Letters to the Editor

Editor's note:

Dear readers,

After the article "Sixteen years of canine hepatic copper concentrations within normal reference ranges in dogs fed a broad range of commercial diets," by Amundson et al,[1] was published online in *JAVMA* on March 7, I received Letters to the Editor expressing concern about the scientific methodologies used in the research. Per our journal's standard processes, we shared these Letters with the authors of the article, which caused them to revisit their data and ultimately request that *JAVMA* retract their article. *JAVMA* published the retraction[2] and linked it to the article; the PDF version of the article is now watermarked as "RETRACTED." It should be noted that retractions are very rare in veterinary medicine, and this is to my knowledge a first in the history of *JAVMA*. As such, our team followed contemporary best practices established by several editorial associations and chose not to publish the original Letters to the Editor once the article had been retracted. However, in subsequent communication with the authors of the Letters, I became aware of their concerns about the methodology of the article that are not readily apparent to the reader in the retraction. To concisely convey these concerns to *JAVMA*'s readers, the Letter authors collaborated and collated their multiple Letters into a single Letter from all authors, the result of which follows this introduction. Thank you, Dr. Sharon Center, for spearheading this collaboration among the Letter authors. The all-author Letter to the Editor that appears herein was shared with Dr. Amundson and her coauthors, and their response follows the Letter.

I appreciate the time my many colleagues took to share their thoughts, and I thank everyone for their commitment during this process.

Best regards,

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1. Amundson MD, Motsinger LA, Brejda J, Hancock L. RETRACTED: Sixteen years of canine hepatic copper concentrations within normal reference ranges in dogs fed a broad range of commercial diets. *J Am Vet Med Assoc*. 2024;262:1–6. doi:10.2460/javma.23.11.0621
2. Notice of Retraction: Amundson et al. Sixteen years of canine hepatic copper concentrations within normal reference ranges in dogs fed a broad range of commercial diets. *J Am Vet Med Assoc*. Published online March 7, 2024. doi:10.2460/javma.2024ret

Synopsis of four Letters to the Editor regarding Admundson MD et al

A synopsis of critical points raised by 8 board-certified veterinarians (5 small animal internists, 1 clinical nutritionist, 2 pathologists) and 2 laboratory directors in 4 independently composed Letters to the Editor regarding the March 7 *JAVMA* article by Admundson et al[1] is herein provided to avoid duplicative commentary. Each critique recognized the reporting of implausibly low liver copper concentrations, concerning scientific methodologies, inadequate nutritional details, insufficient histologic evaluations, and inappropriate statistical analyses.

Frozen liver samples (n = 336) were retrospectively retrieved from a Hill's Pet Foods internal colony (90%, purpose-bred Beagles, likely with limited genetic variance) and fewer pet dogs from external veterinary clinics. Samples were derived from 2 separate collection intervals; 1 sample set (ie, 37 Beagles, 12 Labrador Retrievers, and 6 Labrador mixes) had been previously used in a published Hill's study.[2]

The absence of dietary details, especially from colony dogs, where food intake (type, amount) should be recorded, is noncompliant with good scientific practices or reflects incomplete retrieval of historical information. The authors acknowledge that colony dogs "were likely fed various commercialized diets including both Hill's and a range of competitors' diets … but may have been limited to an array of therapeutically intended diets associated with mitigation of chronic diseases." Thus, limited-to-restricted copper diets may have been fed. This is troublesome because dietary and hepatic copper concentrations are positively correlated in Labrador Retrievers,[3] and there is relevant variability in copper content among commercial dog foods.[4]

That approximately 38% of liver samples were unexpectedly reported as having subnormal copper concentrations underscores our concerns regarding the nondisclosed levels of dietary copper as well as the accuracy of tissue copper measurements. None of us have ever identified a case of clinical copper deficiency in an adult dog eating a commercial diet. Rather, discovery of subnormal liver copper concentration might reflect consumption of a copper-deficient diet, a genetic mutation compromising enteric copper uptake, severe liver fibrosis or parenchymal extinction replacing hepatocyte mass where copper accumulates, or the chronic administration of a copper chelator. The reported finding of Labrador Retrievers having significantly lower liver copper concentrations than Beagles is in stark contradiction to studies[5,6] reporting high liver copper concentrations in Labrador Retrievers

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https://www.avma.org/journals/javma-instructions-authors

with and without hepatitis and in Labrador Retrievers as compared to other breeds since 1997.

