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Circulating miR-216a as a biomarker of metabolic alterations and obesity in women

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ABSTRACT

Obesity leads to an amplified risk of disease and contributes to the occurrence of type 2 diabetes, fatty liver disease, coronary heart disease, stroke, chronic kidney disease and various types of cancer. MicroRNAs (miRNAs), small non-coding RNA molecules of 20–25 nucleotides, can remain stable in plasma and have been studied as potential (predictive) biomarkers for obesity and related metabolic disorders. The aim of this study was to identify circulating miRNAs as biomarkers for obesity status and metabolic alterations in women. Circulating miR-216a and miR-155–5p were selected by miRNA expression profiling and validated by real time quantitative PCR in a validation cohort of 60 obese women and 60 normal weight-age-matched control women. This was supplemented by correlation analysis of the candidate miRNA and anthropometric variables, blood biochemistry and lipid profile markers. Circulating miR-216a was validated as a biomarker of obesity status with significantly reduced levels in obese women. Interestingly, this was associated with a negative correlation between the plasma miR-216a content and body mass index (BMI), waist circumference, mean arterial pressure (MAP), triglycerides, ratio of total cholesterol/high density lipoprotein (HDL)-cholesterol and high sensitivity-C reactive protein (hs-CRP).Taken together, we provide evidence for an abnormally expressed circulating miRNA, miR-216a, with additive value as a predictive marker for obesity that correlates with metabolic alterations presented by lipid profile and inflammatory markers.

1. Introduction

Obesity, a condition of excessive fat accumulation, has been widely acknowledged as the cause for an amplified risk for disease which contributes to an increased all-cause mortality and a reduced life expectancy with an approximated 3.3–18.7 years of life lost [1–5]. In fact, obesity is increasingly prevalent worldwide, and this trend represents an alarming health issue as it is associated with many comorbidities such as insulin resistance, glucose intolerance, dyslipidemia and hypertension, which is classified together as the Metabolic Syndrome (MetS) [6]. As a consequence, obesity contributes to the occurrence of type 2 diabetes (T2D), non-alcoholic fatty liver disease, cardiovascular disease, chronic kidney disease and various types of cancer [2,7]. If overweight and obesity remain unabated, it is projected to reach 89% and 85% in males and females by 2030 shown in a UK model, which is directly linked to an increase of obesity related prevalence of coronary heart disease and stroke by 97%, cancers by 61% and T2D by 21% [8]. The harmful effects of obesity are partially mediated by the obesity-associated high blood pressure, high total cholesterol and the increased fasting plasma glucose [9].Timely diagnosis of obesity-related alterations is of paramount importance for proper disease management and containment of obesity related complications.

Lately, microRNAs (miRNAs) a class of small non-coding RNA molecules of 20–25 nucleotides that negatively regulate gene expression

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through mRNA binding and subsequent transcript degradation or repression of translation, are increasingly being studied [10]. Particularly, mature miRNAs that are formed within cells and secreted from the cytoplasm into the circulation known as circulating miRNA are highly stable and resistant to storage handling. The diagnostic and predictive potential of circulating miRNAs emerges from the non-invasive accessibility from body fluids (plasma, serum, urine) and the presence of disease specific circulating miRNA signatures [11]. Interestingly, distinct circulating miRNA profiles were reported between patients with metabolic disorders and healthy individuals [12–16]. Only a subset of patients with obesity will develop metabolic disease. As such, miRNA could be of additive value to predict patients at risk for future disease. We therefore aimed to identify circulating miRNAs as biomarkers for obesity status and its correlation with metabolic parameters in women.

2. Material and methods

2.1. Study participants

Participants for both screening and validation cohorts were recruited from the Women Day care Centre at the Maastricht University Medical Centre, undergoing post-partum cardiovascular risk assessment in the context of the Queen of Hearts study. Approval for use of human subjects was obtained from the Medical Ethics Committee (METC-14-2-013) and the Board of Directors from the Maastricht University Medical Centre. The study protocol complies with the principles set by the Declaration of Helsinki and is registered in clinicaltrials.gov with number NCT02347540. Prior to participation all subjects gave written informed consent. Subsequently, all participants underwent history assessment, physical examination, biochemical analysis and plasma sampling for miRNA-content analysis. The body mass index (BMI), the body mass divided by the square of the body height, is a metric used for the classification of overweight and obesity. For the initial miRNA screening, plasma RNA was isolated from 10 female participants and subdivided in two RNA pools of 5 samples each; 1) 5 women with overweight and obesity defined by BMI $\geq$25 kg/m^2 and 2) 5 healthy women with normal weight 18 kg/m$^2\leq$ BMI $\leq$24.9 kg/m^2. The validation set of the cohort consisted of an obese group (n = 60), BMI$\geq$ 30 kg/m^2 and age matched lean control group (n = 60) with 18 kg/m^2 $\geq$ BMI $\leq$ 24.9 kg/m^2.

2.2. Clinical examination

Clinical examination included automated blood pressure measurement, anthropometric measurements, venipuncture for blood biochemistry and miRNA content analysis and was performed in one morning session after an overnight fast in standardized environmental conditions. Blood pressure (BP) was measured in sitting supine position by a semiautomatic oscillometric device with a cuff size appropriate for arm circumference (Dinamap Vital Signs Monitor 1846; Critikon, Tampa, FL) every 3 min. The median value of 11 measurements was reported, as a proper alternative for extended ambulatory blood pressure measurements. The presence of hypertension was defined as Systolic Blood Pressure (SBP) $\geq$ 140 mmHg and/or Diastolic Blood Pressure (DBP) $\geq$ 90 mmHg or use of antihypertensive medication. BMI was calculated by dividing the body weight in kilogram by the squared height in meters. Prior to clinical attendance, all participants performed 24-h urine collection, which was examined for albumin, creatinine and micro-albuminuria. Blood biochemistry entailed measurement of fasting glucose, insulin, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), uric acid, leucocyte, high sensitivity C-Reactive Protein (hs-CRP), fibrinogen, fibronectin. Renal function, defined by the glomerular filtration rate (mL •min^{-1}•1.73 m^{-2}), was calculated by the MDRD equation. The diagnosis of MetS and its score were based on criteria set by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III). Accordingly, the diagnosis of MetS is made if three or more of the following five criteria are met: waist circumference > 88 cm, blood pressure over 130/85 mmHg or use of blood pressure medication, fasting triglyceride > 1.7 mmol/L, HDL cholesterol < 1.3 mmol/L, and fasting glucose > 5.6 mmol/L [17].

