RESPONSES TO REVIEWERS

Dear Dr. Guidali,

Thank you for your Stage 1 submission. I have received three insightful reviews on your proposed study. The reviewers see merit in the scientific question, and the design of the study to target the associated hypotheses, however there are some concerns about the theoretical support behind some of the hypotheses. To highlight some of the critical concerns:

Our anonymous reviewer requests you provide additional empirical and theoretical support for your hypotheses. For example, you already cite a few articles to support why the modulation of P30 and P60 align with empirical studies suggesting they are a valid marker for PAS-induced modulation, I suggest you expand on these citations and potentially add more to further support this hypothesis. Equally, the other hypotheses would benefit from further motivation. The anonymous reviewer and Dr. Veniero request clarity on the connectivity analysis and how it will result in evidence of connectivity modulation rather than change in cortical excitability. Finally, the anonymous reviewer highlighted that hypothesis 5 could simply result from subthreshold stimulation at 90% resting motor threshold. It is my interpretation that you hypothesize a modulation of the P60, with no impact on P30 – this would suggest 90% rMT is sufficient to activate the corticospinal tract. If that is the case, interpretation of a P60 modulation for H5 should be contingent on a lack of P30 modulation to preclude insufficient stimulation. A significant repeated measures ANOVA does not control for the lack of P30 modulation.

Dr. Oberman is concerned about the heterogeneity of your sample in producing expected PAS-LTP and PAS-LTD MEP effects. Dr. Oberman suggests a pre-test to include participants which show the expected potentiation and depreciation MEPs. I share Dr. Oberman's concern and request that you consider this pre-test, finally including the number of participants necessary to power the expected results. Alternatively, you should write a contingency plan: How will you interpret your TEP results (hypotheses H1-H5) if the MEP analysis (H0) shows no significant difference between the PAS-LTP and PAS-LTD MEPs? Along these lines, please could the authors complete the final column of the study design table. It may be more relevant to consider this final column as the alternatives to the hypothesized outcome which can elucidate the best approach for future analyses.

Dr. Veniero and Dr. Oberman highlight that you plan to collect the MEP and TEP data in serial rather than concurrently. As the MEP analysis acts as a positive control for following analyses on the TEP data, it seems sensible to consider collecting the data concurrently. Dr. Veniero also noted that the language surrounding the hypotheses should be more direct, which is the standard for registered reports. Wording like "we could speculate…" on page 5, should be replaced by "we hypothesize", and each hypothesis should be supported with the relevant empirical literature. Any hypotheses which seem more exploratory will need to be removed from the Stage 1 – please see my further comments on this below. Of course, this does not preclude these analyses from being performed in an exploratory section of the Stage 2.

From my position as a recommender, I have a few notes regarding the registered reports formatting.

Resp: We thank Dr. Edwards for giving us the opportunity to revise and improve our manuscript. We believe that we have addressed all the issues raised in Editor's and Reviewers' comments, providing point-to-point responses, which include references to relevant changes in the manuscript (highlighted in red in the revised text).

Hypotheses:

Hypothesis three is currently not strongly formulated, making targeted analyses, and the powering of these analyses very difficult. Please evidence your hypothesis for a particular connectivity effect following PAS. If there are multiple avenues to examine, you may want to consider running connectivity analyses in a more exploratory manner, reserved only for Stage 2. The same could be said for hypothesis four. As is currently written in the manuscript, you say effects could remain or disappear (page. 9) and that you could not state a priori how these profiles would progress over time for each stimulation protocol (page 6). For a registered report, a more solid hypothesis is critical, especially for powering your expected effect size.

Resp: We thank Dr. Edwards for point out these issues in our hypothesis. Following also the suggestions of Dr. Veniero and 3, we decided to remove connectivity analysis (i.e., **H3**) from planned analysis given the difficulty to make a clear a-priori hypothesis. Hence, connectivity analysis will be presented as exploratory analysis at the time of *Stage 2* submission. Considering **H4** (now **H3**), we fully agree that our a-priori hypothesis was ambiguous so, we have rephrased the Introduction and Sample size estimation paragraph to clearly state the direction of the expected effect (i.e., plastic effects should disappear after 30 minutes for both protocols) and we have updated Table 1 consequently.

Power analyses:

For hypotheses H2 - H5 the authors indicate there are no prior studies which provide evidence for a hypothesized effect size. This is an understandable and common concern when running power analyses. At PCIRR, we recommend that the authors examine the literature for effects sizes which align with their effect of interest, for example regarding H2: TEP modulation for N100 with LTD like stimulation protocols (e.g. Casula et al., 2014). The same can be said for effect sizes associated with H3 to H5. Assuming a medium effect size for these hypotheses is risky and may mean you will not have the power to examine the effects of interest. Using an assumed small effect size would reduce this risk. Alternatively, if you believe there is no way to predict the effect size of interest for these hypotheses, these analyses become more exploratory in nature. In which case these four sets of analyses should be removed from the registration.

Resp: We agree with Dr. Edwards that assuming a medium effect size for our power analysis could be risky and lead to underpowered investigations, especially when there is no previous literature hinting about the expected effect sizes. Hence, we have updated, to the best of our knowledge, our sample size estimations for

H2 to **H4** (and Table 1 accordingly) basing them on previous literature aligning with our effects of interest. In detail:

