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Peer Review File

α-ATP suppression in 31P MR spectroscopy by a Double Echo Modified z-Filter (DEMz): An approach to quantify NADH and NAD+ at 3T in vivo

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

NCOMMS-23-08754

The authors present novel and excellent data on non-invasive NAD+/NADH detection and quantitation in skeletal muscle. Clinical strength magnetic field and advanced 31P MRS acquisition protocol was applied to partially suppress adjanced alpha-ATP signal which enabled previously impossible distinction of NAD+/NADH signals. Rigorous post-processing of the spectra allowed for robust determination of NAD+ and its reduced form NADH. Authors the method in the phantom solution, determined the reproducibility in vivo, detected the expected changes in NAD+/NADH ratio during ischemic challenge tested its clinical value in cross sectional study on trained and untrained groups of older adults.
The approach to the measurement is innovative, the study is well conducted and presented, and the results are important to the field of clinical physiological and metabolic research. The conclusions are based on the results. Never-the-less, certain points in the method description, graphical data presentation and discussion should be improved.
Following points should be addressed:
1. Extensive and exact prior knowledge for the MRS line deconvolution is obviously crucial for the method. Authors mention this point in the Method, Discussion and Conclusion section without giving details on it. Please provide the prior knowledge and reference the source – own experiments and/or literature.

2. Proton decoupling have been shown and used to improve the 31P MRS line shapes at 3T. Could it be incorporated in this sequence to improve the distinction between NAD+ and NADH?

3. RF pulse shapes and durations should be given, and authors should discuss, if there were any issues with specific absorption rate. Here and for the above-mentioned point the summarizing table from recent consensus paper on reporting standards by Lin et al. NMR Biomed 2021 could be appropriate guideline.

4. In the issue of non-perfect the B1-homogeneity and thus compromised effectiveness of alpha-ATP suppression, authors should also discuss if and how the use of half volume or multiple-channel coils could improve the issue together with possible reductions (if necessary) of SAR.

5. For better comprehension of ischemic changes and differences in the cross-sectional study example spectra including fitted lines in pre- & post- ischemic conditions and trained vs. normally active adults should be given in the Figure 4 and 5, respectively.

Reviewer #2 (Remarks to the Author):

During the last two decades, NAD+ metabolism has been consistently shown to influence age-related physiological decline and metabolic disease in mice and humans. Methods to measure NAD+ in a non-invasive fashion, however, remain scarce in the clinical setting. In this work, Mevenkamp and colleagues exploit 31P-MRS for this purpose by using J-difference-based spectral editing to manipulate MRS signals and unmask NAD metabolites that are generally overshadowed by the spectral overlap with the (alpha-)ATP signal. The authors validate this possibility by evaluating the changes in NAD and NADH content in human muscle after ischemia, as well as differences between physically active vs. sedentary individuals.

This work convincingly demonstrate that NAD+ and NADH levels might be approached in human skeletal muscle through the use of ATP suppression editing in 31P-MRS. The use in two separate cohorts, plus the relatively homogenous readouts in both, suggest that the method is robust and reproducible. This said, a few aspects would be needed to strengthen the manuscript and validate their technique. My main suggestions are below:

- Introduction: in line 36, the authors state that NAD+ was shown to be an important regulator of mitochondrial biogenesis. This is a bit of a biased statement. The manuscript cited does not really demonstrate that. While multiple correlations exist, several studies have already shown that NAD+ supplementation does not have per se any major effect on mitochondrial content or respiratory capacity in human skeletal muscle, including obese and aged individuals. The authors might want to clarify the scope of their sentence or provide a more balanced view.

- Line 218. The authors mention that the signal loss of NAD+ between FID and HB edited spectra was 22.9%. How consistent was this loss across in spectra from human skeletal muscle. For the non-experts in the field, this seems like a great loss of signal, considering that changes in NAD+ levels are relatively subtle. Could the authors discuss this point, and its implications, a bit more in the text?

- Figure 3: It is unclear to me how the NADH and NAD+ signals seem to selectively differ between the FID and HB edited spectra. One should assume that the alpha-ATP signal masked the NAD+, but not the NADH signal. Could the authors use a phantom sample with alpha-ATP, NADH and NAD+ (in the lines of Figure 2) to truly show that this is the case?

- Figure 4: This figure raises some questions. First, how were the NAD+/NADH ratios calculated? Some of the numbers don't seem to match. For example, if we take the darker line, NADH/gATP value is around 1, while the NAD+/gATP value is 3. One should expect a NAD+/NADH ratio of 3, yet is is 4. Some patients did not present changes in their NAD+/NADH ratio (see the second lighter line, for example), albeit showing a decline in NAD+ (and no change in NADH). Second, NADH does not seem to increase, but actually, decrease in a couple samples. Was there any particularity in these two patients, or could this be simply due to the lower ability of this technique to accurately measure NADH? Finally, some of the drastic decreases in NAD+ cannot be accounted by increases in NADH, suggesting NAD+ degradation. Is this what is expected in 10 minutes of ischemia?

- The authors often talk about measuring NAD+ or NADH concentrations. Early in the text, the authors should clarify that they always refer to relative concentrations, not absolute concentrations.

- In my opinion, the main weakness of this work is that the authors do not benchmark their method to other technical approaches in general. This leaves the reader with not many tools to judge the accuracy and advantages of this method vs. other. Could the authors experimentally approach a comparison of their technique to, for example, standardised enzymatic or MS methodologies in order to fully show that their results are consistent? Second, and in line with this point, the authors might want to do a table to compare the different approches to evaluate NAD/NADH in humans, highlighting their pros and cons. This is, to some degree, done in the discussion, but a table would help visualising how this new approach could contribute to a better clinical implementation of NAD+ metabolism evaluation.

- Through the discussion, some references seem off. For example, in line 323, they mention that the high NAD+ contractions in the more active group are in line with recently published evidence, citing Grevendonk et al. However, in that paper no NAD+ measurement is done. Also, the prefer to results in preclinical models supporting that physical activity is important to maintain NAD+ levels. While the statement is well supported in the literature, none of the two papers cited explore this point. Hence a thorough revision of their references would be strongly encouraged.

Reviewer #3 (Remarks to the Author):

In this paper, Mevenkamp et al. report a new 31P MRS pulse sequence enabling them to access in vivo concentrations of NADH and NAD+ in skeletal muscle at a clinical MRI field strength of 3T. Under these experimental circumstances, the NADH/NAD+ responses of interest are overlapped with the dominant ATP 31P alpha-resonance. This complicates quantification of NADH and NAD+. Yet, in vivo NADH/NAD+ ratios provide physiologically important and clinically relevant information, and it would indeed be very useful to access these. The authors demonstrate their sequence first on phantoms, then in vivo. Finally, they applied their new experiment in a cross-sectional study involving older adults.

Note: this reviewer is a specialist in pulse sequence design for liquid NMR spectroscopy (not MRS or MRI), and is not involved in clinical research. I will therefore refrain from commenting on the setup and results of the cross-sectional study.

