Agency: National Institute of Justice

Award # 2012-DN-BX-K018

Project Title: **Body fluid Identification using epigenetic methylation markers and pyrosequencing**

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Final Summary Report
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Body fluid Identification using epigenetic methylation markers and pyrosequencing

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A. Introduction

The discrimination of body fluids present in crime scenes can provide valuable information regarding the circumstances leading to the deposition of DNA. Current methods used in forensic laboratories rely on colorimetric detection of enzymes. Due to the unspecific presence of such enzymes in multiple body fluids, current tests are merely presumptive and may also require the use of big portion of the sample collected (1). Enzymes are the product of specific gene expression in certain body tissues and body fluids. Regulatory mechanisms in the human genome are capable of activating or silencing certain genes in specific tissues in order to promote or silence the expression of gene products, such as proteins, depending whether they are necessary for the biological functions of each specific tissue. Epigenetics is the term used to define such regulatory mechanisms. One example of an epigenetic regulation mechanism is called DNA methylation which involves the addition of a methyl group in the 5' carbon of specific cytosines within the genome. The presence or absence of a methyl group dictates whether transcription factors bind or not the DNA thus promoting or inhibiting transcription (2) . Even though methylcytosines are present throughout the entire human genome, specific regions called tissue-specific differentially methylated regions (tDMRs) are known to present methylation patterns typical of specific body fluids (3). The goal for this project was to identify and study some of those regions in order to use DNA methylation information for body fluid discrimination.

B. Identification of new genomic loci with tissue-specific DNA methylation patterns

The majority of methylated cytosines in the human genome are present in the dinucleotide CG. To avoid confusion with base pairing, potential sites for methylation are called CpGs. There are over 28 Million CpGs dispersed on the human genome. Within the known tDMRs, there is little information regarding how the methylation status of an individual or subsets of CpGs influence tissue-specific cell expression. Some assays designed to identify tDMRs may have single CpG resolution but typically do not offer results for all the CpGs present in the human genome thus focusing on a smaller subset (4). The majority of those CpGs have been previously linked to health studies or environmental factors. Nonetheless, the use of arrays with single-CpG resolution has proved efficient in mapping tDMRs (5) and even to identify CpGs relevant for body fluid identification (6). Since such arrays produce large amounts

of data, its success depends greatly on proper experimental design and statistical analysis. Ideally the tDMRs used for forensic purposes must have a methylation pattern specific for the target body fluid, however running all commonly found body fluid types in arrays would imply decreasing the number of samples per body fluid or prohibitively increase the experimental cost. For this reason, determining new loci for body fluid identification in forensics must constitute a multi-team effort. If a limited number of samples is used, some of the CpGs may relate to specific differences in the small subpopulation rather than presenting tDMRs. Due to these constraints, the CpGs identified by an array study must be confirmed using a method capable of detecting sequence differences (6). One such method is pyrosequencing. Because it is capable of confirming the methylation status of specific CpGs, this method can also be developed and validated to analyze body fluid samples from crime scenes at forensic laboratories. Determining the capability of pyrosequencing for body fluid discrimination in forensic laboratories was another goal of this project.

