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## Salivary extracellular RNA biomarkers for insulin resistance detection in hispanics

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### ARTICLE INFO

#### Article history:

Received 19 October 2016

Received in revised form

15 December 2016

Accepted 4 July 2017

Available online 19 July 2017

#### Keywords:

Salivary biomarker

Extracellular RNA

Insulin resistance

### ABSTRACT

**Aims:** Insulin resistance (IR) detection is challenging and no test is currently used in clinical practice. We developed salivary biomarkers that could be used for IR detection.

**Methods:** We collected saliva from 186 healthy and 276 pre-diabetic participants, divided them into high and low IR groups based on a HOMA cutoff of 2.5. We profiled extracellular transcriptome by microarray in saliva supernatant from 23 high IR and 15 low IR participants, and pre-validated the top ten extracellular mRNA (exRNA) markers in a new cohort of 40 high and 40 low IR participants. A prediction panel was then built and validated in an independent cohort of 149 high and 195 low IR participants.

**Results:** Transcriptomic analyses identified 42 exRNA candidates differentially present in saliva of high and low IR participants. From the top ten candidates, six were individually validated (PRKCB, S100A12, IL1R2, CAMP, VPS4B, CAP1) ( $p < 0.01$ ) and yielded AUC values ranging from 0.66 to 0.76. Body mass index (BMI) was significant higher in high compared to low IR group with AUC of 0.66, and showed no correlation with any of candidate biomarkers. The combination of four exRNA markers (IL1R2, VPS4B, CAP1, LUZP6) with BMI achieved excellent results in the prediction panel building dataset (AUC = 0.79, sensitivity = 79%, specificity = 64%). The prediction model was validated in an independent cohort (AUC = 0.82, sensitivity = 63%, specificity = 92%).

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<http://dx.doi.org/10.1016/j.diabres.2017.07.008>

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Conclusions: A panel of four salivary exRNA biomarkers (IL1R2, VPS4B, CAP1, LUZP6) and BMI was validated that can distinguish high and low IR participants, overall and in subgroups of healthy and pre-diabetic participants.

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## 1. Introduction

Insulin resistance (IR) is a condition in which cells become less sensitive and resistant to the activity of insulin, causing glucose to build up in the blood, leading to hyperglycemia. IR and hyperglycemia are risk factors for type 2 diabetes, metabolic syndrome [1], and coronary heart disease [2]. Increasing evidence shows that IR occurs before impaired glucose tolerance or impaired fasting glucose levels [3]. Hence, it is important to identify IR early to enable timely intervention. The gold standard method for measuring IR is the euglycemic insulin clamp (EIC) test [4]; however it is clinically impractical because of cost and time constraints [5]. A variety of alternative simpler measures have been in use, the most common being homeostasis model assessment (HOMA). Other measures include Matsuda index and insulinogenic index [6–9]. All these indices are limited by their accuracy, cost and require blood drawing [10]. Hence, unlike hyperglycemia testing, IR is rarely evaluated clinically. Validated and practicable tests and biomarkers for IR are urgently needed. For this study we used HOMA to classify the subjects initially for IR status. We also cross-validated our findings from HOMA, with the Matsuda insulin sensitivity index, an alternative standard metric [4] strongly correlated with clamp measures [11,12].

Saliva has omics constituents that can be harnessed for biomarker development, and can be obtained non-invasively. Salivary biomarkers have been developed by our group for oral and systemic diseases [13–15], including type 2 diabetes [16]. This study focused on development of salivary biomarker for IR detection in a prospective study design, using high-throughput RNA discovery to identify salivary extracellular RNA (exRNA) biomarkers, with the goal of early intervention and prevention of diabetes and its complications.

## 2. Material and methods

### 2.1. Study design

This study is an ancillary project to the ongoing San Juan Overweight Adult Longitudinal Study (SOALS), and was approved by the Institutional Review Board at the University of Puerto Rico. The study followed the principle of prospective sample collection and retrospective blinded evaluation (PRoBE) design [17].

### 2.2. Study population

SOALS participants included overweight and obese participants, aged 40–65 years, and free of major cardiovascular

disease and diabetes (reported or based on evaluations using cutoffs from the American Diabetes Association diagnostic guidelines [18]). Details of study participants and pre-diabetes classification are provided in the [Supplemental Tables 1 and 2](#).

Participants were classified into high IR group (HOMA-IR value  $\geq 2.5$ ) or low IR group (HOMA-IR value  $< 2.5$ ). We also replicated the analyses using the Matsuda index to validate our findings from HOMA. The Matsuda Index was derived from 0, 30, 60, and 120 min time points of oral glucose tolerance test (OGTT) measures.

### 2.3. Sample collection

Participants provided blood samples that were drawn at fasting, and after 75 g glucose load at 30, 60, and 120 min. Glucose and insulin were assessed from blood samples at all time-points, and hemoglobin A1c (HbA1c) was also assessed. During the baseline visit, participants were asked to refrain from eating, drinking, smoking or oral hygiene procedures for at least two hours prior to the saliva collection. Five ml of unstimulated whole saliva samples were collected, stabilized and preserved from all consenting participants as previously described [19]. Saliva samples were centrifuged at  $2600 \times g$  for 15 min at 4 °C. Supernatants were removed from the pellet and immediately mixed with the RNase inhibitor SUPERase-In (Ambion, Austin TX).

