Response to Antoniou's response

To restate our major concern with this paper – we found that the data presented in these papers represent a peptidomic quantification, not proteomic quantification. The authors do not combine peptides from the same protein to calculate protein levels. While the manuscripts describe a protein quantification, they instead hold each peptide separate for quantification, in such a way that the behavior of any of these peptides is held as descriptive of the behavior of the entire protein, even when peptides from the same protein show opposite behaviors. These methods are not found in any other TMT protein quantification papers and are flatly not in line with accepted quantification standards in the proteomic community. A protein is composed of many peptides sequences. The combined measurements of different peptides that match the same protein should be used to quantify proteins in a proteomics analysis.

Peptide vs. protein quantification

The authors' claim that they have performed protein-level proteomics analyses is simply false and misleading. This issue applies to no other proteomics papers that we know of, and is completely specific to these two papers. It has been shown that the combination of quantitative data across peptides leads to more accurate quantitation at the protein level. While in some applications peptides are used as a biomarkers, the use of peptides as a proxy for protein levels must be clearly stated and not confused for a proteomic quantification.

In their response, Antoniou *et al.* assert that our complaint is generally applicable to TMT proteomics experiments. However Antoniou highlights TMT proteomics papers from the King's College London Proteomics facility that actually correctly carry out either an explicit peptidomic quantification, or protein level quantitation by combining peptide-level data: Russell 2017 quantifies peptides, Ashton 2015 quantifies proteins, and Höttlä 2015 quantifies both peptides and proteins in separate analyses. These papers are useful to highlight where Mesnage 2016 and Mesnage 2017 diverge from a standard method. If the authors had simply followed the protein quantification methods in Höttlä 2015 or Ashton 2015, their analyses could have been fine. See below quotes from these two papers (Höttlä 2015 and Ashton 2015), two other recent TMT proteomics papers, and guidance from Rauniyar and Yates 2014 where consistent methods for combining peptide-level data to calculate protein-level are described:

Höttlä 2015: *"The relative difference in the abundances of endogenous peptides in AD, reported in Table 3, was calculated as the ratio of a peptide's median TMT reporter ion ratios in the AD and the control group, respectively.* ***For proteins, their relative abundances were calculated by taking the median of the median TMT reporter ion ratios of all the tryptic peptides identified from the given protein****."*

Ashton 2015: *"Ratio scores for each peptide are then calculated (Step 2) by calculating the ratios of the normalised data for each peptide by dividing it by the reference intensity. Ratios corresponding to the same source protein, peptide sequence and gel fraction are then summed.* ***Protein level data is derived from these summed peptide scores (Step 3) by taking either the mean or median of all peptide scores from the same source protein and gel fraction****."*

Liua 2018: *"Notably, the proteins were identified by at least one unique peptide, and* ***protein quantification was calculated with the median ratio of its corresponding unique peptides*** *and then normalized by taking the median of all quantified proteins from all experiments. To confirm the identification of those proteins with one unique peptide, representative MS/MS spectra from four proteins were shown in Fig. S3."*

Paulo 2018*: "The PSMs were identified, quantified, and collapsed to a 1% peptide FDR and then collapsed further to a final protein-level FDR of 1%. Moreover, protein assembly was guided by principles of parsimony to produce the smallest set of proteins necessary to account for all observed peptides.* ***Proteins were quantified by summing reporter ion counts across all matching PSMs.****"*

Rauniyar & Yates 2014**: *"Peptide abundance ratios are calculated by combining data from multiple fractions across MS runs and then averaging across peptides to give an abundance ratio for each parent protein."***

In contrast, Mesnage 2016 and Mesnage 2017 calculated fold change ratios for individual peptides and claimed that this was a protein quantification. No peptides were combined to calculate parent protein ratios. The authors have cited post-translational modifications and isoforms as their reason for not combining peptide measurements from the same protein:

Antoniou response 2018: "*McWhite argues that because different peptides from the same protein measured either and increase or decrease in levels that are results are flawed. However, McWhite fails to take into account that proteins can exist as different isoforms with different post- translational modifications, which can be present in different quantities. Thus, peptides from the same protein may give opposing readings regarding amounts present depending upon the isoform from which they were derived. The results we present reflect the fact that we took into consideration differences in post-translational modifications."*

Antoniou here acknowledges that their analyses quantified peptides, yet the publications repeatedly misleadingly claim to quantify proteins (See quotes below from Mesnage 2016). While post-translational modifications may be a reason to quantify peptides separately in a peptide-level quantification, the authors describe their analyses in-text as protein quantifications. There is no reason to quantify all peptides that map to the same protein separately in a protein quantification just because they could have come from different proteoforms. There is not support for keeping all peptides separate anywhere in other TMT protein quantification papers.

- "**Changes in proteins** and metabolites..."
- "We have performed proteomics and metabolomics analyses of NK603 (sprayed or unsprayed with Roundup) and isogenic maize kernels (Fig. 1). We used a TMT10plex™ isobaric mass tag labelling method and **quantified proteins** by Liquid chromatography-tandem mass spectrometry (LC-MS/MS)"
- "**The list of proteins and metabolites having their levels significantly disturbed** is given in Additional files 5 and 6, respectively."
- "**a total of 117 proteins** and 91 metabolites have been altered in maize by the genetic transformation process"
- "Among them, pyruvate kinase (B4F9G8), enolase (ENO1), and three glyceraldehyde-3-phosphate dehydrogenases (GAPC1, GAPC2, GAPC3) had their levels increased in NK603 maize."
- "Additionally, while **proteins associated with glycolysis were overexpressed**..."