C. Bisulfite modification of DNA and pyrosequencing

Genomic DNA is the template used in forensic laboratories in order to match a suspect to a crime scene. Several methods have been used and validated by forensic laboratories to extract genomic DNA from cells left at crime scenes. For forensic STR typing, the extracted DNA is amplified using primers capable of annealing to the flanking regions of each STR. DNA amplification is performed by a DNA polymerase *in vitro* in a similar fashion to what happens *in vivo*. The enzyme wraps around the template DNA strand and catalyzes the addition of complementary nucleotides. In that process, the cytosine on the template DNA is 'matched' with a guanine on the newly formed strand due to three hydrogen bonds shared between both nucleotides (7). The DNA polymerase will add guanine to the complementary strand of a cytosine regardless of its methylation status. This is a problem as the procedure which would result in a loss of information on the presence of DNA methylation patterns. For that reason, bisulfite modification must be performed prior to PCR amplification. The modification of unmethylated cytosines with bisulfite leads to a chemical modification of the nucleotide base to uracil, whereas the presence of a methyl group in the 5'-carbon of methylated cytosines protect them from bisulfite conversion. The result is a bisulfite-modified DNA (bDNA) that will contain uracils or cytosines depending on initial methylation state of the CpGs. The bDNA is then added as template on a PCR reaction where the uracils are copied as thymines as the amplification continues (8). At the end of PCR, each individual CpG is analyzed, and each cytosine in the initial genomic template is matched to a cytosine (methylated CpG) or a thymine (unmethylated CpG). Since each DNA strand analyzed contains methylation data from a multiplicity of cells and there are two copies of each strand, it is more common to find partial methylation and not a complete methylated or unmethylated CpG. This fact results in intermediate levels of

methylation in CpGs which may pose difficulty in analysis of some forensic samples, namely those that contain mixtures of body fluids.

The analysis of PCR products can be performed by pyrosequencing which provides quantitative results for each individual CpG. The method relies in the annealing of a single stranded PCR product to a sequencing primer, followed by a single nucleotide dispensation, in the presence of DNA polymerase and other enzymes. When the nucleotide dispensed is complementary to the template strand, the DNA polymerase catalyzes its incorporation on the newly forming strand which causes the liberation of a pyrophosphate. The sulfurylase enzyme present in the reaction catalyzes the conversion of that pyrophosphate into ATP which is then converted to light by a luciferase enzyme. A CDD camera detects the light and displays it as a peak in a pyrogram. Before a new nucleotide is dispensed, any dNTP and ATP present in the well is degraded by apyrase. For each CpG both cytosine and thymine are dispensed and the final pyrogram displays a methylation quantification (in percentage) depending on the relative height of peaks formed for each dispensation of T and C. Furthermore, pyrosequencing allows the simultaneous quantification of several CpG as long as they are present in a given PCR product (9) . In this project we were able to successfully obtain results for 10 genomic loci, each containing several CpGs. The results were published in three peer-reviewed journals ((10) D.S.B.S. Silva et al. / Forensic Science International: Genetics 23 (2016) 55–63, (11) J. Antunes et al. / Electrophoresis 37 (2016) 2751–2758 and (12) K. Balamurugan et al. / Electrophoresis 35 (2014) 3079–3086). Each loci has specific methylation patterns for each of the body fluids: semen, saliva, blood and vaginal epithelia (Table 1 and Figures 1-2). For simplicity we only show the histograms of a vaginal marker PFN3A on Figure 1 and of a semen marker custom_B_SPTB_03 on Figure 2. The remaining markers are published in the references cited.

Table 1 – Methylation percent for three loci showing specific percent of methylation for blood, semen and saliva. (10)

Marker	**Body Fluid**	**CpG (Mean % Methylation ± SD)**				
		CpG 1	CpG 2	CpG 3	CpG 4	CpG 5
ZC3H12D	Semen	5.4±4.0	5.3±3.9	6.7±4.3	6.3±4.0	5.1±3.7
Specific for	**Threshold*	*14*	*13*	*15*	*14*	*13*
semen	Blood	94.0±1.6	94.1±2.7	100.0±0.0	97±7.9	85.9±3.5
	Saliva	81.5±4.1	78.0±4.5	98.8±2.1	79.2±4.9	82.3±3.4
cg06379435		CpG 1	CpG 2	CpG 3	CpG 4	CpG 5
specific for	Blood	23.8±7.8	21.8±6.7	33.2±7.4	30.4±8.2	49 ±12
blood	**Threshold*	*8.2*	*8.4*	*18*	*14*	*25*

