CLINICAL INVESTIGATIONS

Utility of Salivary Biomarkers for Demonstrating Acute Myocardial Infarction

C.S. Miller^{1*}, J.D. Foley III², P.N. Floriano³, N. Christodoulides³, J.L. Ebersole¹, C.L. Campbell², A.L. Bailey², B.G. Rose⁴, D.F. Kinane⁴, M.J. Novak¹, J.T. McDevitt³, X. Ding⁵, and R.J. Kryscio^{5,6}

Abstract: *The comparative utility of serum and saliva as diagnostic fluids for identifying biomarkers of acute myocardial infarction (AMI) was investigated. The goal was to determine if salivary biomarkers could facilitate a screening diagnosis of AMI, especially in cases of non-ST elevation MI (NSTEMI), since these cases are not readily identified by electrocardiogram (ECG). Serum and unstimulated whole saliva (UWS) collected from 92 AMI patients within 48 hours of chest pain onset and 105 asymptomatic healthy control individuals were assayed for 13 proteins relevant to cardiovascular disease, by Beadlyte technology (Luminex®) and enzyme immunoassays. Data were analyzed with concentration cut-points, ECG findings, logistic regression (LR) (adjusted for matching for age, gender, race, smoking, number of teeth, and oral health status), and classification and regression tree (CART) analysis. A sensitivity analysis was conducted by repetition of the CART analysis in 58 cases and 58 controls, each matched by age and gender. Serum biomarkers demonstrated AMI sensitivity and specificity superior to that of saliva, as determined by LR and CART.*

The predominant discriminators in serum by LR were troponin I (TnI), B-type natriuretic peptide (BNP), and creatine kinase-MB (CK-MB), and TnI and BNP by CART. In saliva, LR identified C-reactive protein (CRP) as the biomarker most predictive of AMI. A combination of smoking tobacco, UWS CRP, CK-MB, sCD40 ligand, gender, and number of teeth identified AMI in the CART decision trees. When ECG findings, salivary biomarkers, and confounders were included, AMI was predicted with 80.0% sensitivity and 100% specificity. These analyses support the potential utility of salivary biomarker measurements used with ECG for the identification of AMI. Thus, saliva-based tests may provide additional diagnostic screening information in the clinical course for patients suspected of having an AMI.

Key Words: diagnosis, coronary disease, biological markers, saliva, serum, early diagnosis.

Introduction

Acute myocardial infarction (AMI) is a life-threatening complication of coronary heart disease (CHD) and one of the most

frequent causes of death in the United States. Current estimates are that 620,000 new AMIs and 295,000 recurrent attacks occur annually in the United States, resulting in 151,000 deaths (Go *et al.*, 2014). In the emergency setting, patients suspected of having an AMI receive an electrocardiogram (ECG) and measures of serum biomarkers to detect or exclude myocardial necrosis (Ting *et al.*, 2008). While cardiac troponins (T and I; TnT, TnI) serve as a key criterion for the diagnosis of AMI, diagnostic information is also obtained from serum creatine kinase-MB (CK-MB), total CK, and myoglobin (MYO) concentrations (Motiwala *et al.*, 2013). Rapid acquisition of this diagnostic information is critical to the management strategy for, and survival of, the patient.

Despite tremendous progress in the development of new diagnostic and screening procedures (Sabatine *et al.*, 2002; Morrow and Braunwald, 2003; Storrow *et al.*, 2006; Wu, 2007; Straface *et al.*, 2008), significant numbers of AMI cases are missed or diagnosed too late for optimal therapy to be offered. Contemporary emergency departments are overwhelmed with patients, resulting in frequent delays in care. For instance, 25% of AMI patients wait at least

DOI: 10.1177/0022034514537522. ¹Department of Oral Health Practice, Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, KY, USA; ²Division of Cardiovascular Medicine, Gill Heart Institute, University of Kentucky, Lexington, KY, USA; ³Department of Bioengineering and Chemistry, Rice University, Houston, TX, USA; ⁴School of Dentistry, University of Louisville, Louisville, KY, USA; current address, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA; ⁵Department of Biostatistics, College of Public Health, University of Kentucky, Lexington, KY, USA; and ⁶Department of Statistics, College of Arts & Science, University of Kentucky, Lexington, KY, USA; *corresponding author, cmiller@uky.edu

60 minutes prior to seeing a physician (Wilper *et al.*, 2008). Every minute of delay in treating AMI increases the mortality rate (Diercks *et al.*, 2006). Likewise, there is a compelling need to improve triage and minimize delays associated with seeking care, transportation, and performance of catheter- or pharmacological-based reperfusion.

