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FINAL SUMMARY OVERVIEW TO THE NATIONAL INSTITUTE OF JUSTICE
Postmortem Interval Determination: A Metabolomics / Lipidomics Approach
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PURPOSE

The goal of this project is to provide the forensic community with validated equations to estimate postmortem interval (PMI) using biomarkers from human skeletal muscle tissue. The study expands upon our preliminary findings that structural glycerophospholipids (GPLs) of human skeletal muscle are accurate biomarkers of slower degenerative processes in the postmortem period (Wood and Shirley 2013). Skeletal muscle tissue is utilized because it is one of the last of the soft tissues to break down during decomposition (Gill-King 1997, Vass et al. 2002). Skeletal muscle is also easily sampled with minimal disturbance to the corpse. While previous studies have evaluated biomarkers of short-term PMI and focused on small molecular metabolites not specific to the human corpse, this study utilizes mass spectrometry on biomarkers of longer term PMI that are specific to the corpse and not invading microbes.

PROJECT DESIGN AND METHODS

Sampling

Tissue samples were obtained from the University of Tennessee Anthropology Research Facility (ARF). Only donors with a confirmed and documented date and time of death were used for this study. Bodies were placed at the ARF within a caged enclosure to prevent scavenger access. Vastus lateralis muscle was sampled because it is the most commonly and easily biopsied muscle due to its size. Samples were obtained 4-6 inches proximal to the patella and lateral to the femur and stored in a -40°C freezer within 5 minutes of sample collection. Biopsy needles were autoclaved overnight after sampling events. At the time of sample collection temperatures of the ARF entry gate, the incision site, and a designated nearby tree were taken with an infrared thermometer and recorded. The nearest National Weather Service Station

temperature data, which is located less than one mile away on the University of Tennessee Agricultural Campus, was also recorded. Average temperature data and time of daily tissue collection was used to calculate Accumulated Degree Days (ADD) and Accumulated Degree Hours (ADH).

A two-tier sampling strategy was employed to accomplish the project goal:

1. Test samples:

Serial tissue samples were collected daily from 17 bodies placed at the ARF. These samples were used to develop regression equations for estimating PMI. Donors were selected for daily sampling if the postmortem interval was 72 hours or less upon placement at the ARF outdoor decomposition research facility. Muscle biopsy needles were used to obtain 50mg of vastus lateralis muscle. Placement at ARF followed within 24 hours of intake, and another sample was taken at placement. Samples were taken with a biopsy needle unless poor muscle quality required the use of a scalpel to obtain an adequate amount of muscle; sampling method was recorded for each sample collected (needle or scalpel); the sampling sites were resealed with a piece of tape when possible. Daily biopsy samples were taken using the original puncture point until 2000 ADH had been reached or until no muscle was available to sample. If the original sample site became unusable due to insect activity, a new incision or puncture was made to obtain a tissue sample. A total of 495 samples were collected from 22 donors. Data from 5 donors was lost due to a power outage and freezer thaw (74 samples); the remaining 421 samples from 17 donors was utilized for the mass spectrometry analyses.

2. Validation samples:

Single samples were obtained from 16 donors with known time of death and storage conditions prior to placement at the ARF in order to validate the equations derived from the test samples. Approximately 50mg of skeletal muscle tissue was obtained with a scalpel from the right vastus lateralis muscle unless there was damage or obstruction to the right leg. In those instances, a sample was taken from the left leg, and this discrepancy was recorded. Samples were taken within 24 hours of donor intake at the UT Forensic Anthropology Center.

Mass Spectrometry Analyses

For the lipid extraction, 20 to 40 mg of muscle tissue were polytroned in 1 mL water and 1 mL of methanol containing stable isotope internal standards ([$^2H_{31}$]PtdE 34:1, [$^2H_{54}$]PtdE 28:0, [$^2H_{31}$]PtdC 34:1, [$^2H_{54}$]PtdC 28:0) and bromocriptine. Next, 2 mL of methyl-tert-butyl ether were added and the tubes were vigorously shaken at room temperature for 30 minutes. Samples were centrifuged at 5,000 xg at room temperature for 15 minutes, and 1 mL of the upper organic extracts were dried by centrifugal vacuum evaporation and dissolved in isopropanol : methanol : chloroform (4:2:1) containing 7 mM ammonium acetate. Constant infusion lipidomics were performed utilizing high-resolution (140,000 at 200 amu) data acquisition, with sub-millimass accuracy on an orbitrap mass spectrometer (Thermo Q Exactive) with successive switching between polarity modes.

