nature portfolio

Peer Review File

Development and application of GlycanDIA workflow for glycomic analysis

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This work presents a workflow that combines higher energy collisional dissociation (HCD)-MS/MS with staggered windows for glycomic analysis. Also, the authors developed a generic search engine called GlycanDIA Finder, which incorporates an iterative decoy searching for confident glycan identification from DIA data. To demonstrate the feasibility of GlycanDIA, glycomic analysis covering different types of oligosaccharides, including N-glycans, O-glycans, human milk oligosaccharides (HMOs) as well as N-glycans on RNA extracted from cultured human cell lines and mouse tissues was performed. Overall, the manuscript presents the first application of DIA for quantification of N-glycans, with the goal to enhance the detection and quantification capability of various glycan compounds. However, there are several major issues and concerns that need to be addressed before the manuscript can be considered for publication in Nature Communications.

1. Many of the conclusions, including the accuracy of quantification and the differentiation of isomers, are based on the assumption that all glycans are fully separated by liquid chromatography (LC), without considering co-elution and co-isolation of glycans.
2. Selection of MS/MS Ions for Quantification
The criteria for selecting ions used in quantification are not adequately justified. Given that many glycans have similar structures, quantification based on smaller fragment ions can be significantly distorted due to co-elution. Although larger ions tend to provide more specific information and are less likely to be distorted, their generation efficiency is generally low, especially under the NCE 20 conditions listed by the authors. At these conditions, the y1/y2 ion generation efficiency is minimal, or these ions are entirely absent. (Particularly for larger glycans. For NCE, the fragmentation energy increases with the mass of the glycan. However, larger glycans have higher brunches and can be more unstable).
3. The justification for the NCE selection is based on the majority of N-glycans. It would be beneficial to include data or discussion on whether this optimization is equally effective for less common or more complex glycans. And whether it could be better using stepped HCD?
4. There is limited discussion on the potential limitations or challenges of the decoy method. For instance, how does the decoy mode perform with highly complex or novel glycan structures?
5. The identification of isomers based on the ratio/presence of small glycan fragment ions is highly unreliable in DIA due to the large fragmentation windows used. The small glycan fragments the author used for distinguishing isomers can also be generated by other glycans, and co-isolation of those glycans can easily distort the ratio/presence of glycan fragment ions. For instance, the authors use diagnostic peaks of Neu5Ac and Neu5Gc fragments to identify H6N4FNeu5Ac1, H5N4Neu5Ac1Neu5Gc1, and H5N4F2Neu5Gc1.
However, co-fragmenting of other sialylated glycans can also produce Neu5Ac or Neu5Gc fragments, thereby affecting the identification results.
6. Manual Input for Glycan Structures. The software used by the authors lacks the capability for de novo construction of glycan structures and requires manual input of glycan MS/MS fragments for searches. Thus, this approach necessitates prior knowledge of glycan structures, as glycan structural isomers and can influence the b/y ion fragmentations generation of glycans. This limitation needs to be discussed.
7. The comparison between DDA and DIA glycan identification numbers is flawed due to inconsistent software usage. For DDA results, GlycoNote and Agilent MassHunter Qualitative Analysis software (v.B08.22) were used, while for DIA results, GlycoWorkbench (v2.1) was used for glycan fragment prediction, ProteoWizard MSconvrt(v3.0) for demultiplexing the spectrum, and Thermo Scientific FreeStyle (v1.8) and Skyline (v21.0) software for viewing MS1 and MS2 information.

GlycanDIA Finder was used for glycan identification and quantification.
Comparing the identification numbers obtained from these different software tools is not rigorous as they may not use the same criteria for glycan identification. Additionally, the specific conditions for DDA should be listed, and it should be clarified whether the same MS/MS cycle time was used for both DDA and DIA.
8. It is recommended for the authors to provide a detailed description and systematic discussion of previous software tools such as GlycoDeNovo2 and GlycoNote before introducing GlycanDIA Finder. Also, the reviewer wondered whether the same data sets were compared among GlycoDeNovo2, GlycoNote and Glycan DIA Finder?
9. Also, the parameters adopted from GlycoNote and embedded into the decoy mode of GlycanDIA Finder are unclear, as well as the rationale behind why the authors chose these specific parameters.
10. The analysis of N-glycans from RNA extracts is a novel application and highlights the sensitivity of GlycanDIA. It might be good to include some discussion on the biological implications of these findings, particularly how the differences in glycan profiles might affect cellular functions.
11. Only one glycan (Hex(5)HexNAc(5)Fuc(1)) is hard to conclude whether GlycanDIA method outperform the traditional DDA analysis and the possibility of developing a "library-free" software from using the MS2-centric method. Do the authors consider validating GlycanDIA's performance by using the mixture of different glycan types or glycan from complex samples? Furthermore, did the authors compare results between the library-free and library-based approaches within the GlycanDIA workflow?
12. I would recommend that the authors present detailed quantitative performance metrics in a tabular format for both DIA and DDA conditions. This should include details such as linear range, limit of quantification (LOQ), coefficients of variation (CVs), and other relevant parameters.
13. The number of detected glycans decreased with the increased dilution factor, and it seems that only high abundance glycans could be detected after dilution. The authors should show glycan characterization by DDA before dilution and confirm that GlycanDIA did not lead to reduced detection of low concentrations of glycans.

Some more specific minor comments:
14. Figure 2B is not well described. It is unclear what different curves (blue, purple, red) in plots represent. If they represent extracted ion chromatograms for glycan mass, the mass should be mentioned. In Figure 2, while the improvement of DIA is evident, the manuscript could include a more detailed information. i.e. are the cutoffs the same to DDA and DIA data?
15. In Figure 3B, the Venn diagram seems not in proportion for '76' and '56' and might be misunderstood. The second figure in Figure 3E is a bit confusing and doesn't explain thoroughly in the manuscript. Do these glycans reflect their abundances or frequencies in each group?
16. The specific setting parameters for the mass tolerance, intensity threshold, and maximum charge range are unclear.
17. The authors chose to set the False Discovery Rate (FDR) at 2% instead of the typical 1%. It would be beneficial to explain why they made this decision and the impact on the results compared to using a 1% FDR threshold.
18. The CVs of the technical replicates were less than 5% isn't strange, how was the CVs of the technical replicates in DDA under same conditions.
19. It is important to ensure that the sample loading amounts are consistent when comparing different samples. If the loading amounts differ, normalization should be considered to accurately compare differences or similarities between them. The author stated that "the relative abundance of different N-glycan subtypes was also distinct.", which could potentially be attributed to differences in injection amounts. Did the authors determine concentration before LC/MS analysis? The authors should give more details (e.g., the amount of starting materials used for RNA extraction, et al) in the section of profile of glycans on RNA from different mouse tissues.
20. The authors should clarify certain conditions, such as the pH in N-glycan sample preparation and specify the amount of purified glycans injected for LC-MS/MS across all different sample types in the appropriate section.
21. The authors should provide detailed explanations or criteria used for categorizing glycan subtypes such as high mannose, neutral, fucosylated, sialylated, sialofucosylated, and Neu5Gc-containing glycans.
22. In Figure 4, clarify how many biological replicates were used for each error bar.
23. In Figure S5-8, why did the same MS1 and MS2 show different retention time under different DIA strategies? Additionally, clarify why the y-axis scales are not consistent during the comparison of the two different DIA strategies.
24. Please verify carefully multiple formatting errors in the references.
25. There is a typo in the legend of figure 4, "sialyation" should be "sialylation".
26. In material and methods in supplemental information, "Magnesium sulfate (MgSO2)" should be corrected to "Magnesium sulfate (MgSO4)."
27. There is a typo in Figure S25. 'Neu5Ac-conatining' should be 'Neu5Ac-containing'.
28. Ensure consistent use of terms and abbreviations throughout the manuscript, such as "Neu5Ac" versus "NeuAc".

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors of this manuscript report that they have developed an analytical method for the qualitative and quantitative determination of N-and O-linked glycans that are present as modifications on glycoproteins, small human milk oligosaccharides, and N-linked glycans that they have assigned as low abundance modifications of RNA.-The method utilizes fixed-voltage HCD of selected, sequential segments along the m/z axis of the MS1 mass spectral scan and generates structural assignments and quantitative determinations based on the resultant MS2 data. It seems likely that the scheme presented in this manuscript could ultimately offer advantages over previously published analytical methods, in

terms of correct assignment of structures, as well as quantitative precision and accuracy. In their BioRxiv preprint (Ref. 3), the authors used a similar SWATH strategy for detection of glycoRNA, but have developed the approach much more fully while carrying out the work described in this manuscript. However, while the data obtained for standards and the examples of applications presented here verify precision, they do not provide sufficient evidence that all (or most) structural assignments for milk, plasma and glycoRNA components are correct, nor that the quantitation is accurate. In only one experiment is a quantitatively defined standard mixture employed during the method development, in this case, it is applied as a standard addition to the sample mix and quantitative values are reported only for the labeled standards. No independently determined reference quantitative values are included as comparisons with results obtained here for the human milk oligosaccharides, nor for the components of the plasma extract, the bovine mucin, or the glycoRNA samples.
The analysis method and GlycanDIA workflow and novel search engine Glycan DIA Finder described in this manuscript are based on the generation and comparison of glycosidic fragments. Cross-ring fragmentation that defines linkage positions is not used for assignments, yet, throughout the manuscript and the supplementary material, the authors label the peaks in their mass spectra and chromatograms with structures that have specific linkages not defined by their data. Although they state on page 4, line 17, that the method discriminates among and correctly assigns structures to isomeric glycans that differ only in linkage, they do not provide evidence that their method reliably obtains and correctly assigns different MS2 spectra for isomeric glycans that vary in their branching patterns. To give a straightforward and well-documented example, they could have provided an illustration showing the separation, assignments and quantification for the Man7 isomers from the (presumably bovine) RNase B that was one of their standard samples. In fact, even the relative abundances of the RNase B homologs (irrespective of isomer assignments) were not compared to reference data.
On page 5, lines 14-15, the authors assert that all possible N-glycan precursors can be detected below m/z 2000 in ESI-MS and that scanning the range below m/z 1800 is sufficient to detect all N-glycans. References 18 and 19 that are cited to support this choice for the scan range but these papers did not report any results obtained by exploring scan ranges that extended above m/z 2000. Because glycans tend to generate molecular ions in lower charge states than do peptides of the same molecular weight, signals from larger glycans can fail to appear within the range below m/z 2000.
The authors claim that their approach will allow a “library-free” software, but the method depends heavily on reference LC elution behavior, pattern matching of the mass spectra and MatchMS library.
Figures 2 and 3 leave many questions to be answered. How are peak heights/areas calculated? How is the baseline assigned for peaks in regions where the signal is far above the y-axis on both sides of the peak? For the MS2 spectrum shown in Figure 2, section A, all of the peak assignments above m/z 657 are incorrect. The reader is left to wonder whether assignments in the many spectra not presented herein have been verified.
On Page 14, line 20, the authors make the comment that glycans, unlike peptides, generate low m/z ions that are indicative of composition (oxonium ions). Peptides do have analogous peaks, immonium ions, that provide evidence for amino acid composition. A bit later, they state that the CID and HCD MS2 spectra included in most glycan databases have been recorded in the negative-ion mode. This is also untrue. What is true is that negative-ion MSn spectra of glycans show a higher level of retention of sialylated glycans and sulfation. Such labile negatively-charged features are much more easily lost in the positive-ion mode, especially for protonated species; this point is not addressed in their discussion.
The Methods section contains information primarily regarding the preparation and analysis of glycoRNA samples. No or very little information is provided on the sources of, or details of the glycan release from, the glycoprotein standards, the human serum, or the bovine mucin (what type?). Likewise, no figures or tables are presented to verify the quantitative reference values or quantitative accuracy achieved for these determinations. For the plasma sample, there is a comparison with the results from a Q-TOF MS DDA analysis, but there is no independent information to establish reference values. Why was the DDA analysis not performed on the same instrument system used for the DIA analysis? That would have provided a better comparison.
The inclusion of the long section on glycoRNAs in this methods paper has questionable value in terms of establishing the quantitative accuracy of the method. First, the abundance distributions of RNA glycoforms in various sources have not yet been determined by any established reference method. It may be argued that this is due to the very low levels at which such compounds are present. The method presented here is shown to have sufficient sensitivity to detect the species of interest, and that will contribute to further studies of this emerging area. However, the assignments of specific structures and the quantitative accuracy cannot be verified without independent determinations or at least the synthesis and analysis of reference standards. It remains very curious that the glycosyltransferases which add N-glycans to nascent proteins and build up complex glycans and the glycosidases which selectively remove all or parts of N- or O-glycans from glycoproteins have high specificity for their actions, yet this and related works postulate that their activities are compatible with the presence of an RNA substrate instead of the well-defined protein structural elements. Since the authors have shown that they are able to generate synthetic nucleosides bearing N-linked glycans, they are strongly encouraged to demonstrate that PNGase F and the additional glycosidases Endo F2 and Endo F3 used in Ref 3 can release the N-glycans from their synthetic nucleosides or well-defined RNA segments containing the modified bases. Labelling with 18O during release is necessary but not sufficient proof, since it has not yet been shown that there is no 18O exchange on unmodified bases under the experimental conditions used for release of the N-linked glycans. (In Ref. 3, the authors do show evidence for retention of the inner HexNAc during Endo F2 and Endo F3 treatment.)
The presence of a trace level of protein cannot be excluded on the basis of a “clean” channel on an SDS-PAGE gel, such as that shown in Fig. S20A. If there is a very low level of protein (<< 2%) in the recovered RNA, it would not be detected on the gel with a Coomassie stain, if that is what was used. There is no information on either the stain or the amount of sample loaded on the gel.
Should there be a low level of protein associated with the RNA, it would seem likely that the binding is specific and it is a single protein, or a set of closely related proteins bearing a specific amino acid sequence or glycan epitope whose glycoform distribution(s) are not accurately represented by determining the average composition of the whole glycan pool from the cell extract.
In Fig 4 (and elsewhere), splitting glycans into the categories of Neutral, Fucosylated, High Mannose and Sialylated, Sialofucosylated, and Sialylated-NeuGc seems artificial and the results broken down this way could be ambiguous or so

insufficiently defined as to be biologically irrelevant. What is here termed “neutral” would include both hybrid and complex glycans. Fucosylated glycans that lack sialic acid are also neutral. Furthermore, fucose can be located on the core and/or antenna(e), and some glycans could contain both NeuGc and Fuc. Despite this reviewer’s objection given above to using the glycoform distribution for the protein pool, to be consistent, Section C in Figure 4 should also show the glycoform distributions found during the protein analysis.

Other Comments on the Supplementary Information.
In their interpretation of the data shown in Figure S18, the authors calculate the relative abundances of Neu5Ac and an acetylated form on the basis of the peak heights of the oxonium ions characteristic of these two species. (p.11, l.13) In fact, the peak assigned to the acetylated species (m/z 334) would be the C-type fragment, not the (B-type) oxonium ion. However, it is likely that the acetylated fragment at m/z 699 easily eliminates ketene (CH2=C=O) t generates the peak at m/z 657 and the NeuAc oxonium ion signal similarly originates via decomposition of the fragment at m/z 334, in a one- or two-step elimination of acetic acid or ketene and water, respectively. Reference standards containing acetylated forms of sialic acid need to be analyzed under the selected conditions in order to determine the typical yields of each type of oxonium ion and the m/z 657 and m/z 699 species. In any case, it does not seem safe to estimate the relative amounts of the two glycoforms when one of the candidate species is so prone to eliminate the modification which differentiates them.
The authors present Figure S21 to show the release of only trace amounts of N-linked glycans from the high MW RNA but do not provide an equivalent figure for release from the low MW RNA which is more pertinent to the discussion, since they assert that only the low MW RNA has N-glycosylation at a level that could be biologically important. The data presented in this manuscript do not provide an estimate of the frequency of occurrence of glycoRNA species in the examined samples.

