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Atherosclerotic plaque fissuration and clinical outcomes In pre-diabetics vs. normoglycemics patients affected by asymptomatic significant carotid artery stenosis at 2 years of follow-up: role of MIcRoRNAs modulation. The ATIMIR study

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ABSTRACT

BACKGROUND AND PURPOSE—Atherosclerotic plaque instability and rupture in patients with asymptomatic carotid artery stenosis (ACAS) is a leading cause of major adverse cardiac events (MACE). This could be mainly evidenced in patients with pre-diabetes. Indeed, the altered glucose homeostasis and insulin resistance could cause over-inflammation of atherosclerotic plaque, favoring its conversion to unstable phenotype with rupture and MACE. Notably, the metformin therapy reducing the metabolic distress and the inflammatory burden, could lead to reduction of MACE in ACAS patients with pre-diabetes. In this setting, microRNAs (miRs) could be used as molecular biomarkers of atherosclerosis progression, plaque rupture and worse prognosis in normoglycemics (NG) vs. pre-diabetics metformin users (PDMU) vs. pre-diabetics non metformin users (PDNMU). However, the aim of our study was to investigate a wide miRNA panel in peripheral blood exosomes from patients with ACAS divided in NG vs. PDMU vs. PDNMU, and to associate the circulating miRNA expression profiles with MACE at 2 years of follow-up after endarterectomy.

METHODS—The study included 234 patients with ACAS divided in NG (n 125), PDNMU (n 73) and PDMU (n 36). The miRs' expression profiles of circulating exosomes were determined at baseline and at 2 years of follow-up by Affymetrix microarrays from plasma samples of the patients from any study cohort. Then we collected and analyzed MACE at 2 years of follow-up in NG vs. PDMU vs. PDNMU.

RESULTS—prediabetics vs. NG had over-inflammation ($p<0.05$) and over expressed miR 24 and miR 27 at baseline. At 2 years of follow-up PDNMU vs. NG, PDMU vs. NG and PDNMU vs. PDMU over-expressed inflammatory markers and miR 24, miR 27, miR 100, miR 126 and miR 133 ($p<0.05$). Finally, at follow-up end we observed a significant difference about MACE comparing PDNMU vs. NG (n 27 (36.9%) vs. n 8 (6.4%); $p<0.05$), PDNMU vs. PDMU (n 27 (36.9%) vs. n 6 (16.6%); $p <0.05$),

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and PDMU vs. NG (n 6 (16.6%) vs. n 8 (6.4%); p<0.05). Admission glucose values (HR 1.020, CI 95% [1.001-1.038], p 0.029), atheromatous carotid plaque (HR 5.373, CI 95% [1.251-11.079], p 0.024), and miR 24 (HR 3.842, CI 95% [1.768-19.222], p 0.011) predicted MACE at 2 years of follow-up.

CONCLUSIONS—Specific circulating miRs could be over-expressed in pre-diabetics and specifically in PDNMU vs. PDMU after endarterectomy. MiR24, hyperglycemia and atheromatous plaque could predict MACE at 2 years of follow-up.

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BACKGROUND

The rupture of atherosclerotic plaque is a leading cause of major adverse cardiac events (MACE) in patients with asymptomatic carotid artery stenosis (ACAS), (1). Indeed, from one side the significant atherosclerotic plaque narrowing, and from the other side the conversion of the atherosclerotic plaque from a stable to an unstable phenotype could both contribute to the plaque ulceration and rupture (1, 2). Then, the atherosclerotic plaque ulceration and rupture could cause the MACE (3). Notably, the shifting from stable to unstable plaque phenotype with its consequent rupture could involve multiple processes via the over inflammation (1-3). To date, in these patients the endarterectomy is a recommended treatment, to remove the atherosclerotic plaque and to ameliorate the clinical outcomes (4). On other hand, a higher percentage of asymptomatic patients, and particularly high risk patients as those with diabetes mellitus, could experience worse prognosis despite endarterectomy (4). Indeed, in diabetics vs. normoglycemics the altered glucose homeostasis and the insulin resistance could cause the over-expression of inflammatory molecules at level of atherosclerotic plaque, then leading to unstable plaques' rupture and MACE (4). Intriguingly, similar mechanisms have been reported for patients with pre-diabetes (5). The pre-diabetes is an intermedia clinical condition between the normoglycemia (NG) and the diabetes mellitus, characterized by altered glucose homeostasis, insulin resistance and over-inflammation (5). Intriguingly, in pre-diabetics the amelioration of glucose homeostasis and of insulin resistance induced by hypoglycemic drugs as the metformin, could lead to the reduction of the inflammatory burden, that results in the control of atherosclerotic processes (5, 6). Clinically, all these effects metformin induced could lead to the reduction of MACE in patients with pre-diabetes (6). On other hand, there are not conclusive data about the molecular (anti-inflammatory), metabolic (amelioration of glucose homeostasis and insulin resistance) and clinical effects (MACE) induced by metformin in pre-diabetics with ACAS after carotid endarterectomy.

