Low leukotriene B4 receptor 1 leads to ALOX5 down-regulation at diagnosis of chronic myeloid leukaemia.

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Running title: *ALOX5* and CML

Word count: abstract 202, main text 2894, tables 1 and figures 4.

Key words: ALOX5, LTB4, LTB4R1 (BLT1), CML, Chronic myeloid leukaemia

ABSTRACT

ALOX5 is implicated in chronic myeloid leukaemia development in mouse leukaemic stem cells, but its importance in human chronic myeloid leukaemia is unknown. Functional *ALOX5* was assessed using an leukotriene-B4 ELISA and *ALOX5* and leukotriene-B4 receptor 1 mRNA expression was determined via a TaqMan gene expression assay. leukotriene-B4 receptor 1 and 5-LOX protein levels were assessed by cell surface flow cytometry analysis. At diagnosis *ALOX5* was below normal in both blood and CD34+ stem cells in all patients. On treatment initiation, *ALOX5* levels increased in all patients except those who were destined to progress subsequently to blast crisis. leukotriene-B4 levels were increased despite low *ALOX5* expression, suggesting that the arachidonic acid pathway is functioning normally up to the point of leukotriene-B4 production. However, the leukotriene-B4 receptor protein in newly diagnosed patients was significantly lower than after a period of treatment ($p<0.0001$). The low level of leukotriene-B4 receptor 1 at diagnosis explains the down-regulation of *ALOX5.* In the absence of leukotriene-B4 receptor 1, the arachidonic acid pathway intermediates (5-HEPTE and LTA4) negatively regulate *ALOX5. ALOX5* regulation is aberrant in chronic myeloid leukaemia patients and may not be important for the development of the disease. Our data suggest caution when extrapolating mouse model data into human chronic myeloid leukaemia.

INTRODUCTION

Chronic myeloid leukaemia (CML) is a malignant disease of a primitive haematological cell, characterised by inappropriate expansion of myeloid cells. Although the disease is readily controlled by imatinib, approximately one third of patients will eventually fail treatment,[1, 2] and a significant proportion of these will progress towards blast crisis which is usually fatal. However, the factors that contribute towards CML growth and progression are not well understood. Recently, Chen *et al*[3] reported that Arachidonate 5-lipoxygenase *(ALOX5)* is up-regulated in mouse leukaemic stem cells, and this up-regulation is not inhibited by imatinib treatment. Furthermore, mice transplanted with *ALOX5* deficient BCR-ABL1 positive bone marrow cells were resistant to CML induction. *ALOX5* deficiency had no effect on the growth of BCR-ABL1 negative cells, suggesting that it may be essential for malignant but not normal haemapoietic stem cell growth, through an unknown mechanism.

The *ALOX5* gene encodes a member of the lipoxygenase gene family and plays a role in the synthesis of leukotrienes from arachidonic acid (Figure 1). *ALOX5* catalyses the conversion of arachidonic acid to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HEPTE), and subsequently to the allylic epoxide 5(S)-trans-7,9-trans-11,14-cis-eicosatetraenoic acid (leukotriene A4/LTA4). LTA4 is unstable and is converted to LTB4 which is more stable. 5-HEPTE and LTA4 negatively regulate *ALOX5* expression; positive regulation of *ALOX5* occurs when LTB4 binds to its receptor LTB4R1 (also known as BLT1) to mediate its positive feedback on *ALOX5*.[4-9]

The role of *ALOX5* in human CML is unknown. The hypothesis proposed in this study was that CML cells have high levels of *ALOX5* and that the level of *ALOX5* predicts a patient's response to imatinib treatment. If this hypothesis were correct, then *ALOX5* may be a new therapeutic target.