Editorial comments:
Overall, the presentation is incoherent and needs significant editing; one or more of the senior authors should have read through the entire assembled manuscript prior to submission.
1. The text is often rather confusing to read – even within a single paragraph of the experimental section on extraction of glycoRNAs from membranes, the verb tenses repeatedly change back and forth between present and past.
2. It would appear that the different sections of this manuscript were prepared independently since the styles and the voice (first person, third person) change abruptly from the extraction section to the mouse tissue section.
3. The mouse tissue section on methods for preparation of glycoRNAs has a style that would be more appropriate for Nature Protocols than it is for Nature Communications.
4. There are no page numbers. This reviewer has numbered the pages starting with the title page.
5. As noted above, the Methods section focuses on the glycoRNA experiments. For the bovine mucin, the type, the source and the amount analyzed are not defined. For the human milk samples, the sample amount is not given and the procedure is only available in the cited reference. For neither of these are tables provided to compare previously reported quantitative glycoform distributions with the experimental data obtained using the new workflow. For LC-MS analyses, the sample volume is given, but there is no information on the concentration. The comparative method used Agilent MassHunter Qualitative Analysis for quantification.

Abstract, l. 20: ….and there are also…

Introduction
line 1. : Proteins have post-translational modifications, but lipids are not translated from a template so modifications to their initial structures are not PTMs
line 20: Neither labelling nor derivatization decreases sample complexity. Perhaps the authors meant glycosidase treatment rather than derivatization, but then they should acknowledge that this step results in a loss of biologically relevant structural information, e.g., locations and linkages of sialic acid residues. Labelling, e.g., by 18O, or introduction of a chromophore or other blocking group at the reducing end, can help to differentiate fragments that include the non-reducing end from those that retain the reducing end, but again, although they may assist in assignment of fragment ion peaks in the MS2 spectra, these do not reduce sample complexity.

p.4.l.6. The sentence starting on line 5 needs to be rewritten.
p.4.l.27. “amenable” is not an appropriate adjective in this context
p.5, l.1-2 and lines 3-4: ….the signals corresponding to the sequence-defining fragments….
p.5, l. 22: ..of the peaks for most glycans eluting from the PGC column…
P.5, l.27 and elsewhere: “decent” reflects an opinion or value judgement; it is not a scientific term
p.6: here and elsewhere, all signals assigned to specific species should be described with their charge state as well as the observed m/z value
p.6, l. 14 (and elsewhere) peaks corresponding to (not “standing for”)
p.6.: complementary and synchronized information [at the same time] – (redundant).
p.7, l.2: using set parameters
p.7., l.5: ..the glycan peaks can be assigned by…
p.7., l.16: efficiency (not efficacy)
and many more….

Figure 2 caption: glycans of interest, not “interested glycans”

Figure S11. The x-axis range on the three spectra should be kept consistent. Why are Seq Int and Cov Int only reported for DIA analysis?

Figure S13. What was the starting concentration for each glycan? Only the sample volumes are indicated on the plots.

Figure S15. According to the methods section, the released O-glycans were not reduced. The structures of assigned to several peaks are ambiguous. All assignments should be verified.

Figure S17. Part B. Compositions only should be indicated for each m/z value and these should be aligned with the values. The fragment ion structures shown at the bottom are by no means the most likely to occur. The cited paper made the assignments shown in Part A but is not the source for the fragment ion assignments; the figure in that paper which indicates nomenclature for fragments generated at various cleavage sites allows but gives no preference to these structures.

Figure S25 caption should be: Neu5Ac and Neu5Gc were present at different levels among the mouse tissues. The monosaccharide oxonium ions are marked in colors and indicate the presence of these sugars in the precursor structure: HexNAc containing (red, 204.08 (not 292.10) m/z), Neu5Ac-containing (not contaning) glycans (green, 292.10 (not 204.08) m/z), and Neu5Gc- containing (not contaning) glycans (blue, 308.09 m/z).

The authors should consider using the relevant Symbol Nomenclature for Glycans (SNFG) colors for the oxonium ion labels, and for the cartoons and charts, to the extent feasible. Furthermore, the abstract graph and the larger cartoon are misleading since the accepted letter codes for glycan residues do not correspond to the letters above them and the plot included in the figure mimics a mass spectrum but thereby suggests that isomeric residues have different m/z values.

(Remarks on code availability)
I did not attempt to review or use the code. I hope another reviewer has more expertise with evaluating code, installing and testing. I also wonder whether accessing this site would leave a trail that would identify the reviewer.

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors proposed a DIA-based glycomic workflow to identify and quantify released glycans. The workflow GlycanDIA is claimed to provide improved sensitivity in identification and quantification by utilizing the emerging modern data-independent acquisition (DIA). The manuscript contains many experiments to evaluate the effectiveness of the proposed workflow, though some areas require additional details. Major revisions are needed to 1) illustrate the details to prove its novelties when comparing with DDA approach, to 2) describe more precisely when the author claimed that GlycanDIA can “determine” the isomers, and to 3) provide more comparison to proof the effectiveness of FDR control.

Major points:
1.When demonstrating the performance of GlycanDIA workflow in glycan identification, the manuscript claimed that DIA mapped 70 more glycans than DDA method. Which DDA software was used to do the comparison? Was the two search space (i.e. glycan database) comparable? It will be necessary to provide concrete examples that the glycans identified from DIA only are correct and discuss the possible reason why DDA method can’t characterize those glycans. The glycan library used is expected to be provided such that the results presented in the manuscript are reproducible.

2.The authors claimed in the manuscript that GlycanDIA can “determine” isomers (including linkage and composition) and modified glycans. However, since the GlycanDIA Finder only takes account for the composition information in the glycan library, it is hard to identify the glycan isomers under such circumstance. Based on my understanding of the examples and results shared on github, GlycanDIA just provided the possible glycan isomers or modified glycans, which still requires experts to do manual checks in order to determine the structures. Substantial justifications are required to solid prove that GlycanDIA is able to identify glycan isomers.

Latest Glycan identification software tools (e.g. PEAKS GlycanFinder https://doi.org/10.1038/s41467-023-39699-5) designed for DDA data can distinguish the structure difference by utilizing the signature ions explained in the Figure S16. For example, the existence of signature ion 512.20, 308.09 m/z can be leveraged in a scoring function to suggest the best matched glycan isomer for each spectrum.

3.In the manuscript, the authors state that the decoy library is generated by randomly mass shifting. It is a widely used method for glycan FDR control in glycoproteomics. However, it is not a proper decoy as it underestimates the rate of false matches to glycans with shared fragment ions. The manuscript is expected to note this general limitation of this glycan FDR method.
It is said that “100 decoy sets were generated for each aligned tandem MS”. It seems the search space of target and decoy are quite different. Then how is the FDR calculation impacted? If the decoy library is much larger than glycan library, the rate of random matches towards decoy library will be biased.
Usually, 1% FDR is widely used as the cutoff when reporting. Why GlycanDIA choose 2% FDR? When comparing the sensitively with DDA, what was the FDR cutoff for each method/software tool?

Minor points:
1.In the introduction of the manuscript, the authors stated that “the application of DIA for glycomic analysis has not been established”. There were several attempts developed to do identification and quantification of DIA data although they mainly focused on intact glycan instead of released glycan. It is suggested to mention those work in the manuscript.

GproDIA: https://doi.org/10.1038/s41467-021-26246-3
Glyco-DIA: https://doi.org/10.1038/s41592-019-0504-x
Data-Independent Acquisition-Based Mass Spectrometry (DIA-MS) for Quantitative Analysis of Intact N-Linked Glycopeptides: https://doi.org/10.1021/acs.analchem.1c01659

2.The annotation for glycans are not consistent in the manuscript. In Table S3, it is said that “the annotation for glycans in this manuscript is as follows: Hex_HexNAc_Fuc_Neu5Ac_Neu5Gc”. However, Hex(5)HexNAc(4)Sia(1) was used in several annotations, such as in Figure S3, S18. It is better to keep all the annotation consistent.

3.In Figure S16(B) spectrum annotation, the peak 512.20 m/z should not exist in the spectrum if the proposed structure is the core fucose structure.

4.The glycan library used in GlycanDIA Finder only contains glycan composition and possible B/Y fragments. Can you discuss the limitation of such library used during search. What is the difficulty of adding ion intensity and iRT in the library and why they are not considered in the library search?

5.In GlycanDIA workflow, the differentiation of isomers highly depend on the RT difference. What if the RTs for different isomers are very close, how can we distinguish the isomers in this situation?

6.The authors conducted several experiments to find the optimal HCD fragmentation energy and selected 20% NCE in the final state. However, from the Figure S2-3, it looks like NCE @ 15% generated more abundant fragments than NCE @ 20%. Why NCE @ 15% is not chose?

7.There is a typo of glycan in the table title of Figure S11. It is expected to be Hex(5)HexNAc(5)Neu5Ac(1) instead of Hex(5)HexNAc(5)Fuc(1).

(Remarks on code availability)
The README file includes detail instructions that for running the code, and an example dataset with a library were provided. The code at first had an exception after running but easy to fix.
Since I didn't find the glycan library used in the manuscript, I think the results of the paper are not easy to reproduce. It is expected to include these libraries in the dataset.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I would like to commend the authors for their great effort in revising the manuscript. They have effectively addressed most of my previous concerns. Given the significance and potential broad impact of the DIA glycomics workflow in the field, I would like to recommend for its publication in Nature Communications.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have addressed most of the previous comments and provided additional details and examples to demonstrate how GlycanDIA resolves co-elution issues. These revisions have greatly improved the manuscript. However, several points still require further clarification:

1.The introduction states that GlycanDIA retains the ability to discriminate different glycan isomers (including composition and linkage isomers). The differentiation based on composition or distinct fragment ions is straightforward using diagnostic ions, which can be achieved in DDA. However, for isomers that yield the same B/Y fragments—such as the Man8 isomers—if cross-ring cleavages are not employed, how are the structural differences resolved in MS2?
In Figure S20, please clarify what specific fragment patterns are being used and how they enable the differentiation among Man8 structures.
It may be more precise to state that GlycanDIA provides information that aids in differentiation rather than directly determining glycan isomer structures.

2.Figure S11B effectively illustrates the effectiveness of the FDR control, but it is necessary to specify which dataset this figure is derived from. Additionally, does the O-linked glycan dataset exhibit similar FDR performance with the same decoy method? A comparison would be helpful.

3.In response to previous reviewer comments on FDR cutoff selection, the authors referenced a figure from GlycoNote, which is based on DDA data. However, since GlycoNote uses DDA while this study focuses on DIA, it is not reasonable to

assume identical performance, even if the same decoy generation method is applied. It would be more appropriate to present evidence directly from the research data in this manuscript rather than relying on external studies.

4.In response to Reviewer 3 Question 5, the authors stated that "the incorporation of ion mobility as an additional dimension of separation is expected to efficiently reduce coelution, thereby enhancing the accuracy and reliability of glycan isomer identification. The corresponding discussion is included in our revised manuscript (Page 12)." However, this discussion is not found on Page 12 of the revised manuscript as indicated. Please clarify whether this section was inadvertently omitted.

5.The workflow in Figure S11A appears to be missing the input glycan library component.

6.The manuscript states that larger fragments are preferable for quantification but does not specify the fragment selection for quantification. Are the top N fragments chosen, or are all fragments included?

7.In the data analysis section, NeuGc is not listed in the GlycoNote settings. Please clarify if it should be included.
8.Additional details are needed to clarify Figure S12. Please provide a more comprehensive explanation of each subfigure.

9.The title of Figure S4(B) mentions B/Y ions, while the caption refers to BCYZ ions. Please clarify the intended description to ensure consistency.

10.The MS2 spectra at 8.64 min and 13.71 min in Figure S20 do not have peak annotations.

11.The Methods section states that detailed MS setup parameters are available in Table S5. However, Table S5 contains precursor and product ions instead. Please update the reference.

(Remarks on code availability)
The provided code includes a clear README file with installation and execution instructions. It also includes example data and glyco libraries from previous publications, which are beneficial for users to get started.

Reviewer #4

(Remarks to the Author)
This manuscript has been very thoroughly evaluated by three competent reviewers and the revised version is therefore in a much better state than the original. The approach in itself deserves to be presented and made open to discussion but it should really be made clear that a lot of room for improvement remains.

Overall, the authors have addressed most of the reviewers' concerns and I would like to raise only a few remaining points:

1) The authors put forward that they are first to include DIA in glycomics while noting (only after being told) that the technique is more commonly applied in glycoproteomics (one more reference is missing, Ben Schulz introduced DIA prior to all cited references). Given that glycans are attached, it seems somehow more attractive to find out not only which glycans are present in what amount but also where they are attached. Hence the trend. Of course for free glycans, the method is compelling. Some aspects are still somehow toned down in the response and the revised version and the point is not to demonstrate that the authors are the first to introduce DIA in glycomics but to show that glycanDIA can yield finer analyses of glycans than DDA approaches. In particular, the notorious topic of co-elution and the challenge of isomer differentiation cannot be fully solved (raised by reviewers and slightly too assertively addressed). DIA only helps. And it is rather disappointing that the authors gave up on the variable window option.

2) The authors are using Thermo instruments but a large number of DIA people use SWATH (I have no interest in either companies). It is surprising that there isn't the slightest comment on other DIA techniques or at least a comment on the technical dependency of the workflow (raised by Reviewer 2 but not picked in the reply). Moreover, if you come later in the piece in applying DIA, then recent improvements offer benefits that are worth mentioning and these are not in the manuscript, only in the response to reviewers. Comments on the use of ion mobility even if only as a prospect would be of interest and belong to the main text.

3) The main critical point is the selection of the glycan library. As pointed by the authors, their focus is mainly on N-glycans. Regarding the blood sample analysis, why should ref.27 be the only source? Lots of work was published on serum glycans and glycome comparison in health and disease. It is surprising that a 10 years-old paper is considered as the ultimate. Issues in comparison were also raised by reviewers. Maybe more caution statements should be included.

Minor:
The conclusion could be improved.
page 18, line 22-23: "reported in cases of cancer, diabetes, and Alzheimer's disease" tends to imply that only these diseases are at stake. A sentence should be added to evoke the possibility of more reports to come on other diseases.
page 19, line 1: "...can also be revealed" is slightly overstating. ", in many cases" would make the statement more accurate.
A few more sentences to highlight the worth of shifting to DIA for deciphering glycosylation would make sense. Likewise with prospects mentioned above.

(Remarks on code availability)

Reviewer 1:

This work presents a workflow that combines higher energy collisional dissociation (HCD)-MS/MS with staggered windows for glycomic analysis. Also, the authors developed a generic search engine called GlycanDIA Finder, which incorporates an iterative decoy searching for confident glycan identification from DIA data. To demonstrate the feasibility of GlycanDIA, glycomic analysis covering different types of oligosaccharides, including N-glycans, O-glycans, human milk oligosaccharides (HMOs) as well as N-glycans on RNA extracted from cultured human cell lines and mouse tissues was performed. Overall, the manuscript presents the first application of DIA for quantification of N-glycans, with the goal to enhance the detection and quantification capability of various glycan compounds. However, there are several major issues and concerns that need to be addressed before the manuscript can be considered for publication in Nature Communications.