- **H2**: following Dr. Edwards suggestion, and the clarification asked by Dr. Veniero and Reviewer 3, we based our sample size estimation on the work of Casula and colleagues (2014) exploiting inhibitory rTMS and we used the lowest reported N100 pre *vs.* post difference for electrodes which potentially encompass the M1 ROI of our study (i.e., fronto-central ones). In the revised version of our *Stage 1* manuscript (see Introduction, p. XX), we decided to focus **H2** only on PAS_{LTD} due to the controversial evidence present in the literature for PAS_{LTP}; the latter does not allow us to make a clear a-priori hypothesis on the expected direction of modulation after the excitatory protocol. Therefore, the effects of PAS_{LTP} on the N100 will be investigated in an exploratory manner at the time of *Stage 2* submission. We now state (p. 9): "*Based on previous literature related to LTD and M1-TEPs (Casula et al., 2014), we hypothesize an enhancement (i.e., greater negativity) of the late N100 TEP component after delivering* PAS_{LTD}*. Given the absence of a similar comparison in previous TMS-EEG PAS studies (Costanzo et al., 2023; Huber et al., 2008), we based our estimate on the work by Casula et al. (2014) who found an enhancement of the M1-TEP N100 after low-frequency (i.e., inhibitory) repetitive TMS. The authors reported a difference in N100 amplitude over fronto-central electrodes of* $1.88 \pm 0.66\ \mu V$*, which corresponds to a Cohen's d of 2.85 (Casula et al., 2014). As for the previous estimations, to account for potential publication bias, we have considered half of the reported d (i.e., d = 1.42) for our power analysis. Here, the estimated sample size for a one-tailed dependent sample t-test is 10 participants.*"
- **H3** (**H4** in the previous version of our manuscript): here we now refer to the TMS-EEG study of Costanzo et al. (2023) who found a significant main effect of 'Time' after PAS_{LTP} administration in their work. Crucially, they exploited 3 timepoints as in our study. We now state (pp. 9-10): '*Our sample size estimation is based on the work by Costanzo and colleagues (2023), reporting a significant main effect of 'Time'* ($F_{2,30} = 4.679$, $p = 0.047$, $\eta_p^2 = 0.238$) *after* PAS_{LTP} *administration and exploiting three timepoints as in our study. As for the previous estimations, to account for potential publication bias, we have considered half of the reported* η_p^2 *(i.e.,* $\eta_p^2 = 0.119$*) for our rmANOVA power analysis. The estimated sample was found to be 18 participants.*'
- **H4** (**H5** in the previous version of our manuscript): for this hypothesis, we decided to still adopt a medium effect size value due to the absence of previous TMS-EEG studies investigating the effect of sub- *vs.* supra-threshold stimulation with a pre- *vs.* post-intervention experimental design as the present ones (i.e., 2 X 2). Nevertheless, we believe that, given previous literature on the contribution of MEP reafference to TEP components (Fecchio et al., 2017; Lioumis, Kičić, Savolainen, Mäkelä, & Kähkönen, 2009; Petrichella, Johnson, & He, 2017), our a-priori hypothesis is strong enough to be a planned one, and adopting a medium effect size allows us to have enough statistical power to detect the effects of interest. For instance, Lioumis and colleagues (2009), in a work investigating M1-TEP peak differences when TEPs were recorded sub- or supra-threshold (i.e., recorded at 90% and 110%

rMT, as in our study), reported (*a*) for P30, a mean amplitude of 2 ± 3.3 μV (subthreshold) and 6.1 ± 4.8 μV (suprathreshold), corresponding to a *d* of 1.76 for P30; and (*b*) for P60, a mean amplitude of 4.7 ± 5.6 μV (subthreshold) and 13.7 ± 9.3 μV (suprathreshold), corresponding to a *d* of 1.83 (Lioumis et al., 2009). With these values, and accounting for publication bias, the estimated sample size would be 20 participants. We have now clarified this at page 11: "*Considering only PAS_{LTP}, in the absence of a comparison between supra- vs. sub-threshold TEPs in previous TMS-EEG PAS studies (Costanzo et al., 2023; Huber et al., 2008) and previous TMS-EEG literature testing the effects of stimulation intensity in a pre- versus post-intervention experimental design as ours, we run a 2 X 2 rmANOVA power analysis hypothesizing a medium effect size (η_p^2 = 0.06)* (Fritz, Morris, & Richler, 2012). *Notably, given the effect sizes found in previous TMS-EEG literature that has explored M1-TEP modulations by applying TMS below or above the individual rMT (Lioumis et al., 2009), as well as in trials with or without MEPs (Petrichella et al., 2017), this value is configured as sufficient to detect statistically significant effects of interest."*

Minor

Please specify exactly where the EOG channels will be placed to pick up the blink and saccade artifacts, i.e. vertical and horizontal EOG.

Resp: We have now specified EOG channels position at p. 15: "*Two additional electro-oculographic (EOG) channels will be placed near the eyes (i.e., one above the right eyebrow and the other over the left cheekbone) to detect ocular artifacts due to eye movements and blinking (as done in, e.g., Bianco, Arrigoni, Di Russo, Romero Lauro, & Pisoni, 2023; Pisoni, Romero Lauro, Vergallito, Maddaluno, & Bolognini, 2018; Romero Lauro et al., 2014).*"

Reviewed by Domenica Veniero, 03 Oct 2023 12:41

This is a nice written report with a straightforward research plan and experimental design. I don't really have any major issues with the methodology or planned statistics. I only have one theoretical question and a few minor points.

Resp: We are grateful to Dr. Veniero for appreciating our study and for her helpful comments and suggestions.

1- one of the aims of the study is to investigate changes in connectivity following LTP-PAS and LTD-PAS. However, we do know that at some level the excitability of the motor cortex changes as well. Part of this report is set to test which specific components reflect pure cortical changes in M1. My point is if there is a change in the responsiveness of this cortex, would this not be why there is less spread of activation?

Resp: We thank Dr. Veniero for the pertinent remark, which, however, we agree with only in part. A lower signal spread in relation to a change in cortical excitability is certainly a plausible outcome of PAS. Indeed, a

study by Kuo et al. (2007) showed that PAS leads to focal increases in excitability by strengthening the synaptic connections of somatosensory and motor cortical neurons, as opposed to other non-invasive protocols inducing diffuse LTP/LTD-like neural effects (such as transcranial electrical direct current stimulation – tDCS). This suggests that PAS-induced effects are rather selective and possibly restricted to a specific neural population. However, to the best of our knowledge, there are few studies suggesting possible effects of PAS outside the motor cortex. For instance, Huber et al. (2008) reported an increase in TEPs at contralateral sites following PAS_{LTP} and a reduction in TMS-evoked activity anterior to the stimulation site following PAS_{LTD}. Lu et al. (2009) showed PAS effects on motor preparation as indexed by motor-related cortical potentials. They found that the late phase of the Bereitschaftspotential was reduced after PAS administration, suggesting that M1 PAS_{LTP} may reduce effective connectivity between premotor regions and the stimulated M1 (while not reporting any statistically significant effects of PAS_{LTD}).

