Apoptotic profiling of chronic myeloid leukaemia patients' platelets *ex vivo* before and after treatment with Imatinib

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Abstract

Chronic myeloid leukaemia (CML) is a malignancy of the hematopoietic stem cells. The first line of treatment for CML, especially in developing countries, remains the first generation tyrosine kinase inhibitor, Imatinib. Patients with CML are frequently diagnosed with platelet abnormalities. However, the specific mechanism of platelet abnormalities in CML, remains unclear and poorly understood. The aim of this study was therefore to determine the apoptotic profiles of chronic myeloid leukaemia patients *ex vivo* on platelets before and after treatment with Imatinib. Blood samples of healthy volunteers and CML patients at diagnosis and after six months treatment with Imatinib were collected. Platelet counts, viability and activation were determined. Results showed that CML patients' platelet counts were elevated upon diagnosis and these levels statistically significantly decreased after 6 months of treatment. Platelet activation was significantly increased after 6 months of treatment compared to levels at diagnosis (*P*-value < 0.05). Similarly, platelet apoptosis was also increased after 6 months of treatment. Abnormalities in platelet functioning found in this study may partly be due to clonal proliferation of haematopoietic cells in CML patients, specifically of megakaryocyte precursors as well as the inhibition of platelet tyrosine kinase's and the inhibition of platelet derived growth factor.

Keywords: Apoptosis, chronic myeloid leukaemia, *ex vivo*, Imatinib, platelets

1 Introduction

Chronic myeloid leukaemia (CML) is a malignancy of the hematopoietic stem cells resulting in the hyperproliferation of leukocytes[1]. The pathogenesis of CML results from the translocation of the Philadelphia (Ph) chromosome from stem cells in the bone marrow. This translocation causes the collocation of the *Abelson murine leukaemia viral oncogene homolog 1 (ABL1)* gene from chromosome 9, as well as the *breakpoint cluster region protein (BCR)* gene from chromosome 22 resulting in the formation of the BCR-ABL fusion protein[1-4]. The formed BCR-ABL protein has active cytoplasmic tyrosine kinase activity implicated in abnormal cell proliferation and differentiation of hematopoietic cells[5].

CML is characterised by three phases of disease progression, the early chronic stable phase, accelerated phase and the blast phase[1-4]. The majority of patients are coincidentally diagnosed in the chronic phase of the disease as this phase lasts approximately 4 to 6 years and is indicated by various symptoms including fatigue, weight loss, leukocytosis and splenomegaly[4]. Progression from the stable chronic phase to the accelerated phase and subsequently the blast phase is accompanied by uninhibited proliferation, loss of differentiation, decreased response to regular control mechanisms and the gradual increase of immature myeloblasts. The progression is thought to be as a result of additional mutations resulting in uncharacteristic phosphorylation of intracellular proteins and reduced rates of apoptosis[1-5].

Treatment of CML is primarily through administration of tyrosine kinase inhibitors (TKIs)[3]. TKI treatment has significantly improved the prognosis of CML through the introduction of first, second, third and fourth generation TKIs. The first line of treatment for CML, especially in developing countries, remains the first generation TKI, Imatinib. TKIs function through inhibiting the function of the BCR-ABL gene and subsequent formation of the BCR-ABL fusion protein[3].

Patients with CML are frequently diagnosed with platelet abnormalities, specifically thrombocytosis[6-7]. In addition, bleeding abnormalities have been reported in 20.9% of patients treated with Imatinib[6]. Bleeding abnormalities are reported to be due to irregular platelet function owing to the clonal proliferation of hematopoietic cells. However, the specific mechanism of platelet abnormalities in CML, whether due to disease pathogenesis or TKI treatment, remains unclear and poorly understood[8].

There are two platelet markers of interest when determining platelet viability and activation including human cluster of differentiation 41 (CD41) and 62 (CD62P). CD41 or integrin alpha chain 2b is a membrane protein expressed on the surface of platelets and megakaryocytes used to isolate platelet populations as it serves as a receptor for cellular adhesion and platelet aggregation[9]. CD62P or P-selectin is a cell adhesion molecule which becomes externalized from α-granules after activation of platelets resulting in platelet aggregation[10]. CD62P or P-selectin is found in the α-granule membrane of platelets[9]. When platelets are activated, CD62 is translocated to the outer surface of the platelet. This is an indication of platelet degranulation and secretion[9]. The CD62 platelet marker can thus be used to quantify the extent of activation of platelets by measuring the amount of CD62 expressed on the surface of the platelet.

The aim of this study was therefore to determine platelet activation and the apoptotic profiles of chronic myeloid leukaemia patients *ex vivo* on platelets before and after treatment with Imatinib. There is very limited literature available on the involvement of platelets in CML progression. The current study provides the first insight of the impact of CML pathogenesis as well as Imatinib treatment on the activation and apoptosis of platelets in CML patients.

2 Materials and Methods

2.1 Collection of blood

Informed consent was obtained from participants to ensure ethical standards were upheld during the course of the study and each participant was compensated with R150 per blood collection interval. Blood was collected from 30 healthy volunteers as a control group aged between 30-60 years who did not smoke or use any chronic medication in 6 standard ethylenediamine tetra acetic acid (EDTA) tubes or 30 ml of blood was obtained from each control participant ‘once-off’. Blood was also collected from 6 chronic myeloid leukaemia patients according to staging and exclusion- and inclusion criteria (indicated below) determined by medical doctors at the Steve Biko Academic Hospital and the Department of Haematology, University of Pretoria, Pretoria, South Africa at the two stages of testing, namely at diagnosis and after 6 months of treatment with Imatinib.

Blood samples of 30 healthy volunteers and 6 CML patients at diagnosis and after six months treatment with Imatinib was collected after an 8-hour period of fasting between 08:00-09:00 AM. Whole blood was collected in EDTA tubes as per standard diagnostic blood drawing procedure by the Department of Haematology, University of Pretoria, Pretoria, South Africa. Platelet-rich plasma (PRP) was obtained from whole blood by centrifuging the blood at 12 300 xg for 2 min and collecting plasma from the separated blood. PRP samples were stored in 1 ml eppendorf tubes within 5 min of separation by freezing at -70°C[11].