	Semen	3.4±1.7	2.4±1.8	2.7±1.1	1.8±1.3	3.13±1.2
	Saliva	8.7±7.0	2.6±1.4	6.0±3.9	3.5±2.6	7.7±4.7
		CpG1	CpG4	CpG5	CpG6	CpG7
BCAS4	Saliva	63.6±7.1	27.5±5.6	16.3±4.5	44.5±6.7	11.5±2.7
Specific for	**Threshold*	*49*	*16*	*7.2*	*31*	*6.1*
saliva	Blood	6.1±1.4	3.2±2.8	2.7±1.3	6.4±1.9	2.4±1.6
	Semen	3.9±1.6	2.5±1.1	3.0±1.1	5.6±1.6	2.3±0.9

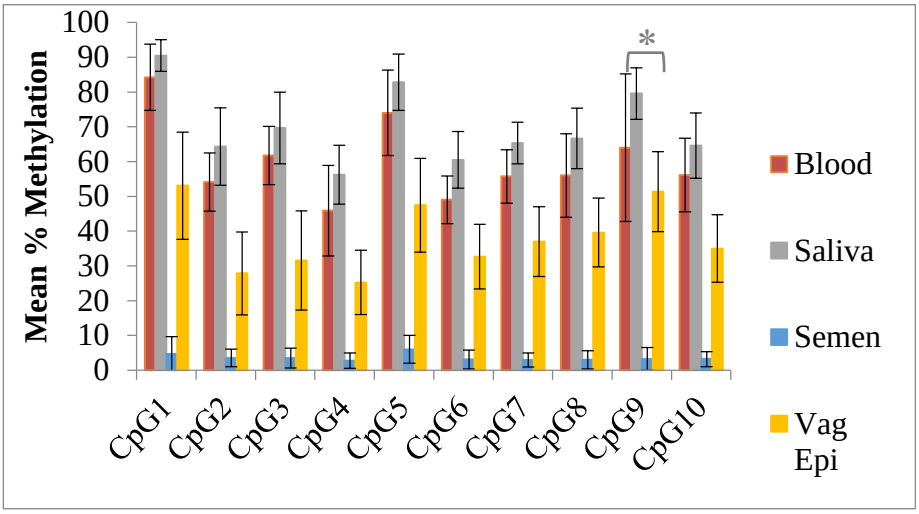

Figure 1 - Graph showing mean percent of methylation on the locus PFN3A for samples of blood, saliva, semen and vaginal epithelia. *CpG where the difference in methylation levels is not statistically significant ($p<0.05$) between vaginal epithelia and blood. (11)