Saliva is a potential source for facilitating an earlier diagnosis of AMI in the emergency setting. Saliva is rapidly, easily, and non-invasively procured and contains thousands of biomolecules that are derived from the local capillary bed (Hu *et al.*, 2005; Xiao and Wong, 2011). Several biomarkers associated with CHD and AMI [*i.e.*, TnI, TnT, CK-MB, active matrix metalloproteinase (MMP)-8, and C-reactive protein (CRP)] can be detected in saliva at concentrations distinct from those in healthy individuals and that correlate with serum concentrations (Floriano *et al.*, 2009; Miller *et al.*, 2010; Buduneli *et al.*, 2011; Mirzaei-Dizgah and Jafari-Sabet, 2011; Foley *et al.*, 2012b; Mirzaei-Dizgah *et al.*, 2012; Mirzaei-Dizgah and Riahi, 2013a,b). However, the clinical utility of these biomarkers for AMI assessment, alone or as a multimarker panel, is not fully understood. In this report, we used logistic regression (LR) and classification and regression tree (CART) analyses to test the hypothesis that specific salivary biomarkers show clinical utility for the assessment of AMI, especially in cases of non-ST elevation MI (NSTEMI), where the diagnostic assessment requires biomarker information.

Materials & Methods

Study Design, Patient Recruitment, and Sample Collection

A cross-sectional case-controlled study was implemented at the University of Kentucky (UK) and the University of Louisville (UL) hospitals. Of 104 consecutive persons recruited, 92 patients who had sustained an AMI as diagnosed by ECG or elevated serum troponin I (TnI) were enrolled. Patients were enrolled if samples could be

obtained within 48 hr of onset of cardiac symptoms. One hundred and five control individuals (*i.e.*, negative for a history of AMI) were enrolled for comparison. These 2 study populations have been described in part, in a previous report (Miller *et al.*, 2010). Recruitment was coordinated with the cardiac care team and balanced with the needs of the patient, including pain management, reperfusion, and family support issues. Exclusion criteria were fever, accompanying stroke, immune system disorders, use of steroidal medications, major organ complications/failure, inability to provide saliva, and age < 18 yr. The study was approved by the institutional review boards of the 2 participating sites. In all cases, informed consent was granted prior to sample collection, and all samples tested were de-identified to ensure the privacy rights of all study participants.

Demographic information was obtained, medical records were reviewed, an oral evaluation was performed, and biological fluids were obtained [*i.e.*, blood and unstimulated whole saliva (UWS)] by a single examiner (*i.e.*, study coordinator) at times when the patient was considered stable, *per* our previously described method (Foley *et al.*, 2012b). Samples were transported to the local laboratory on ice, centrifuged, and stored at -80°C until analyzed. Samples from UL were shipped on dry ice to the UK on a bimonthly basis. All samples were analyzed in duplicate in a blinded manner within 3 mo of storage.

Oral Health Scoring System

Oral health was scored as either good, fair, or poor based on presence of symptoms, level of mucosal inflammation, number of loose or decayed teeth, and obvious tooth or gingival infection as determined by a single examiner *per* a modification of the oral health scoring system (Burke *et al.*, 2003). The categories were: Good – no complaints, no obvious mucosal inflammation, no reported loose teeth or symptoms of disease; Fair – may have complaints, localized areas of mucosal inflammation,

areas of visible decay, no obvious tooth or gum infection; and Poor – generalized areas of mucosal inflammation, multiple broken down teeth, obvious tooth or gum infection, or loose teeth reported. The examiner was a registered dental hygienist who had held certification as a study coordinator for over 10 years. The clinical assessments were performed bedside with illumination.