Response to general comments: We thank the reviewer for taking the time and effort to review our manuscript. We appreciate the valuable comments and suggestions provided. Herein, we would like to clarify the novelty of our work. As the reviewer noted, we reported the first DIA method for glycomics. The current differences between glycomics methods (negative vs. positive, native vs. derivatization, etc.), the cost and availability of glycan standards, limited glycan MS spectral libraries (particularly for high-resolution MS), as well as the lack of software development, have hindered the advancement of glycomics. Thus, we developed the GlycanDIA method, which can bring new opportunities and insights for glycomics, although DIA for glycomics is still in its early stages. To develop the GlycanDIA method, we established a complete workflow including optimized instrumentation methods, data analysis, and software development. Furthermore, we extended this workflow to enable more generalizable analysis of different types of oligosaccharides, including N-glycans, O-glycans, and human milk oligosaccharides (HMOs). Our data and examples confirmed the performance of GlycanDIA in glycan identification and quantification, particularly for low-abundance and isomer identification. As an application of the workflow, we characterized the N-glycans from novel glycoRNA molecules and obtained the first map of the N-glycome of mouse tissue glycoRNAs.

For this revision, we have made major efforts to further resolve the co-elution and co-isolation of glycans following reviewer's suggestion. The high efficiency of the PGC column to separate isomeric and anomeric glycans is important for GlycanDIA, which can largely reduce co-elution and co-isolation from the chromatography aspect. In addition, with a 24-m/z staggered DIA window (12 m/z after demultiplexing) setup, we investigated the frequency of co-isolation in different windows without considering LC separation. We found that glycans in different MS regions mostly have featured ions that can be used for identification and quantification. By integrating these features, the problems of co-elution and co-isolation can be minimized, and we can indeed identify different glycan isomers and anomers, as demonstrated in the newly added example of Man8. Notably, as shown in the example (Hex(5)HexNAc(5)Fuc(1)Neu5Ac(1)), co-elution and co-isolation are also concerns for common DDA analysis, since the two isomers with the same m/z were not completely resolved by the PGC column, and only one MS2 event was triggered between the two peaks, leading to ambiguous identification. Interestingly, GlycanDIA can resolve

this challenging task because DIA acquires MS2 information across the isomeric peaks that can be used for differentiation.

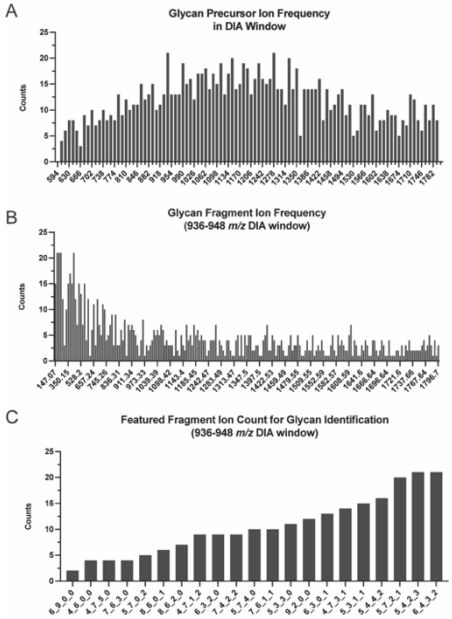
The manuscript has been substantially revised according to the reviewers' comments and suggestions. All the corresponding changes have been highlighted in the marked revised manuscript.

Point-by-point responses:

1. Many of the conclusions, including the accuracy of quantification and the differentiation of isomers, are based on the assumption that all glycans are fully separated by liquid chromatography (LC), without considering co-elution and co-isolation of glycans.

Response: we thank reviewer for the comments. We agree that co-elution and co-isolation of glycans, particularly for isomers, can lead to inaccurate quantification and differentiation of isomers. To address this issue, we would like to provide further clarification on our GlycanDIA workflow and the measures we have taken to minimize potential co-elution and co-isolation problems:

(a) From the LC aspect, we employed a PGC column to separate isomeric and anomeric glycans based on their molecular structure, hydrophobicity, and polarity. This has enabled us to efficiently separate glycans with different subtypes, such as high-mannose type, and those with different degrees of fucosylation and sialylation. Therefore, the LC method in GlycanDIA workflow, although not able to fully sperate all glycans, has largely minimized co-elution thus reduced co-isolation for MS to handle.

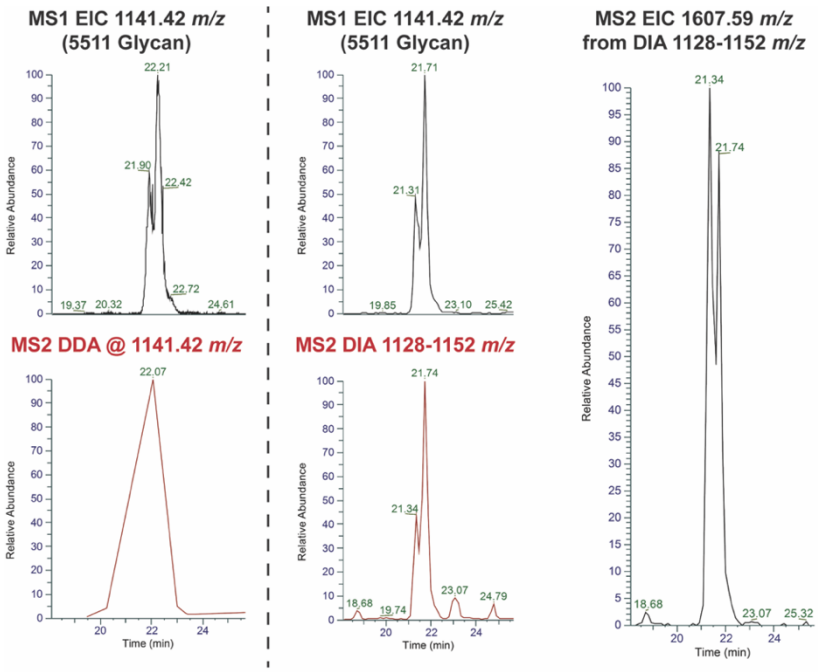


(b) From the MS aspect, we chose a 24 m/z mass window as the optimal for data acquisition, and we obtained the glycan fragments from a 12 m/z window after demultiplexing. To evaluate the 12 m/z window in detail, we examined the frequency of N-glycan precursors (from a general library) with a 12 m/z interval from 600-1800 m/z, without considering LC separation. As shown in the Figure above (Figure S10 in the revision), the 936-948 m/z region is most crowded, with 21 potential precursors. Upon checking their theoretical fragments, we found that, except for some small glycan fragments shared by all glycans (such as 163 and 204 m/z) that can be detected from all

21 glycans, the fragments larger than 500 m/z are mostly shared by five or less glycan precursors, and each glycan has at least two featured fragment ions (shared by three or less glycan precursors). These results demonstrate that 12 m/z DIA window is optimal for GlycanDIA.

(c) For data analysis, the identification and quantification in our pipeline are performed on the apex of the precursor and the fragment ion peaks. In this case, for each peak, only the most abundant glycan is considered regardless the potential co-elution and co-isolation. We also incorporated a random mass shift approach to generate decoy glycans, which were used to indicate whether a glycan match is random and eliminate false identifications in samples. This glycan decoy approach, inspired by pGlyco (Nat. Commun. 8, 438, 2017) and GlycoNote (Anal. Chem. 95, 8223, 2023), has been successfully demonstrated in glycoproteomic DIA using GproDIA (Nat. Commun. 12, 6073, 2021) and has helped to improve glycan identifications. With the confident identifications, we provided peak height values in our software, which can give optimal quantitative values.

To be noted, co-elution and co-isolation also happens in common DDA glycomic



analysis when two isomers are not well separated. In the example provided below (Figure S22 in the revised SI), the anomeric peak of the glycan Hex(5)HexNAc(5)Fuc(1)Neu5Ac(1) was not fully resolved by PGC. In DDA analysis (left), the MS2 fragmentation was triggered between the two peaks, resulting in ambiguous identification and requiring the integration of the two peaks together for quantification. However, with GlycanDIA (right), the MS2 fragmentation was triggered across the two peaks, capturing valuable diagnostic information (such as 1607.59 *m/z*) that enables the identification between the two glycan anomers and allows for more accurate quantification of each anomer separately.

Meanwhile, we are also exploring the use of ion mobility for GlycanDIA analysis to achieve further improvements. The incorporation of ion mobility as an additional dimension of separation is expected to reduce co-elution and co-isolation, thereby

enhancing the accuracy and reliability of glycan identification and quantification. In our revised manuscript, we have updated the relevant figures and added a more detailed text to explain these points.

2. Selection of MS/MS Ions for Quantification. The criteria for selecting ions used in quantification are not adequately justified. Given that many glycans have similar structures, quantification based on smaller fragment ions can be significantly distorted due to co-elution. Although larger ions tend to provide more specific information and are less likely to be distorted, their generation efficiency is generally low, especially under the NCE 20 conditions listed by the authors. At these conditions, the y1/y2 ion generation efficiency is minimal, or these ions are entirely absent. (Particularly for larger glycans. For NCE, the fragmentation energy increases with the mass of the glycan. However, larger glycans have higher brunches and can be more unstable).

Response: we thank the reviewer for the valuable comment. As illustrated in the Figure S10, fragments larger than 500 m/z are not commonly shared by different glycan precursors, with less than 5 precursors sharing each ion out of 21 precursors examined. Additionally, each glycan has at least two featured fragment ions, which further supports the use of larger fragment ions for quantification. We selected NCE at 20% in order to balance the fact that generating large specific fragments while producing glycan fragments with high efficiency for identification and quantification. In our revision, we provided additional analysis to evaluate the MS2 ion performance of Man8 and 6511 glycans (Supplementary Data 8). As expected, individual large MS2 ions exhibited better quantitative performance compared to the smaller ones; importantly, different Man8 isomers and anomers can be identified and quantified using their diagnostic ions. We included these results in our revision (Page 8).

3. The justification for the NCE selection is based on the majority of N-glycans. It would be beneficial to include data or discussion on whether this optimization is equally effective for less common or more complex glycans. And whether it could be better using stepped HCD?

Response: We thank the reviewer for the suggestion. The selection of NCE at 20% was based on our monitoring of different glycan types under various NCE conditions ranging from 15%–35% (Figure S1-3). We chose NCE at 20% to ensure sufficient fragmentation information and adequate efficiency for generating ions for both identification and quantification. NCE at 20% has also been found to be the optimal setting for fragmentation of glycan chains in previous glycoproteomics analyses (Mass Spec Rev., 42, 1261, 2023). Although the absolute fragmentation energy is relatively higher for large glycans, which generate more small fragments (as mentioned by the reviewer), the identification and quantification of large glycans at NCE 20% are more ideal for general glycans across a wide range of m/z values. For the identification of less common glycans, we considered NCE 20% to be a generally applicable starting point, especially given its effectiveness in identifying acetylated N-glycans.

Regarding stepped HCD, it has not been used for DIA analysis in general due to the complicated data processing and the requirements for a fast cycle in DIA analysis (to obtain enough data points for identification and quantification). In addition, stepped HCD leads to ion signal dilution through more fragmentation pathways, while the total ion

capacity remains similar to experiments performed at a single energy. These features make it challenging for glycomic DIA analysis, leading us to continue using normal HCD for our current GlycanDIA setup. We have added corresponding discussions to our text (Page 5).

4. There is limited discussion on the potential limitations or challenges of the decoy method. For instance, how does the decoy mode perform with highly complex or novel glycan structures?

Response: We thank the reviewer for pointing out this. In our decoy mode, which involves a random mass shift, the theoretical fragment ions of a target glycan are shifted by a small random amount in the matching process. By this method, we can estimate the score that the assigned glycan has matched to a given spectrum by chance. However, since glycans often share the same fragment ions, particularly for N-glycans, which share a common chitobiose core, this calculation may lead to an underestimate of the score for distinguishing between similar glycans, and the degree of underestimation increases as the similarity of the glycans increases. These limitations are described in our revised manuscript, Page 18.

5. The identification of isomers based on the ratio/presence of small glycan fragment ions is highly unreliable in DIA due to the large fragmentation windows used. The small glycan fragments the author used for distinguishing isomers can also be generated by other glycans, and co-isolation of those glycans can easily distort the ratio/presence of glycan fragment ions. For instance, the authors use diagnostic peaks of Neu5Ac and Neu5Gc fragments to identify H6N4FNeu5Ac1, H5N4Neu5Ac1Neu5Gc1, and H5N4F2Neu5Gc1. However, co-fragmenting of other sialylated glycans can also produce Neu5Ac or Neu5Gc fragments, thereby affecting the identification results.

Response: We thank the reviewer for the question. We would like to clarify that the examination of the presence of diagnostic peaks for distinguishing between isomers relies on confirming that the apex MS2 ion peak aligns with the corresponding isomer precursors. Specifically, for the example of Hex(6)HexNAc(4)Fuc(1)Neu5Ac(1), Hex(5)HexNAc(4)Neu5Ac(1)Neu5Gc(1), and Hex(5)HexNAc(4)Fuc(2)Neu5Gc(1), the software first identifies the precursor peaks of these isomers. Once the precursor peaks are confirmed, the software searches for MS2 fragment ions that must appear within the nearby cycle of the precursor peak. The Neu5Ac and Neu5Gc diagnostic ions are used to help distinguish between different isomers, but they are only considered reliable diagnostic markers when they appear at the apex of the fragment ions corresponding to the precursor ion peaks. This is because these diagnostic ions must be part of the fragmentation pattern specifically associated with the precursor ion of the isomer, and their presence at the apex confirms the isomer's identity and rules out the possibility that they are present due to the co-isolation of other glycans. Furthermore, our GlycanDIA software allows for customization of the library and glycan fragments to be monitored, enabling users to define their own diagnostic ions when necessary. This clarification is described in more detail in Page 10-11.

6. Manual Input for Glycan Structures. The software used by the authors lacks the capability for de novo construction of glycan structures and requires manual input of

glycan MS/MS fragments for searches. Thus, this approach necessitates prior knowledge of glycan structures, as glycan structural isomers and can influence the b/y ion fragmentations generation of glycans. This limitation needs to be discussed.
Response: We agree the current software is not optimal for *de novo* analysis glycans, and we have added this point in our revision (Page 18).

7. The comparison between DDA and DIA glycan identification numbers is flawed due to inconsistent software usage. For DDA results, GlycoNote and Agilent MassHunter Qualitative Analysis software (v.B08.22) were used, while for DIA results, GlycoWorkbench (v2.1) was used for glycan fragment prediction, ProteoWizard MSconvrt(v3.0) for demultiplexing the spectrum, and Thermo Scientific FreeStyle (v1.8) and Skyline (v21.0) software for viewing MS1 and MS2 information. GlycanDIA Finder was used for glycan identification and quantification. Comparing the identification numbers obtained from these different software tools is not rigorous as they may not use the same criteria for glycan identification. Additionally, the specific conditions for DDA should be listed, and it should be clarified whether the same MS/MS cycle time was used for both DDA and DIA.
Response: We thank the reviewer for the question. We agree that it would be better to compare DDA and DIA using the same platform, as this would allow for a more direct and fair comparison of their respective performances. The comparison between DDA and GlycanDIA presented in our Figure 2 was performed on the same Orbitrap instrument. However, DDA was analyzed using GlycoNote, while DIA data was analyzed using GlycanDIA Finder. As a result, we had to employ two different software for our analysis and comparison. Fortunately, both software used random mass shifts as a decoy mode for FDR control, allowing us to assess the sensitivity, specificity, and overall accuracy of both acquisition modes in a similar algorithm, which ensured a better comparison in the absence of a unified tool. We have included an additional figure for the glycan identified from DIA but not DDA (Figure S13). Meanwhile, we realized it's not a rigorous comparison only between two MS acquisition methods. However, this information is helpful for researchers who just want to apply one established workflow with user friendly computational tools, and thus a more rigorous comparison between the two MS methods for identifying glycans is illustrated in Figure 2F with spiked-in standards. We set up an experiment with isotope-labeled standards spiked into a glycan mixture as another way to evaluate DDA and GlycanDIA. As shown in Figure 2F, 60% (6 out of 10) of DDA injections had at least one missed identification of spiked glycan, while all ten DIA injections were able to detect all three glycan standards. We have added more information to better describe these points in our revision (Page 9).

