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The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 February 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are enclosed below. As you will see, both referees express interest in your study, and are broadly in favour of publication, pending satisfactory revision. I would therefore like to invite you to submit a revised version of your manuscript, addressing the concerns raised by the reviewers.

Most of their criticisms relate to the presentation of the results, and I have to say that I agree with these concerns: given the large amount and density of data, the manuscript is not easy to digest. It would therefore be important to undertake a significant re-write of the text (particularly the first part of the results) to better highlight the major conclusions. There is also a very large amount of supplementary data, and I would encourage you to consider whether this can be reduced and/or simplified to any degree. In terms of additional analysis, the only point requiring further experiments is referee 2's point 3.

I also have a few points from an editorial point of view:
- Please can you ensure that the 'n' numbers are clearly stated in the figure legends for all experiments where statistics are presented?
- We require both an Author Contributions and a Conflict of Interest statement (after the acknowledgments section).
- While I see that you have deposited your microarray and ChIP-Seq data in appropriate databases,

we noticed that NCBI has announced discontinuation of SRA (http://www.ncbi.nlm.nih.gov/About/news/16feb2011), and therefore wonder whether it might be better to deposit these data in GEO: this being the database we now recommend for deposition of ChIP-Seq data.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time (although I hope in this case that you can proceed faster than that!), and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors of this manuscript have used various genome-wide approaches together with metabolic profiling to examine androgen-dependent events in prostate cancer. Through these multidisciplinary means, the authors identified an anabolic transcriptional network involving the androgen receptor (AR) as the core regulator. They next searched for potential "druggable" targets that could be used for prostate cancer treatment and identified CAMKK2 as the candidate. Experiments performed in a xanograft model support the notion that targeting CAMKK2 attenuates prostate cancer growth.

General concerns on presentation

1. The authors have performed extensive genome-wide studies and characterized both AR binding sites and androgen-responsive genes in great detail in various cell models for prostate cancer. Most of the data appear to be reliable and the experiments carefully controlled. However, the authors have done a significant disservice to themselves by trying to include all their results at hand in this manuscript. Since a lot of the data, especially in the supplementary information, is not really germane to the main trust of the manuscript, the authors need to think seriously of leaving out all tangential information. As it stands now, the manuscript is very difficult to read, and the most important message is perhaps buried by the rather peripheral data.

2. Each figure comprises multiple panels; perhaps too many of them. Once again, this is a significant hardship for clarity. The authors need to address this issue by reorganizing the figures and rendering them more reader-friendly. Some panels in the figures are redundant to those shown in the supplementary information. Maybe the number of figures need to be increased.

3. Most of the Results section reads like Results and Discussion, but then there is a separate Discussion section. This latter section is quite general and fairly wordy for its content.

4. The typescript is full of abbreviations. At least some of them need to be spelled out to help uninitiated readers.

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Specific scientific concerns

1. What was the rationale for using antibody specific for Ser5 phosphorylated RNA polymerase II (RNAP II) instead of holo-RNAP II? There were over 15,000 "androgen dependent RNAP II regions" (on p. 6 -- page numbers are missing!) but only less than 10% of them (1,283) overlap with AR binding sites? How is this explained?

2. In general, what did the authors learn from their studies on RNAP II with regard to androgen regulated genes that was not obvious from their careful transcript profiling experiments? In addition, the snapshots on RNAP II recruitment show also overlaps with distant AR binding sites. Was this taken into account in the overlap calculations mentioned on p. 6?

3. Are the GSEA analyses shown in Fig. 1e-h really robust enough to permit the conclusion (p. 7) that a distance of 25 kb is the most significant?

4. On p. 12, there is some discussion on the role of FASN as an oncogene in prostate cancer. While it may not be pertinent here in the first place, the authors should nevertheless add appropriate citations into the text.

5. The results on CAMKK2 are important and interesting and form the main trust of this work. It is, however, somewhat obscure in the manuscript as to how the authors stumbled on this gene. Did the information come from a genome-wide perspective or from previous gene expression profiling studies on prostate cancer?