The new pulse sequence works by exploiting the difference in homonuclear J-modulation behaviour of the ATP, NAD+ and NADH 31P signals. The authors call their sequence “homonuclear BIRD” editing, but I find this is a misnomer. Firstly, BIRD homonuclear editing as introduced by Garbow et al. in 1982 works by exploiting a J-coupling interaction to a heteronuclear spin, providing a composite 180° magnetisation inversion or refocusing only if this heteronuclear interaction is present. If only one of the homonuclear coupling partners experiences such heteronuclear coupling, the 180° inversion becomes selective for that spin (without ever having used rf-selective pulses). However, this is not the case here, as no such heteronuclear coupling is exploited. One could envision a homonuclear spin to play the role of the

heteronuclear coupling partner using rf-selective pulses, but again, this is not what is happening here. Secondly, the total spin echo (TE) delay is equal to 1/2J rather than 1/J as in the original BIRD sequence. The authors have done this intentionally in order to generate antiphase magnetisation and to limit the duration of the time spent in the transverse plane by the magnetization. However, it further shows that a different mechanism than actual BIRD homonuclear editing is used.

A more accurate way to describe the pulse sequence, would be as the spin-echo with z-filter experiment by Richard Ernst et al. (Sørensen, O. W.; Rance, M.; Ernst, R. R. J. Magn. Reson. 1984, 56, 527-534; http://dx.doi.org/10.1016/0022-2364(84)90317-2). Firstly, a simple homonuclear J-modulation occurs during the spin echo, with a delay that should make the ATP magnetisation antiphase and along the x-axis at the end of the echo (90°x-tau-180°-2tau-180°-tau). Secondly, the antiphase magnetisation is purged by using a z-filter element (90°(-x)-delay-90°x) before acquisition. Indeed, the first figure in the Ernst 1984 paper shows a conceptionally identical pulse sequence to the one proposed in the current manuscript, albeit without gradients and adiabatic pulses. Furthermore, in Ernst's spin-echo and z-filter experiment, the complications from zero-quantum coherences are removed in an identical manner as what the authors propose, namely by varying the delay between the 90° pulses and signal averaging.

Taken together, I believe it is much more appropriate to associate this sequence with the z-filtered spin echo by Sørensen, Rance and Ernst than with BIRD.

I have some further concerns with the experiment. The alpha-response from ATP is a clear doublet, due to the other 31P nuclei resonating at very different chemical shifts. For NADH, the response is a singlet, since the two 31P signals are fully chemical shift degenerate. (I disagree with the statement in the introduction that it is 'uncoupled' — rather, the homonuclear coupling is not expressed in the spectrum due to the (near-)complete chemical shift degeneracy). The 31P spins in NAD+ form a strongly coupled AB spin system, meaning its two 31P signals feature as two closely resonating doublets with strongly attenuated outer signals. The product operator analysis discusses correctly the situation for ATP (weakly coupled doublet) and NADH (singlet), but it does not discuss how the NAD+ signals would respond to the sequence. If the two 31P nuclei are indeed not fully degenerate as the authors mention, I would expect some signal loss taking place due to J-modulation for NAD+ also. The experiment with phantoms only compares the signal losses of NAD+ and ATP, but a similar experiment comparing NAD+ and NADH signal losses was not performed. This should be included to my opinion to at least verify no dissimilar signal attenuation occurs, which is what the authors implicitely assume.

In the discussion, the authors mention some possible future improvements of the sequence to improve the ATP signal suppression. They speculate T1 relaxation during the spoil gradient delay (the z-filter) as a possible reason for the remaining ATP signal. If this would be the case, the way to mitigate this would be a simple two-step phase cycle of the first 90° pulse (x,-x) and receiver phase (x,-x) during the signal averaging, which should be easy to test on the phantoms.

Another possibility that the authors mention is the incomplete ATP signal suppression could be due to a mismatched TE delay with ATP's 31P-31P J-coupling, then could the authors not have conducted experiments on the phantoms (or even the initial in vivo study) with different values of the TE delay to determine the optimal delay?

The paragraph discussing future improvements on the sequence is not so clear to read. “ATP spins not fully aligned with x after TE or not fully excited and refocussed will be affected by the final 90°-x pulse…”. And later: “… spoiler gradients … could remove these unaligned spins”. This is all somewhat poorly formulated. What is probably meant is that the ATP magnetisation (not individual spins) may not be perfectly aligned with the x-axis before the second 90° pulse, and that phase cycling or gradient pulses, could improve the performance of the pulse sequence by imposing the coherence transfer pathway shown in Figure 1 (which is not mentioned in the figure caption). They make a number of suggestions, but it is not clear why they have not tested these simple modifications in the first place? For instance, again, on the phantoms.

The same paragraph also mentions “aforementioned B1 inhomogeneity”, while this concept was not previously mentioned.

Finally, did the authors consider the use of 1H decoupling during the FID to further narrow the 31P resonances of NAD+, NADH and ATP (cf. as shown in reference 36)?

Although I find the work interesting and its application appears important, I am left with the impression that the new pulse sequence has not been properly optimised for performance as much as it could have been. In addition, a verification using the phantoms that the output NADH and NAD+ ratios obtained are actually unbiased by the pulse sequence (T2, J-modulation) is lacking. I therefore find this work somewhat too preliminary for Nature Communications.

Some minor remarks:
*The abbreviation HB is used before it is introduced.
*Reference 20 lacks the journal name.

Reviewer # 1

Q: *The authors present novel and excellent data on non-invasive NAD+/NADH detection and quantitation in skeletal muscle. Clinical strength magnetic field and advanced 31P MRS acquisition protocol was applied to partially suppress adjanced alpha-ATP signal which enabled previously impossible distinction of NAD+/NADH signals. Rigorous post-processing of the spectra allowed for robust determination of NAD+ and its reduced form NADH. Authors the method in the phantom solution, determined the reproducibility in vivo, detected the expected changes in NAD+/NADH ratio during ischemic challenge tested its clinical value in cross sectional study on trained and untrained groups of older adults. The approach to the measurement is innovative, the study is well conducted and presented, and the results are important to the field of clinical physiological and metabolic research. The conclusions are based on the results. Never-the-less, certain points in the method description, graphical data presentation and discussion should be improved. Following points should be addressed:*

"Extensive and exact prior knowledge for the MRS line deconvolution is obviously crucial for the method. Authors mention this point in the Method, Discussion and Conclusion section without giving details on it. Please provide the prior knowledge and reference the source – own experiments and/or literature."

A: Our prior knowledge included starting values for relative chemical shifts and fixed T_2 values for all resonances of interest. The prior knowledge was added to the manuscript and is listed in the table below (table S7 in supplementary of manuscript). The T_2 values for all fitted resonances, aside from NADH and NAD^+, are based on the values reported by Bogner et al (1). From the range of values given by Bogner et al., we chose T_2 values within the reported 95% confidence interval that best fit the linewidth of our data (that showed the least residual).