Therefore, PAS may have to some extent the potential to induce complex patterns of modulation at both the network (within the sensorimotor system), and local (by modulating M1 cortical reactivity) levels.

Critically, changes in M1 connectivity following PAS have never been systematically assessed at the whole-brain level, especially with a multimodal approach such as TMS-EEG co-registration. Previous evidence shows that the proposed approach is particularly suitable to test and to disentangle effects that occur locally, from network effects occurring in distant regions following the administration of the two PAS protocols. In earlier studies by our group (Pisoni, Romero Lauro, et al., 2018; Romero Lauro et al., 2016, 2014), indeed, offline plastic changes by anodal tDCS were related to an enhancement of cortical excitability of the stimulated region together with an increase in signal spread within a network of structurally interconnected areas, reflecting the increased activity within a distributed network.

Nonetheless, we have reconsidered including **H3** among the planned analyses. Indeed, given the substantial lack of data in the literature to guide our hypotheses more specifically, perhaps it would be better to proceed in an exploratory manner at *Stage 2*.

2- I was wondering why you have to collect MEP and then TMS/EEG in different blocks, you will have 150 MEPs from this block.

Resp: To be honest, this choice was dictated by some practical needs related to our equipment. In our experience, when we concurrently record MEP and TEP in our laboratory, we often spot electrical noise in the EEG at the 50 Hz frequency. This is possibly due to electrical leakage due to the experimental setup. Typically, this is completely canceled out by applying a notch filter during preprocessing steps, and in no case, this issue has particularly affected further steps of signal processing and the following statistical analysis. In this case, since we intend to make the data public on open repositories (and thus, re-analyzable/re-usable), we believe that it is crucial to prioritize the acquisition of high-quality raw data by separating MEP and TEP recordings.

Moreover, we believe that there are also advantages in terms of experimental design in keeping the recording of MEPs separate from TEPs. Since MEPs analysis is our *positive control*, collecting these trials in a short, separate session that will always be collected at the same timepoints across participants (regardless of the

counterbalancing of TEP blocks recorded at different intensities – see **Figure 1**) will allow us to have an unbiased measure of the effects on corticospinal output. In addition, the number of trials planned for MEP analysis is in line with previous PAS studies (e.g., Costanzo et al., 2023; Stefan et al., 2000; Wolters et al., 2003), making our *positive control* more similar to recordings made in previous literature – which is a critical condition for replicating previous effects. Finally, considering the controversial results found in literature on MEP-TEP relationship when they are recorded together (see e.g., Bonato, Miniussi, & Rossini, 2006; Mäki & Ilmoniemi, 2010), we have not considered to conduct any pre-planned analysis comparing MEP and TEP effects, hence making the co-registration of MEPs and TEPs optional for our Registered Report.

3- Is the coil position identical in the Mep-block and TMS/EEG block?

Resp: Yes, we have now added this specification at p. 14: "*Coil positioning will be the same during EMG and EEG blocks*".

4- In the methods section it is stated that to be considered effective, the 90%MT will have to generate no MEPs in 10 trials. I am assuming this is still in the APB muscle. However, it is possible that the pulse will activate other hand muscles at least in some participants. I would encourage recording from additional hand muscles to make sure there are no MEPs.

Resp: We agree with Dr. Veniero that 90% stimulation of M1-ABP can likely activate other muscles, in particular the FDI, given their contiguous cortical representation in M1. However, the presence of MEPs with a 90% rMT stimulation is a remote possibility, as the 90% of rMT intensity should be enough to prevent the induction of MEPs overall. Nevertheless, to prevent this from happening, during rMT determination we will test the absence of MEP also by recording EMG from participants' FDI muscle. If we record MEPs of at least 50 μV in this second muscle, we will repeat the hunting procedure by refining motor hotspot searching. We have added this clarification in the Methods section at p. 14: "*To ensure that 90% rMT stimulation will not induce corticospinal tract response, a sanity check will be performed, in which no MEPs should be recorded in 10 consecutive trials from both APB and a cortical adjoining muscle (i.e., first dorsal interosseus – FDI) (Reijonen et al., 2020). If MEPs are present in one of these muscles at 90% rMT, motor hotspot searching will be refined until the sanity check is fulfilled.*" At p. 15, we state: "*Only during the sanity check for 90% rMT condition, MEPs from FDI muscle will be recorded to assess the absence of MEPs also in this second muscle (active electrode will be placed over the muscle belly and reference electrode over the metacarpophalangeal joint of the index).*"

5- I suggest replacing "we could hypothesise" with "we hypothesise". This is repeated a few times.

Resp: We have now rephrased the sentences accordingly.

6- Could you be clearer as to which direction you are expecting the N100 to change?

Resp: Following the suggestion of Reviewer 3, we have now made clearer the direction of N100 modulation (please see responses to Reviewer 3)

7- Page 16: The electrodes to be included in the ROI will be verified by visual inspection of the greatest response amplitude after the TMS pulse. Could you explain what this means? Is it a component, a global mean field power, or the first response? Also, if you are planning to change electrodes based on the biggest response in each participant, it should be clearly stated.

Resp: We acknowledge that we have been unclear on this point. Our choice of the electrodes to be included in the ROI is based on previous literature that has investigated the TMS-evoked response of M1 (e.g. Fecchio et al., 2017) and it is made to capture the regional effects of PAS protocols on the stimulated region. However, we will verify by visual inspection the greatest response in the grand average of all participants whether the selected ROI is adequate to measure the components of our interest. If the visual check suggests that we need to expand or modify our electrode selection, this will be reported and discussed. After that, the ROI will be kept fixed among the participants. We have now rephrased this passage in the methods section as follows (p. 16): "*The electrodes to be included in the ROI will be verified by visual inspection of the greatest response amplitude after the TMS pulse in the grand average of all participants. Then, the ROI will be kept fixed among the participants."*

8- "The possible spread of M1-PAS plastic effects outside the motor cortex has been poorly investigated". What do you mean by poorly?

