months**	100 (43-433)	100 (39-433)	95 (49-274)	130 (50-294)

*$p<0.05$

Table 3A. Median values of total cholesterol, HDL, LDL and tryglicerides.

ABI	WHOLE COHORT	IMA (n=89)	NILO (n=59)	DASA (n=38)	SBP [mmHg]	WHOLE COHORT	IMA (n=89)	NILO (n=59)	DASA (n=38)
baseline	1 (0-1,3)	1 (1-1,33)	1 (0-1,3)	1 (1-1,3)	**baseline**	125 (100-170)	130 (110-170)	120 (100-140)	120 (105-150)
+1 month	1,085 (0,9-1,28)	1 (0,94-1,2)	1 (0,9-1,28)	1 (1,08-1,25)	**+1 month**	125 (105-180)	120 (115-160)	130 (100-170)	128 (100-180)
+3 months	1,08 (0,86-1,2)	1 (0,86-1,17)	1 (1-1,2)	1 (1,04-1,17)	**+3 months**	130 (105-160)	130 (100-160)	123 (105-150)	130 (100-150)
+6 months	1,1 (0,96-1,28)	1 (0,96-1,2)	1 (1,08-1,22)	1 (1-1,28)	**+6 months**	130 (100-170)	130 (100-170)	125 (110-160)	130 (110-170)
+12 months	1,04 (0,96-1,16)	1 (0,96-1,08)	1 (1-1,16)	1 (1-1,14)	**+12 months**	130 (100-170)	130 (110-170)	130 (100-160)	133 (110-160)
DBP [mmHg]	**WHOLE COHORT**	**IMA (n=89)**	**NILO (n=59)**	**DASA (n=38)**	**Glycemia [mg/dl]**	**WHOLE COHORT**	**IMA (n=89)**	**NILO (n=59)**	**DASA (n=38)**
baseline	80 (57-90)	75 (57-90)	80 (65-80)	80 (60-90)	**baseline**	89 (46-252)	91 (49-150)	87 (48-140)	84 (46-252)
+1 month	75 (50-100)	80 (67-80)	76 (50-84)	70 (69-90)	**+1 month**	99 (74-237)	101 (52-192)	93 (62-129)	93 (48-237)
+3 months	80 (53-100)	80 (53-90)	75 (55-88)	75 (70-90)	**+3 months**	96 (62-236)	96 (70-165)	96 (62-138)	98 (69-236)
+6 months	80 (60-90)	80 (65-90)	80 (67-85)	80 (60-90)	**+6 months**	96 (63-183)	100 (66-149)	95 (63-174)	94 (69-183)
+12 months	80 (50-105)	79 (60-80)	80 (60-100)	80 (60-90)	**+12 months**	94 (60-206)	97 (79-175)	94 (60-206)	92 (70-150)

Table 3B. Median values of total ABI, SBP, DBP and glycemia.

CV risk

The mean 5-year SCORE for the whole cohort (S=8.3±4.6) was in line with that estimated in a general age- and sex-matched reference population (S=8.1±5.2; p=0.094; IS 12.7±5.9 vs 11.9±6.1, respectively; p=0.085). Furthermore, the prevalence of positive history of CV risk factors and/or major cardiovascular events (MACE) in our studied population resulted comparable to that of the general Italian population (combined and cumulative data 13,4% vs 13,9%; p=0.113) [60]. Considering the presence of CV risk separately according to the first-line TKI treatment chosen, nilotinib cohort displayed a significantly lower 5-year SCORE compared to imatinib or dasatinib (nilotinib S=6.8±2.8, imatinib S=9.5±4.8, dasatinib S=8.2±3.3; nilo vs ima p=0.02, nilo vs dasa p=0.043). This result can be explained by the choice, in the current clinical practice, of other second generation TKIs rather than nilotinib in patients with CV risk factors at diagnosis, including age. In fact, median age was significantly lower in nilotinib (51±1.8) and dasatinib (56±3.2) cohorts compared to imatinib (69±1.3; nilo vs ima p<0.001, dasa vs ima p<0.001).

