Title: A randomised Phase II trial of Hydroxychloroquine and Imatinib versus Imatinib alone for patients with Chronic Myeloid Leukaemia in Major Cytogenetic Response with residual disease

Running title: CHOICES (CHlorOquine and Imatinib Combination to Eliminate Stem cells)

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This manuscript is dedicated to Professor Tessa Holyoake, who tragically passed away on 30th August 2017.

Abstract:

In chronic-phase chronic myeloid leukaemia (CP-CML), residual *BCR-ABL1+* leukaemia stem cells are responsible for disease persistence despite TKI. Based on *in vitro* data, CHOICES (CHlorOquine and Imatinib Combination to Eliminate Stem cells) was an international, randomised phase II trial designed to study the safety and efficacy of imatinib (IM) and hydroxychloroquine (HCQ) compared to IM alone in CP-CML patients in major cytogenetic remission with residual disease detectable by qPCR. Sixty-two patients were randomly assigned to either arm. Treatment 'successes' was the primary end-point, defined as ≥0.5 log reduction in 12-month qPCR level from trial entry. Selected secondary study end-points were 24-month treatment 'successes', molecular response and progression at 12 and 24 months, comparison of IM levels, and achievement of blood HCQ levels >2000ng/ml. At 12 months, there was no difference in 'success' rate (p=0.58); MMR was achieved in 80% (IM) vs 92% (IM/HCQ) (p=0.21). At 24 months, the 'success' rate was 20.8% higher with IM/HCQ (p=0.059). No patients progressed. Seventeen adverse events, including four serious adverse reactions, were reported; diarrhoea occurred more frequently with combination. IM/HCQ is tolerable in CP-CML, with modest improvement in qPCR levels at 12 and 24 months, suggesting autophagy inhibition maybe of clinical value in CP-CML.

(200 words)

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative neoplasm that originates from a constitutively active tyrosine kinase, BCR-ABL, resulting from a reciprocal translocation between chromosomes 9 and 22 [1, 2]. Upregulation of BCR-ABL drives disordered myelopoiesis through aberrant metabolism and expression of downstream signalling pathways [3, 4]. Despite a targeted therapeutic approach, disease persistence is driven by a small residual *BCR-ABL1* positive (+) stem cell population [5-9]. This can lead to disease progression to the more acute form, termed blast crisis, which carries a very poor prognosis [10]. Measures to enhance the elimination of residual disease are therefore required to further improve outcomes and increase the number of patients obtaining deep molecular remission (DMR; defined as ≥4-log reduction in *BCR-ABL* transcript levels) who can be considered for discontinuation of TKI treatment and long-lasting treatment-free remission (TFR) [11-13].

Autophagy, an evolutionarily conserved catabolic process [14], is induced following *in vitro* tyrosine kinase inhibition (TKI) of primitive CML cells [15]. While autophagy has been shown to suppress cancer initiation in mouse models, an increasing amount of evidence suggests it plays a critical pro-survival role following therapeutic stress [16]. Furthermore, pharmacological autophagy inhibition, using the non-specific autophagy inhibitor, chloroquine (CQ), enhances the effect of TKI on functionally defined CML stem cells compared to Imatinib (IM) or CQ alone [15].

Based on these findings, we designed the CHOICES (CHlorOquine and Imatinib Combination to Eliminate Stem cells) trial (NCT01227135); a randomised, open-label, phase II clinical trial comparing the combination of IM and hydroxychloroquine (HCQ) with standard-of-care IM in chronic phase (CP)-CML patients in major cytogenetic response (MCyR) with residual disease detectable by qPCR after at least one year of IM treatment. This is the first clinical trial of autophagy inhibition in leukaemia and provides

a proof-of-concept for further development and testing of more potent and/or specific autophagy inhibitors for use in future leukaemia trials [17].

Methods:

Patients:

Eligible patients were 18 years or older with CP-CML. Patients had been treated with, and tolerated, IM for more than 12 months, achieved at least MCyR and remained *BCR-ABL*+ by qPCR. A stable dose of IM for 6 months prior to study entry was a prerequisite. Eligible patients had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2 and adequate end-organ and marrow function, with no uncontrolled significant illness. Informed consent was obtained in accordance with the Declaration of Helsinki and with approval from Greater Glasgow and Clyde NHS Trust Ethics Committee. The "Hospices Civils de Lyon" (Lyon, France) were the sponsors within France. Following enrolment, the Cancer Research UK Clinical Trials Unit, Glasgow, were contacted to verify eligibility and undertake randomisation. Exclusion criteria are listed in **table I**.

Study Design and Objectives:

This was an international multicenter, two-arm parallel, open-label, randomised phase II trial with a safety run-in, designed to study the safety and efficacy of HCQ in combination with IM (NCT01227135). Patients were randomly assigned at a one-to-one allocation ratio to IM in combination with HCQ (IM/HCQ) or IM alone. Random assignment was stratified using a minimisation algorithm, incorporating the following factors:

- Baseline PCR level (<3 logs below baseline, ≥3 logs below baseline)
- Time on IM (12-24 months, 24 - <36 months, ≥36 months)
- Daily IM dose (<400mg, 400 - <600mg, 600 - 800mg)
- Site

All patients continued once daily dosing of IM throughout the 24-month study period. Patients on the IM/HCQ arm received a maximum of 12 four-weekly cycles of combination treatment (48 weeks). Patients were followed-up for a further 12 cycles, taking each patient's total study participation to a

maximum of 96 weeks. Orally administered HCQ was started at 800mg/day as 400mg twice daily. In the case of missed doses, patients were advised to take the drug on the same day if within 6 hours, or the dose was withheld until the next scheduled dose. For dose reduction, 600mg/day was divided into 400mg every morning and 200mg every night, and 400mg/day into 200mg twice daily. Recruitment was temporarily stopped for 6 weeks once 6 patients were randomly allocated to IM/HCQ to monitor for evidence of any dose limiting toxicity (DLT). DLT was defined as i) any grade 3 or 4 non-haematological toxicity that was/possibly was attributed to the study drug, excluding grade 3 nausea, vomiting and diarrhoea controllable by concomitant therapy, or ii) any grade 3 or 4 haematological toxicity that could not be corrected by granulocyte colony-stimulating factor.

Definitions of end points:

The primary study end-point was the proportion of treatment 'successes', defined as patients who had ≥0.5 log reduction (approximately 3-fold reduction) in their 12-month *BCR-ABL1* qPCR levels from trial entry. Patients who withdrew before the 12-month assessment or who had an increase in IM dose prior to the assessment were classified as treatment 'failures' in the primary end-point analysis. To avoid bias in the primary endpoint, the assessment of qPCR levels was performed blind to the study treatment allocation. The secondary study end-points were the proportion of treatment 'successes' at 24 months, molecular response at 12 and 24 months, comparison of IM levels (using metabolite CGP-74588) between study arms at 12 and 24 months (supplemental methods), and the proportion of patients who achieved therapeutic whole blood HCQ levels >2000ng/ml at 12 and 24 months (supplemental methods). Patients who withdrew prior to 24 months were classified as treatment 'failures' in secondary end-point analyses (**figure 1**).

