Reviewer #1:

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Modified in the text as “RT-PCR is used to detect the transcription level of target genes, and DCFH-A probes are used to evaluate intracellular ROS production.”

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3. -Rephrase: „Many studies by Horwitz have shown that nicotinamide-based methods are beneficial for rapid recovery of neutrophils in patients after transplantation” - use the same verbal tense throughout the hole material and methods chapter

We have rephrased the above content in the manuscript based on your suggestions.

4. - I recommend using a short acronym for the sequences of cells “CD34+CD38-CD45RA-CD49f+” and “CD34+CD38-CD45RA-CD49f+CD90+” to make the text easier to read

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1. Cord blood, should be changed by umbilical cord.

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a. Cord blood mononuclear cells (MNCs) were isolated with hydroxyethyl starch (HES) and Ficoll (Tianjin Haoyang. TBD), and then wash and resuspend them in column buffer. The procedure should be described and /or give a reference.

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CD34+ cells were seeded into 24 well plates. We added that to the article.

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With cell surface molecular markers, our goal was to determine the proportion and absolute number of cells with different phenotypes rather than performing cell sorting. After treated for 10-12 days, the cells were collected and washed with PBS, respectively. Then the cells were resuspended with 400 μL PBS and stained with antibodies for 30 minutes at 4 ℃ in dark. After washed with PBS, the absolute number and phenotype analysis were detected by flow cytometry.

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The PE-Annexin V Apoptosis Detection Kit (BD, Biosciences) contains three reagents: PE Annexin V, 7-AAD and 20×binding buffer. And the 20×binding buffer should be diluted with double distilled water to 1×binding buffer before use.

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After many trials, we found that the experimental results were available after the cells were treated with 70% ethanol for 2-6 hours.

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The 0.4 ml propidium staining solution (Biosciences) was composed of 384 μL staining buffer, 15μL of 25×propidium solution and 1μL of RNase (10mg/mL).

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4. Results. a. The effect of different concentrations of NAM on the quality of HSCs. The viability should be presented.

The cell viability data is shown in Figure 1D.

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The vertical axis represents the number of cells, the horizontal axis represents the fluorescence intensity of DCF, and the higher the ROS level, the higher the fluorescence intensity.

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c. The effect of different concentrations of Nicotinamide on the phenotype of cells LT-HSCs, ST-HSCs should be discussed, as well as the possible mechanisms

Based on your suggestion, we explored the effects and potential mechanisms of different concentrations of niacinamide on the phenotypes of LT-HSCs and ST-HSCs in the Results and Discussion section.

Reviewer #3:

Q1. It is very important to change and modify the title. the title is not appropriate.

Modified as “The effect of niacinamide concentration on Hematopoietic Stem Cells cultured in vitro.”

Q2. Are the objectives and the rationale of the study clearly stated?

The purpose of this study is to explore the effects of different concentrations of NAM on the proliferation and differentiation of HSCs during in vitro culture. The proliferation is reflected by TNC expansion folds, differentiation is reflected by phenotype analysis of HSPCs, ST-HSCs and LT-HSCs populations. Apoptosis ratio and cycle distribution reveal the influencing factors of cell

quantity differences, ROS production and cytokine levels reveal the relevant factors of differentiation differences, and RT-PCR detection of genes related to stemness, antioxidant enzymes and chemotactic molecules to explore the molecular mechanism of NAM actions.

Q3. In the abstract, the research gap was not clearly stated. In addition, the authors need to rewrite the study objectives to be more academic writing

We have made careful revisions to the abstract of the manuscript based on your suggestions.

Q4. In the introduction, include the study's significance and novelty. What makes the study different from the rest and what does it add to the current knowledge?.

Q5. In the introduction, the authors should have explained the purpose of this study and the existing gaps in this field and explained why this study was conducted.

Based on your suggestions, we have revised and supplemented the introduction, clarifying the gaps in this field and explaining the significance and novelty of this study.

Q6. Are the methods clear and replicable? Do all the results presented to match the methods described?

We have provided a detailed description of the experimental method to ensure that it matches all the results described.