### 2.4. Study protocol

This study consisted of three phases conducted among independent groups of participants selected from SOALS:

Biomarker discovery phase: RNA was isolated from 330  $\mu$ l of saliva supernatant using RNeasy Mini kit (Qiagen, Valencia, CA). This process was automated using KingFisher mL technology (Thermo Fisher Scientific, Waltham, MA), followed by TURBO™ DNase treatment (Ambion, Austin, TX). The quality of isolated RNA were examined by RT-PCR for three cellular gene transcripts: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin- $\beta$  (ACTB), and ribosomal protein S9 (RPS9).

Extracted RNA was linearly amplified using the RiboAmp RNA Amplification kit (Molecular Devices, Sunnyvale, CA). After purification, complementary DNA (cDNA) was in vitro transcribed and biotinylated using GeneChip Expression 3'-Amplification Reagents for in vitro transcription labeling (Affymetrix, Santa Clara, CA). The Affymetrix Human Genome U133 Plus 2.0 Array was used for mRNA profiling at the UCLA microarray core facility. Using the minimum information about a microarray experiment (MIAME) criteria [20], Expression values for the microarray analysis were calculated and normalized using the Li and Wong method using dChip

[21]. For every probe set, the Wilcoxon rank sum test was applied to identify differential expression between IR high risk and low risk samples. After obtaining the estimates and the P values of each probe set, we corrected the P values for false discovery rate and then ranked the exRNA markers.

Individual salivary exRNA candidate validation phase: Quantifications of selected exRNA biomarkers from discovery phase were performed with the use of reverse transcription PCR (RT-PCR) and droplet digital PCR (ddPCR) performed as singleplex assays adhering to digital minimum information of quantitative digital PCR experiments (MIQE) guidelines [22]. RT-PCR and ddPCR assays were designed using Primer3-Plus (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>) and their sequences were showed in Table S3. All primers and probes were synthesized by Sigma-Genosys (Woodlands, TX). we used universal human reference RNA (Agilent Technology, Santa Clara, CA) as positive control and UltraPure Distilled water (Life Technology, Carlsbad, CA) as negative control.

1.5 ng extracted RNA was converted to DNA and pre-amplified in an GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) by using SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen, Carlsbad, CA) with following cycling conditions: 2 minutes at 60 °C, 30 minutes at 50 °C, 2 minutes at 95 °C, 15cycles each consisting of a 15 s at 95 °C, 30 s at 50 °C, 10 s at 60 °C and followed by a 72 °C at 10 s, and a final 10 min at 72 °C. After reverse transcription and pre-amplification, DNA were purified with ExoSAP-IT (USB Corporation, Cleveland, OH) for 15 min at 37 °C and 15 minutes at 80 °C. 20 $\mu$ L DNA solution (10 $\mu$ L ddPCR Probe Supermix, 900 nM primers, 250 nM probe, and template DNA) was then loaded into Bio-Rad QX-100 emulsification device and droplets were formed following the manufacturer's instructions. Then 40 $\mu$ L droplets were transferred to a 96-well reaction plate and sealed with a pierceable foil heat sealer at 170 °C for 4 s. All digital PCR reactions were done singleplexed in a T100 thermal cycler (Applied Biosystems, Foster City, CA) with the following cycling conditions: 10 min at 95 °C, 40 cycles each consisting of a 30 s denaturation at 94 °C followed by a 60 °C extension for 60 s, and a final 10 min at 98 °C. After cycling, droplets were analyzed immediately. Quantification of each marker was analyzed by QuantaSoft software accompanying the ddPCR equipment (Bio-Rad DX100). Following PCR, each droplet is analyzed in a droplet reader to determine the fraction of PCR-positive droplets. These data were then analyzed using Poisson statistics to determine the absolute initial copy number of each marker in the original samples. To minimize the variation, the same cut-off value was used in all ddPCR reactions for each marker.

Prediction panel building and definitive validation phase: a prediction panel of salivary exRNA biomarkers was built and definitively validated in an independent cohort with the use of RT-PCR and ddPCR techniques which were used in individual salivary exRNA candidate validation phase.

## 2.5. Statistical analysis

Initial analysis using the microarray data and the individual marker evaluation used Wilcoxon rank sums tests to compare exRNA markers between high and low IR groups. Using the

model building cohort, the performance of each exRNA biomarker was evaluated individually using receiver operating characteristic (ROC) curve and computing the area under curve (AUC) by numerical integration of the ROC curve. Next, a logistic regression model was constructed using forward stepwise variable selection to determine the best combination of biomarkers and clinical factors. The predicted probability for each participant obtained from the model was used to construct an ROC curve along with the AUC and its 95% confidence interval (CI). The sensitivity and specificity for biomarker combinations were estimated by identifying the cut-point of the predicted probability that yielded the highest sum of sensitivity and specificity. Finally, the locked down model was directly applied to the validation cohort. Predicted probabilities from the applied model were used to construct ROC curves and compute the AUC. To assess technical reproducibility, variance components models were constructed to partition the variance into patient and experimental variability. In addition, we computed predicted probabilities from the locked down logistic regression model for each aliquot and computed the variability of those probabilities within participants. All analyses were performed in R V3.1.2 ([www.r-project.org](http://www.r-project.org), Vienna, Austria) and SPSS V22 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Patient characteristics

A total of 463 participants were selected for this study. One sample did not pass the microarray analysis quality control because of significant degradation; hence, 462 samples were included, 212 were in high IR group and 250 in low IR group.

