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Peer Review File

Tissue-specific and sex-biased glycoproteomic landscape of Schistosoma mansoni

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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 manuscript describes the glycoproteomic analysis of the adult worm stage of the human parasite S. mansoni, based on a combination of in-depth mass spectrometry analysis of glycopeptides applying a novel software tool and available transcriptomics data. Hundreds of glycosylated proteins were detected and putative site-specific glycosylation is described on the basis of LC-MS/MS data. Using the available single cell gene expression atlas of S. mansoni the glycoproteomics data is presented and discussed in the context of tissue specific expression and vaccine target potential. The study aims to fill a significant knowledge gap in S. mansoni proteomics and glycomics research, which is the lack of a general overview of protein- and site-specific glycosylation patterns that could be relevant for schistosome biology and parasite-host interactions.

Applying a high-end technological approach to glycoproteomics the study provides a thorough glycoproteomic landscape of S. mansoni worms, beyond the identification of site-specific glycosylation patterns so far reported for only a few individual glycoproteins. Numerous glycosylation sites are identified and characterised, which allow a global and integrated transcriptomics, proteomic and glycomics analysis supported by a multitude of largely insightful figures that different angles of analysis. The authors use this data to address a number of different questions with respect to S. mansoni biology. The mass spectrometry based analysis is complemented with a few additional experiments including inhibition of gene transcription of several gens involved in glycosylation to show that N- and O-glycosylation is essential for healthy worm survival in vitro.

I acknowledge the value and relevance of this integrative approach and the data generated for the field. However, I also have a number of concerns in particular with respect to the limitations of the data at the glycan level and the interpretations presented in the manuscript. Moreover, several of the conclusions presented in the Results and Discussion sections, that highlight the relevance of the findings for vaccine target discovery are not or not sufficiently supported by data in my opinion.

MAIN COMMENTS REGARDING EXPERIMENTAL DATA AND APPROACH

Line 108. The authors state that a comprehensive S. mansoni glycan library is absent. But it is not. Several in-depth glycomics analyses of S. mansoni adult worms have been reported (acknowledged in the Refs), including N-glycans that provide an elaborate library. Because these reports analysed released glycans they did not provide glycoproteomic information (expect for a few glycoproteins). Instead, they have characterized glycan structures by applying glycan-specific sequencing methods including different types of MS fragmentations and linkage-specific exoglycosidase sequencing which provides reliable and complementary evidence for structure assignments that often (and in particular for previously unknown glycans) cannot be based on monosaccharide compositions or MS/MS fragment ions alone. I don't understand why the previously identified library has not been used as input for intact glycopeptide analysis software tools. This could then also have been used to validate the outcomes of the pGlycoNovo tool that in addition identifies so far unreported glycans to be present on specific glycosites. (So far unreported perhaps because of low abundance in total glycan pool, of because they were too small to be detected as released glycans such as for short O-glycans; different methods have different widths and depths). In my opinion this is a critical point in the setup of the study.
Whereas I am largely confident about the idenfication of the glycosites I do have concerns about the glycans. Some glycans, present on one or more glycosylation sites, in one or more proteins, are presented that have not been found before in schistosomes. As mentioned, this is very well possible due to the different approaches applied in different studies. However, several glycan compositions and MS/MS-derived glycan structures are presented that do not seem to make sense from a

biological perspective (‘unusual’ glycan). In particular N-glycosylation pathways in schistosomes and other helminths are relatively conserved, at least for the N-glycan precursor and glycan core formation. Yet some of the glycans presented would deviate from anything so far observed in helminths or eukaryotic organisms in general.
For example, in SFig 11a, the N-glycopeptide spectrum suggests an N5X1 fragment. Which would leave H3N1F2 for the N-glycan core. This is very improbable and would mean that a subset of schistosome N-glycans is built on a novel core type not created by the conserved pathway responsible for all other N-glycans presented.

Also, regarding the presence of sialic acid in some of the presented structures: this remains very controversial and one could argue unlikely. The direct proof provided is very limited. It has been shown before that sialic acids – if present – in schistosome preparations are present on host-derived glycoproteins, as acknowledged by the authors. The MS/MS data now indicated the putative presence of sialylated glycans, specifically on Serpin glycopeptides. The structures suggested to contain sialic acid are again very unusual (and not represented in other glycopeptides). As far as I can judge there are no indications for a sialylation pathway in schistosomes (or any other parasite), and the structures suggested that would contain sialic acid are again very unusual. With a composition of H3N2F1S2X1 (Fig 4B), meaning a Man3 N-glycan core carrying F1S2X1, where would the two sialic acids be linked? The evidence provided in Fig4D is not real evidence I would say. To me the images don’t show overlap of Serpin and sialic acid, but rather that the stained areas touch, and in any case the presence of Serpin and sialic acid in the same cells is no direct evidence for their conjugation.

Finally, with respect to other glycan structures presented there are several biosynthetically very unusual (not logical) structures suggested to occur on specific proteins but not in any other protein, also containing elements and monosaccharide sequences not observed before, such as the left upper structure in Fig 4A, the various bisecting GlcNAc containing glycans in Fig 7d, and the unusual Man 5 glycan in Fig 7d, as examples. Also for many of the other glycans presented in Fig 4 and 7 it is not clear how these structures have been derived from the MS/MS data, even though in these cases they contain elements that have been described before. For instance, how have the Fuc residue locations been determined? From a function and/or immunogenic point of view these positions are critical.
To prove (or suggest) the existence of biosynthetically unexplainable and unusual glycan sequences additional glycan structure analysis experiments would be needed. Or could some of these perhaps be explained by the 1% false discovery rate and are they technological artefacts? Have these experiments been carried out in duplicate to check?
In any case, a better way to present the data could be to just present the compositions (as in SFig 17), in order to avoid showing potentially wrong structure suggestions to a (non-expert) audience.

MAIN COMMENTS REGARDING TEXT/DISCUSSION

Line 71-72. There is no rationale behind this suggestion. Omega-1 glycans are important for the immunomodulatory effect of this RNAse as they mediate protein uptake via mannose receptor by recipient cells, but how would that suggest that the glycan modification can be applied for vaccine development?
Line 181-182. Line 249-251. The fact that these proteins are glycosylated doesn’t make the glycans functionally important specifically in these tissues.
The RNAi experiments do show nicely that N- and O-glycans are relevant, but it should be mentioned that the targeted glycosylation enzymes act very early in the process (N-glycan precursor formation, or O-glycan initiation, including O-GlcNAc which occurs on many different nuclear and intracellular proteins as a regulatory factor). From these experiments, although valuable and relevant no conclusions about specific glycan functions on specific glycoproteins can be drawn. Every multicellular organism needs protein glycosylation to occur.
Line 321-323. This is a suggestion without any reasoning or hypothesis or evidence.
Line 376-379. How would the fact that these previously identified vaccine targets are found in this study now become be validated vaccine targets? This study identifies schistosome glycoproteins and glycosites, which is great, but the findings should not be presented as if these unveil vaccine targets. There is no data regarding their vaccine potential. The title is therefore also too ambitious in my opinion.
Line 473-482. This section makes no sense. Why would ‘streamlined’ glycosylation patterns be related to specific neurological functions? Why would moderate complexity be linked to immune evasion. Such unlimited speculations should be restricted.

MINOR COMMENTS

Line 57. This is not entirely correct. Refs 11-13 reported that these glycan elements are antigenic, they did not report their mass spectrometric identification. The latter was reported in Refs 14-16 indeed.
Line 64-66. It should be mentioned here that this is in a vaccination setting.
Line 441. This is not CCA nut CAA (circulating anodic antigen)
Line 443. GlcA has also been found in other parasitic helminths (e.g. dog heartworm)
Line 457-458. Why would N,O-glycoproteins have dual functions?
Line 472. Polysaccharide?
Line 501. H11 is not from Trichinella but from Haemonchus.
Fig 1 is not entirely correct. In the manuscript structures with two core fucoses are listed. If present, these are not released by PNGaseF, so N,O glycopeptides will also remain, next to O-glycopeptides. This could also have an impact on the O-glycopeptide data.

Reviewer #2

(Remarks to the Author)
Schistosomiasis is caused by parasitic trematodes (blood flukes) of male and female that mate to produce eggs within their host, resulting in significant pathology over the long span of infection. Here the authors examined the glycoproteomes of Schistosoma mansoni in terms of tissue-specific expression and male vs. female. The results largely agree on the glycomic side with studies on glycans from these parasites done over the years by many groups. But a key development here is the glycoproteomics and identification of proteins in tissue- and sex-expression expression. The importance of glycosylation overall is highlighted by their studies demonstrating the knockdown of several glycosyltransferases known to be required for animal survival also impair the viability of the worms in vitro. As for potential candidate vaccines, there are no studies in that direction, so the work is not progressed to that level in this study. The descriptive nature of the work is of limited importance overall and probably is most relevant to parasitology field. The demonstration that known essential genes for glycosylation in other organisms, including the 4 tested here, are also essential in schistosomes, is not particularly novel as it is expected. Nevertheless, the work is novel in several other ways and interesting and represents a key advance in our understanding of this glycoproteomics of this novel parasite.