METHODS

Sample collection: Blood samples were collected from 48 patients at initial chronic phase diagnosis and at 3, 6 and 12 months following the commencement of imatinib treatment. Plasma and total leukocytes were prepared. Samples were enriched for CD34+ cells using CliniMACS (Miltenyi Biotec, Surrey, UK) according to the manufacturer's instructions. The study was approved by the Liverpool Central Research Ethics Committee and all patients gave informed consent. Patient characteristics are shown in Table 1. Peripheral blood from healthy volunteers were used as normal controls (n=10)

Clinical response: Following 12 months of imatinib treatment, patients were stratified into three clinical outcomes:

- **Responders – Patients who achieve a** complete cytogenetic response CCR defined as no Philadelphia positive metaphases amongst at least 20 marrow metaphases. In some cases serial cytogenetic data were not available and achievement of CCR is based on a BCR-ABL1/ABL1 transcript ratio of < 1%, which we have previously shown to be tightly correlated with cytogenetically defined CCR. [10]
- **Non-responders** – Patients who had achieved a complete haematological response but not a complete cytogenetic response and who had not progressed.
- **Blast crisis** – patients who presented in chronic phase but who subsequently progressed into blast crisis.

Plasma preparation: 5ml of peripheral blood was collected into EDTA, and centrifuged at 770g for 15 minutes. Plasma was collected from samples, aliquoted and stored at -20°C prior to use.

Leukotriene B4 ELISA: LTB4 assay was performed according to the manufacturer's protocol (Cambridge Biosciences, Cambridge, UK). Briefly 100µl of the specific standard, sample or assay buffer was added in duplicate to appropriate wells of a 96-well plate and a further 50µl of assay buffer to the negative control wells. 50µl of LTB4 conjugate and 50µl of LTB4 antibody, supplied in the kit, were added to the appropriate wells. The plate was incubated at room temperature, with shaking, for 2 hours. Following incubation the plate was washed three times with wash solution (provided in the kit). 5µl of conjugate was added to the total activity wells, followed by 200µl of pNpp substrate solution to every well. The plate was incubated at 37°C with shaking, for 2 hours. 50µl of 2M HCl was then added to all wells. The absorbance was immediately read at 405nm using a BioTeK µQuant plate

reader. The mean optical density of the negative controls was subtracted from the optical density reading of all wells to eliminate "background noise."

***ALOX5* and LTB4R1 expression**: qRT-PCR was performed using cDNA from either total leukocytes or purified CD34+ cells. Pre-designed TaqMan real time PCR assays were used for *ALOX5* Hs01095330_m1, *BLT1 (LTB4R1)* Hs019388704_s1 and *GAPDH* Hs99999905_m1 (Applied Biosciences, UK). In evaluating the mRNA expression data, the comparative Ct method was used, with the $2^{-\Delta\Delta Ct}$ formula to achieve results for relative quantification (RQ).[10] A pool of cDNA from 4 normal individuals was used as calibrator and all the samples were normalised to GAPDH. The RQ Manager software supported by the ABI Prism 7900HT System was used for data analysis.

Measurement of LTB4 receptor 1 (BLT1): 5ml of peripheral blood was collected into EDTA from CML patients attending the CML clinic. Samples were processed within 4 hours of the sample being taken and cells were used fresh. Briefly, erythrocytes were depleted using red cell lysis buffer ((0.1M ammonium chloride, 10mM sodium bicarbonate and 1.3mM EDTA (Sigma-Aldrich, Dorset, UK). Total leukocytes were then incubated with Leukotriene B4 Receptor 1 (BLT1/LTB4R1) (R&D systems, Abingdon, UK.) and anti -mouse IgG1 antibody (BD) as the control antibody, in the dark for 30 minutes with shaking. Cells were then washed in PBS containing 0.5% BSA and analysed by FACS.

