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MicroRNAs modulation and clinical outcomes at 1 year of follow-up after excision of subcutaneous abdominal fat in overweight patients with pre-diabetes treated with metformin vs. placebo

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ABSTRACT

Background: obese pre-diabetics have altered expression of cytokines, and sirtuin-1, that might influence myocardial function via microRNAs (miRs) expression. **Objectives:** to evaluate inflammatory/oxidative stress, miRs' expression and cardiovascular function in obese pre-diabetics randomly assigned to metformin therapy vs. placebo vs. normo-glycemics at 12 months of follow-up. **Materials and methods:** eighty-three obese patients enrolled for abdominoplastic surgery, were divided in pre-diabetics (n 55), normo-glycemics (n 28), and assigned to hypocaloric diet. Pre-diabetics were randomly assigned to metformin (n 23) or to placebo (n 22) plus hypocaloric diet. **Results:** at enrollment, pre-diabetics obese vs. normo-glycemic presented higher values of glucose, insulin resistance (HOMA-IR), inflammatory/oxidative stress markers, miR-195 and miR-27, and lower values of sirtuin-1 ($p<0.05$). At 12 months of follow up, obese pre-diabetics with metformin vs. placebo experienced significant reduction of glucose values, HOMA-IR, and inflammatory/oxidative stress markers, with significant reduction of intima-media thickness (IMT), septum and posterior wall thickness, and left ventricle mass (LVM), ($p <0.05$). At 12 months of follow-up, obese pre-diabetics with placebo vs. normo-glycemics had higher values of inflammatory/oxidative stress markers, higher values of IMT, septum and posterior wall thickness, LVM, and myocardial performance index (MPI), ($p<0.05$). Obese pre-diabetics in metformin vs. placebo, and obese pre-diabetics with placebo vs. normoglycemics, had significant differences about

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IMT, MPI, and LVM ($p<0.05$). Obese pre-diabetics in metformin vs. placebo showed significant reduction of serum miR-195 and miR-27 ($p<0.05$). Obese pre-diabetics in metformin vs. normoglycemics showed higher expression of serum miR-195 and miR-27 ($p<0.05$). Finally, we found inverse relation between IMT and insulin (R -0.351), HOMA-IR (R -0.340), miR-195 (R -0.355), miR-27 (R -0.181); between LVEF and Insulin (R -0.332), HOMA-IR (R -0.142), miR-195 (R -0.297) and miR-27 (R -0.163). We found inverse correlation between LVM and sirtuin-1 (R -0.272), Insulin (R -0.810), HOMA-IR (R-0.183), miR-195 (R -0.446) and miR-27 (R-0.433), and direct correlation with interleukin-6 (R 0.195). MPI inversely linked to miR-195 (R -0.260) and miR-27 (R -0.591).

Conclusion: in obese pre-diabetics metformin therapy significantly reduces inflammation/oxidative stress, circulating miR-195 and miR-27, causing reduction of LVM, IMT and amelioration of cardiac performance.

BACKGROUND

Obese patients could over express inflammatory cytokines, oxidative stress factors, and down regulate the sirtuin1 (SIRT1) at level of abdominal fat tissue (1). All these factors are significantly over expressed in patients with pre-diabetes vs. normoglycemics, and they could negatively condition the cardiac performance (1). In this setting, SIRT1 could regulate the inflammatory/oxidative and the apoptotic pathways in adipose tissue (2). Notably, SIRT1 is also implied in the adipogenesis and tissue growth, such as in the control of glucose homeostasis (3). Thus, the metformin therapy vs. placebo in obese patients with pre-diabetes could ameliorate glucose homeostasis and insulin resistance, via modulation of the inflammatory/oxidative stress and of SIRT1 expression (1). Finally, all these effects metformin induced could then result in the amelioration of cardiac performance (1). Moreover, it could be relevant to identify key factors bridging inflammation/oxidative stress and SIRT1 pathways of the adipose tissue to the cardiovascular system function in obese patients with pre-diabetes. In this setting, few microRNAs (miRs) could be evaluated as key regulators of inflammation, adipose tissue function and expression of SIRT1, and implied in the regulation of insulin resistance and glucose homeostasis (4-8). Indeed, miRs are small endogenous non coding RNAs that regulate gene expression by translational repression or degradation of target mRNAs (4-8). Specifically, miR-195 and miR-27 are differently expressed in overweight and in normoglycemics vs. hyperglycemic patients, and implied in adipose tissue/systemic inflammation and SIRT1 expression (6, 7, 8). Notably, miR-195 and miR-27 could be down-regulated by metformin therapy in patients with diabetes mellitus (9). In this setting, authors evidenced that metformin therapy reduced left ventricle hypertrophy in patients with pre-diabetes by control of systolic blood pressure, body weight and oxidative stress reduction in obese patients with and without diabetes (10, 11). Therefore, metformin therapy has

protective effects on cardiac remodeling (10, 11). Notably and clinically relevant, as first there are not data about metformin effects on molecular inflammatory pathways and miRs' expression in obese patients with pre-diabetes. Secondly, there are not data about metformin effects on cardiac remodeling via the regulation of molecular inflammatory pathways and miRs' expression in obese patients with pre-diabetes. Thus, our study hypothesis was that in overweight normoglycemics vs. pre-diabetics patients there was a significant difference of the adipose tissue expression of inflammatory/oxidative stress molecules, SIRT1, miR-195 and miR-27. Moreover, these patients could differently express circulating levels of inflammatory/oxidative stress factors, and of miR-195 and miR-27 at baseline. Finally, metformin therapy vs. placebo in overweight patients with pre-diabetes could ameliorate the glucose homeostasis and insulin resistance, with the consequent reduction of inflammatory/oxidative stress molecules and of circulating miR-195 and miR-27. Thus, all these favorable anti-inflammatory and metabolic ameliorative effects induced by metformin, could result in the reduction of left ventricle mass (LVM), intima-media wall thickness (IMT), with improvement of left ventricle ejection fraction (LVEF) and of cardiac performance at 12 months of follow-up. Thus, in the present study we evaluated the expression of inflammatory/oxidative stress molecules, SIRT1, miR-195 and miR-27 at baseline in the adipose tissue of overweight with pre-diabetes vs. normoglycemics, and the circulating expression of inflammatory/oxidative stress biomarkers and of miR-195, miR-27 at baseline, and selectively in normoglycemics vs. pre-diabetics randomly assigned to metformin vs. placebo therapy at follow-up of 12 months. Finally, we correlated the values of these biomarkers to modifications of intima-media thickness (IMT), left ventricle mass (LVM), left ventricle ejection fraction (LVEF) and cardiac performance at 12 months of follow-up in overweight normoglycemics vs. overweight with pre-diabetes randomly assigned to metformin vs. placebo therapy.

MATERIALS AND METHODS

Research design and Methods

We prospectively enrolled in a placebo-controlled study, conducted from January 2015 to January 2019 at University of Campania "Luigi Vanvitelli" , 83 obese patients with standard indications to receive an abdominoplastic surgery (6). We defined obese the patients with body mass index (BMI) > 30 kg/m2 (7). All 83 patients underwent abdominoplastic surgery, and after treatment received a hypocaloric diet as previously reported (1). The full description of diet therapy is reported in **supplementary file**.