However, there several major concerns that tremendously lessen the potential impact of this paper at present.

The structures of glycans are shown but the data is mainly compositional and predictive based on the sizes of known monosaccharides. The linkage of sugars and their precise sequences within glycans are not presented here, although the structures are depicted the lead the reader to think that the structural sequences have been defined. The authors do not provide detailed information as to glycan sequence information, and without it, only compositional or predictive structures can be presented, which lessens the impact of the study overall significantly.

A major problem with this work is the purported discovery of sialic acid here, which is extremely doubtful to this reviewer. This issue is paramount to the discoveries in this paper and the authors make it a significant aspect of their overall discoveries here. However, the authors do not describe glycans or specific glycoprotein-derived glycans containing sialic acid, but rather list it as a monosaccharide in Fig. 1g. The note detection of its signal as well of that of NeuGc, by MS, but this is also uncertain as it does not indicate derivation from the parasite directly. Small amounts of sialic acid may even contaminate studies or be derived from host materials, as the authors state they only washed the worms after their removal from the mice. The presence of NeuGc also indicates contamination, as the parasites lack genes encoding enzymes that can produce this monosaccharide. As these parasites were derived by infection of mice, host material is always a potential problem, as the parasites are well known to consume vast amounts of red blood cells from their hosts, and these cells are highly enriched in sialic acid.

The authors oddly include the symbol for sialic acid in Fig. 4A on glycan structures but do not show any glycans defined to have sialic acid in their sequence. Thus, in that figure the symbol should not be present.

Furthermore, the genetic machinery for synthesizing sialic acid and the enzymes (sialyltransferases) that would use does not occur in these worms to the knowledge of this reviewer. The single reference to a smp_171870, which leads this author to a database on lncRNAs, is conflicting with their interpretation. Thus, the authors need to directly identify glycans containing sialic acid and show its presence on them derived from the parasite, or else remove it or highlight it as uncertain. If they find genes within the schistosome genome known to encode enzymes that utilize sialic acid and transfer it to proteins, they should identify this directly. This issue is extremely important for the immunology community that is exploring the roles of sialic acid in infective organisms vis-à-vis host interactions, and to have this work potentially incorrectly identify its present in worms for the first time could significantly detract from the impact of this paper otherwise.

SNA, which is a lectin that can bind to alpha-2-6-linked sialic acid but also to other types of glycans, including sulfated glycans, should not be used as a definitive proof for the presence of sialic acid in a study that relies of mass spectrometry-based technologies to define these organic structures. This evidence of SNA binding parasites in Fig. 4D is not specific evidence for the presence of sialic acid, and the authors did not prove that this sialic acid is removed by neuraminidase, as is typically done to validate SNA binding to sialic acid rather than non-sialic acid containing materials.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
As in my previous review I would like to acknowledge the value of an integrative approach to glycoproteomics and the data presented in the manuscript, keeping in mind limitations with respect to data interpretation and the conclusions that can be drawn from the data. Several major concerns that I raised in my initial review have been addressed at least to some extent in the rebuttal and revised manuscript, such as the removal of hypothetical glycan structures and the strong but unsubstantiated conclusion that schistosomes would be able to produce sialylated glycans. However in my opinion not all of my initial concerns have been dealt with sufficiently, and several of the conclusions presented in the results and discussion sections are still not sufficiently supported by data and should be further revised.

With respect to my major concerns regarding Experimental methodology:

I appreciate the inclusion of the pGlyco3 database to compare to the pGlycoNovo approach. However, while this does add confidence to the glycopeptide assignments made, it doesn't address my comment that a large amount of schistosome glycans (compositions as well as structures) have been reported in literature, together also forming a database of glycan structures that can be used to support and validate (de novo) assignments. The pGlyco3 database does not contain schistosome specific glycans, only those that are shared between schistosome and the species in the database. In the rebuttal it is stated that In addition, we curated the N-glycan libraries from Ref. 11 and Ref. 12 for cross-validation. Where is this shown in the manuscript? Is the reference to Suppl Data 3 correct? Such comparisons to literature are not only relevant to validate the current approach but also to acknowledge the work that has been done before (which for schistosome glycans is a lot, by several groups over the last 2-3 decades).

Line 299: 'The findings indicate that, in addition to the glycan types supported by the glycan library in pGlyco3, pGlycoNovo can identify rare glycans absent from the database, thereby enhancing our understanding of S. mansoni glycan diversity'.
COMMENT This is what I already criticized in my earlier review: the glycan library in pGlyco3 does not contain schistosome glycans. The comparison should be made with a library constructed of previously published schistosome glycans and not just with pGlyco3. It is nice to know that pGlycoNovo identifies additional glycans (glycan compositions) to pGlyco3, but that is rather obvious if pGlyco3 does not contain schistosome glycan data. There should be more emphasis on pGlycoNovo findings in the light of existing schistosome glycomics data.

Line 486: 'A small proportion of sialic acid was also detected in our study. Sialic acids have been reported in several parasitic helminths, including Fasciola hepatica, Echinococcus granulosus, and Taenia solium. However, because schistosomes lack a canonical sialic acid biosynthesis pathway, their presence has been debated, with most studies suggesting absence. The sialic acids detected here are therefore more likely attributable to host contamination. Alternatively, we cannot rule out the possibility that schistosomes utilize a unique biosynthetic pathway or acquires sialic acids from the host via putative transporters, as observed in certain bacteria. Nonetheless, these explanations remain speculative'.
COMMENT The authors are still suggesting that sialic acids may be produced by helminths and the text is not clear enough about this. There are no indications that schistosomes make sialylated glycans and speculating will keep this controversy going. It should be possible to check that the detected sialylated glycopeptides are derived from host proteins (which are likely to be present in the adult schistosome gut in the process of being digested). Was this done?
The 'impossible' glycan structures have been removed and the text and conclusions regarding the de novo identified glycans are formulated more carefully now (mostly, see below).

With respect to my other (earlier) concerns and the changes made in the text:

Title: As explained in my first review, this study does not unveil vaccine targets and I find the title (and some conclusions, see below) not acceptable. Replacing targets with candidates doesn't change this. The study describes that many of previously identified vaccine candidates are glycosylated. This is important information, but the authors should not claim that they have identified vaccine candidates. They have identified or confirmed that several of these candidates are glycosylated, which may or may not be relevant, in addition to creating a sex- and tissue-specific glycoproteomic landscape for schistosomes. This is what the title should reflect.

Line 29-32 'We further identify and characterize several glycoprotein vaccine candidates. This work provides a foundational glycoproteomic resource and advances glycosylation-based strategies for schistosomiasis control.'
COMMENT: No vaccine candidates were identified, in my opinion these are claims that are not supported by the data. Appropriate would be 'We further show that several vaccine candidates are glycoproteins and characterize their glycosylation. This work provides a foundational glycoproteomic resource supporting the development of glycan and glycoprotein-based strategies for schistosomiasis control'. (or see last sentence of introduction, which is also more appropriate)

Line 56: 'critical' = typical

Line 67: 'the key difference' = a possible key difference

Line 80: 'missing key glycan details'
COMMENT This is not correct. The glycan details (structures, linkages etc) in such studies are usually more extensively analysed that in the current study which mainly identifies compositions. The key details that are missing are the protein and site specific glycosylation details. Please rephrase.

Line 97: 'comprehensive glycan library'
COMMENT I don't agree that this is a comprehensive glycan library. The library is comprehensive in the sense of glycan composition and glycosylation sites. The glycans as such are not further characterized in terms of structure, or by combining existing data into the de novo generated database. Please rephrase (e.g. a comprehensive database of protein and site specific glycosylation).

Line 131: 'underwent' = carried

Line 140: 'unclassified'
COMMENT Could you define somewhere what this means? Are these glycans of which a composition has not been reported before? Or are these glycans of which it is not clear to which class they belong? What actually is unclassified?

Line 141: ‘glycan types’ = glycans

Line 149: ‘While these modifications in S. mansoni have been reported, their high prevalence suggests critical functional roles in this parasite’.
COMMENT Functional roles of these glycans (e.g. fucosylated motifs such as LeX, and their high prevalence in the schistosome glycome have been reported several times before, that is not something that follow from this study. Please refer to earlier papers.

Line 155: ‘and previous studies generally suggested the absence of sialic acid modifications in schistosomes, consistent with our findings’.
COMMENT This is not strong enough (see earlier discussions about sialic acids). Previous studies indicated the absence of sialic acid modifications of schistosomes, although some suggested that host-derived sialylated glycoproteins can be present in worm preparations. Also, the previous studies are not consistent with your findings but your findings are consistent with previous studies.

Line 192: ‘of site-specific glycans per glycoprotein and glycosite’
COMMENT What is a site specific glycan per glycosite? Delete ‘site-specific’?

Line 196: ‘Although most tissue-specific N-glycoproteins contained only a single glycosite, glycoproteins in the parenchyma and muscles displayed a broader range of site complexity, spanning all five categories (Fig. 2c). In contrast, glycoproteins in the gut and tegument rarely possessed more than two glycosites (Fig. 2c).’
COMMENT This is not clear to me. Is this about complexity regarding different categories (types?) of glycans on a single site, or single vs two sites, or both? Please consider clarity.