***BCR-ABL1* detection:**

Monitoring for *BCR-ABL1:ABL1* was performed centrally at Imperial Molecular Pathology Laboratory, London, and all *BCR-ABL1:ABL1* ratios were expressed according to the international scale (IS). Baseline

BCR-ABL1:ABL1 was documented from local laboratory analysis (**table 2**) and repeated centrally to enable subsequent longitudinal analysis of response. MMR was defined as 0.1%$^{(IS)}$ or lower, with 10,000 or more *ABL1* control transcripts.

Statistical method:

Using retrospective study data [18], approximately 30% of patients fulfilling the entry criteria were expected to obtain a ≥0.5 log decrease in *BCR-ABL1* qPCR levels after 12 months of IM treatment (treatment 'success'). To detect an increase in the proportion of treatment 'successes' from 30% to 50% required 33 patients per arm (80% power, 20% 1-sided level of statistical significance). Randomisation was undertaken centrally using a computerised algorithm, which incorporated a random element to remove predictability and ensure groups were well-matched, using a minimisation approach (described above). At the end of the randomisation process, the patient's treatment allocation and unique identifier were generated.

Analyses were performed using SPSS 22.0.0.0 (SPSS, Chicago, IL) and were conducted on an intention-to-treat (ITT) basis. The comparisons between the study arms of "successes"/"failures", progression, and molecular response rates used Fisher's exact test. 95% confidence intervals for the difference in proportions were calculated using method 10 in RG Newcombe [19]. Molecular response rates, IM plasma levels and the most severe common terminology criteria of adverse events (CTCAE v4.0) grade observed per patient for individual adverse events over the 12-month study period and the 12-month follow-up period were compared between the study arms using the Mann-Whitney U test. Statistical analyses of *in vitro* data and continuous *BCR-ABL1:ABL1* qPCR data were performed using the 'NADA' package in R (v3.3.3) to allow interpretation of values below the limit of detection [20, 21]. Adjustments for multiple testing were made, where appropriate, using the false discovery rate (FDR) approach [22], using the p.adjust function ('fdr' option) in R.

Results:

Patient characteristics:

From 22 April 2010 to 31 December 2014, 62 patients were randomly assigned to IM (n=30) or IM/HCQ (n=32). Demographic characteristics were similar between arms (**table 2**). Pre-treatment peripheral blood (PB) qPCR was available for all patients enrolled, with median *BCR-ABL1:ABL1* ratio of 0.046% (interquartile range (IQR) 0.011% to 0.118%) in the IM arm, and 0.034% (IQR 0.012% to 0.047%) in the IM/HCQ arm. Duration of IM prior to study entry was similar. Additional chromosomal abnormalities within the Philadelphia + clone were identified at CML diagnosis in 2 patients in the IM arm (one with a variant Philadelphia chromosomal translocation and one with deletion of chromosome 12), and 3 in the IM/HCQ arm (trisomy 21, deletion of chromosome 9, and a double Philadelphia chromosome abnormality). One patient in the IM arm withdrew from the trial prior to trial initiation and received no treatment on study; 6 patients withdrew consent during the study (**figure 1**). Patients were followed-up for a minimum of 24 months.

Molecular efficacy:

No statistical difference was demonstrated in 'success' rate between arms at 12 months (1.2% lower with IM/HCQ vs IM; 95% CI 21.1% lower to 18.4% higher; 1-sided p=0.58; 2-sided p=0.99) (**table 3**). Patients who withdrew before the 12-month assessment (n=11) or who had an increase in IM dose prior to the assessment (n=1) were classified as 'failures' (n=5 with IM; n=7 with IM/HCQ), which may account for this. At 12 months, MMR was achieved/maintained in 66.7% on IM versus 71.9% on IM/HCQ (5.2% higher in the IM/HCQ arm; 95% CI: 17.1% lower to 27.1% higher; 1-sided p=0.43; 2-sided p=0.78).

At 24 months, 'success' rate in the IM/HCQ arm was 20.8% higher than the IM arm (95% CI: 1.5% lower to 40.4% higher; 1-sided p = 0.059; 2-sided p = 0.090). Patients with a sample approximately 90 days prior to the expected 24-month time point, or at any time after, were eligible for analysis, with the closest sample to the scheduled 24-month date (before or after) chosen. The numbers classed as

'failures' due to failure to achieve the appropriate log reduction in *BCR-ABL1:ABL1*^{IS} within the acceptable window of the 24-month expected assessment time was higher with IM (n=19; 76%) compared to IM/HCQ (n=13; 65.0%). At 24 months, DMR/MMR was achieved/maintained in 66.7% with IM, and 75.0% with IM/HCQ (8.3% higher in the IM/HCQ arm; 95% CI: 13.8% lower to 29.7% higher). There was a slight, but not significant, difference in rates of molecular response between the arms (1-sided p=0.33; 2-sided p=0.58) at the 1-sided 20% significance level. There was no significant difference between depth of molecular response at 12 or 24 months. No confirmed or suspected progressions at any time during the study were identified.

In view of the variation of *BCR-ABL1:ABL1* ratio between patients (**table 2**) at trial entry, a post hoc analysis was performed using the median *BCR-ABL1:ABL1* ratio (0.0305%) to determine sub-groups of 'high' and 'low' *BCR-ABL1:ABL1* expression at trial entry. MMR was not used as this led to a significant imbalance in subgroup sizes between the arms and would not have been informative. In the imatinib only arm, 24/30 patients were in MMR or better, and 6/30 not in MMR; in the IM/HCQ arm, 28/30 patients were in MMR, and 5 were not in MMR. At 12 months, within the high baseline group, the 'success' rate in the IM/HCQ arm was 4.7% higher than in the IM alone arm (95% CI: 26.5% lower to 32.2% higher; unadjusted 2-sided p-value > 0.99; FDR adjusted 2-sided p-value > 0.99), and within the low baseline BCR-ABL group, the 'success' rate in the IM+HCQ arm is 10.5% lower than in the IM alone arm (95% CI: 34.6% lower to 16.4% higher; unadjusted 2-sided p-value = 0.61; FDR adjusted 2-sided p-value > 0.99). At 24 months, this difference is more striking, and the 'success' rate in the IM+HCQ arm is 34.6% higher than in the IM alone arm in those with high baseline BCR-ABL (95% CI: 0.5% higher to 58.3% higher; unadjusted 2-sided p-value = 0.066; FDR adjusted 2-sided p-value = 0.26), and 3.8% higher in the low baseline BCR-ABL subgroup (95% CI: 23.4% lower to 32.3% higher; unadjusted 2-sided p-value > 0.99; FDR adjusted 2-sided p-value > 0.99) (**figure 2**). This suggests that the kinetics of response is

191 determined by *BCR-ABL1:ABL1* ratio at trial entry and those with higher baseline levels may benefit
192 more from the addition of HCQ to IM.