Fifty-five obese patients had pre-diabetes according to international guidelines diagnostic criteria (10). Pre-diabetes was diagnosed by evidence of fasting plasma glucose of ≥5.6 mmol/L but <7.0 mmol/L (100–125 mg/dL; impaired

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fasting glucose [IFG]), a 2-hour glucose of ≥7.8 mmol/L but <11.1 mmol/L during a 75 g oral glucose tolerance test (GTT) (140–199 mg/dL; impaired glucose tolerance [IGT]), or a plasma hemoglobin (Hb) A1c of ≥5.7% but <6.5% (10). Patients with pre-diabetes were randomly divided in those (n =28) treated by hypocaloric diet added to metformin therapy vs. those (n =27) treated by hypocaloric diet plus placebo. However, the patients with pre-diabetes received metformin 850 mg twice a day vs. placebo. Twenty-eight patients were obese normo-glycemics and received hypocaloric diet.

All study groups volunteered for repeated clinical evaluations and laboratory analyses as well as echocardiography. In the follow-up period, patients were treated with a multidisciplinary approach consisting of diet, exercise, and behavioral and nutritional counseling as previously described (1). The enrolled patients were followed quarterly on an outpatient basis until 12 months.

Exclusion study criteria were: diagnosis of type 2 diabetes mellitus, cardiovascular disease, psychiatric problems, a history of alcohol abuse, smoking, or any hypoglycemic medication assumption. All patients had normal results for laboratory data (urea nitrogen, creatinine, electrolytes, liver function tests, uric acid, thyroxin, and complete blood count), chest x-rays, and electrocardiograms. All patients were evaluated at baseline, and at 12 months follow up. Each patient provided informed written consent to participate in this study, which was approved by the institutional committee of ethical practice of our institution. The patients subscribed a separate informed consent to undergo abdominoplasty.

Echocardiography

Two experienced physicians (C.S, G.G) full trained in cardiovascular echography, performed a trans thoracic full 2-dimensional and Doppler echocardiography assessment in independent way and blinded to study protocol and treatment groups. The exam was performed at baseline and after 12 months, according to the American Society of Echocardiography recommendations (12). For the echocardiography we used a Philips iE33 echocardiograph (Eindhoven, The Netherlands). LVM was calculated and normalized by both body surface area (BSA) and by height squared correct for the effect of overweight (13). As described previously, we calculated the LVEF, as index of cardiac pump (12). LVEF was calculated, dividing the stroke volume by the volume of blood collected in the left ventricle at the end of diastolic filling as end-diastolic volume (12). The stroke volume was the fraction of chamber volume ejected in systole as the difference between end diastolic volume and end systolic volume (12). Subsequently, we measured doppler velocities and time intervals from mitral inflow and left ventricular outflow recordings. However, mitral early

diastolic flow deceleration time was measured as the time interval between the peak of early diastolic velocity and the end of early diastolic flow (12). The total systolic time interval was measured from the cessation of one mitral flow to the beginning of the following mitral inflow (12). The ratio of velocity time intervals of mitral early and late diastolic flows was then calculated (12). As previously reported (1), we evaluated as myocardial doppler indexes and intervals the left ventricle isovolumetric relaxation time (IRT), left ventricle ejection time (ET), and left ventricle isovolumetric contraction time (ICT) (1, 8). Thus, IRT was the time interval from cessation of left ventricular outflow to onset of mitral inflow, and the ET was the left ventricle ejection time, as an interval from the onset and cessation of left ventricular outflow (1). However, we evaluated the ICT as interval calculated by subtracting ET and IRT from the total systolic time interval (1). Thereafter, from these indexes we obtained the myocardial performance index. In fact, myocardial performance index (MPI) was calculated by using the formula MPI= (IRT +ICT)/ET (1). The MPI, by including both systolic and diastolic time intervals, could be evaluated for assessment of cardiac dysfunction (1).

Finally, we calculated in all study population the LVM using 2-dimensional (2D) echocardiography, and via M-mode as recommended method (12). The used formula to estimate LVM from linear dimensions, based on the assumption of the left ventricle as a prolate ellipsoid of revolution, was to have linear measurements of interventricular septum wall thickness (IVST), as well as left ventricular internal diameter (LVID) and posterior wall thickness (PWT), from the parasternal acoustic window in end-diastole at the level of the LV minor axis (mitral valve leaflet tips) using 2D-targeted M-mode or directly from 2D images (12).

Measuring the IMT of carotid artery

At baseline and at follow-up we evaluated for all study population the IMT of carotid artery by B-mode ultrasound imaging of the carotid arteries, using a Philips iE33 echocardiograph (Eindhoven, The Netherlands) with a 7-MHz linear array transducer used to clearly display both the blood–intima and media–adventitia boundaries on the far walls of the arteries. However, according to authors we used gain settings to optimize image, and to have best quality for visualization of the carotid arteries' lumen (14). However, we obtained the scanning of the right and left common carotid arteries longitudinally in the 5- to 20-mm segment proximal to the carotid bulb and in areas free of plaques (14). Than in the study protocol we used these projections for measuring carotid artery IMT, to locate the lumen-intima and media–adventitia echographic boundaries, and to have IMT measurements offline on a personal computer (14). Finally, we diagnosed the atherosclerotic carotid plaques, as localized echo structures encroaching into the vessel lumen for which the distance between the media-adventitia interface and the internal side of the lesion was>1.5 mm

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(14). All scans and IMT measurements were performed by a two experienced physicians (G.G, R.M) full trained in vascular ultrasound, blinded to the study protocol, and the intra observer coefficient of variation for C-IMT was<3%.

Abdominal Dermolipectomy

As previously reported, the patients underwent conventional abdominoplasty surgical procedure, with umbiliculum transposition and cutaneous adipose mass tissue excision ranging from 200 grams (1). However, 24 h after the intervention the patients were mobilized; anti-inflammatory therapy (non-steroidal anti-inflammatory drugs) was suspended after 48 h and were discharged 72 h following with antibiotic therapy. From excision of adipose tissue authors (G.P, S.F) extracted the specimens of adipose tissue for further analysis.

Analyses of adipose tissue

After the abdominal surgery, the specimens were cut parallel to the long axis into four different parts for the different works-ups. Thus, a first part was frozen in liquid nitrogen for the following enzyme-linked immune-sorbent assay analysis. However, a portion of the other specimens was immediately immersion fixed in 10% buffered formalin. Sections were serially cut at 5 µm, mounted on lysine-coated slides, and stained with hematoxylin/eosin. Finally, the specimens were analyzed by light microscopy.

Western blot analysis for SIRT1 detection SIRT1 expression was analyzed in specimens of adipose abdominal superficial tissue. The samples (200 mg) from obese normoglycemic and obese pre-diabetics patients were cut into small pieces and homogenization was performed by adding to tissues 600 µL of 2D lysis buffer (7 mM urea, 2 mM thiourea, 4% CHAPS [3-([3-cholamidopropyl]dimethylammonio)-1-propane sulfonate] buffer, 30 mM Tris-HCl, pH 8.8, (1). Tissue homogenized with a Precellys 24 system (Bertin Technologies, Montignyle-Bretonneux, France) was centrifuged at 800 xg for 10 min at 4°C to collect the supernatant. Thus, the proteins were then precipitated by adding 100% cold methanol, and the protein extracts (80 µg) were separated by SDS-polyacrylamide gel electrophoresis (7-%) and transferred on to nitrocellulose membranes by Trans-Blot Turbo Transfer System (BioRad). However, the membranes were blocked in 5% w/v milk, 1 X Tris-buffered saline (TBS), 0.1% Tween-20 at 25 °C for 2h with gentle shaking, and then incubated over night at 4°C with antibodies against SIRT1 (1:1.000) (rabbit monoclonal, ab32441, Abcam, Cambridge, UK) or NF-κB (1:1000) (rabbit monoclonal, C22B4, Cell Signaling Technology, Danvers, MA) or Fas (rabbit monoclonal, ab82419, Abcam, Cambridge, UK). After the incubation with the secondary antibody (1:10.000), the membranes were washed three times and bands were detected by the enhanced chemiluminescence kit (Immobilon Western,

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Chemiluminescent HRP Substrate, Millipore, Billerica, MA 01821, USA) and analyzed by using a Scan LKB (Amersham Pharmacia), (1). Finally, we ensured the normalization of results by incubating the nitrocellulose membranes in parallel with the alpha-tubulin antibody (1:1,000) (mouse monoclonal, 3873, Cell Signaling Technology, Danvers, MA).