Table 1
Baseline characteristics screening cohort.

Characteristic	Overweight and Obesity BMI≥ 25 kg/m^2 (n = 5)	Normal weight BMI ≥18–24.9 kg/m^2 (n = 5)
Metabolic Syndrome n (%)	2 (40%)	0
Hypertension n (%)	1 (20%)	0
Demographics (mean ± SD)		
Age	42 ± 1.6	41 ± 2.6
Weight (kg)	90 ± 17	64 ± 7.6
Height (cm)	173 ± 4.5	168 ± 7.6
BMI (kg/m2)	30 ± 4.1	22.7 ± 1.6
Waist circumference (cm)	99.4 ± 15.7	79 ± 2.7
Hemodynamics (mean ± SD)		
SBP (mmHg)	125 ± 18	107 ± 6
DBP (mmHg)	76 ± 11	67 ± 7
MAP (mmHg)	94 ± 13	82 ± 7
Heart rate (bpm)	70 ± 12	63 ± 3
Biochemistry (mean ± SD)		
Total Cholesterol (mmol/L)	5.2 ± 0.8	4.8 ± 1
HDL Cholesterol (mmol/L)	1.7 ± 0.3	1.6 ± 0.2
LDL Cholesterol (mmol/L)	2.8 ± 0.5	2.8 ± 1
Triglycerides (mmol/L)	1.5 ± 1.3	0.86 ± 0.3
Total Cholesterol/HDL Cholesterol	3.1 ± 0.8	3 ± 0.8
Fasting Glucose (mmol/L)	5.3 ± 0.9	4.7 ± 0.3
Insulin (mmol/L)	130 ± 79	29 ± 16
GFR (mL/min/1.73m2)	85.8 ± 10.9	82 ± 9.6
Uric Acid (mmol/L)	0.27 ± 0.07	0.24 ± 0.05
Microalbuminuria (g/mol Creat)	0.34 ± 0.36	0 ± 0
Conventional Biomarkers (mean ± SD)		
hs-CRP (mg/L)	2.2 ± 3.1	1.3 ± 1.8
Leucocytes (10^9/L)	6.6 ± 1.1	5.7 ± 1.6
Fibronectin (mg/L)	386 ± 82.6	321 ± 37
Fibrinogen (g/L)	2.9 ± 0.43	2.9 ± 0.51

Data are presented as means ± standard deviations (SD). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; GFR glomerular filtration rate; hs- CRP, high sensitivity C-reactive protein.

2.3. Plasma and total RNA isolation

Venous blood samples were collected by venipuncture in standard ethylenediaminetetraacetic acid (EDTA) Vacutainer tubes, centrifuged at 2500g for 15 min at room temperature, from which the separated plasma was stored at −80 °C until further processed for total RNA isolation. Total RNA including miRNAs was extracted from 100 µl of plasma according to the manufacturers protocol using the miRNeasy Serum/Plasma kit (Qiagen 217,184). A synthetic cel-miR-39 (219,610, Qiagen) was spiked into each plasma sample after addition of Qiazol for miRNA extraction efficiency.

2.4. miRNA expression profiling

The miRNA expression profiling analysing the expression of 754 miRNAs was performed in technical triplicates of two pooled RNA samples from the screening cohort consisting of RNA from 5 patients

Table 2
Baseline characteristics validation cohort.

Characteristic n (%)	Obese (n = 60)	Normal weight (n = 60)	p value
Metabolic Syndrome	27 (45%)	0 (0%)	< **0.001** #
Hypertension	21 (35%)	0 (0%)	< **0.001** #
Demographics (mean ± SD)			
Age (years)	39.6 ± 9.2	39.6 ± 8.9	0.681†
Weight (kg)	94.8 ± 15.2	62.4 ± 6.8	< **0.001** †
Height (cm)	166.5 ± 6.3	169.9 ± 6.6	**0.006** ††
BMI (kg/m2)	34.1 ± 4.2	21.6 ± 1.6	< **0.001** †
Waist circumference (cm)	104.3 ± 12.3	77.9 ± 6.8	< **0.001** †
Hemodynamics (mean ± SD)			
SBP (mmHg)	122.1 ± 13.2	108.6 ± 10.5	< **0.001** †
DBP (mmHg)	75.4 ± 9.6	67.4 ± 6.2	< **0.001** †
MAP (mmHg)	93.7 ± 10.9	83.0 ± 7.6	< **0.001** †
Heart rate (bpm)	69.4 ± 10.0	64.5 ± 10.1	**0.025** ††
Biochemistry (mean ± SD)			
Total Cholesterol (mmol/L)	5.1 ± 1.0	4.7 ± 0.7	**0.007** ††
HDL Cholesterol (mmol/L)	1.5 ± 0.4	1.8 ± 0.5	< **0.001**†
LDL Cholesterol (mmol/L)	3.1 ± 0.8	2.5 ± 0.5	< **0.001**††
Triglycerides (mmol/L)	1.4 ± 1.0	0.8 ± 0.3	< **0.001** †
Total cholesterol/HDL	3.6 ± 1.1	2.7 ± 0.5	< **0.001**†
Fasting Glucose (mmol/L)	5.3 ± 0.6	5.0 ± 0.4	< **0.001**†
Insulin (mmol/L)	77.2 ± 43.6	31.2 ± 20.8	< **0.001** †
GFR (mL/min/1.73m2)	87.5 ± 16.7	85.3 ± 11.7	0.340 ††
Uric acid	0.3 ± 0.1	0.2 ± 0.0	< **0.001** ††
Microalbuminuria (g/mol creat)	1.3 ± 3.0	0.4 ± 0.7	0.051†
Conventional Biomarkers (mean ± SD)			
hs-CRP (mg/L)	4.7 ± 4.6	1.4 ± 1.5	< **0.001**††
Leucocytes (10^9/L)	6.7 ± 1.4	5.3 ± 1.1	< **0.001**††
Fibronectin (mg/L)	387.3 ± 71.1	325.3 ± 66.3	< **0.001**††
Fibrinogen (g/L)	3.4 ± 0.6	2.8 ± 0.7	< **0.001**††