Measurement of ALOX5 protein by FACS: 5-lipoxygenase (5-Lox) protein was assessed by FACS using the method previously described using 5-LOX antibody (Santa Cruz Biotechnology, California, USA) and mouse IgG1 (Becton Dickinson (BD), Oxford, UK) as an isotypic control.[11]

RESULTS

***ALOX5* mRNA expression and clinical outcome**

In order to determine whether *ALOX5* expression was elevated in CML patients, and if the levels were related to clinical outcome, *ALOX5* expression was measured at diagnosis (chronic phase) and following 3, 6 and 12 months of imatinib treatment. Patients' details are shown in table 1. At diagnosis the expression level of *ALOX5* was below the level observed in normal healthy volunteers cDNA pool. This was the case in responders, non-responders and those who subsequently progress to blast crisis, as defined in the design and methods section (Figure 2). No difference was seen between patients according to their Sokal score (data not shown). In the responder cohort once imatinib treatment had commenced, the expression level of *ALOX5* increased and remained high throughout treatment. The increase in *ALOX5* expression compared with diagnosis was statistically significant at 3, 6 and 12 months ($p=0.003$, $p=<0.001$ and $p=0.024$ respectively). These data suggest that *ALOX5* expression in patients who subsequently achieve a CCR is restored to a level observed in the normal calibrator pool upon treatment (Figure 2A). A similar trend in *ALOX5* expression was observed in the non-responder group (Figure 2B), with *ALOX5* expression increasing once treatment had commenced. Again the increase in *ALOX5* expression compared with diagnosis was statistically significant at 3, 6 and 12 months ($p=0.008$, $p=0.03$ and $p=0.013$ respectively). Conversely, in patients destined to subsequently progress to blast crisis, imatinib treatment failed to increase *ALOX5* expression (Figure 2C).

These data suggest that a failure to increase *ALOX5* expression following three months of imatinib treatment may indicate a higher risk of disease progression (Figure 2C). No difference in *ALOX5* expression was observed at diagnosis between those patients destined to progress to blast crisis and the responder and non-responder groups, suggesting that *ALOX5* expression at diagnosis is not predictive of the outcome of imatinib treated patients.

When the same experiment was repeated on diagnostic CD34+ stem cells (from the same patients), no difference in *ALOX5* expression was observed between responders and non-responders. *ALOX5* expression was lower in CD34+ cells than in total leukocytes when comparing results in figure 2 A and D, although similar overall results were obtained, confirming that *ALOX5* is down-regulated in CML patients in both CD34+ and chronic phase MNC (Figure 2, panels A and D, $p=0.01$).

To determine whether 5-LOX protein levels were a function of *ALOX5* gene expression, 5-LOX protein expression was assessed by FACS. As patients respond to imatinib treatment and achieve a major

molecular response (MMR), there is a trend for an increase in the amount of detectable ALOX5 protein (Figure 2E); this is consistent with the increase in mRNA expression observed in Figure 2A-C.

***ALOX5* function - LTB4 levels in CML patients**

Plasma levels of LTB4 can be used as a marker of *ALOX5* functional activity (as previously described by Chen *et al*[3]). LTB4 levels were measured at diagnosis, three and six months post imatinib treatment in 27 patients of known subsequent clinical outcome (18 responders, 7 non-responders and 2 patients whom subsequently progressed to blast crisis, Figure 3A). LTB4 levels increased in all three patient groups following imatinib treatment. At diagnosis the LTB4 level in patients destined to progress to blast crisis was the lowest of the three patient groups. Conversely, once imatinib treatment commenced the LTB4 level increased to the highest level compared to the other two clinical groups (Figure 3A). The levels of LTB4 in the CML patients were in general higher than normal; this may indicate an accumulation of LTB4 and a potential block in the arachidonic acid pathway.

Since LTB4 levels increased in all patients assessed, it was necessary to confirm whether this was a direct physiological response to imatinib treatment, or if it was due to the disease. To address this issue, MNC from five healthy volunteers were treated with 5µM imatinib for 24 hours and the changes in LTB4 levels were measured. No significant difference was observed between the untreated and imatinib treated normal samples, thus suggesting that changes in LTB4 observed in CML patients may be attributed to the leukaemia and not as a physiological response to imatinib treatment (Figure 3B).

ALOX5 function was assessed using samples from patients diagnosed in chronic phase and from patients actually in blast crisis. LTB4 levels were found to be elevated in samples taken at blast crisis compared to chronic phase samples, but this difference was not statistically significant. The observed increase in LTB4 observed coincided with a decrease in *ALOX5* expression as shown is figure 2C.