8. It is recommended for the authors to provide a detailed description and systematic discussion of previous software tools such as GlycoDeNovo2 and GlycoNote before introducing GlycanDIA Finder. Also, the reviewer wondered whether the same data sets were compared among GlycoDeNovo2, GlycoNote and Glycan DIA Finder?
Response: We thank the reviewer for the suggestion. We included more detailed introduction about GlycoDeNovo2 and GlycoNote in our revised manuscript (Page 8). Regarding the questions about the three software, they were designed for different purposes. Specifically, GlycoDeNovo2 software is designed for de novo analysis without

prior knowledge of glycan structure and has been demonstrated for handling different MS/MS spectra. GlycoNote requires a desired glycan composition range and can only read DDA data, while GlycanDIA Finder needs a library of precursor and fragment ions and can only check DIA data. Therefore, we cannot employ a full investigation using the same data sets to examine the three software. At the same time, since all the glycans are ideally fragmented in the DIA analysis, we expect to extract the fragment ions from the same precursor and apply the GlycoDeNovo2 software algorithm for identification, which can achieve the possible "library-free" identification and provide valuable information for investigating less common glycans in the future.

9. Also, the parameters adopted from GlycoNote and embedded into the decoy mode of GlycanDIA Finder are unclear, as well as the rationale behind why the authors chose these specific parameters.

Response: We thank the reviewer for the question. The random mass shift approach has been widely used to generate decoy glycans, including pGlyco, MSFragger-Glyco, Glyco-Decipher, StrucGP, and GlycoNote software. While all these software were developed for glycoproteomics search, GlycoNote was developed for glycomics search, with optimized settings. Therefore, we adapted parameters from GlycoNote, including mass shift range, number of decoy sets, FDR, tolerance fragment, and tolerance precursor. We chose a mass shift range of 50% because our precursor mass is 600-1800 m/z. The number of decoy sets was set as 100, which is a balance between simulation time and variability of the decoy set. Smaller tolerances for fragment and precursor ions, 20 ppm and 10 ppm, respectively, were used in our software for resolving the data acquired from high-resolution mass spectrometry instruments. An FDR of 2% was chosen as a balance between run time and controlling false-positive identifications, which aligns with the GlycoNote settings. We included the details in our revised text (Page 21).

10. The analysis of N-glycans from RNA extracts is a novel application and highlights the sensitivity of GlycanDIA. It might be good to include some discussion on the biological implications of these findings, particularly how the differences in glycan profiles might affect cellular functions.

Response: We thank the reviewer for recognizing the significance to map the RNA glycome. We added the corresponding discussions in our results and to illustrate the importance of these findings for biology (Page 15).

11. Only one glycan (Hex(5)HexNAc(5)Fuc(1)) is hard to conclude whether GlycanDIA method outperform the traditional DDA analysis and the possibility of developing a "library-free" software from using the MS2-centric method. Do the authors consider validating GlycanDIA's performance by using the mixture of different glycan types or glycan from complex samples? Furthermore, did the authors compare results between the library-free and library-based approaches within the GlycanDIA workflow?

Response: We thank the reviewer for the question. We would also like to clarify that the "library-free" approach has only been tested preliminarily on the Hex(5)HexNAc(5)Neu5Ac(1) glycan, and while there is potential for its broader application in data analysis, we do not claim it as a fully validated method at this stage. Current workflow focused on library-based approaches, and more close investigations

are needed to demonstrate the feasibility and robustness of this approach across a wide range of glycans, requiring extensive datasets for proper validation. Furthermore, the design of an effective algorithm for comprehensive library-free glycan identification is highly complex and goes beyond the scope of our current study. We added more information in our discussion to clarify this point (Page 18).

Regarding the GlycanDIA performance from the complex sample. As mentioned previously, we set up the experiment with isotope-labeled standards spiked into a glycan mixture as another way to evaluate DDA and GlycanDIA. As a result, DIA achieved 100% detection across all ten injections, consistently identifying all three standards, while 60% of DDA injections (6/10) missed one or more spiked glycans.

12. I would recommend that the authors present detailed quantitative performance metrics in a tabular format for both DIA and DDA conditions. This should include details such as linear range, limit of quantification (LOQ), coefficients of variation (CVs), and other relevant parameters.

Response: We agree it is better to also have the quantitative performance in tabular format. And we now have Supplementary Data 7 for showing the information.

13. The number of detected glycans decreased with the increased dilution factor, and it seems that only high abundance glycans could be detected after dilution. The authors should show glycan characterization by DDA before dilution and confirm that GlycanDIA did not lead to reduced detection of low concentrations of glycans.

Response: We thank the reviewer for the suggestion. The glycan characterization by DDA was shown in Figure S15 and we included Figure S16 to show GlycanDIA ability to detect glycans at the low concentrations.

Some more specific minor comments:

14. Figure 2B is not well described. It is unclear what different curves (blue, purple, red) in plots represent. If they represent extracted ion chromatograms for glycan mass, the mass should be mentioned. In Figure 2, while the improvement of DIA is evident, the manuscript could include a more detailed information. i.e. are the cutoffs the same to DDA and DIA data?

Response: We thank the reviewer for the suggestion. We provided more information for Figure 2 and corresponding description. And we included more information in our data analysis (Page 21).

15. In Figure 3B, the Venn diagram seems not in proportion for ‘76’ and ‘56’ and might be misunderstood. The second figure in Figure 3E is a bit confusing and doesn’t explain thoroughly in the manuscript. Do these glycans reflect their abundances or frequencies in each group?

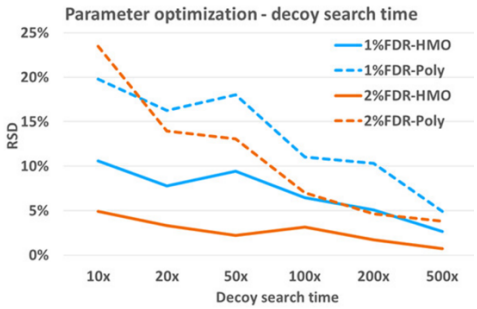
Response: Our Venn diagram is in proportion, and we have included more details and explanation for Figure 3E.

16. The specific setting parameters for the mass tolerance, intensity threshold, and maximum charge range are unclear.

Response: We thank the reviewer for the comments. We included this information in our data analysis (Page 21).

17. The authors chose to set the False Discovery Rate (FDR) at 2% instead of the typical 1%. It would be beneficial to explain why they made this decision and the impact on the results compared to using a 1% FDR threshold.

Response: We thank the reviewer for the suggestions. FDR at 2% is the balance between searching space and false-positive identification, which has been demonstrated in our previous GlycoNote software, and we added this information to our text for clarification (Page 8).



18. The CVs of the technical replicates were less than 5% isn't strange, how was the CVs of the technical replicates in DDA under same conditions.

Response: The quantification in DDA is based on MS1 signal, and the CVs of the technical replicates in DDA is higher compared to DIA (larger than 10%), and we had this information in our text and Figures 2D. We added more information about this point in Page 9.

19. It is important to ensure that the sample loading amounts are consistent when comparing different samples. If the loading amounts differ, normalization should be considered to accurately compare differences or similarities between them. The author stated that "the relative abundance of different N-glycan subtypes was also distinct.", which could potentially be attributed to differences in injection amounts. Did the authors determine concentration before LC/MS analysis? The authors should give more details (e.g., the amount of starting materials used for RNA extraction, et al) in the section of profile of glycans on RNA from different mouse tissues.

Response: We thank the reviewer for the suggestions. We clarify that the concentrations of our samples were measured prior to glycan release, with consistent amounts used for analysis: 1 mg of cell membrane protein or 5 μg of RNA per sample. We included this information in our method section during the revision.

20. The authors should clarify certain conditions, such as the pH in N-glycan sample preparation and specify the amount of purified glycans injected for LC-MS/MS across all different sample types in the appropriate section.

Response: We thank the reviewer for the comments and added this information in our method section.

21. The authors should provide detailed explanations or criteria used for categorizing glycan subtypes such as high mannose, neutral, fucosylated, sialylated, sialofucosylated, and Neu5Gc-containing glycans.
Response: The glycan subtypes were noted based on the previous publications. We included the explanation for these subtypes (Page 22).

22. In Figure 4, clarify how many biological replicates were used for each error bar.
Response: We had three biological replicates and added this information in the Figure 4 caption.

23. In Figure S5-8, why did the same MS1 and MS2 show different retention time under different DIA strategies? Additionally, clarify why the y-axis scales are not consistent during the comparison of the two different DIA strategies.
Response: We thank the reviewer for the question. We conducted separate runs/injections to investigate different DIA methods. Our analysis was performed at the nanoflow rate (300 nL/min), which commonly led to retention time (RT) shifts across different runs. As a result, the peaks were aligned exactly the same between runs, though their intensity levels are generally consistent. For example, the MS1 peaks for the Hex(5)HexNAc(2) glycan are consistently around the 2e8 intensity level across all runs.

24. Please verify carefully multiple formatting errors in the references.
Response: We appreciate the reviewer for carefully checking the details. We have corrected the error in our revision.

25. There is a typo in the legend of figure 4, “sialyation” should be “sialylation”.
Response: Corrected.

26. In material and methods in supplemental information, “Magnesium sulfate (MgSO2)" should be corrected to "Magnesium sulfate (MgSO4)."
Response: Corrected.

27. There is a typo in Figure S25. ‘Neu5Ac-conatining’ should be ‘Neu5Ac-containing’.
Response: Corrected.

28. Ensure consistent use of terms and abbreviations throughout the manuscript, such as "Neu5Ac" versus "NeuAc".
Response: Corrected.

Reviewer 2:

The authors of this manuscript report that they have developed an analytical method for the qualitative and quantitative determination of N-and O-linked glycans that are present as modifications on glycoproteins, small human milk oligosaccharides, and N-linked glycans that they have assigned as low abundance modifications of RNA.-The method utilizes fixed-voltage HCD of selected, sequential segments along the m/z axis of the MS1 mass spectral scan and generates structural assignments and quantitative determinations based on the resultant MS2 data. It seems likely that the scheme presented in this manuscript could ultimately offer advantages over previously published analytical methods, in terms of correct assignment of structures, as well as quantitative precision and accuracy. In their BioRxiv preprint (Ref. 3), the authors used a similar SWATH strategy for detection of glycoRNA, but have developed the approach much more fully while carrying out the work described in this manuscript. However, while the data obtained for standards and the examples of applications presented here verify precision, they do not provide sufficient evidence that all (or most) structural assignments for milk, plasma and glycoRNA components are correct, nor that the quantitation is accurate. In only one experiment is a quantitatively defined standard mixture employed during the method development, in this case, it is applied as a standard addition to the sample mix and quantitative values are reported only for the labeled standards. No independently determined reference quantitative values are included as comparisons with results obtained here for the human milk oligosaccharides, nor for the components of the plasma extract, the bovine mucin, or the glycoRNA samples.

Response to general comments: We sincerely appreciate the reviewer's insightful comments, which has helped guide our efforts to refine our manuscript. We are grateful for the valuable comments and suggestions provided, which have helped us to improve the clarity and accuracy of our work. We would like to highlight the significance of our manuscript, which presents the first DIA method for glycomics. Glycomics is a field that has been hindered by various challenges, including the use of different analysis methods (such as negative vs. positive, native vs. derivatization), the high cost and limited availability of glycan standards, and the lack of software. We have developed the GlycanDIA method, which involves a comprehensive workflow that includes optimized instrumentation methods, data analysis, and software development. Our results demonstrate the effectiveness of GlycanDIA in identifying and quantifying glycans, particularly for low-abundant and isomeric glycans.

In more detail, our N-glycan analysis was performed using glycans released from standard source proteins (RNase B and human serum), which have been fully characterized in our previous studies with respect to their composition and structures. In the initial phase of this study, we injected the same amount of N-glycan samples, which allowed us to directly compare the performance of the previously established method with GlycanDIA. Our results clearly demonstrated the advantages of GlycanDIA in terms of identification accuracy and quantitation efficiency. However, the absolute quantitation in the analysis relied on protein content as a reference, which is a limitation. To address this limitation, we incorporated three isotope-labeled glycan standards into the glycan mixture in subsequent experiments. This not only enabled us to evaluate GlycanDIA's capability for low-abundance glycan identification and quantitation but also further validated its robustness and reliability. The results again demonstrate the performance of GlycanDIA.

As this is the first application of a DIA-based method for glycomic analysis, the majority of this study focused on N-glycans due to their relatively well-characterized information (retention time, fragmentation pattern, etc.), providing a strong foundation for benchmarking GlycanDIA. However, we also expanded the scope to explore its utility for O-glycans released from Bovine Submaxillary Mucin, HMOs standards, and human milk samples. Our findings demonstrated that GlycanDIA can be effectively applied to different glycan types as well, indicating its potential for broader applications in glycomics research. Moving forward, we expect that comprehensive high-quality reference glycan panels with acceptable cost will be available in the future, which can be used to facilitate absolute quantitation and more precise structural elucidation. We believe this step will further enhance the accuracy and utility of GlycanDIA for glycomic analyses.

For the data analysis, we incorporated random mass shift decoy, a popular approach among glycomic and glycoproteomic software developers, to generate decoy glycans, although this approach may underestimate the rate of false matches to glycans with shared fragment ions. We showed more details to evaluate this FDR calculation. To improve the accuracy of GlycanDIA Finder, we added glycan molecule feature examination, which detects glycan isotope peaks and monitors possible misidentification. We further established confidence in the software's identification performance through rigorous manual cross-verification of glycans identified by the software (Supplementary Data 7). We have also developed a new tool, Glycan Product Generator, which calculates possible glycan fragmentations based on the input structure and outputs the glycans in a format that can be directly imported into Skyline for search and evaluation. Additionally, we have provided common glycan libraries to streamline the high-confidence identification and quantification process in GlycanDIA data analysis.

Overall, the GlycanDIA method offers a major advancement in glycomic analysis and allows researchers to address questions in glycobiology from a novel perspective. The manuscript has been substantially revised according to the reviewers' comments and suggestions. All the corresponding changes have been highlighted in the marked revised manuscript. We greatly appreciate if the reviewer can reconsider the manuscript.