In negative ion ESI, the anions of ethanolamine plasmalogens (PlsE) and phosphatidylethanolamines (PtdE), were quantitated and lipid identities validated by MS/MS. In positive ion ESI, the cations of choline plasmalogens (PlsC) and phosphatidylcholines (PtdC) were quantitated and lipid identities validated by MS/MS. The cations and anions of

bromocriptine were used to monitor for potential mass axis drift. Between injections, the transfer line was washed with successive 500 µL washes of methanol and hexane/ethyl acetate/chloroform (3:2:1) to minimize potential ghost effects. R values (ratio of endogenous lipid peak area to the peak area of an appropriate internal standard) per 100 mg of tissue were calculated.

Data Analysis

Mass spectrometry was able to extract lipids from 293 tissue samples with an ADD between 0 and just over 2,000 (ADH up to 47,800). Six biomolecules were extracted with sufficient consistency to be utilized in multivariate analyses: phosphtatidylglycerol (PG) 34:0, ethanolamine plasmalogen (PlsE) 36:4, phosphatidylethanolamine (PtdE) 36:4, choline plasmalogen (PlsC) 34:2, very long chain fatty acid (VLCFA) 24:0, phosphatidylcholine (PtdC) 36:2. Univariate outlier analysis was performed on each variable using the outlier labeling rule (Hoaglin et al. 1986) prior to conducting any multivariate analyses. Curve estimation was performed on each of the variables to determine if they should be transformed prior to multivariate statistical analyses. Four of the variables required a natural log transformation (PG 34:0, PlsE 36:4, PtdE 36:4, PlsC 34:2). The remainder exhibited linear relationships with ADD and ADH (VLCFA 24:0, PtdC 36:2).

Since the goal of this project is to produce easy to apply validated equations for the forensic community, and because the dependent variable is a continuous nominal variable, multiple linear regression was used to analyze the data after log transformations were performed. Stepwise variable selection was utilized to derive a parsimonious model that accounted for a considerable degree of variation in the dependent variable (ADD and ADH). Models were

screened for multicollinearity by examining the variance inflation factor and for multivariate outliers, which were removed from the final regression models. Bootstrapping was used to develop the final models, and analyses and validations were performed in SPSS 25.

RESULTS AND CONCLUSIONS

The most reliable biomarkers of long-term PMI detected in our studies were membrane structural glycerophospholipids (GPLs). This included both choline (phosphatidylcholine 36:2, choline plasmalogen 34:2) and ethanolamine glycerophospholipids (phosphatidylethanolamine 36:4; ethanolamine plasmalogen 36:4) that declined over time during the postmortem period. These metabolites are specific to the corpse and not to invading microbes. The mass spectrometry analyses also detected free very long chain fatty acids, which increase over time, and could have been associated with the muscle cells and/or with invading bacteria. However, VLCFA activity is compromised in cold temperatures (i.e. they stop increasing), so this variable is not as reliable as the cell-membrane specific formulae. Sterol sulfates (e.g. cholesterol) were not consistently extracted from all samples and therefore not included in the analysis.

Tables 1 and 2 present the model summaries for four models for predicting ADH and ADD, respectively. The most consistently extracted lipids were phosphtatidylglycerol (PG) 34:0 and phosphatidylethanolamine (PtdE) 36:4; therefore, regression models were provided for each of these variables individually and combined. The model with PtdE 36:4 and PG 34:0 has slightly high variance inflation factors (VIF) and moderately high multicollinearity, but is still within acceptable tolerance limits. Figure 1 shows the decline of phosphatidylethanolamine 36:4 during the postmortem period. This single glycerophospholipid accounted for a considerable portion of variation in the dependent variables ADD (R-square = 0.413) and ADH (R-square =

0.312). Regression coefficients, coefficient standard error, and error terms for the residuals are provided in Tables 3 and 4 for ADH and ADD, respectively. The standard error of the estimate is the square root of the mean square error of the residuals, also known as the standard error of regression.