Finding subnormal liver copper concentrations in approximately 38% of dogs raises concerns regarding sample preservation, processing, and/or analysis. We query whether liver samples were adequately desiccated to constant weight (no validation provided); inadequate desiccation would erroneously lower measured tissue copper concentrations. We suspect that the described 3- to 4-hour oven (104 °C) tissue drying protocol was insufficient as compared to the standard 24- to 48-hour desiccation protocol.[7] Copper analytic methodologies are incompletely described and were conducted at different times with different instruments and, potentially, different operators in different laboratories. When analytic instruments, operators, or protocols change, it is mandatory to document equivalent outcomes using control samples. This was not reported. Further, it is unclarified whether measurements were performed with an appropriately validated assay in an American Association of Veterinary Laboratory Diagnosticians (AAVLD)–accredited laboratory.

Best practice for documenting liver copper concentration is to reconcile bench measurements with rhodanine staining of ≥ 3 biopsies from spatially distant regions of liver.[8,9] This was not done, and it is not clear whether any tissues remain that might be used to verify subnormal liver copper concentrations. Rhodanine stain is copper specific and confirms the existence of > 200 µg of copper/g of dry-weight liver (dwl), confirms the heterogeneity of tissue copper distribution, and allows for qualitative copper scoring. Rhodanine-stained liver sections also can accurately determine copper concentrations > 200 µg/g of dwl using a digital scanning algorithm.[8,9] Essential stains defining hepatic fibrosis and architectural remodeling were not completed. These assessments are needed to rule out hepatic fibrosis and/or parenchymal extinction as causes of erroneously low bench copper analysis.[8] While liver histopathology is cited as a study objective, there is no board-certified pathologist involved in the study (eg, no pathologist is included as an author or even acknowledged). Thus, it is unclear how histologic assessment of 336 liver samples was accurately completed. There is no information regarding biopsy adequacy (ie, sufficiency of sample size including 12 to 15 portal tracts) or a morphologic grading system. Subdivision of liver histology into "normal" and "abnormal" is vague to meaningless without designation of guidelines for histologic characterization. Mere classification of tissue changes as normal versus abnormal is inconsistent with contemporary histologic standards. One egregious error was classification of nodular hyperplasia, a normal aging change in dogs, as an abnormal finding.

Study findings are confounded by what we suggest are inappropriate statistical analyses with nonparametric data treated as parametric (eg, least-squares mean estimate of observed yearly mean copper, data "smoothing" by reporting SEM) and an inscrutable polynomial regression (Figure 2). Reporting tissue copper concentrations by year of death (sample collection) has no significance unless samples were collected before dietary copper recommendations were modified in 1993.[10]

While all concerned parties agree that more research is needed on dietary copper supplementation, it is clear that copper deficiency, raised as an issue in the Discussion, is not a current risk. A PubMed review reaching back to the 1950s fails to discover any reports of spontaneous copper deficiency in dogs fed commercial food without underlying genetic issues or inappropriate copper restriction during prolonged D-penicillamine chelation.

The authors are encouraged to submit portions of their archived specimens to an external AAVLD-accredited laboratory for histologic evaluation, rhodanine staining, and determination of tissue copper concentrations. However, even if the reported results were substantiated, it would not offset the inability to extrapolate findings from purpose-line-bred research Beagles to pet dogs. Therefore, the limitations of this publication in our opinion outweigh any potential benefits to the veterinary community.

We submit this critique to help clinicians decipher the value of this manuscript and encourage best practices in experimental design, sample collection, data representation, and statistical analyses for complex topics relevant to veterinarians in clinical practice.