Resp: We apologize for the lack of clarity. In the cited passage, we were referring to the paucity of studies that have investigated the effects of motor PAS outside the M1 local circuit. Indeed, the two studies concerning M1 connectivity changes after PAS presented in the introduction (and mentioned at point 1) are the only evidence resulting from our literature search. To date, there are no studies that systematically address potential changes in M1 effective connectivity following PAS interventions. We are aware that the main interest in applying this protocol is the induction of plastic phenomena at the local level. This is why part of this study is set to report the effects strictly related to M1. However, given the growing emphasis on the importance of achieving a more comprehensive understanding of the effects induced by neuromodulation techniques to control their inter-individual variability in the reported outcomes, we believe it is of interest to fill this gap in the literature. We will eventually address this issue in the exploratory analysis at the time of *Stage 2* submission.

9- "At first, trials with artifacts (muscular or background noise) deviating from 200 µV". Do you mean exceeding?

Resp: Yes, we have now corrected the typo.

Reviewed by Lindsay Oberman, 02 Oct 2023 15:22

This sounds like a well thought out study and one that will provide valuable information for the field. I have a few questions/concerns regarding the details of the methodology to ensure that this study will be optimally powered and designed to provide the data that the authors intend.

Resp: We thank Dr. Oberman for appreciating our work and the positive feedback.

1. There is a good deal of heterogenetiy in response to the PAS protocols and in my personal experience, about 50% do not display the expected facilitation to PAS-LTP/suppression to PAS-LTD. Thus, perhaps may be best to do a pre-test to determine whether they will respond to the PAS intervention and/or increase the enrollment numbers to account for this attrition.

Resp: We thank Dr. Oberman for highlighting the issue of variability in PAS-induced effects.

Considering increasing the enrollment number, we believe that we have already adopted a conservative approach in our power analyses: namely, we have considered half of the reported effect size for all our power analyses based on previous literature results (to account for publication bias). Hence, we believe that PAS effects (at least on MEPs) should be detectable even if non-responders will be present in our sample. Nevertheless, at the time of *Stage 2*, based on the results found in the planned analysis, an additional analysis comparing responders to non-responders (i.e., variability of PAS effects) could be performed. Secondly, we believe that the choice to enroll participants based on being a 'responder' at M1-PAS may be detrimental to the aim of our work. First of all, it would introduce a sample bias for the generalization of our results. Indeed, we aim to study M1-PAS effects at a population level (and not in a specific subgroup) and decided to make a Registered Report to better highlight the variability of outcomes of such stimulation protocol – which is still debated in the literature. Then, it is well known that the excitability of the corticospinal tract (i.e., MEPs) may not correspond to the excitability of cortico-cortical pathways (i.e., TEPs) (see, e.g., Guidali et al., 2023). Hence, doing a pre-test session based on PAS effects on MEPs (considering that, to date, this is the main variable to assess PAS responders) could lead to discarding participants who, in principle, may have PAS effects at the cortical level but not at the corticospinal one.

2. Time between visits (48 hours) and between single pulses (2000-2300ms) may not be sufficient to protect against visit-visit or pulse-pulse carryover. I would suggest longer (perhaps a week) between visits and longer (perhaps 5-8 seconds between single pulses).

Resp: Considering the time between visits, we agree that the interval may be too short to protect against possible visit-visit carryover; hence, we now state that (p. 11): "*The study will consist of a within-subjects design in two sessions separated by a washout period of at least one week to avoid PAS carry-over effects*".

Considering inter-pulse interval, previous studies already showed that this jittered time interval should be long enough to avoid pulse-pulse carryover effects (Casarotto et al., 2010; Lioumis et al., 2009; Romero Lauro et al., 2016). Casarotto et al., indeed, proved that, with even faster inter-trial interval, TEPs are a stable and reliable measure of cortical response, with no superadditive influence of pulses on their amplitude, as reported

by Lioumis et al. (2009) with a lower ISI. This point is also discussed in Romero Lauro et al., 2016, where the recording of TEPs in two subsequent blocks with the same inter trial interval and similar number of trials did not result in changes in local or global response to TMS. Hence, we decided to maintain this inter-pulse interval to avoid that the experiment would last too long.

3. They seem to exclude those on anxiolytics and antihistamines. Since these medications are often used PRN, how long are the investigators requiring that the participants abstain from these medications to be included in the study?

Resp: We have now specified that (p. 8): "*participants taking medications known to affect PAS effects (i.e., corticosteroids, anxiolytics, centrally acting ion channel blockers, or antihistamines) will be excluded from the study, unless, at the time of the first session of the experiment, they have not taken such medications for at least one month prior to the assessment (Suppa et al., 2017)*"

4. I am not clear why the investigators separated out the "EMG" and "EEG" blocks rather than measuring both concurrently? If concurrently then the investigators could look pulse by pulse and equate the EMG response to the EEG response of a given trial rather than only averages.

Resp: We thank Dr. Oberman for the insightful comment. As said to the Dr. Veniero, we have preferred to record MEPs and TEPs in separate blocks (*a*) to avoid 50 Hz contamination in the EEG trace, prioritizing the acquisition of high-quality signals and (*b*) to maintain an experimental design in line with previous literature, considering that MEPs are our *positive control* variable. For further details, please see our response to Dr. Veniero's second question who arise the same concern.

Reviewed by anonymous reviewer, 02 Oct 2023 14:57

Arrigoni and colleagues propose to use TMS-EEG to assess the cortical effects of the traditional PAS protocol proposed by Stefan and colleagues. This is an interesting study, and the experiment design suggested by the authors is straightforward. However, I have serious concerns about their methodology and hypotheses. My main critique is that they based their analysis and some of their hypotheses on the recent study by Costanzo et al., 2023 (doi: 10.3390/brainsci13060921). From my point of view, the study by Costanzo et al., 2023 has serious red flags that, unfortunately, were not detected by the reviewers; therefore, in my opinion their results are doubtful. In addition, the work by Arrigoni has a few conceptual interpretations that need to be clarified.

Resp: We thank the Reviewer for the positive feedback and for their suggestions which have undoubtedly enriched the quality of our work.

1. H0 (positive control): Effects of PAS protocols on MEP amplitude. This is valid and has value in the field for replicability of PAS-MEP results published in the literature.

Resp: We thank the Reviewer for this positive feedback.