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Moreover, the identification of biomarkers could be crucial to predict plaque rupture and MACE in prediabetics vs. NG, and specifically in prediabetics under metformin therapy (PDMU) vs. pre-diabetics without metformin therapy (PDNMU). To date, few microRNAs (miRs) have been recently evaluated in this respect (7). The miRs are small, noncoding, regulatory RNAs composed of 18 to 22 nucleotides, that regulate target gene expression at the post-transcriptional level by either inhibiting translation or causing degradation of the corresponding messenger RNA (7). In this context, the miRs have been evaluated in the progression and rupture of unstable carotid artery plaques in in ACAS patients (7, 8). To date, we might speculate that few miRs could be implied in fissuration and rupture of the unstable plaques for ACAS' patients with pre-diabetes vs. normoglycemics. Therefore, our study hypothesis was that metformin therapy, reducing inflammatory burden, could modulate miRs' expression for ACAS' patients, then leading to reduction of MACE after endarterectomy. Moreover, in the present study we evaluated the inflammatory burden and circulating miRs in NG vs. PDNMU vs. PDMU at baseline and at 2 years of follow-up after carotid endarterectomy. Finally, for these three cohorts of patients we evaluated the rate of MACE at 2 years of follow-up after carotid endarterectomy.

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RESEARCH DESIGN AND METHODS

In an observational, multicenter study we investigated consecutive inpatients with ACAS of both sex undergoing carotid endarterectomy as recommended by international guidelines (9). Thus, the ACAS patients undergoing carotid endarterectomy evidenced a stenosis of the carotid artery >70% (9). To evaluate the entity of carotid atherosclerotic plaque-related stenosis and to provide information on the plaques' composition we used the duplex ultrasound and the computed tomographic angiography (9). Then, the degree of carotid stenosis was determined according to the NASCET (North American symptomatic carotid endarterectomy trial) criteria (10). All the enrolled patients received an optimal medical treatment to limit disease progression associates control of atherosclerosis risk factors as smoking, hypertension, diabetes mellitus, and dyslipidemia, with antiplatelet agents, statins, and angiotensin-converting enzyme inhibitors (10). The ACAS patients underwent a baseline clinical examination, gave their medical history, and had never developed neurologic symptoms or cerebral lesions assessed by computed tomography (10). Finally, all the recruited patients before the intervention received computed tomography or magnetic resonance imaging to evaluate the carotid stenosis (10).

Then, from the study population we identified the NG patients and those with pre-diabetes. The pre-diabetes was categorized according to the criteria of the American Association of Clinical Endocrinologists and the American Diabetes Association (11). Furthermore, the patients with pre-diabetes answered a specific questionnaire about the use of metformin before the beginning of the study, the dates of the beginning and the end of treatment, the route of administration, and the duration of use (11).

To date, we classified the PDMU patients as pre-diabetics who used metformin therapy for at least 6 months. The PDNMU were the pre-diabetics that did never assume metformin and/or that assumed metformin for a period <6 months. The study population was recruited from the

Department of Cardiology and Cardiovascular Surgery of the Gemelli Molise, Campobasso, and from the Department of Advanced Medical and Surgical Sciences, University of Campania "Luigi Vanvitelli", Naples, Italy, from January 2016 to June 2018. In the overall population and in three cohorts of study (NG, PDNMU, PDMU) authors performed the carotid sonography by a single ultrasound machine (Toshiba Medical Systems Co, Ltd, Tokyo, Japan). The study was approved by the local Ethics Committee (University of Campania Ethical Committee number 440, date 08.08.2017), and informed written consent was obtained for each patient. The study was performed in accordance with the Declaration of Helsinki.

The study endpoints were evaluated in the 3 study cohorts (NG, PDNMU, PDMU) after endarterectomy at 2 years of follow-up. The enrolled patients respected the following inclusion and exclusion criteria.