6. Given the complex nature of all figures, many of their legends could be expanded to include more details.

Referee #2 (Remarks to the Author):

In this study the authors used an interdisciplinary approach, including genome wide identification of androgen receptor (AR) binding regions, gene expression profiling of androgen responsive genes and comprehensive metabolomics profiling to investigate the effects of androgen signaling in models of prostate cancer.
The major findings from this paper are: 1) Identification of more than 9000 common AR binding regions in two prostate cancer cell lines using ChIP-seq. 2) Identification of 3319 androgen regulated transcripts in one of the cell lines using gene expression profiling. 3.) Gene ontology analysis revealed a significant enrichment of metabolic targets in the core set of direct AR target genes and the metabolomics profiling confirmed the role of AR signaling in stimulating aerobic glycolysis and anabolic synthesis in prostate cancer cells. 4.) Identification of CAMKK2 as a metabolic master regulator downstream of the AR in prostate cancer cells.
These findings reveal important new insights into the complex signaling pathways controlled by AR and this study also provides a good example of applying omics data integration to address fundamental biological questions related to a complex biological system. The biological conclusions of this study are of interest.

Major comment
Importantly, a better strategy needs to be adopted to summarize and present the large amount of the data generated in this project, especially in the first section of the results part. The critical information needs to be clearly delivered to the readers.

Specific comments
1. The authors extracted the common AR binding regions identified in two cell lines, LNCaP and VCaP, as the core binding sites for the further analysis. However, as a large number of the identified binding regions are distinctive for either of the two cell lines, the general features of the binding regions identified in both cell lines and the cell specific binding regions should be discussed. Possible reasons for the much higher number of binding regions identified in Vcap cells should be discussed. Are the distributions of common sites and cell unique sites in relation to gene annotation similar? Are the enriched motifs the same for the common sites and unique sites? From the data presented in the figures (Fig1a and Fig 3a) and supplemental figures (Fig2s, Fig5s), it seems that the

tag count is always lower in the LNCap cells compare to the counts in Vcap cells for the common AR regions. Is this a general phenomenon for these two datasets?

2. The authors integrated the genomic locations of AR binding sites, androgen stimulated recruitment of RNAP II and gene expression profiling to identify direct androgen regulated genes for further investigation, and these are listed in Table S8. However, the criteria for selecting those androgen target genes are not clearly presented to readers. Are these the genes that only contain the 1,283 overlapping androgen dependent RNAP II and AR binding sites in the 25 KB window relative to the gene and for which the mRNA expression was altered in response to androgens?

3. Similar as the confirmation data shown in Fig 6S and Fig 2S B, the selected AR binding regions should also be confirmed by qPCR in LNcap cells, to correlate with gene expression data obtained from this cell line.

4. The authors empirically defined the 25 KB as the optimal genomic window to integrate ChIP and gene expression data. It should be clarified to the readers whether this 25KB is from AR sites to the gene boundary or to the putative TSS of the genes.

5. It should made clear to readers in the results part that Illumina BeadArrays were performed only using the samples from LNCap cells

6. All the tracks for presenting the ChIP-seq data in figures or supplemental figures should be employing the same Y axis scale. For example, for the AR binding regions shown in figure 1 A the scale for LNCap AR is 2 to 33, but the scale for vCap AR is 2 to 42.

1st Revision - authors' response 31 March 2011

Addressing editorial comments:

- Please can you ensure that the 'n' numbers are clearly stated in the figure legends for all experiments where statistics are presented?
Numbers of measurements are now included in figure legends

- We require both an Author Contributions and a Conflict of Interest statement (after the acknowledgments section).
Author contributions now listed in the main text immediately after the Acknowledgements section.