Control participants were included in the study if they complied with the following inclusion criteria: aged between 20-60 years, female and male participants, non-smoking, not on any chronic medication. Control participants were excluded according to the following exclusion criteria: chronic or acute illnesses, autoimmune diseases, hereditary diseases, hypertension, hormonal contraceptive use, smokers, and use of platelet affecting drugs i.e. aspirin.

CML patients were included in the study according to the following inclusion criteria: newly diagnosed with CML in the blastic phase of the disease, treated with Imatinib for six months at 400 mg/day (which is the standard dosage for newly diagnosed patients) daily, adult participants aged 18 years and older, female and male participants.

Exclusion criteria for CML patients were as follows: human immunodeficiency virus (HIV) positive patients, patients using platelet affecting drugs (antiplatelet drugs) i.e. cyclooxygenase-1 inhibitors (aspirin), adenosine diphosphate (ADP) receptor antagonists (clopidogrel and prasugrel), and integrin αIIbβ3 (GPIIb-IIIa) receptor blockers (abciximab, eptifibatide, and tirofiban).

2.2 Materials

EDTA tubes and needles were acquired from Transpharm (Gauteng, SA). Microplates (96 well) were obtained from Separation Scientific (Randburg, Johannesburg, SA). Phosphate-buffered saline (PBS) was purchased from Gibco-BRL (Invitrogen, Carlsbad, CA, USA) and prepared as a tenfold concentrated stock solution consisting of 80 g/l NaCl, 2 g/l KCl, 2g/l KH_2PO_4 and 11.5 g/l Na_2PO_4. The latter was prepared in double distilled water (ddH_2O) and the pH adjusted to 7.4. A 1×- solution of PBS was prepared with ddH_2O as a 1:10 dilution of the 10×- stock and subsequently autoclaved

(120˚C, 15 psi, 20 min) before use. Dimethyl sulphoxide (DMSO) and propidium iodide was supplied by Sigma-Aldrich Co. (St. Louis, USA). All blood-contaminated waste materials were collected and discarded into 5 litre biohazardous waste bins.

2.3 Platelet count and activation determination by human cluster of differentiation 41 and 62P

CD41 is a marker expressed on the surface of platelets and megakaryocytes used to isolate platelet populations as it serves as a receptor for cellular adhesion and platelet aggregation[9]. CD62P or P-selectin is found in the α-granule membrane of platelets[9]. When platelets are activated, CD62 is translocated to the outer surface of the platelet. This is an indication of platelet degranulation and secretion[9]. The CD62 platelet marker was used to quantify the extent of activation of platelets by measuring the amount of CD62 expressed on the surface of the platelet.

Platelets were obtained and platelet samples (20 µl, 500 000 platelets) added to 10 ml tubes containing 1 ml PBS[9]. Subsequently, 20 µl CD41- phosphatidylethanolamine (PE), CD41- fluorescein isothiocyanate (FITC) and CD62P-PE were respectively added and incubated in the dark for 20 min at room temperature[9]. Analysis was conducted with the FC500 system flow cytometer (Beckman Coulter South Africa (Pty) Ltd) equipped with an air-cooled argon laser with an excitation wavelength of 488 nm. Information generated from at least 10 000 cells was analyzed by means of Kaluza Analysis Software (Indianapolis, Indiana, USA).

2.4 Annexin V-fluorescein isothiocyanate apoptosis detection assay

During apoptosis, activation of calcium-dependent phospholipid scramblase causes the symmetry of the phospholipid content of the cell membrane to be lost[12]. This occurs in platelets following activation and results in the externalization of the phospholipid layer which can be detected using the phosphatidylserine (PS)-binding protein, Annexin V-FITC[13].

Platelets were obtained as described and platelet samples were resuspended in 100 µl of the 1 x Binding Buffer. Subsequently, 10 µl of Annexin V-FITC was added and incubated for 15 min in the dark at room temperature as described in supplier manual from MACS (Miltenyi Biotec GmbH)[13]. After incubation, samples were washed with 1 ml 1 x Binding Buffer and centrifuged at 300 × g for 10 min. The supernatant was carefully pipetted off and samples were resuspended in 500 µl 1 x Binding Buffer

solution. Annexin V-FITC fluorescence was measured with a FC500 System flow cytometer equipped with an air-cooled argon laser with an excitation wavelength of 488 nm. Events (10 000 to 30 000) was counted for each repeat and analysis of the data was performed with the use of Kaluza Analysis Software (Indianapolis, Indiana, United States). Data from fluorescence channel for green monomers (Fl1 Log) are represented as histograms on the x-axis.

2.5 Statistics

Data is expressed as a ratio of the value measured for diagnosis samples compared to the post-treatment/remission samples defined as mean relative fluorescence. This involves flow cytometry analysis of at least 10 000 events that was repeated for each control and test participant with the use of Kaluza Analysis Software (Indianapolis, Indiana, USA).

The study was set up in two steps, the first being to establish reference values for the outcome parameters of interest in healthy volunteers and in step two chronic myeloid leukaemia patients were assessed for changes from baseline with respect to the outcome parameters following 6 months treatment with Imatinib.

Reference values (mean ±25D) i.e. normal values have been established for the markers of interest. Onset and endpoint (6 months) data for CML patients may be compared with these reference values. For change from onset to endpoint, the reference values ±25D were employed.

All the outcome parameters measured on a continuous scale and are summarized by group, and also by observation period in the cancer patient group, with descriptive statistics. Reference intervals are reported as mean +/- 2 standard deviations and inside the cancer group change over 6 months treatment employed linear-mixed-model analysis. Testing was at the 0.05 level of significance.

3 Results

Platelet counts and activation of platelets were determined by combining CD41 and CD62 markers. These markers are expressed on the surface of platelets. CD62 is expressed upon release from α-granules in activated platelets and plays an important role in cell adhesion and platelet-neutrophil and -monocyte interaction[14]. The combination of the 2 markers allowed for quantification of platelets (CD41) and further quantification of platelet activation (CD62) of CML patients and control participants. Results are indicated in Figures 1 and 2 with line and bar graphs of platelet counts and their extent of activation is displayed in Figures 3, 4 and 5.