Study inclusion and exclusion criteria

The inclusion criteria were: patients aged >18 and <75 years with indication to receive a carotid endarterectomy for extracranial high-grade (≥70%) internal carotid artery stenosis; patients without confirmed diagnosis of diabetes mellitus; patients without inflammatory chronic disease; patients without neoplastic disease.

Exclusion criteria were: diagnosis of diabetes mellitus; patients with clinical or laboratory evidence of heart failure; patients with previous endarterectomy for significant carotid stenosis; patients with previous stroke, valvular heart defects, malignant neoplasms, or secondary causes of hypertension.

Laboratory Analysis

In overall study population authors measured, after an overnight fast, the plasma glucose, HbA1c, and serum lipid levels by enzymatic assays in the hospital chemistry laboratory. However, the levels of fasting blood glucose were evaluated before surgery. Fasting and post-prandial plasma glucose data were obtained from the average of each assessment. From peripheral blood samples at

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baseline and at follow-up end we evaluated inflammatory markers as C reactive protein (CRP), interleukine 6 (IL6) and tumor necrosis factor alpha (TNFα). Then, at baseline and at follow-up end we evaluated the miRs implied in atherosclerotic carotid plaque instability and rupture for ACAS patients as miR 24, miR 27, miR 100, miR 126 and miR 133 (8, 9).

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

MiRNAs were extracted from blood sample using the miRNeasy Kit Qiagen according to the manufacturer's instructions. Total RNA, including miRNAs, was extracted using Trizol Reagent (Invitrogen) reagents according to the manufacturer's instructions. The RNA yield and concentrations were determined by Bioanalyzer Agilent 2100 and all RNA samples were stored at -80°C until use. MiRNA expression was analyzed using the two-step protocol of TaqMan MicroRNA Assays (Applied Biosystems), according to the manufacturer's instructions. Briefly, 5-10 ng of total RNA were reverse transcribed using miRNA-specific primers for hsa miRNA-24 3p (Applied Biosystems), hsa miRNA-27b-3p (Applied Biosystems), hsa miRNA 100-5p (Applied Biosystems), hsa miRNA 126-5p (Applied Biosystems), hsa miRNA 133a-3p (Applied Biosystems), hsa miRNA 133b (Applied Biosystems), and hsa miRNA 638 (Applied Biosystems) was used to normalize miRs expression combined with reagents (dNTPs, reverse transcription buffer, RNase inhibitor, reverse transcriptase, and H2O nuclease free) from the Taqman miRNA Reverse Transcription kit (Applied Biosystems). The reactions were performed for 30 minutes at 16° C, 30 minutes at 42° C, 5 minutes at 85°C, and then stored at 4° C. Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) was subsequently conducted using specific TaqMan probes of the TaqMan microRNA Assay kits (Applied Biosystems) to quantify miRNA expression. The reactions were incubated in a 96-well plate at the following thermal cycling conditions: 2 minutes at 50° C, 20 second at 95° C, (3

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seconds at 95°C and 30 seconds at 60°C) for 40 cycles. Data analysis Relative quantification of miRNA expression was calculated with the 2- ΔΔCt method (9).

Carotid ultrasound

Two experienced physicians in sonography, blinded to study protocol, performed the carotid ultrasound exam for every enrolled patient. However, for all the study population a high-resolution B-Mode, color Doppler and pulse Doppler ultrasonography of extracranial arteries was performed, at admission to the hospital (before intervention) and at 2 years of follow-up with the ultrasound machine Toshiba Aplio Power Vision (Toshiba Medical Systems Co, Ltd, Tokyo, Japan) equipped with 4–11 MHz linear array transducer. The procedures were all standardized to avoid bias between operators, and the patients were examined in a supine position with the head tilted backwards (9, 10). Then, we classified the ultrasonography findings for each carotid as normal, stenosis <50%, stenosis 50% to 70%, stenosis >70%, and as total occlusion (10). Then we examined the plaque morphology, and the atherosclerotic lesions by their echogenicity as echolucent (soft, lipid rich), moderately echogenic (heterogeneous or fibrotic) and dominantly echogenic (calcified), (10). Finally, operators examined the surface structure of atherosclerotic plaque to individuate the presence of ulcerations or thrombus, and then to define the plaques as ulcerative or smooth and thrombotic, respectively (10).

