

## RESEARCH

# Circulating MIR148A associates with sensitivity to adiponectin levels in human metabolic surgery for weight loss

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## Abstract

**Objective:** We sought to discover secreted biomarkers to monitor the recovery of physiological adiponectin levels with metabolic surgery, focusing on epigenetic changes that might predict adiponectin function.

**Design:** We conducted a prospective observational study of patients undergoing metabolic surgery by Roux-en-Y Gastric Bypass (RYGB) for weight loss in a single center (IRB GHS # 1207-27).

**Methods:** All patients ( $n=33$ ; 27 females; 6 males) signed informed consent. Metabolites, adiponectin and MIR148A were measured in fasting plasma. We followed MIQE for transcript profiles.

**Results:** Patients lost on average  $47 \pm 12\%$  excess BMI (%EBMI) after 12 weeks. Adiponectin pre, post or delta (post minus pre) did not correlate with %EBMI. A decrease in adiponectin following weight loss surgery was observed in a subset of patients, chi-square test of independence rejects the null hypotheses that the liver DNA methyltransferase 1 (DNMT1) and delta adiponectin are independent (**chi-square statistics  $\chi^2=6.9205$ ,  $P=0.00852$ ,  $n=33$** ), as well as MIR148A and delta adiponectin are independent (**chi-square statistics  $\chi^2=9.6823$ ,  $P=0.00186$ ,  $n=33$** ). The presence of plasma MIR148A allows identification of patients that appear to be adiponectin insensitive at baseline.

**Conclusion:** We combined the presence of plasma MIR148A, the concentration of total adiponectin and the expression of DNA methyltransferase 1 (DNMT1) in liver biopsy tissue to identify patients with non-physiological adiponectin. Weight loss and physical activity interventions complemented with the new method presented here could serve to monitor the physiological levels of adiponectin, thought to be important for long-term weight loss maintenance.

## Key Words

- ▶ total plasma adiponectin
- ▶ liver DNA methyltransferase 1 (DNMT1)
- ▶ metabolic surgery
- ▶ RYGB
- ▶ weight loss

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## Introduction

Adiponectin (Adp) is a peptide hormone expressed primarily in adipose tissue (1), which shows paradoxical decreased secretion as adiposity increases (2). Plasma adiponectin

levels in human are not always necessarily physiologically active and the concept of adiponectin insensitivity is well accepted (3). Adiponectin secretion has been suggested

to be regulated by epigenetic mechanisms (4), and interventions that activate its physiological secretion levels hold promise for the treatment of metabolic disorders associated with adipokine and insulin insensitivity (5).

The liver is a major target organ for adiponectin; hepatocytes contain adiponectin receptors 1 and 2 (6). Adiponectin promotes hepatic functions, such as glucose homeostasis and fatty acid oxidation. Further, it has been well documented that the liver function is influenced by signaling interactions with adipose tissue (7, 8). Specifically, DNA methyltransferase 1 (DNMT1) has been described as a key regulator of normal liver function (9). The liver's sensitivity to environmental factors (10), including physical activity and central metabolic functions (11) make it an important mediator of whole body homeostasis.

Beyond the genome, epigenetics can directly affect the function of genes, and the underlying mechanism that increases the susceptibility to develop a disease (12, 13). The coordinated actions of mRNAs with other epigenetic factors (14) are thought to be responsible for critical changes in the expression and function of genes. The presence of MIR148A has been associated with DNA hypomethylation caused by sequence complementarity between MIR148A and DNMT1 (15, 16, 17). MicroRNAs in general have large amounts of methylation-susceptible CpG islands in their promoter regions, and for that they are known to form regulatory loops with DNMT1 in cancer tissue and cancer cell lines *in vitro* (17). In adipocytes, DNMT1 contributes to the expression of adiponectin (18, 19).

We report that high levels of MIR148A in plasma and low levels in liver DNMT1 mRNA transcripts in liver are related to adiponectin insensitivity in patients following metabolic surgery for weight loss at 12 weeks.