Analyses of Blood Samples

Authors obtained for all the patients enrolled in the study the serum samples, to evaluate the inflammatory markers, cytokine levels and miRsì expression. The samples were stored at temperature under 80°C until assayed. Serum concentrations of tumor necrosis factor alpha (TNFα), interleukine 6 (IL6), and Nitrotyrosine were determined in duplicate using a highly sensitive quantitative sandwich enzyme assay (ELISA, Quantikine HS; R&D Systems, Minneapolis, MN). Venous blood samples were drawn for nitrotyrosine evaluation. Nitrotyrosine plasma concentration was assayed by ELISA, after an overnight fast, at breakfast time, and before the sensor insertion. Assays for serum total and high-density lipoprotein cholesterol, triglyceride, and glucose levels were performed in the hospital's chemistry laboratory. Authors assayed the plasma insulin levels by radioimmunoassay technique (Ares, Serono, Italy). However, we assessed the insulin resistance in the fasting state with homeostasis model assessment (HOMA) and calculated with the following formula: fasting plasma glucose (millimoles per liter) times fasting serum insulin (microunits per milliliter) divided by 25, as previously described (1).

MicroRNAs isolation and reverse-transcription Real Time PCR (qRT-PCR)

The miRs were isolated from adipose tissue by using Mirneasy mini kit (Qiagen, Milan, Italy), while serum miRs isolation was performed by using miRNeasy serum/plasma kit (Qiagen, Milan, Italy). Total RNA isolation efficiency was monitored by adding Syn-cel-miR-39-3p miScript miRNA Mimic 5 nM (Qiagen, Milan, Italy) to each sample before the miRNAs isolation. RNA concentration and quality was determined by Nano Drop 2000c spectrophotometer (Thermo Scientific).Mature miRs were converted in cDNAby Gene Amp PCR System 9700 (Applied Biosystems) and MiScript II Reverse Transcription Kit (218161, Qiagen).The triplicate determinations of hsa-miR-195-5p (MIMAT0000461) and hsa-miR-27a-3p (MIMAT0000084)(MIMAT000163) were evaluated through CFX96 Real-Time System C1000 Touch Thermal Cycler (BioRad Laboratories, Inc), by using miScript SYBR Green PCR kit (218073, Qiagen) and specific miScript primer Assays (MS00003703 andMS00003241,Qiagen). Ce_miR-39-3p (MIMAT0000010; MS00019789, Qiagen) was used to normalize miRs expression. The qRT-PCR data were analyzed by using $2^{\wedge -\Delta\Delta Ct}$ method, where

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Cycle threshold (Ct) values were determined by CFX Manager™ Software (BioRad Laboratories, Inc) as previously reported (15).

Study endpoints

The **study endpoints** were:

-to evaluate the expression of miR-195, and miR-27 in abdominal fat tissue biopsy at baseline for obese normoglycemics vs. prediabetics in metformin therapy vs. prediabetics in placebo therapy; - to evaluate the values of circulating inflammatory/oxidative stress biomarkers and of circulating miR-195, and miR-27 at baseline and at follow-up of 12 months in obese normoglycemics vs. prediabetics in metformin therapy vs. prediabetics in placebo therapy.

-to evaluate the values of IMT, LVM, LVEF, and MPI at follow-up end, and their correlation as delta values (difference between baseline and follow-up end values) to circulating levels of inflammatory/oxidative stress biomarkers and of miR-195, and miR-27 in overweight normoglycemics vs. prediabetics in metformin therapy vs. prediabetics in placebo therapy at 12 months of follow-up.

Statistical analysis The data were presented as group means ± SD. One-way analysis of variance (ANOVA) was used to compare baseline data, followed by Scheffe's test for pair wise comparisons. We used simple and partial correlation to evaluate relationships between variables. A linear regression analysis was performed to study the relation between the study variables (BMI, hypertension, WHR, HOMA-IR, glucose, miR 195, miR 27 etc) and the clinical study outcomes. In detail, these study variables were correlated to the delta values of study outcomes, that represented changes between follow up vs. baseline values. The presented data were a combination of obese pre-diabetics in metformin arm, obese pre-diabetics in placebo arm and of obese normoglycemics, and we performed a partial correlation with groups and fasting glucose as a confounder. However, for each study outcome we calculated the R value and the p value. A value of $P<0.05$ was considered significant. Statistical analysis was performed using the SPSS software package for Windows 17.0 (SPSS Inc., Chicago Illinois). We calculated a sample size with 15 participants for each group, with estimated 80% power to detect a change of 0.015 between the mean changes of LVM in the placebo-treated and actively treated groups, at a 5% level of significance. A 20% Loss due to early withdrawals and/or non-evaluable measurements was assumed and, combined with the effect of stratification on analysis, resulted in the requirement to recruit at last 18 patients per treatment group.

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RESULTS

1. Clinical characteristics of study population at enrollment. At baseline, pre-diabetics obese in metformin and in placebo arm of treatment vs. normo-glycemics obese patients presented higher values of glucose (6.64 ± 0.14 vs. 5.31 ± 0.52 mmol/L, and 6.63 ± 0.18 vs. 5.31 ± 0.52 mmol/L, p <0.05), lower values of insulin (19.8 ± 1.9 vs. 22.5 ± 1.9 µU/mL, and 20.0 ± 1.8 vs. 22.5 ± 1.9 µU/mL, p <0.05), and higher values of HOMA-IR (5.1 ± 0.72 vs. 4.1 ± 0.25, and 5.0 ± 0.69 vs. 4.1 ± 0.25, p <0.05). **Table 1.** At enrollment, pre-diabetics obese in metformin and in placebo arm of treatment vs. normo-glycemics obese patients presented higher values of creatinine (101.5 ± 4.1 vs. 83.2 ± 2.9 mmol/L, and 101.8 ± 3.8 vs. 83.2 ± 2.9 mmol/L, p <0.05), and higher values of inflammatory markers, as C reactive protein (CRP, 1.13 ± 0.52 vs. 0.85 ± 0.36 mmol/L, and 1.09 ± 0.49 vs. 0.85 ± 0.36 mmol/L, p< 0.05), IL6 (4.41 ± 0.52 vs. 3.49 ± 0.38 pg/ml, and 4.36 ± 0.43 vs. 3.49 ± 0.38 pg/ml, p<0.05), TNFα (6.96 ± 0.56 vs. 5.56 ± 0.92 pg/ml, and 6.92 ± 0.54 vs. 5.56 ± 0.92 pg/ml, p<0.05), and Nitrotyrosine (5.19 ± 0.68 vs. 1.89 ± 0.24 nmol/L, and 5.20 ± 0.64 vs. 1.89 ± 0.24 nmol/L, p<0.05). **Table 1**. Again, pre-diabetics obese in metformin and in placebo arm of treatment vs. normo-glycemics obese patients expressed higher values of circulating miR-195 (198.82 ± 32.18 vs. 62.18 ± 8.31, and 196.41 ± 46.67 vs. 62.18 ± 8.31, p<0.05), and of miR-27 (116.89 ± 40.31 vs. 93.46 ± 43.76, and 116.81 ± 40.31vs. 93.46 ± 43.76, p<0.05).**Table 1**.