Measurement of Biomarkers by Luminex® and Beckman Access

Standard cardiac biomarkers B-type natriuretic peptide (BNP), CK-MB, MYO, and cTnI were measured in serum and saliva with the Beckman Access®2 in the UK hospital CLIA-certified clinical chemistry laboratory. The Luminex® IS-100 instrument (Luminex® Corp., Austin, TX, USA) was used for the multiplex detection of 9 biomarkers in serum and saliva, with kits available from Beadlyte® Technology (Millipore, Billerica, MA, USA): CRP, interleukin-6 (IL-6), interleukin-1β (IL-1β), myeloperoxidase (MPO), soluble cluster of differentiation ligand (sCD40L), tumor necrosis factor-α (TNF-α), adiponectin (Adip), soluble intracellular adhesion molecule-1 (sICAM-1), and MMP-9. Samples were diluted, if necessary (serum Adip, MMP-9, MPO, sICAM-1 diluted 1:100, and serum CRP 1:5,000) and analyzed in duplicate, and all results were reported within the linearity of the standard curve. The process resulted in a 192-patient dataset for serum and 158 patients for saliva. The inability for sufficient sample volume to be collected for the numerous assays of this study was responsible for the loss of some patients who could have provided a complete set of biomarkers.

Data Analysis

Demographics were compared between groups by 2 sample *t* tests for interval level variables and chi-square tests for categorical variables. The distribution of each saliva and serum biomarker was compared between the control individuals and AMI patients by the Wilcoxon Rank Sum statistic. To avoid inflation of the Type I error rate, we

Table 1.
General Characteristics of Study Participants

	AMI (n = 92)	Controls (n = 105)	p value
Age (mean ± SD), yr	54.2 ± 11.9	48.57 ± 8.6	.0003
Gender (%)	Female: 21.6	Female: 57.1	< .0001
Race (%)	AA: 15.2 White: 84.8	AA: 8.2 White: 86.7 Hispanic: 3.1 Asian: 2.0	.065
SBP (mean ± SD)	119.6 ± 19.1	125.1 ± 15.5	.033
DBP (mean ± SD)	67.1 ± 16.3	76.9 ± 10.4	< .001
BMI (mean ± SD), Kg/m ²	29.11 ± 6.8	28.3 ± 5.6	.35
Smoking (%)	64.7	24.4	< .001
Teeth (mean ± SD)	15.1 ± 11.2	25.4 ± 6.0	< .001
Oral health (%)	Good: 46.7 Fair: 33.3 Poor: 20.0	Good: 87.5 Fair: 9.38 Poor: 3.13	< .0001

AA, African American; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index.

determined statistical significance at the 0.01 level.

To determine if each biomarker was associated with an AMI, we fitted a logistic regression (LR) model to the data adjusted for gender, age, race, smoking, number of teeth, and oral health status, since these differed between the groups; in these models, the log of the biomarker was used to determine the adjusted odds ratio. Next, a non-parametric classification and regression tree (CART) analysis was constructed to determine which subset of the 13 serum or saliva biomarkers best separated cases from control individuals; the CART solutions also included the adjustment variables mentioned above. We conducted a sensitivity analysis by repeating the CART analysis using 58 of the cases who matched 58 control individuals by age (to within 2 yrs) and gender. Data analyses were conducted with PC-SAS version 9.3 (Cary, NC, USA).

Results

Patient Characteristics

The characteristics of the 2 study groups are presented in Table 1. The majority in each cohort were White. Those in the AMI group were predominantly STEMI (63%) and were significantly older, male, smoked tobacco

more frequently, and had significantly fewer teeth and poorer oral health than those in the control group. The mean body mass indices (BMI) reflected the overweight status of both cohorts. More than 80% of the AMI patients received antifibrinolytic/antithrombotic therapy prior to providing their samples, and the majority were receiving antihypertensive and statin drugs (data not shown). The mean time to sample procurement was 23.7 hr after chest pain onset for the AMI patients (range, 10-47 hrs; SD, 10.7).