Arterial Occlusive Events

After a median follow up of 23,3 months from the beginning of TKI treatment, AOEs were assessed in 10 out of 186 (5%) CML patients. The median treatment duration until the occurrence of the events was 19,1 months (range 6-51 months). Regarding the TKI implied at the moment of the AOE, 4 out of 10 patients were treated in first-line with nilotinib, one in third-line with nilotinib after two switches from dasatinib and imatinib, and two were treated with imatinib. The remaining three patients had an AOE during the second-line treatment after a switch from imatinib. In particular, one of them was treated in second-line with dasatinib and the other two patients in bosutinib. ACS was experienced in all the five patients treated with nilotinib and in one of the patients undergoing second-line bosutinib. PAOD was assessed in one of the imatinib treated patients and in the other bosutinib treated patient, who died due to complications after the AOE. The two remaining patients underwent TIA and stroke during treatment with imatinib and dasatinib, respectively. Notably, 6/10 (60%) patients had ≥ 2 CV baseline risk factors and 3/6 (50%) reported a previous AOE before TKI treatment. All patients' characteristics are summarized in Table 4.

In this subgroup of patients, IL-6 and TNFα median values were higher both at diagnosis and at each timepoint evaluated, compared to the whole cohort. Instead, IL-10 and ox-LDL had similar median concentrations in both cohorts, except for ox-LDL at 12 months which resulted higher in patients who experienced AOEs. However, no significant correlation between AOEs and pro/anti-inflammatory cytokines measured plasma levels was detected.

Patient	SOKAL	N° CV risk factors	Previous AOE	Event	TKI at the moment of the AOE	Time to treatment (months)
#1	intermediate	1	no	ACS	nilotinib	15,2
#2	low	2	no	ACS	nilotinib	21,1
#3	low	2	no	ACS	nilotinib	51
#4	intermediate	2	yes	TIA	imatinib	6
#5	high	1	yes	stroke	dasatinib*	17,1
#6	intermediate	1	no	PAOD	imatinib	10,6
#7	intermediate	2	yes	ACS	nilotinib**	24,7
#8	intermediate	3	yes	PAOD	bosutinib*	23,1
#9	intermediate	4	no	ACS	bosutinib*	16,1
#10	intermediate	0	no	ACS	nilotinib	24,9

*switched from first-line imatinib; **switched from second-line imatinib.

Table 4. Patients who experienced AOEs.

Multivariate and relation analyses

In the multivariate analysis we found a significant association between nilotinib treatment and changes in IL-10 levels (HR 1.29, 95% CI 1.04-1.96 $p< 0.05$), IL6/IL10 (1.28, 95% CI 1.08-1.93 $p< 0.05$) and TNFα/IL10 ratios (1.21, 95% CI 1.03-1.90 $p< 0.05$), ox-LDL (HR 1.39, 95% CI 1.18-2.04 $p< 0.01$) and LDL levels (HR 1.22, 95% CI 1.08-1.99 $p< 0.05$). Furthermore, a significant inverse relation between IL-10 and ox-LDL levels was found in nilotinib treated subjects starting from 3 months of treatment ($r= - 0.44$, $p< 0.01$). Regarding MACE, nilotinib treatment showed a 3.1 times increased risk compared to other TKIs (HR 3.1, 95% CI 2.6-4.4 $p< 0.001$), whereas SCORE was not predictive in the whole cohort and subgroups (cumulative HR 0.6, 95% CI 0.33-0.94 $p= 0.094$).

DISCUSSION

Since the introduction of TKIs as CML standard treatment, patients' life expectancy is progressively increased, becoming nowadays comparable to that of the general population. Despite TKIs great efficacy, the occurrence of AEs moved the attention of clinicians toward the safety and toxicity of these drugs. In particular, several studies reported AOEs in association to treatment with second and third generation TKIs, especially with nilotinib. Results from these studies increased the awareness for an accurate TKI selection in the management of CML patients, inducing hematologists to consider at diagnosis the presence of traditional CV risk factors in order to avoid TKI-associated CV toxicity in those patients at higher CV risk. Furthermore, nilotinib-associated CV toxicity is not completely understood yet; an off-target effect has been hypothesized, based on nilotinib-induced upregulation of pro-atherogenic molecules.