Table 1. Clinical characteristics of study population as pre-diabetics obese (metformin therapy vs. placebo), and obese normoglycemics at baseline. In this table are reported the study variables of study population of 83 patients divided in three groups at baseline: group 1, 28 pre-diabetics obese patients treated by hypocaloric diet added to metformin; group 2, 27 pre-diabetics obese patients treated by hypocaloric diet added to placebo; group 3, 28 normoglycemics obese patients treated by hypocaloric diet. In the study variables (first column to the left) are reported clinical variables, biohumoral markers, echocardiographic parameters, and drug therapy for each group of patients. We reported fasting glucose and lipid values.

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Study variables	Pre-DM plus metformin (n 28)	Pre-DM plus placebo (n 27)	NG (n 28)	P value
Clinical variables				
Age	42.5±7	41.8±5	40.6±8	/
Male (%)	8 (28.6)	9 (33.3)	9 (32.1)	/
BMI (Kg/m^2)	33.9±2.8	33.8±2.9	33.7±2.4	/
Systolic arterial pressure (mmHg)	136±13.2	135±12.6	131±12.8	/
Diastolic arterial pressure (mmHg)	83±3.1	84±2.1	85±2.1	/
Heart rate (beats for minute)	72±9	72±10	69±8	/
WHR	0.91±0.008	0.91±0.007	0.91±0.001	/
HOMA-IR	5.1±0.72	5.0±0.69	4.1±0.25	<0.05**, <0.05***
Insulin (µU/mL)	19.8±1.9	20.0±1.8	22.5±1.9	<0.05**, < 0.05***
Glucose (mmol/L)	6.64± 0.14	6.63± 0.18	5.31± 0.52	<0.05**, <0.05***
Cholesterol (mmol/L)	4.49±0.87	4.52±0.88	4.56±0.91	/
HDL(mmol/L)	1.78±0.44	1.79±0.36	1.82±0.41	/
LDL(mmol/L)	3.32±0.63	3.33±0.59	3.16±0.55	/
Triglycerides(mmol/L)	1.89±0.44	1.86±0.55	1.64±0.32	/
Creatinine (mmol/L)	101.5±4.1	101.8±3.8	83.2±2.9	<0.05**, < 0.05***
Biohumoral markers				
CRP (mmol/L)	1.13± 0.52	1.09±0.49	0.85±0.36	<0.05**,<0.05***
IL6(pg/ml)	4.41±0.52	4.36±0.43	3.49±0.38	<0.05**,<.005***
TNFα (pg/ml)	6.96±0.56	6.92±0.54	5.56±0.92	<0.05**,<0.05***
Nitrotyrosine (nmol/l)	5.19±0.68	5.20±0.64	1.89±0.24	<0.05**, <0.05***
miR 195	198.82±32.18	196.41±46.67	62.18±8.31	<0.05**, <0.05***
miR 27	116.89±40.31	116.81±40.31	93.46±43.76	<0.05**, <0.05***
Adipose tissue markers				
SIRT1 (arbitrary units)	0.63±0.29	0.63±0.23	0.88±0.15	<0.05**, <0.05***
NFKb (arbitrary units)	1.18±0.14	1.15±0.11	0.93±0.09	<0.05**, <0.05***
FAS (arbitrary units)	218.49±88.62	221.49±92.28	81.66±22.43	<0.05**, <0.05***
IL6 (arbitrary units)	79.08±3.15	79.15±3.11	48.21±3.07	<0.05**, <0.05***
TNFα (arbitrary units)	36.13±5.88	35.89±6.19	23.43±5.10	<0.05**, <0.05***
Nitrotyrosine (arbitrary units)	51.89±4.87	51.65±5.09	14.23±4.43	<0.05**, <0.05***
miR 195	161.39±53.01	156.22±56.12	68.11±12.49	<0.05**, <0.05***
miR 27	229.14±66.15	223.15±62.59	74.46±41.07	<0.05**, <0.05***
Echocardiographic parameters				
Intima-media tickness	1.02±0.15	1.03±0.17	0.88±0.14	<0.05**, <0.05***
LVEDd (mm)	56±5.8	56±5.1	54±4.2	/
LVESd (mm)	35±4.9	33±4.6	31±5.8	/
LVEF (%)	52±7	52±7	54±5	/
LAD (mm)	45±6	43±6	42±2	/
Septum (mm)	14±2.9	14±2.7	13.2±2.3	/
Posteriorwall (mm)	12±1.3	12±1.8	11±1.2	/
MPI	0.59±0.03	0.58±0.03	0.57±0.03	/
LV mass (g)	217.4±32.65	218.3±42.3	201.6±39.3	/
LV mass/BSA (g/m^2)	94.11±22.13	93.54±21.88	92.08±20.87	/
LV mass/h (m^2)	70.11±17.45	70.34±17.62	69.15±17.10	/
Drug therapy	28	27	28	

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Beta blockers (%)	22 (78.6)	21 (77.8)	22 (78.6)	/
ACE inhibitors (%)	14 (50)	13 (48.1)	12 (42.8)	/
ARS blockers (%)	7 (25)	8 (29.6)	6 (21.4)	/
Calcium channels blockers (%)	3 (10.7)	3 (11.1)	2 (7.1)	/
Loop diuretics (%)	3 (10.7)	3 (11.1)	2 (7.1)	/
Thiazides (%)	8 (28.6)	7 (25.9)	6 (21.4)	/
Statin (%)	11 (39.3)	11 (40.7)	10 (35.7)	/

ACE is angiotensin converting enzyme; ARS is angiotesin renin system; CRP, C reactive protein; h is height; HOMA-IR, homeostasis model for the assessment of insulin resistance; IL6, interleukine 6; LAD is left atrium diameter; LV is left ventricle; LVEF is left ventricle ejection fraction; LVEDd is left ventricle end diastolic diameter; LVESd is left ventricle end systolic diameter; miR: is microRNA; MPI is myocardium performance index; SIRT 1, sirtuin 1; TNFα tumor necrosis factor alpha; WHR, waist hip ratio. The symbol * is indicating a p value <0.05 by the comparison of group 1 vs. group 2; the symbol ** is indicating the p value < 0.05 by the comparison of group 1 vs. group 3; the symbol *** is indicating a p value <0.05 by the comparison of group 2 vs. group 3. The symbol / is indicating a not statistical significant value. The p value < 0.05 is indicating a statistical significant p value.

This trend was confirmed as over inflammation/oxidative stress and miRs' expression also at level of adipose tissue.

Table 1. In addition, there was a statistical significant difference comparing pre-diabetics obese in metformin and in placebo arm of treatment vs. normo-glycemics obese patients about IMT (1.02 ± 0.15 vs. 0.88 ± 0.14, 1.03 ± 0.17 vs. 0.88 ± 0.18, p<0.05). **Table 1**.