Platelet counts were initially high and then decreased statistically significantly in CML patients following 6 months of treatment with Imatinib (Figure 1). Platelet counts of CML patients were furthermore found to be increased in comparison to control participants (Figure 2). Platelet activation was found to be increased for CML patients after 6 months of treatment with Imatinib (Figures 3 and 4). In comparison to control participant's platelet activation, it can be seen that the activation of CML patients' platelet were decreased at the time of diagnosis. After 6 months of treatment with Imatinib, the activation of CML patients' platelets were increased in comparison to the control participants as well as at time of diagnosis for CML patients (Figure 5).

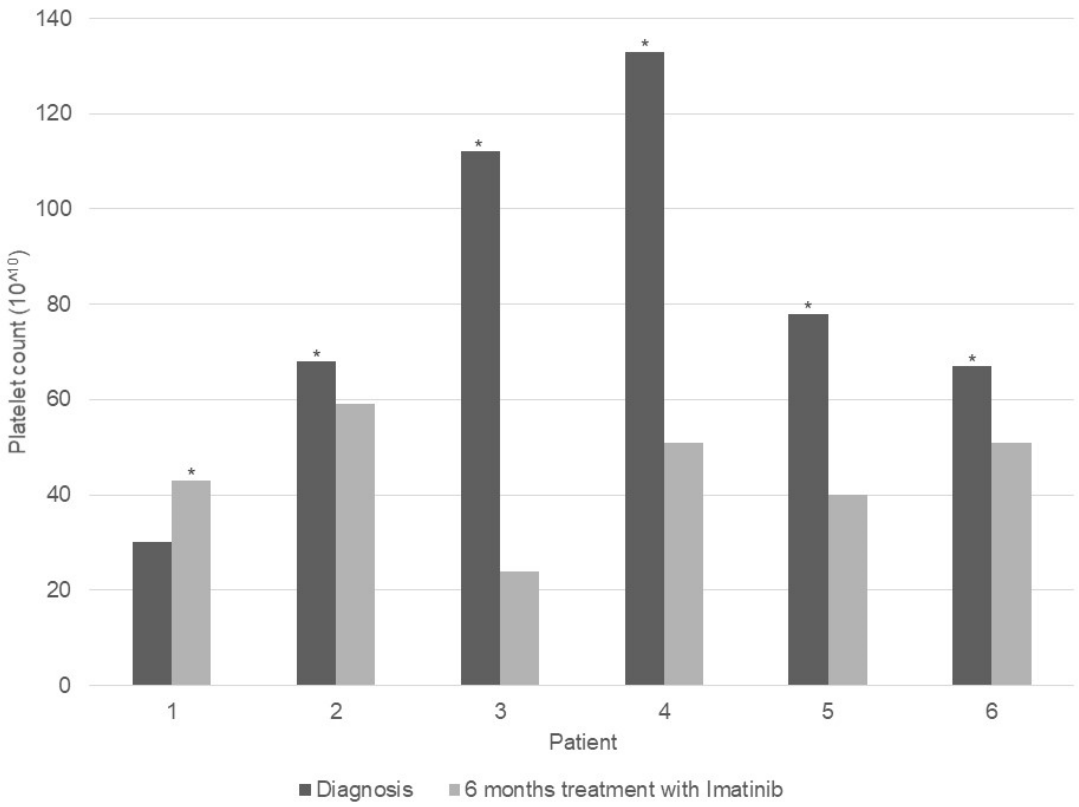

Figure 1: Bar graph of platelet counts of CML patients at diagnosis and after 6 months of treatment with Imatinib. The bar graph indicates the platelet counts for CML patients at diagnosis (dark grey bars) and a statistical significant decrease in platelet counts for all CML patients after 6 months of treatment with Imatinib (light grey bars) (*P*-value <0.05).