193 Similarly, in a post hoc analysing utilising the median *BCR-ABL1:ABL1* ratio at trial entry, we analysed the
194 proportion of patients achieving a deep molecular response (DMR), as defined by MR3, MR4, MR4.5,
195 and MR5, at both 12 and 24 months. There was no significant difference in those achieving DMR
196 between experimental arms of 'high' and 'low' *BCR-ABL1* expressors. However, there was a higher
197 trend for achievement of DMR within the IM/HCQ arm, particularly at 24 months (**table SI**) where the
198 proportion of patients in the 'high' *BCR-ABL1* subgroup achieving MR3 was 26.0% higher in the IM/HCQ
199 arm (95% CI: 7.7% lower to 53.6% higher; unadjusted 2-sided p-value = 0.26; FDR adjusted 2-sided p-
200 value = 0.85); MR4, 17.9% higher in the combination arm (95% CI: 13.9% lower to 43.4% higher;
201 unadjusted 2-sided p-value = 0.41; FDR adjusted 2-sided p-value = 0.85); MR4.5, 16.7% higher in the
202 combination arm (95% CI cannot be computed; unadjusted 2-sided p-value = 0.25; FDR adjusted 2-sided
203 p-value = 0.85); and MR5, 11.1% higher in the combination arm (95% CI cannot be computed;
204 unadjusted 2-sided p-value = 0.50; FDR adjusted 2-sided p-value = 0.85). Interpretation of this needs to
205 be carefully considered as this will be underpowered by the very nature of a post hoc analysis.

206 **Plasma levels:**

207 To ensure that HCQ did not interfere with IM plasma levels, and that patients were achieving an
208 adequate dosage of HCQ, plasma levels of drugs in both study arms were determined. IM plasma levels
209 were assessed in the ITT population, excluding the 12 patients (n=6 in both arms) in the safety run-in
210 period where blood samples were not taken, and those that withdrew consent. Plasma levels were
211 taken 20 to 26 hours after the last dose of drug in cycles 1, 2, 4, 7, 10, and 13. There was no significant
212 difference, with an adjustment for multiple comparisons using the FDR approach, in trough IM levels
213 between the arms at any time-point. However, there was a trend towards increased CGP metabolite
214 (IM metabolite) plasma levels relative to baseline at all time-points in the IM/HCQ arm compared to IM

alone. These differences reached statistical significance at the 2-sided 10% level at cycle 2 (unadjusted 2-sided p=0.032; FDR adjusted 2-sided p=0.090) and cycle 13 (unadjusted 2-sided p=0.036; FDR adjusted 2-sided p=0.090) (**figure S1A**).

HCQ plasma levels were aiming to achieve a trough concentration of >2000ng/ml at the time points described above. Only 47.1% (n=8/17) achieved this trough HCQ plasma concentration at any time point during the 12 months of IM/HCQ treatment. There was no correlation between the likelihood of achieving treatment 'success' and achieving this trough HCQ concentration (**figure S1B**).

Autophagy inhibition was additionally determined *ex vivo* using the lipidated form of microtubule-associated protein 1 light chain 3B (*LC3B-II)* levels as a marker of autophagosomes. Bone marrow and PB samples were collected at baseline, 6 and 12 months (**table SII**). In line with recent findings demonstrating increased autophagy flow in primitive CML cells [23], the number of *LC3B-II* puncta was significantly increased in BM derived CD34+ samples, when compared with PB mononuclear cells (p=0.002) (**figure S2A**). LC3B-II puncta were often undetectable in PB and, as expected, *ex vivo* HCQ treatment was required to determine LC3B-II expression (**figure S2B**). We demonstrated no linear correlation with trough IM/HCQ levels and degree of *LC3B-II* levels (data not shown). We did not demonstrate a reduction in colony-forming cell or long-term culture-initiating cell potentiation with IM/HCQ compared with IM alone (**figure S2C, D**).

Safety analysis:

Recruitment was temporarily stopped for 6 weeks once 6 patients were randomly allocated to IM/HCQ to monitor for evidence of DLTs. No evidence of toxicity at a dose of HCQ 800mg/day was determined.

Toxicity was graded according to the CTCAE v4.0, and the worst grade determined for each patient in the first 12 months of treatment (**figure 3A**) and the 12 months follow-up (**figure 3B**). Treatment was generally well tolerated. During treatment, 4/29 treated patients developed hyponatraemia with IM (3

at grade 3 [1 present at grade 1 pre-treatment] and 1 grade 1), compared with 0/32 on IM/HCQ (p=0.031). Diarrhoea was more common, with higher CTCAE grade, in the IM/HCQ arm with 21/32 patients affected (10 grade 1, 8 grade 2, and 3 grade 3) compared with 7/29 patients on IM alone (6 grade 1 and 1 grade 2; p = 0.00031). Grade 1 musculoskeletal problems were seen with IM (n=8), but not with IM/HCQ (p=0.0015). There were no cases of retinopathy documented within the IM/HCQ cohort.

During the trial period, 17 serious adverse events (SAEs) were reported; four were considered serious adverse reactions (SARs). Within the IM arm, dyspepsia was reported. Three SARs occurred in the IM/HCQ arm, and included one case each of cardiac rhythm disorder, dyspnoea, and heart failure. Cardiac function fully recovered following discontinuation of HCQ in the patient with heart failure.

No dose reductions for IM were recorded for any patients during the study. Eleven patients (n=4 on IM, and n=7 on IM/HCQ) discontinued with 'on trial' IM treatment. The reasons included consent withdrawal (n=6), rising *BCR-ABL1* (n=2), sub-optimal IM plasma levels (n=1), patient choice (n=1 on IM/HCQ), and other medical conditions (depression CTCAE grade 2, n=1). Within the IM/HCQ arm, 6 patients had a total of 8 HCQ dose reductions (4 patients had 1 reduction, 2 patients had 2 reductions). Dose reductions were related to diarrhoea (n=5), fatigue (n=2), and patient choice (n=1). Twenty-five patients completed the 12 cycles of HCQ. Seven patients stopped HCQ before the end of the scheduled 12 cycles, due to withdrawing consent (n=4), treatment-related toxicity (depression and insomnia (both CTCAE grade 2), n=2) and rising *BCR-ABL1* (n=1). Overall the IM/HCQ combination was safe and well tolerated and side effects were manageable.