The samples used in different phases of study were: (1) Biomarker discovery phase: 23 high IR and 15 low IR participants. (2) Individual salivary exRNA candidate validation phase: 40 high IR and 40 low IR. (3) Prediction panel building and definitive validation phase: 149 high IR and 195 low IR. Schematics of the study design are shown in Supplemental Fig. 1, and descriptive statistics for all participants used for discovery, individual marker validation and definitive validation are shown in Table 1.

### 3.2. Salivary transcriptomic biomarker discovery for IR detection

In the discovery phase, after transcriptomic profiling of the cell free saliva, 42 genes showed significant differences between high and low IR groups ( $p < 0.05$ ). Thirty-three genes were up-regulated in high IR group of which eight exhibited over two fold up-regulation. Nine genes were down-regulated in the high IR group of which four exhibited over two fold down-regulation. The microarray data generated in this study have been uploaded to the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>), the access number is GSE67738). The ten exRNA markers that were most significantly different between high IR and low IR were selected as the candidates for individual validation (PRKCB, ADI1, S100A12, KSR, LUZP6, IL1R2, CAMP, COX17, VPS4B, and CAP1).

**Table 1 – Clinical and oral health characteristics of study population.**

	Discovery phase			Individual exRNA marker validation			Definitive validation phase					
	Low IR	High IR	P-value <sup>a</sup>	Low IR	High IR	P-value <sup>a</sup>	Model building			Model validation		
							Low IR	High IR	P-value <sup>a</sup>	Low IR	High IR	P-value <sup>a</sup>
N	15	23		40	40		130	100		65	49	
Age (years)	52.8 ± 8.4	48.7 ± 7.1	0.11	49.8 ± 7.2	50.3 ± 6.3	0.77	50.3 ± 6.6	49.9 ± 6.7	0.66	50.9 ± 6.6	49.5 ± 6.8	0.29
Male (%)	8 (53%)	6 (26%)	0.09	5 (13%)	11 (28%)	0.09	30 (23%)	40 (40%)	0.03	18 (28%)	14 (29%)	0.94
Hispanics (%)	100%			100%			100%			100%		
Healthy/Pre-diabetes	4/11	8/ 15	0.02 <sup>b</sup>	20/20	20/20	1 <sup>b</sup>	60/70	30/70	0.04 <sup>b</sup>	30/35	14/35	0.11 <sup>b</sup>
Hypertension (%)	6 (40%)	10 (44%)	0.83 <sup>b</sup>	14 (35%)	17 (43%)	0.23 <sup>b</sup>	52 (40%)	48 (48%)	0.08 <sup>b</sup>	26 (40%)	23 (47%)	0.11 <sup>b</sup>
Smoker (%)	4 (22%)	5 (27%)	0.73 <sup>b</sup>	8 (20%)	7 (18%)	0.92 <sup>b</sup>	30 (23%)	20 (20%)	0.52 <sup>b</sup>	20 (31%)	10 (20%)	0.09 <sup>b</sup>
Alcohol consumption (g/day)	2.1 ± 1.1	8.1 ± 3.0	0.10	3.7 ± 1.0	4.2 ± 1.3	0.76	6.5 ± 1.0	3.9 ± 0.9	0.07	6.4 ± 1.6	6.8 ± 1.7	0.88
Coffee Consumption (≥1 time/day)(%)	10 (67%)	13(57%)	0.77 <sup>b</sup>	27 (68%)	27 (68%)	0.93 <sup>b</sup>	92 (71%)	63 (63%)	0.46 <sup>b</sup>	42 (65%)	28 (57%)	0.34 <sup>b</sup>
Statins therapy (%)	1 (7%)	2 (9%)	0.82 <sup>b</sup>	4 (10%)	1 (3%)	0.36 <sup>b</sup>	6 (5%)	4 (4%)	0.99 <sup>b</sup>	3 (5%)	2 (4%)	1 <sup>b</sup>
BMI (kg/m <sup>2</sup> )	32.8 ± 2.4	38.9 ± 7.1	0.003	30.2 ± 3.6	36.0 ± 7.2	<0.001	32.0 ± 5.7	35.7 ± 7.4	<0.001	31.3 ± 4.3	35.5 ± 5.7	<0.001
Fasting glucose (mg/dl)	93.1 ± 8.2	98.5 ± 8.7	0.07	88.8 ± 7.3	94.2 ± 7.6	0.002	90.5 ± 9.1	95.9 ± 9.2	<0.001	90.6 ± 8.1	96.7 ± 9.5	<0.001
HbA1c (%)	5.6 ± 0.1	5.8 ± 0.1	0.008	5.6 ± 0.1	5.6 ± 0.1	0.63	5.6 ± 0.03	5.8 ± 0.04	<0.001	5.6 ± 0.04	5.7 ± 0.1	0.09
HbA1c (mmol/mol)	37.2 ± 0.9	40.1 ± 0.6	0.007	37.5 ± 0.6	37.9 ± 0.6	0.64	37.9 ± 0.3	39.5 ± 0.4	0.002	37.8 ± 0.5	39.1 ± 0.5	0.09
HOMA-IR	1.7 ± 0.5	4.3 ± 1.4	<0.001	1.4 ± 0.6	3.7 ± 1.3	<0.001	1.5 ± 0.5	4.2 ± 1.7	<0.001	1.5 ± 0.6	4.1 ± 1.7	<0.001
Matsuda index	5.4 ± 0.6	2.4 ± 0.1	<0.001	7.0 ± 0.6	2.7 ± 0.2	<0.001	6.7 ± 0.3	2.8 ± 0.1	<0.001	6.8 ± 0.5	2.6 ± 0.1	<0.001
Probing depth (PD) (mm)	1.79 ± 0.13	1.85 ± 0.13	0.74	1.55 ± 0.05	1.90 ± 0.07	<0.001	1.85 ± 0.06	1.91 ± 0.08	0.53	1.70 ± 0.05	1.82 ± 0.07	0.14
Clinical attachment loss (CAL) (mm)	1.50 ± 0.25	1.69 ± 0.19	0.55	1.52 ± 0.16	1.89 ± 0.12	0.06	1.91 ± 0.13	1.93 ± 0.13	0.89	1.68 ± 0.11	1.72 ± 0.13	0.81
Bleeding on probing (BOP) (%)	27.8 ± 6.9	26.8 ± 5.4	0.91	18.5 ± 3.4	29.4 ± 3.4	0.03	27.4 ± 2.2	29.8 ± 2.7	0.48	24.0 ± 2.6	28.5 ± 3.9	0.32
Decayed, missing and filled teeth (DMFT)	9.7 ± 1.4	11.0 ± 1.2	0.51	13.2 ± 0.9	12.9 ± 0.9	0.86	13.4 ± 0.6	12.8 ± 0.6	0.50	12.4 ± 0.6	11.3 ± 0.9	0.33