2. Clinical characteristics and comparison between the three groups of patients at 12 months of follow up.

At 12 months of follow up, **pre-diabetics obese treated by metformin vs. pre-diabetics obese treated by placebo** experienced a significant reduction of heart rate (66 ± 8 vs. 71 ± 7, p<0.05), of WHR (0.78 ± 0.003 vs. 0.88 ± 0.006, p<0.05), with a reduction of glucose values (5.45 ± 0.13 vs. 6.67 ± 0.19 mmol/L, p<0.05), and an amelioration of insulin resistance (HOMA-IR, 4.1 ± 0.28 vs. 5.0 ± 0.67, p<0.05; Insulin 22.9 ± 1.6 vs. 20.2 ± 1.6 µU/ml, p<0.05). **Table 2**. About serum inflammatory markers, at 12 months of follow up, pre-diabetics obese treated by metformin vs. pre-diabetics obese treated by placebo experienced a significant reduction of CRP (0.48 ± 0.28 vs. 0.56 ± 0.28 mmol/L, <0.05), IL6 (3.29 ± 0.31 vs. 4.11 ± 0.25 pg/ml, p<0.05), TNFα (5.14 ± 0.38 vs. 6.52 ± 0.49 pg/ml, p<0.05), and of nitrotyrosine (2.148 ± 0.348 vs. 4.520 ± 0.380 nmol/L, p<0.05). **Table 2.**

About echocardiographic parameters, at 12 months of follow up, pre-diabetics obese treated by metformin vs. pre-diabetics obese treated by placebo experienced a significant reduction of IMT (0.85 ± 0.12 vs. 1.02 ± 0.17 mm, p<0.05), septum thickness (10.2 ± 2.3 vs. 13.5 ± 2.2 mm, p <0.05), posterior wall thickness (8.0 ± 1.1 vs. 10.6 ± 1.4 mm,

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p <0.05), LVM (131.8 ± 21.8 vs. 181.2 ± 31.4 grams, p value <0.05), LV mass/BSA (56.13 ± 16.18 vs. 79.81 ± 16.83 g/m2, p<0.05), LV mass/h (46.63 ± 13.24 vs. 63. 24 ± 14.43 m2, p<0.05) and of MPI (0.36 ± 0.03 vs. 0.49 ± 0.04, p <0.05); not significant differences were found about LVEF (p>0.05). **Table 2, figure 1.**

Table 2. Clinical characteristics of study population as obese pre-diabetics (group 1 and 2), and obese normo-glycemics (group 3) at 12 months of follow up.

Study variables	Pre-DM plus metformin (n 28)	Pre-DM plus placebo (n 27)	NG (n 28)	P value (pre-DM plus metformin vs. pre-DM plus placebo)	P value (pre-DM plus metformin vs. NG)	P value (pre-DM plus placebo vs. NG)
Clinical variables						
BMI (Kg/m^2)	31.2±0.5	32.1±0.8	31.5±0.7	/	/	/
Systolic arterial pressure (mmHg)	129±2.3	124±11.3	124±7.6	/	/	/
Diastolic arterial pressure (mmHg)	78±2.8	76±2.3	75±2.2	/	/	/
Heart rate (beats for minute)	66±8	71±7	66±5	<0.05*	/	<0.05***
WHR	0.78±0.003	0.88±0.006	0.81±0.001	<0.05*	/	<0.05***
HOMA-IR	4.1±0.28	5.0±0.67	4.1±0.28	<0.05*	/	<0.05***
Insulin(µU/ml)	22.9±1.6	20.2±1.6	23.1±1.7	<0.05*	/	<0.05***
Glucose (mmol/L)	5.45± 0.13	6.67± 0.19	5.31 ± 0.48	<0.05*	/	<0.05***
Cholesterol (mmol/L)	4.04±0.76	4.15±0.90	4.13±0.79	/	/	/
HDL (mmol/L)	1.81±0.44	1.84±0.38	1.78±0.39	/	/	/
LDL (mmol/L)	3.10±0.53	3.17±0.52	3.11±0.52	/	/	/
Triglycerides(mmol/L)	1.66±0.72	1.70±1.24	1.62±0.41	/	/	/
Creatinine (mmol/L)	103.6±6.4	98.6±3.5	74.2±1.7	/	<0.05**	<0.05***
Biohumoral markers						
CRP (mmol/L)	0.48± 0.28	0.56±0.28	0.46±0.12	<0.05*	/	<0.05***
IL6 (pg/ml)	3.29±0.31	4.11±0.25	3.12±0.38	<0.05*	/	<0.05***
TNFα (pg/ml)	5.14±0.38	6.52±0.49	4.72±0.71	<0.05*	/	<0.05***
Nitrotyrosine (nmol/l)	2.148±0.348	4.520±0.380	0.920±0.110	<0.05*	<0.05**	<0.05***
miR 195	104.54±32.71	164.52±47.44	38.14±17.17	<0.05*	<0.05**	<0.05***
miR 27	62.53±35.34	98.66±30.28	39.48±18.15	<0.05*	<0.05**	<0.05***
Echocardiographic parameters						
Intima-media tickness	0.85±0.12	1.02±0.17	0.81±0.13	<0.05*	/	<0.05***
LVEDd (mm)	54±3.3	55±3.1	53±4.5	/	/	/
LVESd (mm)	31±5.4	32±4.1	29±4.8	/	/	/
LVEF (%)	57±5	54±8	56±6	/	/	/
LAD (mm)	44±2	43±2	41±2	/	/	/
Septum (mm)	10.2±2.3	13.3±2.2	10.1±1.8	<0.05*	/	<0.05***
Posteriorwall (mm)	8±1.1	10.6±1.4	8.4±1.2	<0.05*	/	<0.05***
MPI	0.35±0.03	0.49±0.05	0.36±0.04	<0.05*	/	<0.05***
LV mass (g)	131.8±21.8	181.2±31.4	126.4±21.3	<0.05 *	/	<0.05***

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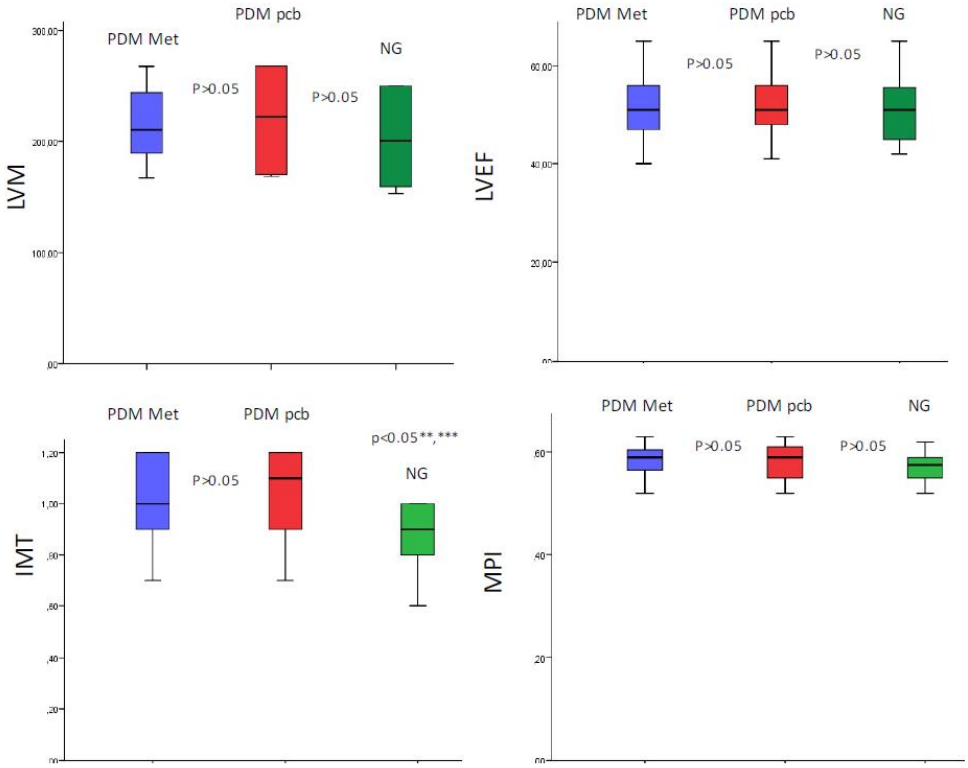
LV mass/BSA (g/m^2)	56.13±16.18	79.81±16.83	56.59±13.83	<0.05 *	/	<0.05***
LV mass/h (m^2)	46.63±13.24	63. 24±14.43	44.73±11.28	<0.05 *	/	<0.05***