Data are presented as means ± standard deviations [SD]. # Indicates usage of McNemar test, †† indicates usage of paired sample T-test, † the usage of non-parametric Wilcoxon Rank Sum Test. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; GFR glomerular filtration rate; hs- CRP, high sensitivity C-reactive protein.

per pool of each group using TaqMan Array Human microRNA cards panels (4,444,750, TaqMan). Pooling of total RNA samples was performed by the addition of 2 μl of each RNA sample belonging to the 5 individual subjects of a group to an end volume of 10 μl. Reverse transcription (RT) and pre-amplification were performed on both RNA pools using Megaplex Primers Human Pool A v2.1 and Pool B v3.0 (4,444,750, Taqman) following the manufacturer protocol. For RT, an input of 3 μl of total RNA from the pooled RNA sample was transferred to the RT reaction mix in a final volume of 7.5 μl using the TaqMan MicroRNA Reverse transcription Kit, (Applied Biosystem 4,366,597) and Megaplex RT Primer Pools. Thermally cycled (T3000 Biometra Thermo cycler) for 2 min at 16 °C, 1 min 42 °C, 1 sec 50 °C for 40 cycles, followed by 85 °C for 5 min. Next, RT-product was pre-amplified prior to real time polymerase chain reaction (PCR) by the addition of 5 μl of RT product to matching PreAmp Primer Pools and TaqMan PreAmp Mastermix to 25 μl cycled for 10 min at 95 °C, 2 min at 55 °C, 2 min at 72 °C followed by 15 s at 95 °C and 4 min at 60 °C for 12 cycles and 99 °C for 10 min. The pre-amplification product was diluted 1:4 in nuclease free water, then diluted 1:25 in TaqMan Universal Mastermix II and loaded on to the eight ports of the 384 well Array Cards A and B, centrifuged and sealed for real time PCR according to the manufacturer's protocol.

2.5. miRNA candidate selection

Data analysis was performed in parallel on Excel 2010 exports from ViiA 7 Real time PCR system, using Quant Studio 6&7 Flex Real time PCR system as well as the Expression Suite Software v1.1 by means of the comparative Cq ($^{\Delta\Delta}$Cq) method [18]. Cq-values of miRNAs reported as 'undetermined' were replaced with a value of 40. Both global normalization and endogenous normalization was used for identification of miRNA candidates. Global normalization involved the use of the mean expression values of all miRNAs as the normalizer. For endogenous normalization, miRNAs known to be stably expressed in plasma are used as normalizers. Best Keeper software was used to identify a miRNA candidate for endogenous normalization based on a list of miRNAs known to be stable in plasma that were present in the Taqman Megaplex panel (hsa-miR-24, hsa-miR-484, hsa-mir-93–5p, hsa-miR-191–5p, hsa-miR-126–3p, hsa-miR-16) [19–22]. Significantly changed (p-value < 0.05) miRNAs between groups that corresponded in both normalization methods were further filtered for the candidate selection and excluded by the following characteristics; (i) Raw Cq values > 30, Fold change < |1.5| for both global and endogenous normalization (iii) False positives or undetermined among triplicates. The presence of haemolysis can distort the actual miRNA content, therefore the degree of haemolysis was assessed in the screening cohort by determining the ratio of miR-451 to miR-23a. Samples with a ratio > 5 were considered at moderate risk whereas samples with a ratio > 7 were considered at severe risk of miRNA contamination by hemolysis [23,24]. Besides this, miRNA candidate selection for validation was ultimately based on whether the miRNA remains stable in case of haemolysis.

2.6. Validation of candidate miRNAs

The expression of the selected candidate miRNAs following miRNA expression profiling was validated by real time quantitative PCR (qPCR) in the 120 individual plasma samples from the validation cohort which was carried out on the Bio-Rad CFX96 Real-Time System. Equal RNA input for all samples was ensured by Nanodrpo 2000 (Thermo Fischer Scientific). cDNA synthesis and pre-amplification were performed according to the manufacturers protocol with the TaqMan Advanced miRNA cDNA synthesis Kit (A28007), which included Poly(A) Tailing, Adapter Ligation, RT and miR-Amp. For Poly(A) tailing, 3 μl of 8 ng RNA sample was added to Poly(A) Reaction Mix to a total volume of 5 μl and polyadenylated for 45 min at 37 °C, and stopped 10 min at 65 °C on a Bio-Rad MyCycler Thermal Cycler 580BR1990. Following, the adaptor ligation was performed by the addition of ligation reaction mix to a total volume of 15 μl and incubated for 60 min at 16 °C. 15 μl of the RT reaction mix was added to a total volume of 30 μl, thermally cycled for 15 min at 42 °C, and inactivated at 85 °C for 5 min. Preamplification was performed by adding 5 μl of RT product to miR-Amp Reaction mix; cycled as follows, 95 °C for 5 min; 14 cycles at 3 sec 95°C and 30 sec 60°C; 99 °C for 10 min. Following the 1:10 dilution in 0.1x TE, 5 μl of the miR-Amp product were loaded on a 96-well plate together with 15 μl PCR reaction mix consisting of TaqMan Fast Advanced Master Mix (4,444,556) and TaqMan Advanced miRNA hydrolysis probes assay (A25576) for qPCR and cycled 20 s at 95 °C; 1 sec 95°C and 20 s 60 °C for 40 cycles. For each individual sample, qPCR reactions were performed in triplicates. The mean Cq values obtained from each sample were normalized to miR-191–5p (477,952_mir) (identified by Bestkeeper Software) Cq value of the respective sample. The qPCR output was analyzed by the comparative Cq ($^{\Delta\Delta}$Cq) method as previously described [20]. Each individual sample from the obese group was matched to an individual sample of the normal weight control by age. If either sample of an obese-control pair was not amplified, the complete pair was excluded from the analysis.