Line 207: ‘Regarding residue modifications’
COMMENT This is an unfamiliar term. What is meant here? Antenna modifications, core modifications? Glycan elements/motifs? Or specific monosaccharides? Please clarify.

Line 212: ‘likely supporting its complex biologic processes and adaptive mechanisms for parasitism’ COMMENT This is an unsupported, vague and general claim. Delete.

Line 225: ‘Furthermore, 468 site specific glycans exhibited a female-specific bias, while 508 showed a male-specific bias’
COMMENT Could you please write this up more precisely. What is meant here by 468 site specific glycans? Is this 468 glycopeptide combinations? (in 468 cases a glycan that is found on a certain glycosylation site is only found on that specific site in females?)

Line 239: ‘the distribution of monosaccharide numbers remained consistent (Supplementary Fig. 8b), suggesting that sex-specific N-glycosylation differences primarily arise from compositional variations—such as differences in the number of specific monosaccharide residues within glycan chains’.
COMMENT This is not clear to me. Consider rephrasing.

Line 292: ‘multiple residue modifications’
COMMENT This is an unfamiliar term. What does this mean precisely. It is quite normal that glycans contain both fucose and xylose and other monosaccharides, perhaps better to delete this phrase.

Line 324: ‘Tissue and sex heterogeneity of O-glycosylation in S. mansoni’.
COMMENT Most O-glycosylation consists of a single HexNAc, which is likely O-GlcNAc. This is a very different type of glycosylation, occurring mostly intracellularly and dynamically (with O-glcNAc transferase and O-glcNAc-ase as regulatory factors), and the data should be discussed with this in mind.

Line 337. ‘These tissue specific O-glycosylation patterns may be correlated with their functional adaptations’.
COMMENT Please delete, this is a vague claim.

Line 370: ‘homologous’ = homologs

Line: 386: ‘These findings demonstrate that key genes in the involved in the early stages of glycosylation of substrate proteins are essential for the survival of schistosomes, emphasizing the importance of protein glycosylation in the parasite.’
COMMENT That is true but if would be fair to add that this is entirely as expected for any multicellular eukaryotic organism.

Line 391 ‘Glycoproteomics-based prediction of vaccine candidates at host-parasite interface in S mansoni’
COMMENT Similar to my comments to the main title: vaccine candidates are not predicted by the data in this manuscript. Merely, glycoproteins are described in terms of glycosylation, and perhaps these glycoproteins could be explored as vaccine candidates.

Line 448: ‘absence of a comprehensive glycan library’
COMMENT Dee earlier comments, better refer to it as ‘ glycoproteomics, or glycosylation database. Or perhaps something like ‘despite a large amount of glycomics data a comprehensive glycoproteomic analysis is lacking”

Line 461: please remove ‘sialic acids’

Line 495: ‘novel’
COMMENT Remove novel. These structural variations may not be in the pGlyco3 database but that have been reported in various glycomics papers and are well know to occur in schistosomes.

Line 499: ‘which may associate with the evolutionary adaptations of these parasite flatworms. Conversely, the simplified O-glycosylation profile may reflect specialized functional requirements in schistosomes. Furthermore, the dramatic differences in modification patterns and functional implications between O- and N glycosylation suggest complementary biological functions during the development of schistosomes, with dual-modified (N- and O-glycosylated) proteins potentially exhibiting multifunctional properties deserving further study. Proteins carrying both modifications may participate in important biological pathways, a possibility that merits further study’.
COMMENT In my opinion this is all over-interpretation and vague suggestions and should be deleted.

Line 508: ‘revealed the highest similarity with Mus musculus’
COMMENT This is of limited value as the pGlyco3 database contains only few species. In any case the discussion should also discuss the clear differences between mouse and schistosome glycans including the absence of sialic acid and the abundance of xylosylation and multifucosylation in the latter.

Line 571: remove ‘NeuAc’

Line 573: ‘These findings provide valuable insights for studies on schistosome and lay the groundwork for glycosylation-based vaccine development in parasitic helminths’.
COMMENT Please adapt in line with my other comments e.g. “These findings provide valuable insights for studies on schistosome glycosylation and provide a resource for development schistosome glycoprotein vaccine candidates”.

Reviewer #2

(Remarks to the Author)
This study is interesting and exciting and provides the first overall glimpses into the complex glycomes of this key human parasite Schistosoma mansoni. Knowledge of the expression of its glycoproteins and their associated glycans in this work, which also identified tissue-specific and sex-biased glycosylation patterns, is key to developing better treatments and diagnostics for this parasite, which continues to infect humans worldwide. The main issues from this reviewer were in regard to the depiction of glycan structures, which the authors have correctly addressed, the lack of hard evidence for sialic acid being expressed on the glycans of the parasite rather than perhaps being low level contaminants from other sources, which the authors have completely and also nicely addressed. Overall, this study is thorough and robust, and uses state-of-the-art methods and technologies and represents a key advance in the field of molecular parasitology and the glycobiology of such parasitic helminths.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study applies a state-of-the-art approach to an extensive glycoproteomics study of a major human parasite, revealing aspects of glycoproteome complexity at a hitherto unachievable depth. The data presented form a valuable resource for the schistosome research community.
The manuscript has been carefully revised according to my comments and recommendations, which have been addressed sufficiently. Data are now more carefully interpreted and the conclusions that can be drawn from the data have now been more carefully phrased, or were removed when lacking foundation. In addition several minor issues with wording or clarity have been solved.

I have one suggestion left (not critical). In Fig 6 it would be great if panel b and c are aligned in such a way that the genes (e.g. OGT) are next to each other in each column. In panel b OGT is the top image, but in panel c it is the second from top. It could be better if for instance the legend in panel b is aligned with the control in panel c, and then below that the 4 genes next to each other in each panel instead of diagonally.

We would like to thank the reviewers for their thoughtful and helpful critiques of our work. The work is greatly improved upon the incorporation of their suggestions.

Reviewer #1 (Remarks to the Author):

This manuscript describes the glycoproteomic analysis of the adult worm stage of the human parasite S. mansoni, based on a combination of in-depth mass spectrometry analysis of glycopeptides applying a novel software tool and available transcriptomics data. Hundreds of glycosylated proteins were detected and putative site-specific glycosylation is described on the basis of LC-MS/MS data. Using the available single cell gene expression atlas of S. mansoni the glycoproteomics data is presented and discussed in the context of tissue specific expression and vaccine target potential. The study aims to fill a significant knowledge gap in S. mansoni proteomics and glycomics research, which is the lack of a general overview of protein- and site-specific glycosylation patterns that could be relevant for schistosome biology and parasite-host interactions.

Applying a high-end technological approach to glycoproteomics the study provides a thorough glycoproteomic landscape of S. mansoni worms, beyond the identification of site-specific glycosylation patterns so far reported for only a few individual glycoproteins. Numerous glycosylation sites are identified and characterised, which allow a global and integrated transcriptomics, proteomic and glycomics analysis supported by a multitude of largely insightful figures that different angles of analysis. The authors use this data to address a number of different questions with respect to S. mansoni biology. The mass spectrometry based analysis is complemented with a few additional experiments including inhibition of gene transcription of several gens involved in glycosylation to show that N- and O-glycosylation is essential for healthy worm survival in vitro.

I acknowledge the value and relevance of this integrative approach and the data generated for the field. However, I also have a number of concerns in particular with respect to the limitations of the data at the glycan level and the interpretations presented in the manuscript. Moreover, several of the conclusions presented in the Results and Discussion sections, that highlight the relevance of the findings for vaccine target discovery are not or not sufficiently supported by data in my opinion.

We sincerely thank the reviewer for recognizing the overall significance of our study and its value to the field of parasitology. In response to the reviewer's concerns regarding the limitations of the glycan-level data, we have undertaken systematic revisions by integrating and validating glycoprotein and glycopeptide information through a combined database- and *de novo*–based approach, and we have revised or removed inaccurate interpretations and descriptions where necessary. In addition, we have adjusted the emphasis on vaccine target discovery in the title, as well as in the Results and Discussion sections. The specific revisions are detailed point by point below.

MAIN COMMENTS REGARDING EXPERIMENTAL DATA AND APPROACH

1. Line 108. The authors state that a comprehensive S. mansoni glycan library is absent. But it is not. Several in-depth glycomics analyses of S. mansoni adult worms have been reported (acknowledged in the Refs), including N-glycans that provide an elaborate library. Because these reports analysed released glycans they did not provide glycoproteomic information (expect for a few glycoproteins). Instead, they have characterized glycan structures by applying glycan-specific sequencing methods including different types of MS fragmentations and linkage-specific exoglycosidase sequencing which provides reliable and complementary evidence for structure assignments that often (and in particular for previously unknown glycans) cannot be based on monosaccharide compositions or MS/MS fragment ions alone. I don't understand why the previously identified library has not been used as input for intact glycopeptide analysis software tools. This could then also have been used to validate the outcomes of the pGlycoNovo tool that in addition identifies so far unreported glycans to be present on specific glycosites. (So far unreported perhaps because of low abundance in total glycan pool, of because they were too small to be detected as released glycans such as for short O-glycans; different methods have different widths and depths). In my opinion this is a critical point in the setup of the study.