2. **H1: Effects of PAS protocols on early positive TEP components (P30 and P60). I am concerned about the results published by Costanzo et al., 2023. Please note that my goal is not to criticize the work by Costanzo et al., 2023 but rather to avoid the same mistakes are repeated. First, Fig. 2 of their article suggests strong artifacts before 100 ms. The amplitude of components P30 and P60 are very large, and they look more like muscle artifacts or deflections produced by inappropriate data analysis. For instance, the deflection right after the time window the data the authors started to analyze is very large. Unfortunately, the time scale does not help. A Fig. showing the TEPs before using current source density (CSD) would have been helpful to assess the quality of the data. I am skeptical of the results reported by Costanzo et al., 2023. Arrigoni and colleagues put so much emphasis on the results by Constanzo et al., 2023. It is OK if Arrigoni and colleagues want to study those components, but a justification and rationale behind selecting those specific components is needed. Second, another critique about the experimental design by Costanzo et al., 2023 is that in one of their conditions, they stimulated with peripheral electrical stimulation alone and delivered 180 pulses, which could have induced modulation by itself. This is a major issue. Therefore, I suggest Arrigoni and colleagues rethink in more detail if they want to focus on investigating the effects of the PAS protocols on P30 and P60. Again, if they want to proceed like that it is O.K., but they need to include a better rationale on why those components want to study.**

Resp: we thank the Reviewer for warning us about some critical limitations spotted in the work of Costanzo and colleagues. As a matter of fact, we share the same concerns about the methodology and results presented in Costanzo et al., 2023. Indeed, the very same concerns mentioned by the Reviewer were the starting point of the present registered report, aimed at testing whether the findings concerning P30 and P60 modulations are indeed replicable in a more controlled design. However, we would like to clarify that the emphasis on Costanzo's work was prompted by the format requested for the registered report.

As Costanzo et al. (2023) is to date the only group study in the available literature that used TMS-EEG to evaluate the effects of a PAS protocol, it was inevitable for us to use the data reported in this paper to provide an estimate of the expected effects and thus ground our power analyses, since it is the study that comes closest to ours in terms of experimental design and other methodological aspects.

With respect to the control condition in Costanzo et al. mentioned by the Reviewer (i.e., peripheral electrical stimulation only), we have to admit that it was unclear to us that the recording was performed before the PAS protocol was applied, as the report is not clear about this. We therefore contacted the authors, who confirmed this methodological choice. We are aware that this approach may have biased the results in some way, so we clearly do not intend to proceed in this way in our work.

Using the reported effects for the application of another neuromodulation intervention (e.g., Casula et al., 2014) or on another neurophysiological outcome measure could still have involved approximation.

Concerning the rationale for expecting P30 and P60 modulations, we agree that it deserves to be expanded.

At pp. 5-6, we added the following information: "*P30 and P60 are often used as biomarkers of cortical excitability in studies aimed at assessing the effects of non-invasive neuromodulation techniques inducing LTD/LTP-like phenomena within the motor system by means of TMS-EEG (for a reviews, see: Cruciani et al., 2023).*

In detail, P30 is thought to reflect fast excitatory mechanisms within the M1 local circuit (Mäki & Ilmoniemi, 2010; Rogasch, Daskalakis, & Fitzgerald, 2013). *Hence, P30 was reported to be positively correlated with MEP amplitude (Ferreri et al., 2011; Mäki & Ilmoniemi, 2010). Corroborating this hypothesis, intermittent (iTBS) and continuous (cTBS) theta-burst TMS –used to transiently increase and suppress motor cortex excitability, respectively – influence P30 amplitude in the same direction of MEP modulations. For instance, inhibition of P30 was found following cTBS (Vernet et al., 2013) and Gedankien and colleagues (2017) showed that changes in N15-P30 TEP and MEP amplitudes following iTBS were significantly correlated* (Gedankien, Fried, Pascual-Leone, & Shafi, 2017).

On the other hand, P60 component has been associated with the activity of recurrent cortico-cortical and cortico-subcortical circuits reflecting glutamatergic signal propagation mediated by AMPA receptor activation (Belardinelli et al., 2021). Previous TMS-EEG evidence showed that the P60 component can be modulated by drugs influencing gamma-aminobutyric acid (GABA) neurotransmission (Gordon, Belardinelli, Stenroos, Ziemann, & Zrenner, 2022), *suggesting that P60 amplitude likely reflects an excitation/inhibition balance of the stimulated region. In fact, different TMS and transcranial direct current stimulation interventions significantly modulated the amplitude of the TMS-evoked P60 after their application (Chung et al., 2019; Mosayebi-Samani et al., 2023).*"

3. H2: Effects of PAS protocols on the N100. Arrigoni and colleagues suggested "Based on previous literature about LTD and M1-TEPs (Casula et al., 2014), we hypothesize a modulation of the late N100 TEP component after delivering PASLTD but not after PASLTP." This is not accurate. Indeed, N100 is a biomarker of inhibition as was suggested by Nikulin et al., 2003 (https://doi.org/10.1046/j.1460-9568.2003.02858.x) and see also the work by Kicic et al., 2008 (https://doi.org/10.1016/j.neuroscience.2008.01.043). However, the interpretation by Arrigone et al. is not accurate. When PAS induces LTP-like effects, it should be expected that N100 decreases because there is more excitation; in contrast, when PAS induces LTD-like effects, the amplitude of N100 should increase or not be affected because LTD reflects more inhibition. Please rethink this hypothesis and consult the literature to support your claims.