LTB4 receptor 1 (BLT1) is low at diagnosis

The original hypothesis proposed in this study was that CML patients would have high levels of *ALOX5*. The data presented thus far are in opposition to both this hypothesis and the study presented by Chen *et al*,[3] which demonstrated that mice transplanted with BCR-ABL1 positive bone marrow cells lacking the *ALOX5* gene were resistant to the induction of CML, suggesting that *ALOX5* was essential for the induction and development of CML.[3] However, that study was performed using a mouse model, and the experiments have not yet been carried out on human CML samples, which could explain the discrepancies between the present data and those of Chen *et al*.[3] In order to further explain the

differences between the two studies, the LTB4 pathway was interrogated further. Figure 1 demonstrates the arachidonic acid metabolism pathway. Prior to conversion to LTB4, arachidonic acid is converted into two intermediate products, 5-HEPTE and then LTA4. Both 5-HEPTE and LTA4 negatively regulate *ALOX5* expression, while LTB4 is known to positively regulate *ALOX5* expression.[12] In order for LTB4 to mediate its positive effect on *ALOX5* expression it must bind to the LTB4 receptor.

In the CML clinical samples investigated it is apparent that *ALOX5* expression is suppressed below normal. Furthermore, the LTB4 levels in the CML samples were elevated compared to normal healthy controls, suggesting an accumulation of LTB4. Since LTB4 needs to bind to its receptor LTB4R1 to positively regulate *ALOX5* expression, these data suggest that LTB4R1 expression or function may be abnormal in CML.

LTB4R1 protein expression was measured by cell surface FACS analysis. LTB4R1 protein was found to be low in newly diagnosed chronic phase CML samples compared to normal (p=<0.0001, Figure 4A). LTB4R1 protein was even lower in a blast crisis sample. We next compared the levels of LTB4R1 protein in patients being treated by imatinib and stratified these data by the patients BCR-ABL1/ABL1 percentage. LTB4R1 protein levels were lower than normal in those patients partially responding to imatinib treatment (defined as a BCR-ABL1/ABL1 ratio of 1-10%) (p=0.04). LTB4R1 levels increased from the diagnosis level once treatment had commenced. Patients partially responding and those patients whom had achieved a complete cytogenetic response (defined as a BCR-ABL1/ABL1 ratio of <1%[13]) had significantly higher levels of LTB4R1 protein compared to the level observed at diagnosis (p=0.02 and p=<0.0001, respectively). These data suggest that there is an inverse relationship between LTB4R1 protein and BCR-ABL1 mRNA expression. LTB4R1 protein is very low at diagnosis but increases towards normal as the BCR-ABL1 mRNA expression decreases.

LTB4R1 mRNA expression

LTB4R1 mRNA expression was found to be low in chronic phase diagnostic samples compared to patients who had received 12 months of imatinib treatment (Figure 4B). In the responder group the LTB4R1 expression increased more than in the non-responder group, although this was not statistically significant. When LTB4R1 mRNA expression was assessed in samples from patients in blast crisis at the time of sampling, LTB4R1 mRNA expression was much lower than the diagnostic chronic phase group and the 12 month treatment samples. LTB4R1 mRNA expression was not statistically significant at diagnosis, nor did it predict a patient's clinical outcome (data not shown).

As patients progress to blast crisis, *ALOX5* gene expression is suppressed, possibly due to an accumulation of pathway intermediates (5-HEPTE and LTA4) and a decrease in LTB4R1. This idea is supported by the LTB4R1 protein data and the accumulation of LTB4 in blast crisis. LTB4R1 mRNA expression was also assessed in CD34+ stem cells taken at diagnosis (Figure 4C). No difference in LTB4R1 expression was observed between those patients who later achieved a CCR and those who did not.

CONCLUSIONS

The identification of proteins which discriminate leukaemic from normal stem cells is a challenging concept. Successful identification and therapeutic targeting of leukaemic stem cells remains the ultimate goal in eradicating CML.