Tables 5 and 6 presents the results of the regression formulae validations for predicting ADD and ADH, respectively. Phosphtatidylglycerol (PG) 34:0 and phosphatidylethanolamine (PtdE) 36:4 were extracted from all of the samples collected for the validation; VLCFA were not extracted from these samples; therefore, the equation with this variable could not be validated. The actual ADD and ADH of all (100%) of the test samples fell within the 95% prediction interval limits for the simple linear regression models with PtdE 36:4 and PG 34:0, respectively; 94% fell within the 90% prediction interval limits for the PtdE model, and all were correctly predicted using the 90% prediction interval for the PG model. Both of these univariate models is suitable for forensic applications, though the prediction intervals for PG were wider, especially for ADD. The PtdE 90% prediction provided the most accurate and precise postmortem interval estimates. The model that combined these two variables failed to predict ADD and ADH accurately for the validation samples, perhaps due to the multicollinearity issue in this model. Consequently, the PtdE + PG model is not recommended for use in practice.

Table 1. *Regression models for predicting Accumulated Degree Hours (ADH).*

Models for Predicting ADH	R	R Square	Adjusted R Square	Std. Error of Estimate	Collinearity Statistics	
					Tolerance	VIF
LN (PtdE 36:4)	.559	.312	.305	8647.750	1.00	1.000
LN (PG 34:0) VLCFA 24:0	.843	.710	.697	7750.259	.792 .792	1.262 1.262
LN (PtdE 36:4) LN (PG 34:0)	.931	.867	.861	5259.058	0.128 0.128	7.835 7.835
LN (PG 34:0)	.554	.307	.297	8696.177	1.00	1.00

Table 2. *Regression models for predicting Accumulated Degree Days (ADD).*

Models for Predicting ADD	R	R Square	Adjusted R Square	Std. Error of Estimate	Collinearity Statistics	
					Tolerance	VIF
LN (PtdE 36:4)	.642	.413	.406	368.441	1.00	1.000
LN (PG 34:0) VLCFA 24:0	.845	.715	.702	323.942	.799 .799	1.251 1.251
LN (PtdE 36:4) LN (PG 34:0)	.925	.856	.850	230.068	.126 .126	7.950 7.950
LN (PG 34:0)	.221	.049	.035	469.750	1.00	1.00

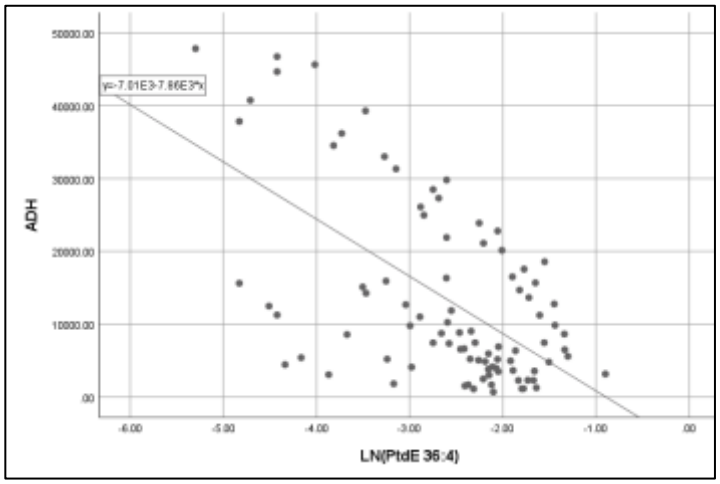

Figure 1. *Decrease of phosphatidylethanalomine 36:4 during the postmortem period The variable is log transformed.*