## Materials and methods

### Patient group and clinical setting

This study complies fully with the 1979 Belmont Report and was approved by the Institutional Review Board (IRB) of The Guthrie Clinic (TGC) # 1207-27. Informed consent was performed on all study patients. The collaborative study also received approval from the Cornell University Office of Research Integrity and Assurance (# 1209003265). All patients scheduled to undergo Roux-en-Y Gastric Bypass (RYGB), referred herein as metabolic surgery were eligible to participate in this study ( $n=33$ ).

All surgeries were performed at Robert Packer Hospital. Patient selection for metabolic surgery followed the 1991 NIH Consensus guidelines for gastrointestinal surgery for severe obesity, as well as the updated 2008 AACE/TOS/ASMBS guidelines (20). Percent excess BMI loss above BMI 25 (%EBMIL), calculated with the formula  $((\text{BMI baseline} - \text{BMI post op}) / (\text{BMI baseline} - 25)) * 100$  was used here to report weight loss change over time. Each patient's baseline before metabolic surgery (pre-RYGB) was used as control for the changes that occurred 12 weeks post RYGB. Metabolic syndrome – as defined by International Diabetes Federation (IDF) criteria (21).

### Blood collection and biochemical analysis

Fasting non-heparinized blood was collected in K<sub>2</sub>EDTA-spray-dried tubes (BD Vacutainer Cat #367841). Plasma was isolated within 30min of collection and aliquoted in 200µL volumes prior to storage at  $-80^{\circ}\text{C}$ . Total adiponectin and insulin were assayed with ELISA kits for human total adiponectin (Millipore Cat # EZHADP-61K) and human insulin (Millipore Cat # EZH1-14K). Blood samples for chemistries were collected separately and chemistry results were later obtained from the patient's clinical records.

### Isolation of liver tissue total RNA

Liver tissue biopsies were collected in the operating room and flash frozen within 2 min, followed by storage at  $-80^{\circ}\text{C}$  until RNA isolation. Total RNA isolation was performed with TRIzol Plus Purification kit (Life Technologies Cat # 12183-555) and included DNase treatment in column with PureLink (Life Technologies Cat # 12185010). Isolated total RNA was eluted from the column and flash frozen within 30s. Samples were stored at  $-80^{\circ}\text{C}$  until reverse transcription.

### Isolation of plasma microRNA

Plasma total microRNA isolations were achieved using 200µL of plasma with the miRNeasy plasma kit (Qiagen Cat # 217184). The spike-in-control *C. elegans* miR-39 (miR-39) was added to every lysate to a final  $4 \times 10^7$  copies/µL.

### Real-time qPCR for relative quantification of liver transcripts

First-strand cDNA was obtained using 2µg of total RNA with Reverse Transcription kit (Qiagen Cat # 205311) that

included an additional gDNA removal step. SYGR real-time qPCR was performed in triplicates of 10  $\mu$ L reactions using Roche LightCycler 480 and ready-to-use SYBR Green 1 master (Roche Cat # 04707516001). Primers for target genes are as follows: DNA (cytosine-5)-methyltransferase 1 (DNMT1, Qiagen Cat # QT00034335). Relative quantification was performed using the  $\Delta\Delta$ CT-method with expression normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase – housekeeping reference). Transcript absence is reported as ND (not detected).

### Real-time qPCR for relative quantification of microRNAs

Reverse transcription was achieved with miScript II RT kit synthesis (Qiagen Cat #218160), with miScript HiSpec buffer specific for downstream quantification of mature mRNAs. Primers for mature mRNAs are as follows: Hs\_miR-148a\_1 miScript Primer Assay (Qiagen Cat #MS00003556) and reference Hs\_RNU6-2\_11 miScript Primer Assay (Qiagen Cat # MS00033740).

Real-time polymerase chain reaction (qPCR) was done with miScript SYBR green master mix kit (Qiagen cat # 218073), using a Roche LightCycler 480 system with 384 block. Amplification curves were generated with an initial denaturing hot start step at 95°C for 15 min, followed by 45 cycles of 94°C for 15 s, 55°C for 30 s and 70°C for 30 s as the temperature was increased at 1°C per second. Melting curves were generated using the following program: PCR products were denatured at 95°C and cooled to 65°C at 20°C per second. The fluorescence signals at a wavelength of 530 nm were then collected continuously from 65°C to 95°C as the temperature was increased at 0.2°C per second. Advanced relative quantification second derivative max using the Roche LC480 software release SW 1.5.1.  $\Delta\Delta$ CT-Method normalized ratio = ((Cq target/Cq reference) sample / (Cq target/Cq reference) calibrator). The expression of the mRNAs was normalized to RNU6 (RNA, U6 small nuclear 6, pseudogene) and the results are reported as relative units. Transcript absence is reported as ND (not detected).