Briefly, the significant carotid stenosis (stenosis >70%) was diagnosed by the measurement of an increase in the peak systolic velocity (PSV) > 2.1 m/s and the end-diastolic velocity (EDV) > 0.7 m/s, (9, 10). Finally, the degree of carotid stenosis was also assessed by measuring the vessel diameter at the point of maximal stenosis compared with the plaque-free vessel diameter distally from the lesion (9, 10). The follow-up of patients included clinical visits and carotid ultrasound at 2 years of follow-up, to detect possible vascular events and to assess stenosis progression, respectively.

Intervention: endarterectomy and carotid revascularization

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After the ultrasonography the ACAS patients received the carotid endarterectomy. This intervention was made at the operator's discretion, preferentially using the eversion technique with peri-procedural shunt whenever feasible (9). The treated patients underwent intervention under general anesthesia, and monitored by transcranial Doppler ultrasonography (TDU) and electroencephalographic (EEG) registration. However, operators used a shunt on the basis of EEG and TCD criteria (9). Before cross-clamping, an intravenous bolus of heparin (5000 IU) was administered and all endarterectomies were open with careful dissection of the bifurcation into the internal and external carotid arteries (9). The operators then used venous patches in case of indicated patch closure (9). To date, they used a Dacron patch only in case of insufficient venous material. After intervention, the plaques were categorized into 3 groups based on their overall appearance: fibrous, fibro-atheromatous, or atheromatous (10). In addition, in peri-procedural medical management all patients received optimal medical treatment and anti-platelet therapy as recommended by international guidelines (10)

Clinical visits, data collection and analysis

Physicians evaluated at baseline and at follow-up the clinical characteristics of the study population as NG vs. PDNMU vs. PDMU. The data were collected and analyzed during clinical visits 10 days after clinical discharge, and at 2 years of follow-up by the treating physician, by telephonic interviews, hospital admissions, and discharge schedules (10). Therefore, at follow up visits, the physicians blinded to study protocol evaluated the clinical status of each patient, and performed a physical examination with collection of vital signs, and of adverse events (10). Thus, physicians evaluated the adherence to drug therapy, and any clinical symptom referred by any patient (10). Therefore, the authors evaluated specifically the MACE at follow-up end, collecting the data prospectively from electronic medical records, used in clinical setting at participants' Institutions. However, authors used the electronic systems for data capture, collection and monitoring, with on-site and real timing

data entry. To date, authors collected the patients' files in each participating Institution, that were then analyzed.

Major Adverse Cardiac Events definition

The MACE were evaluated at 2 years of follow-p in the three cohorts of study as the PDNMU, the PDMU and the NG. The MACE were defined as a composite end point indicating cardiovascular disease events, hospital admissions for heart failure and ischemic cardiovascular events. The cardiovascular disease events were diagnosed by evidence of ischemic heart disease, peripheral arterial disease, stroke/transitory ischemic attack, or revascularization procedure (10, 12). Finally, the study investigators reported all the events with the potential to be adjudicated as one of the predefined study end points, regardless of the opinion of the investigator (12). In the case of identification of a suspected unreported event by a reviewer, authors asked to the reviewer to make a note back to the investigator (12). Thereafter, MACE were collected during patients' interview, visits, and by hospital discharge schedules (12).

Study endpoints

The research was conducted in overall population and selectively in the three different cohorts, as NG, PDNMU and PDMU. However, in the NG, PDNMU, and PDMU patients the authors evaluated the different expression of circulating inflammatory molecules (CRP, IL-6, and TNFα), and of circulating miRs (miR 24, miR 27, miR 100, miR 126 and miR 133) implied in inflammatory alterations of atherosclerotic carotid plaque, and in the plaque instability (7, 8). The inflammatory molecules and miRs were evaluated at baseline before the intervention and at 2 years of follow-up after endarterectomy. Finally, in the three different cohorts we evaluated the rate of MACE at 2 years of follow-up.

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Statistical analysis

A qualified physician with expertise in statistics performed all the statistical analyses by SPSS version 23.0 (IBM statistics, Chicago, USA). Therefore, the categorical variables were presented as frequencies (percentages), and continuous variables as mean ± SD. Then, the Chi-squared analysis or Fisher's-exact test were used to compare categorical data between groups. The independent samples Student t-test or ANOVA test was used to compare normally-distributed continuous data between groups and the Mann-Whitney U test was used to compare the distribution of skewed continuous data between groups. Correlation performed using Pearson's correlation analysis and Spearman's correlation analysis in the case of skewed variables. The MACE rates were derived as Kaplan-Meier estimates, and compared by log-rank test. Multivariate Cox regression analysis was performed for estimating the relationships among study variables (age, bmi, hypertension etc.) and MACE at 2 years of follow-up. Overall survival and event-free survival were assessed by Kaplan-Meier survival curves and compared by the log-rank test. The resulting hazard ratios (HRs) and 95% CIs were reported. Two-tailed P values <0.05 were considered statistically significant.