Table 3. *Regression models for predicting ADH.*

MODEL	LN (PtdE 36:4) (ADJ. R^2=.305)		LN (PG 34:0) & VLCFA 24:0 (ADJ. R^2= .703)			LN (PtdE 36:4) & LN (PG 34:0) (ADJ. R^2= .850)			LN (PG 34:0) (ADJ. R^2= .297)	
	Constant	LN(PtdE 36:4)	Constant	LN(PG 34:0)	VLCFA 24:0	Constant	LN(PG 34:0)	LN(PtdE 36:4)	Constant	LN(PG 34:0)
B	6943.186	1595.901	-16248.162	-14173.460	-9921.581	21790.137	21997.856	-23723.978	13100.500	-2444.961
B std error	-3897.785	603.304	4207.106	1444.646	1271.000	3395.898	1743.897	2442.125	1135.223	448.612
Std error of estimate	8647.750		7750.259			5259.058			8696.177	

Table 4. *Regression models for predicting ADD.*

MODEL	LN (PtdE 36:4) (ADJ. R^2=.406)		LN (PG 34:0) & VLCFA 24:0 (ADJ. R^2= .703)			LN (PtdE 36:4) & LN (PG 34:0) (ADJ. R^2= .850)			LN (PG 34:0) (ADJ. R^2= .035)	
	Constant	LN(PtdE 36:4)	Constant	LN(PG 34:0)	VLCFA 24:0	Constant	LN(PG 34:0)	LN(PtdE 36:4)	Constant	LN(PG 34:0)
B	297.675	-206.625	-667.311	-599.419	-416.969	905.541	907.401	-990.279	706.968	-44.909
B std error	66.927	25.299	172.922	59.649	52.870	147.471	106.773	76.285	60.451	23.884
Std error of estimate	368.441		323.943			230.068			469.750	

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Table 5. *Validation results for ADD prediction models. Predicted value (pred) of ADD with 90% and 95% upper (UL)and lower (LL) prediction interval limits.*

	LN (PtdE 36:4) & LN (PG 34:0)			LN (PtdE 36:4)				LN (PG 34:0)				ADD
UT ID	Pred	95%LL	95%UL	Pred	90%LL 95%LL	90%UL	95%UL	Pred	90%LL 95%LL	90%UL	95%UL	Actual
24-17	2301.9	1498.2	3105.4	167.0	0	328.8	915.7	606.1	0	1524.3	1562.2	40
10-17	2487.9	1651.3	3324.4	171.6	0	335.3	920.2	598.0	0	1515.4	1555.5	240
29-17	3199.3	2296.6	4102.1	349.1	0	584.9	1090.8	604.9	0	1523.0	1561.2	320
19-17	2668.5	1853.4	3483.5	310.6	0	530.4	1053.5	622.1	0	1542.0	1575.6	400
18-17	2537.2	1701.3	3373	196.3	0	369.8	943.8	601.4	0	1519.2	1558.3	720
14-17	2449.8	1621.7	3277.9	174.8	0	339.8	923.2	600.6	0	1518.3	1557.7	200
54-16	2695.8	1834.9	3556.7	209.5	0	388.2	956.4	596.6	0	1514.0	1554.4	200
57-16	2618.8	1816.9	3420.7	320.2	0	544.1	1062.9	626.8	0	1547.4	1579.7	120
56-17	3002.6	2112.5	3892.6	284.6	0	493.7	1028.4	599.3	0	1516.9	1556.6	2114
59-16	3331.4	2422.6	4240.2	399.1	0	655.9	1139.3	610.3	0	1528.9	1565.7	40
60-16	2483.4	1662.4	3304.4	208.1	0	386.4	955.1	606.9	0	1525.2	1562.9	40
58-16	2939.6	2102.2	3777	385.1	0	636.0	1125.7	626.4	0	1546.9	1579.3	80
61-16	2701.7	1860.1	3543.4	259.7	0	458.7	1004.6	608.3	0	1526.7	1564.1	40
75-16	3191	2284.8	4097.2	336.5	0	567.1	1078.6	602.3	0	1520.2	1559.1	240
05-17	2724.2	1860.3	3588.1	215.6	0	396.8	962.2	596.7	0	1514.1	1554.5	80
92-16	2831.4	1948.8	3713.9	220.9	0	404.3	967.3	592.6	0	1509.7	1551.2	80

Table 6. *Validation results for ADH prediction models. Predicted value (pred) of ADH with 90% and 95% upper (UL)and lower (LL) prediction interval limits.*