Discussion:

It has been estimated that 30% of patients on TKI therapy fail to achieve a major molecular response at 2 years [24]. Furthermore, the incidence of progression to blast crisis under TKI treatment ranges between 0.7 and 4.5% per annum [25-27]. One mechanism postulated to contribute to this lack of TKI response is the phenomenon of disease persistence, which suggests that despite a targeted therapeutic approach, BCR-ABL-independent mechanisms are being exploited to sustain the survival of CML LSCs [5, 28, 29]. Autophagy has emerged as a critical factor in resistance to a number of chemotherapeutic agents and is an attractive approach in targeting CP-CML LSCs [15, 16]. In CML, reports suggest that BCR-ABL is a negative regulator of autophagy, with autophagy being induced following *in vitro* TKI treatment, and *in vitro* pharmacological autophagy inhibition enhances the effect of TKI on functionally defined CML stem cells [15, 30]. Other studies have demonstrated that BCR-ABL promotes autophagosome formation and that autophagy is essential for BCR-ABL-dependent leukemogenesis [31, 32], suggesting that BCR-ABL may affect autophagy differently during malignant transformation and progression, as has been suggested in other malignancies [33]. Together, this suggests that combination treatment with TKI and autophagy inhibition may lead to higher rates of sustained molecular response and reduced rates of molecular and clinical progression.

This phase II clinical trial was designed to compare the combination of IM and HCQ, with standard-of-care IM in CP-CML patients in MCyR with residual disease detected by qPCR. IM was used as, internationally, it remains the most commonly administered first-line therapy in CP-CML, and at the time of trial opening in 2010 and during early recruitment, it was the only approved TKI for first-line therapy in the UK. To date, and to our knowledge, this has been the largest autophagy trial in any malignancy and the first in leukaemia.

The primary study end-point was defined as patients who had ≥0.5 log reduction in their 12-month *BCR-ABL1* qPCR levels from trial entry ('successes'). This endpoint is not conventionally used as a criterion clinically to evaluate efficacy of treatment response in a CML population. However, it is well documented that in CML patients with an IM-induced complete cytogenetic response, a minimum of a half-log increase in BCR-ABL RNA (including loss of MMR) is a significant risk factor for future loss of complete cytogenetic response [34]. It was, therefore, felt that a reduction of this magnitude would be clinically significant. There was no statistical difference in 'success' rates between IM and IM/HCQ arms at 12 months. However, there was an increasing trend towards MMR in the IM/HCQ arm, and the number of 'successes' was 20.8% higher with IM/HCQ at 24 months (1-sided p=0.059 2-sided p = 0.090).

A major difficulty in the interpretation of combination treatment efficacy is the significant heterogeneity of *BCR-ABL1:ABL1* transcripts at trial entry in both experimental arms, despite the depth of response being taken into consideration during the randomisation process. This is particularly relevant in view of the kinetic response that exists during TKI therapy, with a steeper slope and 'faster' kinetics noted until MMR is achieved. At trial entry, 47.2% and 31.3% of patients were not in MMR in IM and IM/HCQ arms, respectively. As stated above, however, combination treatment demonstrated a higher proportion of treatment 'successes', which is therefore likely to represent clinical significance. To evaluate this further, in a post hoc analysis, we demonstrated that those patients with 'high' expression of *BCR-ABL1* (defined as >0.0305%, as based on the median level at trial entry) in the combination treatment arm were more likely to achieve both treatment 'success' and DMR at 12 and 24 months, suggesting that further research into autophagy inhibition in combination with TKI is warranted in those patients not achieving optimal treatment milestones on TKI alone.

Our results demonstrate that there may be a clinical advantage for 48 weeks IM/HCQ treatment on prolonged follow-up, with greatest effect noted at 24 months. This is intriguing as patients at 24 months were no longer taking combination treatment, suggesting that the effect of autophagy inhibition was long-lasting. We could hypothesise that this is due to alterations in the quiescent phenotype of the CML LSC leading to greater TKI response with prolonged use. This is similar to other trials targeting CML-LSCs where deeper and significant *BCR-ABL1* transcript response was seen on prolonged follow-up (5 years) [35]. However, we did not establish autophagy inhibition in *in vitro* assays at 12 or 24 months, and in future work in this field, perhaps extending *ex vivo* assays to later timepoints, as well as including alternative cellular mechanisms, such as senescence, could be considered to more clearly define the changes in the functional properties of CML stem cells as a result of prolonged treatment of patients with autophagy inhibitors and continuing subsequent therapies.

As this was a randomised phase II trial, albeit with relatively small sample size, small treatment improvements will not be detected, and therefore the increasing trend towards MMR could be clinically significant. Furthermore, as described above, differences in TKI kinetic response needs to be considered in future clinical trials in this field, as well as the challenges in recruitment and trial dropouts (or 'failures') which meant the power to drive a robust statistical response was not achieved. There are increasing barriers in recruitment to CP-CML studies. Firstly, this is generally a 'well' population, who tolerates TKI treatment, has few follow-up appointments, and is challenged with a low rate of progression. Clinical trials in CP-CML confer increased hospital attendance, with more procedures, including bone marrow aspirates that are psychologically unappealing. However, as demonstrated by the frequent molecular recurrence seen in patients attempting TFR [11, 12, 36, 37], there is an unmet clinical need to develop therapies capable of targeting the CML LSC which is believed to be the cause of molecular recurrence, and enable more patients to obtain DMR and successfully maintain TFR.

Importantly, the combination of IM/HCQ was well tolerated and no DLTs were observed, although increased numbers of patients developed grade 1-3 diarrhoea, consistent with previous clinical trials using HCQ [38-41]. Diarrhoea and fatigue were the main reasons for dose reduction of HCQ, both recognised adverse effects [39, 42]. Interestingly, compared with IM alone, no patients developed musculoskeletal AEs with IM/HCQ compared with 8/29 on IM, in keeping with its known clinical utility in rheumatological disorders [43]. To our surprise, 4/29 patients developed hyponatraemia with IM alone. Although not identified as a significant toxicity in the IRIS clinical trial (NCT00006343) [44], hyponatraemia is recognised as an uncommon adverse event (>1:1000 to < 1:100) of imatinib therapy [45].

Measuring autophagy flux accurately in PB is difficult, and functional assessment is therefore problematic. Plasma levels of HCQ were taken to determine therapeutic dosing, with target trough levels >2000ng/ml. However, very recently published *in vitro* data from our group indicates that even if this was accomplished, at this trough concentration (equivalent to 5.9µM) complete autophagy inhibition may not be achieved [23]. This data was not available when the trial was conducted. Furthermore, consistent HCQ plasma concentrations were not achieved within our trial population and large interpatient variability in HCQ levels has been demonstrated in a recent clinical trial, in combination with everolimus, in renal cell cancer [38]. Together, this perhaps explains the lack of correlation with *in vitro* assessment; an issue that has been previously demonstrated within solid tumours [46-48]. A major drawback to HCQ dose optimisation and ultimate achievement of autophagy inhibition is the risk of adverse effects when using higher doses for longer durations, particularly retinopathy [39, 49]. Retinopathy is unlikely to occur with dosages less than 6.5mg/kg/day within the first 10 years of therapy [40]; we demonstrated no cases of retinopathy.