We thank the reviewer for this insightful and constructive comment. Our initial aim was to apply a *de novo*–based search approach (pGlycoNovo) to uncover glycosylation features that may have been overlooked in previous studies, recognizing that glycomics and intact glycoproteomics provide distinct yet complementary information. We greatly value your suggestion that previously established glycan libraries can serve as useful inputs for intact glycopeptide analysis tools and as benchmarks to validate the outcomes of pGlycoNovo.

Accordingly, in the revised manuscript we modified our workflow following the method of Zeng et al. (**Ref. 21**), incorporating database-based pGlyco3 searches to validate pGlycoNovo outputs. We found that most identifications (2,390/2,692, 88.8%) were consistent between the two approaches (**Fig. 4a**), providing strong support for the accuracy of pGlycoNovo. For overlapping entries, we adopted the pGlyco3 assignments and subsequently merged the results. This strategy ensured reliable identifications through pGlyco3 while leveraging pGlycoNovo to explore novel glycan types and maintain comparability with other species.

In addition, we curated the N-glycan libraries from **Ref. 11** and **Ref. 12** for cross-validation. Of the 48 glycan compositions reported in **Ref. 11** (eggs and cercarial secretions), 44 (92%) were identified in our dataset. From the 117 glycan compositions reported in **Ref. 12** across various life stages, 93 (80%) were also detected, including 56 of 59 (95%) glycans from 6-week adult worms. These cross-references further support the robustness of our results. Moreover, our approach identified 251 N-glycan compositions in 6-week adults alone (**Supplementary Data 3**), substantially expanding the known N-glycan repertoire of *S. mansoni*.

Reference:

11. Jang-Lee J, et al. Glycomics analysis of *schistosoma mansoni* egg and cercarial

secretions *. Molecular & Cellular Proteomics 6, 1485-1499 (2007).
12. Smit CH, et al. Glycomic Analysis of Life Stages of the Human Parasite *Schistosoma mansoni* Reveals Developmental Expression Profiles of Functional and Antigenic Glycan Motifs. Molecular & Cellular Proteomics : MCP 14, 1750-1769 (2015).
21. Zeng W-F, Yan G, Zhao H-h, Liu C, Cao W. Uncovering missing glycans and unexpected fragments with pGlycoNovo for site-specific glycosylation analysis across species. Nature Communications 15, 8055 (2024).

2. Whereas I am largely confident about the idenfication of the glycosites I do have concerns about the glycans. Some glycans, present on one or more glycosylation sites, in one or more proteins, are presented that have not been found before in schistosomes. As mentioned, this is very well possible due to the different approaches applied in different studies. However, several glycan compositions and MS/MS-derived glycan structures are presented that do not seem to make sense from a biological perspective ('unusual' glycan). In particular N-glycosylation pathways in schistosomes and other helminths are relatively conserved, at least for the N-glycan precursor and glycan core formation. Yet some of the glycans presented would deviate from anything so far observed in helminths or eukaryotic organisms in general.
For example, in SFig 11a, the N-glycopeptide spectrum suggests an N5X1 fragment. Which would leave H3N1F2 for the N-glycan core. This is very improbable and would mean that a subset of schistosome N-glycans is built on a novel core type not created by the conserved pathway responsible for all other N-glycans presented.

We thank the reviewer for raising this important point. We agree that glycan structures should not be inferred solely from glycan composition and glycopeptide spectra. Accordingly, we have removed **Supplementary Fig. 11a** and the previous emphasis on the N5X1 fragment. After re-analysis using the combined strengths of pGlyco3 and pGlycoNovo, the proportions of truncated and unclassified glycans were reduced from 9.09% and 9.72% to 5.56% and 5.90%, respectively (**Fig. 1f**), thereby minimizing the likelihood of reporting biologically implausible glycan compositions. In addition, in **Fig. 4** we no longer highlight atypical glycans. Instead, we emphasize that our findings are most robust for the 88.8% of glycans consistently identified by both methods, as well as for unclassified glycans with high Glyscores.

3. Also, regarding the presence of sialic acid in some of the presented structures: this remains very controversial and one could argue unlikely. The direct proof provided is very limited. It has been shown before that sialic acids – if present – in schistosome preparations are present on host-derived glycoproteins, as acknowledged by the authors. The MS/MS data now indicated the putative presence of sialylated glycans, specifically on Serpin glycopeptides. The structures suggested to contain sialic acid are again very unusual (and not represented in other glycopeptides). As far as I can judge there are no indications for a sialylation pathway in schistosomes (or any other parasite), and the structures suggested that would contain sialic acid are again very unusual. With a composition of H3N2F1S2X1 (Fig 4B), meaning a Man3 N-glycan core carrying F1S2X1, where would the two sialic

acids be linked? The evidence provided in Fig4D is not real evidence I would say. To me the images don't show overlap of Serpin and sialic acid, but rather that the stained areas touch, and in any case the presence of Serpin and sialic acid in the same cells is no direct evidence for their conjugation.

We thank the reviewer for raising this concern. We carefully revised our search strategy and applied the GlyScore threshold, which reduced the proportion of NeuAc-modified site-specific glycans from 1% to 0.4%, weakening the support for the presence of sialic acids, particularly NeuAc. As suggested, the spectra in **Fig. 4** and the co-localization experiments cannot be considered definitive evidence due to the absence of a biosynthetic pathway and the structural implausibility. Accordingly, we have revised the Results and Discussion sections to clarify that these identifications are more likely derived from host sources or contamination, thereby avoiding potential misinterpretation (lines 427-434).

4. Finally, with respect to other glycan structures presented there are several biosynthetically very unusual (not logical) structures suggested to occur on specific proteins but not in any other protein, also containing elements and monosaccharide sequences not observed before, such as the left upper structure in Fig 4A, the various bisecting GlcNAc containing glycans in Fig 7d, and the unusual Man 5 glycan in Fig 7d, as examples. Also for many of the other glycans presented in Fig 4 and 7 it is not clear how these structures have been derived from the MS/MS data, even though in these cases they contain elements that have been described before. For instance, how have the Fuc residue locations been determined? From a function and/or immunogenic point of view these positions are critical.
To prove (or suggest) the existence of biosynthetically unexplainable and unusual glycan sequences additional glycan structure analysis experiments would be needed. Or could some of these perhaps be explained by the 1% false discovery rate and are they technological artefacts? Have these experiments been carried out in duplicate to check?
In any case, a better way to present the data could be to just present the compositions (as in SFig 17), in order to avoid showing potentially wrong structure suggestions to a (non-expert) audience.

We sincerely appreciate the reviewer's insightful comments. We fully agree that deducing glycan structures solely from glycan compositions and glycopeptide spectra is not sufficiently rigorous. To prevent potential misinterpretation, we have removed all structural glycan diagrams from the manuscript (e.g., those in **Figures 4 and 7**). In **Fig. 4**, we no longer depict putative structures of atypical glycans. Instead, we present only the compositions of unclassified glycans with high GlyScores identified by pGlycoNovo, highlighting—without risk of overinterpretation—that our data suggest the possible presence of novel glycans, while acknowledging that additional studies will be required for definitive confirmation.

MAIN COMMENTS REGARDING TEXT/DISCUSSION

1. Line 71-72. There is no rationale behind this suggestion. Omega-1 glycans are important for the immunomodulatory effect of this RNAse as they mediate protein uptake via mannose receptor by recipient cells, but how would that suggest that the glycan modification can be applied for vaccine development?

Thank you for this helpful comment. In the revised manuscript, we have removed the speculative statement about vaccine development. The sentence now reads: "These insights underscore the significance of glycosylation in parasite-host interactions and immune responses."

2. Line 181-182. Line 249-251. The fact that these proteins are glycosylated doesn't make the glycans functionally important specifically in these tissues.

We thank the reviewer for pointing out that the original sentence may have overstated the functional significance of glycans in specific tissues without sufficient supporting evidence. To address this concern, we have revised the statement to remove any definitive causal inference. Instead, we now describe the observed enrichment of glycoproteins in these metabolically active tissues as a potential indication of biological relevance, and explicitly note that further functional studies are required to confirm this possibility.

3. The RNAi experiments do show nicely that N- and O-glycans are relevant, but it should be mentioned that the targeted glycosylation enzymes act very early in the process (N-glycan precursor formation, or O-glycan initiation, including O-GlcNAc which occurs on many different nuclear and intracellular proteins as a regulatory factor). From these experiments, although valuable and relevant no conclusions about specific glycan functions on specific glycoproteins can be drawn. Every multicellular organism needs protein glycosylation to occur.

Excellent point. In the revised manuscript, we now explicitly emphasize that the targeted glycosylation enzymes (e.g., OGT, C1GALT1) function at the very early stages of their respective pathways (N-glycan precursor formation or O-glycan initiation). We agree that, given current technical limitations, it remains challenging to directly dissect the roles of specific glycans on individual glycoproteins. Nonetheless, while enzymes such as OGT have been extensively characterized in other species, their functional importance in *S. mansoni* has not yet been established. Therefore, we believe that our RNAi experiments—despite their broad effects—still provide valuable insights into the essentiality of these early glycosylation events in *S. mansoni*.