Resp: We agree with the Reviewer about the neurophysiological meaning of the N100. Indeed, this was clearly stated in our Introduction (p. 6): "*it is well known that the N100 is a marker of inhibitory processing mediated by GABA receptors and different studies related the modulation of this component to the induction of inhibitory-like phenomena or plastic effects* (Bonnard, Spieser, Meziane, De Graaf, & Pailhous, 2009; Casula

et al., 2014; Premoli et al., 2018, 2014; Rogasch et al., 2013). *Similarly, we expect that the N100 is influenced by PAS_{LTD} administration.*"

We also agree with the Reviewer that, theoretically, both PAS protocols can have effects on the amplitude of this specific component – and not only PAS_{LTD}, as we stated in the original version of our manuscript. However, considering the Registered Report format, we reasoned that our hypothesis on the PAS_{LTP} protocol should be grounded on the findings of the only available TMS-EEG report that analyzed N100 modulation after PAS (LTP) administration. Here, previous evidence did not document any significant modulation of this component (i.e., Costanzo et al., 2023). Furthermore, current TMS-EEG literature is controversial about N100 modulation after excitatory TMS protocols, like intermittent or continuous theta burst stimulation (Bai, Zhang, & Fong, 2021; Chung et al., 2019; Desforges et al., 2022; Goldsworthy et al., 2020). Altogether, this evidence suggests us to be more cautious in have a strong a-priori hypothesis on the N100 after PAS_{LTP}.

Hence, we have now clearly stated in the Introduction that the planned **H2** will be focused only on the investigation of N100 effects after PAS_{LTD}. Conversely, N100 modulations after PAS_{LTP} administration will be investigated exploratorily at the time of *Stage 2* submission. We have now rephrased the paragraph as follows (p. 6): "*Hence, considering the inhibitory nature of this component, we hypothesize that PAS_{LTD} administration would lead to a greater (negative) amplitude of this component. Noteworthy, Costanzo et al. (2023) did not find any significant modulation of the N100 after PAS_{LTP}. So, given the controversial literature on N100 modulations after the administration of excitatory TMS protocols (Bai et al., 2021; Chung et al., 2019; Desforges et al., 2022; Goldsworthy et al., 2020), PAS_{LTP} effects will be investigated exploratory.*"

4. H3: Effects of PAS on cortico-cortical connectivity patterns. It is unclear how the authors will study cortico-cortical connectivity. If they aim to analyze the spatial distribution "topoplots" produced by the TEPs, this is not cortico-cortical connectivity but cortical excitability. They discussed in page 16, Section "Source reconstruction" they will do a source-level analysis. However, it is unclear or lacking how they will assess cortico-cortical connectivity. So far, the description is very generic.

Resp: We apologize for the lack of clarity in the description of the analytical procedures to assess changes cortico-cortical connectivity after PAS intervention. However, we never state in the manuscript that **H3** testing will entail the analysis of "the spatial distribution (topoplots)". Conversely, we plan to perform TEP source reconstruction to analyze signal propagation from left M1 (precentral gyrus) to other cortical parcels in the 400 ms after TMS pulse. This approach has been extensively used in TMS-EEG literature to assess effective connectivity. Importantly, this method was already applied to test the changes in connectivity (Massimini et al., 2005), and this approach has also been applied to track changes in effective connectivity following neuromodulatory interventions (Pisoni, Mattavelli, et al., 2018; Romero Lauro et al., 2016, 2014). However, given the concerns raised also by Dr. Veniero and Dr. Edwards, and rethinking about the exploratory nature of this analysis, we reconsidered the inclusion of **H3** in the planned analysis. Instead, we will remove **H3** from *Stage 1* and will perform the analysis exploratorily at the time of *Stage 2* submission.

5. H4: Temporal evolution of induced plasticity. This is acceptable how is.

Resp: We thank the Reviewer for the feedback.

6. H5: Effects of TMS pulse intensity on the modulation of P30 and P60 after PASLTP. Please see the comment about H1. I did not understand the goal of this approach. It seems the authors want to investigate whether an intensity of 90% has a minor effect on reafferent fibers compared to 110%. For studying only TEPs, this approach is valid. However, if the goal is to investigate the effect of PAS on TEPs, this approach could be problematic. Mainly because using subthreshold intensities for PAS may not be enough to activate the corticospinal tract and, together with electrical stimulation, will not affect cortical modulation. The authors may want to revise this approach.

Resp: We believe that the Reviewer misunderstood the passage concerning the use of a 90% rMT intensity in our experimental procedures. Indeed, this intensity will be used only for TEPs recording to test **H5**. As stated by the Reviewer, this is a valid method for disentangling the contribution of reafferent processing in TEPs signals. On the other hand, we have never planned to use 90% rMT for TMS intensity during the PAS administration. As a matter of fact, applying a subthreshold stimulation could prevent from eliciting contingent M1 activation driven by endogenous (i.e., peripheral stimulation) and exogenous (i.e., TMS) paired stimulations. To improve readability, we now rephrased the sentence at page 12 as follows: "*During PAS administrations, TMS will be always set at 110% rMT*". Moreover, we have now reported the stimulation parameters also in the Figure summarizing the experimental procedures.

Other comments:

7. Introduction, page 3: "In detail, when the ISI matches the timing in which the afferent sensory signal from the median nerve electrical stimulation reaches M1 (i.e., 25 ms), LTP is induced (PASLTP), with an increase in post-PAS MEPs amplitude (Conde et al., 2012; Fratello et al., 2006; Nitsche et al., 2007; Stefan et al., 2000; Wolters et al., 2003; Ziemann et al., 2004)." To my knowledge, this statement is not accurate. To induce LTP the ISI should be slightly longer than the connection time. Please see in detail the introduction in the review by Suppa et al., 2017, and the work by Brzosko et al., 2019 (https://doi.org/10.1016/j.neuron.2019.05.041)

Resp: We agree with the Reviewer and thus rephrased the sentence as follows (p. 3): "*when the ISI closely resembles the timing*".

8. EEG preprocessing, page 15-16. "Independent Component Analysis (FastICA, pop_tesa_fastica, 'tahn' contrast) after PCA compression to 30 components (pop_tesa_pcacompress) will be performed to remove blinks, eye movements, residual electrical artifacts, and spontaneous muscular activity by visual inspection." You will first compress and then apply FastICA? If that is the case, please rewrite this sentence because it reads that you first will do FastICA and then PCA compression, which would

be incorrect. Also, add the corresponding citation when discussing PCA compression (doi:10.1016/j.jneumeth.2012.05.029).

Resp: We apologize for the misunderstanding. Undoubtedly, we plan to perform PCA compression before FastICA application. We rephrased the sentence as follows (p. 16): "*Independent Component Analysis (FastICA, pop_tesa_fastica, 'tanh' contrast) will be performed after Principal Component Analysis (PCA) compression to 30 components (pop_tesa_pcacompress). FastICA will be applied to remove blinks, eye movements, residual electrical artifacts, and spontaneous muscular activity by visual inspection (Hernandez-Pavon et al., 2012)*".