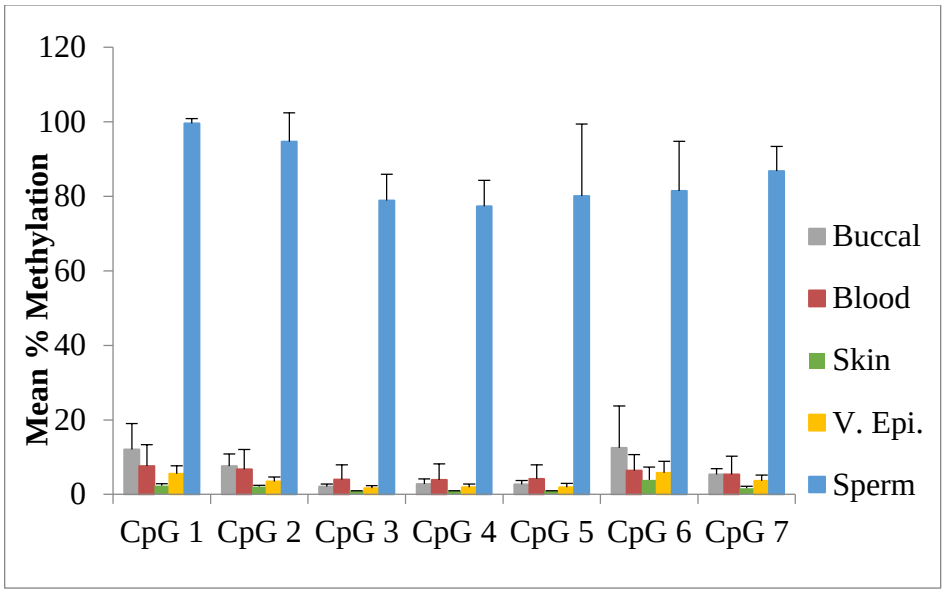

Figure 2 - Graph showing mean percent of methylation on the locus Custom_B_SPTB_03 for samples of blood, buccal cells, semen, skin and vaginal epithelia. (12)

Some loci were validated through the use of samples mimicking those found in forensic cases including mixture of body fluids, samples degraded by heat and UV light, samples with non-human DNA, samples with low amount of genomic DNA, samples containing substances known as PCR inhibitors and samples analyzed in two separate laboratories using pyrosequencing for reproducibility studies. Overall the loci analyzed show promising results to be used in forensic laboratories. The reproducibility studies provided similar results within laboratories (Figure 3) and the exposure to degrading agents such as heat and UV light do not cause differences in methylation in the samples analyzed.

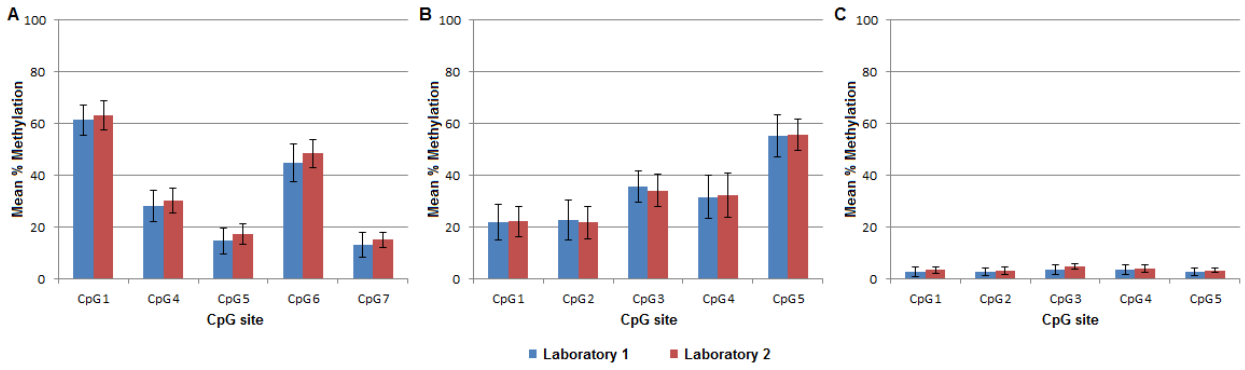

Figure 3 – Comparison between pyrosequencing data generated by two independent laboratories: Laboratory 1 (Florida International University) and Laboratory 2 (University of Southern Mississippi). A)

Methylation data of marker BCAS4 (saliva samples n=8). B) Methylation data of marker cg06379435 (blood samples n=8). C) Methylation data of marker ZC3H12D (semen samples n=8). (10)

Regarding the presence of inhibitors that may be co-extracted with the DNA, the cleanup step after bisulfite treatment seems to be efficient at removing the two inhibitors tested, humic acid and hematin, which are typically found in DNA extracted from soil and blood, respectively. When DNA from non-humans was tested, only the samples corresponding to non-human primates were amplified and from those only some samples for some of the loci actually provided pyrograms that could be mistakenly interpreted as belonging to human DNA. The observed results for primates were expected due to the close evolutionary relationship to humans. No other species' DNA was amplified therefore the primers designed for the loci are considered primate-specific. The mixture of body fluids provided consistent methylation patterns in which the methylation percent was intermediate between the two body fluids mixed, as expected. Regarding sensitivity, one of the primers used was able to amplify and produce reliable results with as little as 0.1 ng of DNA (ZC3H12D), whereas the remaining primers need about 5 to 10 ng of initial genomic DNA template. The fact that such high amounts are necessary is due to a combination of increased fragmentation of DNA due to the harsh chemical modification using bisulfite and the difficulty of finding primers that are specific enough for a template that now contains a bigger percentage of thymines and sometimes a high presence of CpGs with varying methylation status where the primers need to anneal. The fact that the Z3H12D primers were particularly sensitive, indicates a direction for future research in primer design, bisulfite modification, and PCR enhancement to increase sensitivity across all loci.