With respect to the T_2 values for NAD+ and NADH we based our assumptions on reports of Lu et al (2). They describe that based on the similar molecular structure and electronic environment of the phosphate groups within NADH, NAD+, and α-ATP their NMR properties are naturally similar too. Therefore, we assumed the T_2 values to be similar to the α-ATP resonance. When applying the fit to measured data, it became apparent that the fit of the combined NADH and NAD^+ resonances improved when NAD^+ was fitted with a slightly shorter T_2 than NADH. Therefore, this is what we implemented.

The table below presents the T_2 values for all fitted resonances in our fitting model and the starting values for their chemical shifts. This table is included in the supplementary material of the current version of the manuscript, together with explanations regarding the basis of the prior knowledge.

Table S7: Fitting prior knowledge. From reference (1), various T_2 values within the 95% confidence interval were tested and the T2 that resulted in the best fit (lowest residual) was chosen for further implementation. Furthermore, T_2 values for NADH and NAD^+ were assumed to be similar to the T2 of ATP (Lu et al.) and further optimization showed that a slightly shorter T2 for NAD+ resulted in a better fit (less residual) which is the basis for the prior knowledge as given in the table. The same prior knowledge was used for all in vivo measurements.

Metabolite	**Assumed T2 (ms)**		**Relative Chemical Shift at 3T (Hz)**	
		Source		***Source***
Pi	148	(1)	-258.5	(3)
GPE	44		-165.46	
GPC	28		-142.71	
PCr	334	(1)	0.0	(3)
γ-ATP	62		128.23	
α-ATP	47		388.82	
NADH	42		425.85	(4)

NAD+	32	Estimated	437.59	
β-ATP	55	(1)	835.0	(3)

1. Bogner W, Chmelik M, Schmid AI, Moser E, Trattnig S, Gruber S. Assessment of 31P relaxation times in the human calf muscle: A comparison between 3 T and 7 T in vivo. Magnetic Resonance in Medicine. 2009;62(3):574--82.

2. De Graaf RA. In vivo NMR spectroscopy: principles and techniques. John Wiley & Sons; 2013.

3. Graaf RA, Feyter HM, Brown PB, Nixon TW, Rothman DL, Behar KL. Detection of cerebral NAD+ in humans at 7T. Magnetic Resonance in Medicine. 2017;78(3):828--35.

Additionally, to better explain the exact fitting procedure, we added the following passage to the manuscript on page 11: *"Furthermore, homonuclear coupling patterns of NAD^+ resonances were added to the fitting model. NAD^+ was modelled as a strongly coupled quartet based on the findings of Lu et al. (2). The chemical shift between the two NAD^+ resonances at 3T was also calculated based on Lu et al. and deduced to be 24.43 Hz. α-ATP and γ-ATP were modelled as doublets of which the respective coupling constants were determined and fitted for each participant individually based on their acquired reference FIDs."*

The rationale behind this is that the 90-x RF pulse at the end of the editing sequence creates a z-component of α-ATP magnetization that was not aligned with the x-axis at time point TEJ. Consequently, this z-component is unaffected by the spoiler gradient for magnetization in the transversal plane, including its acquired phase relative to the rest of the magnetization in the subsequent Free Induction Decay (FID) as shown in the figure below (and in the manuscript on page 7). In principle, this could be prevented by individually adjusting TEJ. However, we decided to keep TEJ at 27.5ms for all scans so that T_2 relaxation introduced by the double spin echo during spectral editing for NADH and NAD^+ remains constant between participants. Moreover, with smaller J-coupling constants TEJ increases to the point where the strongly coupled NAD^+ resonances are more affected by our editing sequence and start to lose intensity as shown in Figure 5 & Figure 6 for reviewer#3. This would introduce additional variation between volunteers. Regarding the additional phase, we added the following passage to the manuscript on page 10/11:

"To accommodate for the expected phase deviation caused by individual α-ATP J-coupling constant not meeting the condition TEJ = 1/2J we calculated the phase deviation according to the following equation and added it to the fitting model for the α-ATP resonance:

$$\Delta\phi_J = 2\pi \cdot TE_j \cdot \Delta J$$

$\Delta J = 18.18Hz - J_{Measured}$

TEJ = 27.5*10^-3 s"

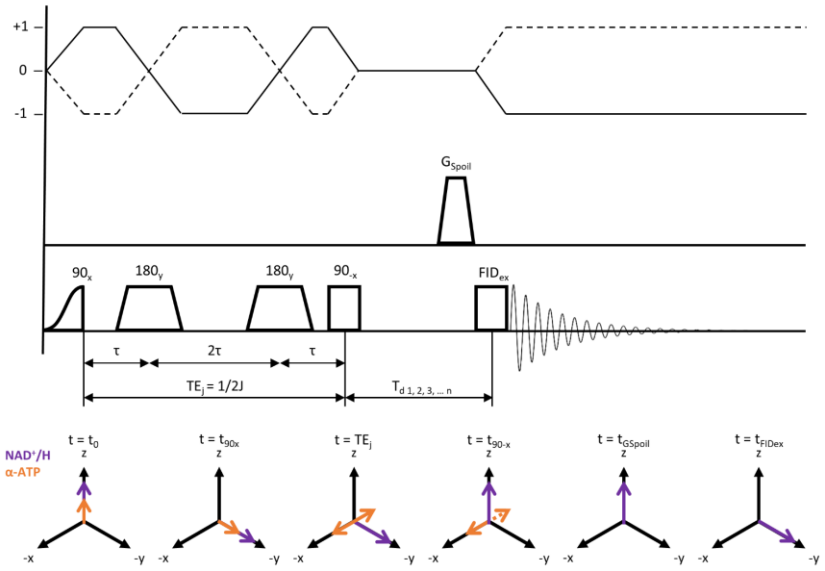

Figure 1: Scheme of the double echo modified z-Filter (DEMz) sequence as used in the current approach. After an initial adiabatic half passage hard pulse and two adiabatic full passage pulses, J-coupled α-ATP spins acquire a phase of 90° with respect to uncoupled spins at t= TE_j = 1/2J and end up aligned with x (t = TE_j). At $t_{90\text{-}x}$ a non-selective 90° block pulse flips spins with J-coupling constants different from that of α-ATP about the x-axis back towards the z-axis. Spins remaining in the x-y plane are then dephased by G_{Spoil}. A varying delay reduces potential phase distortions caused by zero quantum coherences (ZQC).

Q: *"Proton decoupling has been shown and used to improve the 31P MRS line shapes at 3T. Could it be incorporated in this sequence to improve the distinction between NAD+ and NADH?"*

A: We thank the reviewer for providing this valuable suggestion. Indeed, we did explore the effect of incorporating proton decoupling in our sequence, as it has been shown to be helpful in measurements in the brain (Lu et al.). However, our experience is that no visible benefits on spectral resolution of the α-ATP resonance is achieved by using 1H decoupling in our set-up in skeletal muscle. As a result, we decided not to apply proton decoupling in our study. This is incorporated in the discussion on page 22: "Efforts to further enhance spectral resolution at lower field strength involved the utilization of ^{1}H decoupling in ^{31}P spectra (5). Despite the promising results reported in the brain (5), we did not observe a reduction in ATP and NAD+ resonance linewidths when applying ^{1}H decoupling at 3T in the skeletal muscle."