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1. Amundson MD, Motsinger LA, Brejda J, Hancock L. RETRACTED: Sixteen years of canine hepatic copper concentrations within normal reference ranges in dogs fed a broad range of commercial diets. *J Am Vet Med Assoc*. 2024;262:1–6. doi:10.2460/javma.23.11.0621
2. Corsato Alvarenga I, Aldrich CG, Jewell DE. Influence of liver condition and copper on selective parameters of post-mortem dog tissue samples. *Animals (Basel)*. 2018;8(12):237. doi:10.3390/ani8120237
3. Fieten H, Hooijer-Nouwens BD, Biourge VC, et al. Association of dietary copper and zinc levels with hepatic copper and zinc concentration in Labrador Retrievers. *J Vet Intern Med*. 2012;26(6):1274–1280. doi:10.1111/j.1939-1676.2012.01001.x
4. Copper in Dog Foods Expert Panel. *Final Report With Recommendations to the Pet Food Committee*. Association of American Feed Control Officials; 2022. Accessed July 2, 2024. https://www.aafco.org/wp-content/uploads/2023/01/Copper_in_Dog_Foods_Expert_Panel_Report_to_the_PFCkv2136684-2136685.pdf
5. Johnston AN, Center SA, McDonough SP, Wakshlag JJ, Warner KL. Hepatic copper concentrations in Labrador Retrievers with and without chronic hepatitis: 72 cases (1980–2010). *J Am Vet Med Assoc*. 2013;242(3):372–380. doi:10.2460/javma.242.3.372
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7. Radke SL, Ensley SM, Hansen SL. Inductively coupled plasma mass spectrometry determination of hepatic copper, manganese, selenium, and zinc concentrations in relation to sample amount and storage duration. *J Vet Diagn Invest*. 2020;32(1):103–107. doi:10.1177/1040638719894988
8. Miller AJ, Center SA, Randolph JF, Friesen CH, Miller AD, Warner KW. Disparities in hepatic copper concentrations determined by atomic absorption spectroscopy, inductively coupled plasma mass spectrometry, and digital image analysis of rhodanine-stained sections in dogs. *J Am Vet Med Assoc*. 2021;258(4):395–406. doi:10.2460/javma.258.4.395
9. Center SA, McDonough SP, Bogdanovic L. Digital image analysis of rhodanine-stained liver biopsy specimens for calculation of hepatic copper concentrations in dogs. *Am J Vet Res*. 2013;74(12):1474–1480. doi:10.2460/ajvr.74.12.1474
10. Center SA, Richter KP, Twedt DC, Wakshlag JJ, Watson PJ, Webster CRL. Is it time to reconsider current guidelines for copper content in commercial dog foods? *J Am Vet Med Assoc*. 2021;258(4):357–364. doi:10.2460/javma.258.4.357

The authors respond:

We appreciate the opportunity to clarify points from Center et al regarding our retrospective study of canine liver copper concentrations. Previous investigators have hypothesized that the observed increased liver copper accumulation in dogs may be associated with increased dietary copper intake. As such, the overarching aim of this retrospective study was to examine liver copper concentrations in dogs fed a representative sampling of commercially available dog foods throughout their lives. The authors sought to understand whether there is a relationship between a population of dogs fed foods representative of the pet food market and liver copper concentrations, as a function of age, sex, breed, histopathology, and year of death, as previous investigators have observed an increasing prevalence of copper-associated hepatopathy.

Upon further examination, to accurately respond to concerns, our investigation uncovered a conflicting data issue when combining the original dataset from Corsato Alvarenga et al[1] and the additional data provided in the new manuscript.[2] We determined that non-normalized data from the first study[1] were inadvertently combined with normalized data from the additional samples.[2] After further analysis, we will determine whether the original conclusion that showed an increasing trend in liver copper concentrations over time bears scrutiny. We feel responsible to provide the corrected data to resolve concerns raised in the Letters to the Editor and will submit the revised publication in a timely manner.

Sample population and dietary history

Our study sought to examine hepatic liver concentrations in a population of dogs fed a representative sampling of commercially available dog foods throughout their lives. In contrast, previous studies examined dogs with histories of copper storage disease. Additionally, the present study was not a prospective study designed to assess reasons for elevated liver copper concentrations in dogs or solely in breeds frequently affected with copper-associated hepatitis. The population from the present study is a useful baseline of liver copper concentrations from a large population of dogs whose husbandry was maintained for optimum health throughout their lives.

