**Postmortem Interval Determination from Bone:**
**A Metabolomics and Lipidomics Approach**

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EIN

**Summary**

This project aimed to expand the capacity to approximate time since death from skeletal remains using quantitative lipidomic data, in an effort to provide a methodology that extends beyond current observation-based methods. Bone biopsy samples of fresh and skeletal donors with varying postmortem intervals (<1 year-30 years) were subjected to high resolution mass spectrometry to identify preserved lipid biomolecules in bone to explore their capacity to accurately predict long term postmortem intervals (e.g. years or decades) from skeletal remains.

**PURPOSE**

This project aimed to expand the ability of forensic practitioners (namely anthropologists and pathologists) to approximate time since death from skeletal remains using quantitative lipidomics. This study intended to identify lipid candidates in bone marrow that survived initial soft tissue decomposition processes and were retained for long term postmortem intervals (PMI). This study is novel in the attempt to use quantitative methods to trace the molecular degradation of lipid compounds using high resolution mass spectrometry. This approach is an effort to expand on forensic methods that utilize observational markers of bone decomposition patterns such as weathering and bleaching to predict time since death.

This study expanded on previous quantitative research that explored the utility of skeletal muscle metabolites for the prediction of postmortem interval based on soft tissue decomposition. Lipids have shown to be preserved in bones for long term intervals. Archaeological and forensic case studies have reported identification of lipids in bone with time since death ranging from months to years, decades and centuries. Quantification of lipid degradation in bone marrow will aid in designing a quantitative method for estimating the postmortem interval of skeletonized remains. Successful bone lipid extraction after an interval of seven years provided preliminary data that was used to create a protocol for extracting bone marrow lipids housed in trabecular bone. The intent being to utilize quantitative lipid degradation data to construct statistical prediction methods for the postmortem interval of severely decomposed, mummified and skeletal remains with greater precision and accuracy than observation-based methods provide. The broad goal of this project was to expand lipid data on postmortem intervals of less than one year to roughly thirty years since death. This approach to estimating postmortem interval using quantitative, biomolecular data is novel, and thus this study proved to be largely preliminary and exploratory.

**METHODS**

Bone biopsy samples of fresh and skeletal donors with varying postmortem intervals (<1 year to 30 years) were subjected to high resolution mass spectrometry using a Thermo Scientific Q Exactive Orbitrap to identify preserved lipid biomolecules in bone. The degradation of the identified compounds was tracked for 24 months using relative quantitation methods. Bone biopsies were performed using CareFusion DJ6011X Jamshidi bone marrow biopsy needles (11G x 15cm). The biopsy sites of medial calcaneus, proximal tibia and vertebral body

(fourth lumbar) were selected because of high trabecular bone and marrow content and ease of location by examining surface anatomy. The left tibia was sampled, distal to the medial condyle. The left calcaneus was sampled on the medial side, typically just inferior to attachments of the deltoid ligament. The fourth lumbar vertebra was sampled roughly midline on the anterior surface of the vertebral body. Bone plugs were transferred to 7 mL tubes and placed on ice for transport to the Metabolomics Laboratory at Lincoln Memorial University and were stored in a -80C freezer until extraction.

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***Experimental Sample***

Twenty fresh human donors were placed at the University of Tennessee Anthropological Research Facility (ARF) in two cohorts. Ten freshly frozen donors were placed January 23 2018, and an additional ten freshly frozen donors were placed July 26 2018. The six-month interval between the placement of cohorts was to account for differences in daily temperature and humidity averages. Both cohorts decomposed in the outdoor environment of the ARF and bone biopsy samples were collected every 6 months. This resulted in a total PMI of 24 months for the experimental cohort.

***Cross-Sectional Sample***

The cross-sectional sample consisted of skeletal remains of known PMI, curated in the William M. Bass Donated Collection at the University of Tennessee. Bone biopsies were taken from 130 individuals from three skeletal sites with high trabecular bone content (medial

calcaneus, proximal tibia and vertebral body). Skeletal donor samples from the Bass Donated Collection ranged in postmortem interval from 37 years to 3 years since death (Table 1).