Analyte Concentrations between Groups

The distribution of the serum biomarkers CRP, MPO, Adip, MYO, CK-MB, TnI, and BNP differed significantly between AMI and control individuals (Fig. 1A). Of these, serum TnI showed the greatest discriminatory capacity. In saliva, discrimination for AMI was represented by significant differences in concentrations of CRP, sCD40L, sICAM-1, and TNF- α (Fig. 1B).

Logistic Regression

Results of the LR models of the significant biomarker concentrations detected in serum and saliva, with adjustments made for age, gender, race,

smoking, number of teeth, and oral health, are shown in Table 2. In serum, TnI was most associated with AMI, with BNP, CK-MB, MYO, and CRP also attaining significance. In UWS, CRP was most associated with AMI, with both Adip and sICAM-1 attaining significance as well. These results also held in the sensitivity analysis based on the matched dataset. The LR models in Table 2 examined the significance of each biomarker considered alone and assumed that the relationship between the log odds of an AMI and the biomarker was linear in the log of the biomarker.

CART Analysis

The CART procedure was applied to data derived from analyses of 13 serum biomarker concentrations. Fig. 2A shows the resulting decision tree in which 90 AMI cases and 102 control individuals were included; the CART model could have adjusted for the confounders but failed to identify any as significant. CART selected serum TnI as the biomarker most likely to distinguish AMI from control individuals. A decision rule was made at 0.065 ng/mL, where concentrations above this cut-point were accurate for the diagnosis of AMI in 85 of 85 cases (100%). When serum TnI was lower than this concentration, serum BNP $\geq$ 38.05

Figure 1.

Box plots showing analyte levels expressed as log values in serum (A) from 90 acute myocardial infarction (AMI) patients and 102 non-acute myocardial infarction adult control individuals, and in unstimulated whole saliva (B) from 73 AMI and 85 control individuals. Concentrations are pg/mL for all analytes, except Adip, sICAM-1, CRP, TnI, CK-MB, and MYO, which are ng/mL. Samples were obtained within 48 hr of chest pain onset and analyzed in duplicate. Seventeen AMI patients were unable to provide sufficient saliva for analysis. The *p* values shown above the bars indicate significant differences between the 2 groups as determined by Wilcoxon Rank Sum statistics.

pg/mL identified 4 remaining AMIs, but captured 4 false-positives. If BNP was < 38.05 pg/mL, then TNI > 0.045 ng/mL identified the remaining case. Overall,

these 2 serum biomarkers provided 98.9% sensitivity and 96.1% specificity for AMI, regardless of incorporation of the ECG data into the diagnostic

algorithm. Additional sensitivity analysis of the CART by restricting to cases who had only matching healthy control individuals (*n* = 58, each group) and included the confounders of gender, age, race, smoking, number of teeth, and oral health was performed. This analysis produced the same 2 first nodes with only slightly different cut-points, a revised tertiary node being MYO, and near-identical sensitivity and specificity (data not shown).

CART was next applied to the entire dataset derived from the UWS biomarker concentrations with inclusion of confounders in the analysis. CART selected tobacco smoking as the first node, salivary CRP at 4.755 ng/mL and gender as second nodes, and salivary sCD40L at 35.4 pg/mL and the number of teeth as tertiary nodes (Fig. 2B). This CART algorithm provided 82.2% sensitivity and 84.7% specificity. When we further restricted the CART analysis to cases and matched control individuals and included the potential confounders in the analysis, Fig. 2C resulted. Here the decision nodes were similar (*i.e.*, smoking, salivary CRP, and the number of teeth) with the addition of salivary CK-MB discriminating AMI with 71.1% sensitivity and 98.0% specificity. When ECG was considered in a sensitivity analysis, ECG first identified 66.7% of the AMIs (Fig. 2D). The remaining NSTEMI cases were identified with salivary CRP as the secondary decision node, with oral health and age as tertiary nodes (80.0% sensitivity and 100% specificity).