Q7. If relevant are the results novel? Does the study provide an advance in the field? Is the data plausible?

Our study proposes for the first time that different concentrations of NAM have different effects on HSC proliferation and differentiation by regulating different molecular pathways. In addition, based on this study, we can provide NAM reference doses according to different HSC expansion requirements and predict small molecules that synergistically promote HSC expansion with NAM.

Q8. References are relevant, correct, and not recent. The number of references should be increased. please add some references. since this is a scientific review, all the sentences need to be supported with references. This study is very beautiful. I liked the sequence and enjoyed reading. Please add more references on similar studies.

Based on your suggestion, we have supplemented and revised the references.

Q9. There are a lot of grammatical errors. This must be taken care of and addressed.

We carefully checked and corrected grammar errors, and conducted language polishing.

Q10. What are the limitations of the study? A description of limitations is missing at the end of the discussion section

Based on your suggestion, we have supplemented the limitations of this study in the discussion.

Responses to specific comments

The authors have not adhered to the standard revision practices for scientific manuscripts, failing to furnish - point-by-point - meticulous rebuttals in response to the reviewer's comments (Refer to all three reviewers' reports below after EIC comments) and neglecting to include tracked changes in their revisions.

We completed the point-by-point response in December 2023. And we added the details of the answer on page 12.

Specific comments:

1) Page 4: "RESULTS

Compared with the control group, the proportion and expansion folds of hematopoietic stem and progenitor cells (CD34+CD38-) incubated with 5 mmol/L or 10 mmol/L of NAM were significantly increased (all $P < 0.05$)." Neither logic nor meticulousness were manifested in the context: concrete statements have been used. How did they differentiate "hematopoietic stem and progenitor cells?" What was the proportion?

CD34+CD38- are typical surface markers of HSPCs, and we have added the definition of HSPCs in the Background section on page 3. The proportions of HSPCs, ST-HSCs and LT-HSCs refer to the percentage of CD34+CD38- cells, CD34+CD38-CD45RA-CD49f+ cells and CD34+CD38-CD45RA-

CD49f+CD90+ cells in total nucleated cells, respectively. The specific values are supplemented in the Results section of the main text on page 11.

2) Pages 5-6: "NAM may mediate the metabolic transition of aging stem cells by upregulating the expression of young genes such as sirtuin 1 (SIRT1), thereby maintaining stemness." Definition of young? Timelines? Influential factors of aging? Citations?

Under young homeostasis conditions, HSCs are in a state of metabolic inactivity, mainly glycolysis and quiescence, but can temporarily transition to a metabolic active, oxidative phosphorylation driven proliferation state as needed to supplement the blood system.
Upon ageing, HSCs undergo functional and phenotypic changes, including impaired regenerative potential, loss of quiescence, and increased metabolic activity, accompanied by significant expansion of HSC pool and differentiation biases towards myeloid lineage. NAM can weaken age-related metabolic and functional changes in HSC, restoring youthful metabolism[16-20].
In mice, the most primitive HSC are believed to cycle only once every ~4 months.With each cell division, daughter cells lose developmental (long-term repopulating) potential, such that each daughter is less potent than its ancestor(Figure 1). Cell cycle times decrease with developmental stage. In young mice, the pool of stem cells is small, but the potency of each stem cell is high. In aged mice, the pool of stem cells has expanded, but their functionality is restricted[21-22].