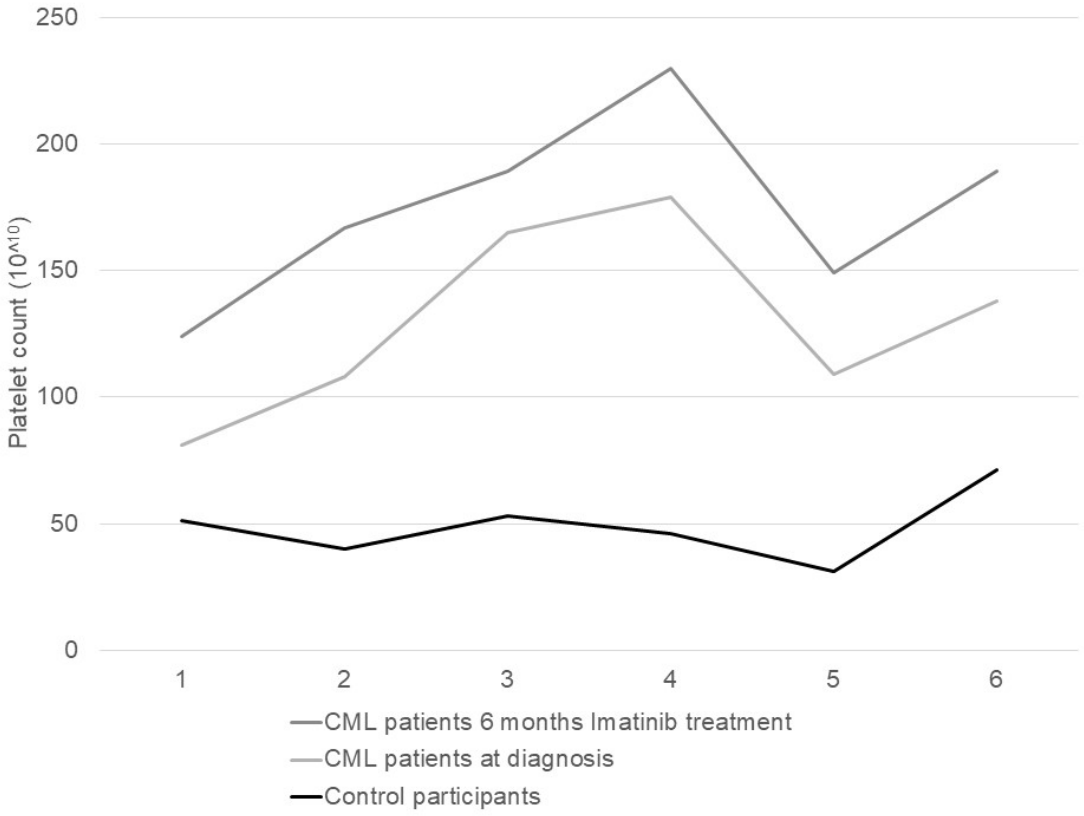

Figure 2: Line graph comparison of platelet counts of CML patients at diagnosis, after 6 months of treatment with Imatinib and control participants. The line graph indicates platelet counts of CML patients at diagnosis (light grey line), CML patients after 6 months of treatment with Imatinib (dark grey line) and control participants (black line). From the comparison of platelet counts, it can be seen that CML patients' platelet counts were elevated at the time of diagnosis compared to the control participants. After 6 months of treatment with Imatinib, CML patients' platelet counts were further elevated in comparison to the control participants as well as patient platelet counts at diagnosis.

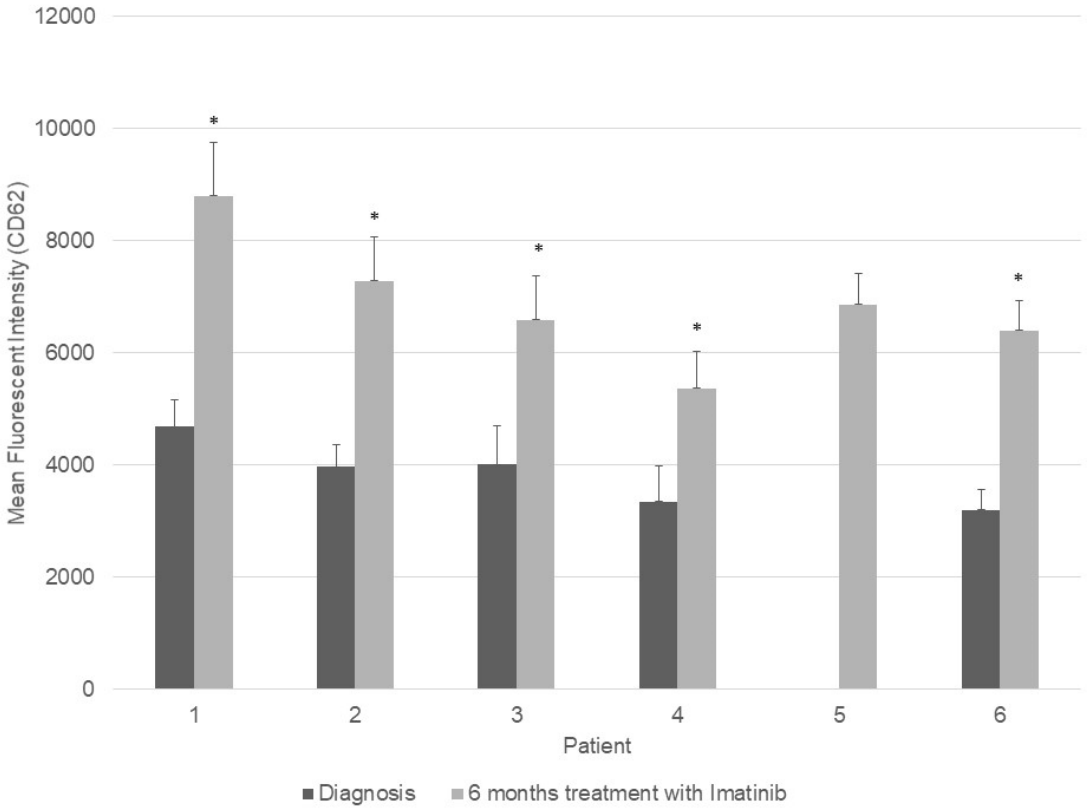

Figure 3: Bar graph of mean fluorescent intensity of CML patients at diagnosis and after 6 months of treatment with Imatinib with the CD62 marker. The bar graph indicates platelet activation for CML patients at diagnosis (dark grey bars) and a statistically significant increase in platelet activation for all CML patients after 6 months of treatment with Imatinib (light grey bars) (*P*-value < 0.05). Patient 5's sample was hypercoagulable before treatment with Imatinib (resulting in the coagulation of the diagnosis sample), but not after treatment.