The present data suggest that although *ALOX5* has been identified as being essential for the development of CML in mice, this is not the case in human CML. Initial assessment of *ALOX5* expression pre and post imatinib treatment in human CML demonstrated that *ALOX5* was down-regulated below normal, unlike the mouse model of CML which showed that *ALOX5* was up-regulated. Following imatinib treatment, *ALOX5* mRNA expression increased in the responder and non-responder groups. In patients who subsequently progressed to blast crisis, no change in *ALOX5* expression was observed. The failure to increase *ALOX5* expression following 3 months of imatinib treatment may identify patients at high risk of disease progression. However whether changes in ALOX5 expression offer any additional value beyond those obtained from measuring early cytogenetic or molecular responses remains unknown and would need to be tested further as part of a clinical trial.

ALOX5 function was assessed by measuring plasma LTB4. LTB4 was found to increase in all three groups of patients following imatinib treatment. LTB4 was also found to be increased in blast crisis compared to chronic phase. These data suggest that the arachidonic acid pathway (Figure 1) is functionally active to the point of LTB4 production.

To determine the differences between the *ALOX5* pathway observed in mice and the clinical samples studied, the LTB4 receptor (BLT1) was investigated. LTB4 acts by binding to LTB4R1 in order to mediate its positive role on *ALOX5*. At diagnosis of CML patients had very low levels of LTB4R1 protein compared to normal samples. Following imatinib treatment LTB4R1 levels increased towards normal and in a case in blast crisis LTB4R1 protein was further suppressed. Using the Bayesian model

averaging (BMA) method on a large microarray data set Radich *et al*[14] identified six genes that discriminate between early chronic phase, late chronic phase, accelerated phase and blast crisis, one of these genes was LTB4R1. The authors found this to be down-regulated during blast crisis which is in agreement with our data. These findings help to explain the data seen in CML patients. At diagnosis of CML, *ALOX5* gene expression was found to be suppressed and there was an accumulation of LTB4. The accumulation of LTB4 is likely due to lack of the LTB4 receptor. A lack of LTB4R1 allows the namely 5-HEPTE and LTA4 intermediates of the pathway to suppress *ALOX5* gene expression, and thus LTB4 accumulates as it has no receptor to bind to. Following imatinib treatment LTB4R1 protein increases; thus LTB4 can bind to its receptor and positively regulate *ALOX5* as demonstrated by an increase in 5-LOX protein and gene expression.

In patients with atherosclerosis the binding of LTB4 to LTB4R1 induces the rapid phosphorylation of mitogen activated protein kinases (MAPK, ERK1/2 and JNK1/2) and PI3K/AKT, and also increases NF-κB activation,[15] which are all targets of BCR-ABL1. It is therefore interesting to speculate that CML cells attempt to switch off excessive signalling via these pathways by down-regulating LTB4R1 in order to try and maintain cellular homeostasis.

Our conclusions are based on work carried out using both MNC and CD34+ cells. We would like to extend these observations by assessing ALOX5 and LTB4R1 expression in primitive CD34+CD38- or CD34+CD38-CD90+ stem progenitor cells, but like most laboratories we do not have access to such sorted primitive sub populations. We hope these can be the subject of future studies.

In conclusion, it is apparent that two different pathways exist with regards to *ALOX5* between the CML mouse model and CML patients. This accounts for the difference between the clinical samples studied herein and the results of the Chen *et al*[3] paper. These data suggest caution when extrapolating mouse model data into human CML.

ACKNOWLEDGEMENTS

We would like to thank the Alison Holcroft, Rachael Fowler and Andrea Davies for their help and support during this project. AG was supported by a generous donation in memory of a CML patient.

AUTHORSHIP AND DISCLOSURES

CML and RJH contributed equally to this study and share first authorship.

CML, RJH and REC designed the study and wrote the manuscript.

CML, AG, and EM performed the laboratory work for this study.

All authors report no conflict of interest.

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Table 1. Patient characteristics.