D. Alternative methods for DNA methylation analysis – High resolution melt analysis

The analysis of bisulfite modified DNA can be performed by looking at specific properties of the PCR product. An unmethylated PCR product will contain a higher content of adenine-thymine double hydrogen bonds, whereas a methylated PCR product of the same region will contain higher guanine-cytosine triple hydrogen bonds. For that reason, one can use different melt temperatures of both PCR products to distinguish levels of methylation. For example, as shown in Table 1, the marker ZC3H12D shows low levels of methylation in semen when compared to blood and saliva. For that reason, a melt curve of the PCR product amplified with the ZC3H12D primers in DNA extracted from semen samples will have a lower melting temperature when compared to the PCR product obtained from the amplification of the same locus in DNA extracted from blood and saliva. Figure 4 shows the melt curves for those samples.

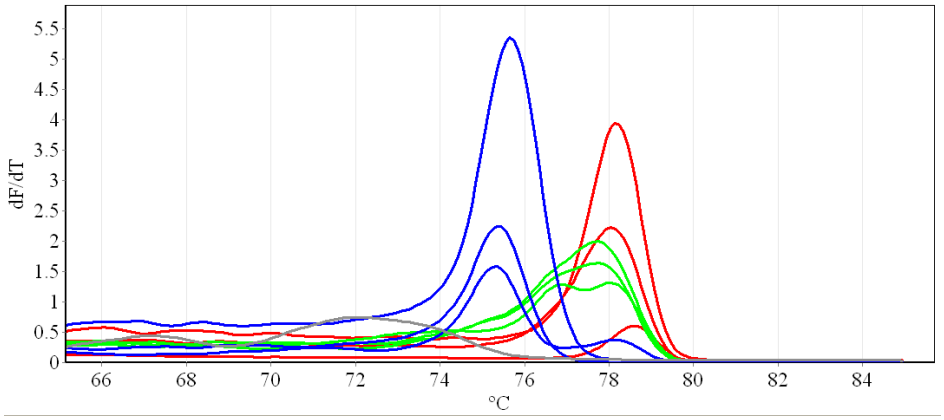

Figure 4 – Graph showing the first derivative of the fluorescence over temperature versus temperature (ºC) for 3 samples of each body fluid: semen (blue), blood (red) and saliva (green). The peak indicates the melting temperature of each curve read in the x-axis for the PCR products obtained after amplification of the ZC3H12D locus.

These results were obtained as a way to validate the use of high-resolution melt (HRM) analysis for forensic purposes. HRM analysis can be performed in any real-time instrument with melt capacity, which is widely available in forensic laboratories. The results are visualized directly as a melt curve such as the one displayed in Figure 4, so little interpretation is necessary. The easiness and lower cost of use for this method makes it attractive for laboratories that have no means to train personnel or to invest in new instruments. The results of this research were published in the peer-reviewed journal Analytical Biochemistry (13).