- While I see that you have deposited your microarray and ChIP-Seq data in appropriate databases, we noticed that NCBI has announced discontinuation of SRA (http://www.ncbi.nlm.nih.gov/About/news/16feb2011), and therefore wonder whether it might be better to deposit these data in GEO: this being the database we now recommend for deposition of ChIP-Seq data.
Processed data have been deposited in GEO under GSE28126 (accessible from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28126). These are .bed files which can be uploaded into the UCSC Genome Browser. The raw data are deposited under SRA012454 (http://www.ncbi.nlm.nih.gov/sra?term=SRA012454).
The Illumina gene expression data are accessible through GSE18684 (http://www.ncbi.nlm.nih.gov/gds?term=GSE18684).

Referee #1:

General points

1. The authors need to think seriously of leaving out all tangential information… the most important message is perhaps buried by the rather peripheral data.
Given the wide range of data generated in this study and the potential interest to researchers in different fields we have attempted to present our data as comprehensively as possible to inform future studies. However, we are grateful for the constructive feedback from the reviewer and to

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address this major point we have made significant changes to the main text, leaving out discussion of the detailed analysis of different subsets within our genomics data (e.g. separate descriptions of motif analysis on AR, RNAP II and 'AR + RNAP II' sites; GO analysis of transcript only and 'ChIP + transcript' data). These points we made form page 6 onwards in our original submission and have now gone. Instead, we have attempted to describe the main findings from our genomics data succinctly and progress to the main novel findings: 1/ the AR drives a coordinated metabolic program in Prostate Cancer; 2/ CAMKK2 is a critical effector of this signalling pathway.

2.1 Each figure comprises multiple panels; perhaps too many of them.
The authors need to address this issue by reorganizing the figures and rendering them more reader-friendly. Some panels in the figures are redundant to those shown in the supplementary information. Maybe the number of figures need to be increased.
We have reduced the number of panels in each figure by streamlining the data being presented and by including an additional figure. Specifically we have:

Figure 1: we have re-ordered the figure and streamlined the GSEA data by presenting a summary graph in place of the four GSEA plots.

Figure 2: unchanged.

Figure 3: we have moved four graphs (showing AR ChIP with qPCR) and one Western blot panel to Supplmentary Figure 6.

Figure 4: we have moved the schematic to Figure 5h, reorganised the remaining panels and enlarged these for clarity

Figure 5: we have moved the CAMKK2 IHC scores, proliferation and xenograft data to the new Figure 6, allowing us to enlarge the remaining panels for clarity.

Figure 6: newly added to reduce the number of panels presented in each figure

Supplementary Figures have been dramatically altered by removing all overlaps with the main text figures, streamlining the data presented and combining figures under common themes for clarity. As a result we have reduced the number of supplementary figures by over a third, from 16 supplementary figures down to 10. We have also reduced the number of Supplementary Tables presented with the manuscript, although the majority of Supplementary Tables are included to provide usable data resources.

3. Most of the Results section reads like Results and Discussion, but then there is a separate Discussion section. This latter section is quite general and fairly wordy for its content.
Where possible we have removed discursive elements from the results section and included succinct summaries of the main points in the discussion section.

4. The typescript is full of abbreviations. At least some of them need to be spelled out to help uninitiated readers.
Common abbreviations are now listed at the start of the text and we have spelled out abbreviations everywhere that it is practical to do so (e.g. "glucose transporter 1 (GLUT1)"), but have not done so for lists of genes grouped under common functional headings (e.g., those encoding G1/S cell cycle regulators), because this would make the text unreadable and listing the extended names of these genes would not to the readers understanding of the manuscript.

Specific scientific concerns

1.1 What was the rationale for using antibody specific for Ser5 phosphorylated RNA polymerase II (RNAP II) instead of holo-RNAP II?
We included ChIP-seq for RNAP II to allow us to home in on the subset of AR binding sites which may regulate transcription in prostate cancer cells (a point now made explicitly in the main text). Serine 5 phosphorylation of RNAP II CTD repeats indicates the recruitment of the RNAP II cofactor complex TFIIH (including CDK7) and therefore ChIP for this epitope allows the identification of

sites of initiated transcription (whereas ChIP for total RNAP II would also include sites where transcription was not initiated). Genomic regions enriched for phospho-Ser5 RNAP II in the absence of any other data would include sites of active transcription and also sites of paused or stalled transcription (a point now spelled-out in the main text). However, we integrated AR ChIP-seq, Ser5-phospho RNAP II ChIP-seq and detailed expression profiling, all within a treatment contrast design (vehicle versus androgen treatment). This allowed us to identify sites where the AR recruits the transcriptional machinery, evidenced by the specific enrichment of androgen up-regulated genes adjacent to sites co-occupied by the AR and RNAP II in response to androgens.