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RESULTS

The overall population was comprised of 234 patients with ACAS, divided in NG (n 125), PDNMU (n 73) and PDMU (n 36). At baseline, NG vs. PDNMU, and vs. PDMU presented a significant lower rate of hypertension, hypercholesterolemia, ischemic heart disease, BMI, systolic blood pressure and diastolic blood pressure ($p<0.05$). **Table 1**. Notably, PDNMU vs. NG, and PDNMU vs. PDMU had higher values of HbA1C, glucose and of the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), as for total cholesterol, HDL/LDL cholesterol, and triglycerides ($p<0.05$). **Table 1**. Regards atherosclerotic plaque morphology, we found a higher rate of Atheromatous plaque in PDNMU vs. NG (n 36 (49.3) vs. n 45 (36); $p<0.05$), and in PDMU vs. NG patients (n 16 (44.4) vs. n 45 (36); $p<0.05$). **Table 1**.

Finally, a significant higher percentage of PDNMU vs. NG and of PDMU vs. NG were under anti-hypertensive medications as ACEi, ARBs, diuretics, and calcium blockers, and under statins' therapy ($p<0.05$). **Table 1**.

Regards **circulating miRs' at baseline**, we found a higher expression of miR24 comparing PDNMU vs NG (1120,04±206.64 vs. 182.15±57.86 A.U.; $p<0.05$), PDNMU vs. PDMU (1120,04±206.64 vs. 458,97±167.74 A.U.; $p<0.05$), and PDMU vs. NG (458,97±167.74 vs. 182.15±57.86 A.U.; $p<0.05$). **Table 2, figure 1**. This trend was confirmed for miR 27, comparing PDNMU vs NG (2.06±0.21 vs. 0.91±0.04 A.U.; $p<0.05$), PDNMU vs. PDMU (2.06±0.21 vs. 1.54±0.30 A.U.; $p<0.05$), and PDMU vs. NG (1.54±0.30 vs. 0.91±0.04 A.U.; $p<0.05$). **Table 2, figure 1**. At baseline, PDNMU vs. NG (1.76±0.22 vs. 0.77±0.04 A.U.; $p<0.05$), and PDNMU vs. PDMU (1.76±0.22 vs. 0.80±0.12 A.U.; $p<0.05$) over-expressed miR 100. **Table 2, figure 1**.

Regards circulating miRs' at 2 years of follow-up after endarterectomy, we found an over-expression of miR 24, miR 27, miR 100, miR 126 and miR 133 comparing PDNMU vs. NG, PDMU vs. NG, and PDNMU vs. PDMU ($p<0.05$). **Table 2**, **figure 2**. Notably, we found a significant down

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regulation of miR 24 (NG: 11.21±6.36 vs. 182.15±57.86 A.U.; PDNMU: 98.25±21.08 vs. 1120,04±206.64 A.U.; PDMU: 51.24±17.88 vs. 458,97±167.74 A.U.; p<0.05), miR 27 (NG: 0.13±0.02 vs. 0.91±0.04 A.U.; PDNMU: 0.95±0.10 vs. 2.06±0.21 A.U.; PDMU: 0.58±0.09 vs. 1.54±0.30 A.U.; p<0.05), miR 100 (NG: 0.028±0.0004 vs. 0.77±0.04 A.U.; PDNMU: 0.089±0.006 vs. 1.76±0.22 A.U.; PDMU: 0.059±0.001 vs. 0.80±0.12 A.U.; p<0.05), miR 126 (NG: 2.79±1.98 vs. 45.64±23.49 A.U.; PDNMU: 29.55±5.89 vs. 108.65±42.71 A.U.; PDMU: 10.43±5.84 vs. 134.22±70.58 A.U.; p<0.05), and miR 133 (NG: 0.30±0.06 vs. 10.82±1.44 A.U.; PDNMU: 1.74±0.28 vs. 14.96±2.22 A.U.; PDMU: 0.94±0.28 vs. 9.14±2.16 A.U.; p<0.05), comparing their values at years of follow-up vs. baseline expression for each cohort of study.