### Statistical analysis

Comparisons between pre and post-op groups were analyzed by paired *t*-test. Nonparametric Spearman's Rho analysis was used for associations. Chi-square statistics tests were used to determine independence where a *P* value <0.05 was considered statistically significant. All statistical analysis was performed using JMP Pro 11 and graphs were

prepared using GraphPad Prism 6.05. Data are presented as mean  $\pm$  s.d., *n* = 33, unless otherwise specified.

## Results

### Characteristics of participant's pre and post RYGB metabolic surgery

Table 1 summarizes the descriptive characteristics on 33 patients included in our study. The patient population is rural, residing in the southern tier region of New York and north-central counties in Pennsylvania of the United States of America. Our population was from low-income socioeconomic backgrounds and geographically fixed (non-transient), with an average obesity rate of 28.82% compared to the American national average of 27.35% reported by the CDC in 2009. The participants included in this study comprised 27 females and 6 males with baseline BMI  $45 \pm 5.7$ . Average age was 43 years for the females and 42 years for males; comorbidities in these patients include metabolic syndrome (MS +/-) and type 2 diabetes (T2D +/-). Significant differences were observed in all of the hormonal parameters between the pre and post RYGB. Out of the metabolic parameters, significant differences are observed in all but high-density lipoprotein cholesterol (HDL-C). Total plasma adiponectin levels increased significantly despite large variations ( $12.7 \pm 6.2$  pre-RYGB,  $17.1 \pm 9.8$  post-RYGB, *P* = 0.0302). In spite of the significant average increase in the levels of plasma adiponectin post weight loss, we observed that 30% of the patients in our cohort presented decreased adiponectin levels post weight loss. To better understand the change in adiponectin levels, we focused on the delta adiponectin (difference between, plasma adiponectin post-RYGB, minus, plasma adiponectin pre-RYGB).

### The change in adiponectin correlates with plasma MIR148A, and liver DNA methyltransferase 1 (DNMT1) post metabolic surgery

Metabolic surgery is well recognized as an effective weight loss procedure. The results in this study are similar to those observed in the general population of patients in terms of weight loss following metabolic surgery. High variability is observed during weight loss even after correction for BMI, age, gender and comorbidities. At 4 weeks post-op, average %EBMIL is 26 (min 10 to max 42), at 12 weeks postop %EBMIL is 49 (min 17 to max 82) and at 24 weeks post-op %EBMIL is 70 (min 29 to max 100).