In our retrospective cross-sectional study, including 110 CP-CML patients treated with imatinib or nilotinib, we supposed that an inflammatory status in nilotinib patients, together with genetic pro-atherothrombotic predisposition, may have a role in the increased incidence of vascular events. The study revealed the strong negative impact of the detrimental allele of OLR-1 gene polymorphism (encoding LOX-1 receptor) and dyslipidemia in nilotinib treated patients. In addition, biochemical analyses showed that these patients present a pro-inflammatory/oxidative status, with lower IL-10 levels and higher ox-LDL levels compared to imatinib cohort [55].

Based on these premises, we started the prospective multicenter KIARO study to furtherly investigate a possible pro-atherothrombotic effect behind CV toxicity in first-line imatinib, nilotinib and dasatinib treated CP-CML patients. In this study, including 186 CP-CML patients, we confirmed and reinforced data from our retrospective analysis. In particular, we showed significantly increased levels in a pro-inflammatory direction in the nilotinib cohort compared to imatinib or dasatinib, as demonstrated by the higher IL-6/IL-10 and TNFα/IL-10 ratios in this group of patients. These results seem to be mostly related to the different course of the anti-inflammatory cytokine IL-10, which decreases during nilotinib treatment. Notably, IL-6/IL-10 ratio has been previously associated with incidence and severity of AOEs [61]. Additionally, we observed a statistically significant increase in ox-LDL levels at 12 months during nilotinib treatment and not in imatinib and dasatinib cohorts.

The combination of inflammatory and oxidative mechanisms, that are closely related in atherogenesis and atherothrombotic complications [62], may eventually be responsible for

nilotinib-associated endothelial activation and the consequent expression of pro-atherogenic adhesion molecules as reported by Hadzijusufovic et al. [53]. Indeed, it is well known that inflammation and oxidation are main players in atherosclerosis [63], with effects on endothelium and macrophages activation and increased LOX-1 expression by these same cells [64]. LOX-1, then, mediates ox-LDL accumulation in the intima, with foam cell formation and atherosclerotic plaque growth but it also increases transcription and activity of proteases which will be ultimately responsible for plaque instability and rupture [65-68].

Although our results showed a pro/anti-inflammatory imbalance, particularly in a pro-inflammatory direction in nilotinib subgroup, we documented an AOE only in 5% of all patients and, mostly, we did not find any significant correlation between AOEs and TKIs. Furthermore, due to the low number of events, it was not possible to establish a correlation between the pro/anti-inflammatory cytokine levels and AOEs.

A possible explanation for this condition, without an apparent correlation between pro/anti-inflammatory imbalance and AOEs, could reside in the clinical status of the patients of each arm. Indeed, we also evaluated clinical data of the patients. We applied the CV SCORE in all CML patients enrolled to better identify patients with high risk to experience an AOE, but this parameter was not predictive for our cohort, being in line with that of the general population. The strategic choice of TKI treatment according to patients' age may explain this result, which can be also documented by the lower median age in nilotinib cohort (51 years) compared to imatinib and dasatinib cohorts (69 years and 56 years, respectively). An additional possible reason could be that a higher percentage of imatinib treated patients carried ≥2 traditional CV risk factors compared to nilotinib and dasatinib cohorts, as reflected by the baseline SCORE index. Indeed, differently from our retrospective study, TKI treatment at diagnosis was chosen also according to the presence of traditional CV risk factors and previous CV history.

Even if our results showed a pro-inflammatory status in nilotinib subgroup during the first year of treatment, considering that AOEs occurred after a median treatment duration of 19,1 months, we believe that the evaluation of pro/anti-inflammatory cytokines should be performed for an extended treatment time to better investigate the correlation between pro-atherothrombotic status and AOEs. Based on these observations, we hypothesize that, in our selected cohort, the time to develop an AOE could be longer, compared to patients with a higher baseline CV risk.

Our data confirm that nilotinib-associated CV toxicity remains a serious issue even in the real life setting with already carefully selected patients, while dasatinib and imatinib, despite the latter is prescribed to the majority of patients at higher baseline CV risk, seem to exert a

protective effect against AOEs with respect to nilotinib. However, the reason why this happens is still poorly understood.