	No. patients	M/F	Mean age (range)	Sokal score			
				Low	Intermediate	High	Unknown
Responders	25	11/14	43 (19-60)	15	3	5	2
Non-responders	17	8/9	49 (23-67)	4	3	7	3
Blast crisis	6	5/1	37 (24-59)	1	2	2	1
Total	48	24/24	44 (19-67)	20	8	14	6

FIGURE LEGENDS

Table 1. Patient characteristics

Figure 1. Arachidonic acid metabolism pathway.

5-LOX is encoded by the *ALOX5* gene and is activated by the enzyme FLAP. Arachidonic acid is converted to 5-HEPTE by 5-LOX which is further converted to LTA4 by 5-LOX. Both 5-HEPTE and LTA4 negatively regulate *ALOX5* expression. LTA4 is unstable and is further converted to LTB4. The binding of LTB4 to its receptor LTB4R1 positively regulates *ALOX5* expression.

Figure 2. *ALOX5* expression

Panel A: *ALOX5* expression levels in the responder group (n=25). **Panel B:** *ALOX5* expression levels in the non-responders group (n=17). **Panel C:** *ALOX5* expression levels in patients destined to progress into blast crisis (n=6). **Panel D:** *ALOX5* expression levels in CD34+ cells taken at diagnosis and stratified by the patients' eventual clinical response. The normal level was determined using four healthy volunteers (n=6). **Panel E:** 5-LOX protein expression. 5-LOX protein was assessed by FACS using fresh leukocytes (n=9). 5-LOX expression is higher in CML patients in major molecular response as determined by qRT-PCR.

Figure 3. LTB4 levels following imatinib treatment.

Panel A. Mean plasma LTB4 levels of 27 patients stratified by clinical outcome; 18 responders, 7 non-responders and 2 blast crisis. Samples were taken during chronic phase at diagnosis, 3 and 6 months after commencement of imatinib treatment. Normal level was determined using plasma from 15 healthy volunteers. **Panel B.** MNC from five healthy volunteers were treated with 5μM imatinib for 24 hours and the changes in LTB4 levels were measured. No significant difference was observed between the untreated and imatinib treated normal samples.

Figure 4. LTB4 (BLT1) receptor expression.

Panel A: LTB4R1 surface expression was measured in newly diagnosed chronic phase CML patients (n=5), a patient in blast crisis (n=1), patients currently responding to imatinib treatment (n=6), patients in CCR (n=10) and healthy volunteers (n=10). LTB4R1 cell surface expression was low in newly diagnosed chronic phase CML patients compared to patients responding to treatment or healthy volunteers. P values denoted with *refer the comparison made with normal blood. **Panel B:** LTB4R1 mRNA expression was measured in newly diagnosed chronic phase CML patients (n=24), patients treated with imatinib for twelve months who achieved a CCR (n=10) and non-responders (n=8), as well

as patients who were in blast crisis (n=6). **Panel C:** LTB4R1 mRNA expression in diagnostic CD34+ cells stratified by the patient's clinical outcome.

Figure 1. Arachidonic acid metabolism pathway.