Point-by-point responses:

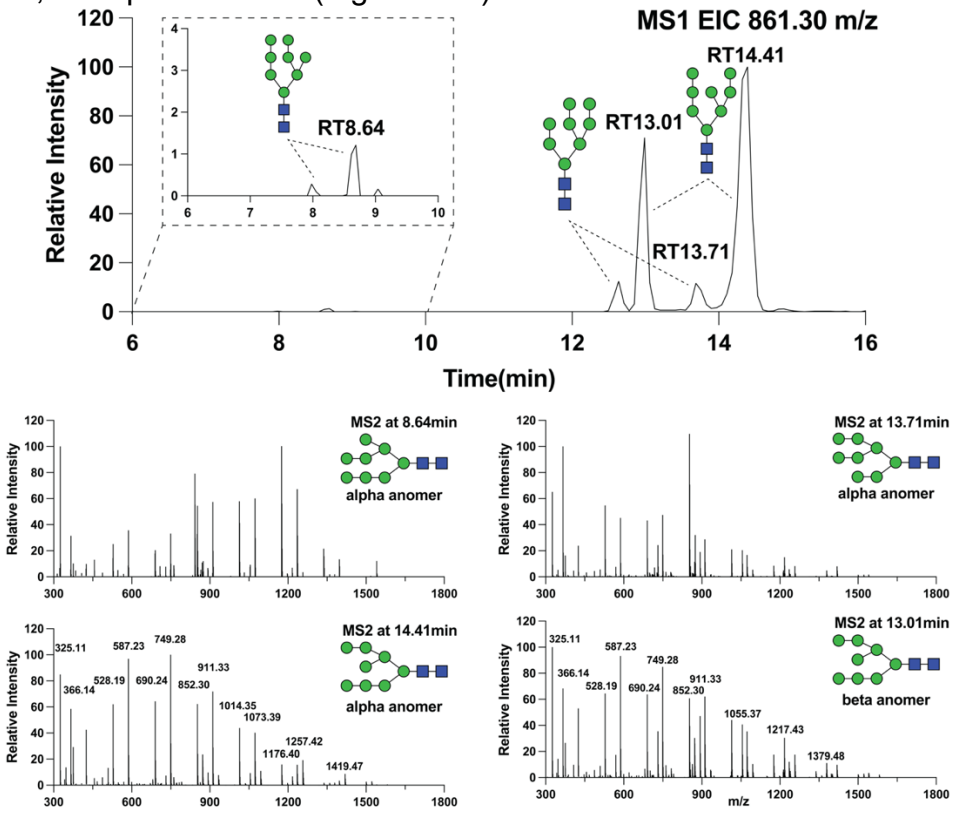
The analysis method and GlycanDIA workflow and novel search engine Glycan DIA Finder described in this manuscript are based on the generation and comparison of glycosidic fragments. Cross-ring fragmentation that defines linkage positions is not used for assignments, yet, throughout the manuscript and the supplementary material, the authors label the peaks in their mass spectra and chromatograms with structures that have specific linkages not defined by their data.

Response: We thank the reviewer for the comment and agree that our GlycanDIA data indeed have limitations in resolving glycan linkage information. However, it should be noted that the GlycanDIA workflow was first developed using the N-glycans released from a mixture of RNase B and human sera, in which we have obtained glycan profiles containing composition and structure information in previous studies (Anal. Chem. 92, 14038, 2020; Anal. Chem. 87, 7754, 2015). The linkage-specific annotations in our figures and tables are inferred from reference datasets. At the same time, while GlycanDIA's HCD-MS/MS spectra primarily yield B/Y ions, these data still enable robust differentiation of compositionally distinct isomers through relative fragment ion ratios and

chromatographic separation. For example, the diagnostic fragments 512.20/657.24 m/z (Figure S19) allowed us to propose structural assignments for Hex(6)HexNAc(4)Fuc(1)Neu5Ac(1) isomers. However, we agree that α2,3- vs. α2,6-linked sialic acid differentiation (Figure S21) requires further validation with linkage-specific workflows. To clarify these points, we have added more information and discussions in our revised text (Pages 8 and 17).

Although they state on page 4, line 17, that the method discriminates among and correctly assigns structures to isomeric glycans that differ only in linkage, they do not provide evidence that their method reliably obtains and correctly assigns different MS2 spectra for isomeric glycans that vary in their branching patterns. To give a straightforward and well-documented example, they could have provided an illustration showing the separation, assignments and quantification for the Man7 isomers from the (presumably bovine) RNase B that was one of their standard samples. In fact, even the relative abundances of the RNase B homologs (irrespective of isomer assignments) were not compared to reference data.

Response: We thank the reviewer for their valuable suggestion, and following this, we examined the GlycanDIA performance for Man8 isomers, including their separation, identification, and quantification (Figure S20).



(a) Separation: We observed three isomers and their corresponding anomers, which is consistent with the findings of a previous study by She et al. (Anal. Chem. 92, 14038, 2020). This suggests that our method using PGC at nanoflow can efficiently separate the native glycan isomers and anomers.

(b) Identification: The previous publication reported the identification of glycan isomers and anomers using the fragmentation pattern generated by low-energy CID, and we also

obtained such information from our GlycanDIA method using HCD at 20% NCE. As a result, we were able to distinguish the six Man8 isomers and anomers based on their different fragmentation patterns. These results highlight the enhanced analytical capabilities of our GlycanDIA method.

(c) Quantification: As shown in Figure S14 and Supplementary Data 8, we demonstrated that the Man8 glycans (isomers and anomers) can be quantified with good linearity (R2 > 0.99) using the large product ions. This indicates that our method can provide reliable quantification for the glycans.

Overall, our example data for Man8 demonstrate the reliability and efficiency of the GlycanDIA method, and the integrated separation, identification, and quantification capabilities of this method highlight its potential for advancing glycomics research.

On page 5, lines 14-15, the authors assert that all possible N-glycan precursors can be detected below m/z 2000 in ESI-MS and that scanning the range below m/z 1800 is sufficient to detect all N-glycans. References 18 and 19 that are cited to support this choice for the scan range but these papers did not report any results obtained by exploring scan ranges that extended above m/z 2000. Because glycans tend to generate molecular ions in lower charge states than do peptides of the same molecular weight, signals from larger glycans can fail to appear within the range below m/z 2000.

Response: We thank the reviewer for the comment. The scanning range larger than *m/z* 2000 is indeed beneficial for EThcD-based glycoproteomic analysis (Anal. Chem. 2019, 91, 10401). However, we would like to clarify that the mass range we designed is sufficient for the common glycans analyzed. Although glycans typically have lower charge states (normally 2-3 charges) compared to peptides, their *m/z* values generally fall within our designated window. For instance, the highly branched glycan with the composition Hex(7)HexNAc(6)Fuc(3)Neu5Ac(2) has an *m/z* of 1696.62 and 1131.41 when two and three charges are added, respectively, which is within our set range. For a glycan to have an *m/z* larger than 2000, it would require a composition larger than Hex(9)HexNAc(8)Fuc(6)Neu5Ac(7), which is not commonly observed in most studies. To better demonstrate our claim about the mass range, we have revised the original text to provide further clarification (Page 5).

The authors claim that their approach will allow a “library-free” software, but the method depends heavily on reference LC elution behavior, pattern matching of the mass spectra and MatchMS library.

Response: We thank the reviewer for the comment. We would like to clarify that while our preliminary investigation of the "library-free" approach (demonstrated for the Hex(5)HexNAc(5)Neu5Ac(1) glycan in Figure S31) shows promise for glycan discovery without relying on predefined spectral libraries, we explicitly avoid positioning it as a fully validated method in its current form. The "library-free" approach holds significant potential for de novo glycan characterization, as it eliminates the need for prior knowledge of glycan compositions or structures. However, developing a robust algorithm for comprehensive library-free identification requires sophisticated de novo sequencing capabilities. Therefore, we used “library-based” method in our analysis, and our results suggested the better performance of GlycanDIA compared to DDA. We made corresponding changes in our results to clarify this point (Page 18).

Figures 2 and 3 leave many questions to be answered. How are peak heights/areas calculated? How is the baseline assigned for peaks in regions where the signal is far above the y-axis on both sides of the peak? For the MS2 spectrum shown in Figure 2, section A, all of the peak assignments above m/z 657 are incorrect. The reader is left to wonder whether assignments in the many spectra not presented herein have been verified.

Response: We thank the reviewer for the comment. The glycan peaks were shown in Figure 2B and Figure S5-8. The signal-to-noise (S/N) ratio above 5 was chosen for quantification (peak area), and the apex of the peak was used as the peak height. Figure 3A presents the MS/MS signal of m/z 204.08, which was used to show the total degree of N-glycans in two samples. We have re-labeled the MS/MS peaks based on the reviewer's suggestion and provided detailed information for quantification in the method section (Page 21).

On Page 14, line 20, the authors make the comment that glycans, unlike peptides, generate low m/z ions that are indicative of composition (oxonium ions). Peptides do have analogous peaks, immonium ions, that provide evidence for amino acid composition.

Response: We thank the reviewer for pointing out the problem in our description. We have revised the relevant sentence to better focus on glycans, removing the unnecessary reference to peptides. The revised sentence now reads: "This is because glycans often generate common fragments…." (Page 16).

A bit later, they state that the CID and HCD MS2 spectra included in most glycan databases have been recorded in the negative-ion mode. This is also untrue. What is true is that negative-ion MSn spectra of glycans show a higher level of retention of sialylated glycans and sulfation. Such labile negatively-charged features are much more easily lost in the positive-ion mode, especially for protonated species; this point is not addressed in their discussion.

Response: We thank the reviewer for the comment. We have revised our text to remove the claim about established glycan databases mainly based on negative-ion mode. Additionally, we have included more information on the analysis of glycans using positive and negative modes, highlighting our selection for using positive mode for GlycanDIA. Corresponding changes have been made on Page 5 in the revised manuscript: "Electrospray ionization in positive mode facilitates the detection of neutral glycans, while still allowing for the identification of sialylated glycans and sulfation, although with reduced efficiency. Therefore, to obtain a more comprehensive glycan profile, we chose the positive mode for our GlycanDIA."

The Methods section contains information primarily regarding the preparation and analysis of glycoRNA samples. No or very little information is provided on the sources of, or details of the glycan release from, the glycoprotein standards, the human serum, or the bovine mucin (what type?). Likewise, no figures or tables are presented to verify the quantitative reference values or quantitative accuracy achieved for these determinations.

Response: We thank the reviewer for the comment. We now provide more information about our standard glycoproteins, N-glycans, and HMOs, including the amount, source,

vendor, and catalog numbers (Table S2). We have added the following statement to our Methods section: "Details regarding our standard glycoproteins, N-glycans, and human milk oligosaccharides (HMOs) are provided in Table S2 for reference." Additionally, we have included the quantitative information we obtained in Supplementary Dataset 7.

For the plasma sample, there is a comparison with the results from a Q-TOF MS DDA analysis, but there is no independent information to establish reference values. Why was the DDA analysis not performed on the same instrument system used for the DIA analysis? That would have provided a better comparison.

Response: We thank the reviewer for the question. The comparison between DDA and GlycanDIA presented in our Figure 2 was performed on the same Orbitrap instrument. However, DDA was analyzed using GlycoNote, while DIA data was analyzed using GlycanDIA Finder. As a result, we had to employ two different software for our analysis and comparison. Fortunately, both software used random mass shifts as a decoy mode for FDR control, allowing us to assess the sensitivity, specificity, and overall accuracy of both acquisition modes in a similar algorithm, which ensured a better comparison in the absence of a unified tool. We have included an additional figure for the glycan identified from DIA but not DDA (Figure S13). Meanwhile, we realized it's not a rigorous comparison only between two MS acquisition methods. However, this information is helpful for researchers who just want to apply one established workflow with user friendly computational tools, and thus a more rigorous comparison between the two MS methods for identifying glycans is illustrated in Figure 2F with spiked-in standards. We set up an experiment with isotope-labeled standards spiked into a glycan mixture as another way to evaluate DDA and GlycanDIA. As shown in Figure 2F, 60% (6 out of 10) of DDA injections had at least one missed identification of spiked glycan, while all ten DIA injections were able to detect all three glycan standards. We have added more information to better describe these points in our revision (Page 9).

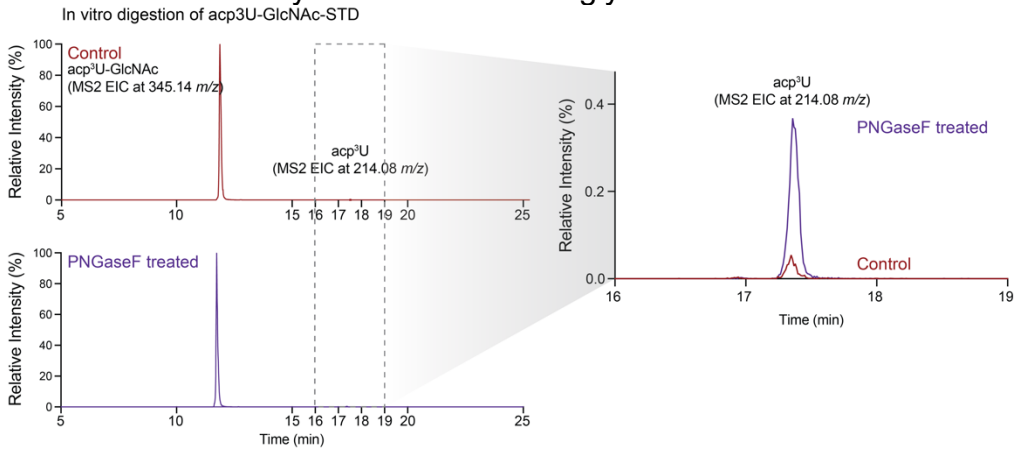
The inclusion of the long section on glycoRNAs in this methods paper has questionable value in terms of establishing the quantitative accuracy of the method. First, the abundance distributions of RNA glycoforms in various sources have not yet been determined by any established reference method. It may be argued that this is due to the very low levels at which such compounds are present. The method presented here is shown to have sufficient sensitivity to detect the species of interest, and that will contribute to further studies of this emerging area. However, the assignments of specific structures and the quantitative accuracy cannot be verified without independent determinations or at least the synthesis and analysis of reference standards.

Response: We thank the reviewer for the comment. The glycan profile from glycoRNA was annotated based on the glycan information we obtained from glycoproteins, using retention time information in PGC and fragmentation patterns as references. The quantification values are relative, calculated by dividing each glycan's abundance by the total abundance. We have added this information to our revised manuscript for clarification (Page 14). We agree with the reviewer that the assignments of specific structures and quantitative accuracy would be better addressed using synthetic standards. As the biosynthetic pathway of glycoRNA is further elucidated in the future, we expect to build a reference glycan library for glycoRNA molecules. This will enable more accurate

and reliable analysis of glycoRNA. As the value of GlycanDIA in identifying and quantifying low abundance glycans, thus can be a good pioneer method for this new field of glycoRNAs.

It remains very curious that the glycosyltransferases which add N-glycans to nascent proteins and build up complex glycans and the glycosidases which selectively remove all or parts of N- or O-glycans from glycoproteins have high specificity for their actions, yet this and related works postulate that their activities are compatible with the presence of an RNA substrate instead of the well-defined protein structural elements. Since the authors have shown that they are able to generate synthetic nucleosides bearing N-linked glycans, they are strongly encouraged to demonstrate that PNGase F and the additional glycosidases Endo F2 and Endo F3 used in Ref 3 can release the N-glycans from their synthetic nucleosides or well-defined RNA segments containing the modified bases. Labelling with 18O during release is necessary but not sufficient proof, since it has not yet been shown that there is no 18O exchange on unmodified bases under the experimental conditions used for release of the N-linked glycans. (In Ref. 3, the authors do show evidence for retention of the inner HexNAc during Endo F2 and Endo F3 treatment.)