To overcome both inconsistent autophagy inhibition and mitigation of side effects, more potent and specific autophagy inhibitors are required. These are beginning to be assessed in pre-clinical models [23, 50, 51]. CQ derivatives, such as Lys05, have been shown to be 3- to 10-fold more potent and have good effect in CML models. Within murine models, however, higher doses, led to Paneth cell dysfunction and intestinal obstruction [23, 51]. As yet, these have not been translated to clinical trial.

We conclude that while HCQ (at 400-800mg daily) in combination with IM is a safe and tolerable treatment option in CP-CML, the primary endpoint of this study was not met, in part due to difficulties in recruitment and retention within the trial and in part due to failure to achieve adequate HCQ plasma levels. Our study suggests that clinically achievable doses of HCQ are unlikely to achieve a sufficient trough plasma concentration to accomplish meaningful autophagy inhibition. However, with more potent and specific autophagy inhibitors on the horizon and in preclinical development, this may be worth pursuing in future clinical trials with the aim to eradicate the CP-CML LSC.

Figure Legends

Figure 1. Trial CONSORT diagram. IM = Imatinib; IM/HCQ = Imatinib and Hydroxychloroquine; Rx = treatment

Figure 2. Plot of median *BCR-ABL1:ABL1* ratio (with upper and lower quartiles denoted by vertical bars) over the study period, split by treatment arm. Separate trend lines are shown for each treatment arm, for patients with baseline BCR-ABL greater than ("high" group) and less than or equal to ("low" group) the overall median value. Individual patient data (jittered) are overlaid. Values that are recorded as undetectable (zero) have been censored at 0.001% – the censored ranges are denoted by dotted lines

Figure 3. (A) Butterfly plot illustrating prevalence of selected haematology and biochemistry toxicities and adverse events during the first 12 months of treatment. Toxicities and adverse events present at any grade and at worse grade (≥ 2) are presented and restricted to toxicities and adverse events where at least 10% of patients on either arm experience worse grade during the first 12 months of treatment. **(B) Butterfly plot illustrating prevalence of selected haematology and biochemistry toxicities and adverse events during the 12 months follow-up period.** Toxicities and adverse events present at any grade and at worse grade (≥ 2) are presented and restricted to toxicities and adverse events where at least 10% of patients on either arm experience worse grade during the 12 months follow-up period. The 2-sided p-value from a Mann-Whitney test comparing the distribution of grades between treatment arms is presented for each CTCAE-defined toxicity. Significant change between arms are depicted (*).

Table I. Exclusion criteria

Table II. Baseline demographics and disease characteristics. Data are presented as median or n (%). IM = Imatinib; HCQ = Hydroxychloroquine; ECOG = Eastern Cooperative Oncology Group.

Table III. Molecular response rates at 12 and 24 months in IM versus IM/HCQ arms. 'Success' rates were determined by ≥0.5 log reduction in *BCR-ABL1:ABL1* ratio between arms. Patients who withdrew before assessment or who had an increase in dose prior to assessment were classified as 'failures'. Complete molecular response (CMR) was defined as undetectable *BCR-ABL1* in the presence of at least 10,000 *ABL1* control transcripts. Major molecular response (MMR) was defined a *BCR-ABL1:ABL1* ratio consistently ≤0·1%. IM = Imatinib; IM/HCQ = Imatinib and hydroxychloroquine.

Supplemental figure legends (online only)

Figure S1. Ratio of CGP metabolite to IM, and HCQ plasma levels. (A) Ratio of current to baseline CGP to IM levels over sequential cycle follow-up. No correlation was detected between ratio and treatment cohort. (B) HCQ concentration (ng/ml) did not correlate with 'success' or 'failure' rates. IM = Imatinib; HCQ = Hydroxychloroquine.

Figure S2. In vitro autophagy and functional response on HSPC population. (A) Percentage of LC3B-II puncta positive cells by IF in CD34+ BM cells versus unselected PB (p=0.002). (B) Western blotting of LC3B-II and GAPDH in 3 patient samples (pt 42.6 – BM; pt 47 – BM; pt 60 – PB and BM) untreated and treated *in vitro* with HCQ. (C) Change from baseline in percentage of colonies by CFC analysis from CD34+-selected BM populations at 6 and 12 months in IM and IM/HCQ cohort. (D) Change from baseline in the percentage of colonies by LTC-IC analysis from CD34+-selected BM populations at 6 and 12 months in IM and IM/HCQ cohort. HSPC = haemopoietic stem and progenitor cell; IM = Imatinib; HCQ = Hydroxychloroquine.

Table SI. Proportion of DMR split by 'high' and 'low' baseline *BCR-ABL1:ABL1* ratio according to median ratio at trial entry

Table SII. Sample number used in *in vitro* experiments

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Competing Interests

ALL: honoraria (Kite a Gliead Company), speakers bureau (Kite a Gliead Company) and consulting or advisory role (Jazz Pharmaceuticals). JB: honoraria (Novartis, Pfizer) and speakers bureau (Novartis, Pfizer, Jazz Pharmaceuticals, Alexion). GS: research funding (Novartis, Pfizer, Ariad). SK: honoraria (Novartis, BMS, Pfizer, Incyte, Roche, AOP Pharma, Janssen, Bayer) and consulting or advisory role (Pfizer, Incyte, Novartis, AOP Pharma, BMS, CTI, Roche, Bayer). SK: research funding (Novartis, BMS, Janssen). THB: consulting or advisory role (Novartis, Pfizer, Janssen, Merck, Takeda) and research funding (Novartis, Pfizer). PS: honoraria (BMS, Novartis, Alexion, MerckSerono, Pfizer, MSD, Roche, Gilead) and consulting or advisory role (BMS, Novartis, Merck Serono, Alexion, Pfizer). PG: honoraria (BMS). FT: consulting or advisory role (bionomics) and research funding (Roche, Lilly, AstraZeneca). REC: honoraria (Novartis, Pfizer, BMS), consulting or advisory role (Novartis, Pfizer, Jazz pharmaceuticals, Abbvie) and research funding (Novarits, BMS). DM: consultancy and honoraria (ARIAD, Bristol-Myers Squibb, Novartis, Pfizer, Incyte) and speakers bureau (Incyte). FEN: consulting or advisory role (Incyte, Sun Pharma Ltd) and speakers bureau (Incyte, BMS, Novartis). TLH (sadly passed away): research funding (Novartis, BMS), advisory board member (Novartis, Incyte), and honoraria (BMS, Novartis, Incyte). MC: research funding (Novartis, BMS, Cyclacel, Incyte), advisory board member (BMS, Novartis, Incyte, Pfizer), and honoraria (Astellas, BMS, Novartis, Incyte, Pfizer, Takeda, Celgene). The other authors have no competing financial interests to disclose.