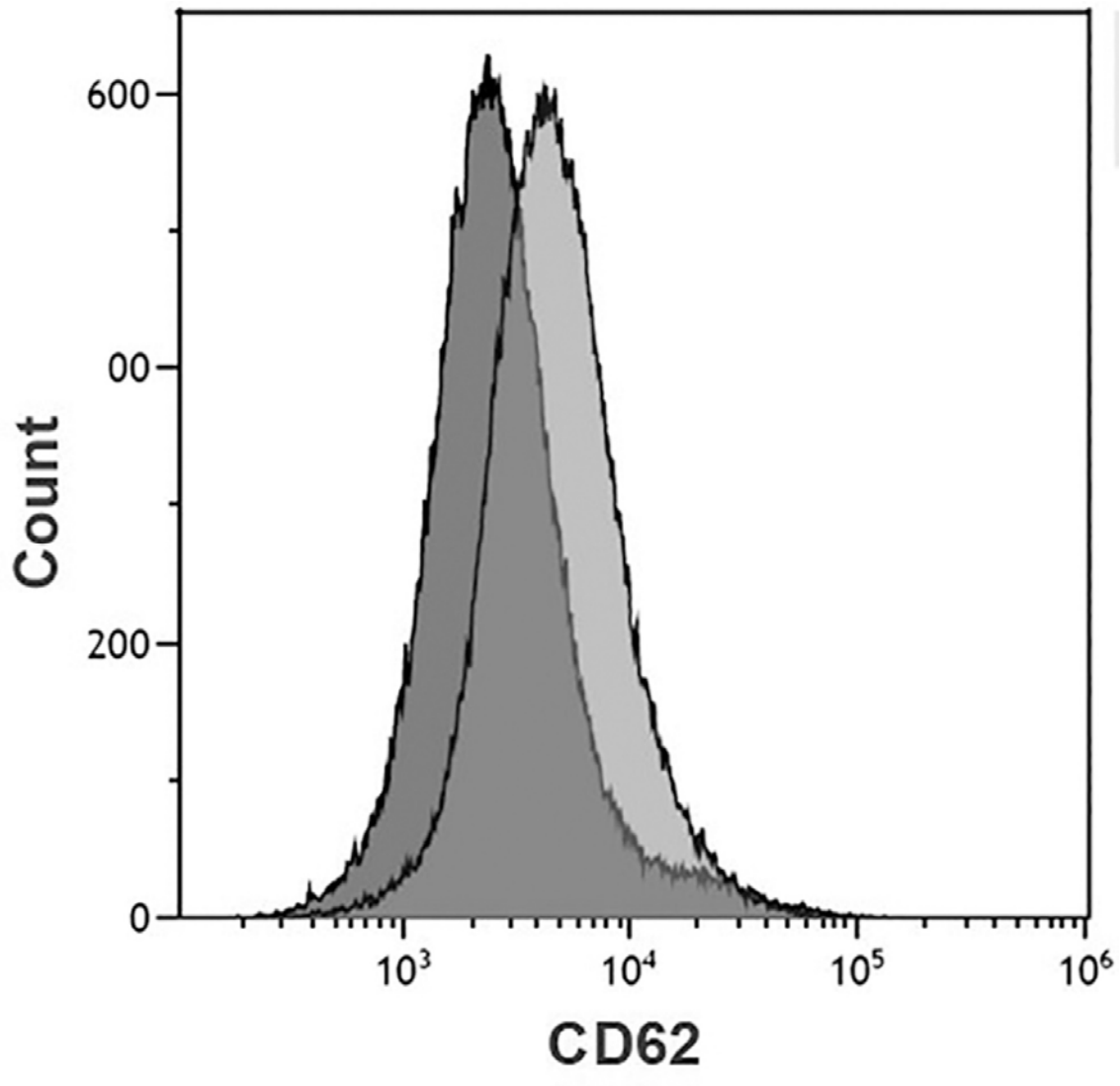

Figure 4: Representative overlay histogram of the mean fluorescent intensity of CML patients at diagnosis and after 6 months of treatment with Imatinib with the CD62 marker. The overlay histogram of platelets from CML patients indicates the occurrence of platelet activation was increased after 6 months of treatment with Imatinib with a mean fluorescence intensity shift of 2041. CML patients at diagnosis is indicated by the dark grey histogram and CML patients after 6 months of treatment with Imatinib is indicated by the light grey histogram.

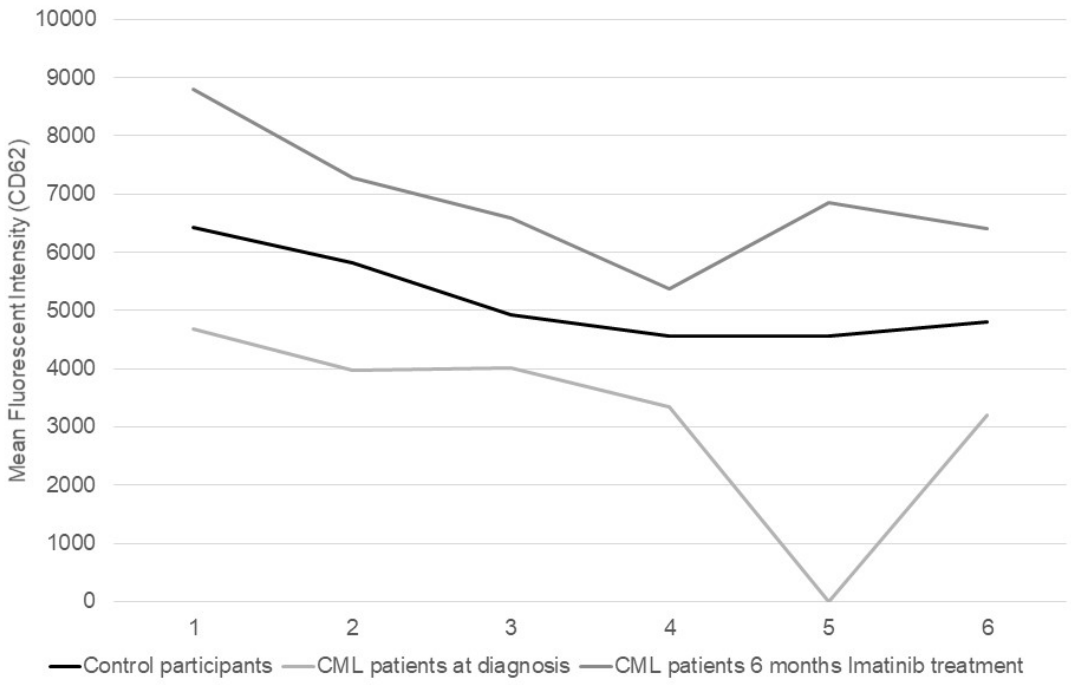

Figure 5: Line graph comparison of mean fluorescent intensity of CML patients at diagnosis, after 6 months of treatment with Imatinib and control participants with the CD62 marker. The line graph indicates platelet activation of CML patients at diagnosis (light grey line), CML patients after 6 months of treatment with Imatinib (dark grey line) and control participants (black line). From the comparison of platelet activation, it can be seen that the activation of CML patients' platelet were decreased at the time of diagnosis compared to the control participants. After 6 months of treatment with Imatinib, the activation of CML patients' platelets were increased in comparison to the control participants as well as at time of diagnosis for CML patients. Patient 5's sample was hypercoagulable before treatment with Imatinib (resulting in the coagulation of the diagnosis sample), but not after treatment.

