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Methylation age as a correlate for allele burden, disease status and clinical response in myeloproliferative neoplasm patients treated with Vorinostat

S. McPherson[1], G. Greenfield[1]*, C. Andersen[2], J. Grinfeld[3], H. Hasselbalch[2], J. Nangalia[3], K.I. Mills[1], MF. McMullin[1,4]

[1]Blood Cancer Research Group, Centre for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, United Kingdom, [2]Department of Haematology, Roskilde Hospital, Roskilde, Denmark, [3]Department of Haematology, Cambridge Institute for Medical Research and Wellcome Trust/MRC Stem Cell Institute, University of Cambridge, Cambridge, United Kingdom; Centre for Medical Education, Queen's University Belfast, Belfast, United Kingdom

*Corresponding author

Graeme Greenfield – **g.greenfield@qub.ac.uk**

Blood Cancer Research Group

Centre for Cancer Research and Cell Biology

Queen's University Belfast

University Rd

Belfast BT7 1NN

United Kingdom

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Highlights

- **DNA Methylation age varies dependent on MPN phenotype**
- **Increased *JAK2*V617F mutation burden correlates with increased DNA methylation age**
- **Vorinostat therapy can alter DNA methylation age in PV and ET patients**

Abstract

The myeloproliferative neoplasms (MPNs) are a heterogeneous group of clonal neoplastic disorders. Driver mutations in *JAK2, CALR* and *MPL* genes have been identified in the majority of cases. Alongside these, an increasing number of genes are repeatedly identified as mutated in MPN. These, including, *ASXL1, TET2, DMNT3A, EZH2* have key roles in epigenetic regulation. Dysregulation of epigenetic processes is therefore a key feature of MPN. Vorinostat is a pan histone deacetylase inhibitor (HDACi) which has been investigated in MPN. DNA methylation (DNAm) is a well-defined epigenetic mechanism of transcription modification. It is known to be affected by ageing, lifestyle and disease. Epigenetic ageing signatures have been previously described allowing calculation of a methylation age (MA). In this study we examined the effect of vorinostat on MA in MPN cell lines and in Polycythaemia Vera (PV) and Essential Thrombocythemia (ET) patients treated with vorinostat as part of a clinical trial. An older MA was observed in patients with a higher *JAK2* V617F allele burden and those with a longer duration of disease. PV patients had a MA which was older than predicted whilst MA was younger than predicted in ET. Treatment with vorinostat resulted in a younger MA in PV patients and older MA in ET patients, in both cases a trend towards the normal chronological age. When MA change was compared against response, non-response was associated with a younger than predicted MA in ET patients and a higher than predicted MA in PV patients. The link between MA and *JAK2* mutant allele burden implies that allele burden not only has a role in clinical phenotype and disease evolution in MPN patients but in the overall methylation landscape of the mutated cells.

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Introduction

The myeloproliferative neoplasms (MPNs) are a group of clonal hematological disorders, where there is a change from the polyclonal hematopoiesis seen in health, to an abnormal monoclonal proliferation of blood cells. Polycythemia vera (PV) and essential thrombocythemia (ET) are characterized respectively by the excess production of red blood cells and platelets. Identification of the *JAK2* V617F driver mutation, in 95% of PV cases and 50% of ET cases, causing constitutive activation of the JAK/STAT pathway has revolutionized our understanding of the pathogenesis of these conditions.(1) In *JAK2* V617F negative cases driver mutations in *MPL* and *CALR* have been identified in the majority of remaining ET cases.(2, 3) There is now evidence that MPNs are the result of combined genetic and epigenetic dysregulation, with mutations in co-operating genes increasingly reported.(4) These include genes involved in cell signalling pathways (*LNK, CBL, NRA* and, *NF1),* epigenetic regulation (*ASXL1, EZH2, TET2, DNMT3A, IDH1* and *IDH2),* transcriptional regulation (*TP53, RUNX1)* and mRNA processing (*SF3B1, SRSF2, U2AF1, ZRSR2*). These mutations dictate the way genes are expressed, and are not MPN specific, being found in a wide variety of myeloid disease phenotypes and clonal hematopoiesis of indeterminate potential.(5) In addition, they are not mutually exclusive, making the hierarchy complex and unpredictable. Increasing evidence suggests that the order of acquisition of mutations can determine the phenotype of the disease.(6) Other mechanisms of epigenetic dysregulation have been identified in MPN. JAK2 V617F protein localises to the nucleus mediating phosphorylation of Histone H3 and the arginine methyltransferase PRMT5. (7, 8)