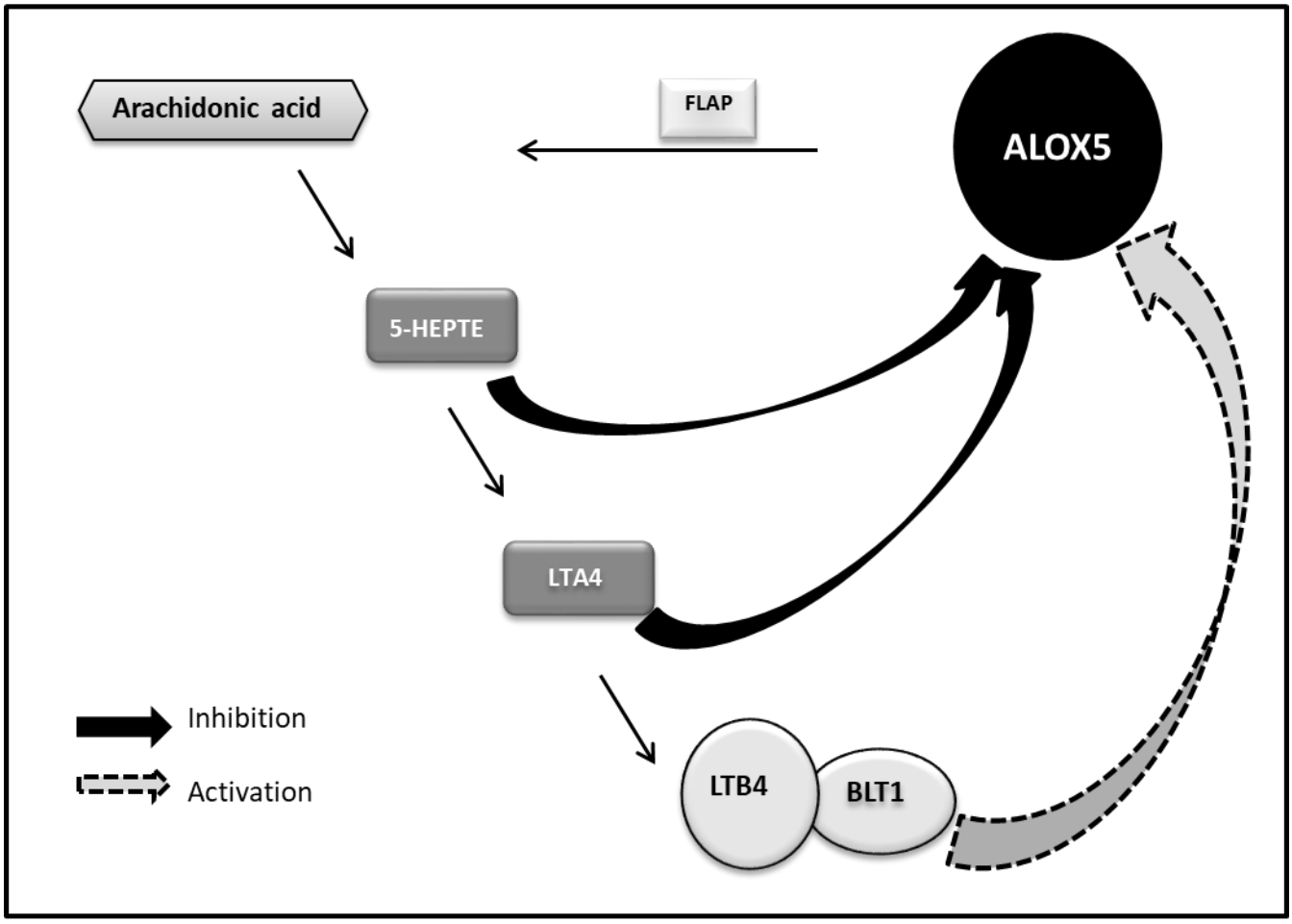

Figure 2. *ALOX5* expression

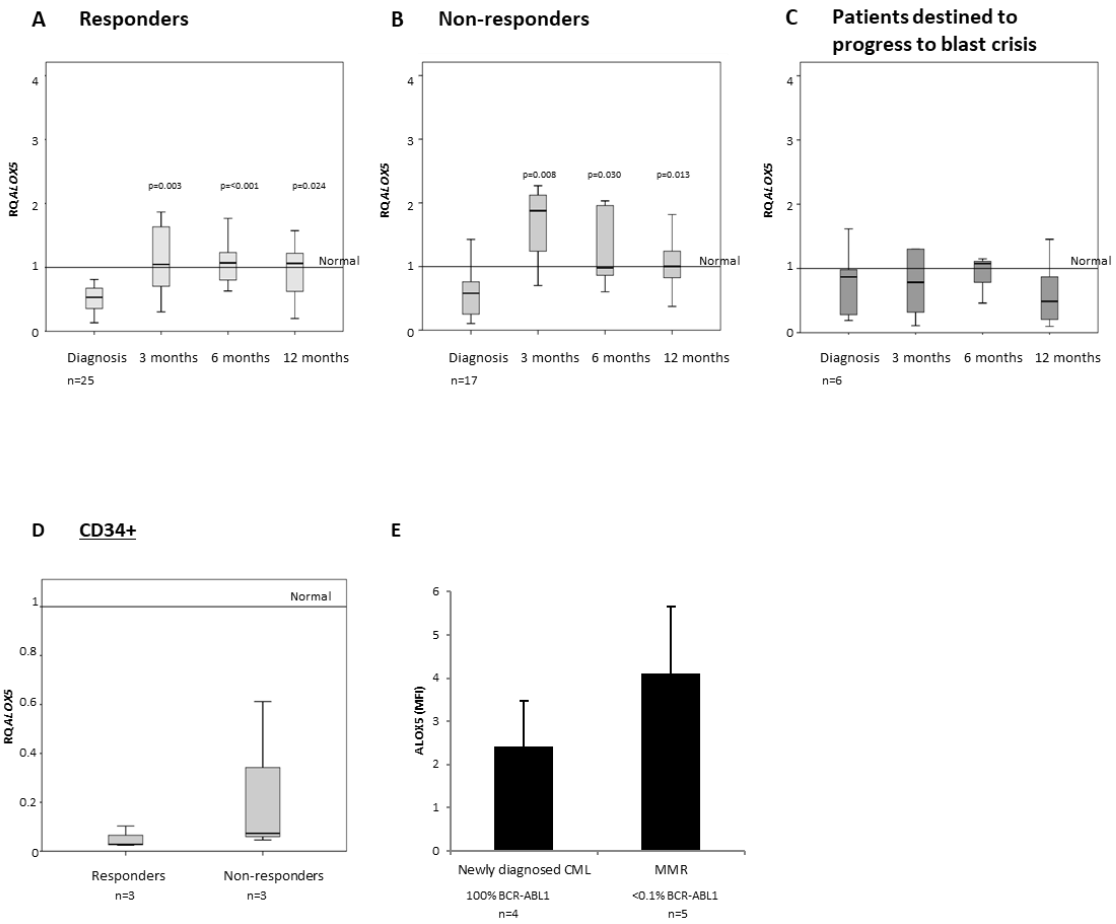