9. In general, the literature reviews should be deepened: please add more complete original papers where needed.

Resp: We thank the Reviewer for this suggestion. We believe that the edited version of the *Stage 1* manuscript provides now a more detailed literature background to back up the rationale of the proposed study.

Reference

Bai, Z., Zhang, J., & Fong, K. N. K. (2021). Intermittent theta burst stimulation to the primary motor cortex reduces cortical inhibition: A TMS-EEG study. *Brain Sciences*, *11*(9). https://doi.org/10.3390/brainsci11091114

Belardinelli, P., König, F., Liang, C., Premoli, I., Desideri, D., Müller-Dahlhaus, F., … Ziemann, U. (2021). TMS-EEG signatures of glutamatergic neurotransmission in human cortex. *Scientific Reports*, *11*(1), 1–14. https://doi.org/10.1038/s41598-021-87533-z

Bianco, V., Arrigoni, E., Di Russo, F., Romero Lauro, L. J., & Pisoni, A. (2023). Top-down reconfiguration of SMA cortical connectivity during action preparation. *IScience*, *26*(8), 107430. https://doi.org/10.1016/j.isci.2023.107430

Bonato, C., Miniussi, C., & Rossini, P. M. (2006). Transcranial magnetic stimulation and cortical evoked potentials: A TMS/EEG co-registration study. *Clinical Neurophysiology*, *117*(8), 1699–1707. https://doi.org/10.1016/j.clinph.2006.05.006

Bonnard, M., Spieser, L., Meziane, H. B., De Graaf, J. B., & Pailhous, J. (2009). Prior intention can locally tune inhibitory processes in the primary motor cortex: Direct evidence from combined TMS-EEG. *European Journal of Neuroscience*, *30*(5), 913–923. https://doi.org/10.1111/j.1460-9568.2009.06864.x

Casarotto, S., Lauro, L. J. R., Bellina, V., Casali, A. G., Rosanova, M., Pigorini, A., … Massimini, M. (2010). EEG responses to TMS are sensitive to changes in the perturbation parameters and repeatable over time. *PLoS ONE*, *5*(4). https://doi.org/10.1371/journal.pone.0010281

Casula, E. P., Tarantino, V., Basso, D., Arcara, G., Marino, G., Toffolo, G. M., … Bisiacchi, P. S. (2014). Low-frequency rTMS inhibitory effects in the primary motor cortex: Insights from TMS-evoked potentials. *NeuroImage*, *98*, 225–232. https://doi.org/10.1016/j.neuroimage.2014.04.065

Chung, S. W., Sullivan, C. M., Rogasch, N. C., Hoy, K. E., Bailey, N. W., Cash, R. F. H., & Fitzgerald, P. B. (2019). The effects of individualised intermittent theta burst stimulation in the prefrontal cortex: A TMS-EEG study. *Human Brain Mapping*, *40*(2), 608–627. https://doi.org/10.1002/hbm.24398

Costanzo, M., Leodori, G., Cutrona, C., Marchet, F., De Bartolo, M. I., Mancuso, M., … Fabbrini, G. (2023). Motor Cortical Correlates of Paired Associative Stimulation Induced Plasticity: A TMS-EEG Study. *Brain Sciences*, *13*(6), 921. https://doi.org/10.3390/brainsci13060921

Cruciani, A., Mancuso, M., Sveva, V., Maccarrone, D., Todisco, A., Motolese, F., … Capone, F. (2023). Using TMS-EEG to assess the effects of neuromodulation techniques: a narrative review. *Frontiers in Human Neuroscience*, *17*(August), 1–15. https://doi.org/10.3389/fnhum.2023.1247104

Desforges, M., Hadas, I., Mihov, B., Morin, Y., Rochette Braün, M., Lioumis, P., … Tremblay, S. (2022). Dose-response of intermittent theta burst stimulation of the prefrontal cortex: A TMS-EEG study. *Clinical Neurophysiology*, *136*, 158–172. https://doi.org/10.1016/j.clinph.2021.12.018

Fecchio, M., Pigorini, A., Comanducci, A., Sarasso, S., Casarotto, S., Premoli, I., … Rosanova, M. (2017). The spectral features of EEG responses to transcranial magnetic stimulation of the primary motor cortex depend on the amplitude of the motor evoked potentials. *PLoS ONE*, *12*(9), 1–15. https://doi.org/10.1371/journal.pone.0184910

Ferreri, F., Pasqualetti, P., Määttä, S., Ponzo, D., Ferrarelli, F., Tononi, G., … Rossini, P. M. (2011). Human brain connectivity during single and paired pulse transcranial magnetic stimulation. *Neuroimage*, *54*(1), 90–102.

Fritz, C. O., Morris, P. E., & Richler, J. J. (2012). Effect size estimates: Current use, calculations, and interpretation. *Journal of Experimental Psychology: General*, *141*(1), 2–18. https://doi.org/10.1037/a0024338

Gedankien, T., Fried, P. J., Pascual-Leone, A., & Shafi, M. M. (2017). Intermittent theta-burst stimulation induces correlated changes in cortical and corticospinal excitability in healthy older subjects. *Clinical Neurophysiology*, *128*(12), 2419–2427. https://doi.org/10.1016/j.clinph.2017.08.034

Goldsworthy, M. R., Rogasch, N. C., Ballinger, S., Graetz, L., Van Dam, J. M., Harris, R., … Ridding, M. C. (2020). Age-related decline of neuroplasticity to intermittent theta burst stimulation of the lateral prefrontal cortex and its relationship with late-life memory performance. *Clinical Neurophysiology*, *131*(9), 2181–2191. https://doi.org/10.1016/j.clinph.2020.06.015

Gordon, P. C., Belardinelli, P., Stenroos, M., Ziemann, U., & Zrenner, C. (2022). Prefrontal theta phase-dependent rTMS-induced plasticity of cortical and behavioral responses in human cortex. *Brain Stimulation*, *15*(2), 391–402. https://doi.org/10.1016/j.brs.2022.02.006