Q: *"RF pulse shapes and durations should be given, and authors should discuss, if there were any issues with specific absorption rate. Here and for the above-mentioned point the summarizing table from recent consensus paper on reporting standards by Lin et al. NMR Biomed 2021 could be appropriate guideline."*

A: We agree with the reviewer that incorporating this information will enhance the clarity of the manuscript. Therefore, we have included the relevant details regarding the RF pulse shapes and durations in table S5 in the supplementary material of the manuscript:

Pulse Shape	Duration in ms	Philips Name
Half Passage Adiabatic	4.6528	hs_ex_500_400_100
Full Passage Adiabatic	3.4620	oit_800_6500
Block	0.096	BLOCK

Table S5: Details of the pulses used

The Specific Absorbance Rate (SAR) according to the calculations of the MR-scanner is <7.3 W/kg, which is well below the allowable limit of 20 W/kg for extremity scans. As can be expected based on these calculations, we did not encounter any SAR-related issues in any of the measurements in this study. This was added to the methods section on page 6.

Q: *"In the issue of non-perfect B1-homogeneity and thus compromised effectiveness of alpha-ATP suppression, authors should also discuss if and how the use of half volume or multiple-channel coils could improve the issue together with possible reductions (if necessary) of SAR."*

A: As described by Adriany & Gruetter 1996 JMR, the use of half volume coils has the potential to enhance B1-homogeneity and SNR. Multichannel coils would improve SNR and B1 homogeneity as described by (6) and indeed could both be beneficial for the current protocol. Unfortunately, half volume coils or X-nuclei multi channel coil arrays and receiver amplifiers are not widely available, also not at our institute. Our intention was to develop a method, which can be widely applicable at other sites and is not dependent on very specialized coils and therefore, we think it is essential that we showed the feasibility using standard coils. However, we completely agree with the reviewer that such coils could lead to future improvements of the method and we have addressed this option and its potential benefits in the 'discussion' section of the manuscript (p. 23), by adding the following sentence: "The ATP suppression that is currently reached is not absolute. A possibility to improve the suppression could be to use 1/J as TEj, which would allow to nul residual ATP signals by tuning the spoiler delay to a fixed value based on the T_1 relaxation of ATP. However, this comes at the cost of a prolonged TE_j which leads to increased T_2 signal loss of NAD^+ and NADH, and is therefore not beneficial. The current (non-complete) suppression of ATP appears to be sufficient to uncover and quantify NAD metabolites, while sparing NAD+ and NADH signal intensity."

Q: *"For better comprehension of ischemic changes and differences in the cross-sectional study example spectra including fitted lines in pre- & post- ischemic conditions and trained vs. normally active adults should be given in the Figure 4 and 5, respectively."*

A: We have adapted Figures 4 and 5 according to the reviewer's suggestion on page 14 and 16-17 of the current version of the manuscript to include the information as suggested by the reviewer.

Reviewer # 2

Q: *During the last two decades, NAD+ metabolism has been consistently shown to influence age-related physiological decline and metabolic disease in mice and humans. Methods to measure NAD+ in a non-invasive fashion, however, remain scarce in the clinical setting. In this work, Mevenkamp and colleagues exploit 31P-MRS for this purpose by using J-difference-based spectral editing to manipulate MRS signals and unmask NAD metabolites that are generally overshadowed by the spectral overlap with the (alpha-)ATP signal. The authors validate this possibility by evaluating the changes in NAD and NADH content in human muscle after ischemia, as well as differences between physically active vs. sedentary individuals.*

This work convincingly demonstrate that NAD+ and NADH levels might be approached in human skeletal muscle through the use of ATP suppression editing in 31P-MRS. The use in two separate cohorts, plus the relatively homogenous readouts in both, suggest that the method is robust and reproducible. This said, a few aspects would be needed to strengthen the manuscript and validate their technique. My main suggestions are below:

Q: *"Introduction: in line 36, the authors state that NAD+ was shown to be an important regulator of mitochondrial biogenesis. This is a bit of a biased statement. The manuscript cited does not really demonstrate that. While multiple correlations exist, several studies have already shown that NAD+ supplementation does not have per se any major effect on mitochondrial content or respiratory capacity in human skeletal muscle, including obese and aged individuals. The authors might want to clarify the scope of their sentence or provide a more balanced view."*

A: We agree that NAD+ supplementation has not shown very pronounced effects in humans. However, this can be due to many reasons and importantly, the lack of effect of supplementation does not mean that NAD+ is not a crucial regulator of mitochondrial function. Indeed, the references were off, we thank the reviewer for noticing and replaced them by appropriate references.

Q: *"Line 218. The authors mention that the signal loss of NAD+ between FID and HB edited spectra was 22.9%. How consistent was this loss across spectra from human skeletal muscle? For the non-experts in the field, this seems like a great loss of signal, considering that changes in NAD+ levels are relatively subtle. Could the authors discuss this point, and its implications, a bit more in the text?"*

A: To determine this experimentally, in vivo, signals with and without editing would need to be compared. However, it is impossible to quantify NAD in-vivo without editing, due to the overlapping α-ATP resonance in the in-vivo FID spectrum, which in fact highlights the necessity of the editing sequence. Therefore, the editing-induced signal loss was determined in a phantom with ATP and a high concentration of NAD+ and NADH (3.5 mM). With the high concentration, combined with better shimming in phantoms, it was possible to quantify the NADH and NAD^+ signals in both the FID and the edited spectra and determine the editing-induced signal loss. As we used the same TEJ for all measurements, we expect that the signal loss due to the editing sequence was consistent across all participants and conditions (normoxia, ischemia, trained and sedentary). Potential other variations in signal loss could be based on variations in T2-associated signal loss. However, there is no reason to expect major differences in T2 relaxation times across individuals, as generally these differences are small as documented by Bogner at al. (1). Furthermore, the NAD^+ and NADH content is given as a ratio, relative to ATP and any other variations in signal intensity is expected to affect both (NAD metabolites and ATP) similarly, leaving the ratio unchanged and therefore comparable between measurements.

This is added to the methods part of the manuscript on page 11: *"As we used the same TEJ for all measurements, we expect that the signal loss due to the editing sequence was consistent across all participants and conditions (normoxia, ischemia, trained and sedentary). Potential other variations in signal loss could be based on variations*

in T2-associated signal loss. However, there is no reason to expect major differences in T2 relaxation times across individuals, as generally these differences are small as documented by Bogner at al. (1). Furthermore, the NAD^+ and NADH content is given as a ratio, relative to ATP and any other variations in signal intensity is expected to affect both (NAD metabolites and ATP) similarly, leaving the ratio unchanged and therefore comparable between measurements."