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Overexpression of the transcription factor in NFE2 in PV results in elevated levels of JMJD1C, a histone demethylase, and subsequent global reductions in H3K9me1 and H3K9me2 levels.(9) In addition, DNA methylation patterns have been demonstrated to be abnormal in chronic phase MPN and change further in transformation to blast phase disease.(10)

Best available therapies (BAT) have remained unchanged for PV and ET patients for many years and include low dose aspirin and cytoreductive agents such as hydroxycarbamide. These therapies have no effect on modifying the underlying disease process. Recent developments include the use of specific JAK inhibitors including ruxolitinib, a direct JAK1 and JAK2 inhibitor. Studies have demonstrated improved hematocrit control, spleen volume reduction and modest but sustained allele burden reduction in PV patients resistant or intolerant to HU.(11, 12) However, no benefit over BAT was established for ET in the same second line setting.(13) As the role of epigenetic dysregulation in MPN becomes increasingly established, epigenetic therapies have been trialed in MPN. Vorinostat (MK-0683) is a pan histone deacetylase inhibitor (HDACi) which has been shown to induce tumour cells to undergo growth arrest, differentiation or apoptotic cell death(14-16). In PV and ET, vorinostat has demonstrated efficacy in MPN. Discontinuation of therapy over a six month treatment phase was high with only 48% of patients completing the treatment course. A majority of patients had decreased leucocyte or platelet counts on treatment, with a reduction in the prevalence of splenomegaly and pruritus observed. Very modest reductions in the *JAK2* V617F burden in positive patients were observed with no relation to response.(17)

DNA methylation (DNAm) is known to be altered by ageing and can reflect the effect of diet, lifestyle or disease on cellular processes(18). Changes in DNAm influence the relative transcription profile of the cell by activating or inactivating gene transcription. 'Methylation age' (MA) may be a more accurate reflection of disease than chronological age (CA). Using an ageing signature composed by Weidner *et al* to generate individual MA,(19) we hypothesised that DNAm may be altered in MPN patients resulting in a change in MA. Further, we hypothesised that the use of an epigenetic modifier would alter MA in PV and ET patients. Therefore, we set out to investigate the effect of vorinostat on MA in a clinical trial setting.

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Methods

Tissue culture and drug treatment

UKE-1, SET-2 and HEL cell lines were cultured in line with standard practices. Vorinostat was solubilized in DMSO. Specified concentrations were added to a cell suspension obtained at $2x10^5$ cells/mL. Cells were then incubated at 37°C for required time frames.

DNAm age calculation

To validate the DNAm ageing signature previously described, the granulocyte fraction of whole blood obtained in EDTA from healthy volunteers was obtained by centrifugation, separation of buffy coat, addition of PBS and further centrifugation. DNA extraction was performed using the *Quick*-gDNA™ Miniprep Kit (Zymo research, California) as per manufacturer instructions. Bisulfite conversion of DNA was performed using Epitect bisulfite kit (Qiagen) as per manufacturer instructions. This converts unmethylated cytosine to uracil leaving methylcytosine residues unaffected. PyroMark PCR kit (Qiagen) was used to amplify DNA for regions within *ASPA*, *ITGA2B* and *PDE4C* genes using primers with biotinylation of the 5' sequence. DNA gel electrophoresis confirmed adequate PCR product. Pyrosequencing was undertaken using the PyroMark Gold Q24 reagents kit, Qiagen and PyroMark Q24 sequencer machine. DNAm levels were inserted into the epigenetic ageing signature previously described by Weidner *et al.* (19)

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Clinical trial samples

Samples were available from PV and ET patients in a non-randomised open label phase II multicentre study of Vorinostat (EudraCT #2007-005306-49). At trial enrollment, patients had consented to the collection, storage and analysis of additional peripheral blood samples for use in research associated with the trial. Quantitative analysis of *JAK2* V617F was performed as previously described.(17) Clinicohematological parameters used to assess response as previously described.(17)

Statistical analysis

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GraphPad Prism version 5 software was used to calculate all statistical values including IC_{50}, R^2 and p values using the paired/unpaired t-test as appropriate (***p<0.001, **p<0.01, *p<0.05, n.s not significant).