4. Line 321-323. This is a suggestion without any reasoning or hypothesis or evidence.

We have removed this point in the revised manuscript.

5. Line 376-379. How would the fact that these previously identified vaccine targets are

found in this study now become be validated vaccine targets? This study identifies schistosome glycoproteins and glycosites, which is great, but the findings should not be presented as if these unveil vaccine targets. There is no data regarding their vaccine potential. The title is therefore also too ambitious in my opinion.

We apologize for the confusion. Our study identified glycoproteins and glycosylation sites expressed at the host–parasite interface, some of which have previously been reported as potential vaccine targets, although none have been validated in clinical trials. Our intention was to emphasize that proteins located at this interface may hold promise as vaccine candidates. To avoid any misleading statements, we have rephrased the relevant sentences in lines 350–351, lines 368-374 and also revised the title to "Tissue-specific and sex-biased glycoproteomic landscape of *Schistosoma mansoni* unveils vaccine candidates".

6. Line 473-482. This section makes no sense. Why would 'streamlined' glycosylation patterns be related to specific neurological functions? Why would moderate complexity be linked to immune evasion. Such unlimited speculations should be restricted.

Thank you. We have removed these statements.

MINOR COMMENTS

1. Line 57. This is not entirely correct. Refs 11-13 reported that these glycan elements are antigenic, they did not report their mass spectrometric identification. The latter was reported in Refs 14-16 indeed.

We have removed Refs 11–13 from this statement and replaced them with Refs 14–16.

2. Line 64-66. It should be mentioned here that this is in a vaccination setting.

Added.

3. Line 441. This is not CCA nut CAA (circulating anodic antigen)

Thanks for your carefully review. Corrected.

4. Line 443. GlcA has also been found in other parasitic helminths (e.g. dog heartworm)

Thanks. We have added this point in lines 422-423 and cited.

5. Line 457-458. Why would N,O-glycoproteins have dual functions?

We have revised the statement in lines 445-446 to minimize speculation.

6. Line 472. Polysaccharide?

Thanks. We have revised the sentence to avoid any inappropriate descriptions.

7. Line 501. H11 is not from Trichinella but from Haemonchus.

Corrected.

8. Fig 1 is not entirely correct. In the manuscript structures with two core fucoses are listed. If present, these are not released by PNGaseF, so N,O glycopeptides will also remain, next to O-glycopeptides. This could also have an impact on the O-glycopeptide data.

Thank you. We have revised **Figure 1** accordingly and emphasized in the O-glycoproteomics data processing section that N-glycans were removed to avoid their interference with the O-glycopeptide data.

Reviewer #2 (Remarks to the Author):

Schistosomiasis is caused by parasitic trematodes (blood flukes) of male and female that mate to produce eggs within their host, resulting in significant pathology over the long span of infection. Here the authors examined the glycoproteomes of Schistosoma mansoni in terms of tissue-specific expression and male vs. female. The results largely agree on the glycomic side with studies on glycans from these parasites done over the years by many groups. But a key development here is the glycoproteomics and identification of proteins in tissue- and sex-expression expression. The importance of glycosylation overall is highlighted by their studies demonstrating the knockdown of several glycosyltransferases known to be required for animal survival also impair the viability of the worms in vitro. As for potential candidate vaccines, there are no studies in that direction, so the work is not progressed to that level in this study. The descriptive nature of the work is of limited importance overall and probably is most relevant to parasitology field. The demonstration that known essential genes for glycosylation in other organisms, including the 4 tested here, are also essential in schistosomes, is not particularly novel as it is expected. Nevertheless, the work is novel in several other ways and interesting and represents a key advance in our understanding of this glycoproteomics of this novel parasite.

However, there several major concerns that tremendously lessen the potential impact of this paper at present.

We sincerely thank the reviewer for recognizing the novelty and significance of our study and for acknowledging its contribution to advancing schistosome glycoproteomics. In the revised manuscript, we have improved the data analysis and modified the figures, removing potentially misleading aspects related to sialic acids and glycan structures. Regarding vaccine targets, we have revised the title as well as the Results and Discussion sections to avoid overstatement. Finally, we have carefully addressed all major concerns raised by the reviewer, with detailed point-by-point responses provided below.

1. The structures of glycans are shown but the data is mainly compositional and predictive based on the sizes of known monosaccharides. The linkage of sugars and their precise sequences within glycans are not presented here, although the structures are depicted the lead the reader to think that the structural sequences have been defined. The authors do not provide detailed information as to glycan sequence information, and without it, only compositional or predictive structures can be presented, which lessens the impact of the study overall significantly.

We greatly appreciate the reviewer's insightful comment. We fully agree that our data do not provide definitive structural information and that inferring glycan structures solely from compositions and predictions is not accurate. To avoid any potential misrepresentation, we have removed all glycan structure illustrations from the manuscript (including **Figure 4 and 7**). In **Figure 4**, we no longer depict putative structures of atypical glycans. Instead, we present the compositions of high-GlyScore unclassified glycans identified by pGlycoNovo.

2. A major problem with this work is the purported discovery of sialic acid here, which is extremely doubtful to this reviewer. This issue is paramount to the discoveries in this paper and the authors make it a significant aspect of their overall discoveries here. However, the authors do not describe glycans or specific glycoprotein-derived glycans containing sialic acid, but rather list it as a monosaccharide in Fig. 1g. The note detection of its signal as well of that of NeuGc, by MS, but this is also uncertain as it does not indicate derivation from the parasite directly. Small amounts of sialic acid may even contaminate studies or be derived from host materials, as the authors state they only washed the worms after their removal from the mice. The presence of NeuGc also indicates contamination, as the parasites lack genes encoding enzymes that can produce this monosaccharide. As these parasites were derived by infection of mice, host material is always a potential problem, as the parasites are well known to consume vast amounts of red blood cells from their hosts, and these cells are highly enriched in sialic acid. The authors oddly include the symbol for sialic acid in Fig. 4A on glycan structures but do not show any glycans defined to have sialic acid in their sequence. Thus, in that figure the symbol should not be present. Furthermore, the genetic machinery for synthesizing sialic acid and the enzymes (sialyltransferases) that would use does not occur in these worms to the knowledge of this reviewer. The single reference to a smp_171870, which leads this author to a database on lncRNAs, is conflicting with their interpretation. Thus, the authors need to directly identify glycans containing sialic acid and show its presence on them derived from the parasite, or else remove it or highlight it as uncertain. If they find genes within the schistosome genome known to encode enzymes that utilize sialic acid and transfer it to proteins, they should identify this directly. This issue is extremely important for the immunology community that is exploring the roles of sialic acid in infective organisms vis-à-vis host interactions, and to have this work potentially incorrectly identify its present in worms for the first time could significantly detract from the impact of this paper otherwise. SNA, which is a lectin that can bind to alpha-2-6-linked sialic acid but also to other types of glycans, including sulfated glycans, should not be used as a definitive proof for the presence of sialic acid in a study that relies of mass spectrometry-based technologies to define these organic structures.

This evidence of SNA binding parasites in Fig. 4D is not specific evidence for the presence of sialic acid, and the authors did not prove that this sialic acid is removed by neuraminidase, as is typically done to validate SNA binding to sialic acid rather than non-sialic acid containing materials.

This is a critical point. We carefully revised our search strategy and applied the GlyScore threshold, which reduced the proportion of NeuAc-modified site-specific glycans from 1% to 0.4%, weakening the support for the presence of sialic acids. Considering the absence of known biosynthetic pathways and structural inconsistencies, we have removed the emphasis on sialic acids throughout the manuscript, including annotated spectra and SNA co-localization experiments in **Figure 4**. As the reviewer noted, trace sialic acid signals are likely due to contamination or host origin. Accordingly, we have revised the Results and Discussion sections to clarify that these identifications are more likely derived from host sources or contamination, thereby avoiding potential misinterpretation.

We would like to thank the reviewers for their thoughtful and helpful critiques of our work. The work is greatly improved upon the incorporation of their suggestions.

Reviewer #1 (Remarks to the Author):

As in my previous review I would like to acknowledge the value of an integrative approach to glycoproteomics and the data presented in the manuscript, keeping in mind limitations with respect to data interpretation and the conclusions that can be drawn from the data. Several major concerns that I raised in my initial review have been addressed at least to some extent in the rebuttal and revised manuscript, such as the removal of hypothetical glycan structures and the strong but unsubstantiated conclusion that schistosomes would be able to produce sialylated glycans. However in my opinion not all of my initial concerns have been dealt with sufficiently, and several of the conclusions presented in the results and discussion sections are still not sufficiently supported by data and should be further revised.

We sincerely thank you for the thorough evaluation of our revised manuscript. We have carefully addressed all of your comments and revised the manuscript accordingly. Your in-depth assessment has undoubtedly improved the quality and clarity of our work. Please find our detailed, point-by-point responses below.