In this table are reported the study variables of study population of 58 patients divided in three groups at 12 months of follow up: group 1, 20 pre-diabetics obese patients treated by hypocaloric diet added to metformin; group 2, 20 pre-diabetics obese patients treated by hypocaloric diet added to placebo; group 3, 18 normoglycemics obese patients treated by hypocaloric diet. In the study variables (first column to the left) are reported clinical variables, biohumoral markers, echocardiographic and parameters for each group of patients. . We reported fasting glucose and lipid values. Abbreviations: CRP, C reactive protein; g, grams; m, meters; h height; HOMA_IR, homeostasis model for the assessment of insulin resistance; IL6, interleukine 6; LAD,left atrium diameter; LV, left ventricle; LVEF, left ventricle ejection fraction; LVEDd, left ventricle end diastolic diameter; LVESs, left ventricle end systolic diameter; MPI, myocardium performance index; TNFα tumor necrosis factor alpha; WHR, waist hip ratio. The symbol * is indicating a p value <0.05 by the comparison of group 1 vs. group 2; the symbol ** is indicating the p value < 0.05 by the comparison of group 1 vs. group 3; the symbol *** is indicating the p value < 0.05 by the comparison of group 2 vs. group 3. The symbol / is indicating a not statistical significant value. The p value < 0.05 is indicating a statistical significant p value.



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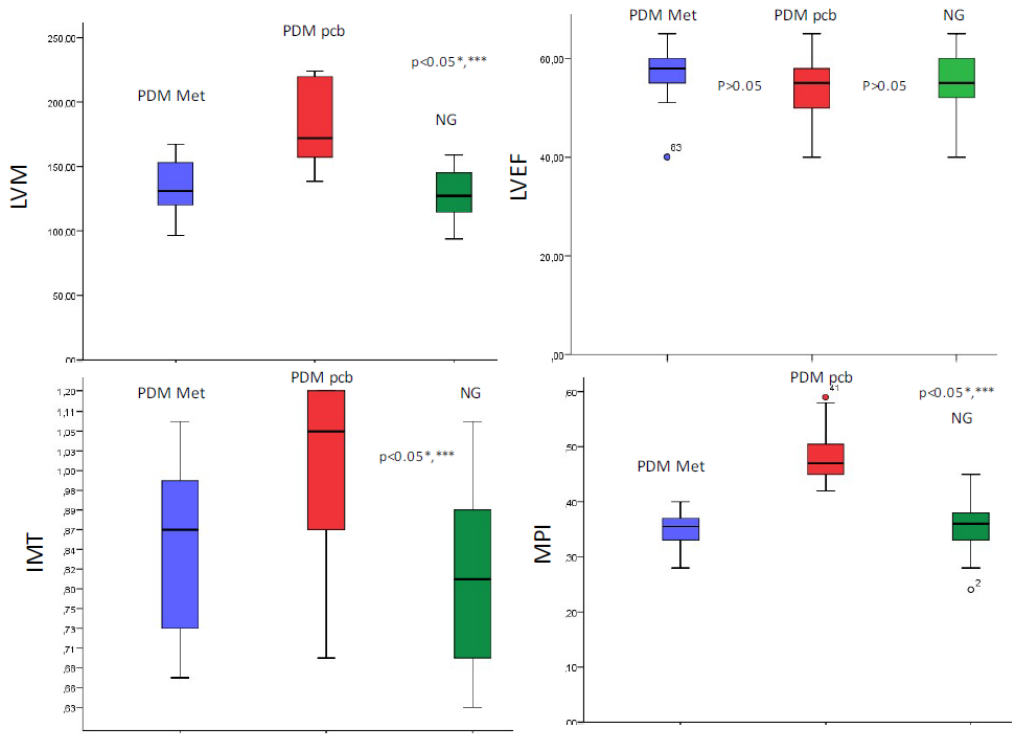

Figure 1. In this figure the baseline (upper part) and follow-up (lower part) values of left ventricle mass (LVM, grams), left ventricle ejection fraction (LVEF in %), intima-media tickness (IMT…) and myocardial performance index (MPI) in obese with pre-diabetes under metformin therapy (PDM Met in violet color), obese with pre-diabetes under placebo therapy (PDM pcb in red colour), and obese with normoglycemia (NG in green colour).
In upper part for baseline values of **IMT:** ** is for p<0.05 comparing PDM Met vs. NG; *** is for p<0.05 comparing PDM pcb vs. NG.
In lower part for follow-up values the symbol *is for p<0.05 comparing PDM Met vs. PDM pcb; *** is for p<0.05 comparing PDM pcb vs. NG.

At 12 months of follow up, **pre-diabetics obese treated by metformin vs. normo glycemics obese** had higher serum values of creatinine (103.6 ± 6.4 vs. 74.2 ± 1.7 mmol/L, p<0.05), and of nitrotyrosine (2.148 ± 0.348 vs. 0.920 ± 0,110 nmol/L, p <0.05). **Table 2.**

At 12 months of follow up, **pre-diabetics obese treated by placebo vs. normo glycemics obese** had higher values of heart rate (71 ± 7 vs. 66 ± 5, p<0.05), of WHR (0.88 ± 0.006 vs. 0.81 ± 0.001, p <0.05), of glucose values (6.67 ± 0.19 vs. 5.31 ± 0.48 mmol/L, p<0.05), with insulin resistance (HOMA-IR, 5.0 ± 0.67 vs. 4.1 ± 0.28, p<0.05; Insulin, 20.2 ± 1.6 vs. 23.1 ± 1.7 µU/ml, p<0.05), and higher serum values of creatinine (98.6 ± 3. 5 vs. 74.2 ± 1.7 mmol/L, p <0.05). **Table 2.**
Again, about serum markers of inflammation and oxidative stress**,** at 12 months of follow up, pre-diabetics obese treated by placebo vs. normo glycemics obese had higher values of CRP (0.56 ± 0.28 vs. 0.46 ± 0.12 mmol/L, p <0.05), IL6 (4.11 ± 0.25 vs. 3.12 ± 0.38 pg/ml, p<0.05), TNFα (6.52 ± 0.49 vs. 4.72 ± 0.71 pg/ml, p<0.05), and nitrotyrosine (4.520 ± 0.380 vs. 0.920 ± 0.110 nmol/L, p<0.05). **Table 2.**