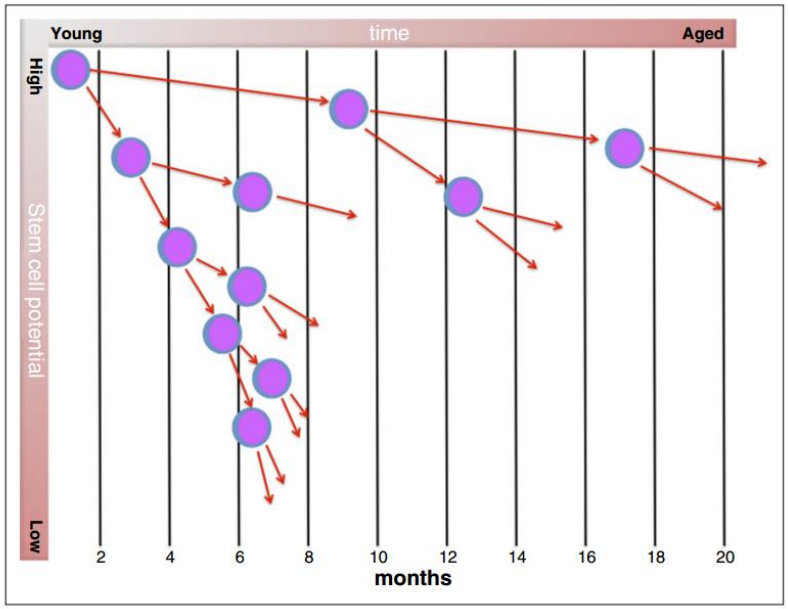

Figure1： Hypothetical tracing of the offspring of a single HSC during aging.[doi: 10.1182/blood-2017-06-746412.]

3) Page 7: "Based on the different concentrations of NAM, the cells were divided into control group (0 mmol/L), low concentration group (5 mmol/L), and high concentration group (10 mmol/L)." How did these relate to physiological conditions in vivo? Citations?

The normal plasma concentration of nicotinamide is around 5 μM. High dosage of nicotinamide is often required in clinical treatment, and the concentration in serum could reach the millimolar range. Researchers have an inconsistent understanding of the specific concentration at which NAM exerts its effects, such as 0.1mM in Son MJ et al.[25], and 2.5mM in Horwitz ME et al[26]. However, our previous experimental results showed that NAM only played a role when the concentration reached

5mM(Figure 2). And this part of the results, we also added in the article and supplementary materials. As for the difference in the concentration, we suspect that it may be related to the purity of NAM and experimental conditions.

Evidence provided by Son MJ et al. suggests that NAM have positive effects on pluripotency control of human pluripotent stem cells at concentrations well above the physiological range.[25] Therefore, we explored the effect of different concentrations of NAM (0mM,5mM,10mM,15mM) on HSC differentiation.

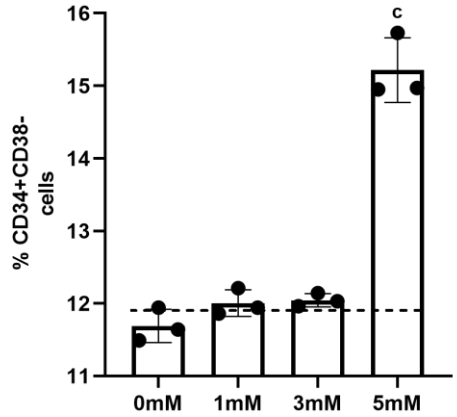

Figure 2 The minimum concentration of NAM play a role.

4) Fig 1 D Cell size for each group of cells. How did NAM affect the cell sizes?

With aging and differentiation, HSCs display an enlarged morphology.[27] Therefore, cell size can serve as an indirect indicator of the degree of differentiation of HSCs. When NAM inhibits the differentiation of HSCs, the proportion of HSPCs, ST-HSCs or LT-HSCs increases, and the average cell diameter also decreases accordingly.

5) Fig 1 E: Viability of each group of cells. There were no effects of 10 mmol/L on viability, which contradicted the apoptosis data. Why?

Cell viability and apoptosis are two completely different detection methods. Using trypan blue to detect cell viability, it can only pass through incomplete cell membranes and then dead cells were dyed clear blue. When cells undergo apoptosis, membrane integrity is not disrupted, but rather manifested as phosphatidylserine (PS) flipping from the inner side of the cell membrane to the cell surface, which can be detected by Annexin V. So the results of these two experiments are not contradictory.

6) Fig 2B: Fold expansion of total nucleated cells. How did they determine the data – Excel spreadsheets of duplications? Why did they not do proliferation assays?