Organic extraction methods attempted to analyze the three skeletal sites of calcaneus, tibia and vertebra. However, due to the inability to elute singular compounds in the tibial and vertebral samples, only the calcaneus bone biopsies were successfully analyzed using HR-MS/MS.

**Table 1.** Summary of postmortem interval cohorts from cross-sectional skeletal sample curated at the William M. Bass Collection at the University of Tennessee.

| Bass Collection Donors: Biopsy Summary | | | | |
|---|---|---|---|---|
| PMI Interval Years | Year of Death Cohort | Calcaneus n= | Tibia n= | Vertebra (L4) n= |
| 37-30 | 1983-1990 | 20 | 20 | 20 |
| 29-25 | 1991-1995 | 14 | 14 | 14 |
| 24-20 | 1996-2000 | 13 | 13 | 0 |
| 19-15 | 2001-2005 | 29 | 29 | 29 |
| 14-10 | 2006-2010 | 32 | 32 | 32 |
| 9-5 | 2011-2015 | 20 | 20 | 20 |
| <5 | 2016-2017 | 2 | 2 | 2 |
| Total n= | | 130 | 130 | 117 |

**RESULTS**

***Experimental and Cross-Sectional Studies***

For the analysis of PMI in human calcaneus bone (40 – 80 mg wet weight) we performed an extraction with methyl-tertbutylether (MTBE). This extract subsequently underwent flow infusion with electrospray sample introduction for high-resolution (140,000; < 2 ppm mass error) mass spectrometric analysis (Orbitrap). Scans from 300 to 1200 amu was performed in both positive and negative ion modes. As with our prior findings for PMI in human muscle (1-2), we observed that phosphatidylcholines (PC) provided the most robust signal to noise and indicated that these glycerophospholipids declined in bone between 1 and 6 months post entry into the study (Table 2). The individual PCs were validated by MS/MS which generates phosphocholine (184.0738; < 2 ppm) as the product cation. These data clearly indicate that bone phosphatidylcholines deteriorate in bone between 1- and 6-months postmortem.

**Table 2.** Postmortem relative levels of phosphatidylcholines (PC) in human calcaneus bone. The peak intensity of each endogenous PC was divided by the peak intensity of an appropriate stable isotopic internal standard to generate the relative levels, which was subsequently corrected for wet weight. Peak intensities were obtained with full scans from 300 to 1200 amu in positive ion mode.

| Lipid | Time 0 | 1 Month | 6 Months | Ratio (T6/T1) |
|---|---|---|---|---|
| PC 34:1 | 0.48 | 0.59 | 0.024 | 0.040 |
| PC 34:2 | 0.63 | 0.59 | 0.017 | 0.029 |
| PC 36:1 | 0.55 | 0.88 | 0.031 | 0.034 |
| PC 36:2 | 0.24 | 0.26 | 0.042 | 0.16 |
| PC 36:4 | 0.16 | 0.13 | 0.0018 | 0.014 |

**Study of Methods Improvement: Solvent Washing Procedure**

While the initial full scan studies yielded data, many samples also were not useful due to significant ion suppression. Ion-suppression is a well-documented obstacle in liquid-chromatography and tandem mass spectrometry in which multiple compounds elute from a chromatographic column (3). This inhibits or severely limits specific and accurate identification of, in this study, lipid species. Due to the extreme ion-suppression challenges encountered in the tibial and vertebral samples, only the calcaneus bone biopsies were successfully analyzed.

Since many of these samples were highly colored, we next investigated a washing procedure for the MTBE extracts with water:acetonitrile (1:1). This yielded valuable new data regarding the 6- and 12-month PMI intervals (Table 3); however, a significant number of samples still were limited by ion suppression. All PCs were validated by MS/MS which generates phosphocholine (184.0738; < 2 ppm) as the product cation.