**Table 1** Descriptive characteristics of patient's pre and post RYGB metabolic surgery.

n=33	Pre RYGB		Post RYGB		t test
	n	Mean±s.d.	n	Mean±s.d.	P-Value
Demographic characteristics					
Female age (years)	27	42.5±6.6			
Male age (years)	6	41.7±8.9			
Anthropometric characteristics					
<b>Weight (kg)</b>	<b>33</b>	<b>122±19</b>	<b>33</b>	<b>97±17</b>	<b>&lt;0.0001</b>
<b>BMI (kg/m<sup>2</sup>)</b>	<b>33</b>	<b>45±5.7</b>	<b>33</b>	<b>36±5.4</b>	<b>&lt;0.0001</b>
Hormonal parameters					
<b>FPI (μIU/mL)</b>	<b>33</b>	<b>14.5±9.7</b>	<b>33</b>	<b>4.3±2.2</b>	<b>&lt;0.0001</b>
<b>HbA1c</b>	<b>32</b>	<b>5.9±1.2</b>	<b>32</b>	<b>5.3±0.5</b>	<b>0.0131</b>
<b>HOMA-IR</b>	<b>33</b>	<b>4.2±4.9</b>	<b>33</b>	<b>1.0±0.5</b>	<b>0.0003</b>
<b>tAdp (μg/mL)</b>	<b>33</b>	<b>12.7±6.2</b>	<b>33</b>	<b>17.1±9.8</b>	<b>0.0302</b>
Metabolic parameters					
<b>FPG (mg/dL)</b>	<b>33</b>	<b>104±32</b>	<b>33</b>	<b>91±16</b>	<b>0.0467</b>
<b>Cholesterol (mg/dL)</b>	<b>32</b>	<b>196±43</b>	<b>33</b>	<b>154±29</b>	<b>&lt;0.0001</b>
HDL-C (mg/dL)	32	43±11	33	40±9	0.1538
<b>LDL-C (mg/dL)</b>	<b>31</b>	<b>120±33</b>	<b>33</b>	<b>92±25</b>	<b>0.0004</b>
LDL/HDL ratio	31	4.1±7.3	33	2.4±0.8	0.1870
<b>Cholesterol/HDL ratio</b>	<b>32</b>	<b>4.7±1.1</b>	<b>33</b>	<b>4.0±0.9</b>	<b>0.0073</b>
<b>Triglyceride (mg/dL)</b>	<b>32</b>	<b>159±113</b>	<b>33</b>	<b>107±42</b>	<b>0.0154</b>

Total number of patients  $n=33$  ( $n=27$  females;  $n=6$  males); NAFLD (– and +); MS (– and +); T2D (– and +). **Significant differences between pre and 12 weeks post RYGB calculated with paired t-test, P-values <0.05 are shown in bold and italic font.**

BMI, body mass index; FPI, fasting plasma insulin; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, insulin resistance by homeostatic model assessment; LDL-C, low-density lipoprotein cholesterol; tAdp, total fasting plasma adiponectin.

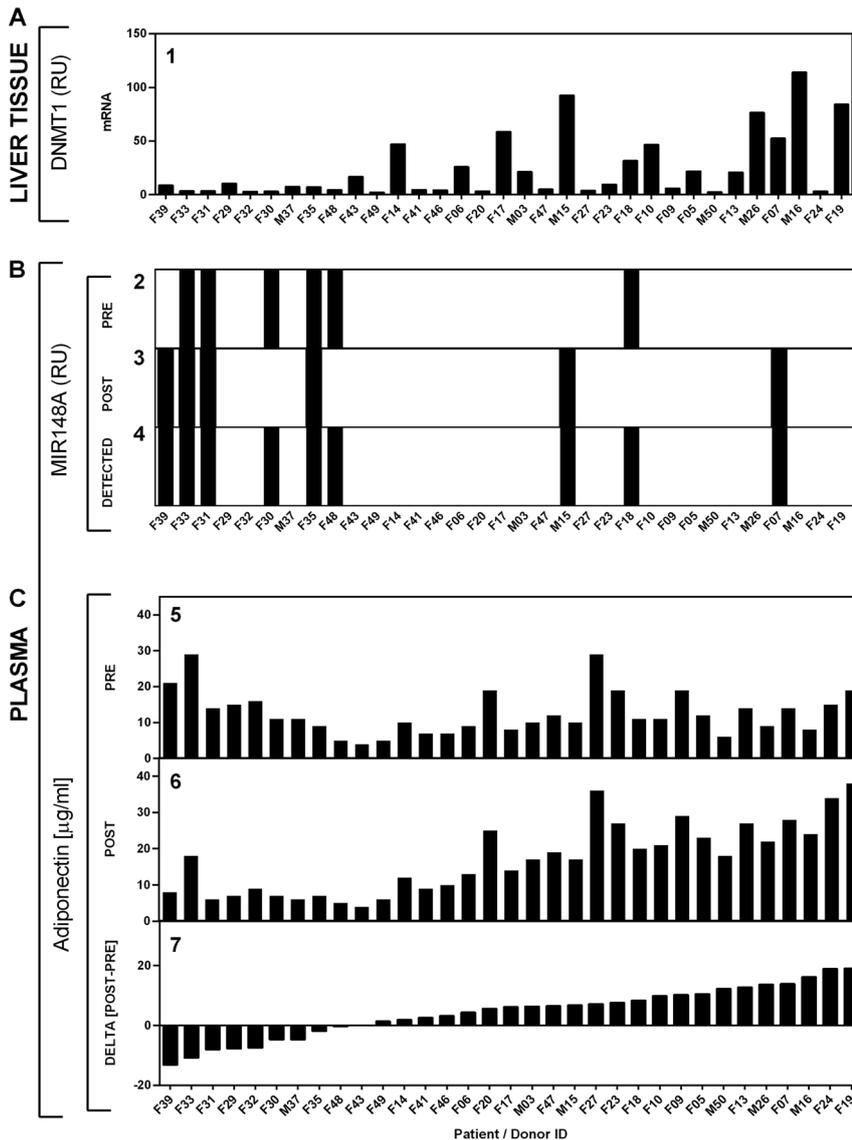
We next sought to analyze our baseline available clinical, gene expression levels of DNMT1 in liver and circulating mRNA levels and adiponectin for potential correlations of clinical interest. Using baseline information, we note a positive correlation of delta adiponectin with liver DNMT1 ( $Rho=0.4402$ ,  $P=0.0104$ ,  $n=33$ ) and a negative correlation with plasma MIR148A ( $Rho=-0.4363$ ,  $P=0.0111$ ,  $n=33$ ). The clinical parameters for insulin sensitivity HOMA-IR (insulin resistance by homeostatic model assessment) and HbA1c (glycated hemoglobin) showed a positive association ( $Rho=0.5885$ ,  $P=0.0004$ ,  $n=33$ ). Plasma MIR148A showed a negative association with HbA1c ( $Rho=-0.3940$ ,  $P=0.0256$ ,  $n=33$ ) and adiponectin post op ( $Rho=-0.4263$ ,  $P=0.0134$ ,  $n=33$ ). A positive association for adiponectin post op and pre op ( $Rho=0.5086$ ,  $P=0.0025$ ,  $n=33$ ). Adiponectin pre, post or delta did not correlate with %EBMIL. Neither did any other parameters included in this study.