The initial number of cells in each group is equal (50000/ml, 0.5ml). After the same time of in vitro culture, the total number of cells in each group is measured (concentration multiplied by volume), and the TNC fold expansion is calculated by dividing the latter by the former. The experiment should

be repeated independently at least 3 times. In addition, the proliferation of hematopoietic stem cells is significantly influenced by cell concentration, so the proliferation assays cannot objectively reflect the effect of NAM on HSC proliferation.

7) Fig 2A: Scale bars should be embedded in Figures.

We have made relevant corrections.

8) Fig 2A: All four panels of cell images looked like HSCs in suspension culture: Neither the morphological nor the adherent justified like 40X magnification of any microscope - rechecking these images should be a must if not suspension culture (Refer to "Establishment of an adherent cell layer from human umbilical cord blood" DOI:10.1590/S1415-47572000000300002Corpus ID: 1999346). If yes, they should mark in the method! Utilizing the colony-forming unit (CFU) cell assay, also known as the CFC assay, is mandatory when investigating the proliferation and differentiation of HSCs. Have they examined CFU assays?

It is well known that HSC is a kind of cell in suspension culture. Unlike the above article, we did not establish a system for co-culture with adhesion cell layer.
We supplemented the CFU assays(Figure1F, 1G).

9) How did they define ST- and LT-HSCs with biomarkers in a continuous in vitro culture relevant to in vivo microenvironment? Citations?

The HSPCs, ST-HSCs and LT-HSCs phenotypes used in this study were functional phenotypes defined by the researchers who sorted different subgroups using CD34, CD38, CD45RA, CD49f and CD90 molecular markers and then performed in vivo transplantation[10-15].

10) Fig 3C: Why did they not plot the data of 15 mmol/L NAM?

The 15 mmol/L NAM group has very few cells and a high proportion of apoptosis, making it unsuitable for cycle analysis.

11) Figure 4 Effect of different concentrations of nicotinamide on reactive oxygen species content, cytokine levels, and gene expression in cultured hematopoietic stem cells. How did NAM affect these molecules? Why did CXCR4 not change? How did they define functional HSCs without CXCR4 [PubMed PMID: 21466480] and engraftment?

Hematopoietic stem cells are in a physiological hypoxic environment in the bone marrow, and intracellular ROS production as well as cytokine levels in the medium are important factors affecting the maintenance, differentiation and proliferation of HSCs[28-33]. The expression levels of

stemness, chemotaxis, hypoxia pathways and antioxidant enzymes genes are common indicators for exploring the mechanisms of action of different small molecule compounds in HSC amplification systems[34-39].

NAM has always been considered as a SIRT1 inhibitor, regulating HSC amplification through epigenetic modifications, while there are few reports on its involvement in other pathways mentioned above. The results of this study indicate that 5 mmol/L NAM reduces ROS levels by upregulating the expression of antioxidant enzymes, and the IL-6 levels in the NAM group are also lower than those in the control group, indicating that NAM delays the differentiation of HSCs by reducing ROS and IL-6 levels. In addition, 5 mmol/L NAM upregulates the hypoxia pathway SIRT1-HIF1A, which is beneficial for the survival and proliferation of HSCs under hypoxic environmental conditions.

Figure 4C shows that the expression of CXCR4 in both 5mmol/L and 10mmol/L NAM groups is significantly higher than that in the control group.

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12) Fig 4A: the tickmarks are missing in the Y-axis.

We used Kaluza software (Beckman) to draw similar figure on the basis of many references.[doi: 10.3389/fphar.2022.806837 doi: 10.1002/sctm.19-0199] At the same time, in order to make the experimental results more clear and specific, we supplemented the bar chart.

13) Page 14: "CONCLUSION

In conclusion, different concentrations of NAM have distinct effects on proliferation and differentiation of HSCs." Where were their data sets for either one – how did they do differentiation of HSCs? Cell cycle analysis is not sufficient to claim that.

We have already explained the above questions in the introduction section on page 6. This study mainly evaluates the differentiation of HSCs by the percentage of HSPCs，ST-HSCs and LT-HSCs, and evaluates the proliferation of HSCs by the expansion folds of TNC, HSPCs，ST-HSCs and LT-HSCs, cell cycle is only an auxiliary indicator reflecting cell proliferation, as the proliferation of HSCs is significantly affected by cell density and is not suitable for proliferation assays.