2.7. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (version 25. Armonk, NY: IBM Corp). Graphs were generated by Prism 6. Quantitative variables are presented as means and standard

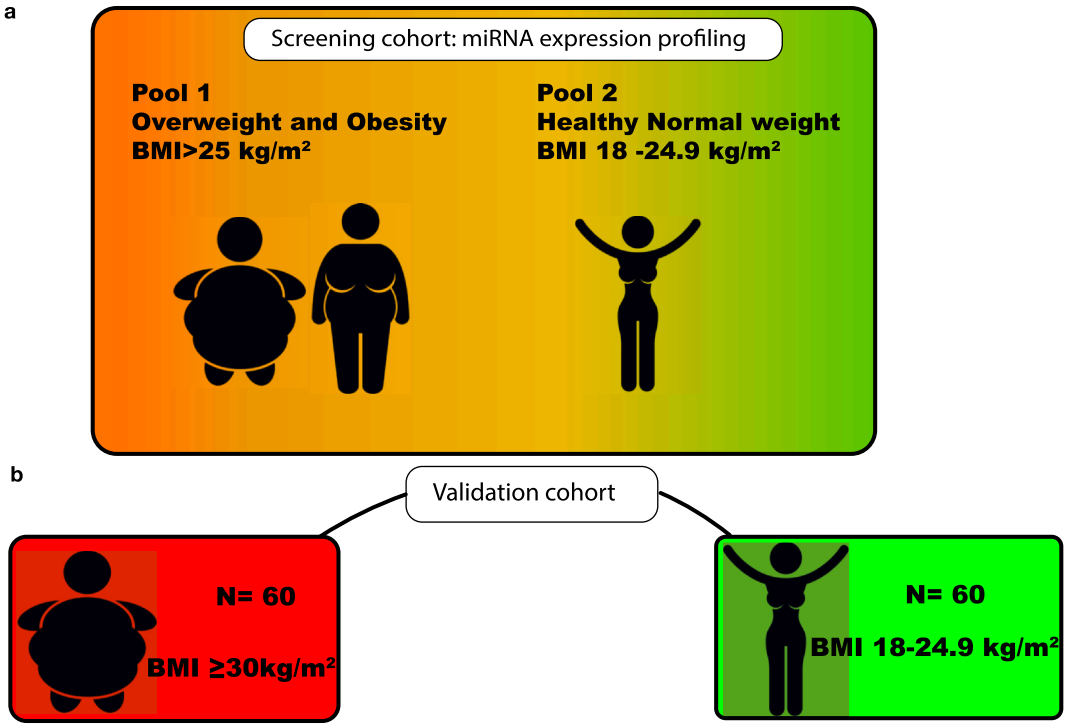

Figure 1. Experimental setup for biomarker identification. (a) Overview of the screening cohort and miRNA expression profiling strategy. Every pool is organized in a group; the relative characteristics and inclusion criteria are described in the main text, while the images represent the main characteristics of each pool. Plasma RNA from 10 patients is subdivided in two pools by pooling RNA from 5 patients together within a group. Pool 1 consists of plasma RNA from 5 female patients with BMI ≥ 25kg/m^2, forming a pool with obesity and overweight. Pool 2 consists of RNA from 5 healthy control women with normal weight BMI 18.5- 24.9 kg/m^2. **(b)** The lower panels show an overview of the validation cohort consisting of 60 obese (BMI ≥ 30kg/m^2) and 60 normal weight (BMI 18.5- 24.9 kg/m^2) female subjects matched by age. miRNA candidates were validated by qPCR in plasma RNA of each individual sample (n = 120). BMI, Body Mass Index; qPCR, quantitative Polymerase Chain Reaction.

deviations while categorical variables are presented as frequencies and percentages. Significance level was 0.05. Differences in categorical variables of the validation cohort were analyzed using McNemar test for matched samples. Numerical quantitative data of the validation cohort were analyzed using the Wilcoxon signed rank test for non-parametric distributions, while paired samples t-test is used for parametric distributions. For the screening cohort the sample size of each group (n = 5) is too small, for which statistical analysis of baseline characteristics is omitted from the analysis. Spearman rank correlations were performed to test for the association between candidate miRNA expression and blood biochemistry and anthropometric variables within the complete cohort.

3. Results

3.1. Study characteristics

Approval for studies on human tissue samples was obtained from the Medical Ethics Committee of Maastricht University Medical Centre, The Netherlands. All patients gave written informed consent before usage of their material. Clinical characteristics and demographics of the screening and validation cohorts are presented in Table 1 and Table 2, respectively. Within the screening cohort, the average age for the overweight and obesity group was 42 years and for the control group was 41 years. Within the overweight and obesity group, 40% of subjects exhibited the MetS, indicating significant metabolic alterations in this screening group. The control subjects of the validation cohort were free of MetS, and hypertension, while among the obese cases, 45% had MetS and 35% had hypertension (Table 2). Overall, the obese women included in the validation cohort exhibited significantly higher BMI, cholesterol, fasting glucose, higher blood pressure, increased inflammatory markers, such as leucocytes and hs-CRP compared to normal weight controls (Table 2).

3.2. miRNA expression profiling and miRNA candidate validation

The first phase of miRNA expression profiling involved the analysis of the expression of 754 miRNAs by real time PCR between the two pools of the screening cohort, from which merely 221 miRNAs were rendered detectable (Fig. 1). Two independent normalization methods were used for the identification of relatively changed miRNA's between pools (each corresponding to a group). All plasma samples of the screening cohort were shown to be at moderate or severe risk for hemolysis with miR ratios ranging from 6 to 11.5 (Table A1). Best Keeper software was used to screen for the most stably expressed miRNAs present within the screening platform. miR-191–5p and miR-484 were identified as the best normalizers, of which miR-191–5p least affected by hemolysis, thus most suitable as endogenous normalizer in a cohort exposed to hemolysis (Table A2) [21,24].

The comparison between pool 1 (overweight and obesity) and pool 2 (normal weight control) generated 23 miRNAs significantly changed by global normalization, 64 by endogenous normalization, with 15 miRNAs corresponding between both methods (Table A3). The significant miRNAs corresponding between both normalization methods were further screened as previously described (Fig. 2a).