Results

Validation of ageing signature

In 2014, Weidner *et al* performed a comprehensive analysis of 102 age related CpG sites in blood.(19) They described how the measurement of DNAm levels at CpG's within 3 key genes, *ASPA, ITGA2B, PDE4C* enabled the determination of a reliable MA that reflected CA in normal individuals using an "aging signature" calculation (Figure 1A). To ensure the ageing signature calculation presented in the publication was representative of CA in normal individuals in our hands, samples were obtained with verbal consent from 5 healthy volunteers. The mean age of the volunteers was 39 years (range 23-60) and included 3 females and 2 males. The granulocyte fraction was obtained from peripheral blood samples obtained in EDTA with subsequent DNA extraction. Pyrosequencing of each gene of interest was performed in turn following bisulfite conversion of the DNA and PCR cycling. 1 site of CpG methylation was analysed within *ASPA,* 3 sites within *ITGA2B* and 4 sites within *PEDE4C*. MA was calculated for each of the volunteers using the ageing signature calculation. Using the mean of DNAm values at site 1 and 3 within *PDE4C*, the mean of DNAm at all sites in *ITGA2B* and the DNAm value for the one site in *ASPA* resulted in a MA that closely aligned with the chronological age of the volunteers as a whole group as shown in Figure 1B (*r* =0.987, CI 0.81-1.0).

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A)

$$\textbf{Predicted age (in years)} = 38.0 - 26.4\,\alpha - 23.7\,\beta + 164.7\,\gamma$$

α *ASPA*

β *ITGA2B*

γ *PDE4C*

B)

Validation of ageing signature in healthy volunteers

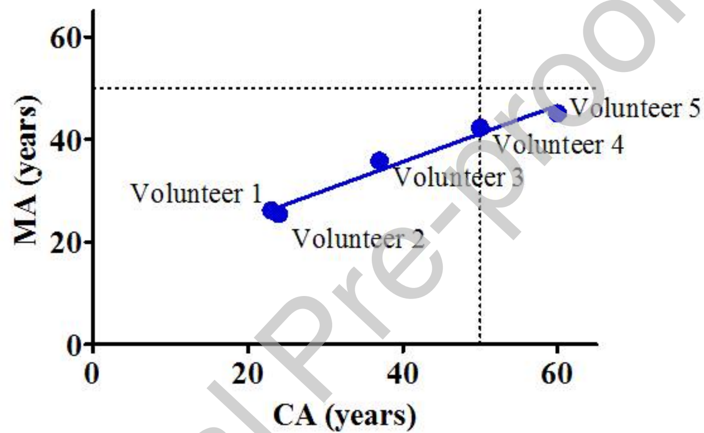

Figure 1

A) **Calculation prepared Weidner *et al* that predicted CA in normal individuals by assessing DNAm levels within 3 genes: *ASPA*, *ITGA2B* and *PDE4C***

B) **Correlation of Methylation age (MA) to Chronological age (CA) in healthy volunteers showing final MA and CA closely correlated in a healthy volunteer group.**

Effect of vorinostat on MA in clinical trial samples

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Vorinostat was tested as a therapeutic strategy in a group of PV and ET patients as part of an investigator initiated non-randomised open label phase II multicentre study (EudraCT #2007-005306-49). This study included 63 patients from 15 centres across Europe. Vorinostat was given at a dose of 400mg orally once daily for 24 weeks. Response rate (RR) to vorinostat (complete response (CR) and partial response (PR)) on an intention-to-treat basis was 35%, with a decrease in the incidence of splenomegaly and constitutional symptoms (in particular pruritus). There was however a high discontinuation rate (52%) due to side-effects (most commonly diarrhea, fatigue and renal impairment) or lack of response. (17)

DNA samples from 22 PV and 18 ET patients from this trial were available for research purposes. The gender split was 23 female patients and 17 male patients with a mean age of 62 years at trial entry (range 29-81 years). Time from diagnosis to trial entry was available in all but 1 patient and was on average 347 weeks (range 0-1428). All patients received a wash out period before enrolment and were drug free at time of trial entry. Figure 2A shows the demographics of the trial participants, relative *JAK2* V617F allele burdens and response rates per trial criteria.

Samples, originating from peripheral blood, were taken at trial enrolment prior to receiving vorinostat and after 3 months of therapy. In 21 of the 40 patients, DNA was also available after 6 months of vorinostat treatment. MA was determined for each patient at each sample time point. A predicted MA was also generated for each patient, using their known CA and the ratio established in the healthy volunteer group. We were then able to correlate MA with clinical parameters including known CA, gender, disease group, mutational profile and therapeutic response.

The correlation of MA to CA at each time point is shown for all patients in Figure 2B. The trend line established in healthy volunteers allowed the patients in whom the calculated MA was older or younger than expected to be clearly visualized. At baseline, a trend towards a higher than predicted MA was observed averaging 0.5 years older than expected for CA (range 31.9 years younger to 49.8 years older). After 6 months therapy vorinostat had altered MA, with a trend towards a lower than expected MA when compared to CA (1.0 years younger, range 26.2 years younger to 43.9 years older).