14) Page 15: "Research motivation

The effects of different concentrations of NAM on the proliferation and differentiation of HSCs, as well as whether it affects sirtuin 1 (SIRT1) transcription levels, have not been reported yet. This is crucial for selecting the working concentration of NAM and predicting small molecules that have a

synergistic effect with NAM."Where is their data for SIRT1 synergistic effects? How did they define SIRT1 synergistic effects? Synergic with what?

There are various small molecule substances used for in vitro culture of HSCs (including UM171, SR1, VPA, NAM, ID8), which may affect the maintenance, proliferation, differentiation, and homing of HSCs by regulating different pathways, and different molecular pathways may have synergistic effects. This study aims to provide a theoretical basis for the future joint application of multiple small molecules by elucidating the molecular mechanism of NAM.[49-51]

15) Page 16: "Research conclusions

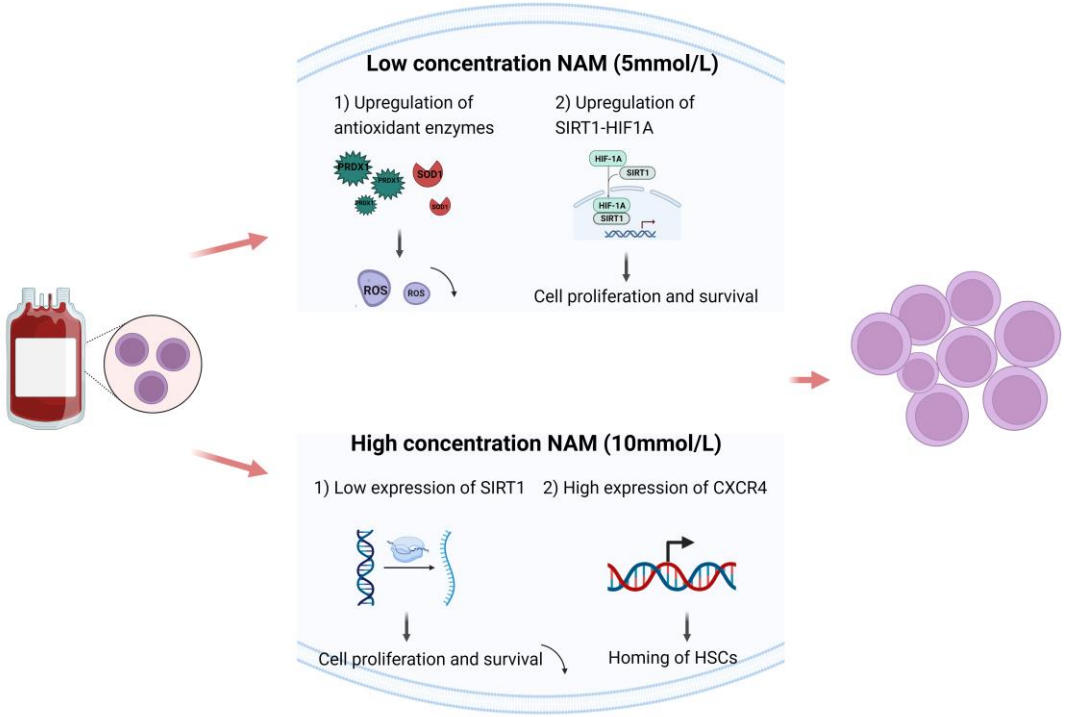
Low concentration NAM did not inhibit but upregulated the transcription of SIRT1, promoting cell proliferation by activating the SIRT1-HIF1A pathway." Where is the data of the hypoxia SIRT1-HIF1A pathway (page 12) (did they use The hypoxia condition)? A schematic diagram should be provided to illustrate that pathway coupled with the data.

Not sufficient alternatives related to stem cell reports were discussed, such as NAM-regulated ROCK and casein kinase 1[doi: 10.1016/j.stemcr.2018.10.023].