This additional filtering yielded 5 miRNAs candidates for validation (*miR-216a, miR-106b, miR-203, miR-155, let-7e*). Hierarchical clustering of the miRNA profiles showed segregation according to cases and controls (Fig. 2b). Upon exclusion of the miRNAs unstable in case of hemolysis [25,26], 2 miRNA candidates were further selected for the validation, including miR-155–5p, miR-216a (Fig. 2b). Further validation of the two miRNAs identified by the screening was performed by real time-qPCR in the validation cohort (n = 60 obese women, age matched to n = 60 normal weight controls). Significantly decreased

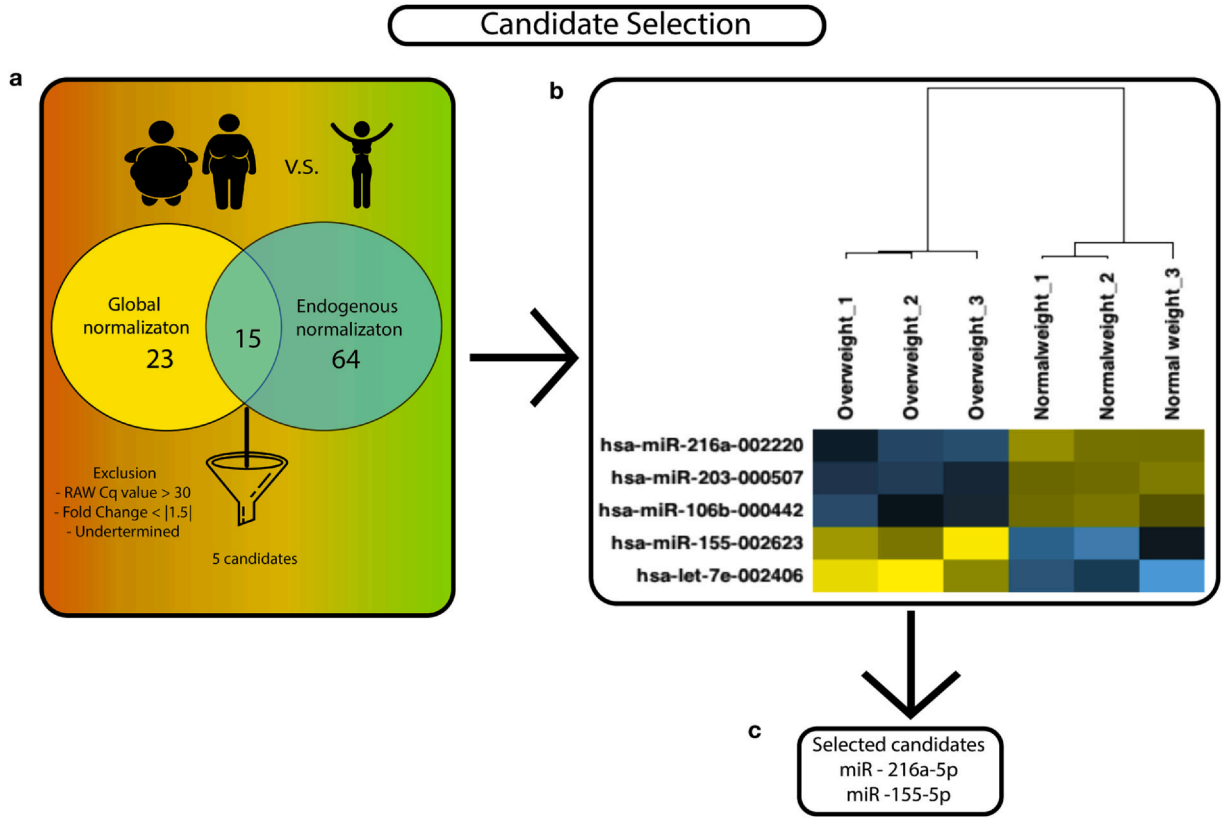

Figure 2. Workflow Candidate selection. **(a)** Overview of the workflow of miRNA candidate selection following miRNA expression profiling. The general workflow includes the comparison of significantly deregulated miRNAs between the two pools using both global and endogenous normalization methods (miR-191-5p as endogenous normalizer). miRNAs corresponding between both methods following additional filtering (Raw Cq values > 30, fold change < |1.5|, false positives with undetermined values) were identified as biomarker candidates for validation. Pool 1 vs Pool 2; showed 23 significantly deregulated miRNAs by global normalization, 64 by endogenous normalization, and 15 corresponding between both methods, with 5 candidates after final filtering. **(b)** Hierarchical clustering and heatmap showing the ultimate list of miRNA candidates. Blue indicates fold changes < 0, reduced expression, and yellow indicates fold changes > 0, increased expression. **(c)** After considering miRNAs that remain stable after hemolysis, two candidates were chosen for validation: miR-216a-5p and miR- 155-5p.

expression levels of miR-216a ($p = 0.008$) and miR-155–5p ($p = 0.008$) was observed among obese women, compared with healthy control subjects following non-parametric testing for paired samples (Fig. 3). For the expression of miR-216a in 53 of the cases were detectable in both age-matched obese and normal weight paired samples, where 34 of the obese-normal weight pairs had an increased expression of miR-216a in normal weight compared to obesity. For the expression of miR-155–5p, 39 of the obese normal weight pairs were both detectable, where 27 of the obese-normal weight pairs showed an increased expression in normal weight compared to obese. This confirms that the observed significance is not driven by outliers. Expression levels of miR-216a showed the same trend observed in the screening, while miR-155–5p expression showed the complete opposite trend. In order to analyze whether miR-216a and miR155–5p are related, since they show similar patterns of deregulation, Spearman correlation on the expression was performed, showing that there was no significant correlation between the two miRNAs ($p = 0.855$).