Data are means ± SD or number (%).

<sup>a</sup> Univariate analysis with unpaired student's t test, for means.

<sup>b</sup> Chi-square test for frequencies

**Table 2 – Performance of validated salivary exRNA biomarkers and other factors.**

Individual exRNA marker validation			Prediction panel building		
Candidate Markers	P-value	AUC value (95%CI)	Candidate Markers	P-value	AUC value (95%CI)
PRKCB	0.002	0.71 (0.59–0.82)	<b>IL1R2</b>	<b>0.004</b>	<b>0.61 (0.54–0.69)</b>
S100A12	<0.001	0.74 (0.63–0.85)	<b>VPS4B</b>	<b>0.002</b>	<b>0.62 (0.55–0.69)</b>
<b>IL1R2</b>	<b>0.001</b>	<b>0.72 (0.61–0.83)</b>	<b>CAP1</b>	<b>&lt;0.001</b>	<b>0.63 (0.56–0.71)</b>
CAMP	0.011	0.66 (0.55–0.78)	ADI1	0.22	0.55 (0.47–0.62)
<b>VPS4B</b>	<b>&lt;0.001</b>	<b>0.76 (0.65–0.86)</b>	KSR	0.48	0.53 (0.45–0.60)
<b>CAP1</b>	<b>&lt;0.001</b>	<b>0.73 (0.62–0.84)</b>	LUZP6	0.36	0.54 (0.46–0.61)
ADI1	0.189	0.59 (0.46–0.71)	<b>BMI</b>	<b>&lt;0.001</b>	<b>0.66 (0.59–0.73)</b>
KSR	0.686	0.53 (0.40–0.65)			
LUZP6	0.603	0.53 (0.41–0.66)			
COX17	0.062	0.62 (0.50–0.74)			
<b>BMI</b>	<b>&lt;0.001</b>	<b>0.76 (0.65–0.87)</b>			
Smoke	0.747	0.52 (0.47–0.72)			
Gender (male)	0.248	0.58 (0.45–0.70)			
Hypertension	0.141	0.60 (0.47–0.72)			

Using a new independent cohort of 40 high IR and 40 low IR saliva samples, six of the ten candidate exRNAs were significantly different ( $p < 0.05$ ) between high IR and low IR (PRKCB, S100A12, IL1R2, CAMP, VPS4B, CAP1) (Fig. 2A). For these six markers their expression patterns in the independent cohort were consistent with that observed the microarray data. These validated exRNA markers yielded individual AUC values ranging from 0.66 to 0.76 (Table 2).

The correlation structure of the six validated salivary exRNAs showed that PRKCB and CAMP were correlated with IL1R2 ( $r = 0.71$  and  $0.69$ ,  $p < 0.001$ ); S100A12 and CAMP were correlated with CAP1 ( $r = 0.80$  and  $0.77$ ,  $p < 0.001$ ) (Supplemental Table 4). Therefore only three markers (IL1R2, VPS4B and CAP1) are effectively useful for multivariate modeling.

We found no association between these variables (smoke, gender, hypertension) and IR status except for BMI, which alone yielded an AUC value of 0.76 (Table 2) and had no correlation with any of the 10 candidate biomarkers in the individual marker validation cohort (Supplemental Table 5). We decided to include BMI in our model building because of its strong association with IR, lack of correlation with our biomarkers, and its ease of measuring clinically.