### Plasma MIR148 and liver DNMT1 levels are dependent of adiponectin sensitization in obese patients undergoing surgical weight loss

We reasoned that change in total circulating adiponectin levels following metabolic surgery might provide insight into factors that associate with adiponectin sensitivity. To this end, we sorted the response of our patients in Fig. 1,

from low to high delta adiponectin. The level of expression of liver DNMT1 at baseline for each patient is presented in Fig. 1 panel A. The presence of MIR148A at levels above 1000-fold relative units was considered positive if detected at any time point (see panel B4), for detection at baseline only (see panel B2) or 12 weeks post-op only (see panel B3). Plasma adiponectin levels at baseline, 12 weeks post-op and delta adiponectin are shown in Fig. 1, panel C5, C6 and C7 respectively. Furthermore, for chi-square test of independence, we used binary frequencies (negative or positive delta adiponectin with cutoff zero, liver DNMT1 negative or positive with cutoff 10 fold, and plasma MIR148A negative or positive with cutoff 1000), the distributions and results are presented in Table 2.

A total of nine patients were identified with negative delta adiponectin levels. The patients with negative delta adiponectin had low levels of liver DNMT1 and some of them showed the presence of MIR148A. To clarify this observation was not occurring by chance in this population, the chi-square test of independence was performed. The analysis revealed that indeed, liver DNMT1 and delta adiponectin are dependent (**Chi-square statistics  $\chi^2=6.9205$ ,  $P=0.00852$ ,  $n=33$** ) (Fig. 1, panels A1 and C7, and Table 2). Likewise, the frequency in which plasma MIR148A and the negative delta adiponectin are detected are also dependent on each



**Figure 1**  
Decreased adiponectin levels post metabolic surgery is dependent on plasma MIR148A and liver DNMT1. Bar graphs showing the distribution of patients sorted by delta adiponectin (post-pre). (A) Liver DNMT1 transcription levels in relative units, only baseline data available; (B) MIR148A in relative units, presence in plasma at levels over 1000-fold is indicated with black bars; (B2) pre; (B3) post; (B4) consolidated detection panel indicates presence either pre or post (used for Chi-square statistics); (C) plasma adiponectin; (C5) pre; (C6) post; (C7) delta adiponectin (post-pre) (used for chi-square statistics). Pre stands for the baseline at the time of metabolic surgery; post stands for 12 weeks post-op. ( $n=33$ ).