With respect to my major concerns regarding Experimental methodology:

1. I appreciate the inclusion of the pGlyco3 database to compare to the pGlycoNovo approach. However, while this does add confidence to the glycopeptide assignments made, it doesn't address my comment that a large amount of schistosome glycans (compositions as well as structures) have been reported in literature, together also forming a database of glycan structures that can be used to support and validate (de novo) assignments. The pGlyco3 database does not contain schistosome specific glycans, only those that are shared between schistosome and the species in the database. In the rebuttal it is stated that In addition, we curated the N-glycan libraries from Ref. 11 and Ref. 12 for cross-validation. Where is this shown in the manuscript? Is the reference to Suppl Data 3 correct? Such comparisons to literature are not only relevant to validate the current approach but also to acknowledge the work that has been done before (which for schistosome glycans is a lot, by several groups over the last 2-3 decades).

Thank you for this valuable suggestion. We fully agree that incorporating *Schistosoma*-specific glycan compositions from the literature is essential for rigorous validation of our assignments and for appropriately acknowledging prior work in the field.

To address this, we combined **174 glycan compositions** previously reported from *S. mansoni* eggs, cercariae (and their secretions), adult worms, and other life-cycle stages **(Refs. 11-13, 23-27)**, and integrated them into the pGlyco3 database for re-analysis. Using this expanded database, we found that **90.3% (2441/2703)** of the site-specific glycans

identified by pGlycoNovo were consistent with the database-dependent pGlyco3 results. Comparison with published compositions further showed that **69% (120/174)** of reported glycans were detected in our 6-week adult *S. mansoni* glycoproteomic dataset, with **106** identified by both pGlycoNovo and pGlyco3. These cross-validation results enhance confidence in our assignments and support the accuracy of the *de novo* approach. The published glycan compositions are summarized in **Supplementary Data 1**, and the glycan compositions identified by the two search strategies in this study are provided in **Supplementary Data 11**.

We also re-validated our newly obtained N-glycan compositions from adult *S. mansoni* against datasets from the literature. Of the 117 compositions reported across multiple life stages by Smit *et al.* **(Ref. 12), 99 (84.6%)** were present in our dataset, including all **59 (100%)** compositions previously reported for 6-week adult worms. Likewise, we detected **45 of the 49 (91.8%)** glycans in egg secretions described by Lee *et al.* **(Ref. 11)**, **33 of 34 (97%)** egg N-glycans reported by Khoo *et al.* **(Ref. 23)**, and **all 33 (100%)** cercarial N-glycans reported by Khoo *et al.* **(Ref. 24)**. These published glycans have also been incorporated into **Supplementary Data 1**.

Together, these literature-based comparisons validate the robustness and accuracy of our dataset and reinforce the reliability of the earlier glycan-characterization studies. The descriptions of these cross-validation analyses and the relevant citations have now been added to the manuscript (**Lines 273-278, clean version**).

Reference:

11. Jang-Lee J, *et al*. Glycomics analysis of *schistosoma mansoni* egg and cercarial secretions *. *Molecular & Cellular Proteomics* 6, 1485-1499 (2007).
12. Smit CH, *et al*. Glycomic Analysis of Life Stages of the Human Parasite *Schistosoma mansoni* Reveals Developmental Expression Profiles of Functional and Antigenic Glycan Motifs. *Molecular & Cellular Proteomics*: MCP 14, 1750-1769 (2015).
13. Wuhrer M, Koeleman CAM, Fitzpatrick JM, Hoffmann KF, Deelder AM, Hokke CH. Gender-specific expression of complex-type N-glycans in schistosomes. *Glycobiology* 16, 991-1006 (2006).
23. Khoo K-H, Chatterjee D, Caulfield JP, Morris HR, Dell A. Structural mapping of the glycans from the egg glycoproteins of *Schistosoma mansoni* and *Schistosoma japonicum*: identification of novel core structures and terminal sequences. *Glycobiology* 7, 663-677 (1997).
24. Khoo KH, Huang HH, Lee KM. Characteristic structural features of schistosome cercarial N-glycans: expression of Lewis X and core xylosylation. *Glycobiology* 11, 149-163 (2001).
25. Wuhrer M, Koeleman CAM, Deelder AM, Hokke CH. Repeats of LacdiNAc and fucosylated LacdiNAc on N‐glycans of the human parasite *Schistosoma mansoni*. *The FEBS Journal* 273, 347-361 (2006).
26. Hokke CH, Deelder AM, Hoffmann KF, Wuhrer M. Glycomics-driven discoveries in schistosome research. *Experimental Parasitology* 117, 275-283 (2007).

27. Hokke CH, Van Diepen A. Helminth glycomics - glycan repertoires and host-parasite interactions. *Molecular and Biochemical Parasitology* 215, 47-57 (2017).

2. Line 299: 'The findings indicate that, in addition to the glycan types supported by the glycan library in pGlyco3, pGlycoNovo can identify rare glycans absent from the database, thereby enhancing our understanding of S. mansoni glycan diversity'.
COMMENT This is what I already criticized in my earlier review: the glycan library in pGlyco3 does not contain schistosome glycans. The comparison should be made with a library constructed of previously published schistosome glycans and not just with pGlyco3. It is nice to know that pGlycoNovo identifies additional glycans (glycan compositions) to pGlyco3, but that is rather obvious if pGlyco3 does not contain schistosome glycan data. There should be more emphasis on pGlycoNovo findings in the light of existing schistosome glycomics data.

Thank you for this important comment. We have removed the statement in the revised manuscript. In response, we incorporated previously reported *S. mansoni* glycans into the pGlyco3 glycan library and re-performed the comparison with pGlycoNovo. Following this re-analysis, we identified **262** site-specific glycans detected exclusively by pGlycoNovo, and the top 38 with the highest GlyScores are shown in **Fig.4b**.

We have also added more details of these comparisons in **Lines 268-272**: At the glycan-composition level, **69% (120/174)** of previously reported *S. mansoni* compositions were recovered in our 6-week adult worm dataset, including **106** identified by both pGlycoNovo and pGlyco3. In addition, **84** compositions were uniquely identified by pGlycoNovo, **15** were unique to pGlyco3, and **25** were detected by both tools but have not been reported previously **(Supplementary Fig. 13** and **Supplementary Data 11)**. These findings expand the glycan composition library of *S. mansoni* and provide new insights into the diversity of schistosome N-glycans.

3. Line 486: 'A small proportion of sialic acid was also detected in our study. Sialic acids have been reported in several parasitic helminths, including Fasciola hepatica, Echinococcus granulosus, and Taenia solium. However, because schistosomes lack a canonical sialic acid biosynthesis pathway, their presence has been debated, with most studies suggesting absence. The sialic acids detected here are therefore more likely attributable to host contamination. Alternatively, we cannot rule out the possibility that schistosomes utilize a unique biosynthetic pathway or acquires sialic acids from the host via putative transporters, as observed in certain bacteria. Nonetheless, these explanations remain speculative'.
COMMENT The authors are still suggesting that sialic acids may be produced by helminths and the text is not clear enough about this. There are no indications that schistosomes make sialylated glycans and speculating will keep this controversy going. It should be possible to check that the detected sialylated glycopeptides are derived from host proteins (which are likely to be present in the adult schistosome gut in the process of being digested). Was this done?

We agree that there is no solid evidence that schistosomes produce sialylated glycans. Although we were unable to directly assign these sialylated glycopeptides to specific host proteins, we acknowledge this limitation and now cite studies in parasitic helminths in which detected sialic acids were attributed to host **(Refs. 33-36)**. We also note that host proteins can be present in schistosome digestive contents **(Ref. 43)**. To avoid confusion, we have removed the speculative statements and clarified that the small amount of sialic acid detected in our dataset is derived from the host.

The revised paragraph now reads: “A small proportion of sialic acid was also detected in our study. However, schistosomes lack the canonical sialic-acid biosynthesis pathway, and previous studies have consistently reported an absence of sialylation. Instances of sialic acids detected in helminth preparations have generally been attributed to host contamination. We therefore consider the small amount of sialic acid in our dataset to be most likely host-derived. This interpretation is further supported by the fact that schistosomes ingest host blood, and host proteins have previously been identified in worm vomitus.” (**Lines 442-448)**

Reference:

33. Ravidà A, Aldridge AM, Driessen NN, Heus FAH, Hokke CH, O’Neill SM. *Fasciola hepatica* Surface Coat Glycoproteins Contain Mannosylated and Phosphorylated N-glycans and Exhibit Immune Modulatory Properties Independent of the Mannose Receptor. *PLOS Neglected Tropical Diseases* 10, e0004601 (2016).
34. Coff L, *et al*. Profiling the glycome of *Cardicola forsteri*, a blood fluke parasitic to bluefin tuna. *International Journal for Parasitology* 52, 1-12 (2022).
35. Khoo K-H, Nieto A, Morris HR, Dell A. Structural characterization of the N-glycans from *Echinococcus granulosus* hydatid cyst membrane and protoscoleces. *Molecular and Biochemical Parasitology* 86, 237-248 (1997).
36. Lee JJ, Dissanayake S, Panico M, Morris HR, Dell A, Haslam SM. Mass spectrometric characterisation of Taenia crassiceps metacestode N-glycans. *Molecular and Biochemical Parasitology* 143, 245-249 (2005).
43. Hall SL, Braschi S, Truscott M, Mathieson W, Cesari IM, Wilson RA. Insights into blood feeding by schistosomes from a proteomic analysis of worm vomitus. *Molecular and Biochemical Parasitology* 179, 18-29 (2011).