Methods

As stated in the Methods section of the manuscript, the present study utilized all liver samples available from a bioarchive collection composed of internal colony dogs and external dogs, which included samples from a breed with a known predisposition of copper-associated hepatopathies. The samples from dogs utilized in the present study provided insight into a population of dogs that were fed various brands and forms of dog food throughout their lives, including a wide variety of commercially available products. Foods offered also included a variety of forms such as extruded, wet, fresh, wellness, and veterinary diets available on the market. Thus, dogs utilized in this study were fed foods representing the current pet food market selections containing various copper concentrations and sources. Additionally, considering the utility and husbandry of the population utilized in the present

study, veterinary diets were not routinely fed long term, although there were exceptions when indicated for the individual veterinary needs. Exact diet histories are of limited value for the 336 dogs in this study. Dogs, in many cases, were offered hundreds of different diets throughout their lives that did not account for total nutrient concentrations due to the extensive use of commercial pet food. This limitation, along with the limitation of the unknown diet histories of the external dogs, is acknowledged in the Discussion section of the manuscript. Additional research is needed to evaluate correlations between popular dietary trends and liver copper concentration in dogs. Furthermore, additional research on predisposed and non-predisposed breeds is necessary to represent the broader population of dogs in the US, as this study was a starting point for understanding baseline liver copper concentrations in healthy dogs.

Data from a previously published study[1] were combined with new data from an additional 281 samples for this analysis. The data from the previous study utilized the same laboratory, instrumental parameters, and methodology, but differed in sample preparation for copper quantification. Data from the previous study were analyzed on a wet basis, then back calculated to a dry-matter basis using moisture data. New data samples were prepared by first blotting to ensure excess fluid was removed, followed by heating each of the samples in their entirety (0.5 to 2.5 g) at 104 °C to dryness (constant weight). Once dry, each sample was ground until homogeneous, and then a known weight (approx 0.25 g) was digested to completion in nitric acid using a microwave digestion system (Multiwave 7000; Anton Paar GmbH). Samples were then diluted to 50 mL using polished deionized water, followed by inductively coupled plasma optical emission spectroscopy (5100 ICP-OES; Agilent Technologies) using a 5-point calibration line. We have shown that both sample preparation methods are not statistically different. This methodology uses multiple control samples to ensure that the sample preparation and instrumentation stay consistent between runs and has been proven to be highly reproducible with a relative SD of < 5%.

Liver histopathologies for the 336 samples were completed by board-certified veterinary pathologists at an American Association of Veterinary Laboratory Diagnosticians–accredited laboratory over a 16-year period. Pathologists provided liver conditions as "normal" and "abnormal" with additional comments if the sample was deemed abnormal. Since this study was to investigate the hypothesis of increasing prevalence of hepatic copper as stated in previous publications, additional information regarding the histopathology report was only provided for 4 samples in Table 3 of the manuscript because they were categorized as abnormal and also above the normal reference range.

Statistical evaluation

Liver copper concentrations are continuous data, and it is appropriate to summarize these data using means. The use of yearly means in the regression analysis eliminated unwanted sampling variability. The central limit theorem states that the sampling distribution of the mean will always be normally distributed if the sample size is large enough, regardless of whether the population has a normal, Poisson, binomial, or any other distribution. The normality of the residuals is a key assumption underlying the *F* tests in the regression analysis; therefore, we chose the yearly means as an appropriate summary statistic to use in the analysis. The yearly mean is an unbiased estimate of the sample mean for the dogs used in this study for each collection year. The variance around that mean is sampling variance, not true residual error variance. The true error variance is the difference between the sample mean and the predicted mean for each year from the regression equation. Thus, using yearly means does not constitute data smoothing, as suggested.

Concerning our use of year as the predictor variable, responses over time are common in biological systems. Our objective was to describe changes in liver copper concentrations over time (years); as such, this was an appropriate predictor variable to use in the regression model. A related concern might be whether the dogs used in this study were randomly sampled each year and whether the sample population is sufficiently large. This was a retrospective study. The dogs used in this study came from a database of dogs whose end of life was between 2006 and 2022 and that had liver biopsies performed after death. No effort was made to censor this data or introduce bias. However, we also did not have the luxury of randomly selecting dogs for inclusion in the study due to its retrospective nature. Concerning the sample size for each collection year, we were limited to what was available in the database. This situation often arises in retrospective studies when researchers look for relationships in data that have already been collected for other purposes.