Specific data related to the SIRT1-HIF1A pathway are shown in Figure 4C. In hematopoietic stem cells, upregulation of SIRT1-HIF1A pathway promotes cell proliferation and differentiation.[47-48] Atmospheric (in vitro) oxygen concentration is around 150 mm Hg (20% O2, normoxia), whereas physiologic (in vivo) oxygen is much lower, ranging up to 5% in cartilage, 4–7% in bone marrow,

10–13% in arteries, lungs and liver.[DOI 10.1007/s13770-017-0068-8] The in vitro culture environmental differences can alter the characteristics of the stem cells. In this article, all cells were cultured in the hypoxia chamber that was filled with a gas mixture of composition 5% O2 and 5% CO2, balanced with N2(see in MATERIALS AND METHODS- cell culture).

We drew the schematic through the BioRender software.



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16) Grammar errors crawl around pages (A corrected form is within square brackets […]):

e.g.,

i. Page 3: “cultured for 10-12 d in [a] serum-free medium.”
ii. Page 5: “Hematopoietic stem cells (HSCs) ~~are cells that~~ can self-renew and give rise to mature cells of all hematopoietic lineages following extensive proliferation and differentiation[1,2].”
iii. Page 7: “Based on the different concentrations of NAM, the cells were divided into [the] control group (0 mmol/L),”
iv. Page 12: “The basic HSCs in vitro culture system consists of [a] serum-free medium supplemented with cytokines.”
v. Editorial Certificate manifested in a typo: “Paper Title:
The effect of different concentrations of niacinamide [nicotinamide] on hematopoietic stem cells cultured in vitro
Author:
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Modified as “The effect of niacinamide concentration on Hematopoietic Stem Cells cultured in vitro.”

Q2. Are the objectives and the rationale of the study clearly stated?

The purpose of this study is to explore the effects of different concentrations of NAM on the proliferation and differentiation of HSCs during in vitro culture. The proliferation is reflected by TNC expansion folds, differentiation is reflected by phenotype analysis of HSPCs, ST-HSCs and LT-HSCs populations. Apoptosis ratio and cycle distribution reveal the influencing factors of cell quantity differences, ROS production and cytokine levels reveal the relevant factors of differentiation differences, and RT-PCR detection of genes related to stemness, antioxidant enzymes and chemotactic molecules to explore the molecular mechanism of NAM actions.

Q3. In the abstract, the research gap was not clearly stated. In addition, the authors need to rewrite the study objectives to be more academic writing

We have made careful revisions to the abstract of the manuscript based on your suggestions.

Q4. In the introduction, include the study's significance and novelty. What makes the study different from the rest and what does it add to the current knowledge?.

Q5. In the introduction, the authors should have explained the purpose of this study and the existing gaps in this field and explained why this study was conducted.

Based on your suggestions, we have revised and supplemented the introduction, clarifying the gaps in this field and explaining the significance and novelty of this study.

Q6. Are the methods clear and replicable? Do all the results presented to match the methods described?

We have provided a detailed description of the experimental method to ensure that it matches all the results described.

Q7. If relevant are the results novel? Does the study provide an advance in the field? Is the data plausible?

Our study proposes for the first time that different concentrations of NAM have different effects on HSC proliferation and differentiation by regulating different molecular pathways. In addition, based on this study, we can provide NAM reference doses according to different HSC expansion

requirements and predict small molecules that synergistically promote HSC expansion with NAM.

Q8. References are relevant, correct, and not recent. The number of references should be increased. please add some references. since this is a scientific review, all the sentences need to be supported with references. This study is very beautiful. I liked the sequence and enjoyed reading. Please add more references on similar studies.

Based on your suggestion, we have supplemented and revised the references.

Q9. There are a lot of grammatical errors. This must be taken care of and addressed.

We carefully checked and corrected grammar errors, and conducted language polishing.

Q10. What are the limitations of the study? A description of limitations is missing at the end of the discussion section

Based on your suggestion, we have supplemented the limitations of this study in the discussion.