### 3.3. Prediction panel building and validation

To build the prediction panel, we subjected the three individually validated (IL1R2, VPS4B, CAP1) and three potential performance enhancing non-validated (KSR, LUZP6, ADI1) salivary exRNA markers to a second individual marker validation step using an independent cohort of 100 high IR and 130 low IR subjects (Table 1). The three validated markers (IL1R2, VPS4B, and CAP1) continued to show significant differences between two groups (Fig. 1B) yielding AUC values from 0.61 to 0.63 (Table 2, prediction panel building). BMI also showed a significant difference between two groups (Table 1) yielding an AUC value of 0.66. After logistic regression analysis, among the different models of exRNA biomarkers chosen by stepwise variable selection and the addition of a “non-validated” marker LUZP6, the combination of IL1R2, VPS4B, CAP1, LUZP6, and BMI yielded a best AUC value of 0.79 with

79% sensitivity and 64% specificity (Table 3). This is the locked down prediction panel.

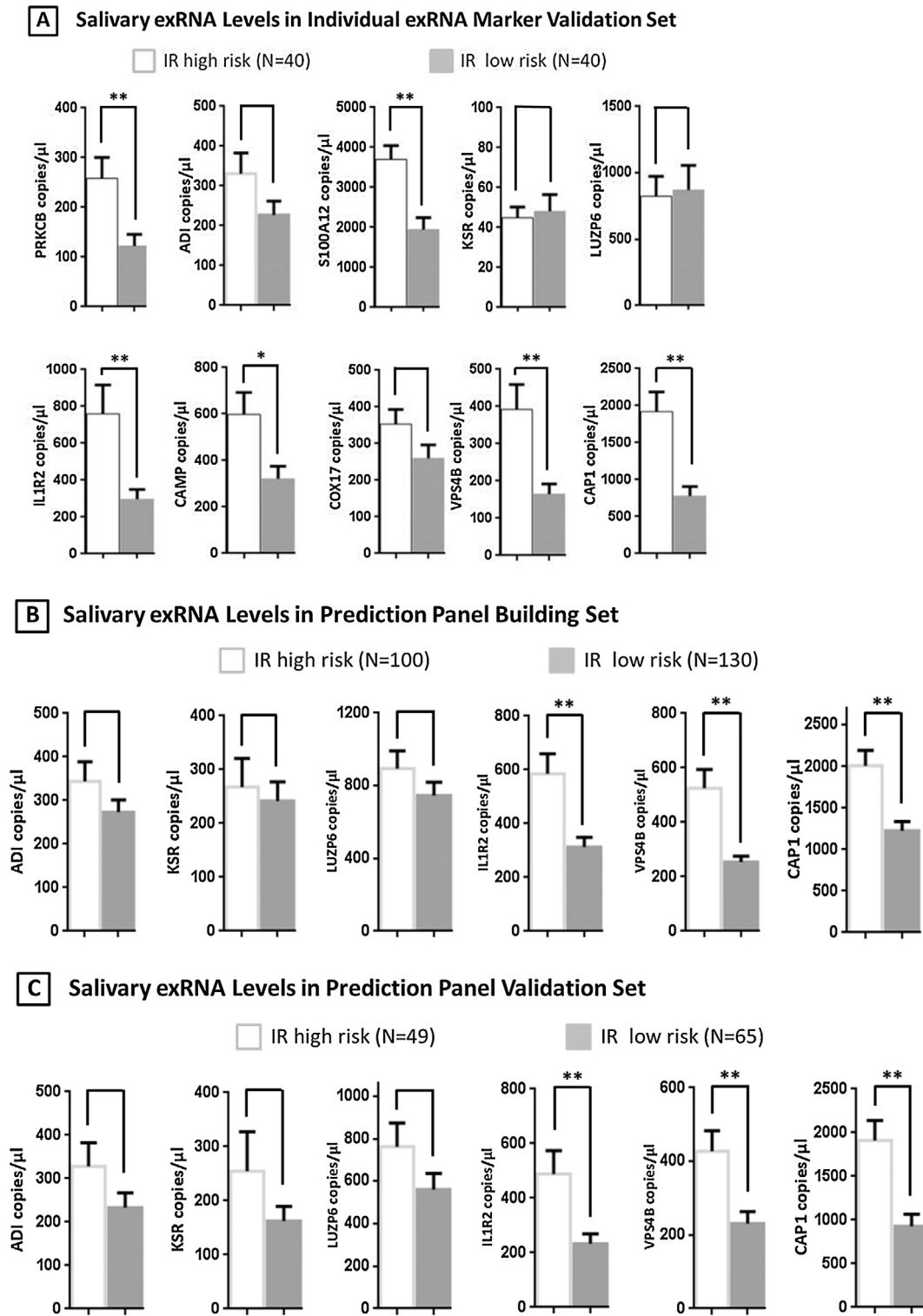
The locked down predictive panel was further evaluated in an independent cohort of 65 low IR and 49 high IR individuals (Table 1 and Fig. 1C). The discriminatory performance of the prediction panel achieved an AUC value of 0.82, a sensitivity of 63% and a specificity of 92% (Fig. 2A). BMI alone achieved an AUC of 0.66. The four salivary exRNA biomarkers enhanced the discriminatory AUC to 0.82, an increase of 0.16. When we stratified the results by diabetes status, the model had an AUC value of 0.88 with 93% sensitivity and 78% specificity among healthy participants and an AUC value of 0.76 with 69% sensitivity and 89% specificity among pre-diabetic participants (Fig. 2B and C).