other (**Chi-square statistics**  $\chi^2=9.6823$ , **P=0.00186**,  $n=33$ ) (Fig. 1, panels B4 and C7, and Table 2). The rejection of the null hypotheses of independence for delta adiponectin with liver DNMT1 and plasma MIR148A post metabolic surgery suggests that obese patients with negative delta adiponectin are undergoing a sensitization process of the adipokine induced by the lifestyle changes during weight loss.

## Discussion

The present study was undertaken to discover secretory biomarkers in plasma that might act as episenors of adiponectin sensitivity. Detailed metabolic characterization of our obese patients included clinical

parameters, circulating microRNA profiling, intraoperative liver biopsy transcriptomic analysis and total plasma adiponectin profiled pre and 12 weeks post RYGB. We observed that in a subset of our patients, adiponectin levels post RYGB decreased despite following the normal weight loss process. This subgroup had normal adiponectin levels at baseline and critically low levels of DNMT1 transcripts in liver. The decrease in adiponectin following weight loss surgery is not due to chance as the chi-square test of independence rejects the null hypotheses that the liver DNMT1 and delta adiponectin are independent (**Chi-square statistics**  $\chi^2=6.9205$ , **P=0.00852**,  $n=33$ ), as well as MIR148A and delta adiponectin are independent (**Chi-square statistics**  $\chi^2=9.6823$ , **P=0.00186**,  $n=33$ ). Specifically, the unexpected decrease in plasma adiponectin post weight loss appears to be related to low

**Table 2** Association between delta adiponectin and the presence of liver DNMT1 and plasma MIR148A.

	Delta adiponectin (post-pre)		$\chi^2$	P-Value	Row totals
	Negative n (%)	Positive n (%)			
Liver DNMT1					
Negative	8 (4.64) (2.44)	9 (12.36) (0.92)	6.9205	<b>0.00852</b>	17
Positive	1 (4.36) (2.59)	15 (11.64) (0.97)			16
Column total	9	24			33 grand total
MIR148A					
Negative	3 (6.55) (1.92)	21 (17.45) (0.72)	9.6823	<b>0.00186</b>	24
Positive	6 (2.45) (5.12)	3 (6.55) (1.92)			9
Column total	9	24			33 grand total

Chi-square test of independence was used to evaluate the association between delta adiponectin and liver DNMT1, and delta adiponectin and plasma MIR148A. Adiponectin and MIR148A were assayed on plasma, at baselines and 12 weeks post-op. Delta adiponectin (difference between baseline and 12 weeks post-op) (positive/negative cutoff zero). DNMT1 assayed in liver biopsied samples only at baseline (positive/negative cutoff 10 fold). MIR148A considered positive if detected either at baseline or 12 weeks post-op (positive/negative cutoff 1000 fold). Within round parentheses (the expected cell totals) and within square parentheses (the Chi-square statistic for each cell). All *P* values were two-sided and *P* < 0.05 was considered statistically significant (*n* = 33).

expression of liver DNMT1 and the presence of MIR148A. In some patients, low expression of liver DNMT1 could be linked to the presence of plasma MIR148A compared to other patients in the cohort. The plasma MIR148A has sequence complementarity to the promoter region of the DNMT1 gene (15, 16, 17).

Since liver DNMT1 plays a major role in global methylation patterns in mammals (9, 22) and due to the fact that delta adiponectin is dependent on both liver DNMT1 and plasma MIR148, we hypothesize that together plasma MIR148A and adiponectin can be used as secretory episensors of the human epigenome.