3.3. Correlations analysis

Spearman rank correlation coefficients between plasma levels of miR-216a, miR-155 and anthropometric variables and blood biochemistry are calculated for the complete validation cohort (Table 3). In the complete validation cohort, a significant negative correlation was observed between miR-216a expression in plasma and BMI ($r = -0.200$, $p = 0.042$), Waist circumference ($r = -0.223$, $p = 0.023$) DBP ($r = -0.202$, $p = 0.039$), MAP ($r = -0.206$, $p = 0.036$), Triglycerides ($r = -0.201$, $p = 0.041$), ratio Total cholesterol/HDL ($r = -0.225$, $p = 0.021$) and hs-CRP ($r = -0.271$, $p = 0.006$) (Fig. 4a–g). On the other hand, miR-155–5p only showed significant negative correlation with fibrinogen ($r = -0.237$, $p = 0.040$).

4. Discussion

Obesity has reached endemic proportions and represents a major health crisis with an amplified risk for metabolic complications such as diabetes, fatty liver disease, chronic kidney failure, cardiovascular disease, and cancer. In our study, miRNA profiling of pooled samples of a high-risk female population consisting of overweight and obesity, and healthy normal weight women, led to the identification of miRNA candidate biomarkers for a high-risk metabolic profile [27]. Next, miR-216a and miR-155–5p expression patterns observed in the screening cohort were validated in a larger validation cohort by RT-qPCR. This led to the identification of miR-216a as a biomarker for obesity status and a high-risk metabolic profile where a significant decrease in plasma levels of miR-216a was observed among obese patients, confirming the trend observed in the screening. Beside this, miR-155–5p, significantly deregulated within the validation cohort, showed an opposite trend compared to that observed in the screening. Aside from the fact that miR-216a and miR155–5p show similar patterns of deregulation, they did not correlate with one another and we confirmed that the two miRNAs are localized on completely different regions within the genome as miR-216a is located on chromosome 2p16.1, while miR-155 is located on chromosome 21q21.3 (NCBI). Several miRNAs are known to be differentially expressed in patients with obesity as compared to healthy individuals and are candidates for non-invasive metabolic

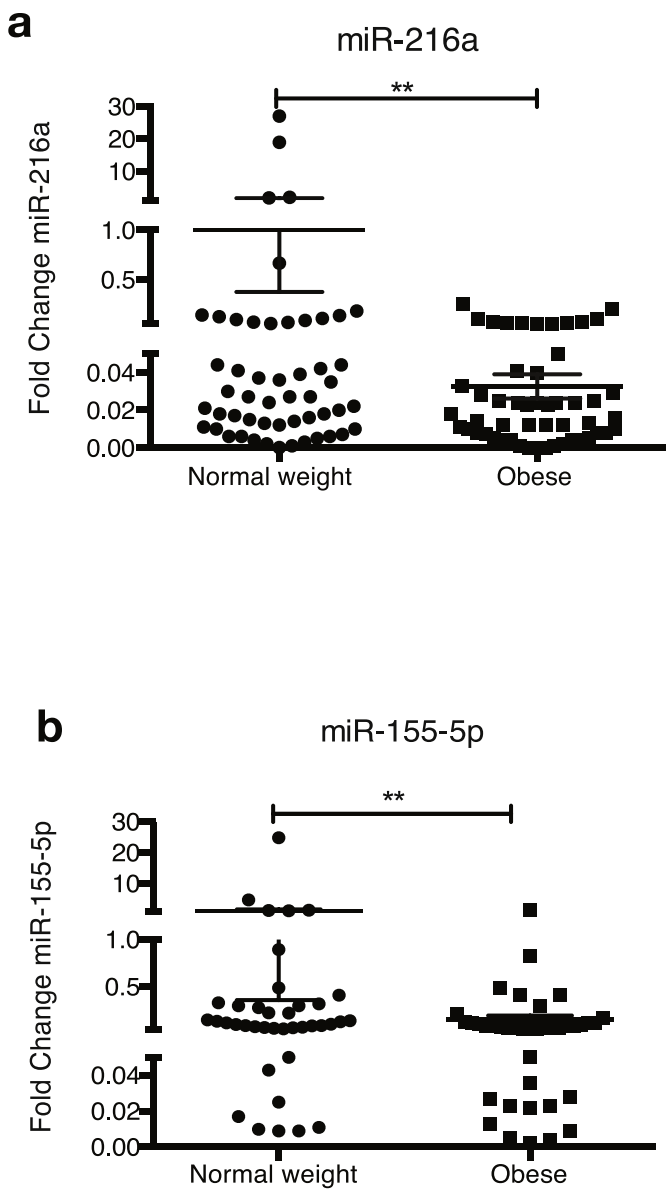

Figure 3. Plasma miRNA expression levels in the validation cohort. The dot plots show the individual fold changes with the means and SEM for **(a)** miR-216a and **(b)** miR-155-5p, by real time qPCR in plasma samples of the validation cohort (n = 60 obese and n = 60 normal weight controls paired by age). Every dot in the dot plot represents the expression corresponding to an individual subject, presented together with the mean and standard error of means per group. Not all miRNAs of a sample pair could be detected in every sample; for miR-216a, n = 53 paired samples, thus 106 individual samples were rendered detectable, miR-155, n = 39 paired samples, thus 78 individual samples were rendered detectable. The relative miRNA expression levels were normalized to miR-191-5p. Statistical analysis included nonparametric Wilcoxon signed rank test for paired samples **P < 0.01; SEM, standard error of means.

biomarkers [14,28–32]. Nevertheless, circulating miR-216a in plasma is validated for the first time as a differentially expressed circulating plasma miRNA in obesity adding a novel marker to obesity miRNA signatures. Genome wide association studies has led to the discovery of novel obesity susceptibility loci [33]. Moreover, studies on comparative genetics, heritability analysis and DNA methylation show similarities in genomic regions of interest for studying obesity [34–36].

Interestingly, the highly conserved miR-216a gene is present at such obesity related region of interest and was validated for the first time in an obese cohort in the present study. In line, miR-216a has several obesity-related target genes including phosphatase and tensin homolog

Table 3
Spearman rank correlation analysis.