Results and Discussion

Referencing the dataset combination error described in the opening of this response, we uncovered a data normalization error when combining 2 datasets, warranting a revision of the data analysis and our manuscript. While our initial findings suggested an increasing trend in liver copper concentrations, the new data necessitate a reevaluation and/or reinterpretation depending upon the results. We are committed to providing reliable, valid data and analysis of the results and will submit a new manuscript in a timely manner.

Conclusion

We agree that more research is needed on food interactions and their impact on liver copper concentrations. Our study serves as a starting point, providing a baseline for future investigations. We encourage further research

with detailed dietary histories, appropriate biological end points, and comprehensive histopathological evaluations to better understand the potential factors influencing liver copper concentrations in dogs.

We appreciate the constructive feedback and are committed to improving the quality and reliability of our research. Thank you for the opportunity to address these concerns.

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1. Corsato Alvarenga I, Aldrich CG, Jewell DE. Influence of liver condition and copper on selective parameters of post-mortem dog tissue samples. *Animals (Basel)*. 2018;8(12):237. doi:10.3390/ani8120237
2. Amundson MD, Motsinger LA, Brejda J, Hancock L. RETRACTED: Sixteen years of canine hepatic copper concentrations within normal reference ranges in dogs fed a broad range of commercial diets. *J Am Vet Med Assoc*. 2024;262:1–6. doi:10.2460/javma.23.11.0621

Comments on stem cell therapy and regenerative medicine research

Thank you very much for the recent important and informative *JAVMA* Supplementary Issue: The Current State of Veterinary Regenerative Medicine (June 2024, Volume 262, Issue S1).

I read the various papers with great interest. I have some important comments about studies with stem cell injections into animals (including humans) and their use in therapy for various diseases. My stem cell therapy research experience is with mice and rats injected with such cells and from reading many publications in this evolving field of medicine. Of course, human hematopoietic stem cell injection is successful in humans given immunosuppressive treatments and in immunodeficient mice. My problem with the entire field of stem cell therapy/regenerative medicine in humans and animals is that there are too many unproven studies and many unscientific studies that are often poorly controlled.

1. Most studies do not use an important control—injection of nonstem cells—like normal cell culture cells, normal tissue cells, etc. I have much experience with that process in rodents.
2. Since most stem cell studies of domestic animals are usually preliminary, often untreated disease controls are not used.
3. As you know, the use of mesenchymal stem or mesenchymal stromal cells for any such cells is controversial, but in the *JAVMA* issue, both terms are used singularly or together in the various articles. The terminology of such cells has been discussed.[1]
4. Histopathology of injected cells is rare in any published studies of humans or animals. Thus, the histopathogenesis of stem cell injection and possible therapy are rare. Injection of any live cell (or even dead cells or cell products) into an animal evokes an inflammatory response. Cells often die within a few days whether injected IV, SC, IP, or into any tissue or organ. Most published papers have not performed histopathology after injection (at day 1, 2, 3, or 5, etc) and thus have little idea what is going on biologically and histologically, despite the other assays performed (imaging, lab assays, etc). Acute and chronic lesions occur after injection and may last an extended period.
5. Histocompatibility problems can arise. Often, stem cells injected are from various animal strains, breeds, or stocks of animals that differ from the injected animals. Immune-related inflammation will occur.

I hope that all those involved in these important studies would please consider my comments and suggestions. More scientific studies and outcomes should occur.

Thank you for listening.