The results of platelet apoptosis as determined by the Annexin-V FITC marker for CML patients and control participants are shown in Figures 6, 7 and 8. As previously mentioned, phosphatidylserine (PS) is externalised in platelets during apoptosis and activation. This occurs in platelet apoptosis through a Bak/Bax-caspase-mediated pathway independent of activation of platelets[15]. As shown in the platelet activation results (Figures 3 and 4), activation of CML patients platelets were increased after 6 months of treatment with Imatinib. This implies that treatment with Imatinib in CML patients affects platelet activation which could be through activation of apoptotic

mechanisms not only in leukocytes, but concurrently with platelets as an unforeseen consequence. These findings were corroborated by a slight increase of apoptosis in CML patient's platelets after 6 months of treatment with Imatinib (Figures 6 and 7). When comparing platelet apoptosis levels of control participants to CML patients' at the time of diagnosis, an increase in apoptosis was observed. After 6 months of treatment with Imatinib, platelet apoptosis of CML patients' were further increased in comparison to the control participants as well as at time of diagnosis for CML patients (Figure 8).

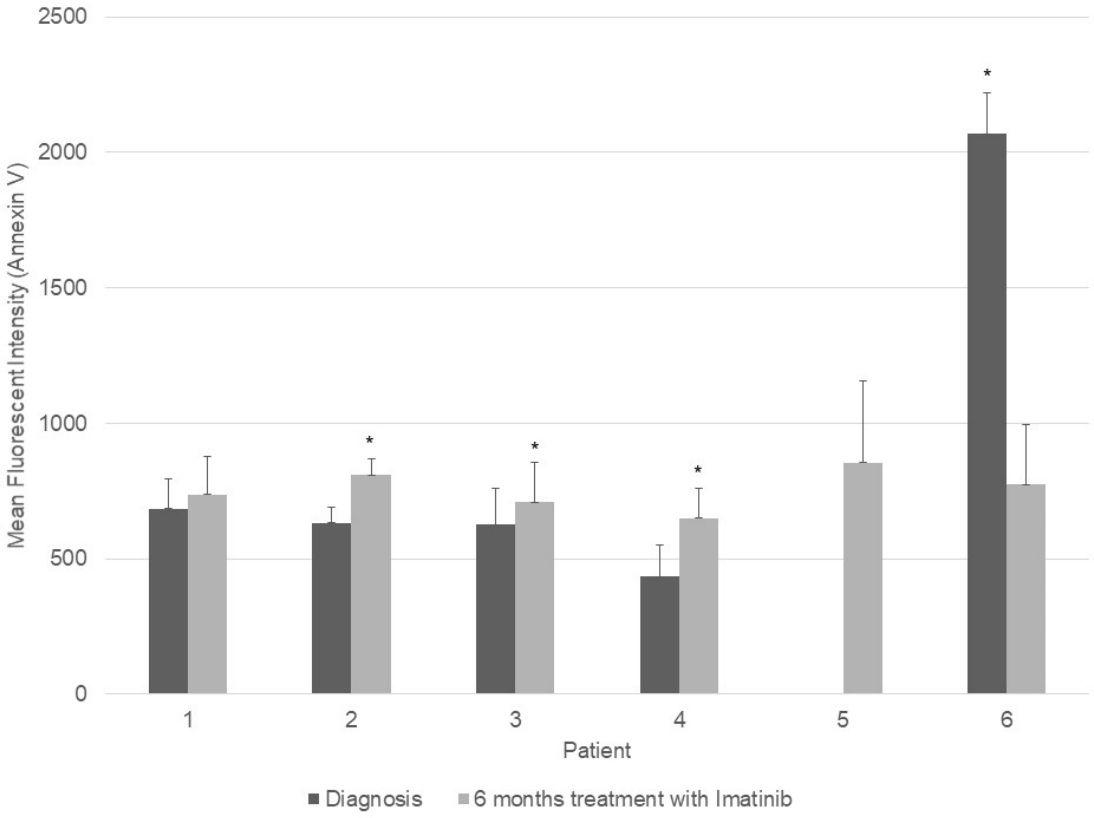

Figure 6: Bar graph of mean fluorescent intensity of CML patients at diagnosis and after 6 months of treatment with Imatinib with the Annexin-V FITC marker. The bar graph indicates platelet apoptosis for CML patients at diagnosis (dark grey bars) and an increase in platelet apoptosis in CML patients after 6 months of treatment with Imatinib (light grey bars) (*P*-value >0.05). In the majority of patients, platelet apoptosis was insignificantly increased after 6 months of treatment with Imatinib. Patient 5's sample was hypercoagulable before treatment with Imatinib (resulting in the coagulation of the diagnosis sample), but not after treatment.