Lack of data curation

We were disappointed by the lack of a response to some of our main concerns regarding data quality and curation, reprinted below.

McWhite & Boutz: *"There are several known sources of quantification error for TMT labelling quantification, including 1) efficiency of labeling, 2) isotopic contamination of isobaric mass tags, and 3) convolution of multiple co-isolated TMT signals within the isolation window (Sandberg 2014). These are well known issues with TMT-based quantitation. Approaches to correct for these errors are well established in the proteomics community, but were not addressed by the authors."*

One of the papers cited by Antoniou (Höttlä 2015) clearly describes filtering for co-isolation, *"MS/MS spectra with >30% coisolation (interference) in the precursor selection step were excluded from quantification."* This important quality control step was apparently not performed in Mesnage 2016 and 2017, and we find that for example that 35% of the peptides presented as perturbed in the results of Mensage 2016 have isolation interference above 30%.

McWhite & Boutz: *"Common ambiguous and isobaric peptide assignments are not corrected. In certain cases, multiple entries are included in the results table based on the observation of a single shared or isobaric peptide (isoleucine/leucine ambiguity, deamidation, etc.). In other cases, non-unique peptides that match multiple proteins in the maize or rat proteome are listed with a single protein ID. While protein groups are commonly assigned, how the major protein was selected from multiple possible is unclear. As stated by Rauniyar and Yates, 2014, "Significant quantification errors arise if a quantified peptide is not unique to its corresponding protein"."*

While in some cases degenerate peptides can be confidently assigned to single proteins with consideration, we find that the authors do not consider uniqueness at all. As an example of the scale of this issue, in Mesnage 2016, 80% of "perturbed" peptides (described as perturbed proteins in supplementary results) are non-unique. The results of Mesnage 2016 include a peptide assigned to Globulin S (Uniprot ID: P15590) that matches 61 different proteins. The manuscripts do not mention peptide or protein identification false-discovery rates. This lack of rigor and failure to correct for known causes of error in TMT quantification also includes disregard for isoleucine/leucine isobaric ambiguity and deamidation. Finally, we note that the authors chose to not respond to the following major technical points from our letter, quoted below.

McWhite & Boutz: *"In Mesnage 2016, the database of protein sequences used to identify peptides is described as the maize proteome, however, supplementary tables inexplicably identify fungal maize pathogens. There is no mention of a common contaminant database, or why non-maize proteins were included in the proteomic database."*

McWhite & Boutz: *"Details regarding the calculation of peptide/protein abundances are completely missing. While Mesnage 2017 mentions an uncited SumScale normalization, Mesnage, 2016 does not discuss how quantitative TMT data was normalized or fold change ratios calculated."*

Single observations

As a final technical point, Antoniou claims "There was not a single observation" and "The peptide must have been present in all samples". To clarify, the unit of observation in a TMT analysis is a single spectrum composed of multiple peaks corresponding to the base mass of the peptide plus individual peaks offset by the different mass tags. A set of these peaks that corresponds to an expected peptide mass is one peptide-spectral match, and thus intensities for all conditions are contained in one peptide-spectral match (i.e. one observation). As an example of a single

spectra observation being used to extrapolate protein levels, in Mesnage 2016, an oleosin (Uniprot ID: B6SIZ2) is listed as the second most depleted in one condition solely based on one spectrum from one of their two replicates, with 47% isolation interference. Even though there are 23 other unused quantifiable peptides that map to this oleosin. Only looking at single peptide species discards information from other peptides in the protein, hugely reducing the power and confidence of the analysis. Drawing conclusions using single spectrum out of thousands of spectra without multiple hypothesis correction is just not robust statistically.

Misleading

Finally, it is misleading to present a peptide quantification as a protein quantification. An informed reader cannot simply look at these papers and know that 1) the fold change quantifications are for peptides and 2) the peptides may match multiple proteins. The text of Mesnage 2016 and Mesnage 2017 both describe results as protein quantifications when they are really peptide quantifications. Given the knowledge that the presented fold changes are for peptides, and how low/rare peptides with fold changes over 0.5 are in their data, we are not convinced that the limited variation across samples that is observed is out of the range of normal biological noise. If done properly, it is unclear if any of the proteins are going to show significance for differential change, but the presentation of hundreds of changes of what they call proteins but are actually individual peptides is simply misleading.

We are strictly focusing on the technical merits of the proteomics. However in reference to Antoniou suggestion that we consider features outside of proteomics, there are numerous other legitimate reasons to doubt the solidity of these papers that have been brought up by others, including (1) the use of animals with extreme tumor loads and dimensions much over animal welfare and likely Nature Publishing Group standards[1] (tumors >25% body weight and 8 centimeters long), (2) the claim that two different non-isogenic hybrid maize lines[2] with similar catalog number are isogenic, and (3) the fact that the range of natural inherent variability between TMT measurements of different conventionally bred maize strains is unknown. Despite all of this, we have restricted our criticism to the technical aspects of the proteomics analysis, which is what initially drew out attention to Mesnage 2016 as a rare example of advance crop plant proteomics (an area of particular interest to our group).

Any conclusions drawn from the analysis described as protein quantification in these papers are unsupported and not scientifically sound.

Any future paper that attempts to use the same quantification method will be flawed and unreliable.

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&

Daniel Boutz

References

[1] https://www.nature.com/news/welfare-breach-prompts-nature-to-update-policy-on-publishing-animal-experiments-1.18384

[2] http://themadvirologist.blogspot.com/2017/01/what-is-isogenic-line-and-why-should-it.html

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