**Table 3.** Postmortem relative levels of phosphatidylcholines (PC) in human calcaneus bone. The peak intensity of each endogenous PC was divided by the peak intensity of an appropriate stable isotopic internal standard to generate the relative levels which was subsequently corrected for wet weight. Peak intensities were obtained with full scans from 300 to 1200 amu in positive ion mode.

| Lipid | 6 Months | 12 Months | Ratio (T6/T12) |
|---|---|---|---|
| PC 34:1 | 0.046 | 0.0068 | 0.15 |
| PC 36:1 | 0.49 | 0.034 | 0.069 |
| PC 36:2 | 0.022 | 0.0020 | 0.091 |

**Study of Methods Improvement: Dry Weight Extraction Protocol**

On the sampling dates, the postmortem bone samples were stored in 7mL polystyrene tubes with lids, and remained in these same tubes until extraction. While the postmortem bone samples from the January 2018 Cohort timepoint 0 (initial sampling before freezing donors) and timepoint 1 (initial sampling after thawing donors) were initially stored in laboratory-grade methanol, the remaining postmortem bone samples were placed into empty tubes without any solvent. To minimize the impact of freeze-thaw cycles on the integrity of the lipids in the bone plugs, samples were weighed in batches in advance of lipid extraction. Pre-weighed postmortem bone samples were stored in a -80C freezer until extraction.

The extraction process followed the standard protocol for lipid extraction (4) with some notable exceptions due to the high incidence of ion suppression. Modifications to the standard lipid extraction protocol were designed specifically to address the distinct challenge of processing postmortem bone samples, which has never been attempted to our knowledge. Many of the bone samples retained the shape of the bone biopsy needle. As such, these samples were transferred to a foil packet to minimize contamination and were impacted with a standard hammer to fragment the bone for further processing. This mechanical fragmentation resulted in pulverization most in samples with low water content. Bone samples with higher water content tended to flatten rather than fragment. Next, all bone samples were transferred into smaller 6mL tubes containing 1mL of methanol and stable isotope internal standards. One mL of distilled water was added to each sample prior to sonication that employed a Fisherbrand Model 50 Sonic Dismembrator with thicker probe (Model 4435, 0.63cm diameter). For samples originally stored in methanol, no new methanol was added and only the internal standards and

water were added to maintain consistent volume across samples. The use of the shorter tubes and thicker probe were specific modifications to the standard extraction protocol to aid in further breakdown of bone samples. Further, two separate sonication steps, consisting of 30sec each, were required for breakdown of the bone. This time interval was short enough to avoid unnecessary heating of the samples through sonication.

After double-sonication, the bone sample and liquid solvents were decanted into the original 7mL polystyrene tubes with lids. Subsequent steps followed the standard lipid extraction protocol: 2mL of tert-butylmethylether were added to samples before shaking at room temperature for 30min and centrifugation at 4000 xg for 30min at room temperature. To minimize contamination, only 1mL of the upper organic layer was transferred to a 96-deep-well-plate, and the samples were dried by vacuum centrifugation for a minimum of 3.5hrs. Then, 200μL of infusion solvent containing 2-propanol:methanol:chloroform (8:4:4) + 5 mM ammonium chloride was added to optimize the formation of anions for detection. Finally, after sealing the 96-deep-well-plate, one final centrifugation for 15min was the last modification of the standard protocol to encourage separation of any remaining ion suppressants from the infusion solvent for analysis.

The modifications to the standard lipid extraction protocol were created specifically to manage the ion suppression in the postmortem bone samples. This novel protocol successfully detected lipids from samples close to 35 years since the death of the individual. However, more work is needed on cleaning the samples so the utility of subsequent processing can be maximized. The high prevalence of ion suppression in the postmortem bone samples precludes untargeted relative quantitation at this time due to the high background levels masking the

signals of lipids and overall increasing the variance in the levels. In response to the ion suppression, tandem mass spectrometry (MS2) was exclusively employed to maintain ion detection at high resolution (140,000; < 2 ppm mass error) and low variance. For MS2 analyses, an isolation window of 0.4 amu and collision energy of 30NCE were used in positive ionization.