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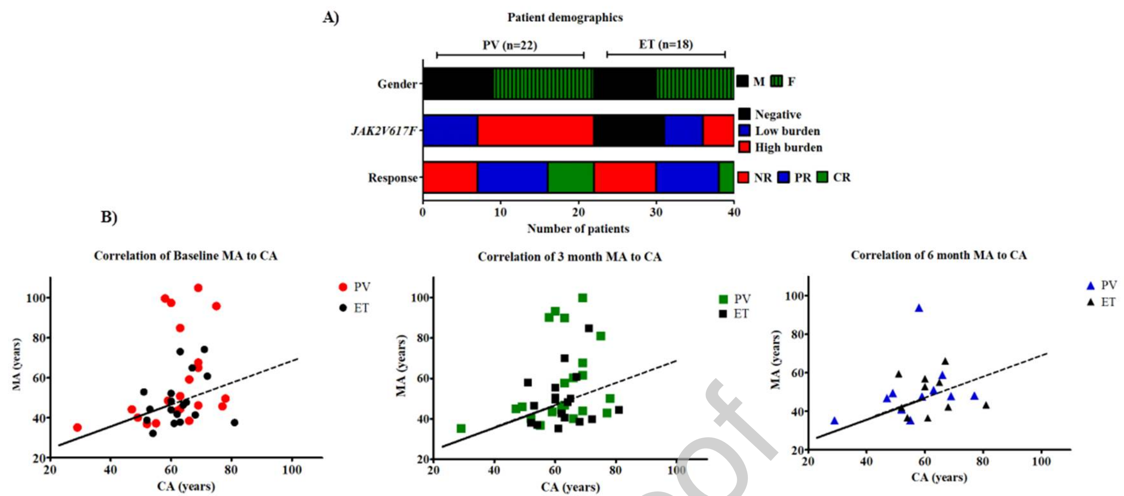

Figure 2

A) Gender, JAK2 mutant status and response classification for each disease category. In 66.7% (n=6/9) of the *JAK2* wild type patients *CALR* was confirmed as the driver mutation.

B) Correlation of MA to CA in Vorinostat treated patients. Calculated MA at each time point compared to known CA of patients with trend line as established in healthy volunteers. Patients below the line are younger than expected for CA, while those above the line are older than expected for CA

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After surveying the entire cohort, each disease group (PV and ET) was scrutinised separately. We observed a tendency towards a higher than expected MA at all time points in PV whilst the opposite was true in the ET cohort. This was despite having a similar mean CA (PV 61.8 years, range 29 - 78 and ET 62.6 years, range 51 - 81). Amongst PV patients mean MA was 58.1 years (range 35.2 – 104.9) at baseline, 57.5 years (range 35.5 – 99.9) at 3 months and 50.5 years (range 35.3 – 93.7) at 6 months. Amongst ET patients mean MA was 48.7 years (range 32.3 – 74.1) at baseline, 49.6 years (range 35.3 – 84.9) at 3 months and 49.1 years (range 36.6 – 66.1) at 6 months. The difference between the groups (PV vs. ET) was statistically significant at trial entry (5.04 years older vs. 5.1 years younger, p=0.01), but not after 3 months (4.44 years older vs. 4.16 years younger) or 6 months vorinostat therapy (1.8 years older vs. 4.0 years younger). Figure 3A demonstrates the delta change in age of the entire cohort and disease groups separately. In this way, all patients start at a baseline CA of 1.0 and their calculated MA displayed as a ratio of CA. The change in MA after 3 months vorinostat was not significant and reflected the change expected from normal chronological ageing (mean change of +0.1 years, range -20.9 to +10.8). When the follow-up data on the 21 patients who had longer term samples available was analysed, a significant increase in MA was noted from baseline to 6 months (mean change +2.8 years, range -5.9 to + 9.4, p=0.0036, paired student t test). When disease groups were analysed separately, the change from baseline to 6 months was only significant among ET patients (mean change +4.2 years, range -0.6 to +8.9, p=0.0021) and not within the PV group (mean change +1.6 years, range -5.9 to +9.4. An MA score was calculated by subtracting the predicted MA from the observed MA. At baseline, this MA score was 0.45 for the entire cohort, -5.27 for ET group and 5.52 for the PV group. At six months of therapy, the MA score was -1.04 (p = 0.002) for the whole cohort, -4.2 for the ET group (p = 0.0013) and 1.8 for the PV group (not significant).