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About echocardiographic parameters, at 12 months of follow up the pre-diabetics obese treated by placebo vs. normo glycemics obese had higher values of IMT (1.02 ± 0.17 vs. 0.81 ± 0.13 mm, p<0.05), septum thickness (13.3 ± 2.2 vs. 10.1 ± 1.8 mm, p<0.05), posterior wall thickness (10.6 ± 1.4 vs. 8.4 ± 1.2 mm, p<0.05), LVM (181.2 ± 31.4 vs. 126.4 ± 21.3 grams, p<0.05), LVM/BSA (79.81 ± 16.83 vs. 56.59 ± 13.83 g/m^2, p<0.05), LVM/h (63. 24 ± 14.43 vs. 44.73 ± 11.28 m^2, p<0.05), and MPI (0.49 ± 0.04 vs. 0.36 ± 0.02 p<0.05); not significant differences were found about LVEF (p>0.05). **Table 2, figure 1.**

The study results and comparison between clinical characteristics of obese pre-diabetics treated by hypocaloric diet added to metformin at 12 months of follow up vs. baseline condition, of obese pre-diabetics treated by hypocaloric diet added to placebo at baseline vs. 12 months of follow up, and of obese normoglycemics treated by hypocaloric diet at baseline vs. 12 months of follow up were reported in **supplementary file**.

Study outcomes

At follow-up end the enrolled patients experienced a modification in **circulating miRs' values** with significant difference comparing the three cohorts of study. Indeed, comparing pre-diabetics obese in metformin vs. pre-diabetics obese in placebo we found a significant reduction of miR-195 (104.54 ± 32.71 vs. 164.52 ± 47.44, p<0.05), and of miR-27 (62.53 ± 29.71 vs. 98.66 ± 30.28, p<0.05), while pre-diabetics obese in metformin vs. normoglycemics obese patients showed a higher expression of serum miR-195 (104.54 ± 32.71vs. 38.14 ± 17.17, p<0.05), and miR-27 (62.53 ± 35.34 vs. 39.48 ± 18.15, p<0.05). **Table 2.** Finally, pre-diabetics obese in placebo therapy vs. normoglycemics obese patients showed a higher expression of serum miR-195 (164.52 ± 47.44 vs. 38.14 ± 17.17, p<0.05), and miR-27 (98.66 ± 30.28 vs. 39.48 ± 18.15, p<0.05). **Table 2.** Again, comparing pre-diabetics in metformin vs. placebo arm of treatment and pre-diabetics treated with placebo vs. normoglycemics obese, we found significant differences about **IMT** (0.85 ± 0.12 vs. 1.02 ± 0.17, p<0.05; 1.02 ± 0.17 vs. 0.81 ± 0.13, p<0.05), **MPI** (0.35 ± 0.03 vs. 0.49 ± 0.05, p<0.05; 0.49 ± 0.05 vs. 0.36 ± 0.04, p<0.05), and **LVM** values (131.8 ± 21.8 vs. 181.2 ± 31.4 grams, p<0.05; 181.2 ± 31.4 vs. 126.4 ± 21.3, p<0.05). **Figure 1**. Finally, we evaluated by the regression analysis the relationship between study variables and delta (difference between baseline and follow-up values) of IMT, LVEF, LVM and MPI. Thus, we found a reverse relation between **IMT** and insulin (R -0.351, p=0.017), HOMA-IR (R -0.340, p 0.022), miR-195 (R -0.355, p =0.001), miR-27 (R -0.181, p=0.005). **Table 3.** For **LVEF** we found an inverse relation with Insulin (R -0.332, p=0.031), HOMA-IR (R -0.142, p=0.021), miR-195 (R -0.297, p=0.005) and miR-27 (R -0.163, p=0.001). **Table 3**. For **LVM**

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we found an inverse relation with SIRT1 (R -0.272, p=0.005), Insulin (R -0.810, p=0.001), HOMA-IR (R-0.183, p=0.001), miR-195 (R -0.446, p=0.005) and miR-27 (R -0.433, p=0.001), and a direct correlation with IL6 (R 0.195, p=0.008).

Table 3. For **MPI** we found an inverse relation with miR-195 (R -0.260, p=0.049) and miR-27 (R -0.591, p=0.026). **Table 3**.

Table 3. In the table linear regression analysis for study variables and study outcomes. For each study outcome is reported the R value and the p value.

Variables	Δ IMT		Δ LVEF		Δ LV MASS		Δ MPI	
	R value	P value	R value	P value	R value	P value	R value	P value
IL6	0.105	0.268	0.044	0.210	0.195	0.008*	0.312	0.059
TNFα	0.094	0.390	0.079	0.054	0.169	0.193	0.023	0.868
SIRT1	0.088	0.440	0.034	0.356	-0.272	0.005*	0.082	0.660
Cholesterol	0.149	0.150	0.086	0.057	0.128	0.584	0.211	0.102
Insulin	-0.351	0.017*	-0.332	0.031*	-0.810	0.001*	-0.210	0.248
HOMA-IR	-0.340	0.022*	-0.142	0.021*	-0.183	0.001*	-0.221	0.227
Systolic arterial pressure	0.258	0.797	0.019	0.623	0.209	0.814	0.146	0.261
Glucose	0.285	0.059	0.184	0.051	0.104	0.494	0.029	0.899
miR 195	-0.355	0.001*	-0.297	0.005*	-0.446	0.005*	-0.260	0.049*
miR 27	-0.181	0.005*	-0.163	0.001*	-0.433	0.001*	-0.591	0.026*

The study outcomes are expressed as delta (Δ) values, that represents changes between follow up vs. baseline values. The symbol * is indicating a p value <0.05. The p value < 0.05 is indicating a statistical significant p value. CRP, C reactive protein; HOMA_IR, homeostasis model for the assessment of insulin resistance; IL6, interleukine 6; MPI, myocardium performance index; SIRT1, sirtuin 1; TNFα tumor necrosis factor alpha.

Discussion

In the present study, the hyperglycemia and the insulin resistance in obese with pre-diabetes vs. obese normoglycemics patients were linked to over expression of inflammatory cytokines, and lower expression of SIRT1 in the subcutaneous abdominal fat. Parallely, the subcutaneous abdominal fat of these patients over-expressed miR-195 and miR-27. This trend was confirmed by the evaluation of circulating inflammatory/oxidative stress molecule and miR-195/miR-27 at baseline. In this setting, the metformin therapy vs. placebo was effective to reduce hyperglycemia, insulin resistance and inflammatory burden. Furthermore, in our study the metformin therapy significantly reduced circulating miR-195 and miR-27. Indeed, in obese patients with pre-diabetes the serum inflammatory/oxidative stress molecules, as circulating miR-195 and miR-27 were significantly down regulated by metformin therapy added to hypocaloric diet vs. placebo at 12 months of follow-up. Furthermore, we might speculate that these effects might lead

to the significant reduction of IMT, LVM, and MPI at follow-up end. In addition, here we evidenced an inverse relation between the abdominal adipose tissue expression of miR-195 and miR-27, and study outcomes as delta values of IMT, LVM, LVEF and MPI. Finally but not less relevant, lowest abdominal adipose tissue expression of SIRT1 were inversely correlated to highest LVM, while highest IL6 abdominal adipose tissue values were directly linked to highest LVM values.