Variable		Validation cohort (n = 120)	
		miR-216a (n = 106)	miR-155 (n = 78)
Metabolic Syndrome	r	−0.104	−0.047
	p	0.292	0.687
Metabolic Syndrome score	r	−0.185	−0.152
	p	0.060	0.192
Age (years)	r	0.107	0.033
	p	0.279	0.776
Weight (kg)	r	−0.165	−0.195
	p	0.095	0.093
Height (cm)	r	0.061	0.046
	p	0.541	0.695
BMI (kg/m2)	r	**−0.200**	−0.161
	p	***0.042**	0.168
Waist circumference (cm)	r	**−0.223**	−0.161
	p	***0.023**	0.168
SBP (mmHg)	r	−0.170	−0.064
	p	0.084	0.584
DBP (mmHg)	r	**−0.202**	−0.750
	p	***0.039**	0.523
MAP (mmHg)	r	**−0.206**	−0.052
	p	***0.036**	0.655
heart rate (bpm)	r	−0.07	0.005
	p	0.483	0.967
Total Cholesterol (mmol/L)	r	−0.107	−0.048
	p	0.281	0.681
HDL Cholesterol (mmol/L)	r	0.190	0.086
	p	0.054	0.464
LDL Cholesterol (mmol/L)	r	−0.145	−0.096
	p	0.141	0.415
Triglycerides (mmol/L)	r	**−0.201**	−0.168
	p	***0.041**	0.149
Total cholesterol/HDL	r	**−0.225**	−0.144
	p	***0.021**	0.218
Fasting Glucose (mmol/L)	r	0.004	0.108
	p	0.966	0.354
Insulin (mmol/L)	r	−0.162	−0.115
	p	0.101	0.330
GFR (mL/min/1.73m2)	r	−0.017	−0.099
	p	0.862	0.400
Uric Acid (mmol/L)	r	−0.165	−0.110
	p	0.094	0.348
Microalbuminuria (g/mol Creat)	r	−0.040	−0.210
	p	0.686	0.071
hs-CRP (mg/L)	r	**−0.271**	−0.179
	p	****0.006**	0.130
Leucocytes (10^9/L)	r	−0.076	−0.145
	p	0.449	0.221
Fibronectin (mg/L)	r	−0.114	−0.021
	p	0.257	0.858
Fibrinogen (g/L)	r	−0.154	**−0.237**
	p	0.121	***0.040**

Relationship between miR-216a, miR-155, and factors constituting metabolic risk in the validation cohort (n = 120). miR-216a was detectable in 106 samples of the validation cohort, while miR-155–5p was detected in 78 samples of the validation cohort. r, Spearman rank correlation coefficient; p, p-value; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; GFR glomerular filtration rate; hs- CRP, high sensitivity C-reactive protein.

(*PTEN*), a gene implicated in T2D, and obesity-related predicted target genes such as adiponectin receptor1 (*ADIPOR1*), caveolin1 (*CAV1*), caveolin2 (*CAV2*) and peroxisome proliferator-activated receptor gamma (PPARG) and has previously been implicated in obesity-related pathways [37,38]. In addition, it was shown that CpG islands of the miR-216a locus contained differential DNA methylation levels between obese and non-obese children suggesting an involvement of epigenetic regulation of miR-216a for the development of obesity [39]. Similar to what was observed in other studies screening for obesity related biomarkers, the association between miR-216a and obesity status is further