### 3.4. Correlation analysis between HOMA and Matsuda index

In the prediction panel building phase, we found a strong relationship between HOMA and Matsuda index (Spearman's correlation =  $-0.917$ ,  $p < 0.001$ , Table 4). Then, we correlated the indices with the individual salivary markers, the Spearman's correlation between HOMA-IR and individual markers were: ADI1: $\rho = 0.089$  ( $p > 0.05$ ), KSR: $\rho = 0.041$  ( $p > 0.05$ ), LUZP6: $\rho = 0.067$  ( $p > 0.05$ ), IL1R2: $\rho = 0.169$  ( $p < 0.05$ ), VPS4B: $\rho = 0.160$  ( $p < 0.05$ ), CAP1: $\rho = 0.204$  ( $p < 0.05$ ); the correlation between Matsuda index and individual markers were similar: ADI1: $\rho = -0.104$  ( $p > 0.05$ ), KSR: $\rho = -0.037$  ( $p > 0.05$ ), LUZP6: $\rho = -0.097$  ( $p > 0.05$ ), IL1R2: $\rho = -0.180$  ( $p < 0.01$ ), VPS4B: $\rho = -0.180$  ( $p < 0.01$ ), CAP1: $\rho = -0.192$  ( $p < 0.01$ ) (Table 4).

### 3.5. Correlation analysis between salivary ExRNA biomarkers panel and HOMA for IR detection

As HOMA is commonly used for IR evaluation, we evaluated if the individual markers from the validated salivary IR prediction model are associated with HOMA using linear regression. The correlation coefficient for IL1R2 was 0.18 ( $p = 0.001$ ) (Supplemental Fig. 2A), and similar results were obtained for VPS4B ( $r = 0.19$ ,  $p < 0.001$ ) and for CAP1 ( $r = 0.23$ ,  $p < 0.001$ ) (Supplemental Figs. 2B and 2C).



**Fig. 1** – Panel A shows the mean ( $\pm$ SE) salivary levels of ten candidate markers in individual exRNA marker validation set, six of them (PRKCB, S100A12, IL1R2, CAMP, VPS4B, CAP1) showed significant difference between high IR and low IR groups. Panel B and C show levels of six candidate markers used in prediction panel building (B) and validation of the prediction model (C). In Panel A, B, and C, a single asterisk denotes  $P < 0.05$  for the between-group comparison; double asterisks denote  $P < 0.01$ .

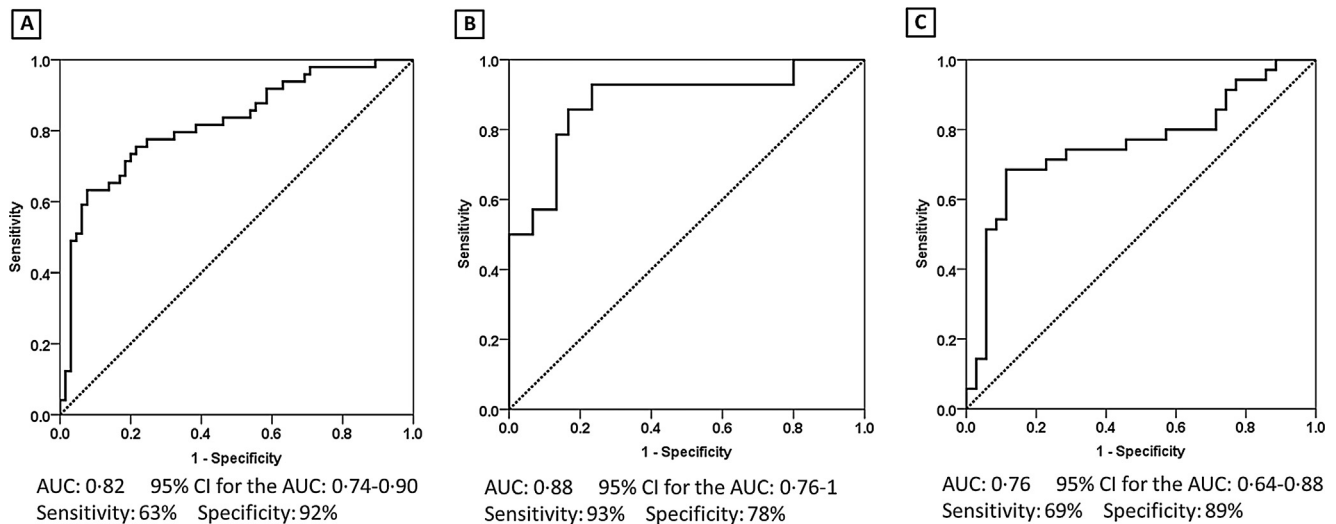
#### 4. Discussion

People with IR have a high risk of developing type 2 diabetes and other metabolic disorders [23]. Effective non-invasive IR

measurements could serve as an early and strong predictor for metabolic disorders. The gold standard for detecting IR is the EIC test. EIC is clinically impracticable because of the high cost, time needed and invasiveness. Other tests based