MA and Mutational status

An ANOVA analysis was performed to pursue any associations between MA and the mutational spectrum seen in the cohort. This included the presence of driver mutations (wild type or mutant *JAK2*, *JAK2* mutant allele burden, *CALR*, *MPL*) and any additional co-operating mutations (*ASXL1, TET2, EZH2, DNMT3, SRSF2*). A statistically significant link between MA and *JAK2* allele burden was seen. *JAK2* allele burden was independently associated with MA score at baseline using linear regression (p=0.01). Compared to patients with low *JAK2* allele burden, patients with high *JAK2* (>60% variant allele frequency at baseline) had an older MA at baseline (64.2 years vs. 44.8, p=0.008) and after 3 months therapy (64.3 years vs. 44.1, p=0.0002). PV and ET patients were examined separately. PV patients with a high *JAK2* allele burden compared to the low burden group

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had a mean MA of 66.0 years vs. 46.2 at baseline (p=0.0149) and 61.5 years vs. 46.2 after 3 months (p=0.0077). Within ET, high burden patients compared to low burden patients had a mean MA of 57.3 years vs. 50.0 years at baseline (not significant) and 61.5 years vs. 47.8 after 3 months (not significant). However, after 6 months vorinostat therapy, this relationship between allele burden and MA was not significant in the overall cohort with a mean MA in high burden patients 54.7 years vs. 45.8 years in low allele burden group. Significance was also lost in the PV group with the high burden mean MA 48.5 years vs 43.2 years in the low burden group. There were insufficient remaining low burden patients in the ET group to draw any conclusion. When *JAK2* negative patients were included in the low burden group, the results were similar across all phenotypes and time points with only the 3 month ET time point displaying significance were it had not been previously. Figure 3B demonstrates these results. There was no correlation between JAK2 burden at baseline and change in MA on therapy. There was no association between change in allele burden and MA on vorinostat treatment.