Discussion

In patients with chest pain, an important goal is to diagnose or rule out AMI efficiently, to minimize infarct size and myocardial damage. For STEMI, medical intervention and reperfusion are recommended within 30 to 90 min of first medical contact (Antman *et al.*, 2004; Jacobs *et al.*, 2007). However, there are substantial obstacles for achieving this goal that involve issues with transportation, hospital facilities, admission wait times, and diagnostic testing. Because time delays contribute

Table 2.
Logistic Regression Model Selection with *p* values Determined by Chi-square

Fluid	Analyte	Odds Ratio	Lower 95% CI	Upper 95% CI	<i>p</i> value
Serum	Log Tnl	> 999.99	> 999.99	> 999.99	.0088
Serum	Log BNP	10.64	4.40	25.75	< .0001
Serum	Log CK-MB	20.40	5.96	69.76	< .0001
Serum	Log MYO	6.56	2.63	16.39	< .0001
Serum	Log CRP	2.78	1.87	4.13	< .0001
UWS	Log Adip	1.68	1.17	2.40	.0049
UWS	Log sICAM	1.92	1.26	2.93	.0026
UWS	Log CRP	1.68	1.33	2.13	< .0001

p values were generated by logistic regression using the entire dataset (*n* = 90 AMI, *n* = 102 controls) after adjustment for age, gender, race, smoking, number of teeth, and oral health.

Figure 2.

CART decision trees. (A) Based on serum biomarkers in which 90 cases and 102 control individuals were included for the primary endpoint of AMI. Two cases and 3 control individuals had insufficient sample volume for analysis of all biomarkers. (B) The CART decision tree based on unstimulated whole saliva (UWS) biomarkers in which 73 cases and 85 control individuals were included for the primary endpoint of AMI; analysis done without ECG. Nineteen cases and 20 control individuals had insufficient sample volume for analysis of all biomarkers. (C) CART analysis with UWS biomarkers from 45 cases and 51 matched control individuals with the primary endpoint of AMI. (D) Forty-five cases and 51 matched control individuals, UWS biomarkers, confounders, and ECG to discriminate STEMI and NSTEMI.

to poor outcomes (De Luca *et al.*, 2013), improvements that lead to reduced time to diagnosis and therapy continue to be sought, especially with respect to NSTEMI, where biomarkers serve as the main diagnostic decision point.

In this study, we evaluated 92 patients who presented with AMI and provided serum and saliva samples within 48 hr of chest pain and compared fluid biomarker concentrations with those from a similar number of control individuals. Biomarkers were selected for their documented involvement in atherosclerosis, cardiovascular disease, and tissue damage and remodeling (Sabatine *et al.*, 2002; Morrow and Braunwald, 2003; Shen *et al.*, 2012), and data were analyzed by 2 different approaches, LR and CART. In serum, LR showed that the standard cardiac panel (TnI, CK-MB, MYO, and BNP) provided excellent diagnostic capacity. CART identified a similar set of markers, but narrowed the significant biomarkers to TnI and BNP and identified optimal cutoff concentrations. These 2 biomarker concentration cutoffs identified all AMIs with 4 false-positives (3.9%), suggesting that this combination is important for identifying AMI in emergency settings. Of note, the Wilcoxon analysis of the serum biomarker profiles also demonstrated that concentrations of CRP and MPO were significantly elevated and Adip was significantly lower during the post-AMI interval. However, these concentrations did not improve the diagnostic capacity of the standard cardiac panel for AMI diagnosis, but may provide important insight into the biochemical processes involved during post-AMI events. This could be clinically important, because, although trends indicate improvements in door-to-balloon times nationally, in-hospital mortality for AMI has not improved. Thus, additional strategies that address processes that contribute to earlier diagnoses, risk stratification, and reduced mortality are needed (Menees *et al.*, 2013).