Figure 3. LTB4 levels following imatinib treatment.

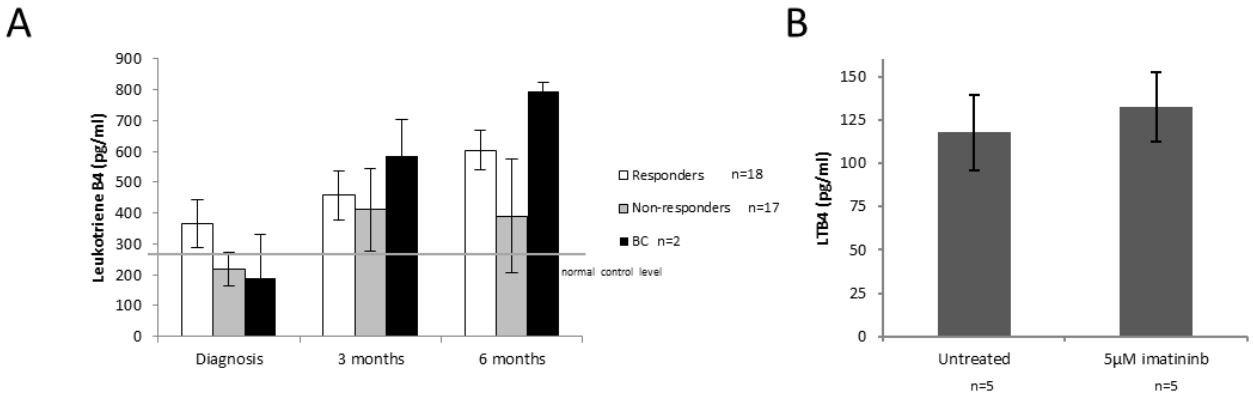

Figure 4. LTB4R1 protein and mRNA expression.