1.2 There were over 15,000 "androgen dependent RNAP II regions" (on p. 6 -- page numbers are missing!) but only less than 10% of them (1,283) overlap with AR binding sites? How is this explained?
As mentioned for point 1.1, these RNAP II enriched sites include actively transcribed regions, sites of transcriptional initiation and also sites of stalled or paused transcription. Therefore, the remaining 90% of androgen stimulated RNAP II sites (+/- androgen at 4h) will include peaks of transcription progressing through activated genes and also sites of paused or stalled transcription. Indeed, our integrated analysis (Figure 1d) highlighted the presence of androgen stimulated RNAP II sites adjacent to both up- and down-regulated genes, whereas overlapping AR-RNAP II sites were specifically associated with androgen up-regulated genes.

2.1 In general, what did the authors learn from their studies on RNAP II with regard to androgen regulated genes that was not obvious from their careful transcript profiling experiments?
As mentioned above, the integration of AR and RNAP II enriched regions allowed us to identify a core set of sites where the AR recruits the transcriptional machinery, which were specifically associated with androgen up-regulated genes. Therefore, we used the overlapping AR-RNAP II sites to determine the characteristics of these core 'functional' AR binding sites, finding the most significant enrichment for 6bp AR half-site motifs and co-enrichment of motifs for known AR cooperating transcription factors (e.g. forkhead and NF-1), as well as enrichment for novel AR associated transcription factors (e.g. CREB, AhR). Therefore, these data identify a core sub-set of direct AR targets (now annotated in Supplementary Table 7) and highlight CREB and AhR transcription factors for future studies investigating AR signalling.

2.2 In addition, the snapshots on RNAP II recruitment show also overlaps with distant AR binding sites. Was this taken into account in the overlap calculations mentioned on p. 6?

All AR and RNAP II sites were included in the overlaps shown in Figure 1B. However, in practice few RNAP II sites were distant from genes (e.g. 4% over 25kb distant from genes) and so distant RNAP II sites represent only a small proportion of the AR-RNAP II overlapping regions.

3. Are the GSEA analyses shown in Fig. 1e-h really robust enough to permit the conclusion (p. 7) that a distance of 25 kb is the most significant?
We have changed the main text to clearly explain the method and interpretation of our GSEA analysis of AR binding sites and androgen-regulated genes (now summarised in Figure 1C and Supplementary Figure 3C-D). In this analysis we generated gene sets using a range of genomic windows around AR binding sites (e.g. identifying all genes within 25kb of an AR binding site) and then used GSEA to assess the enrichment of androgen regulated genes identified in our detailed gene expression profiling experiments. Therefore, the maximal enrichment score for androgen regulated genes within 25kb of an AR binding site reflects the largest proportion of androgen-regulated genes and the lowest proportion of genes not responsive to androgens. Therefore, smaller genomic distances from AR binding sites will include a higher proportion of false-negatives (i.e. will miss more androgen regulated genes) and greater genomic distances will include a higher proportion of false positives (i.e. will include more genes not regulated by androgens). Therefore, we feel justified in our statement and selection of this genomic window.

4. On p. 12, there is some discussion on the role of FASN as an oncogene in prostate cancer. While it may not be pertinent here in the first place, the authors should nevertheless add appropriate citations into the text.
We have removed this comment from the Results section.