Q: *"Figure 3: It is unclear to me how the NADH and NAD^+ signals seem to selectively differ between the FID and HB edited spectra. One should assume that the alpha-ATP signal masked the NAD+, but not the NADH signal. Could the authors use a phantom sample with alpha-ATP, NADH and NAD+ (in the lines of Figure 2) to truly show that this is the case?"*

A: We acknowledge the reviewer's observation that figure 4 may suggest that the NADH and NAD^+ signals seemingly selectively differ between FID and edited spectra. This is however misleading, as the quantification of NADH and NAD^+ in the (non-edited) FID is not reliable. Therefore, the high NADH signal in the FID and the seemingly high signal loss when compared to the edited spectrum, in fact only shows the difficulty of quantifying NADH and NAD when no editing is used and ATP is strongly overlapping (with both, NADH and NAD+). In the edited spectra, which show a far lower α-ATP intensity, the NADH and NAD^+ fit becomes more reliable. To address the question of the individual signal loss of the two resonances, we quantified the signal loss for both, NAD+ and for NADH in phantoms, where quantification in the FID is possible and found very consistent signal loss for the two metabolites (22.9% and 22.7% respectively). This is shown in figure 2 of the current version of the manuscript. In this figure the overlap of α-ATP with both, NADH & NAD+, is clearly visible. NAD+ however is overlapping slightly less with α-ATP compared to NADH because of NAD^+'s lower chemical shift frequency, but still visibly overlapping. This, in combination with broader lines in the in-vivo situation, makes editing for NAD detection clearly necessary for in vivo quantification.

Q:*"Figure 4: This figure raises some questions. First, how were the NAD+/NADH ratios calculated? Some of the numbers don't seem to match. For example, if we take the darker line, NADH/gATP value is around 1, while the NAD+/γATP value is 3. One should expect a NAD+/NADH ratio of 3, yet is is 4. Some patients did not present changes in their NAD+/NADH ratio (see the second lighter line, for example), albeit showing a decline in NAD+ (and no change in NADH). Second, NADH does not seem to increase, but actually, decrease in a couple samples. Was there any particularity in these two patients, or could this be simply due to the lower ability of this technique to accurately measure NADH? Finally, some of the drastic decreases in NAD+ cannot be accounted by increases in NADH, suggesting NAD+ degradation. Is this what is expected in 10 minutes of ischemia?"*

A: The NAD+/NADH ratio was calculated by $NAD_{rat} = AU_{NAD+}/AU_{NADH.}$

We appreciate the reviewer for bringing to our attention the inconsistency in figure 4 (with some cases where NAD+/NADH is not changing, while NAD+ appears to decline, while showing no change in NADH). We noticed that this apparent contradiction was due to a mistake in the color-coding scheme of the graph. We have now corrected the graph, which is figure 6, in the current version of the manuscript on 14 and can be found on page 18.

The two cases that show decreasing concentrations during ischemia are not due to bad spectral quality or any other peculiarity in the spectra. We have added the following paragraph to the discussion on page 23, as an explanation for the apparent NADH increase during ischemia: *"In two participants we observed a rise in NADH relative concentrations of 6.7% and 7.9% respectively during ischemia, which was unexpected. However, this*

increase lies within the 10% variation of fitted NADH amplitudes as shown by its CRLB and therefore falls within the expected variation."

Q: *"The authors often talk about measuring NAD+ or NADH concentrations. Early in the text, the authors should clarify that they always refer to relative concentrations, not absolute concentrations."*

A: We agree with the reviewer. We have now clarified this early on in the manuscript at all instances that mentioned concentrations earlier.

Q: *"In my opinion, the main weakness of this work is that the authors do not benchmark their method to other technical approaches in general. This leaves the reader with not many tools to judge the accuracy and advantages of this method vs. other. Could the authors experimentally approach a comparison of their technique to, for example, standardised enzymatic or MS methodologies in order to fully show that their results are consistent? Second, and in line with this point, the authors might want to do a table to compare the different approches to evaluate NAD/NADH in humans, highlighting their pros and cons. This is, to some degree, done in the discussion, but a table would help visualising how this new approach could contribute to a better clinical implementation of NAD+ metabolism evaluation."*

A: In order to show the linearity and the quantitative nature of the MRS method, we performed measurements on phantoms in a range of different concentrations and added this to the manuscript in figure 3. Furthermore, in a group of volunteers we performed both, the MRS measurements and metabolomics in a muscle biopsy. This data shows reasonable agreement of the in vivo and ex vivo method, as shown below and in figure 8. We summarized the pros and cons of the currently available techniques to determine NAD metabolites in a table, as shown below and added to the manuscript as table 1. The main advantage of the MRS method clearly is the non-invasive nature of the technique, for all other techniques, muscle biopsies need to be taken.

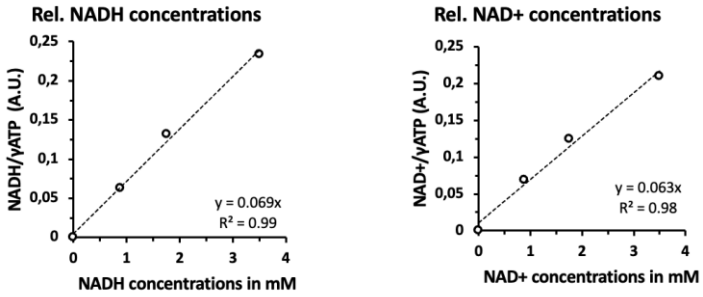

Figure 2: Relative concentrations resulting from DEMz spectra of NADH (A) and NAD^+ (B) phantoms over actual NADH and NAD^+ concentrations of phantom compartments in mM.

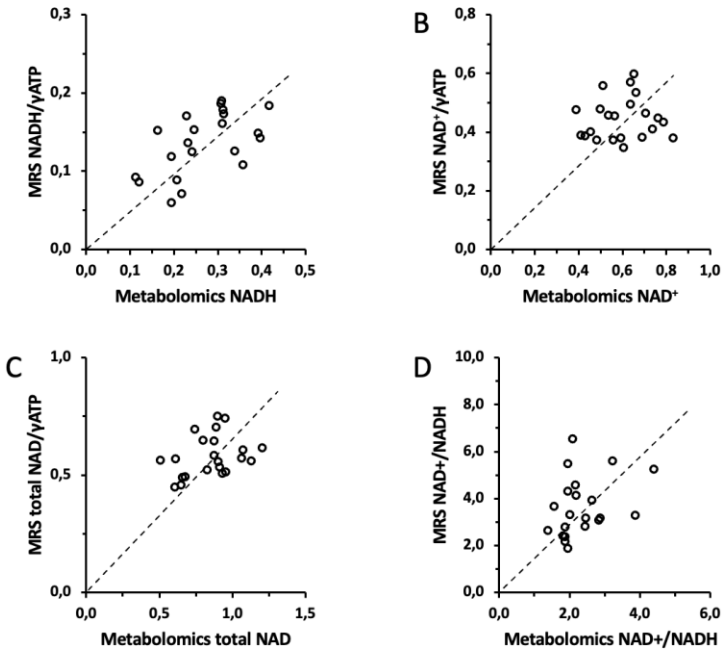

Figure 3: NADH (A), NAD^+ (B), NAD^+/NADH ratio (C) and total NAD (D) quantified by metabolomics plotted against their respective MRS results and trend line (stippled line).