**Table 3 – Performance of biomarker combinations in the prediction panel building phase.**

exRNA biomarkers combination	AUC (95% CI)	Sensitivity	Specificity
IL1R2 + VPS4B + CAP1	0.62 (0.55–0.69)	0.29	0.93
IL1R2 + VPS4B + CAP1 + BMI	0.70 (0.63–0.77)	0.58	0.74
IL1R2 + VPS4B + CAP1 + KSR	0.64 (0.57–0.71)	0.35	0.89
IL1R2 + VPS4B + CAP1 + LUZP6	0.71 (0.64–0.77)	0.91	0.42
IL1R2 + VPS4B + CAP1 + ADI1	0.62 (0.54–0.69)	0.32	0.92
IL1R2 + VPS4B + CAP1 + LUZP6 + BMI	0.79 (0.72–0.84)	0.79	0.64

**Fig. 2 – ROC curves for final prediction panel for IR in: (A) non-diabetic and pre-diabetic; (B) non-diabetics; (C) pre-diabetics.****Table 4 – Correlations analysis between exRNA markers and HOMA-IR as well as the markers with Matsuda in the prediction panel building phase.**

Spearman's $\rho$		HOMA-IR	Matsuda	ADI1	KSR	LUZP6	IL1R2	VPS4B	CAP1
HOMA-IR	$\rho$		–0.917	0.089	0.041	0.067	0.169	0.160	0.204
	p-value		<0.001	0.18	0.53	0.31	0.01	0.02	0.002
Matsuda	$\rho$	–0.917		–0.104	–0.037	–0.097	–0.180	–0.180	–0.192
	p-value	<0.001		0.12	0.58	0.14	0.005	0.005	0.004

on fasting blood (insulin-to-glucose ratio, and HOMA), OGTT derived indices (Matsuda Index [24] and Mari Index [9,25]) have been developed. Serum biomarkers (fetuin-A, gamma-glutamyltransferase,  $\alpha$ -Hydroxybutyrate, ferritin, and calprotectin) for IR have also been developed; however, these tests lack insulin measurements. Verification and validation and lack of standardized insulin assays [26], also limit the value of OGTT derived indices such as HOMA for routine IR assessment.

We have previously developed omics-based salivary biomarkers for detection of oral and systemic diseases [13,15,27], including type 2 diabetes [28]. The major goal of this study was to develop an effective non-invasive saliva-based test to screen non-diabetic and prediabetic individuals for their IR status. To reach this goal, we evaluated whether the salivary exRNAs varied across groups with high and low IR as assessed by HOMA; whether salivary exRNAs identified by microarray data could be validated in an independent

cohort; and the discriminatory ability of a validated panel of salivary biomarkers. Our results confirmed that saliva biomarkers are effective non-invasive tools for IR detection.

A total of 42 genes exhibited differential expression, 33 were up-regulated and 9 were down regulated. We selected the top ten exRNA markers for individual validation, where six of ten genes (60%) were validated ( $p < 0.01$ ,  $>1.5$ -fold); and were up-regulated in high compared to low IR group, yielding AUC values from 0.71 to 0.76. One salivary exRNA that were not significant individually but were chosen in the final models by stepwise selection, because many of the validated individual exRNA markers are correlated while these non-validated markers are not correlated and subsequently complement the prediction panel by enhancing the discriminatory performance (AUC). These gene subsets were determined to be the most predictive overall indicators of IR.

Obesity is commonly recognized as a high risk factor for IR [29]. Lee confirmed the direct correlation of BMI with IR [30].

Although all our study participants were overweight (BMI  $\geq 25.0$  kg/m<sup>2</sup>) or obese (BMI  $\geq 30.0$  kg/m<sup>2</sup>), participants' BMI in high IR group were still higher compared to the low IR group in all phases ( $p < 0.01$ ). Since BMI showed no correlation with candidate biomarkers and exhibited strong correlation with IR [31], we included BMI as an independent predictor.

The final locked down panel of IL1R2, VPS4B, CAP1, LUZP6, and BMI had a predicted performance of AUC = 0.79. The actual validated performance of the prediction panel was AUC = 0.82, confirming the discriminatory power and validity of salivary exRNA biomarkers for IR detection. In the final panel validation phase, BMI alone had an AUC of 0.66 whereas the combination of the four salivary exRNA biomarkers and BMI had an AUC of 0.82, a strong performance improvement of 0.16 AUC over BMI alone.

This study was restricted to high risk overweight/obese who would be most likely to benefit from this diagnostic test, given the very low prevalence of IR ranging from 0% to 16% [32] in lean or normal weight individuals [33]. Participants in this study were healthy or pre-diabetic. This is of importance, as diabetes interventions are more effective at early stages. In the individual marker validation phase, we found that salivary biomarkers yielded similar AUC values in healthy (0.93) and pre-diabetic (0.94) participants. In the definitive validation, we assessed the prediction power of the validated panel among healthy and pre-diabetic participants separately. The validated panel performed well in both groups (healthy: AUC = 0.88, sensitivity = 93%, specificity = 78%; pre-diabetes: AUC = 0.76, sensitivity = 69%, specificity = 89%), which indicates that our panel is a valuable tool in detecting IR among both healthy and pre-diabetic participants. While it is known that gender differences can impact gene expression, but gender did not impact salivary exRNA levels in our study.