5. The results on CAMKK2 are important and interesting and form the main trust of this work. It is, however, somewhat obscure in the manuscript as to how the authors stumbled on this gene. Did the information come from a genome-wide perspective or from previous gene expression profiling studies on prostate cancer?
We have now made this connection explicit in the main text. CAMKK2 was included in the 1140 direct AR regulated genes we identified (ranked 43rd by autocorrelation score) and while it was significantly over-expressed in all studies the mean rank was 40th over all the clinical expression data sets. Therefore, CAMKK2 was only highlighted to us by combining our detailed AR studies and clinical expression profiling. In subsequent literature searches we identified the hypothalamic role of CAMKK2-AMPK signalling and this further highlighted CAMKK2 as a candidate metabolic regulator downstream of the AR and this was borne out by our functional studies.

6. Given the complex nature of all figures, many of their legends could be expanded to include more details.
All figure legends have been extended to aid interpretation, including detailed descriptions of the experimental design and comprehensive descriptions of the data presented. This allows them to be viewed either in conjunction with or in isolation from the majority of the main text.

Referee #2 (Remarks to the Author):

Major comment:

Importantly, a better strategy needs to be adopted to summarize and present the large amount of the data generated in this project, especially in the first section of the results part. The critical information needs to be clearly delivered to the readers.

To address this main point we have made significant changes to the main text. In particular the presentation of the data in the original submission was, we now realise, somewhat delighted by the discourse on motif coenrichments and gene ontologies from around page 6 onwards. This has now been removed to allow us to describe the main findings from our genomics data succinctly and progress to the main novel findings: 1/ that the AR drives a coordinated metabolic program in Prostate Cancer; 2/ CAMKK2 is a critical effector of this signalling pathway. In particular we feel that we have clarified the steps taken to transition from whole-genome ChIP-seq and expression data to metabolic control and the target, CAMKK2, on which we focus.

Specific comments:

1.1 The authors extracted the common AR binding regions identified in two cell lines, LNCaP and VCaP, as the core binding sites for the further analysis. However, as a large number of the identified binding regions are distinctive for either of the two cell lines, the general features of the binding regions identified in both cell lines and the cell specific binding regions should be discussed.
We appreciate that this is an interesting question but believe that a discussion of the AR binding profiles in each cell line and the core overlapping AR binding profiles is not pertinent to the main thrust of the paper and would certainly be out of place in the revised, focussed first Results section. However, we have included a brief reference to this in the main text and now include a comparative analysis of AR binding sites identified in LNCaP, VCaP or those found in both cell lines in Supplementary Figure 1D-F. In short this shows little difference in the general characteristics of each AR binding set, for sequence conservation, location with respect to genes and enrichment of binding motifs for the AR and other cooperating transcription factors.

1.2 Possible reasons for the much higher number of binding regions identified in Vcap cells should be discussed.
VCaP cells harbour a genomic amplification of the AR locus and express higher levels of AR protein, compared to LNCaP cells. Therefore the most likely explanation is that the increased levels of AR in VCaP cells alters the equilibrium of AR binding and/or the efficiency of AR ChIP enrichment in this cell line. While of general interest with regard to AR binding in Castrate Resistant Prostate Cancer (where AR gene amplification is common), this point is not germane to

the focus of the manuscript. Therefore we have mentioned this possible explanation in the main text and included two panels in Supplementary Figure 1A-B to highlight this for future studies.

1.3 Are the distributions of common sites and cell unique sites in relation to gene annotation similar? Are the enriched motifs the same for the common sites and unique sites?
Yes, the conservation, location and motif enrichment are the same for the different sets (summarised in Supplementary Figure 1E-F).

1.4 From the data presented in the figures (Fig1a and Fig 3a) and supplemental figures (Fig2s, Fig5s), it seems that the tag count is always lower in the LNCap cells compare to the counts in Vcap cells for the common AR regions. Is this a general phenomenon for these two datasets?
Yes, AR ChIP in VCaP cells had greater enrichment (higher tag counts) at almost every site compared to LNCaP cells. We also found this to be true for AR ChIP with qPCR assessment of enrichment and suggest that the most likely explanation for this observation is the higher levels of AR expression in VCaP cells (as outlined in point 1.2).