Regards **inflammatory markers**, PDNMU vs. NG, and PDMU vs. NG over-expressed at baseline CRP, IL6 and TNFα, (p<0.05). **Table 2**. **At 2 years of follow-up** PDNMU vs. NG, PDMU vs. NG and PDNMU vs. PDMU over-expressed CRP, IL6 and TNFα, (p<0.05). **Table 2**.

Finally, at follow-up end we observed a significant difference about MACE (n 41 (17.5%)), comparing PDNMU vs. NG (n 27 (36.9%) vs. n 8 (6.4%); p<0.05), PDNMU vs. PDMU (n 27 (36.9%) vs. n 6 (16.6%); p <0.05), and PDMU vs. NG (n 6 (16.6%) vs. n 8 (6.4%); p<0.05). **Table 2**.

From the Cox regression analysis, admission glucose values (HR 1.020, CI 95% [1.001-1.038], p 0.029), atheromatous carotid plaque (HR 5.373, CI 95% [1.251-11.079], p 0.024), and miR 24 (HR 3.842, CI 95% [1.768-19.222], p 0.011) predicted MACE at 2 years of follow-up. **Table 3**.

The Kaplan curve showed the significant difference regards freedom from MACE at 2 years of follow-up (24 months) comparing PDNMU vs. NG, PDMU vs. NG, and PDNMU vs PDMU (p<0.05). **Figure 3**.

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DISCUSSION

In our study, at baseline the pre-diabetics vs. the NG over-expressed the inflammatory molecules and the circulating miR 24 and miR 27, while the PDNMU vs. PDMU over expressed circulating miR 100. Notably, at follow-up end the PDNMU vs. NG, the PDMU vs. NG and the PDNMU vs. PDMU over expressed the inflammatory markers and the circulating miR 24, miR 27, miR 100, miR 126 and miR 133. Conversely, all the study cohorts evidenced a significant miRs down regulation at 2 years of follow-up after endarterectomy, that was higher in NG vs. both cohorts of pre-diabetics, and in PDMU vs. PDNMU. The same down-regulative trend was observed for inflammatory markers. Notably, both cohorts of pre-diabetics vs. NG, and PDNMU vs. PDMU experienced higher rate of MACE at 2 years of follow-up after endarterectomy. Intriguingly, MACE were predicted by highest values of miR 24, by highest glucose blood values at hospital admission and by diagnosis of atheromatous carotid plaque.

Moreover, taken together our study results could indicate an ameliorative effect induced by endarterectomy in the three different study cohorts, and more evidenced in the cohort of pre-diabetics under metformin therapy as compared to non-metformin users. From previous study, the over-inflammation could promote the atherosclerotic processes in ACAS' patients with pre-diabetes (6). Consequently, these pathogenic processes could lead to plaque instability and rupture (6). Then, this could result in higher rate of MACE in pre-diabetics vs. NG.

In this context, the metformin therapy by the amelioration of glucose homeostasis and the insulin resistance, could lead to the significant reduction of inflammatory burden in pre-diabetics (13). Parallely to this effect, we observed, in addition to endarterectomy, a significant reduction of miRs in ACAS' patients with pre-diabetes. Thus, we might speculate that the pleiotropic effects of metformin, via the reduction of inflammatory burden and circulating miRs implied in plaque instability/rupture, might consequently reduce the MACE in ACAS' patients with pre-diabetes.

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Conversely, despite the significant miRs' down regulation seen in any cohort of study after endarterectomy, this effect was inferior comparing pre-diabetics vs. NG, and specifically PDNMU vs. PDMU patients. Moreover, we might say that the metformin therapy enhanced the effects of endarterectomy in pre-diabetics with ACAS, leading to a more significant inflammatory/miRs down regulation and reduction of MACE.

Intriguingly, in our study the miR 24 was a predictor of MACE at 2 years of follow-up after endarterectomy, with an increased 3.8 folds higher risk to have MACE in the ACAS' population. In this context, the miR 24 is implied in complex atherosclerotic processes and angiogenesis (14). Indeed, miR 24 is a regulator of angiogenesis-related functions, via up-regulation of matrix metalloproteinase 7 (MMP7), vascular endothelial growth factor receptor 1 (VEGFR1), and hepatocyte growth factor (HGF) and down-regulation of vascular endothelial growth factor-A (VEGF-A), (14). In detail, miR 24 is involved in the production of angiostatin and endostatin, which are linked to recurrent ischemic cerebral events (14). However, we might monitor the circulating miR24 as a promising diagnostic and prognostic marker for ACAS' patients with pre-diabetes. On other hand, also the miR 27, miR 100, miR 126 and miR 133 are part of a cluster of miRs linked to carotid atherosclerotic plaque instability and the worse prognosis in ACAS patients (7, 8). Indeed, these miRs are regulators of the inflammatory cascade, and promoters of changing of vascular smooth muscle cells (VSMCs) from the contractile phenotype to the active synthetic phenotype (7, 8). Notably, the VSMCs proliferation and migration from the media to the intima of carotid vessels, could lead to the excessive production of extracellular matrix that could secondary lead to the transformation of atherosclerotic carotid plaque then favoring its rupture (7). Therefore, all together these miRs are epigenetic regulators of endothelial progenitors' cells migration and proliferation in the atherosclerotic cap (14). Therefore, these miRs might be evaluated as monitors and predictors of adverse clinical events caused by plaque instability and rupture in ACAS patients