Serum biomarkers of CHD are detectable in saliva (Floriano *et al.*, 2009; Miller *et al.*, 2010; Foley *et al.*, 2012a), and recent reports indicate that concentrations of select salivary

markers are elevated early post-AMI and after myocardial damage (Buduneli *et al.*, 2011; Mirzaei-Dizgah and Jafari-Sabet, 2011; Foley *et al.*, 2012b; Mirzaei-Dizgah *et al.*, 2012; Shen *et al.*, 2012; Mirzaei-Dizgah and Riahi, 2013a; Toker *et al.*, 2013). Despite a host of markers accumulating from these studies, the ability to discriminate which biomarkers would be best for facilitating a screening diagnosis of AMI, especially in cases of NSTEMI, has been lacking. Our initial screen using Wilcoxon Rank Sum statistics revealed a panel of biomarkers (*i.e.*, CRP, sICAM-1, and MPO) that were elevated in the AMI group. LR also pointed to salivary CRP and sICAM-1 as well as Adip as discriminators of AMI. The CART identified smoking as the primary decision node and salivary CRP as a secondary decision node, in analyses of both the entire dataset and sensitivity analysis. The number of teeth also served as a consistent tertiary decision node for predicting a screening diagnosis of AMI. Additional tertiary decision nodes were UWS sCD40L, CK-MB, oral health, and age. These outcomes, which showed smoking, tooth loss, poor oral health, and aging as CART predictors of AMI, are consistent with the epidemiological literature identifying these as important risk factors of AMI (Orford *et al.*, 2002; reviewed in Zoellner, 2011), and lend support to the importance of the salivary biomarkers identified in the CARTs. Our findings, combined with those of others, suggest that important salivary markers of AMI could be CRP, sCD40L, and CK-MB, as well as creatine phosphokinase, TnT, and TnI (Mirzaei-Dizgah and Jafari-Sabet, 2011; Mirzaei-Dizgah *et al.*, 2012; Mirzaei-Dizgah and Riahi, 2013a,b).

One reason for using CART is that the algorithm offers the advantage of classifying patients at risk for a disease while allowing for visualization of the interaction between and among predictor covariates. Second, CART provides specific concentration cut-points. Here, the overlapping findings of the LR and CART, as well as the overlapping findings within CARTs (*i.e.*, sensitivity analyses), are strengths of this study. Additional strengths include the use of a well-

characterized clinical population that experienced an AMI, a control population that lacked a history of AMI, the precise and routine collection of biological fluids within a time period coincident with the rise and fall of serum and salivary biomarkers, and the measurement of analyte concentrations by standardized immunoassays. However, it is of note that our CART did not identify salivary TnI as a decision node, whereas salivary TnT and TnI have been identified as biomarkers of AMI (Mirzaei-Dizgah and Riahi, 2013a,b). Explanations for the contrasting results between our laboratories may include the fact that Mirzaei-Dizgah and Riahi sampled patients more proximal to the AMI event and did not enroll NSTEMI patients, whereas 37.8% of our patients had NSTEMI, which led to lower Tn levels, and we used LR and CART, and they used correlations and Student's *t* test without adjustment for potential confounders (tobacco use, number of teeth, oral health).

Potential limitations of the study are the study size, the biomarker panel being limited to 13, antihypertensive medications not being fully documented, and the limited procurement period being between 10 and 47 hr after chest pain onset. A larger study size with additional biomarkers could have allowed for a more robust CART analysis. Also, earlier time-points or serially obtained samples could have yielded different diagnostic information and potentially improved discriminatory power. Finally, inclusion of control individuals who complained of non-cardiac-related chest pain would have provided a more robust generalization to the findings.

In summary, the synergistic use of multiple biological markers in LR and CART analysis helped to define optimal markers, their sequence for use, and clinically relevant concentration thresholds for predicting a screening diagnosis of AMI. Although biomarkers in saliva were less sensitive and specific than those in serum, the ease of salivary acquisition and its discriminatory power were evident, based on single hospital measurements. Analysis of these data and the accumulating literature suggests that

saliva could provide important screening diagnostic information that could be critical for initially directing patients to facilities that can perform primary percutaneous coronary intervention, which has been proven to improve patient outcomes (Eitel *et al.*, 2010), as well as subsequent diagnostic decisions. Thus, the present study may provide proof-of-principle that saliva, when used with additional clinical information, can assist clinicians in making decisions important in AMI diagnosis that can be validated in future clinical studies.

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