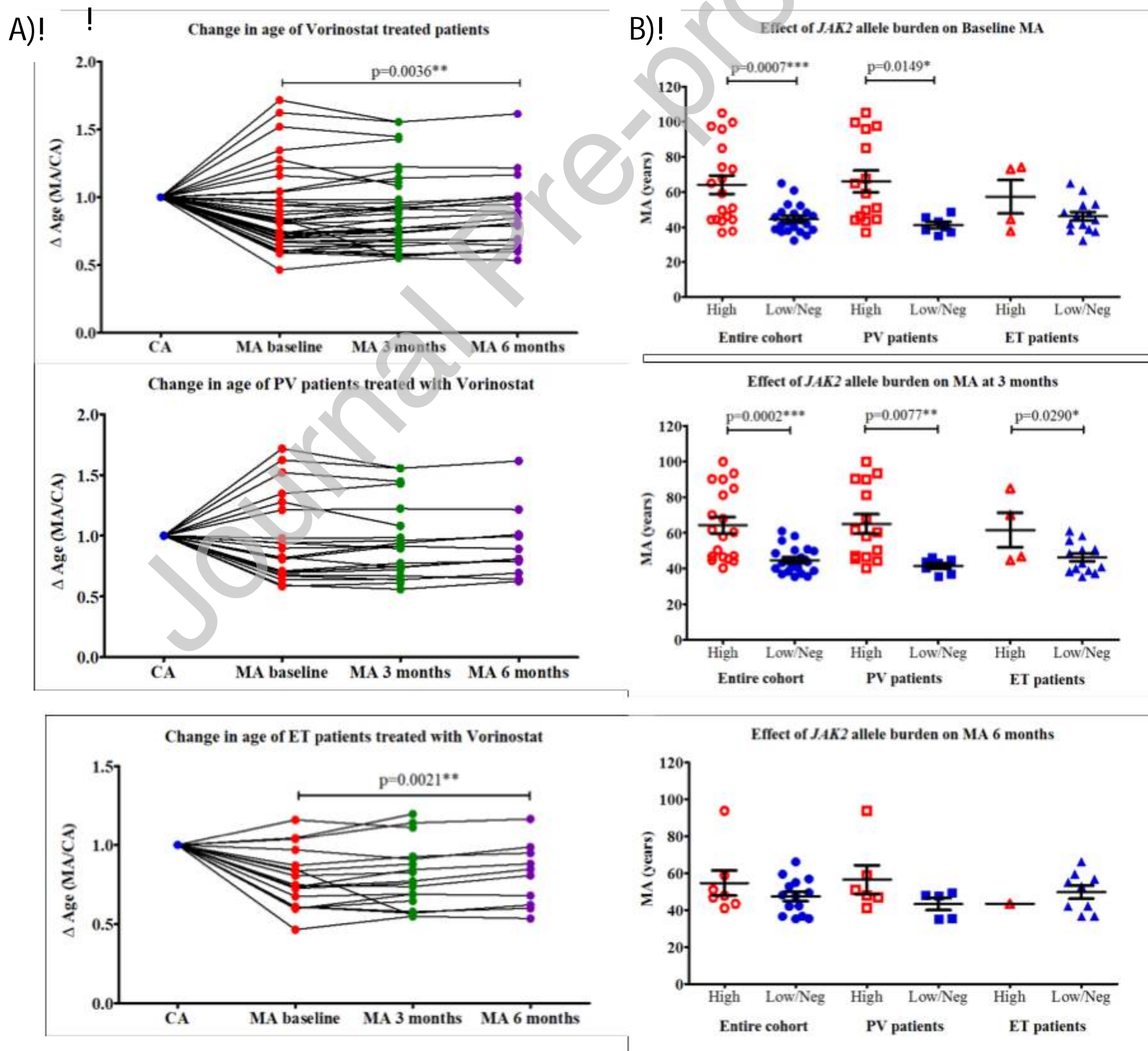

Figure 3

A) Vorinostat treated patient, change in MA with treatment. For entire cohort and each disease group separately, the change in MA as a ratio of CA over time. A significant change in MA was noted from baseline to 6 months in the cohort overall, and when ET patients were analysed separately.

B) Effect of *JAK2* allele burden on MA. MA at all time points of the entire cohort and each disease group, separated into those with high *JAK2* allele burden and those with low burden/wild type *JAK2*. Patients with high burden were significantly older by MA at baseline and after 3 months therapy.

We examined the effect of time to enrolment in the study on *JAK2* V617F allele burden and MA. Time from diagnosis to enrolment was available for all but 1 patient. There was a significant association between high allele burden and longer diagnosis to enrolment time. Amongst the high burden patients mean time to enrolment was 530 weeks (range 0-1428) compared to 171 weeks (range 0-776) in the low burden group (p=0.02). From this, we investigated if MA was correlated with time to enrolment. At baseline and 3 months a positive correlation was evident (R^2: 0.1291 & 0.2344; respectively). However, by 6 months the variables showed no correlation. When the MA score, the difference between calculated and predicted MA, was analysed, the same correlations were evident. Therefore, following Vorinostat administration, the MA and MA score were now independent of time to enrolment.

Additional co-operating oncogenic mutations were detected in several patients (*ASXL1* n=4/40, *TET2* n=6/40, *EZH2* n=3/40), with three patients having more than one mutation. No statistically significant effect was seen on MA or MA score in relation to these mutations.

MA and Response

MA was analysed in parallel with the known response rates of the cohort (20% CR, 42.5% PR and 37.5% Non response (NR)). In the cohort overall, NR compared to CR was associated with a younger MA after 6 months therapy (38.4 years vs. 57.8, p=0.01 unpaired t test). This evidence linking patients with a younger MA with non-response was also evident separately, within the ET patient group; , NR compared to PR was associated with a younger MA after 3 months therapy (41.4 years vs. 56.3, p=0.0156); and NR compared to CR was associated with a younger MA after 6 months therapy (38.5 years vs. 59.5, p=0.0158) (Figure 4A).

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When the effect of MA score on response was examined, further correlations were noted. Although the cohort size was small, by 6 months NR compared to CR was associated with a MA that was younger than that expected for CA (i.e. a negative MA score) (-11.42 years vs. +7.97, p=0.0477). This was also noted separately within the ET group, were again NR compared to CR was associated with a MA that was younger than expected for CA at 6 months (-13.9 years vs. + 5.0, p=0.0161). In addition, within PV, NR compared to PR was associated with a MA that was older than expected for CA at baseline (+18.0 years vs. -5.1, p=0.0279) and after 3 months therapy (+16.2 years vs. -4.7, p=0.0293) (Figure 4B).