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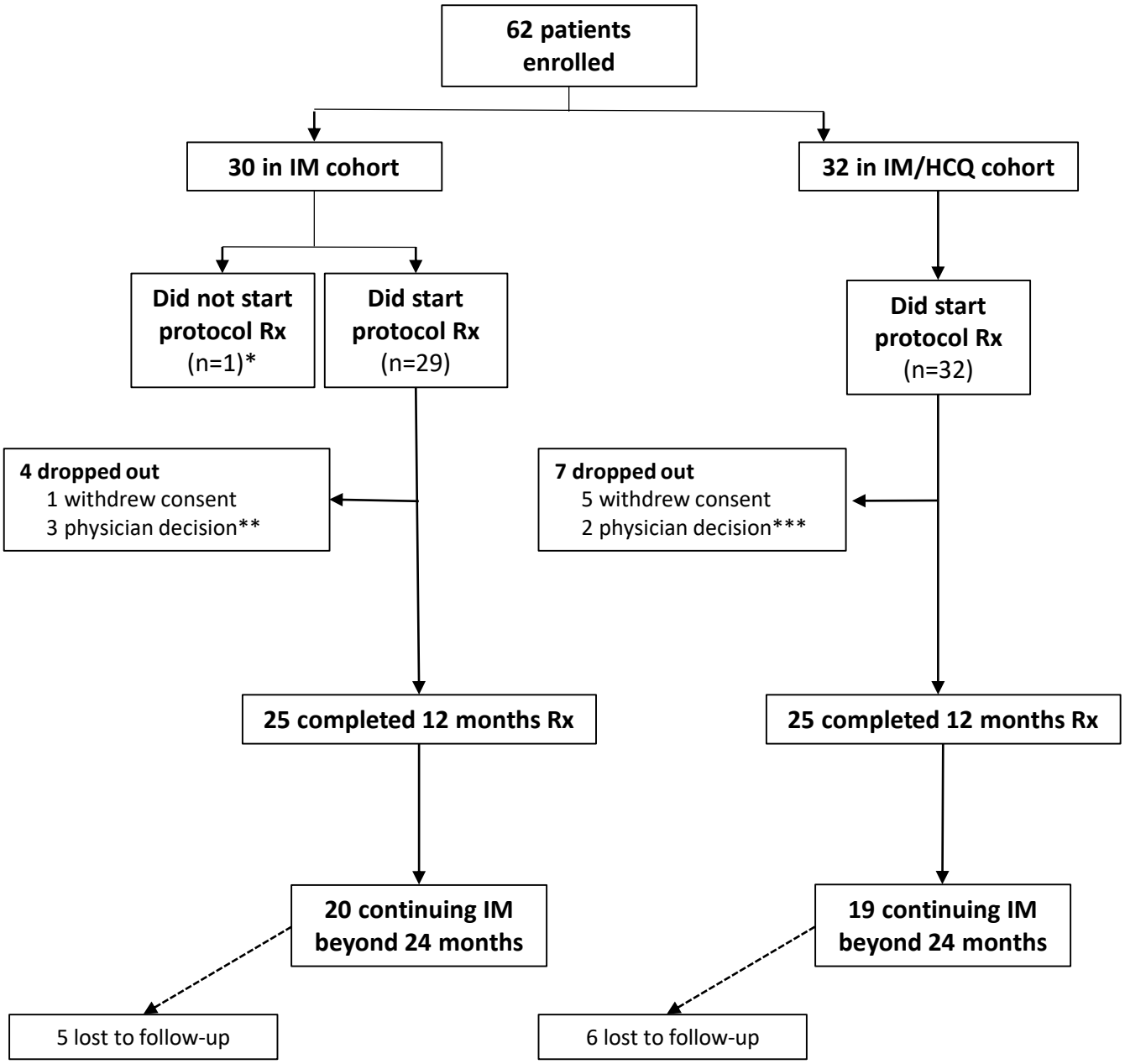
Figure 1. CONSORT diagram



*withdrew consent prior to initiation of study; received no treatment on study
** 1 due to rising BCR-ABL PCR, 1 due to low IM plasma levels leading to increased dose, and 1 change to second generation TKI
*** 1 due to rising BCR-ABL PCR, and 1 due to another co-morbidity (depression)

Figure 2. Plot of median BCR-ABL % (with upper and lower quartiles denoted by vertical bars) over the study period, split by treatment arm.