with pre-diabetes (7, 8). In this context, highest glucose blood values at hospital admission predicted the risk to have MACE after endarterectomy at 2 years of follow-up. Indeed, the atherosclerosis could be accelerated in patients with hyperglycemia (4, 5). Moreover, the hyperglycemia could induce pro-atherogenic effects, interfering with arterial remodeling, angiogenesis and apoptotsis by a complex multi-factorial interplay and cross talking with inflammatory, angiogenetic and apoptotic pathways (4). Therefore, hyperglycemia might contribute to the plaque instability and rupture, with consequent worse prognosis in ACAS' patients. In addition, in our study the pre-diabetics vs. NG had higher rate of atheromatous plaques. Notably, the diagnosis of atheromatous plaques increase of 5.4 folds the risk to have MACE at 2 years of follow-up after endarterectomy. This concept has been previously investigated (4). Indeed, the atheromatous plaques have higher risk of rupture and subsequent thromboembolic/ischemic events and strokes in overall population, and specifically in pre-diabetics (4, 9). To date, taken together our data evidenced an increased risk for atherosclerotic carotid plaque progression and rupture in patients with pre-diabetes, and especially in those with worse glycemic control and insulin resistance as those without metformin therapy. Notably, form recent meta-analysis the patients with pre-diabetes have higher rate of atherosclerotic disease and plaques progression of carotid arteries (16). To date, this could contribute to a more severe progression of the cardiovascular disease with increased risk of recurrent stroke and worse prognosis (16). In our opinion, this could involve the expression of miRs implied in atherogenesis, via over-inflammation and alteration of angiogenetic pathways. Then, metformin therapy by the reduction of glycaemia and insulin resistance could modulate the expression of these miRs and of miR24, that could be seen as a predictor of worse prognosis in ACAS' patients. Therefore, we might suggest the metformin therapy to reduce the risk of cardiovascular disease progression and worse prognosis in ACAS patients with pre-diabetes.

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This study could present few limitations. As first, we did not perform magnetic resonance imaging exams to study the atherosclerotic plaque stable vs. unstable phenotype. In addition, we did not have hemodynamic and biomechanical stress data, and we cannot assess their association with plaque data. Again, the loss of ex vivo model cannot furnish us conclusive data regards the metformin induced effects on inflammation, angiogenesis and apoptosis and miRs' expression in atherosclerotic stable/unstable cap of humans. Again, we did not practice the analysis of inflammatory burden and miRs expression at level of plaques' specimens, but this was outside of the scope of the present analysis. Thus, we believe that future prospectively randomized, large-scale clinical trials are required to further clarify the existing relationship between pre-diabetes, inflammatory burden and circulating miRs in ACAS patients. In addition, further investigations are required to determine whether metformin therapy could significantly reduce the inflammatory burden, miRs' expression and MACE in ACAS' patients.

CONCLUSIONS

In ACAS' patients the pre-diabetes might cause an over expression of inflammatory molecules and miRs, implied in carotid plaque instability, rupture and MACE at 2 years of follow-up after endarterectomy. In this context, the circulating miR 24 could monitor these atherosclerotic processes, and predict clinical outcomes for ACAS' patients. Notably, the metformin therapy by reduction of inflammatory burden and significant down regulation of miR 24, miR 27, miR 126 and miR 133, could then ameliorate clinical outcomes in pre-diabetics with ACAS.

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Figures and tables legend.