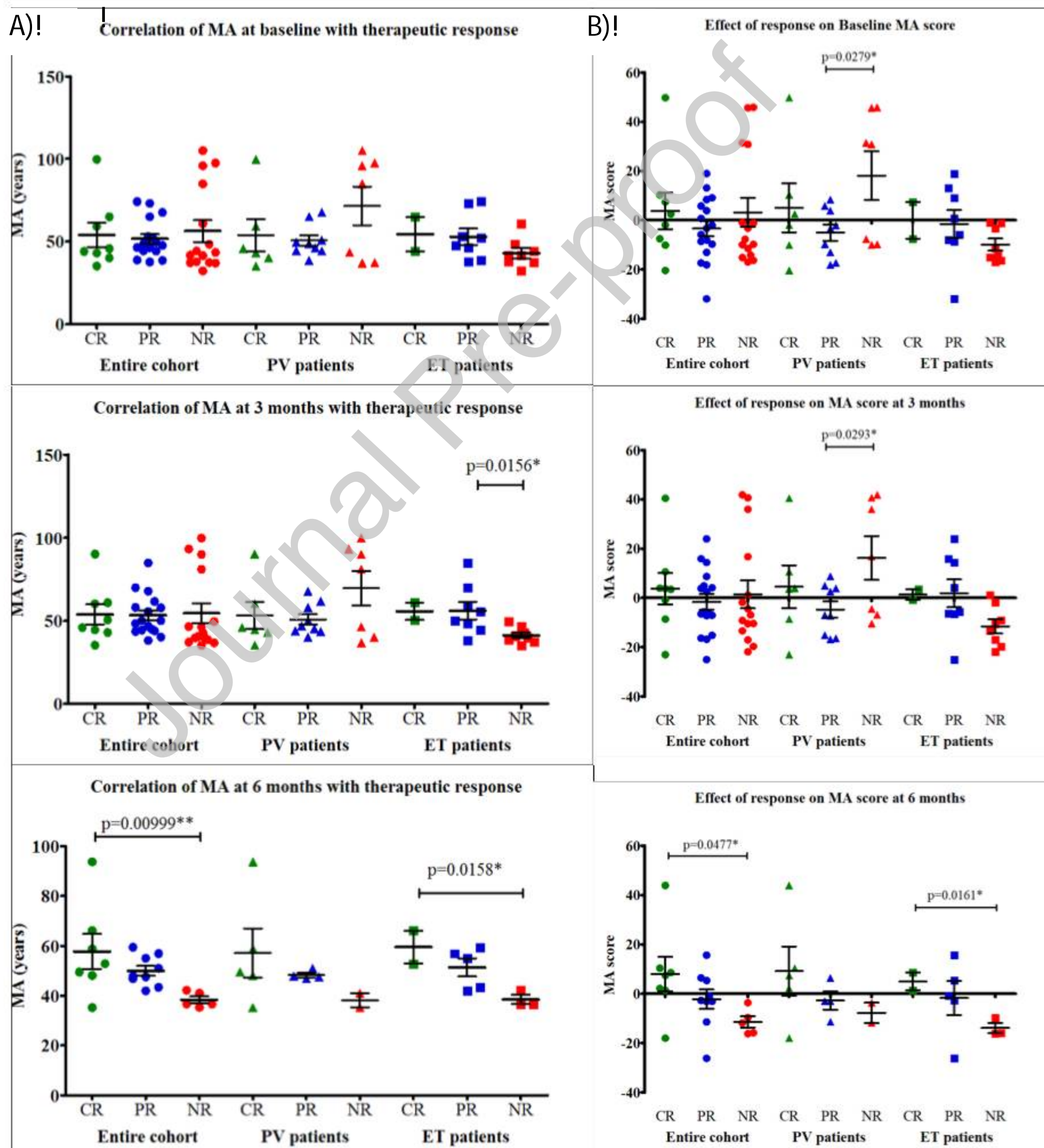

Figure 4

A) At each time point, the MA of the entire cohort and each disease group separated by response (CR, PR and NR).

B) At each time point, the MA score of the entire cohort and each disease group separated into by response (CR, PR and NR).

Discussion

The role of epigenetic dysregulation in MPN pathogenesis has been increasingly defined.(20) Using a epigenetic ageing signature based on DNAm at three genes (*ASPA, ITGA2B, PDE4C)* designed specifically for peripheral blood, which has been validated for changes in cellular composition between individuals,(19) we set out to investigate the impact on DNAm resulting from the use of a histone deacetylase inhibitor, vorinostat, in real world clinical trial samples. DNAm is perhaps the best described epigenetic mechanism of transcription regulation. The effect of ageing on DNAm levels has been referred to as the 'epigenetic clock' and is a concept that has been widely accepted for over fifty years(18) The phenomenon of a diverging epigenome landscape in aging individuals has been associated with neoplastic diseases(21) and DNAm changes have also been implicated in myeloid malignancy.(22) Previous studies have demonstrated an observable change in DNAm patterns in chronic phase MPN samples compared to normal samples and a further change during transformation to blast phase disease.(10)