The metformin is a hypoglycemic drug with ameliorative effect on glucose homeostasis, insulin resistance, and inflammatory burden in obese with pre-diabetes (1). Indeed, metformin therapy resulted in a significant amelioration of glucose homeostasis and insulin resistance, with down regulation of circulating inflammatory/oxidative stress pathways (1, 16). These metabolic and anti-inflammatory effects induced by metformin therapy could be linked to the reversion of left ventricle hypertrophy and LVM in patients with pre-diabetes (11). Indeed, these different pathways are implied in cardiac hypertrophy and fibrosis, with increase of LVM and IMT, and alteration of the cardiac performance (1, 16). A detailed description of these pathways is reported in **Supplementary files**. The novelty of our study is that in obese pre-diabetics the metformin vs. placebo could determine all these ameliorative metabolic and molecular effects, via the significant down-regulation of circulating miR-195 and miR-27. Indeed, in the present research for the first time in literature we identified in humans' obese with pre-diabetes vs. normoglycemics the different expression of miR-195 and miR-27 as epigenetic regulators and therapeutic target of inflammation/oxidative stress at level of adipose tissue and of cardiovascular system. Indeed, it is well known that the adipose tissue in obese patients could cause an altered synthesis and relapse of miR-195 (17). The miR-195 could cause the over-expression and secretion of inflammatory molecules in human adipose tissue and in obese patients (17). Again, the hyperglycemia could increase miR-195 levels and decrease SIRT1 expression in a cultured cellular model of diabetes (18). To date, the transfection with miR-195 antagomir in these cells forced expression of SIRT1, preventing the SIRT1 mediated tissutal damage via hyperglycemia and insulin resistance (18). In this setting, miR-195 caused endothelial dysfunction and insulin resistance via over expression of inflammation/oxidative stress and down regulation of SIRT1 (19). In addition, in diabetic mice model the knockdown of miR-195 increased myocardial capillary density and improved maximal coronary blood flow, leading to the reduction of diabetic cardiomiopathy (20). Therefore, metformin therapy could cause down regulation of miR-195, beyond improving glycemic control and insulin resistance, and leading to cardio-protective effects (21). Similarly, the expression of miR-27 is increased in fat tissue of obese mice, and in patients with metabolic syndrome and type 2 diabetes mellitus (23). Indeed, miR-27 is implied in molecular pathways regulators of lipid metabolism, vascular signaling, and glucose homeostasis (23). Furthermore, in

peripheral blood increased levels of miR-27 are linked to over expression of inflammatory markers as IL-6, TNF-α, and to reduced expression of SIRT1 (24). Notably, miR-27 has a regulatory role in increasing insulin sensitivity (25). Thus, in cellular lines with insulin resistance, authors found an altered expression of PPAR-γ, PI3K/Akt signaling pathway and GLUT4 expression after transfection with antagomiR-27 (25). However, the down regulation of miR-27 levels in cellular and mice model enhanced glucose uptake (glucose lowering effect) in time- and dose-dependent manners (25).

Therefore, taken together our study results might evidence, in obese patients with pre-diabetes, the implication of miR-195 and miR-27 as regulators of glucose homeostasis and insulin resistance, and of inflammation/oxidative stress pathways which are implied in cardiovascular remodeling (17-25). Notably and clinically relevant, the metformin in these patients could modulate these metabolic and inflammatory pathways, leading to cardio-protective effects. Furthermore, miR-195 and miR-27 could become targets for the treatment of obesity and insulin resistance, and to revert the pathological shifting from over inflammation/oxidative stress to cellular hyperplasia and hypertrophy, as seen in obese with pre-diabetes. Indeed, here we provide for metformin therapy an important regulative function on the control of these pathogenic mechanisms, by significant reduction of inflammatory/oxidative stress markers, as CRP, IL6, TNFα, and nitrotyrosine (1, 16), and by down regulation of circulating miR-195 and miR-27, (21, 25). Finally, an inverse correlation exists between miR-195, miR-27 and cardiovascular parameters as IMT, LVEF, LVM and MPI. Again, in our study lowest SIRT1 adipose tissue expression and highest circulating IL6 values linearly marked to highest LVM values. However, we might speculate that, patients with an induced over expression of SIRT1 and down regulation of IL6, could experience a significant reduction of LVM, as in the case of obese pre-diabetics under metformin therapy vs. placebo. Indeed, SIRT1 is implied in glucose metabolism, insulin resistance, and it plays an anti-remodeling effect during adaptive cardiac hypertrophy (1). Intriguingly, metformin therapy could work as direct SIRT1 inductor (1). Therefore, we might say that metformin therapy reduces the inflammation/ oxidative stress, and secondary this might reduce myocardial wall thickness, and myocardial mass, leading to the improvement of the cardiac function in pre-diabetics obese patients (1). Therefore, the significant reduction of IL6, that could be seen as anti-inflammatory effect induced by metformin therapy, could linearly mark with reduction of LVM in obese pre-diabetics.

Conclusion Abdominal fat tissue in pre-diabetics obese patients over expresses inflammatory/oxidative metabolites that are linked to reduced expression of SIRT1, and to increased expression of miR-195 and miR-27 at level of adipose abdominal tissue.

All these molecules could influence IMT, LVM, LVEF and MPI via systemic effects, that might be linked to adipose tissue and circulating expression of miR-195 and miR-27. Intriguingly, in pre-diabetics obese patients the metformin therapy might significantly reduce the inflammatory/oxidative stress metabolites, and the expression of circulating miR-195 and miR-27 at 12 months of follow-up. Notably, these effects are associated to the reduction of IMT, LVM, and MPI, and are linked to the amelioration of LVEF. Moreover, we might say that metformin therapy in addition to hypocaloric diet vs. placebo looks to be a relevant treatment to reduce hyperglycemia and insulin resistance, and to revert the systemic inflammation/oxidative stress in obese pre-diabetics via down regulation of circulating miR-195 and miR-27. Future studies are needed to further assess the effects of metformin in pre-diabetics obese patients, and its possible correlation with clinical outcomes via modulation of miRs.

Study limitations The present study evidences few limitations, as the short duration of follow up that might affect the long term outcomes. Again, the small sample size of pre-diabetics obese might affect the study results, and the loss of animal or cellular models could not test the human study results obtained by peripheral blood analysis (baseline and follow-up end analysis) and by direct analysis of samples by abdominal fat tissue biopsy (baseline analysis). Again, in the present study we did not use the magnetic cardiac resonance to evaluate the LVM, and so we did not compare this technique to echocardiography. On other hand, also in absence of this innovative technique for the analysis of LVM we could refer to the echocardiography, which did not show its inferiority to evaluate LVM as compared to magnetic heart resonance (12, 13). Again, we did not use other techniques to evaluate the overall percentage of abdominal fat deposit for study population. On other hand, the evaluation of entire volumetry of fat tissue for each cohort of study is outside th scope of the present study. Therefore, we could conclude that further long-term studies in a larger population of pre-diabetics obese will be needed to confirm our finding and to evaluate the effects of metformin at molecular, cellular, epigenetic and clinical level. Thus, in future studies we could evaluate the effects of metformin therapy added to abdominoplastic surgery and hypocaloric diet on the abdominal fat regulation of SIRT1/miRs, and of cytokine blood levels and circulating miRs, and to translate these information in a clinical setting for pre-diabetics patients.

Ethical approval: The study was approved by University of Campania "Luigi Vanvitelli"ethical committee. (approval number: 01.0012016)

DISCLOSURE

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Conflict of Interest: none declared.

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