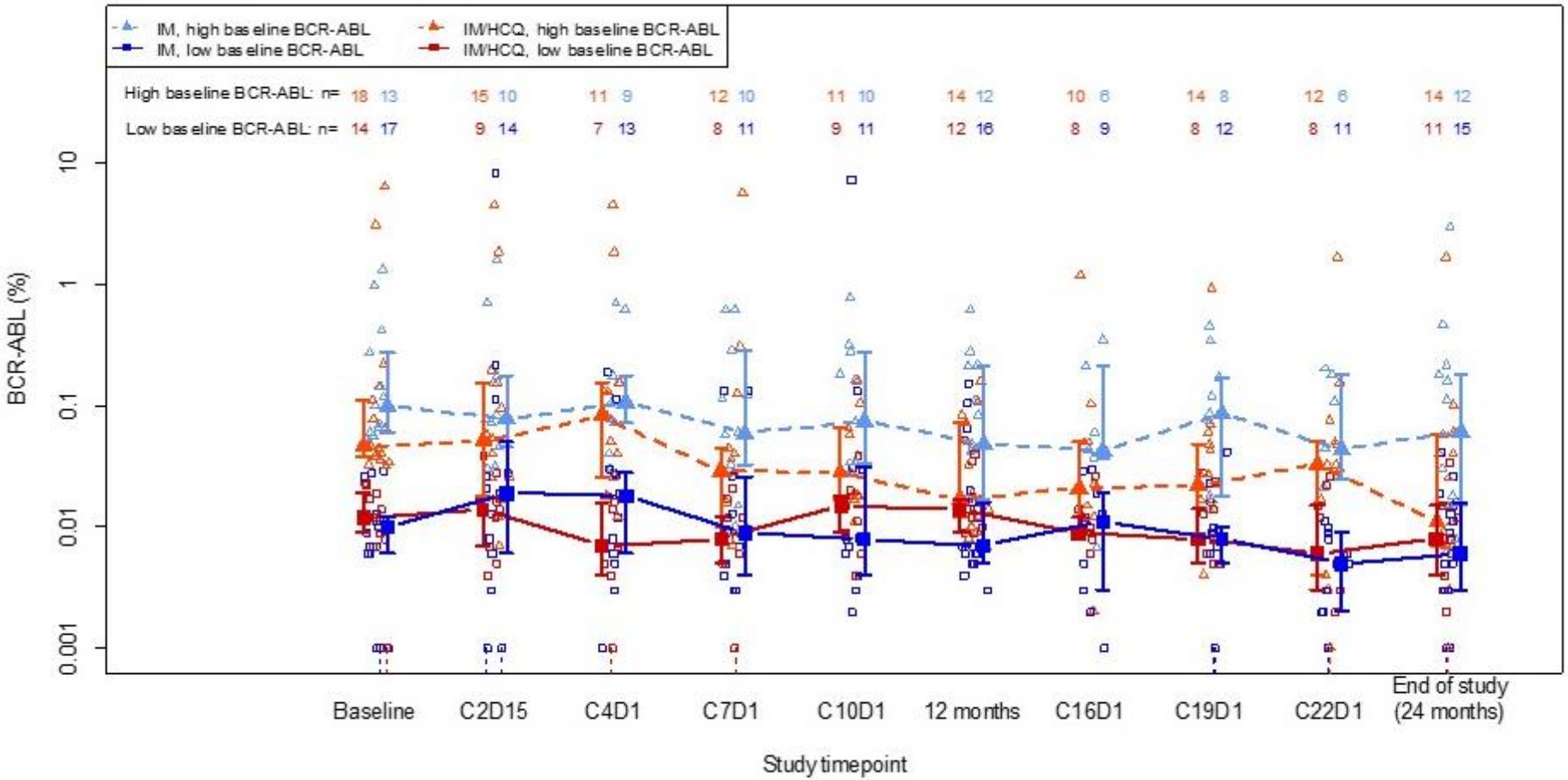
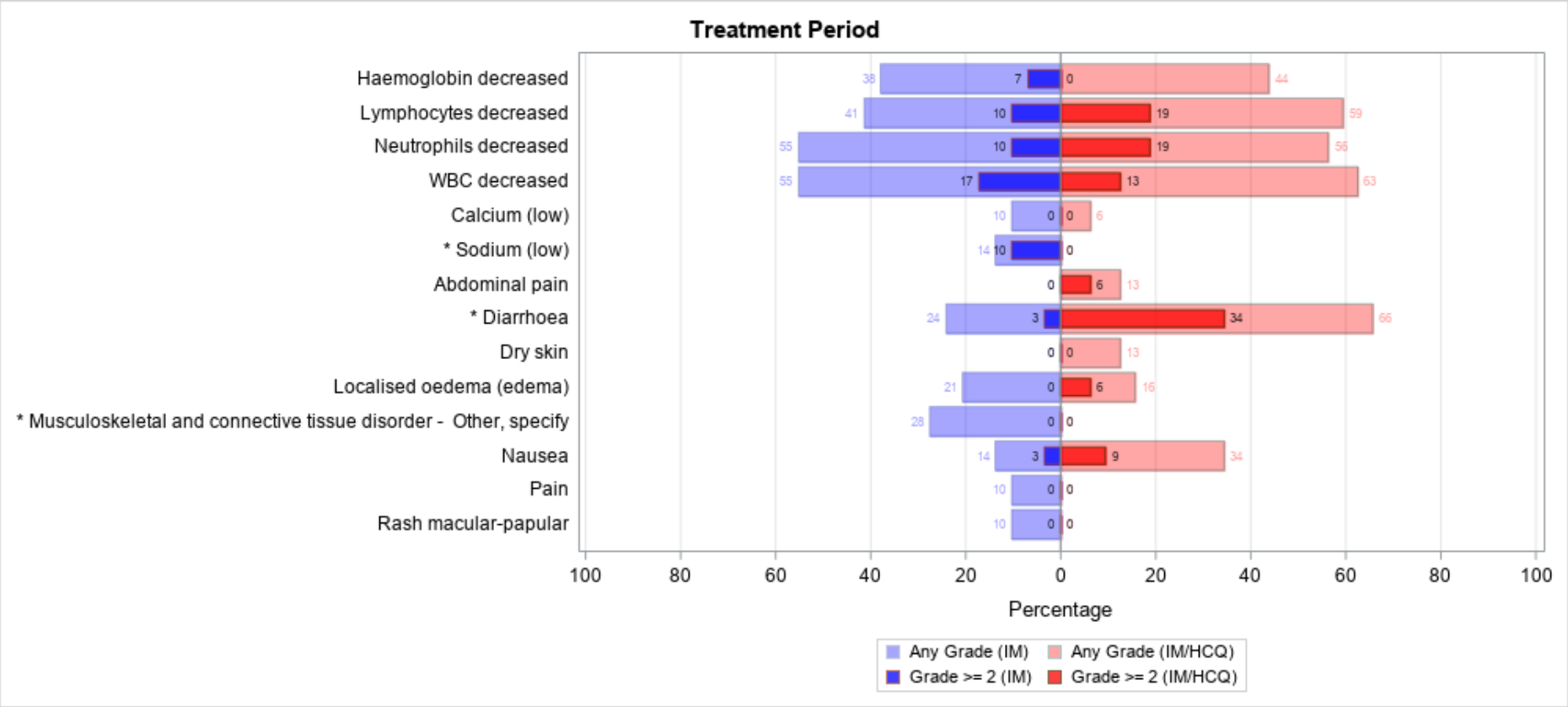


Figure 3A. Butterfly plot illustrating prevalence of selected haematology and biochemistry toxicities and adverse events during the first 12 months of treatment.



* denotes a statistically significant difference in the distribution of worst grades over the period between the arms at the 2-sided 5% significance level, assessed by the Mann-Whitney U test.

Figure 3B. Butterfly plot illustrating prevalence of selected haematology and biochemistry toxicities and adverse events during the 12 months follow-up period.