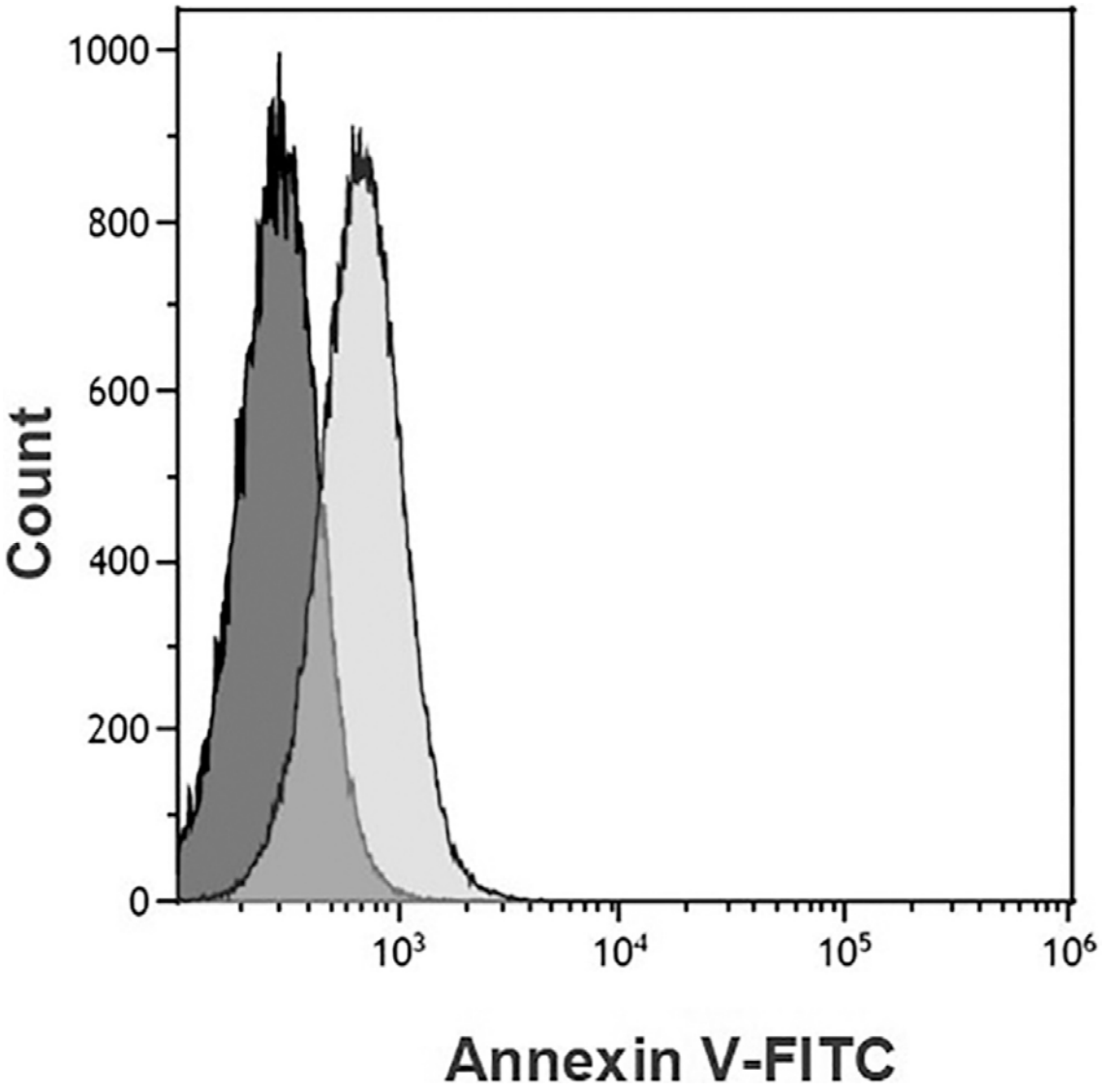

Figure 7: Representative overlay histogram of the mean fluorescent intensity of CML patients at diagnosis and after 6 months of treatment with Imatinib with the Annexin-V FITC marker. The overlay histogram of platelets from CML patients indicates the occurrence of platelet apoptosis was increased after 6 months of treatment with Imatinib with a mean fluorescence intensity shift of 439. CML patients at diagnosis is indicated by the dark grey histogram and CML patients after 6 months of treatment with Imatinib is indicated by the light grey histogram.

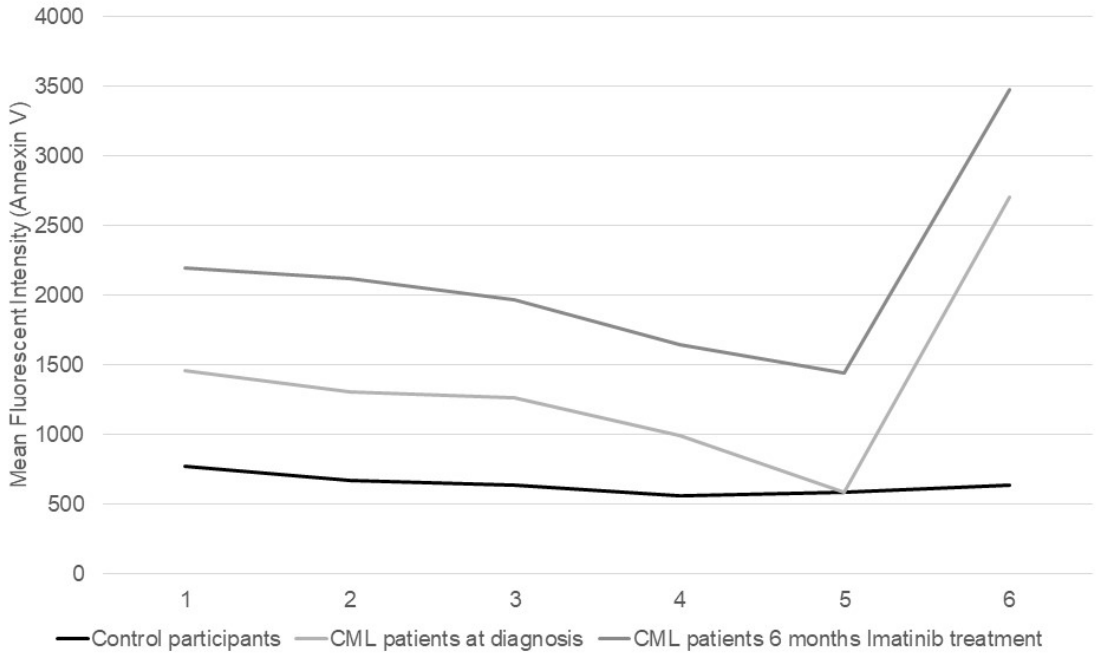

Figure 8: Line graph comparison of mean fluorescent intensity of CML patients at diagnosis, after 6 months of treatment with Imatinib and control participants with the Annexin-V FITC marker. The line graph indicates platelet apoptosis of CML patients at diagnosis (light grey line), CML patients after 6 months of treatment with Imatinib (dark grey line) and control participants (black line). From the comparison of platelet apoptosis, it can be seen that apoptosis of CML patients' platelets were increased at the time of diagnosis compared to the control participants. After 6 months of treatment with Imatinib, platelet apoptosis of CML patients' were further increased in comparison to the control participants as well as at time of diagnosis for CML patients.

Discussion

Chronic myeloid leukaemia's treatment with TKIs has altered the progression of the disease to a chronic, treatable level without the disease progressing to the blast crisis phase. However, as with any diseases treatment, resistance remains a clinical setback and has been found to be on the increase[16]. Patients treated with TKIs are required to endure a lifetime of treatment to maintain the disease in its chronic stable phase, usually associated with significant side effects, the most common being nausea, vomiting, and diarrhoea. Treatment with Imatinib delivers acceptable responses in about 60% of patients; with 20% discontinuing therapy due to intolerance of side effects and 20% developing drug resistance[17]. Resistance to TKIs is problematic and

usually requires follow-up treatment with second or third generation TKIs including, but not limited to, ilotinib, dasatinib, bosutinib and ponatinib, which has financial implications since first-line Imatinib treatment remains the most cost effective strategy[18-19].