**Final Protocol: MS/MS Relative Quantitation**

To simplify the analyses as much as possible and to reduce the issue of ion suppression we settled on utilizing MS/MS as the optimal mode in regard to the experimental samples. The results are presented in Table 4. We also switched from normalization of wet bone weight to dry weights to minimize the long-term effect of hydration or dehydration on bone samples. An example of potential confounding effects of storage include the fact that from the initial time of weighing, compared to the dry weight, which only accounts for evaporation while in the freezer, the calcaneus samples lost an average of 17.87mg. A duration of 4-month storage frozen intervals indicated that the samples lost 28.6% of their weight.

For this approach, the extracted samples were dried at 80$^{o}$C and the weights of the dry powders recorded. Specifically, this involved drying the samples in their respective 7mL tubes at 80$^{o}$C after the upper organic layer of interest was removed. This dry weight not only accounts for differential decomposition of the donors but also accounts for the extended freezer storage while minimizing lipid degradation. Overall, this offers a more accurate measurement to normalize the relative levels of the lipids.

The results of these analyses indicate that the major deterioration of PCs occurs in the first three months postmortem (Table 4). Furthermore, analysis of bone samples approximately 35 years postmortem (range of 20 – 35 years), stored at room temperature in sample

collections, revealed that the PC levels remain stable at these low levels. These MS/MS data also suggest that our observations in the washed samples (Table 2), of a decline in PC levels between 6- and 12-months postmortem, are suspect and were probably affected by ion suppression of the internal standard.

**Table 4.** Post-mortem relative levels of phosphatidylcholines (PC) in human calcaneus bone. The peak intensity of each endogenous PC was divided by the peak intensity of the stable isotopic internal standard to generate the relative levels, which was subsequently corrected for dry weight. Peak intensities of the MS/MS product cation 184.0738 were obtained for the individual PCs and the internal standard [$^{2}H_{31}$]PC 16:0/18:1. The 420 month samples ranged from 20 to 35 years PMI. Data are presented as mean ± SEM.

| PMI Months (N) | PC 34:2 | PC 36:1 | PC 36:2 |
|---|---|---|---|
| 1 (14) | 2.7 ± 0.62 | 18.9 ± 6.1 | 1.27 ± 0.40 |
| 3 (9) | 0.19 ± 0.029 | 4.04 ± 0.48 | 0.42 ± 0.063 |
| 12 (20) | 0.15 ± 0.018 | 3.42 ± 0.42 | 0.28 ± 0.028 |
| 18 (19) | 0.19 ± 0.045 | 2.66 ± 0.16 | 0.24 ± 0.027 |
| 24 (10) | 0.25 ± 0.043 | 2.52 ± 0.24 | 0.19 ± 0.022 |
| 48 (9) | 0.13 ± 0.019 | 4.14 ± 0.64 | 0.39 ± 0.071 |
| 420 (54) | 0.060 ± 0.0041 | 2.27 ± 0.13 | 0.19 ± 0.019 |

**Conclusion and Proposed Next Steps**

Our data indicate that the PMI between 0 and 3 months is the critical period for degradation of bone phosphatidylcholines and that these compounds are preserved in bone at low levels for decades. Therefore, the next steps will be to investigate the 0 to 3-month time interval in more detail. We also suggest that this analytical approach should establish and utilize an absolute rather than relative quantitation method, such that a valuable database can be established between different laboratories. We also suggest continuing the use of dry-bone weights for normalization. While this study established the linear degradation of several phosphatidylcholines housed in trabecular bone, more data is needed to establish regression equations to predict PMI that extends beyond three months. This study has been one of the first to attempt to quantify decomposition in bone via biomolecules identified and retained in bone marrow following the soft tissue decomposition process. The data produced, although too sparse to be useful for building regression equations in tandem with accumulated degree day data, are still informative. We hope to utilize the preliminary results of this study to bring attention to the exploration of skeletal decomposition at the cellular level. Namely, our results indicate that exploration of absolute quantitation is necessary to establish and identify lipid candidates that are retained in bone and slow to degrade. The identification of preservation of phosphatidylcholines housed in trabecular bone in individuals with postmortem intervals of several decades illustrates the potential for future identification of decomposition metabolites in bone. The prevalence of ion suppression in the cross-sectional bone samples precludes untargeted relative quantitation as it inhibits our ability to identify single compounds, as more than one compound elutes. However, the amended protocol was successful in extracting