E. Determination of suspect age

Preliminary work was performed in this grant to determine the application of epigenetics for suspect age determination. To do this CpG sites in genes GRIA2 and NPTX2 were examined [14,15]. Although both genes had been previously analyzed for their correlation with age, they had not been sequenced for methylation content. Thus we decided to investigate different CpG sites in GRIA2 and NPTX2 using pyrosequencing as an alternative analysis method. Our results indicated multiple CpG sites in both genes which provided information on suspect age [16]. We then examined the methylation level of two age markers, GRIA2 and NPTX2, in blood and saliva samples from 44 donors with ages ranging from 5 to 72 years. Three CpG sites were analyzed for GRIA2 and six out of the twelve sites were analyzed for NPTX2. Results with the locus GRIA2 showed a strong correlation with chronological age (R^2=0.801, $p<0.0001$) indicating an average difference of 6.9 years when compared to the suspects

known age. Interestingly, the results in saliva were more accurate than those found in blood samples, thus further affirming the importance of body fluid analysis when using epigenetic studies to determine a suspect's age.

F. Conclusions

We propose that the use of epigenetic information on the DNA can be used as a confirmatory test for the determination of body fluid type in forensic laboratories. DNA methylation can present individual patterns in certain loci in the human body which are tissue-specific. In this project we were able to identify 10 new genome locations that present patterns specific for semen, saliva, blood and vaginal epithelia. Each genome location contains several CpGs whose methylation percent was individually quantified. The quantification is possible due to a specific sequencing technology such as pyrosequencing. Moreover some loci were tested using challenging samples such as those commonly found in crime scenes. We were able to show that those loci show reproducible results, can provide information when low amounts of DNA is used, are primate-specific, can withstand degradation by UV light and presence of inhibitors. We also demonstrate that the presence of body fluid mixtures can be deconvoluted when multiple epigenetic loci are utilized and that body fluid identification is important in suspect age determination. Overall, we believe that this research represents an important advance in the forensic applications of DNA methylation as a confirmatory test for body fluid identification and age.

Implications for criminal justice policy and practice:

The results in this project illustrate the potential of DNA methylation markers as a confirmatory test for body fluid discrimination in forensics. Our results demonstrate that DNA methylation analysis is human specific, is stable for at least 20 years, is resistant to PCR inhibition and can indicate the presence of mixtures. Sensitivity up to 0.1 ng was demonstrated and the process easily fits within the current workflow of forensic laboratories. DNA methylation can also be used to determine a suspects age if combined with body fluid analysis. Continued work on identifying new markers and increasing sensitivity is necessary, however we believe this process has a strong potential to increase the capability of forensic analysis.

Impact on crime laboratories

Our process provides crime laboratories with a new method for body fluid analysis. In particular our work with real time PCR indicates a simple test can be implemented immediately using high resolution melt techniques for body fluid determination. Next gen sequencing methods are also compatible with our procedures. Thus we believe the future is bright for the implementation of these new techniques.

Impact on Technology Transfer

We have collaborated with Qiagen in the development of these epigenetic markers and they have published a user developed protocol for distribution to interested parties and produced a webinar on the topic, October 26, 2016. The marker PFN3A for vaginal epithelia discrimination has been submitted to the Office of Technology Management and Commercialization at Florida International University to be considered for a patent.

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Appendix 1
Publications and Presentations

PUBLICATIONS

Silva DS, Antunes J, Balamurugan K, Duncan G, Alho CS, McCord B. Developmental validation studies of epigenetic DNA methylation markers for the detection of blood, semen and saliva samples. Forensic Science International: Genetics. 2016;23:55-63.

Antunes J, Silva DS, Balamurugan K, Duncan G, Alho CS, McCord B. Forensic discrimination of vaginal epithelia by DNA methylation analysis through pyrosequencing. Electrophoresis. 2016;37(21):2751-2758.

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PRESENTATIONS

Joana Antunes, Deborah Silva, Kuppareddi Balamurugan, George Duncan, Clarice S. Alho, Bruce McCord. High Resolution Melt analysis of DNA methylation to discriminate semen in biological stains. Poster presentation at the 68th Annual meeting of the American Academy for Forensic Sciences, Young Forensic Science Forum, Las Vegas, Nevada. February 22-27, 2016.