Response: We thank the reviewer for their comment. To confirm the direct reaction of PNGaseF on N-glycoRNA, we previously conducted an experiment to evaluate the enzymatic activity of PNGaseF towards glycoRNA in vitro (Xie et al, Cell. 187, 19, 2024). Specifically, we incubated acp3U-GlcNAc-STD in buffer with PNGaseF and compared the results to a control reaction with buffer only. Our findings demonstrated a significant release of acp3U in the PNGaseF-treated sample compared to the control, providing evidence for the direct activity of PNGaseF on N-glycoRNA.



The presence of a trace level of protein cannot be excluded on the basis of a “clean” channel on an SDS-PAGE gel, such as that shown in Fig. S20A. If there is a very low level of protein (<< 2%) in the recovered RNA, it would not be detected on the gel with a Coomassie stain, if that is what was used. There is no information on either the stain or the amount of sample loaded on the gel.

Response: We thank the reviewer for the comment. In our revision, we added proteomic analysis of extracted RNA and found that only common proteins known as contaminants (such as keratins) were detected, with no glycoproteins identified (Supplementary Data

9). We revised the sentence to read: "... from the SDS-PAGE gel with 5 μg loaded RNA using Coomassie staining. We further performed proteomic analysis, and only detected common contaminant peptides corresponding proteins, such as keratins, and no glycoproteins or RNA-binding proteins (RBPs) were identified, which suggests the high specificity of our glycoRNA preparation."

Should there be a low level of protein associated with the RNA, it would seem likely that the binding is specific and it is a single protein, or a set of closely related proteins bearing a specific amino acid sequence or glycan epitope whose glycoform distribution(s) are not accurately represented by determining the average composition of the whole glycan pool from the cell extract.

Response: We thank the reviewer for the comment. We agree that RNA is often associated with RNA-binding proteins (RBPs). To address this, our workflow for preparing RNA samples includes treatment with proteases and mucinases to remove molecules that may bind to RNAs. Additionally, in the glycan release step, we heated the RNA sample to 100°C for three minutes prior to PNGase F treatment, which should be sufficient to dissociate any interactions between peptides and glycan epitopes. In our revision, we performed proteomic analysis on glycoRNA samples and only detected common contaminant peptides/proteins (e.g., keratins, Supplementary Data 9), confirming the specificity of our preparation protocol. Overall, our RNA sample preparation workflow is specifically designed to minimize the impact of contaminants that may bias the deciphering of glycan profiles on RNAs.

In Fig 4 (and elsewhere), splitting glycans into the categories of Neutral, Fucosylated, High Mannose and Sialylated, Sialofucosylated, and Sialylated-NeuGc seems artificial and the results broken down this way could be ambiguous or so insufficiently defined as to be biologically irrelevant. What is here termed “neutral” would include both hybrid and complex glycans. Fucosylated glycans that lack sialic acid are also neutral. Furthermore, fucose can be located on the core and/or antenna(e), and some glycans could contain both NeuGc and Fuc.

Response: We thank the reviewer for the comment. We have refined our classification of glycan subtypes and have information about different subtypes in our method section to provide better clarity (Page 22).

Despite this reviewer’s objection given above to using the glycoform distribution for the protein pool, to be consistent, Section C in Figure 4 should also show the glycoform distributions found during the protein analysis.

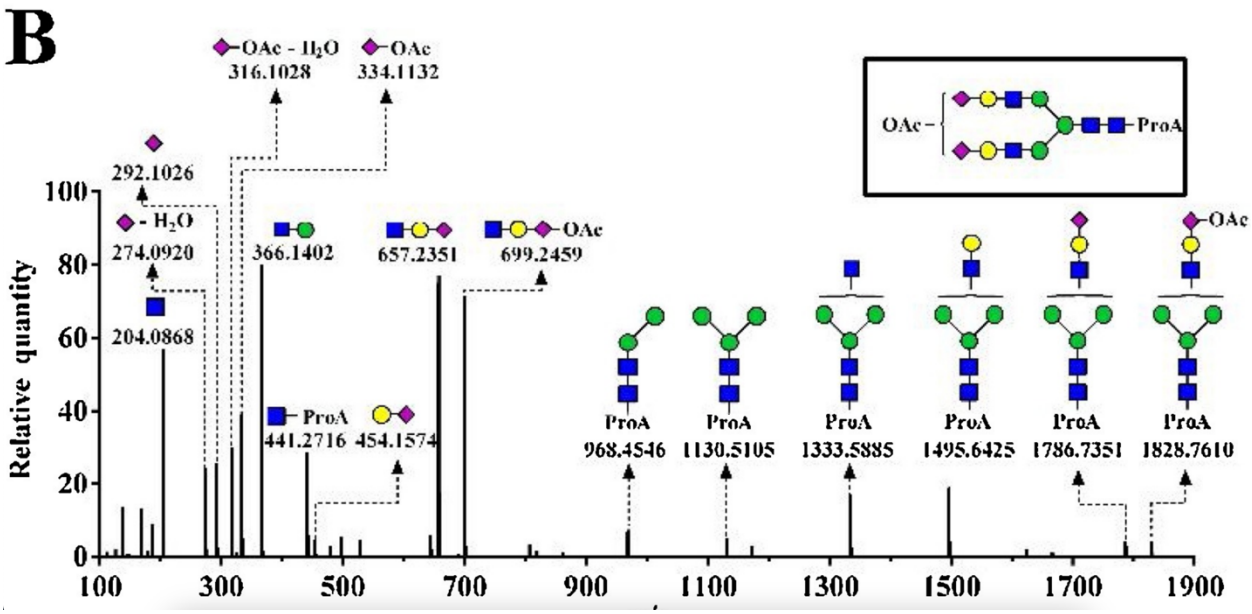
Response: We thank the reviewer for the comment. The glycomic analysis of proteins from mouse tissue has been previously reported by Otaki et al. (Nat. Commun. 15, 9725, 2024; Sci. Rep., 12, 17804, 2022). We have highlighted the findings in our description to provide comparison with our RNA results.

Other Comments on the Supplementary Information.

In their interpretation of the data shown in Figure S18, the authors calculate the relative abundances of Neu5Ac and an acetylated form on the basis of the peak heights of the oxonium ions characteristic of these two species. (p.11, l.13) In fact, the peak assigned

to the acetylated species (m/z 334) would be the C-type fragment, not the (B-type) oxonium ion. However, it is likely that the acetylated fragment at m/z 699 easily eliminates ketene (CH2=C=O) to generates the peak at m/z 657 and the NeuAc oxonium ion signal similarly originates via decomposition of the fragment at m/z 334, in a one- or two-step elimination of acetic acid or ketene and water, respectively. Reference standards containing acetylated forms of sialic acid need to be analyzed under the selected conditions in order to determine the typical yields of each type of oxonium ion and the m/z 657 and m/z 699 species. In any case, it does not seem safe to estimate the relative amounts of the two glycoforms when one of the candidate species is so prone to eliminate the modification which differentiates them.

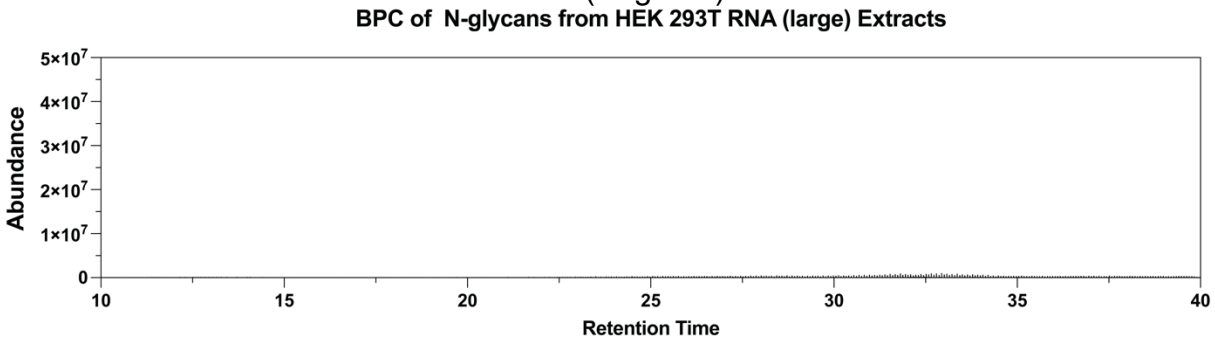
Response: We thank the reviewer for the question. The tandem MS/MS spectrum of the acetylated sialyloglycans have been reported by Park et al. (shown in bottom, J. Pharm. Biomed. Anal. 169, 2019), in which they investigated ProA-labeled acetylated N-glycans using HCD and positive mode, yielding comparable results to ours. Notably, their findings revealed that the m/z 334, corresponding to [Neu5Ac_OAc]+, and m/z 699, [GalGlcNAcNeu5Ac_OAc]+, cannot generate m/z 657 through the mechanism proposed by the reviewer. Furthermore, our examination of the alignment between precursor and product ions only revealed alignment for m/z 699, suggesting the specific generation of diagnostic ions from acetylated glycan species. This observation highlights the applicability of GlycanDIA for analyzing modified glycans.

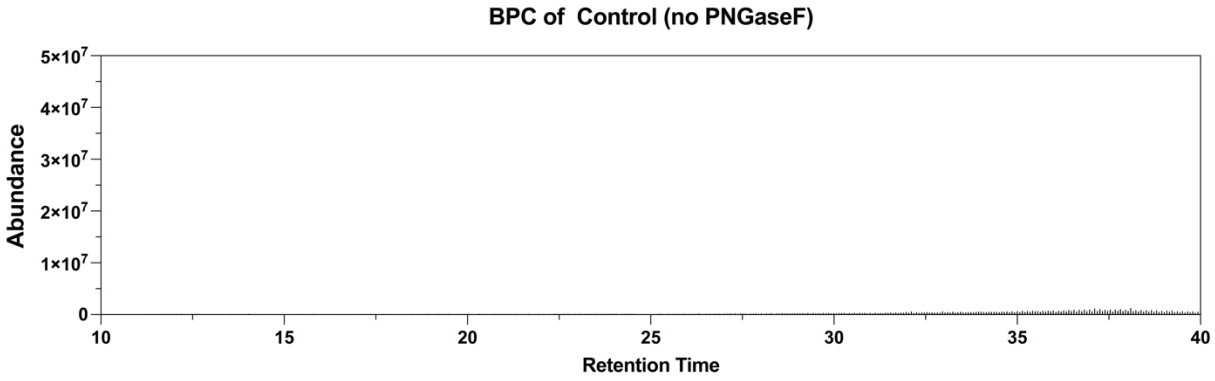


The authors present Figure S21 to show the release of only trace amounts of N-linked glycans from the high MW RNA but do not provide an equivalent figure for release from the low MW RNA which is more pertinent to the discussion, since they assert that only the low MW RNA has N-glycosylation at a level that could be biologically important. The data presented in this manuscript do not provide an estimate of the frequency of occurrence of glycoRNA species in the examined samples.

Response: We thank the reviewer for the comment. The glycan signal from low MW RNA was shown in Figure 3A (green). To address the reviewer's point, we adjusted the absolute signals of the large MW RNA and control samples to the low MW RNA sample.

As the figure we show below, the result revealed that the glycan signal in large MW RNA at the background level (control sample), which is consistent with previous observations. With this example data, we also would like to emphasize the advantageous feature of GlycanDIA, in which we can estimate the level of glycosylation and sialylation, etc., using the diagnostic signals (similar work has been reported by Sutherland et al., J. Proteome Res. 2024, 23, 12, 5606, recently for glycoproteomic analysis). The amount of glycosylated RNA has been estimated in the previous glycoRNA publication, where approximately 20 pmol of glycans per μg of total RNA was determined, and we have included this information in our main text (Page 13).





Abstract, l. 20: ….and there are also…
Response: Corrected.

Introduction
line 1. : Proteins have post-translational modifications, but lipids are not translated from a template so modifications to their initial structures are not PTMs.
Response: We thank the reviewer for the comment. We corrected our statement.

line 20: Neither labelling nor derivatization decreases sample complexity. Perhaps the authors meant glycosidase treatment rather than derivatization, but then they should acknowledge that this step results in a loss of biologically relevant structural information, e.g., locations and linkages of sialic acid residues. Labelling, e.g., by 18O, or introduction of a chromophore or other blocking group at the reducing end, can help to differentiate fragments that include the non-reducing end from those that retain the reducing end, but again, although they may assist in assignment of fragment ion peaks in the MS2 spectra, these do not reduce sample complexity.

Response: We would like to thank the reviewer for pointing out the confusion about our statement. Our intention was to highlight that the reducing end labeling and derivatization method can generate potential problems, including sample loss and preventing the separation of glycan isomers in the chromatograph. We have revised our statements about this point (Page 3).

p.4.l.6. The sentence starting on line 5 needs to be rewritten.
p.4.l.27. “amenable” is not an appropriate adjective in this context.
p.5, l.1-2 and lines 3-4: ….the signals corresponding to the sequence-defining fragments….
p.5, l. 22: ..of the peaks for most glycans eluting from the PGC column…
P.5, l.27 and elsewhere: “decent” reflects an opinion or value judgement; it is not a scientific term
p.6: here and elsewhere, all signals assigned to specific species should be described with their charge state as well as the observed m/z value
p.6, l. 14 (and elsewhere) peaks corresponding to (not “standing for”)
p.6.: complementary and synchronized information [at the same time] – (redundant).
p.7, l.2: using set parameters
p.7., l.5: ..the glycan peaks can be assigned by…
p.7., l.16: efficiency (not efficacy)
and many more….
Figure 2 caption: glycans of interest, not “interested glycans”
Response: We thank the reviewer for their careful review of our manuscript. We have corrected the corresponding errors and appreciate the help in ensuring the accuracy of our work.

Figure S11. The x-axis range on the three spectra should be kept consistent. Why are Seq Int and Cov Int only reported for DIA analysis?
Response: We thank the reviewer for the question. We adjusted the figures x-axis range and included the Seq Int and Cov Int information for DDA spectrum (Figure S21).

Figure S13. What was the starting concentration for each glycan? Only the sample volumes are indicated on the plots.
Response: We thank the reviewer for the comments. The glycans were released from 1mg protein standard concentration, we included this information in our method (Page 20).

Figure S15. According to the methods section, the released O-glycans were not reduced. The structures of assigned to several peaks are ambiguous. All assignments should be verified.
Response: We thank the reviewer for the comment. In our O-glycan analysis, we employed beta-elimination with sodium hydroxide to release the glycans, followed by reduction with sodium borohydride to prevent potential peeling reactions from the reducing end. To provide further clarification, we have added more details to the methods section. Additionally, we have annotated the glycan peaks based on previously identified O-glycans from library, incorporating more structural assignment of the observed glycans.

Figure S17. Part B. Compositions only should be indicated for each m/z value and these should be aligned with the values. The fragment ion structures shown at the bottom are by no means the most likely to occur. The cited paper made the assignments shown in Part A but is not the source for the fragment ion assignments; the figure in that paper which indicates nomenclature for fragments generated at various cleavage sites allows but gives no preference to these structures.
Response: We thank the reviewer for the suggestion. We now listed the compositions corresponding to the observed m/z values, rather than assigning specific structures.