2. The authors integrated the genomic locations of AR binding sites, androgen stimulated recruitment of RNAP II and gene expression profiling to identify direct androgen regulated genes for further investigation, the criteria for selecting those androgen target genes are not clearly presented to readers.
The criteria are now stated in the main text and each gene in this core set of direct AR regulated genes is fully annotated for all AR and AR-RNAPII binding sites within 25kb (Supplementary Table 7).

3. Similar as the confirmation data shown in Fig 6S and Fig 2S B, the selected AR binding regions should also be confirmed by qPCR in LNcap cells, to correlate with gene expression data obtained from this cell line.
We have added the data from LNCaP AR ChIP with qPCR for these sites to the Supplementary Figures (now in Figure S2B and Figure S5B).

4. The authors empirically defined the 25 KB as the optimal genomic window to integrate ChIP and gene expression data. It should be clarified to the readers whether this 25KB is from AR sites to the gene boundary or to the putative TSS of the genes.
We identified all genes whose boundaries were within 25kb of an AR binding site and have made this explicit in the text. In addition we have included a more comprehensive description of the approach and interpretation of the results. We chose to use the distance to gene boundaries in order to include instances where the AR binds within an androgen regulated gene, but at a distance >25kb from the TSS. Clearly there are alternative methods to annotate binding sites with genes (e.g. finding the nearest genes or within set distances to the TSS), however, no method is without fault and the criteria we chose were supported by the specific, significant enrichment in our GSEA profiles.

5. It should made clear to readers in the results part that Illumina BeadArrays were performed only using the samples from LNCap cells
This is now explicitly stated in the main text.

6. All the tracks for presenting the ChIP-seq data in figures or supplemental figures should be employing the same Y axis scale. For example, for the AR binding regions shown in figure 1 A the scale for LNCap AR is 2 to 33, but the scale for vCap AR is 2 to 42.
We have adjusted all binding profiles to show the same y-axis to satisfy the reviewer. However, we would suggest that the criteria of sequence tag pile-up (the data which determines the y-axis scale for these plots) is not a definitive measure for AR binding at any given locus. The total number of tags contributing to a peak, the peak shape and a range of statistical tests (e.g. FDR, p-value) together with tag pile-up provides a better assessment of true binding events. Therefore, while we accept the reviewers comment and have adjusted the graphs we are presenting, we would argue that setting a single y-axis scale for enrichment plots is not essential in every case and indeed may reduce the readers ability to interpret peak shape and/or the total number of tags contributing to a given peak.

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2nd Editorial Decision 19 April 2011

Many thanks for submitting the revised version of your manuscript. It has now been seen again by both referees, whose (brief) comments are enclosed below. As you will see, both now find the paper substantially improved and suitable for publication here. I am therefore pleased to be able to tell you that we will be able to accept your manuscript. There is, however, just one issue from the editorial side. Supplementary table 10 is what seems to me a rather odd mix of tables and figure panels, with no explanatory legend, and to be honest I can't follow what it's supposed to show. I think this needs to be modified and/or simplified - perhaps the easiest way forwards would be to split it into an additional supplementary figure (with legend) and a table (or set of tables), showing uniformly presented data. Also, the excel file you provide appears to contains links, which I get a warning message about when I open the file; this needs to be fixed.

If you could just attend to this last remaining issue, and submit an appropriately revised version of the manuscript, we should then be able to accept the paper for publication here.

Many thanks for choosing EMBOJ for publication of this study, and congratulations on a fine piece of work!

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The revised version is much improved over its predecessor. The authors have addressed my major concerns in a satisfactorily fashion.

Referee #2 (Remarks to the Author):

The authors have responded to our suggested revision in an appropriate way.

2nd Revision - authors' response 20 April 2011

Further to your email of the 19th April 2011 we are delighted to hear that we have satisfied the reviewers' comments on our manuscript and that the subsequent amendments to Supplementary Table 10 have suitably clarified this for you. We enclose the accepted versions of the figures, main text and supplementary items together with amended Supplementary Table 10.

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