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1. Berglund AK, Fortier LA, Antczak DF, Schnabel LV. Immunoprivileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells. *Stem Cell Res Ther*. 2017;8(1):288. doi:10.1186/s13287-017-0742-8

The guest editor responds:

A Letter to the Editor in response to the recent *JAVMA* Supplementary Issue: The Current State of Veterinary Regenerative Medicine (June 2024, Volume 262, Issue S1), brought up some very important points about this field of clinical medicine and research. One of the most important points that was raised centered around the randomized controlled trials and lack of histopathologic outcomes. Dr. Ward points out the lack of a nonstem cell–based control group, as it is clear that injection of cells incites an immune response in host. This is an excellent point and one that is worthy of further investigation. Any cell-based treatment could incite an inflammatory or immunomodulatory effect such that injection of a cell that is not characterized as a mesenchymal stem or stromal cell (MSC) would be an important control. Unfortunately, the use of larger animal models, which are necessary if translation from bench to the clinic is to be achieved, often precludes additional control groups due to the increased costs of conducting large animal models. It should be noted that cellular control groups are not needed in all arms of regenerative medicine research. There are numerous regenerative medicine products, including autologous blood-based therapies or hemoderivatives, with clearly demonstrated therapeutic benefits, especially for treatment of musculoskeletal diseases, in which the final product is acellular. That being said, a nonstem cell–based control group should be considered for all studies and included when possible.

Dr. Ward also astutely points out the lack of untreated or randomized control groups in animals with naturally occurring disease versus animals being used in experimental models. Overall, veterinary medicine continues to lack randomized controlled trials due to the difficulty enrolling animals into trials where they may receive placebo treatment. Recognition of this shortcoming in veterinary medicine has led researchers to strive to conduct more randomized controlled trials, and I believe we are starting to see more of these studies in the literature. For example, Burk et al[1] recently published their results of treatment of naturally occurring tendon disease with allogeneic MSCs in a randomized, controlled, triple-anonymized study in which treatment horses were treated with MSCs in serum and control horses were treated with serum. I am hopeful that we are entering the age of more such trials as the veterinary community begins to truly embrace evidence-based medicine.

The interchangeable use of mesenchymal stem or mesenchymal stromal cell (both referred to as MSC) continues to be an issue. The terminology of these cells has been discussed at length by the International Society for Cell and Gene Therapy (ISCT), with the group urging researchers to preserve the term "stem" for cells with demonstrated self-renewal and differentiation capabilities and to use the term "stromal" for other cells that have immunomodulatory and secretory functions. Despite the ISCT's recommendations, many researchers and published studies continue to use the term "stem." Because many studies do not put the cells through rigorous tests of self-renewal and differentiation, authors should be urged to use the term "stromal."

Histopathology is a critical component of understanding the impacts of all regenerative medicine therapies. Upon examination of the literature, the vast majority of experimental trials do in fact include histopathology. In large animal models, there is a dearth of histopathology at multiple time points due to the expense of conducting these studies with large animal numbers. That being said, large animal models do offer clear benefits over small animal models in which large animal numbers are more feasible. Large animal models allow for larger fluid and tissue samples for evaluation, can withstand more robust testing, and provide more realistic biomechanical loading of tissues among others. Histopathology is clearly missing from clinical studies for obvious reasons. Imaging and lab assays are minimally invasive tests that can be used to partially evaluate response to treatment. However, in clinical patients, histopathology should be used whenever possible to examine the response to regenerative medicine therapies. This could include minimally invasive biopsy of tissues.

Finally, there continues to be unanswered questions about the histocompatibility of allogeneic regenerative medicine therapies. Many researchers continue to make great progress in this arm of regenerative medicine. For example, Dr. Lauren Schnabel at North Carolina State University has published several studies examining the immunogenicity of allogeneic MSCs, including a review article entitled, "Immunoprivileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells."[2] I believe we will continue to see more studies investigating the impact of allogeneic regenerative medicine products.

As with any scientific pursuit, there are endless questions that need to be answered. I believe the veterinary regenerative medicine community is tackling these important research and clinical questions head on and is making impressive headway in the field. The researchers in the field are also clearly committed to pushing the boundaries of our knowledge while improving the care of animals and people.

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1. Burk J, Wittenberg-Voges L, Schubert S, Horstmeier C, Brehm W, Geburek F. Treatment of naturally occurring tendon disease with allogeneic multipotent mesenchymal stromal cells: a randomized, controlled, triple-blinded pilot study in horses. *Cells*. 2023;12(21):2513. doi:10.3390/cells12212513
2. Berglund AK, Fortier LA, Antczak DF, Schnabel LV. Immunoprivileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells. *Stem Cell Res Ther*. 2017;8(1):288. doi:10.1186/s13287-017-0742-8