Figure S25 caption should be: Neu5Ac and Neu5Gc were present at different levels among the mouse tissues. The monosaccharide oxonium ions are marked in colors and indicate the presence of these sugars in the precursor structure: HexNAc containing (red, 204.08 (not 292.10) m/z), Neu5Ac-containing (not contaning) glycans (green, 292.10 (not 204.08) m/z), and Neu5Gc- containing (not contaning) glycans (blue, 308.09 m/z).
Response: We thank the reviewer for the detailed problems. We corrected these errors.

The authors should consider using the relevant Symbol Nomenclature for Glycans (SNFG) colors for the oxonium ion labels, and for the cartoons and charts, to the extent feasible.
Response: We would like to thank the reviewer for their comment. We generated the glycan structure and labels using GlycoWorkbench which follows the SNFG color scheme (Table S4).

Furthermore, the abstract graph and the larger cartoon are misleading since the accepted letter codes for glycan residues do not correspond to the letters above them and the plot included in the figure mimics a mass spectrum but thereby suggests that isomeric residues have different m/z values.
Response: We thank the reviewer for the comment. We adjusted the abstract graph to remove the potential misleading information.

Reviewer 3:

In this manuscript, the authors proposed a DIA-based glycomic workflow to identify and quantify released glycans. The workflow GlycanDIA is claimed to provide improved sensitivity in identification and quantification by utilizing the emerging modern data-independent acquisition (DIA). The manuscript contains many experiments to evaluate the effectiveness of the proposed workflow, though some areas require additional details. Major revisions are needed to 1) illustrate the details to prove its novelties when comparing with DDA approach, to 2) describe more precisely when the author claimed that GlycanDIA can “determine” the isomers, and to 3) provide more comparison to proof the effectiveness of FDR control.

Response to general comments: We thank the reviewer for taking the time and effort to review our manuscript. We really appreciate the valuable comments and suggestions and have modified our manuscript accordingly.

Our GlycanDIA analysis was established using glycans released from standard source proteins (RNase B and human serum), which have been fully characterized in our previous studies. We injected the same amount of N-glycan samples, allowing us to directly compare the performance of the previously established method with GlycanDIA. We showed an example of a glycan that was detected by GlycanDIA but not by DDA. We also examined the details of Man8, showcasing the ability to distinguish between different isomers and anomers. Furthermore, we evaluated the performance of different ions and demonstrated that featured glycan fragments can be used to quantify glycan isomers and anomers with high accuracy. These results again demonstrated the performance of GlycanDIA in glycomic analysis.

For the data analysis, we presented more detailed results and discussions about the use of random mass shift decoy, the current popular approach among glycomic and glycoproteomic software developers. As shown in Figure S12, this decoy method demonstrated robust performance in specificity, sensitivity, FDR, and F1. In addition, we incorporated glycan molecule feature examination, which detects glycan isotope peaks, to improve the accuracy of our analysis. We rigorously examined the software's identification performance by systematically cross-referencing all glycans identified by the software against raw MS data, thereby establishing high-confidence validation as detailed in Supplementary Data 7. Besides, we introduced the new tool, Glycan Product Generator, which calculates possible glycan fragmentations based on the input structure and outputs the glycans in a format that can be directly imported into GlycanDIA Finder search and further Skyline evaluation. We included established common glycan libraries to streamline the high-confidence identification and quantification process in GlycanDIA data analysis.

The manuscript has been substantially revised according to the reviewers' comments and suggestions. All the corresponding changes have been highlighted in the marked revised manuscript.

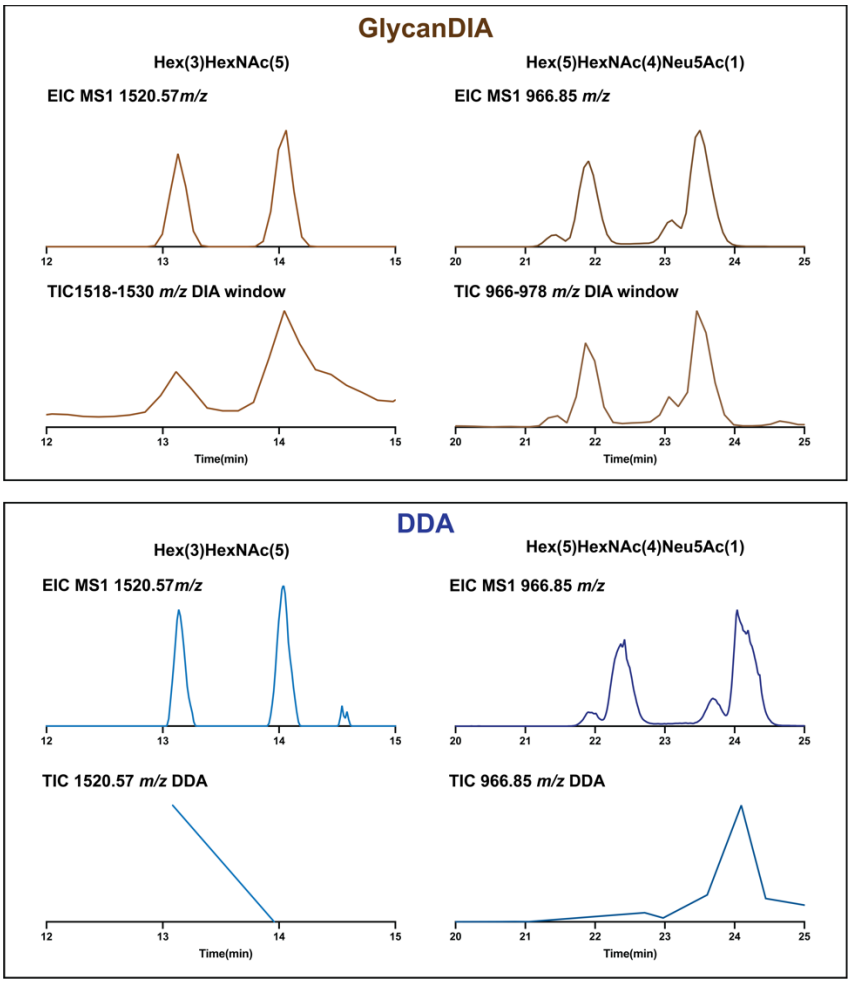
Point-by-point responses:

Major points:

1.When demonstrating the performance of GlycanDIA workflow in glycan identification, the manuscript claimed that DIA mapped 70 more glycans than DDA method. Which DDA software was used to do the comparison? Was the two search space (i.e. glycan database) comparable? It will be necessary to provide concrete examples that the glycans identified

from DIA only are correct and discuss the possible reason why DDA method can't characterize those glycans. The glycan library used is expected to be provided such that the results presented in the manuscript are reproducible.

Response: We thank the reviewer for the valuable question. We analyzed the DDA results using GlycoNote. GlycoNote allows for a flexible search input, such as a range of monosaccharide compositions (e.g., Hex 2-15; HexNAc 2-10; dHex 0-5; Neu5Ac 0-4) for general N-glycan analysis. In contrast, our GlycanDIA Finder software requires specific information on glycan composition and theoretical or experimental fragments for identification. This more focused approach enables more accurate and confident glycan identification.

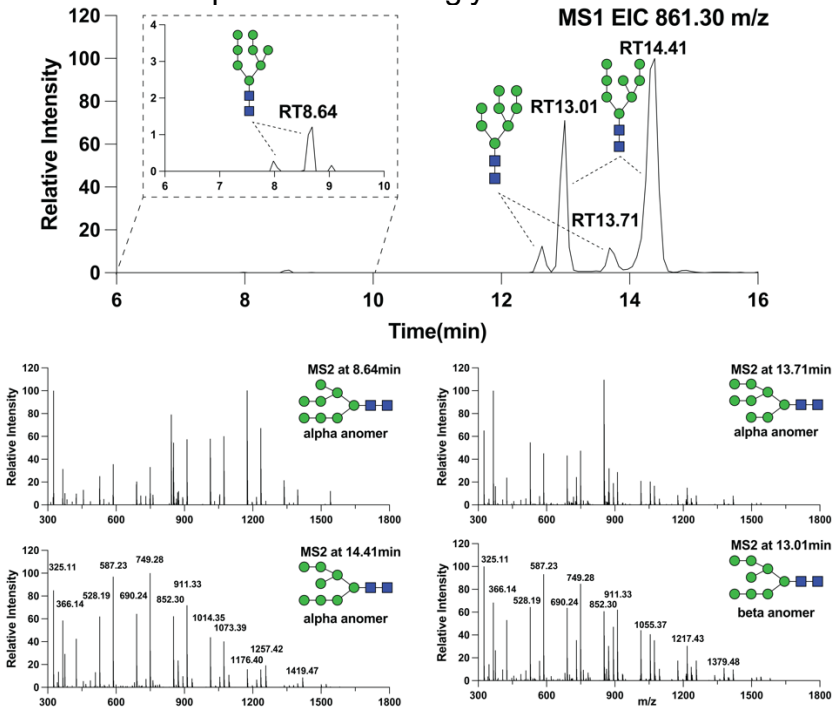


We agree that showcasing examples of glycans identified by DIA but not DDA would be beneficial. To this end, we present a new Figure above (Figure S13), where glycans isomers detected by DIA but missed by DDA due to the lack of MS2 triggering for the precursor. In contrast, the pre-set mass window from DIA allows for the generation of MS2 information, enabling glycan identification. This example highlights the advantage of DIA in detecting glycans that may be missed during DDA. We have included the results and discussion of this example in our revised manuscript (Page 8).

For the glycan library, we have introduced an additional function to our software that enables researchers to generate a theoretical library and format it for input into Skyline and GlycanDIA Finder for further application. We have also provided the common library

used for N-glycan and O-glycan from previous pGlyco publications in our revision (Nat. Commun. 8, 438, 2017). These features aim to make GlycanDIA more accessible to a broader range of researchers, including those without extensive experience in glycomics. By providing an automated library generation, we hope to empower researchers to explore the complex world of glycomics with confidence. Corresponding information was added to our revised manuscript.

2.The authors claimed in the manuscript that GlycanDIA can “determine” isomers (including linkage and composition) and modified glycans. However, since the GlycanDIA Finder only takes account for the composition information in the glycan library, it is hard to identify the glycan isomers under such circumstance. Based on my understanding of the examples and results shared on github, GlycanDIA just provided the possible glycan isomers or modified glycans, which still requires experts to do manual checks in order to determine the structures. Substantial justifications are required to solid prove that GlycanDIA is able to identify glycan isomers.
Latest Glycan identification software tools (e.g. PEAKS GlycanFinder https://doi.org/10.1038/s41467-023-39699-5) designed for DDA data can distinguish the structure difference by utilizing the signature ions explained in the Figure S16. For example, the existence of signature ion 512.20, 308.09 m/z can be leveraged in a scoring function to suggest the best matched glycan isomer for each spectrum.
Response: We thank the reviewer for the valuable comment. The previous publication reported the identification of glycan isomers and anomers using the fragmentation pattern generated by low-energy CID, and we also obtained such information from our GlycanDIA method. As a result, we were able to distinguish the six Man8 isomers and anomers based on their different fragmentation patterns. Importantly, different Man8 glycans can be quantified using their product ions with high linearity (R2 > 0.99). This indicates that our method can provide reliable quantification for glycan isomers.



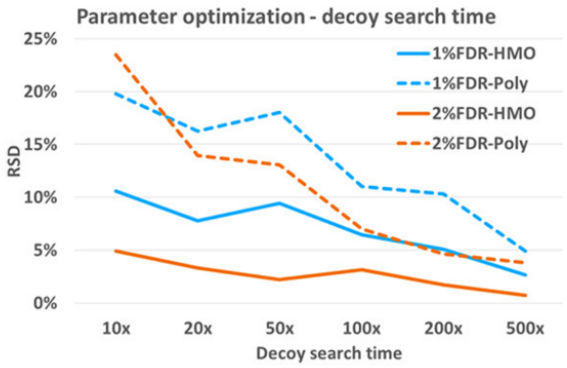
We appreciate the reviewer's suggestion to incorporate a scoring function, which would indeed enhance the ability to distinguish glycan isomers using the software. However, the complexity of glycan structures, including high mannose, fucosylated, and sialylated types, generates intricate information that requires consideration of multiple composite factors. This complexity makes the development of a comprehensive scoring function a nontrivial task at this stage. Our GlycanDIA software allows for customization of the library and glycan fragments to be monitored, enabling users to define diagnostic ions when necessary. Nevertheless, we recognize the importance of this feature and plan to address it in future improvements of GlycanDIA Finder. The creation of a reference library using synthetic standards will be fundamental in achieving this goal, and we will prioritize this aspect in our ongoing efforts to enhance the software.

3.In the manuscript, the authors state that the decoy library is generated by randomly mass shifting. It is a widely used method for glycan FDR control in glycoproteomics. However, it is not a proper decoy as it underestimates the rate of false matches to glycans with shared fragment ions. The manuscript is expected to note this general limitation of this glycan FDR method.

It is said that “100 decoy sets were generated for each aligned tandem MS”. It seems the search space of target and decoy are quite different. Then how is the FDR calculation impacted? If the decoy library is much larger than glycan library, the rate of random matches towards decoy library will be biased.

Usually, 1% FDR is widely used as the cutoff when reporting. Why GlycanDIA choose 2% FDR? When comparing the sensitively with DDA, what was the FDR cutoff for each method/software tool?

Response: We thank the reviewer for the comment, and we have added more information about the limitation of random mass shifting method in our text (Page 18). In our implementation, we ensure that the decoy library is constructed to match the complexity of the target glycan library in terms of size and fragmentation characteristics. Each decoy is generated with random mass shifts while preserving the general distribution of intensity and sequence coverage, as shown in Figure S11A. This approach minimizes bias by ensuring that random matches are distributed comparably between the target and decoy libraries. Although the decoy library is larger in terms of the number of decoy sets (100 sets per aligned tandem MS), the iterative decoy searching process averages the distributions, reducing the potential for an inflated rate of random matches. The FDR is calculated using these averaged distributions, ensuring a rigorous estimation of false positives while maintaining sensitivity and specificity. To further address the concern, the robustness of our FDR calculation is validated by comparing the score distributions of target and decoy searches (Supplementary Figure S11B). The distinct separation between high-quality target identifications and low-quality decoy matches demonstrates that the method effectively controls the rate of random matches, even with a larger decoy library. At the same time, because of the large decoy set, we find that 2% FDR is optimal for the search in terms of the balance between searching time and identification accuracy. More details about this information can be found in our previous GlycoNote software. In our revised manuscript, we have added the functionality for users to define the FDR for the search, and we have provided more information in the corresponding context.



Minor points:

1.In the introduction of the manuscript, the authors stated that "the application of DIA for glycomic analysis has not been established". There were several attempts developed to do identification and quantification of DIA data although they mainly focused on intact glycan instead of released glycan. It is suggested to mention those work in the manuscript.
GproDIA: https://doi.org/10.1038/s41467-021-26246-3
Glyco-DIA: https://doi.org/10.1038/s41592-019-0504-x
Data-Independent Acquisition-Based Mass Spectrometry (DIA-MS) for Quantitative Analysis of Intact N-Linked Glycopeptides: https://doi.org/10.1021/acs.analchem.1c01659

Response: We thank the reviewer for the suggestion. We have added the relevant references and expanded the introduction to provide more context and background information on these methods (Page 4).

2.The annotation for glycans are not consistent in the manuscript. In Table S3, it is said that "the annotation for glycans in this manuscript is as follows: Hex_HexNAc_Fuc_Neu5Ac_Neu5Gc". However, Hex(5)HexNAc(4)Sia(1) was used in several annotations, such as in Figure S3, S18. It is better to keep all the annotation consistent.