The ‘impossible’ glycan structures have been removed and the text and conclusions regarding the de novo identified glycans are formulated more carefully now (mostly, see below).

With respect to my other (earlier) concerns and the changes made in the text:

1. Title: As explained in my first review, this study does not unveil vaccine targets and I find the title (and some conclusions, see below) not acceptable. Replacing targets with candidates doesn’t change this. The study describes that many of previously identified vaccine candidates are glycosylated. This is important information, but the authors should

not claim that they have identified vaccine candidates. They have identified or confirmed that several of these candidates are glycosylated, which may or may not be relevant, in addition to creating a sex- and tissue-specific glycoproteomic landscape for schistosomes. This is what the title should reflect.

Thank you for this suggestion. We agree, and have revised the title accordingly to:"**Tissue-specific and sex-biased glycoproteomic landscape of *Schistosoma mansoni***" as suggested by you and the editor.

2. Line 29-32 'We further identify and characterize several glycoprotein vaccine candidates. This work provides a foundational glycoproteomic resource and advances glycosylation-based strategies for schistosomiasis control.'
COMMENT: No vaccine candidates were identified, in my opinion these are claims that are not supported by the data. Appropriate would be 'We further show that several vaccine candidates are glycoproteins and characterize their glycosylation. This work provides a foundational glycoproteomic resource supporting the development of glycan and glycoprotein-based strategies for schistosomiasis control'. (or see last sentence of introduction, which is also more appropriate)

We are grateful for this valuable comment and have revised the text according to your suggestion. (**Lines 28-31)**

3. Line 56: 'critical' = typical

Thank you. Revised.

4. Line 67: 'the key difference' = a possible key difference

Revised.

5. Line 80: 'missing key glycan details'
COMMENT This is not correct. The glycan details (structures, linkages etc) in such studies are usually more extensively analysed that in the current study which mainly identifies compositions. The key details that are missing are the protein and site specific glycosylation details. Please rephrase.

Thank you for pointing this out. We have removed "missing key glycan details" from the manuscript. (**Line 77)**

6. Line 97: 'comprehensive glycan library'
COMMENT I don't agree that this is a comprehensive glycan library. The library is comprehensive in the sense of glycan composition and glycosylation sites. The glycans as such are not further characterized in terms of structure, or by combining existing data into the de novo generated database. Please rephrase (e.g. a comprehensive database of

protein and site specific glycosylation).

Thank you. We have changed the phrase to “comprehensive database of protein and site-specific glycosylation” as recommended. (**Lines 92-93)**

7. Line 131: ‘underwent’ = carried

Corrected.

8. Line 140: ‘unclassified’
COMMENT Could you define somewhere what this means? Are these glycans of which a composition has not been reported before? Or are these glycans of which it is not clear to which class they belong? What actually is unclassified?

Apologies for the confusion. We followed the classification method consistent with Zeng *et al.* **(Ref. 21).** In this context, “unclassified” refers to any N-glycan composition that is not included in existing glycan database-dependent search engines. Zeng *et al.* combined N-glycan compositions from Byonic, MSFragger, and pGlyco3, resulting in a set of 1,461 non-redundant N-glycan compositions. Any N-glycan composition not matching these 1,461 entries is therefore defined as “unclassified.” To clarify the point, we have now added this definition to the Methods section in **Lines 635-637**: “Any composition that did not match the 1,461 non-redundant N-glycan compositions in the glycan database constructed by Zeng *et al.* was designated as “unclassified” in this study.”

Reference:
21. Zeng W-F, Yan G, Zhao H-h, Liu C, Cao W. Uncovering missing glycans and unexpected fragments with pGlycoNovo for site-specific glycosylation analysis across species. *Nature Communications* 15, 8055 (2024).

9. Line 141: ‘glycan types’ = glycans

Thanks. Corrected.

10. Line 149: ‘While these modifications in S. mansoni have been reported, their high prevalence suggests critical functional roles in this parasite’.
COMMENT Functional roles of these glycans (e.g. fucosylated motifs such as LeX, and their high prevalence in the schistosome glycome have been reported several times before, that is not something that follow from this study. Please refer to earlier papers.

Thank you for this critical comment. We agree that the high prevalence and functional roles of these glycans have been well established in previous studies. We have revised the sentence accordingly and cited appropriate references **(Refs. 29-30)**.

The revised text now reads:

“These fucosylated glycans (including structures such as LeX and LDN-F) and xylosylated glycans have been widely reported to be highly prevalent in *S. mansoni* and are known to play important functional roles.” (**Lines 143-145)**

Reference:

29. Remoortere AV, H.Hokke C, Dam GJV, Die IV, M.Deelder A, Eijnden DHVD. Various stages of Schistosoma express Lewisx, LacdiNAc, GalNAc 1-4 (Fuc 1-3)GlcNAc and GalNAc 1-4(Fuc 1-2Fuc 1-3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins. Glycobiology 10, 601-609 (2000).
30. Faveeuw C, Mallevaey T, Paschinger K, Wilson IB, *et al*. Schistosome N‐glycans containing core α3‐fucose and core β2‐xylose epitopes are strong inducers of Th2 responses in mice. *European Journal of Immunology* 33, 1271-1281 (2003).

11. Line 155: ‘and previous studies generally suggested the absence of sialic acid modifications in schistosomes, consistent with our findings’.
COMMENT This is not strong enough (see earlier discussions about sialic acids). Previous studies indicated the absence of sialic acid modifications of schistosomes, although some suggested that host-derived sialylated glycoproteins can be present in worm preparations. Also, the previous studies are not consistent with your findings but your findings are consistent with previous studies.

We appreciate your suggestion. We have incorporated relevant evidence and reports **(Refs. 33-36)** to strengthen this point and have clarified that our findings are consistent with previous studies.

The sentence has been revised to:
“Previous studies have reported that schistosomes lack endogenous sialic acid modifications and suggested that any detected sialylated glycoproteins in worm preparations are likely host-derived. The sialic acid detected in our dataset aligns with these observations and is most plausibly attributable to host contamination.” (**Lines 149-152)**

Reference:

33. Ravidà A, Aldridge AM, Driessen NN, Heus FAH, Hokke CH, O’Neill SM. *Fasciola hepatica* Surface Coat Glycoproteins Contain Mannosylated and Phosphorylated N-glycans and Exhibit Immune Modulatory Properties Independent of the Mannose Receptor. *PLOS Neglected Tropical Diseases* 10, e0004601 (2016).
34. Coff L, *et al*. Profiling the glycome of *Cardicola forsteri*, a blood fluke parasitic to bluefin tuna. *International Journal for Parasitology* 52, 1-12 (2022).
35. Khoo K-H, Nieto A, Morris HR, Dell A. Structural characterization of the N-glycans from *Echinococcus granulosus* hydatid cyst membrane and protoscoleces. *Molecular and Biochemical Parasitology* 86, 237-248 (1997).
36. Lee JJ, Dissanayake S, Panico M, Morris HR, Dell A, Haslam SM. Mass spectrometric

characterisation of Taenia crassiceps metacestode N-glycans. *Molecular and Biochemical Parasitology* 143, 245-249 (2005).

12. Line 192: 'of site-specific glycans per glycoprotein and glycosite'
COMMENT What is a site specific glycan per glycosite? Delete 'site-specific'?

Thank you. Deleted.

13. Line 196: 'Although most tissue-specific N-glycoproteins contained only a single glycosite, glycoproteins in the parenchyma and muscles displayed a broader range of site complexity, spanning all five categories (Fig. 2c). In contrast, glycoproteins in the gut and tegument rarely possessed more than two glycosites (Fig. 2c).'
COMMENT This is not clear to me. Is this about complexity regarding different categories (types?) of glycans on a single site, or single vs two sites, or both? Please consider clarity.

Thank you for the valuable comment. In the original manuscript, the phrase "five categories" may have been misleading, as it was intended to represent glycoproteins containing 1, 2, 3, 4, or ≥5 glycosites. To avoid any confusion, we have revised the sentence to clarify that **Fig. 2c** illustrates the distribution of glycosite numbers per tissue-specific glycoprotein.

The updated sentence now reads:
"Although most tissue-specific N-glycoproteins contained only a single glycosite, glycoproteins in the parenchyma and muscles exhibited a broader distribution of glycosite numbers per protein (Fig. 2c). In contrast, glycoproteins in the gut and tegument rarely contained more than two glycosites (Fig. 2c)." (**Lines 190-193)**

14. Line 207: 'Regarding residue modifications'
COMMENT This is an unfamiliar term. What is meant here? Antenna modifications, core modifications? Glycan elements/motifs? Or specific monosaccharides? Please clarify.

Thank you for pointing this out. "Residue modifications" refers to "specific monosaccharides", and we have clarified this throughout the manuscript.