Guidali, G., Zazio, A., Lucarelli, D., Marcantoni, E., Stango, A., Barchiesi, G., & Bortoletto, M. (2023). Effects of transcranial magnetic stimulation (TMS) current direction and pulse waveform on cortico-cortical connectivity: A registered report TMS-EEG study. *European Journal of Neuroscience*, *58*(8), 3785–3809. https://doi.org/10.1111/ejn.16127

Hernandez-Pavon, J. C., Metsomaa, J., Mutanen, T., Stenroos, M., Mäki, H., Ilmoniemi, R. J., & Sarvas, J. (2012). Uncovering neural independent components from highly artifactual TMS-evoked EEG data. *Journal of Neuroscience Methods*, *209*(1), 144–157. https://doi.org/10.1016/j.jneumeth.2012.05.029

Huber, R., Määttä, S., Esser, S. K., Sarasso, S., Ferrarelli, F., Watson, A., … Tononi, G. (2008). Measures of cortical plasticity after transcranial paired associative stimulation predict changes in electroencephalogram slow-wave activity during subsequent sleep. *Journal of Neuroscience*, *28*(31), 7911–7918. https://doi.org/10.1523/JNEUROSCI.1636-08.2008

Lioumis, P., Kičić, D., Savolainen, P., Mäkelä, J. P., & Kähkönen, S. (2009). Reproducibility of TMS - Evoked EEG responses. *Human Brain Mapping*, *30*(4), 1387–1396. https://doi.org/10.1002/hbm.20608

Mäki, H., & Ilmoniemi, R. J. (2010). The relationship between peripheral and early cortical activation induced by transcranial magnetic stimulation. *Neuroscience Letters*, *478*(1), 24–28. https://doi.org/10.1016/j.neulet.2010.04.059

Massimini, M., Ferrarelli, F., Huber, R., Esser, S. K., Singh, H., & Tononi, G. (2005). Neuroscience: Breakdown of cortical effective connectivity during sleep. *Science*, *309*(5744), 2228–2232. https://doi.org/10.1126/science.1117256

Mosayebi-Samani, M., Agboada, D., Mutanen, T. P., Haueisen, J., Kuo, M. F., & Nitsche, M. A. (2023). Transferability of cathodal tDCS effects from the primary motor to the prefrontal cortex: A multimodal TMS-EEG study. *Brain Stimulation*, *16*(2), 515–539. https://doi.org/10.1016/j.brs.2023.02.010

Petrichella, S., Johnson, N., & He, B. (2017). The influence of corticospinal activity on TMS-evoked activity and connectivity in healthy subjects: A TMS-EEG study. *PLoS ONE*, *12*(4), 1–18. https://doi.org/10.1371/journal.pone.0174879

Pisoni, A., Mattavelli, G., Papagno, C., Rosanova, M., Casali, A. G., & Romero Lauro, L. J. (2018). Cognitive Enhancement Induced by Anodal tDCS Drives Circuit-Specific Cortical Plasticity. *Cerebral Cortex*, *28*(4), 1132–1140. https://doi.org/10.1093/cercor/bhx021

Pisoni, A., Romero Lauro, L. J., Vergallito, A., Maddaluno, O., & Bolognini, N. (2018). Cortical dynamics underpinning the self-other distinction of touch: A TMS-EEG study. *NeuroImage*, *178*(March), 475–484. https://doi.org/10.1016/j.neuroimage.2018.05.078

Premoli, I., Király, J., Müller-Dahlhaus, F., Zipser, C. M., Rossini, P., Zrenner, C., … Belardinelli, P. (2018). Short-interval and long-interval intracortical inhibition of TMS-evoked EEG potentials. *Brain Stimulation*, *11*(4), 818–827. https://doi.org/10.1016/j.brs.2018.03.008

Premoli, I., Rivolta, D., Espenhahn, S., Castellanos, N., Belardinelli, P., Ziemann, U., & Müller-Dahlhaus, F. (2014). Characterization of GABAB-receptor mediated neurotransmission in the human cortex by paired-pulse TMS-EEG. *NeuroImage*, *103*, 152–162. https://doi.org/10.1016/j.neuroimage.2014.09.028

Reijonen, J., Pitkänen, M., Kallioniemi, E., Mohammadi, A., Ilmoniemi, R. J., & Julkunen, P. (2020). Spatial extent of cortical motor hotspot in navigated transcranial magnetic stimulation. *Journal of Neuroscience Methods*, *346*. https://doi.org/10.1016/j.jneumeth.2020.108893

Rogasch, N. C., Daskalakis, Z. J., & Fitzgerald, P. B. (2013). Mechanisms underlying long-interval cortical inhibition in the human motor cortex: A TMS-EEG study. *Journal of Neurophysiology*, *109*(1), 89–98. https://doi.org/10.1152/jn.00762.2012

Romero Lauro, L. J., Pisoni, A., Rosanova, M., Casarotto, S., Mattavelli, G., Bolognini, N., & Vallar, G. (2016). Localizing the effects of anodal tDCS at the level of cortical sources: A Reply to Bailey et al., 2015. *Cortex*, *74*, 323–328. https://doi.org/https://doi.org/10.1016/j.cortex.2015.04.023

Romero Lauro, L. J., Rosanova, M., Mattavelli, G., Convento, S., Pisoni, A., Opitz, A., … Vallar, G. (2014). TDCS increases cortical excitability: Direct evidence from TMS-EEG. *Cortex*, *58*, 99–111. https://doi.org/10.1016/j.cortex.2014.05.003

Suppa, A., Quartarone, A., Siebner, H., Chen, R., Di Lazzaro, V., Del Giudice, P., … Classen, J. (2017). The

associative brain at work: Evidence from paired associative stimulation studies in humans. *Clinical Neurophysiology*, *128*(11), 2140–2164. https://doi.org/10.1016/j.clinph.2017.08.003

Vernet, M., Bashir, S., Yoo, W. K., Perez, J. M., Najib, U., & Pascual-Leone, A. (2013). Insights on the neural basis of motor plasticity induced by theta burst stimulation from TMS-EEG. *European Journal of Neuroscience*, *37*(4), 598–606. https://doi.org/10.1111/ejn.12069