phosphatidylcholines. We have identified the need to expand on this work using absolute quantitation methods rather than relative, which involves using internal and primary reference standards. While our results are preliminary, these experiments were a necessary first step in the exploration of quantitative, molecular based methods to better quantify decomposition of human skeletal material.

## REFERENCES


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3) Volmer AV and Jessome LL. (2006) Ion Suppression: A Major Concern in Mass Spectrometry. Liquid Chromatography Gas Chromatography North America.
4) Wood PL. (2017) Non-targeted lipidomics utilizing constant infusion high resolution ESI mass spectrometry. In: Wood PL, editor. Springer Protocols Neuromethods: Lipidomics Springer.


## PARTICIPANTS AND COLLABORATING ORGANIZATIONS

### Participants

Name: Beatrix Dudzik

Project Role: Principal Investigator

Nearest person month worked: 24

Contribution to Project: Dr. Dudzik has performed work in the area of grant management, supervision of grant personnel, bone biopsy sampling, donor acquisition, metabolomics mass spectrometry analysis and report writing.

Name: Paul Wood

Project Role: Co-Principal Investigator

Nearest person month worked: 6

Contribution to Project: Dr. Wood has supervised the LMU graduate students doing mass spectrometry processing and validated lipid candidates using MS/MS.

Name: Lee Jantz

Project Role: Co-Principal Investigator

Nearest person month worked: 0.6

Contribution to Project: Dr. Jantz has done work in the area of supervision of the UTK graduate student, donor acquisition and ARF placement.

Name: Stacy Chelf

Project Role: LMU Graduate Student

Nearest person month worked: 2

Contribution to Project: Ms. Chelf has performed work in the area of performing bone biopsy sampling on fresh and skeletal donors, processing tissue samples, mass spectrometry analysis, and coordinating with the UT FAC.

Name: Kathleen Hauther

Project Role: UTK Graduate Student

Nearest person month worked: 24

Contribution to Project: Ms. Hauther has performed work in the area of performing bone biopsy samples on fresh and skeletal donors at the Forensic Anthropology Center, donor acquisition and ARF placement of fresh donors.

**Partner Organizations**

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Participation: collaborative research

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Participation: facilities (Anthropological Research Facility) and collaborative research

**List of products**

An abstract submitted to the American Academy of Forensic Sciences, entitled *"Forens-OMICS": the application of omics sciences to forensic investigations*, was presented as a conference paper at the American Academy of Forensic Sciences Annual Scientific Conference on Thursday, Feb. 20, 2020. A copy of the abstract can be accessed at:
https://aafs.org/common/Uploaded%20files/Resources/Proceedings/2020_Proceedings.pdf

An abstract was submitted to the American Academy of Forensic Sciences, entitled *Using High Resolution Mass Spectrometry Analysis to Investigate Trabecular Bone Metabolomics for Postmortem Interval (PMI) Estimation* and presented as a poster presentation on Friday, Feb. 22, 2019

A copy of the abstract can be accessed at:
https://aafs.org/common/Uploaded%20files/Resources/Proceedings/2019%20_Proceedings.pdf

An abstract was submitted to the American Academy of Forensic Sciences, entitled *Postmortem Interval (PMI) Estimation Using Bone Lipidomics* and presented as a poster presentation on Friday, Feb. 23, 2018

A copy of the abstract can be accessed at:
https://aafs.org/common/Uploaded%20files/Resources/Proceedings/2018_Proceedings.pdf

**Dissemination Activities: N/A**