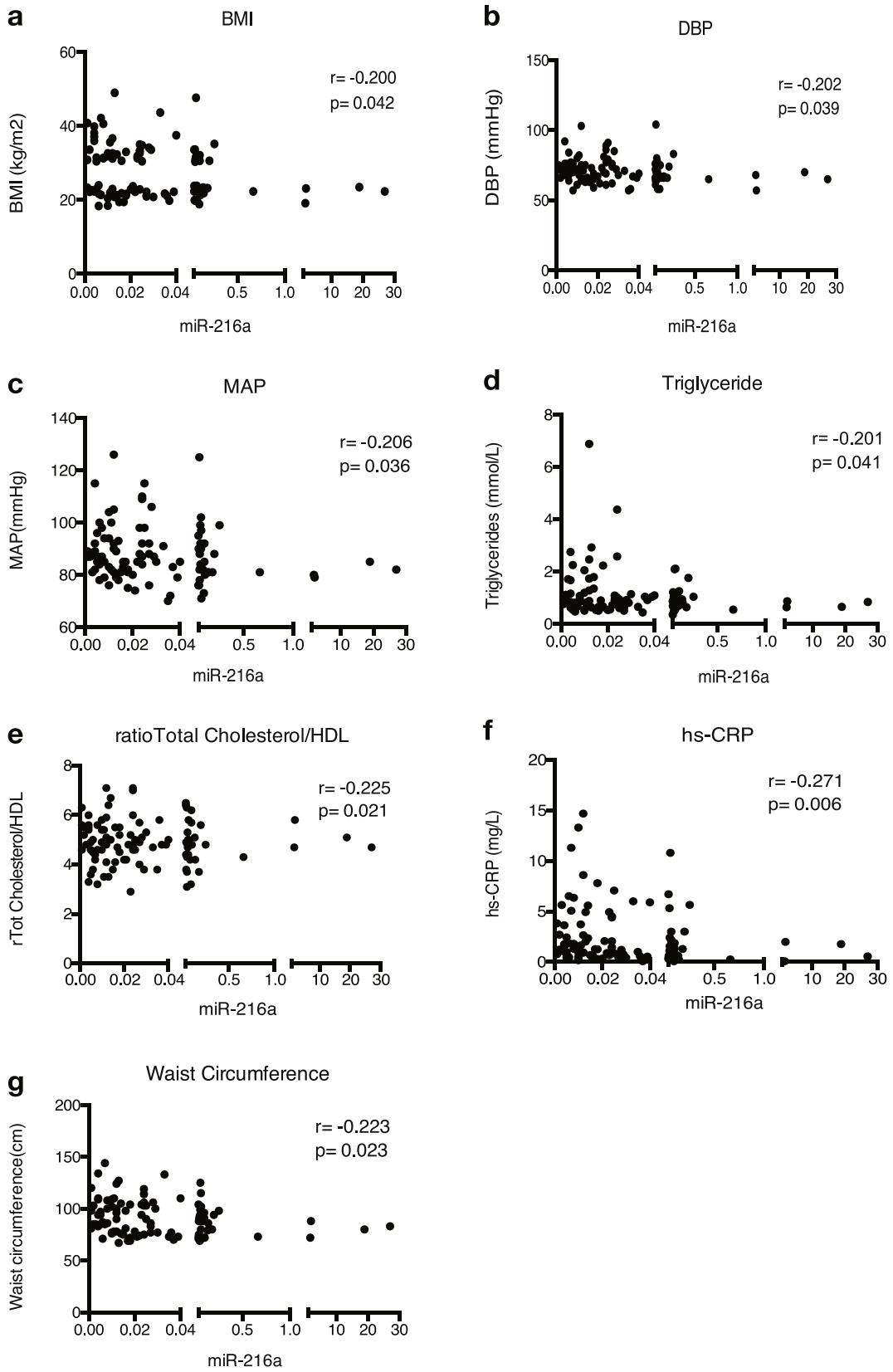

Figure 4. Spearman correlations between miR-216a, and miR-155-5p with anthropometric and biochemical markers. The scatterplots illustrate the correlations between miR-216a-5p fold change, or miR-155-5p fold change, and the following variables: **(a)** BMI (kg/m^2), **(b)** DBP (mmHg), **(c)** MAP (mmHg), **(d)** Triglycerides (mmol/L), **(e)** ratio Total Cholesterol and HDL-cholesterol, **(f)** hs-CRP (mg/L) and **(g)** waist circumference (cm). r = Spearman rank correlation coefficient, p= p-value, BMI, body mass index; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high density lipoprotein; hs-CRP, high sensitivity C-reactive protein.

supplemented by significant correlations with BMI and waist circumference, confirming validity of the identified biomarker. However, this was not the case for miR-155–5p. In addition, we also demonstrate that miR-216a negatively correlated with DBP, MAP, triglycerides, total cholesterol/HDL ratio and hs-CRP which are interrelated components of an increased metabolic disease risk. These findings confirmed the trend that lower expression of miR-216a corresponds with higher levels of BMI, waist circumference, DBP, MAP, triglycerides, total cholesterol/HDL cholesterol ratio and hs-CRP. Obesity characterized by the accumulation of excessive visceral fat is accompanied by excessive lipids, a low grade systemic chronic inflammatory state [40]. Consequently, these mechanisms induce a cascade of events which through increase in insulin resistance, stimulation of RAAS and sympathetic nervous system, impairment in baro-and chemoreflex control, endothelial dysfunction, increase sodium retention and leads to increased blood pressure levels [41].

A previous study demonstrated that reduced levels of urinary miR-216a was associated with diabetic nephropathy and subclinical atherosclerosis [42]. This report is consistent with our finding that obesity is associated with reduced levels of circulating miR-216a, in that it also suggests that the presence of higher levels of this miRNA could provide protection from metabolic disease. Another study supporting protective effects by the miRNA shows that urinary expression of miR-216a correlates with the rate of renal function decline and progression to dialysis-dependent renal failure [43]. Increased levels of miR-216a indicated protection and showed better dialysis-free survival [43].

Contrary to studies addressing the beneficial effects of circulating miR-216a, studies addressing the molecular mechanisms targeted by the miRNA in heart showed that the expression of miR-216a was increased in myocardial biopsies from patients with ischemic heart failure [44,45]. Here, increased levels of the miRNA was linked to diabetic heart failure [45]. The discrepancy between tissue specific expression of the miRNA and circulating miRNA could derive from the fact that different tissues contribute to the circulating miRNA content. Previous studies have shown that adipose tissue-derived circulating miRNAs are an important source of miRNAs present in the circulation which is supported by the discovery of a significant reduction in circulating miRNAs in patients with lipodystrophy [46]. Besides this, adipose tissue specific Dicer knockout mice displayed a lipodystrophic phenotype with reduced levels of circulating miRNAs. Accordingly, a great part of circulating miRNAs originates from adipocytes but could also originate from adipose tissue macrophages, and human studies have shown that the levels of circulating miRNA is influenced by the degree of obesity [46,47].

A limitation of our study is that samples were obtained only from women. Based on gender differences, some miRNA families have expression divergence and opposing deregulation in male and female patients [48]. Even though miR-216a has not been identified among sex-biased miRNAs, it remains uncertain whether the data found in the present study can be extrapolated to a mixed-gender population, therefore our conclusions are limited to female patients [48]. Another limitation of our study is that no adipose tissue biopsies were taken to analyze the correlation between adipose tissue and plasma miRNA content, unlike a previous study where a correlation could be established between plasma levels of circulating miR-17–5p and miR-132, and expression of miRNAs in visceral fat tissue biopsy [28].

5. Conclusions

In conclusion, we have validated plasma miR-216a as a biomarker of obesity status combined with additional metabolic alterations presented by lipid profile and inflammatory markers. Coincidentally, we found the obesity susceptibility locus, harboring the miRNA216a gene that contained CpG sites with differential DNA methylation levels between obese and non-obese children to be associated with differential circulating plasma miRNA-216a levels in obese and non-obese women. Circulating miR-216a is of additive value as a predictive marker for obesity and related metabolic disease in women. Genome wide association studies, heritability analysis and DNA methylation studies have meaningful contributions to the field. Future research can be directed towards extracting and validating loci of interest identified by these studies, as it could lead to valuable discoveries.

CRediT authorship contribution statement

Indira G.C. Vonhögen: Investigation, Formal analysis, Writing - original draft, Visualization. **Zenab Mohseni:** Investigation. **Bjorn Winkens:** Methodology, Data curation, Formal analysis. **Ke Xiao:** Methodology. **Thomas Thum:** Resources, Writing - review & editing. **Martina Calore:** Methodology, Writing - review & editing. **Paula A. da Costa Martins:** Funding acquisition, Supervision, Visualization, Writing - review & editing. **Leon J. de Windt:** Conceptualization, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Marc E.A. Spaanderman:** Resources, Supervision, Writing - review & editing. **Chahinda Ghossein-Doha:** Resources, Writing - review & editing.

Declaration of competing interest

P.D.C.M and L.D.W are co-founders and stockholders of Mirabilis Therapeutics BV. TT is founder and shareholder of Cardior Pharmaceuticals GmbH. All other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2020.08.001.

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