Of the frequent symptoms and side effects of CML and TKI treatment, platelet abnormalities have often been reported[20]. The most common platelet abnormality observed in CML patients is thrombocytosis; the current study's findings are in agreement[21]. The results showed that CML patient's platelet counts were elevated upon diagnosis and these levels decreased significantly after 6 months of treatment with Imatinib (*P*-value <0.05). At the time of diagnosis, CML patients presented with platelet dysfunction that may be as a result of the pathogenesis of CML. After 6 months of treatment with Imatinib these levels decreased, as a result of treatment with Imatinib and subsequent increased apoptosis resulting from treatment with Imatinib. Since Imatinib functions to block the formation of tyrosine kinase proteins, it can be postulated that treatment with Imatinib, therefore, results in atypical tyrosine kinase activity in platelets[17]. Furthermore, Imatinib is a key inhibitor of platelet-derived growth factor (PDGF), a growth factor released from the α-granules of platelets known to play a role in wound healing and repair[22]. These factors could result in the targeting of these ineffective, altered platelets for programmed cell death or apoptosis since their functioning is abnormal.

Platelet apoptosis occurs through the intrinsic apoptotic pathway and this cellular mechanism is understood to regulate the lifespan of platelets[23]. Apoptosis in platelets comprises its ability to maintain and regulate their cellular homeostasis and is of importance in platelets as their lifespan is only about 10 days in humans[24-25]. During this process, PS is externalised on the platelet membrane in order for macrophages to remove apoptotic platelets through phagocytosis[23, 26-27]. Platelet apoptosis is closely associated with platelet activation, as platelet activation is accompanied by PS externalization, cell shrinkage and formation of microparticles which is suggestive of the apoptotic process[28].

Results indicated that platelet activation in CML patients was significantly increased after 6 months of treatment with Imatinib compared to levels at diagnosis (*P*-value <0.05). Similarly, platelet apoptosis was also found to be increased after 6 months of

treatment with Imatinib in CML patients. It can, therefore, be postulated that the decrease seen in platelet counts after 6 months of treatment with Imatinib corresponds to the increased platelet activation and apoptosis observed.

Increased platelet activation and apoptosis are known to occur independently from one another; platelet activation is necessary for the aggregation and clotting functions of platelets, whereas platelet apoptosis functions to clear platelets from the circulation through phagocytosis which is thought to be partly due to platelet ageing[28].

Platelet activation in patients with CML is reported to be associated with increased thrombosis[29-30]. However, the effect of Imatinib on platelets of CML patients is not well understood. Platelets counts and specifically thrombocytosis is widely reported in CML patients; in the current study these reports are corroborated. The current study further reports on a statistically significant decrease in platelet counts after treatment with Imatinib for 6 months which can partly be attributed to the increase in platelet activation and apoptosis detected. This study highlights the fact that platelets are integral to CML pathogenesis and that treatment with Imatinib affects the mechanisms of platelet activation and apoptosis, decreasing platelet counts and increasing the chances for thrombolic events as a result of impaired platelet function. The abnormalities in platelet functioning found in this study could initially be due to the pathogenic clonal proliferation of haematopoietic cells in CML patients, specifically of megakaryocyte precursors[6]. The abnormalities found after 6 months of treatment with Imatinib may be relatively attributed to the inhibition of platelet tyrosine kinase's as well as the inhibition of PDGF which disrupts the normal homeostasis of platelets. However, the exact mechanism of Imatinib-related platelet dysfunction is not fully understood[6-7].

Conclusion

The importance of targeted therapies in CML has recently become evident as the occurrence of tyrosine kinase inhibitor resistance and, specifically Imatinib resistance, increases. This study indicated the impact that Imatinib treatment has on the functioning of platelets activation and apoptotic processes. Results showed that CML patient's platelet counts were elevated upon diagnosis and these levels decreased significantly after 6 months of treatment with Imatinib. Platelet activation in CML patients was significantly increased after 6 months of treatment with Imatinib

compared to levels at diagnosis and platelet apoptosis was also found to be increased after 6 months of treatment with Imatinib. There is very limited literature available on the involvement of platelets in CML progression, the current study provides the first insight of the impact of CML pathogenesis, as well as Imatinib treatment on the activation and apoptosis of platelets in CML patients. Further studies should focus on the possible identification of targets of interest including autophagic and angiogenic mechanisms in platelets of CML patients.

Declarations

Ethics approval and consent to participate

Ethical clearance was obtained from The Research Ethics Committee, Faculty of Health Sciences, University of Pretoria which complies with ICH-GC.P guidelines (Ethics clearance number: 284/2015).

Data Sharing and Data Accessibility

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Authors' contributions

LR was responsible for conducting all experiments, analysing data, literature review and the main contributor to drafting of the manuscript. RP, MK, GT, PB and AMJ assisted in drafting of the manuscript. All authors read and approved the final manuscript.

List of Abbreviations

ABL1 - Abelson murine leukaemia viral oncogene homolog 1

ADP - Adenosine diphosphate

AM - Ante-meridiem

BCR - Breakpoint cluster region protein

CA - California

CD41 - Human cluster of differentiation 41

CD62P - Human cluster of differentiation 62

CML - Chronic myeloid leukaemia

ddH_2O - double distilled water

DMSO - Dimethyl sulphoxide

EDTA - Ethylenediamine tetra acetic acid

FITC - Fluorescein isothiocyanate

HIV - Human immunodeficiency virus

KCl - Potassium chloride

KH_2PO_4 - Monopotassium phosphate

MACS - Magnetic-activated cell sorting

Min - Minute

Na_2PO_4 - Disodium phosphate

NaCl - Sodium chloride

PBS - Phosphate-buffered saline

PDGF - Platelet-derived growth factor

PE - Phosphatidylethanolamine

Ph - Philadelphia

PRP - Platelet-rich plasma

PS - Phosphatidylserine

Psi - Pounds per square inch

SA - South Africa

TKI - Tyrosine kinase inhibitor

USA – United States of America

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