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In keeping with this, we have demonstrated a significant difference in MA between PV and ET with PV patients demonstrating a higher than predicted MA in contrast to ET patients demonstrating a lower than predicted MA. We also demonstrate that patients with a higher *JAK2* V617F allele burden have an increased MA for all patients and the PV only cohorts. This allows us to question the role of DNA methylation change in the pathogenesis of the eventual MPN phenotype. Our understanding of the determination of MPN phenotype remains incomplete. Higher *JAK2* V617F allele burdens are associated with an emphasis of the PV phenotype.(23) Recent work suggests that the order of acquisition of mutations may directly affect the resulting end phenotype,(6) whilst the presence of particular cooperating somatic mutations are observed with varying frequency between MPN phenotypes suggesting a role in the determination. The number of co-existing somatic mutations is also observed to be higher in primary myelofibrosis in comparison to ET or PV.(24) In observing this difference in DNAm between ET and PV we have not defined cause or effect. The older MA may be a reflection of other cellular processes driving the PV phenotype potentially directly related to the

JAK2 allele burden. The association between time from diagnosis to enrolment with *JAK2* allele burden raises the possibility that allele burden may act as a surrogate marker for disease duration. In *JAK2* V617F positive murine models, there is clear exhaustion of the HSC population.(25) It could be hypothesized that exhaustion of this stem cell population in higher *JAK2* allele burden or prolonged diseased settings may impact on the MA of the resulting haematopoietic progenitors.

Alternatively, the change in DNAm which is reflected by the differing MA observed, may directly influence the development of a particular phenotype through regulation of gene transcription favoring a PV or ET type expression profile. Future studies should aim to differentiate these hypotheses.

Our observations of the role of vorinostat in altering DNAm have been limited by the clinical trial design. Unfortunately, the toxicity of the dosing regime used in the trial resulted in a high drop-out rate with many patients failing to complete the 6 months of treatment. Using unpaired analysis we demonstrated a change in the MA of both PV and ET patients. Compared to predicted MA, in general PV patients behaved as might be expected of a disease cohort; ageing older than predicted at trial entry and trended towards getting younger with therapy. In contrast, ET patients actually had a younger than anticipated MA at trial entry and became older on therapy. Both groups trended towards the predicted MA calculated from our normal cohort over the course of treatment, suggesting that there was a normalisation of DNAm patterns resulting from vorinostat therapy. These results are susceptible to bias resulting from the drop out of individuals with MA readings at the extremes of the results, for each cohort. When we looked at paired analysis, the significance of the increase in MA at 6 months therapy in the ET cohort was maintained consistent with a modification effect towards normal resulting from vorinostat therapy. We did not observe any correlation between *JAK2* allele burden at baseline and change in MA on therapy or between the change in allele burden on therapy and MA.

We have demonstrated a correlation of MA to response in the clinical trial. Non response was associated with a younger MA after 6 months of therapy in comparison to complete responders in the ET group. Meanwhile, non-response was associated with an older MA than predicted at baseline in comparison to partial responders in the PV group. This suggests that MA may contribute to treatment resistant biological phenotypes. We speculate that in these non-responders vorinostat is unable to overcome the mechanisms driving altered methylation patterns typical of each disease phenotype. Individuals in whom the methylation patterns are therapeutically manipulated towards normal

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demonstrate an association with disease response rates. It will be interesting to investigate whether this association of normalisation of DNAm patterns and disease response occurs in the setting of other effective therapies for MPN including the JAK inhibitors.

Conclusion

This study investigated the effect of the HDACi, vorinostat, on DNAm at three key genes (*ITGA2B, ASPA* and *PDE4C)* which have previously been validated to produce a epigenetic aging signature in peripheral blood. We observed an older MA at baseline in patients with a higher *JAK2* V617F allele burden. Patients with PV had an observed MA which was older than predicted whilst patients with ET had an observed MA which was younger than predicted. Therefore, DNA methylation patterns may be reflective of, or, causative of the resulting disease phenotype. Non-response in ET patients was associated with a younger than predicted MA after therapy in comparison to patients with a complete response. Meanwhile in PV, non-response was associated with an older than predicted MA in comparison to partial responders prior to therapy. This is suggestive that therapeutic manipulation of the DNAm ageing pattern of cells towards normal may be reflective of response more generally. In comparison, an inability of vorinostat to successfully manipulate DNAm in a number of cases reflects a tendency to non-response.

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