The choice of first-line TKI therapy represents a crucial point in the management of CML patients and should take into account patients' baseline CV profile, in order to drive clinicians toward the most adequate and personalized therapy considering as ultimate goals the possibility of reaching a safe TFR and reducing AOEs-associated comorbidities, at least when imatinib is the drug of choice. Instead, when nilotinib is considered as a possible first-line treatment, other factors should be taken into account considering the lack of predictivity of the SCORE, in a brief period with respect to 5 years assessed risk, as suggested from our data.

We believe that nowadays, in light of the several evidences documented, clinicians should benefit of all available instruments such as evaluation of CV risk factors, lipidic profile and SCORE chart, to offer the best and safe long-term TKI treatment for CML patients. Moreover, we suggest the application of ABI measurement, being a crucial predictive factor of AOEs, although in this study it was difficult to evaluate it in the majority of hematological centers.

We suggest that nilotinib treatment could contribute to causing changes in the pro/anti-inflammatory cytokines balance toward an inflammation status which seems to be maintained stable overtime. Indeed, we have partial results regarding cytokine levels at 18 and 24 months of treatment confirming the permanence of this pro-atherothrombotic status (data not shown). However, it remains unclear if these data are sufficient by themselves to predict AOEs or predispose CML patients to undergo these side effects, but we believe that a possible correlation could be evaluated at a longer treatment follow-up.

In conclusion, further investigations on the biochemical mechanisms involved in pro-atherothrombotic nilotinib associated events are needed to corroborate our findings, with particular involvement of lipidic factors, as demonstrated also by cytokines induced pro-inflammatory status.

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ABBREVIATIONS

ABI, ankle brachial index; ABL1, Abelson; ACA, additional cytogenetic abnormalities; ACS, acute coronary syndrome; AEs, adverse events; ALL, acute lymphoblastic leukemia; alloBMT, Allogeneic bone marrow transplant; AOEs, arterial occlusive events; AP, accelerated phase; BC, blastic crisis; BFORE, Bosutinib Trial in First-Line Chronic Myelogenous Leukemia Treatment; BM, bone marrow; BP, blastic phase; BCR, breakpoint cluster region; BUS, busulphan; CAD, coronary artery disease; CBA, chromosome binding analysis; CC, coiled-coil domain; CCyR, complete cytogenetic response; CML, Chronic myeloid leukemia; CMR, complete molecular response; CNL, chronic neutrophilic leukemia; CP, chronic phase; CV, cardiovascular; CyR, cytogenetic response; DBP diastolic blood pressure; DDR1, discoidin domain receptor 1; ELISA, enzyme-linked immunosorbent assay; ELTS, EUTOS Long Term Survival; EFS, event free survival; ENESTnd, Evaluating nilotinib Efficacy and Safety in Clinical Trials – Newly Diagnosed Patients; EUTOS, European Treatment and Outcome Study for CML; FDA, Food and Drug Administration; FISH, fluorescence in situ hybridization; HDL, high density lipoprotein; HR, hematologic response; HU, hydroxyurea; IFNα, interpheron alpha; IL-6,interleukin-6; IL-10, interleukin-10; IRIS, International Randomized Study of Interferon and STI571; IS, International Scale; KIARO, Kinase Inhibitors induced pro-AtherothROmbotic; LDL, low-density lipoprotein; LSCs, leukemic stem cells; MACE, major cardiovascular events; MDR, multidrug-resistant; MMR, major molecular response; MR, molecular response; MRD, minimal residual disease; OD, optical density; OLR-1, ox-LDL receptor-1; OS, overall survival; ox-LDL, oxidized low-density lipoprotein; PACE, Ponatinib Ph+ ALL and CML Evaluation; PAOD, peripheral arterial occlusive disease; PB, peripheral blood; PDGFR, platelet-derived growth factor receptor; PFS, progression free survival; Ph, Philadelphia; 4-PL, four-parameter logistic; RQ-PCR, quantitative RT-PCR; RT-PCR, reverse transcriptase-polymerase chain reaction; SBP, systolic blood pressure; SCORE, systemic coronary risk evaluation; SH3, SRC homology domain 3; TFR, treatment-free remission; TKI, tyrosine kinase inhibitor; TMB, tetramethylbenzidine; TNFα, tumor necrosis factor alpha; TOPS, Tyrosine Kinase Inhibitor Optimization and Selectivity; VEGF, vascular endothelial growth factor.