15. Line 212: 'likely supporting its complex biologic processes and adaptive mechanisms for parasitism' COMMENT This is an unsupported, vague and general claim. Delete.

Deleted.

16. Line 225: 'Furthermore, 468 site specific glycans exhibited a female-specific bias, while 508 showed a male-specific bias'
COMMENT Could you please write this up more precisely. What is meant here by 468 site specific glycans? Is this 468 glycopeptide combinations? (in 468 cases a glycan that is found on a certain glycosylation site is only found on that specific site in females?)

Apologies for the confusion. Your interpretation of “site-specific glycans” is correct-- it refers to glycopeptide combinations, meaning a specific glycan identified at a defined glycosite. We have revised the manuscript accordingly and replaced the term "site-specific glycans" with this more precise wording.

To avoid misunderstanding, we clarify that the observed “bias” does not refer to the presence or absence of the glycopeptide combination between sexes. Instead, the 468 glycopeptide combinations showed statistically significant higher relative abundance in females than in males. These glycopeptide combinations were identified using strict quantitative thresholds: an adjusted p-value < 0.05 and an absolute $\log_2$(Fold Change) ≥ 1. (**Lines 686-687**)

In the revised manuscript, the sentence has been updated to clearly reflect this distinction: “Furthermore, 473 distinct glycopeptide combinations exhibited a significant female-specific abundance bias, while 525 showed a significant male bias.” (**Lines 218-220**)
Please note that several numerical values have been updated due to the use of the expanded glycan database in the reanalysis.

17. Line 239: ‘the distribution of monosaccharide numbers remained consistent (Supplementary Fig. 8b), suggesting that sex-specific N-glycosylation differences primarily arise from compositional variations—such as differences in the number of specific monosaccharide residues within glycan chains’.
COMMENT This is not clear to me. Consider rephrasing.

To make it clear, we have revised the sentence to: “The numbers of glycans containing each specific monosaccharide were broadly comparable between males and females (Fig. 3e), and the overall glycan size distribution was also similar across sexes (Supplementary Fig. 8b). These observations indicate that sex-specific N-glycosylation differences primarily arise from compositional variations, such as differences in the numbers of specific monosaccharides within glycans.” (**Lines 231-235**)

18. Line 292: ‘multiple residue modifications’
COMMENT This is an unfamiliar term. What does this mean precisely. It is quite normal that glycans contain both fucose and xylose and other monosaccharides, perhaps better to delete this phrase.

Thank you. We have removed this phrase.

19. Line 324: ‘Tissue and sex heterogeneity of O-glycosylation in S. mansoni’.
COMMENT Most O-glycosylation consists of a single HexNAc, which is likely O-GlcNAc. This is a very different type of glycosylation, occurring mostly intracellularly and dynamically (with O-glcNAc transferase and O-glcNAc-ase as regulatory factors), and the data should be discussed with this in mind.

We apologize for the misunderstanding caused by the labeling in **Fig. 5c** and by our accompanying description. In our dataset, O-glycans were grouped solely based on the presence or absence of Hex, and the term “only HexNAc” was not intended to imply that these glycans contain a single HexNAc. Rather, this category includes O-glycans that lack Hex but often contain multiple HexNAc residues and/or other monosaccharides (e.g., N(2), N(1)F(1)), which are incompatible with O-GlcNAc.

To avoid potential misinterpretation, we have renamed the two categories as glycans “with Hex” and “without Hex” **(Fig. 5c)**. Correspondingly, the sentence has been revised to “O-glycans were primarily composed of hexoses (Hex) and N-acetylhexosamines (HexNAc), with a minority of glycans lacking Hex.” (**Lines 290-292**)

20. Line 337. ‘These tissue specific O-glycosylation patterns may be correlated with their functional adaptations’.
COMMENT Please delete, this is a vague claim.

Thank you. Deleted.

21. Line 370: ‘homologous’ = homologs

Corrected.

22. Line: 386: ‘These findings demonstrate that key genes in the involved in the early stages of glycosylation of substrate proteins are essential for the survival of schistosomes, emphasizing the importance of protein glycosylation in the parasite.’
COMMENT That is true but if would be fair to add that this is entirely as expected for any multicellular eukaryotic organism.

We have added a note that this is expected for any multicellular eukaryotic organism in the revised sentence following your suggestion. (**Lines 362-363**)

23. Line 391 ‘Glycoproteomics-based prediction of vaccine candidates at host-parasite interface in S mansoni’
COMMENT Similar to my comments to the main title: vaccine candidates are not predicted by the data in this manuscript. Merely, glycoproteins are described in terms of glycosylation, and perhaps these glycoproteins could be explored as vaccine candidates.

We appreciate your valuable feedback and agree with the concern. Accordingly, we have revised the subheading to “Glycosylation profiling of glycoproteins at the host–parasite interface in *S. mansoni*.” (**Line 366**)

24. Line 448: ‘absence of a comprehensive glycan library’
COMMENT Dee earlier comments, better refer to it as ‘ glycoproteomics, or glycosylation database. Or perhaps something like ‘despite a large amount of glycomics data a

comprehensive glycoproteomic analysis is lacking"

Thank you. We have revised the sentence to: "Despite a large amount of glycomics data, a comprehensive glycoproteomic analysis is lacking." (**Lines 417-418**)

25. Line 461: please remove 'sialic acids'

Removed.

26. Line 495: 'novel'
COMMENT Remove novel. These structural variations may not be in the pGlyco3 database but that have been reported in various glycomics papers and are well know to occur in schistosomes.

Thanks. Deleted.

27. Line 499: 'which may associate with the evolutionary adaptations of these parasite flatworms. Conversely, the simplified O-glycosylation profile may reflect specialized functional requirements in schistosomes. Furthermore, the dramatic differences in modification patterns and functional implications between O- and N glycosylation suggest complementary biological functions during the development of schistosomes, with dual-modified (N- and O-glycosylated) proteins potentially exhibiting multifunctional properties deserving further study. Proteins carrying both modifications may participate in important biological pathways, a possibility that merits further study'.
COMMENT In my opinion this is all over-interpretation and vague suggestions and should be deleted.

Thank you. Deleted.

28. Line 508: 'revealed the highest similarity with Mus musculus'
COMMENT This is of limited value as the pGlyco3 database contains only few species. In any case the discussion should also discuss the clear differences between mouse and schistosome glycans including the absence of sialic acid and the abundance of xylosylation and multifucosylation in the latter.

Thank you for this constructive comment. We have revised the original discussion by adding the text as follows: "Although *S. mansoni* N-glycans appear broadly similar to those of *Mus musculus*, they differ in several major aspects. Notably, schistosomes lack sialylated N-glycans and instead display abundant xylosylation and extensive multifucosylation—features that are largely absent from mammalian glycomes." (**Lines 460-463**)

29. Line 571: remove 'NeuAc'

Removed.

30. Line 573: 'These findings provide valuable insights for studies on schistosome and lay the groundwork for glycosylation-based vaccine development in parasitic helminths'. COMMENT Please adapt in line with my other comments e.g. "These findings provide valuable insights for studies on schistosome glycosylation and provide a resource for development schistosome glycoprotein vaccine candidates".

Thank you. We have revised the sentence following your suggestion. (**Lines 514-516**)

Reviewer #2 (Remarks to the Author):

This study is interesting and exciting and provides the first overall glimpses into the complex glycomes of this key human parasite Schistosoma mansoni. Knowledge of the expression of its glycoproteins and their associated glycans in this work, which also identified tissue-specific and sex-biased glycosylation patterns, is key to developing better treatments and diagnostics for this parasite, which continues to infect humans worldwide. The main issues from this reviewer were in regard to the depiction of glycan structures, which the authors have correctly addressed, the lack of hard evidence for sialic acid being expressed on the glycans of the parasite rather than perhaps being low level contaminants from other sources, which the authors have completely and also nicely addressed. Overall, this study is thorough and robust, and uses state-of-the-art methods and technologies and represents a key advance in the field of molecular parasitology and the glycobiology of such parasitic helminths.

We sincerely appreciate your positive evaluation of our work. Thank you for your time and expertise, which has undoubtedly helped improve the clarity and rigor of this manuscript.

Reviewer #1 (Remarks to the Author):

This study applies a state-of-the-art approach to an extensive glycoproteomics study of a major human parasite, revealing aspects of glycoproteome complexity at a hitherto unachievable depth. The data presented form a valuable resource for the schistosome research community.
The manuscript has been carefully revised according to my comments and recommendations, which have been addressed sufficiently. Data are now more carefully interpreted and the conclusions that can be drawn from the data have now been more carefully phrased, or were removed when lacking foundation. In addition several minor issues with wording or clarity have been solved.

I have one suggestion left (not critical). In Fig 6 it would be great if panel b and c are aligned in such a way that the genes (e.g. OGT) are next to each other in each column. In panel b OGT is the top image, but in panel c it is the second from top. It could be better if for instance the legend in panel b is aligned with the control in panel c, and then below that the 4 genes next to each other in each panel instead of diagonally.

We sincerely thank you for the positive and constructive evaluation of our work. Your professional comments have significantly improved the clarity and quality of the manuscript. Figure 6 has been revised according to your suggestion.