Response: We appreciate the reviewer for carefully checking the details. The glycan composition format has been kept consistent.

3.In Figure S16(B) spectrum annotation, the peak 512.20 m/z should not exist in the spectrum if the proposed structure is the core fucose structure.

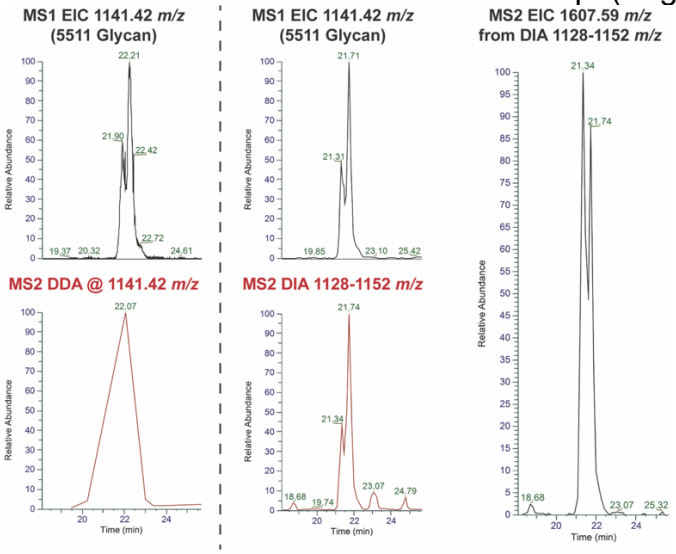
Response: We thank the reviewer for their careful attention to detail. We have removed the peak label in our revision.

4.The glycan library used in GlycanDIA Finder only contains glycan composition and possible B/Y fragments. Can you discuss the limitation of such library used during search. What is the difficulty of adding ion intensity and iRT in the library and why they are not considered in the library search?

Response: We thank the reviewer for the suggestion. We added more discussion about these points in Page 17.

5. In GlycanDIA workflow, the differentiation of isomers highly depend on the RT difference. What if the RTs for different isomers are very close, how can we distinguish the isomers in this situation?

Response: We thank the reviewer for the question. Indeed, the differentiation of isomers relies on the resolution of LC, and when the RTs for different isomers are very close, distinguishing between isomers requires the diagnostic ions for identification. Notably, this is also an issue for common DDA glycomics. In the example provided (Figure S22), the anomeric peak of the glycan Hex(5)HexNAc(5)Fuc(1)Neu5Ac(1) was not fully resolved by PGC. In DDA analysis (left), one MS2 event was triggered between the two peaks, resulting in ambiguous identification. However, with GlycanDIA (right), the MS2 fragmentation was triggered across the two peaks, capturing valuable diagnostic information (such as 1607.59 m/z) that generate different peak distributions and enable differentiation between the two glycan anomers. In addition, the incorporation of ion mobility as an additional dimension of separation is expected to efficiently reduce co-elution, thereby enhancing the accuracy and reliability of glycan isomer identification. The corresponding discussion is included in our revised manuscript (Page 12).



6.The authors conducted several experiments to find the optimal HCD fragmentation energy and selected 20% NCE in the final state. However, from the Figure S2-3, it looks like NCE @ 15% generated more abundant fragments than NCE @ 20%. Why NCE @ 15% is not chose?

Response: We thank the reviewer for the question. We selected NCE at 20% in order to strike a balance between generating large specific fragments while producing glycan fragments with high efficiency for identification and quantification (Page 5).

7.There is a typo of glycan in the table title of Figure S11. It is expected to be Hex(5)HexNAc(5)Neu5Ac(1) instead of Hex(5)HexNAc(5)Fuc(1).

Response: Corrected.

The README file includes detail instructions that for running the code, and an example dataset with a library were provided. The code at first had an exception after running but easy to fix.

Since I didn't find the glycan library used in the manuscript, I think the results of the paper are not easy to reproduce. It is expected to include these libraries in the dataset.

Response: We thank the reviewer for their comment. The code only had an issue with Windows systems, and we have corrected the code and tested it on both Mac OS and Windows systems.

Reviewer #1 (Remarks to the Author):

I would like to commend the authors for their great effort in revising the manuscript. They have effectively addressed most of my previous concerns. Given the significance and potential broad impact of the DIA glycomics workflow in the field, I would like to recommend for its publication in Nature Communications.

Response to general comments: We thank the reviewer for the thorough re-evaluation of our manuscript and for recognizing the improvements made during the revision process. We greatly appreciate the reviewer's positive assessment of the significance of GlycanDIA and its potential impact on the field.

Reviewer #3 (Remarks to the Author):

The authors have addressed most of the previous comments and provided additional details and examples to demonstrate how GlycanDIA resolves co-elution issues. These revisions have greatly improved the manuscript. However, several points still require further clarification:

Response to general comments: We are deeply grateful to the reviewer for their careful re-evaluation of our manuscript and for acknowledging the enhancements made during revision. Furthermore, we have made additional changes based on the reviewer's suggestions.

Point-by-point responses:

1.The introduction states that GlycanDIA retains the ability to discriminate different glycan isomers (including composition and linkage isomers). The differentiation based on composition or distinct fragment ions is straightforward using diagnostic ions, which can be achieved in DDA. However, for isomers that yield the same B/Y fragments—such as the Man8 isomers—if cross-ring cleavages are not employed, how are the structural differences resolved in MS2?

In Figure S20, please clarify what specific fragment patterns are being used and how they enable the differentiation among Man8 structures.

It may be more precise to state that GlycanDIA provides information that aids in differentiation rather than directly determining glycan isomer structures.

Response: We thank the reviewer for the comment. We can distinguish different isomers, such as the Man8 example, based on their characteristic B/Y fragmentation patterns, as demonstrated by She et al. (DOI: 10.1021/acs.analchem.0c02951). We agree that it is more accurate to state that GlycanDIA provides information that helps the differentiation instead of the directly determination. We have revised our statement accordingly and included more details in Figure S20 to illustrate the distinguishing features of the Man8 isomers (Page 12).

2.Figure S11B effectively illustrates the effectiveness of the FDR control, but it is necessary to specify which dataset this figure is derived from. Additionally, does the O-linked glycan dataset exhibit similar FDR performance with the same decoy method? A comparison would be helpful.

Response: The FDR was derived from Supplementary Data 6, and this information has now been updated in Figure S11. Given the greater complexity of the N-glycan mixture, we evaluated the FDR specifically for N-glycans, but not for O-glycans.

3.In response to previous reviewer comments on FDR cutoff selection, the authors referenced a figure from GlycoNote, which is based on DDA data. However, since GlycoNote uses DDA while this study focuses on DIA, it is not reasonable to assume identical performance, even if the same decoy generation method is applied. It would be more appropriate to present evidence directly from the research data in this manuscript rather than relying on external studies.

Response: We thank the reviewer for the comment. We agree that it is important to select the decoy cutoff based on the DIA data instead of the DDA dataset. We would like to clarify that we realized the complication of DIA data, and therefore, to balance between searching space and false-positive identification, we initially referred to the 2% FDR cutoff based on Glyconote parameters from DDA runs. To further test the FDR at 2%, we evaluated decoy mode performance in GlycanDIA Finder. As a result, high Con_Int and Con_Seq confirm the decoy mode's ability to minimize false discoveries and ensure robust glycan identification (as shown in Figure S12), and thereby, we decided to choose 2% FDR for GlycanDIA search.

4.In response to Reviewer 3 Question 5, the authors stated that "the incorporation of ion mobility as an additional dimension of separation is expected to efficiently reduce coelution, thereby enhancing the accuracy and reliability of glycan isomer identification. The corresponding discussion is included in our revised manuscript (Page 12)." However, this discussion is not found on Page 12 of the revised manuscript as indicated. Please clarify whether this section was inadvertently omitted.

Response: We thank the reviewer for carefully checking the text. We unintentionally omitted that sentence during text editing and have now included it in the revised manuscript (Page 12).

5.The workflow in Figure S11A appears to be missing the input glycan library component.

Response: We thank the reviewer for carefully checking the details. We have included the glycan library information in Figure 11A.

6.The manuscript states that larger fragments are preferable for quantification but does not specify the fragment selection for quantification. Are the top N fragments chosen, or are all fragments included?

Response: We thank the reviewer for the comment. We chose top 5 fragments for the quantification, this information has been included in our revised manuscript (Page 22).

7.In the data analysis section, NeuGc is not listed in the GlycoNote settings. Please clarify if it should be included.

Response: We thank the reviewer for the comment. Neu5Gc and potential other glycan component (such as reduction, acetylation, sulfation, and phosphorylation) were searched using the "add on mass" feature when necessary, this information has been included in our revised manuscript (Page 22).

8.Additional details are needed to clarify Figure S12. Please provide a more comprehensive explanation of each subfigure.

Response: We thank the reviewer for the suggestion. We have included the following

details for Figure S12: Robustness of Con_Int and Con_Seq scoring metrics across dilution series in GlycanDIA Finder. The analysis was conducted across three dilution levels (10×, 100×, and 1000×) and evaluated using four key performance metrics: A. False Positive Rate, B. Specificity, C. Sensitivity, and D. F1 Score. Con_Int reflects the agreement between observed and theoretical fragment ion intensities, while Con_Seq measures sequence-level alignment. Panel A shows that the false positive rate (FPR) decreases progressively with dilution, dropping below 0.3 at 100× and approaching zero at 1000×, indicating improved discrimination under lower signal conditions. Panel B highlights a substantial increase in specificity, reaching ~0.9 at 1000× dilution. Panel C shows that sensitivity remains high at all dilutions but gradually declines at higher dilutions. Panel D demonstrates that the F1 score remains consistently high at 10× and 100× dilutions, with a modest decline at 1000×. These results demonstrate that both Con_Int and Con_Seq maintain robust performance under dilution and that the 100× dilution condition offers an optimal trade-off between specificity and sensitivity.

9.The title of Figure S4(B) mentions B/Y ions, while the caption refers to BCYZ ions. Please clarify the intended description to ensure consistency.

Response: We thank the reviewer for the comment. We have corrected the information to “B and Y ions”.

10.The MS2 spectra at 8.64 min and 13.71 min in Figure S20 do not have peak annotations.

Response: We thank the reviewer for pointing out the missing information. We have added the annotation and updated Figure S20.

11.The Methods section states that detailed MS setup parameters are available in Table S5. However, Table S5 contains precursor and product ions instead. Please update the reference.

Response: Corrected.

Reviewer #3 (Remarks on code availability): The provided code includes a clear README file with installation and execution instructions. It also includes example data and glyco libraries from previous publications, which are beneficial for users to get started.

Response: We thank the reviewer for acknowledging the README, example data, and glycan libraries for using GlycanDIA Finder, which we hope will be helpful for general users.

Reviewer #4 (Remarks to the Author):

This manuscript has been very thoroughly evaluated by three competent reviewers and the revised version is therefore in a much better state than the original. The approach in itself deserves to be presented and made open to discussion but it should really be made clear that a lot of room for improvement remains.

Response to general comments: We are deeply grateful to the reviewer for the careful evaluation of our manuscript and previous responses. We particularly appreciate the positive comments of GlycanDIA's significance and potential broad impact on glycomics. We concur that significant room for future development remains, especially regarding

glycan library construction, library-free based search, ion mobility incorporation, and other advancements, as we discussed in our main text.

Point-by-point responses:

Overall, the authors have addressed most of the reviewers' concerns and I would like to raise only a few remaining points: 1) The authors put forward that they are first to include DIA in glycomics while noting (only after being told) that the technique is more commonly applied in glycoproteomics (one more reference is missing, Ben Schulz introduced DIA prior to all cited references). Given that glycans are attached, it seems somehow more attractive to find out not only which glycans are present in what amount but also where they are attached. Hence the trend. Of course for free glycans, the method is compelling. Some aspects are still somehow toned down in the response and the revised version and the point is not to demonstrate that the authors are the first to introduce DIA in glycomics but to show that glycanDIA can yield finer analyses of glycans than DDA approaches. In particular, the notorious topic of co-elution and the challenge of isomer differentiation cannot be fully solved (raised by reviewers and slightly too assertively addressed). DIA only helps. And it is rather disappointing that the authors gave up on the variable window option

Response: We thank the reviewer for the detailed feedback on our revised manuscript. Our initial motivation to apply DIA for glycomics stemmed from development of DIA-based glycoproteomics. However, in our first manuscript, we considered the current glycoproteomics DIA workflow as similar to proteomics with added glycan information, and therefore intentionally excluded this context in our initial manuscript to focus on the novelty of applying DIA for glycomics. We added this information in our previous revision and have also included Ben Schulz's manuscript in our latest references.

We agree that it is more important to highlight the advantages of GlycanDIA, especially in the identification and quantification of low-abundant glycans, particularly for glycosylated RNAs. We would also like to clarify that isomer differentiation is a challenge not unique to DIA. Our example aimed to illustrate that while DDA can sometimes generate ambiguous results, DIA can provide helpful information for distinguishing isomers. As the reviewer mentioned, and we also agree, further work is needed to improve the DIA workflow for glycomic analysis. Ongoing efforts in our current work include adapting variable window acquisition (SWATH) on SCIEX instruments and integrating ion mobility with Bruker instruments. The development of these advancements is beyond the scope of this manuscript, but we hope to present more developed workflows across different platforms in the future.

2) The authors are using Thermo instruments but a large number of DIA people use SWATH (I have no interest in either companies). It is surprising that there isn't the slightest comment on other DIA techniques or at least a comment on the technical dependency of the workflow (raised by Reviewer 2 but not picked in the reply). Moreover, if you come later in the piece in applying DIA, then recent improvements offer benefits that are worth mentioning and these are not in the manuscript, only in the response to reviewers. Comments on the use of ion mobility even if only as a prospect would be of interest and belong to the main text.

Response: We thank the reviewer's suggestion. As we mentioned in the point 1, we

agree that GlycanDIA should be employ to other platform, and therefore we are currently working on SWATH on SCIEX instruments and integrating ion mobility with Bruker instruments, and we aim to present more developed workflows on diverse platforms in future publications. In our revision, we have added more descriptions about ion mobility (Page 12), included more discussions about more recent DIA methods (Page 18).

3) The main critical point is the selection of the glycan library. As pointed by the authors, their focus is mainly on N-glycans. Regarding the blood sample analysis, why should ref.27 be the only source? Lots of work was published on serum glycans and glycome comparison in health and disease. It is surprising that a 10 years-old paper is considered as the ultimate. Issues in comparison were also raised by reviewers. Maybe more caution statements should be included.

Response: We thank the reviewer for the comment. We agree it is important to include more description about serum glycan analysis. Meanwhile, we would like to clarify that we used the exact same serum sample as in Ref. 27 (Sigma-Aldrich, S7023), and thereby used it as a “standard” to demonstrate the performance of GlycanDIA. We have clarified this point on page 8: we characterized the N-glycans released from a mixture of well-characterized glycoprotein source (RNase B and human sera) as the standard.

Minor: The conclusion could be improved.

page 18, line 22-23: "reported in cases of cancer, diabetes, and Alzheimer's disease" tends to imply that only these diseases are at stake. A sentence should be added to evoke the possibility of more reports to come on other diseases.

page 19, line 1: "...can also be revealed" is slightly overstating. ", in many cases" would make the statement more accurate.

A few more sentences to highlight the worth of shifting to DIA for deciphering glycosylation would make sense. Likewise with prospects mentioned above.

Response: We thank the reviewer’s suggestions about our conclusions. We have made corresponding changes in our revised text (Page 18).