Legend: ++ = *Superior* + = *Yes or Possible* - = *No or Not possible*	Detects NAD^+	Detects NADH	Sensitivity	Detects other NAD related metabolites	In-situ measurements	Non-Invasive	Sample degradation during processing	Repetitive measurements	Measures absolute concentrations	Whole muscle NAD determination
Biochemical Detection										
Fluorometric Assays (18)	-	+	+	-	-	-	+	+	+	-
Spectrophotometric Assays(18, 19)	+	+	+	-	-	-	+	+	+	-
High Performance Liquid Chromatography (20)	+	+	+	-	-	-	+	+	+	-
Metabolomics(11)	+	+	+	+	-	-	+	+	+	-
Fluorescence Imaging										
Fluorescence Imaging (21-23)	-	+	+	-	+	-	-	+	+	-
MRS										
^1H(24, 25)	-	+	-	-	+	+	-	++	+	+
^{31}P(15, 26-28)	+	+	+	+	+	+	-	++	+*	+

Table 1: Overview of invasive and non-invasive NAD metabolite detection methods.

Q: *"Through the discussion, some references seem off. For example, in line 323, they mention that the high NAD+ contractions in the more active group are in line with recently published evidence, citing Grevendonk et al. However, in that paper no NAD+ measurement is done. Also, the prefer to results in preclinical models supporting that physical activity is important to maintain NAD+ levels. While the statement is well supported in the literature, none of the two papers cited explore this point. Hence a thorough revision of their references would be strongly encouraged."*

A: Thank you, we referred to the wrong paper, it should be Janssens et al. We also went through the other references and adapted them when necessary. We apologize for this inconsistency.

Reviewer # 3

Q: *In this paper, Mevenkamp et al. report a new 31P MRS pulse sequence enabling them to access in vivo concentrations of NADH and NAD+ in skeletal muscle at a clinical MRI field strength of 3T. Under these experimental circumstances, the NADH/NAD+ responses of interest are overlapped with the dominant ATP 31P alpha-resonance. This complicates quantification of NADH and NAD+. Yet, in vivo NADH/NAD+ ratios provide physiologically important and clinically relevant information, and it would indeed be very useful to access these. The authors demonstrate their sequence first on phantoms, then in vivo. Finally, they applied their new experiment in a cross-sectional study involving older adults.*

Note: this reviewer is a specialist in pulse sequence design for liquid NMR spectroscopy (not MRS or MRI), and is not involved in clinical research. I will therefore refrain from commenting on the setup and results of the cross-sectional study

The new pulse sequence works by exploiting the difference in homonuclear J-modulation behaviour of the ATP, NAD+ and NADH 31P signals. The authors call their sequence "homonuclear BIRD" editing, but I find this is a misnomer. Firstly, BIRD homonuclear editing as introduced by Garbow et al. in 1982 works by exploiting a J-coupling interaction to a heteronuclear spin, providing a composite 180° magnetisation inversion or refocusing only if this heteronuclear interaction is present. If only one of the homonuclear coupling partners experiences such heteronuclear coupling, the 180° inversion becomes selective for that spin (without ever having used rf-selective pulses). However, this is not the case here, as no such heteronuclear coupling is exploited. One could envision a homonuclear spin to play the role of the heteronuclear coupling partner using rf-selective pulses, but again, this is not what is happening here. Secondly, the total spin echo (TE) delay is equal to 1/2J rather than 1/J as in the original BIRD sequence. The authors have done this intentionally in order to generate antiphase magnetisation and to limit the duration of the time spent in the transverse plane by the magnetization. However, it further shows that a different mechanism than actual BIRD homonuclear editing is used.
A more accurate way to describe the pulse sequence, would be as the spin-echo with z-filter experiment by Richard Ernst et al. (Sørensen, O. W.; Rance, M.; Ernst, R. R. J. Magn. Reson. 1984, 56, 527-534; http://dx.doi.org/10.1016/0022-2364(84)90317-2). Firstly, a simple homonuclear J-modulation occurs during the spin echo, with a delay that should make the ATP magnetisation antiphase and along the x-axis at the end of the echo (90°x-tau-180°-2tau-180°-tau). Secondly, the antiphase magnetisation is purged by using a z-filter element (90°(-x)-delay-90°x) before acquisition. Indeed, the first figure in the Ernst 1984 paper shows a conceptionally identical pulse sequence to the one proposed in the current manuscript, albeit without gradients and adiabatic pulses. Furthermore, in Ernst's spin-echo and z-filter experiment, the complications from zero-quantum coherences are removed in an identical manner as what the authors propose, namely by varying the delay between the 90° pulses and signal averaging.
Taken together, I believe it is much more appropriate to associate this sequence with the z-filtered spin echo by Sørensen, Rance and Ernst than with BIRD."

A: We agree with the reviewer's suggestion, and we have now renamed the editing sequence as "Double Echo modified z-Filter (DEMz)" instead of "homonuclear BIRD editing".

Q: *"I have some further concerns with the experiment. The alpha-response from ATP is a clear doublet, due to the other 31P nuclei resonating at very different chemical shifts. For NADH, the response is a singlet, since the two 31P signals*

are fully chemical shift degenerate. (I disagree with the statement in the introduction that it is 'uncoupled' — rather, the homonuclear coupling is not expressed in the spectrum due to the (near-)complete chemical shift degeneracy). The 31P spins in NAD+ form a strongly coupled AB spin system, meaning its two 31P signals feature as two closely resonating doublets with strongly attenuated outer signals. The product operator analysis discusses correctly the situation for ATP (weakly coupled doublet) and NADH (singlet), but it does not discuss how the NAD+ signals would respond to the sequence. If the two 31P nuclei are indeed not fully degenerate as the authors mention, I would expect some signal loss taking place due to J-modulation for NAD+ also. The experiment with phantoms only compares the signal losses of NAD+ and ATP, but a similar experiment comparing NAD+ and NADH signal losses was not performed. This should be included to my opinion to at least verify no dissimilar signal attenuation occurs, which is what the authors implicitely assume"

A: We agree with the reviewer that the description of the NADH and NAD+ spin systems was not precise. We adjusted it based on the reviewer's comment and refer to NADH as a singlet due to (near-)complete shift degeneracy. Furthermore, we performed new measurements with a phantom containing 5 mM ATP and 3.5 mM NADH to compare signal losses with respect to each other. We acquired FID and edited spectra of this phantom, using the same protocol as for the in-vivo studies earlier. Spectra from the FID and edited FID were subsequently fitted and the ratio of the NADH intensity in the edited spectrum to the NADH intensity in the FID was calculated to determine the signal loss. The signal loss for NADH was 22.7%, which was very similar to the signal loss of NAD+ (22.9%). These results confirm that no dissimilar signal attenuation occurs. We added this to the manuscript on page 12 (last paragraph), together with figure 2, as depicted here:

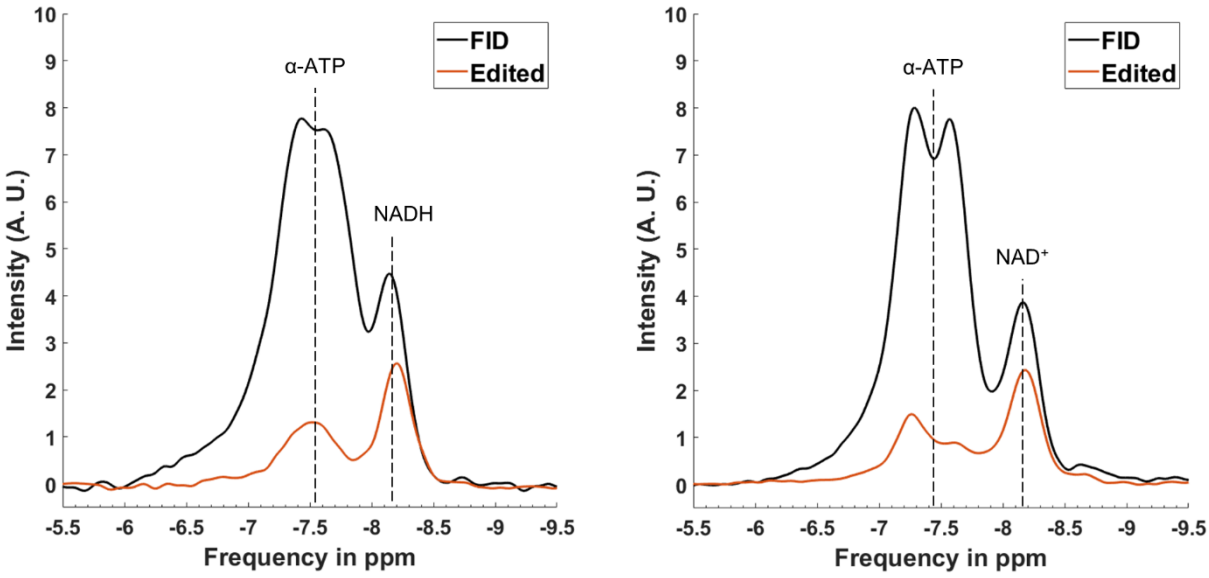

Figure 4: Comparison of spectra resulting from FID and Double Echo Modified z-Filter acquisition in phantoms composed of ATP and NADH (A) or ATP and NAD⁺ (B). α-ATP suppression of 85% with an approximate signal loss of 22.7% for NADH and 22.9% for NAD⁺ were observed

Q: *"In the discussion, the authors mention some possible future improvements of the sequence to improve the ATP signal suppression. They speculate T1 relaxation during the spoil gradient delay (the z-filter) as a possible reason for the remaining ATP signal. If this would be the case, the way to mitigate this would be a simple two-step phase cycle of*

the first 90° pulse (x,-x) and receiver phase (x,-x) during the signal averaging, which should be easy to test on the phantoms"

A: We agree with the reviewer that this might be an approach to remove signal that has returned by T1 recovery. As the current study is a proof of principle study, we acknowledge there is still room for improvement and fine tuning in the future. We agree that for future optimisation, the suggestion by the reviewer should be tested. Importantly, our study shows that the principle of the sequence works. Testing further improvements will not change the data shown and should be done in follow-up research. We incorporated the suggestion of the reviewer in the discussion section (p. 23) as a potential option to refine our methodology.

Q: *"Another possibility that the authors mention is the incomplete ATP signal suppression could be due to a mismatched TE delay with ATP's 31P-31P J-coupling, then could the authors not have conducted experiments on the phantoms (or even the initial in vivo study) with different values of the TE delay to determine the optimal delay?"*

A: Thank you for this suggestion. In fact, we did conduct experiments with varying TEJ on phantoms with 5mM ATP and either 3.5mM NADH or 3.5mM NAD+ and we now added these results to the manuscript. The range of TEJ was chosen to be 13.75ms, 25.0ms, 27.5ms, and 30.0ms. This range started with the shortest possible TEJ of 13.75ms, followed by the actually implemented (and theoretically optimal)27.5ms ± 2.5 ms.

TEJ = 27.5ms showed the best α-ATP suppression in combination with the least signal loss of NADH and NAD+, as shown in Figure 5 and Figure 6. We have added the results of these phantom measurements as figure 4 to the current version of the manuscript.

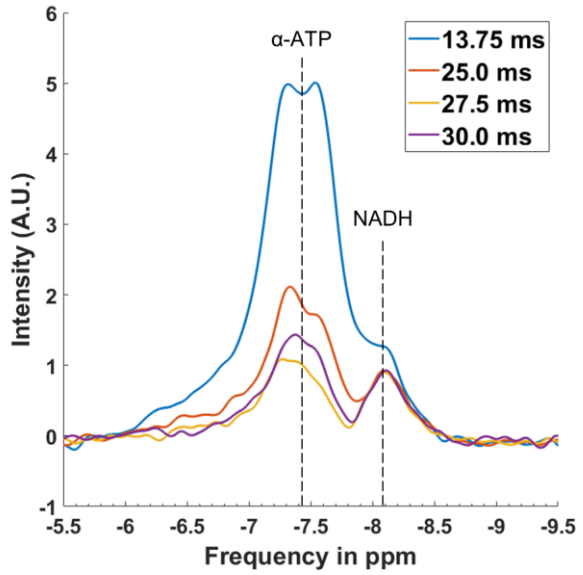

Figure 5: Series of editing echo times (TEJ) in modified z-Filter sequence measuring a phantom composed of 5mM ATP and 3.5mM NADH. NADH intensity remains stable over this range of TEJ. α-ATP residual is smallest at TEJ 27.5ms.

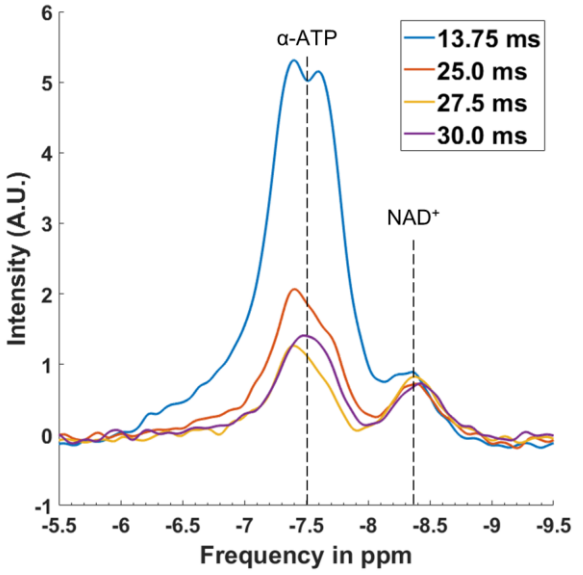

Figure 6: Series of editing echo times (TEJ) in modified z-Filter sequence measuring a phantom composed of ATP and NAD^+. NAD^+ intensity remains stable over this range of TEJ. α-ATP residual is smallest at TEJ 27.5ms.