### Liver DNMT1 and plasma MIR148A and their impact on adiponectin levels

Recently, Kim and colleagues showed how obesity leads to proinflammatory cytokine expression that inhibits adiponectin expression by reducing DNMT1 activity and DNA hypermethylation of R2 (adiponectin promoter methylation position (18)). Notably, suppressing DNMT1 activity with inhibitor RG108 elevated adiponectin levels with improvement in metabolic parameters (18). In our study, we identify a group of patients with presumed epigenetic abnormalities, identified with overexpression of episensor biomarker MIR148A, which is responsible for the downregulation of liver DNMT1. As a consequence of liver DNMT1 downregulation, we predict hypomethylation of the adiponectin gene will produce abnormally high non-physiologic adiponectin levels in plasma. Increased adiponectin levels have been associated with worsening cardiovascular mortality (23). In the subset of patients in which adiponectin decreased post weight loss, the sensitization of adiponectin is thought to

improve cardiometabolic health such that the net effect of weight loss in our intervention is unclear.

### MIR148A as a marker of physiological adiponectin

Increase in circulating adiponectin levels following weight loss would indicate the recovery of the physiological adiponectin benefits. Liver biopsy samples at the time of surgery indicate low levels of DNMT1 were associated with non-physiological plasma adiponectin. Sequence complementarity between MIR148A and DNMT1 could explain the downregulation of liver DNMT1 in the presence of excess MIR148A, which we hypothesize will cause global DNA methylation disruptions (15, 16, 17). Dysregulatory global methylation, in turn will cause the presence of non-functional adiponectin. Limitations in the methods for the detection of adiponectin function are mentioned in publications targeting the presence of the adiponectin molecule itself (24). Liver DNMT1 would be preferable in this method of identification although it is not always available. The plasma MIR148A is particularly useful at times at which liver biopsy samples are not available. Furthermore, circulating MIR148A levels are thought to mediate the effects of liver DNMT1 on adiponectin methylation. Thus, the presence of plasma MIR148A may provide a simple detection method for the identification of non-physiological adiponectin levels mediated by DNMT1 in human patients.

### Conclusions

We identified that plasma episensor biomarker MIR148A, which by itself or in combination with liver DNMT1

gene expression identifies patients with adiponectin-insensitive states as a proxy for epigenetic abnormalities. These patients would be predicted to have a long-term, poor response to metabolic surgery in terms of weight loss as their adipose tissue is not sensitive to adiponectin which in turn limits physiologic remodeling and extra adipose endocrine benefit. Patients' positive for presence of plasma MIR148A would benefit from additional sensitization of adiponectin through epigenetic means including DNMT1 activation. Such an intervention in turn would be predicted to potentiate weight loss through metabolic surgery, making it an attractive candidate for neoadjuvant weight loss treatment leading to improved metabolic health. Our preliminary data suggest this method will allow early diagnosis, as well as the monitoring of interventions, aim for the prevention and treatment of epigenetic abnormalities in accordance with the precision medicine initiative (25), thought to be caused by nutrient bioavailability limitations and physical inactivity.

#### Declaration of interest

M A N is Principal Investigator/Founder-CEO of epiWELL, LLC. In accordance with my ethical obligation as a researcher, I am reporting that I have financial and business interests in my company epiWELL that may be affected by the research reported in the enclosed paper. I have disclosed those interests both to Cornell University and the Guthrie Clinic, as former and current collaborators, and I have in place an approved plan for managing any potential conflicts arising from that involvement. The research results presented here are disclosed on the U.S. Provisional Patent Application Serial No. 62/553452, filed September 1, 2017, for Circulating Mir148a Associates With Sensitivity To Adiponectin Levels In Humans. Inventor: Magnolia Ariza-Nieto. Cornell Reference No. 7352-01-US. LR Reference No. 29543.8010.

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#### Author contribution statement

Magnolia Ariza-Nieto PhD contributed to original study conception, design, experimental assays, results and analysis and is the sole inventor of the functionality of biomarkers and diagnostic kit, and also contributed to manuscript writing. Joshua B Alley MD, Sanjay Samy MD and Laura Fitzgerald MPH, health care providers for the patients in the study, original study conception and design, sample collection and manuscript revisions. Françoise Vermeylen helped in review of the statistical analysis, results and manuscript. Michael L Shuler PhD contributed to overall supervision, analysis and revision of the manuscript. José O Alemán, MD PhD contributed to analysis of information and manuscript writing.

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