Joana Antunes, Deborah Silva, Kuppareddi Balamurugan, George Duncan, Clarice S. Alho, Bruce McCord. High Resolution Melt analysis of DNA methylation to discriminate semen in biological stains. Oral presentation at the Graduate Student Appreciation Week, Miami, Florida, March 2016

Joana Antunes, Deborah Silva, Kuppareddi Balamurugan, George Duncan, Bruce McCord. DNA methylation larmers as a powerful tool for age prediction and to discriminate body fluids from crime scenes. Poster presentation at Statewide Graduate Student Research Symposium, Orlando, Florida, April 2015

Joana Antunes, Deborah Silva, Kuppareddi Balamurugan, George Duncan, Bruce McCord. DNA methylation larmers as a powerful tool for age prediction and to discriminate body fluids from crime scenes. Oral presentation at the Graduate Student Appreciation Week, April 2015, Miami, Florida

Deborah Silva, Joana Antunes, Kuppareddi Balamurugan, George Duncan, Clarice Alho, Bruce McCord. DNA methylation patterns as markers in forensic investigation. Paper presented at the 67th Annual meeting of the American Academy of Forensic Sciences, Orlando FL. February 16-21, 2015.

Joana Antunes, Deborah Silva, Tania Madi, Kuppareddi Balamurugan, Robin Bombardi, George Duncan, Clarice Alho, Bruce McCord. Forensic Epigenetics, A powerful technique to discriminate body fluids present at crime scenes. Oral presentation at the NIJ grantees meeting in conjunction with the 67th Annual meeting of the American Academy of Forensic Sciences, Orlando FL. February 16-21, 2015.

Antunes, Joana; Madi, Tania; Bombardi, Robin; Balamurugan, Kuppareddi; Duncan, George and McCord, Bruce. DNA metylation markers as a powerful technique to discriminate body fluids present in crime scenes. Oral presentation at the 66th annual meeting of the American Academy of Forensic Sciences, Seattle, WA February 17-22, 2014.

Joana P. Antunes, Tania Madi, Kuppareddi Balamurugan, Robin Bombardi, George Duncan, and Bruce McCord. DNA methylation markers as a powerful technique to discriminate body fluids present in crime scenes. Oral presentation at the 24th annual meeting of International Symposium on Human Identification, Atlanta, GA. October 6-10th, 2013.

Joana Antunes, Tania Madi, Kuppareddi Balamurugan, Robin Bombardi, George Duncan, and Bruce McCord. DNA methylation markers as a powerful technique to discriminate body fluids present in crime scenes. Oral presentation at the International Association for Identification (IAI) meeting held in Providence, Rhode Island, August 4-10, 2013.

Joana Antunes, Tania Madi, Kuppareddi Balamurugan, Robin Bombardi, George Duncan, and Bruce McCord. DNA methylation markers as a powerful technique to discriminate body fluids present in crime scenes. Oral presentation at Graduate Student Appreciation Week held in Miami, Florida, March 2013

Joana Antunes, Tania Madi, Kuppareddi Balamurugan, Robin Bombardi, George Duncan, and Bruce

McCord. DNA methylation markers as a powerful technique to discriminate body fluids present in crime scenes. Oral presentation at the 2nd Annual Forensic Symposium of the International Forensic Research Institute, Miami, Florida, March 2013

Bruce McCord, Epigenetic Tissue Typing, NIJ Grantees Meeting, AAFS, Orlando, FL Feb 15-19.

Bruce McCord, New progress in Pyrosequencing for Tissue Sourcing and Age Prediction, Webinar, Qiagen, October, 26 2016.

Bruce McCord Forensic epigenetics, a novel method for body fluid identification and phenotyping, Qiagen Investigator forum, Miami Beach, FL, June 14-16, 2016.

Bruce McCord, Forensic epigenetics, a novel method for body fluid identification and phenotyping. Forensica 2016, Oloumoc, Czech Republic, May 23-25, 2016.