Q: *"The paragraph discussing future improvements on the sequence is not so clear to read. "ATP spins not fully aligned with x after TE or not fully excited and refocussed will be affected by the final 90°-x pulse…". And later: "… spoiler gradients … could remove these unaligned spins". This is all somewhat poorly formulated. What is probably meant is that the ATP magnetisation (not individual spins) may not be perfectly aligned with the x-axis before the second 90° pulse, and that phase cycling or gradient pulses, could improve the performance of the pulse sequence by imposing the coherence transfer pathway shown in Figure 1 (which is not mentioned in the figure caption). They make a number of suggestions, but it is not clear why they have not tested these simple modifications in the first place? For instance, again, on the phantoms."*

A: We agree that the phrasing was unclear. Please note that of course we thought carefully about the sequence at the start of the project and tested various strategies in phantoms and in vivo, but as things can always be improved, we felt we should say something about future improvements in the discussion. However, going back to the discussion and thinking this over, we believe that due to the adiabatic pulses used, the suggested spoiler gradients would in fact not add anything significant and we removed this part. We do still discuss other potential future improvements, as for example the use of dedicated volume coils to improve SNR, as suggested by reviewer 1.

Q: *"The same paragraph also mentions "aforementioned B1 inhomogeneity", while this concept was not previously mentioned."*

A: *We apologize for this and removed the word "aforementioned".*

Q: *"Finally, did the authors consider the use of 1H decoupling during the FID to further narrow the 31P resonances of NAD+, NADH and ATP (cf. as shown in reference 36)?"*

A: We indeed considered the use of proton decoupling. However, we consistently observed an increase in the amount of residual α-ATP when using proton decoupling and we did not observe any reduction in linewidth. We are aware that in the brain, the benefit of decoupling to observe NAD metabolites was demonstrated (5), but we did not observe this in skeletal muscle. As a result, we decided to not utilize proton decoupling in our study. We added this point to the discussion on page 22 (second paragraph).

Q: *"Although I find the work interesting and its application appears important, I am left with the impression that the new pulse sequence has not been properly optimised for performance as much as it could have been. In addition, a verification using the phantoms that the output NADH and NAD+ ratios obtained are actually unbiased by the pulse sequence (T2, J-modulation) is lacking. I therefore find this work somewhat too preliminary for Nature Communications."*

A: In the current version of the manuscript, we added more information about the extensive optimisation and validation that was performed (e.g. shown in figure 2 and 8 of the current version of the manuscript). Furthermore, in order to show the linearity and the quantitative nature of the MRS method, we performed measurements on phantoms in a range of different concentrations of NADH and NAD+ and added this to the manuscript in figure 3. The reviewer can appreciate the good agreement between the known and the measured NADH/ATP and NAD+/ATP respectively. Furthermore, in a group of volunteers we performed both, the MRS measurements and metabolomics in a muscle biopsy. This data shows reasonable agreement of the in vivo and ex vivo method, as shown below and in figure 8. We summarized the pros and cons of the currently available techniques to determine NAD metabolites in a table, as shown below and added to the manuscript as table 1. The main advantage of the MRS method clearly is the non-invasive nature of the technique, for all other techniques, muscle biopsies need to be taken.

We hope that after the above-mentioned additions, the reviewer is also convinced that the uniqueness and the scientific quality of this study would make it of high interest for the readership of Nature Communications.

Q: *"Some minor remarks:*

- *The abbreviation HB is used before it is introduced*
- *Reference 20 lacks the journal name."*

A: As we have changed the name of the sequence to Double Echo Modified z-Filter (DEMz), the first comment is not applicable anymore. We have added the journal name in reference 20.

1. Bogner W, Chmelik M, Schmid AI, Moser E, Trattnig S, Gruber S. Assessment of 31P relaxation times in the human calf muscle: A comparison between 3 T and 7 T in vivo. Magnetic Resonance in Medicine. 2009;62(3):574--82.
2. Lu M, Zhu X-HH, Zhang Y, Chen W. Intracellular redox state revealed by in vivo 31P MRS measurement of NAD+ and NADH contents in brains. Magnetic resonance in medicine. 2014;71(6):1959--72.
3. De Graaf RA. In vivo NMR spectroscopy: principles and techniques: John Wiley \& Sons; 2013.
4. Graaf RA, Feyter HM, Brown PB, Nixon TW, Rothman DL, Behar KL. Detection of cerebral NAD+ in humans at 7T. Magnetic Resonance in Medicine. 2017;78(3):828--35.

5. Lu M, Zhu X-H, Chen W. In vivo 31P MRS assessment of intracellular NAD metabolites and NAD+/NADH redox state in human brain at 4T. NMR in Biomedicine. 2016;29(7):1010--7.
6. Roemer PB, Edelstein WA, Hayes CE, Souza SP, Mueller OM. The NMR phased array. Magn Reson Med. 1990;16(2):192-225.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Thank you very much for taking the effort and addressing all the comments and remarks. In my eyes it has substantially increase the impact of the manuscript.

Never-the-less, I would like to ask you to check on your citation database and the correct uniform way of citing. Throughout the References you have:

- two different ways how to report page range - "-" and "--"

- wrong spelling of "PloS one" (R9); "Cell metabolism" (R11), "Nature communications" (R23)

- three different ways of spelling "NMR in biomedicine" (R13) "NMR Biomed" (R15) "NMR IN BIOMEDICINE" (R18)

- three different ways of spelling " "Magn Reson Med" (R26), "Magnetic resonance in medicine" (R29), "Magnetic Resonance in Medicine: An official ..." (R51)

- missing pages in R38

- wrong spelling of authors in Ref2 and Ref51

and may be some others.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed all my points.

Reviewer #3 (Remarks to the Author):

The authors have addressed the initial concerns that I had, and added additional technical data and verifications that is reassuring. There are indeed aspects of the experiment that can be further improved upon, but as the authors note, this proof of principle is of direct clinical relevance. I am satisfied with the responses they have given.

We would like to thank all reviewers for their careful review.
Reviewer 2 and 3 have no remaining points to be addressed.

Reviewer 1 noticed errors in the reference list. We would like to thank the reviewer for noticing.

The full comment of Reviwer 1 was:

> *Thank you very much for taking the effort and addressing all the comments and remarks. In my eyes it has substantially increase the impact of the manuscript.*
>
> *Never-the-less, I would like to ask you to check on your citation database and the correct uniform way of citing. Throughout the References you have:*
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> *- three different ways of spelling " "Magn Reson Med" (R26), "Magnetic resonance in medicine" (R29), "Magnetic Resonance in Medicine: An official ..." (R51)*
> *- missing pages in R38*
> *- wrong spelling of authors in Ref2 and Ref51*
> *and may be some others.*

We rebuilt the bibliography from online source and thereby have amended all errors.