	LN (PtdE 36:4) & LN (PG 34:0)			LN (PtdE 36:4)				LN (PG 34:0)				ADH
UT ID	Pred	95%LL	95%UL	Pred	90%LL 95%LL	90%UL	95%UL	Pred	90%LL 95%LL	90%UL	95%UL	Actual
24-17	55712.7	37272.8	74152.7	4477.4	0	10759.7	22072.6	7607.2	0	19611.7	25327.1	534.4
10-17	60215.1	41019.8	79410.4	4565.7	0	10895.2	22155.7	7165.4	0	18929.6	24912.5	5762
29-17	77227.3	56521.9	97932.7	7913.7	0	16084.4	25336.5	7544.2	0	19514.2	25267.9	7860
19-17	64414.5	45716.6	83112.5	7186.1	0	14947.9	24640.3	8477.7	0	20963.0	26148.5	9521.2
18-17	61377.9	42198.6	80557.2	5031.4	0	11610.8	22594.6	7352.2	0	19217.7	25087.6	17584.4
14-17	59288.9	40288.0	78289.7	4625.6	0	10987.1	22212.1	7309.4	0	19151.5	25047.4	5068.8
54-16	65204.3	45452.2	84956.5	5279.5	0	11993.0	22829.0	7094.4	0	18820.2	24845.9	4704.8
57-16	63199.9	44804.1	81595.6	7369.0	0	15232.9	24814.9	8737.3	0	21367.9	26394.5	3038.8
56-17	72542.4	52124.2	92960.7	6695.6	0	14184.4	24172.4	7238.7	0	19042.5	24981.1	2114
59-16	80364.2	59521.6	101206.7	8856.9	0	17565.1	26243.3	7835.3	0	19964.8	25541.8	992
60-16	60060.8	41223.9	78897.7	5254.3	0	11954.2	22805.2	7650.5	0	19678.6	25367.8	1234
58-16	70889.5	51682.6	90096.3	8592.4	0	17148.9	25988.5	8711.5	0	21327.6	26370.0	2044.4
61-16	65285.3	45975.4	84595.1	6227.5	0	13457.9	23727.1	7729.4	0	19800.7	25442.1	564.4
75-16	77042.1	56256.4	97827.9	7675.7	0	15712.0	25108.4	7403.1	0	19296.2	25135.3	6495.6
05-17	65884.8	46064.5	85705.1	5395.2	0	12171.3	22938.3	7097.2	0	18824.4	24848.5	1959.6
92-16	68475.3	48227.4	88723.2	5495.2	0	12325.6	23032.9	6876.4	0	18484.7	24641.9	367.2

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The use of targeted metabolomics and lipidomics assay platforms provides quantitative data for biomarkers of postmortem interval estimation. Methods based on visual inspection of decomposition changes (e.g. total body score methods) are not universally applicable across climactic zones and are rendered invalid by scavenging activity. This initial analysis of surface remains in above and below freezing temperatures in the eastern United States suggests that membrane phospholipids offer a more robust predictor of PMI. Future investigations in varying climates and taphonomic conditions (e.g. buried and submerged) are needed to evaluate the validity of lipidomic analyses of structural glycerophospholipids as universal PMI predictors. Furthermore, sampling of multiple tissues will provide a better definition of optimal biomarkers and optimal tissues for sampling.

IMPLICATIONS FOR US CRIMINAL JUSTICE POLICY AND PRACTICE

As purveyors of sound methodologies, scientific researchers must ensure that methods are relevant and reliable and stand up to admissibility standards. Forensic anthropologists have made considerable headway in the past 15 years in terms of updating and improving methodologies and establishing error rates. However, there are not many easily applied methods for accurately estimating PMI, and even fewer that have been validated on independent samples. This study uses analytical data from cadaveric metabolites to offer a validated scientific standard for determining postmortem interval using biomarkers unique to human tissues. The method requires only a small amount of tissue, is less subjective than visual methods such as total body scoring, and is robust to drastic fluctuations in temperature. We predict that sophisticated quantitative methods for estimating PMI utilizing biomolecules

unique to the corpse and the human microbiome will eventually replace more subjective visual inspection techniques for determining time since death.

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