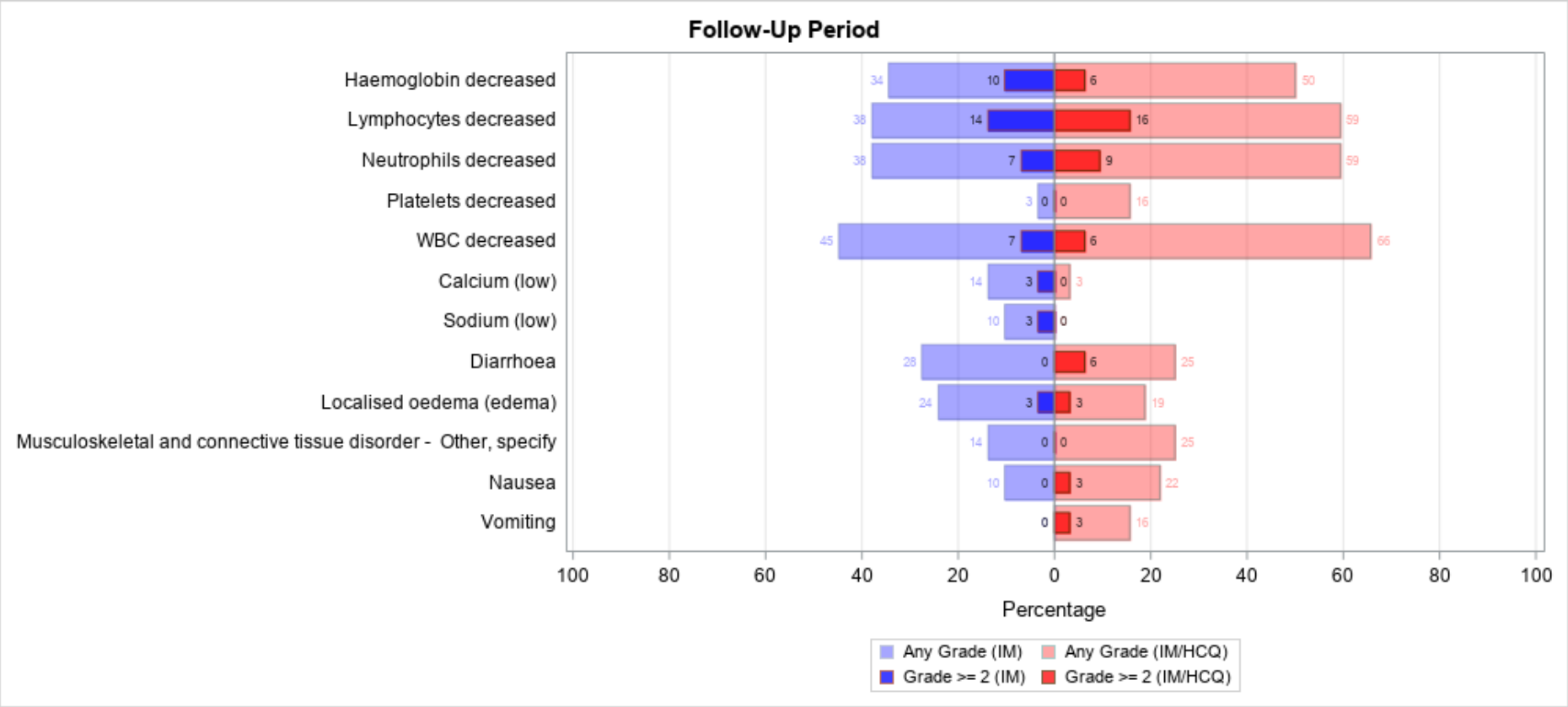


Table I. Exclusion criteria

Exclusion criteria
Patient who have been treated with Imatinib <12 months or patients who have changed dose in previous 6 months
Impaired cardiac function including any one of the following: • Screening ECG with a QTc >450 msec • Patients with congenital long QT syndrome • History or presence of sustained ventricular tachycardia • Any history of ventricular fibrillation or torsades de pointes • Congestive heart failure (NY Heart Association class III or IV) • Uncontrolled hypertension
Patients with severe GI disorder, uncontrolled epilepsy, known G6PD deficiency, known porphyria, moderate or severe psoriasis, known myasthenia gravis or other concurrent severe and/or uncontrolled medical conditions
Patients who have received chemotherapy, any investigational drug or undergone major surgery <4 weeks prior to starting study drug or who have not recovered from side effects of such therapy
Concomitant use of any other anti-cancer therapy or radiation therapy
Patients who have a pre-existing maculopathy of the eye
Female patients who are pregnant or breast feeding or patients of reproductive potential not willing to use a double method of contraception including a barrier method (i.e. condom) during the study and 3 months after the end of treatment. (Patients should continue with standard contraceptive precautions beyond the study period as per Imatinib)
Women of childbearing potential (WOCBP) must have a negative serum pregnancy test within 7 days of the first administration of oral HCQ
Male patients whose sexual partners are WOCBP not willing to use a double method of contraception including condom during the study and 3 months after the end of treatment on study. (Patients should continue with standard contraceptive precautions beyond the study period as per Imatinib)
Patients with any significant history of non-compliance to medical regimens or with inability to grant a reliable informed consent

Table II. Baseline Demographics and Disease Characteristics

Baseline characteristic	IM (n = 30)	IM/HCQ (n = 32)
Median age, years (IQR)	49.5 (42.0 – 66.0)	50.0 (38.5 – 60.5)
Gender		
Female	33.3%	28.1%
Male	66.7%	71.9%
Ethnicity		
White	93.1%	100.0%
Afro /Caribbean	6.9%	0.0%
ECOG		
0	93.1%	87.5%
1	6.9%	12.5%
IM dose at trial entry		
400mg	90.0%	84.4%
600mg	6.7%	12.5%
800mg	3.3%	3.1%
Median time on IM pre-trial Entry, months (IQR)	52.2 (32.8 – 110.0)	49.7 (27.5 – 89.0)
Response to imatinib at trial entry		
Complete haematological response	10.0%	0.0%
Partial cytogenetic response	3.3%	0.0%
Major cytogenetic response	3.3%	6.3%
Complete cytogenetic response	30.0%	25.0%
Major molecular response	50.0%	62.5%
Deep molecular response	0.0%	0.0%
Unknown	3.3%	6.3%
Additional chromosomal abnormalities	6.7%*	9.4%**

NOTE. Data presented as percentage, or median (with IQR).
IM is Imatinib; HCQ is hydroxychroroquine; IQR is inter-quartile range (the 25th and 75th percentiles). * one patient on imatinib only had a variant Philadelphia chromosome translocation, and one had a deletion of chromosome 12. **one patient on IM/HCQ had trisomy 21, one had a double Phliadelphia chromosome abnormality and one had a deletion of chromosome 9.

Table III. Molecular response rates at 12 and 24 months in the IM versus IM/HCQ arms.

		Study arm			
		IM		IM/HCQ	
		No. of patients	%	No. of patients	%
12 month 'success'/'failure' status (1-sided p=0.58; 2-sided p=0.99)	Success	6	20.0%	6	18.8%
	Failure	24	80.0%	26	81.3%
Reason for treatment 'failure' at 12 months	Failed to achieve >0.5 log reduction	19	79.2%	19	73.1%
	Increase in IM dose	1	4.2%	0	0.0%
	Withdrew	4	16.7%	7	26.9%
24 month 'success'/'failure' status (1-sided p=0.059; 2-sided p=0.090)	Success	5	16.7%	12	37.5%
	Failure	25	83.3%	20	62.5%
Reason for treatment 'failure' at 24 months	Failed to achieve >0.5 log reduction	19	76.0%	13	65.0%
	No data	1	4.0%	0	0.0%
	Increase in IM dose	1	4.0%	0	0.0%
	Withdrew	4	16.0%	7	35.0%
Molecular response at 12 months (1-sided p=0.43; 2-sided p=0.78)	CMR	0	0.0%	0	0%
	MMR	20	66.7%	23	71.9%
	No molecular response	5	16.7%	2	6.3%
	Missing data	5	16.7%	7	21.9%
Molecular response at 24 months (1-sided p=0.33; 2-sided p=0.58)	CMR	1	3.3%	2	6.3%
	MMR	19	63.3%	22	68.8%
	No molecular response	4	13.3%	1	3.1%
	Missing data	6	20.0%	7	21.9%