We selected HOMA for this study as it is the most widely used measure of IR, and shows good correlation ( $r = -0.80$ ) with clamp derived measures [34], especially among people without significant hyperglycemia, which is our target population [35]. EIC test is the gold standard but is also not perfect, as it does not accurately reflect physiological conditions as reflected post-meal or after an oral glucose load [36]. The use of measures such as HOMA as the standard is generally likely to underestimate rather than overestimate the diagnostic ability of the saliva markers, due to random misclassification [37]. Hence our results compared to HOMA can be viewed as a conservative estimate.

We cross-validated our findings from HOMA, with the Matsuda insulin sensitivity index, an alternative standard metric [4] strongly correlated with clamp measures [11,12]. There was a strong correlation between HOMA and Matsuda in our prediction model training set showed a strong relationship (Spearman's correlation =  $-0.917$ ,  $p < 0.001$ ), highlighting the validity of HOMA against a more invasive and extensive measure. The individual salivary markers (ADI1, KSR, LUZP6, IL1R2, VPS4B, and CAP1) had very similar correlations with HOMA and with Matsuda (Table 4). The AUC of our final model for HOMA was 0.79 (95% CI 0.73–0.84) and for Matsuda (using above/below the median Matsuda score in our dataset which was 3.97) was 0.77 (95% CI 0.71–0.83). The comparative results for our biomarker panel against two IR assessment

indices (HOMA or Matsuda) and the correlations in the literature relating these indices to EIC, support the validity of our panel as a measure of IR. Future studies should consider evaluation against EIC.

The known biological functions of these validated biomarkers are mainly linked to protein binding, ATP binding and ATPase activity (Supplemental Table 6), some of which were associated with insulin secretion and glucose metabolism. For example, IL1R2 was found to be positively correlated with reduced insulin secretion and higher HbA1c levels [38]; VPS4B was up-regulated in response to the inhibition of glucose stimulated insulin secretion [39]; and CAP1 expression level was associated with insulin sensitivity [40]. These studies corroborate our findings and strengthen the premise that our panel could be used in the clinical detection of IR. Furthermore, identifying the genes that are involved in IR, will allow us to better understand its pathogenesis and mechanism of the diseases, and means for prevention.

This is the first study to evaluate salivary exRNA from non-diabetics and pre-diabetics to obtain a discriminatory panel of salivary biomarkers capable to differentiate IR. We have developed and validated a predictive panel for detection of IR in healthy and pre-diabetic individuals in a high risk Hispanic population. Future studies should evaluate the ability of this panel in predicting progression of IR and hyperglycemia and evaluate potential utility in different populations and ethnic groups. Furthermore, the multi-center evaluation of the panel should be conducted to fully address its clinical predictive value.

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## 5. Conclusions

This study is an important milestone for salivary biomarker development as the first study to complete the biomarker development path for salivary biomarkers, based on prospective study design (PROBE-compliant) for discovery and validation, and the definitive validation of individual and panel of salivary exRNA markers for IR detection with discriminatory clinical performance. A panel consisting of four salivary exRNA biomarkers and BMI was developed and validated that can distinguish high and low IR participants, among non-diabetic people and within subgroups of healthy and pre-diabetes participants.

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## Acknowledgements

The authors acknowledge Dr. Cynthia Perez, Mr. Francisco Munoz, Dr. Maribel Campos, Ms. Elaine Rodriguez, Ms. Sasha Martinez, Mr. Helson Lasanta, and Ms. Nicole Iglesia. The authors would also acknowledge the Puerto Rico Clinical, and Translational Research Consortium nurses (Ms. Barbara Guzman, Ms. Tania Ginebra and Ms. Laritza Berrios) for all of their support. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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## Contribution statement

Y.Z, QX.L, D.C, K.J and D.W wrote and edited the manuscript. Y.Z, K.J and D.W conceptualized the overall strategy and



developed the clinical translation and implementation. K.J designed the clinical component and assessment of insulin resistance and contributed to planning the data collection and processing. J.V enrolled patients to the protocol, and implemented the data collection. Y.Z, J.S, M.P and F.L designed and performed the RNA extraction and mRNA expression measurement. T.G and D.E designed and performed the statistical analyses. K.J and D.W were the study's principal investigators.

### Conflicts of interest statement

David T.W Wong is co-founder of RNameTRIX Inc., a molecular diagnostic company. He holds equity in RNameTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNameTRIX. Intellectual property that David Wong invented and which was patented by the University of California has been licensed to RNameTRIX. All other authors have nothing to disclose.

### Funding sources

This research was funded by the National Institutes of Health (R01DE020111, 2U54MD007587, UH2TR000923, R01CA139596, U54RR026139, U54MD007587), Delta Dental-Michigan #20164106, the Barnes Fund for Head & Neck Cancer Research, and the O'Keefe Foundation.

### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.diabres.2017.07.008>.

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