Figure 1. Pre-intervention values of circulating microRNAs.
In this figure the representation of circulating microRNAs (miRs) for normoglycemics (NG; green color), pre-diabetics non metformin users (PDNMU; red color) and pre-diabetics metformin users (PDMU; yellow color) at baseline before intervention. Values of miR 24, miR 27, miR100, miR 126 and miR 133 are in arbitrary units (A.U.). miR27 and miR100 values are expressed X 100; miR133 values x 10.
* Is for statistical significant (p <0.05) NG vs. PDNMU; ** is for statistical significant (p<0.05) NG vs. PDMU; *** is for statistical significant (p<0.05) PDNMU vs. PDMU.

Figure 2. Post-intervention values of circulating microRNAs.
In this figure the representation of circulating microRNAs (miRs) for normoglycemics (NG; green color), pre-diabetics non metformin users (PDNMU; red color) and pre-diabetics metformin users (PDMU; yellow color) at 2 years of follow-up. Values of miR 24, miR 27, miR100, miR 126 and miR 133 are in arbitrary units (A.U.). miR 27 and miR133 values are x10; miR values are x 100.
* Is for statistical significant (p <0.05) NG vs. PDNMU; ** is for statistical significant (p<0.05) NG vs. PDMU; *** is for statistical significant (p<0.05) PDNMU vs. PDMU.

Figure 3. The Kaplan curves for freedom from Major Adverse Cardiac Events (MACE) at 2 years of follow-up in study cohorts.
Green color: normoglycemics; yellow: PDMU; red color: PDNMU.
NG: normoglycemics; PDMU: pre-diabetics with metformin; PDNMU: pre-diabetics non metformin users. * Is for statistical significant (p <0.05)

Table 1. Characteristics of study population at baseline.
Data are presented as mean ± SD, or number (%). *P<0.05 compared with never current user group. * Is for statistical significant (p <0.05) for NG vs. PDNMU; ** is for statistical significant (p<0.05) for NG vs. PDMU; *** is for statistical significant (p<0.05) for PDNMU vs. PDMU.
ACE = angiotensin-converting enzyme; ARBs: angiotensin receptor blockers; BMI = body mass index; Hb1Ac: glycated haemoglobin; HDL = high-density lipoprotein; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; IHD = ischemic heart disease; LDL = low-density lipoprotein.

Table2. microRNAs (miRs) expression at baseline and at 2 years of follow-up in Normolgycemics vs. pre-diabetics non metformin-users vs. pre-diabetics metformin-users, and Major Adverse Cardiac Events at 2 years of follow-up in Normolgycemics vs. pre-diabetics non metformin-users vs. pre-diabetics metformin-users.
A.U.= arbitrary unit; CRP: C reactive protein; IL6: interleukin 6; MACE: major adverse cardiac events; miR= microRNA; NG= normoglycemics; PDNMU: pre-diabetics never metformin users; PDMU= pre-diabetics metformin users; TNFα: tumor necrosis factor alpha. Data are presented as mean ± SD, or number (%). * Is for statistical significant (p <0.05) for NG vs. PDNMU; ** is for statistical significant (p<0.05) for NG vs. PDMU; *** is for statistical significant (p<0.05) for PDNMU vs. PDMU.
Table 3. Multivariate Cox Regression analysis to predict Major Adverse Cardiac Events (MACE) at 2 years of follow-up.

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ACEi: angiotensin-converting enzyme inhibitors; BMI: body mass index; CI: confidence of interval; HR: Hazard ratio; IL6: interleukin 6; miR 24: microRNA 24. * Is for statistical significant ($p < 0.05$).

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Table 1. Characteristics of study population at baseline

Patients characteristics	NG (n 125)	PDNMU (n 73)	PDMU (n 36)	p
Age, years	66.7±6.5	68.8±7.2	69.7±9.2	0.104; 0.130; 0.891
Males ,n (%)	87 (69.6)	52 (71.2)	25 (69.4)	0.752; 0.562; 0.412
Patients characteristics				
Hypertension, n (%)	86 (64.8)	61 (83.6)	31 (86.1)	0.05*; 0.011**; 0.256
Hypercholesterolemia, n (%)	84 (67.2)	58 (79.5)	29 (80.5)	0.001*; 0.004**; 0.150
Cigarette smoking, n (%)	17 (13.6)	10 (13.6)	5 (13.9)	0.859; 0.870; 0.451
Ischemic heart disease, n (%)	45 (36)	35 (47.9)	18 (50)	0.05*; 0.04**; 0.120
BMI (kg/m2)	26.8±1.9	28.4±1.8	28.0±1.7	0.001*; 0.001**; 0.145
Systolic blood pressure (mmHg)	124.7±10.1	129.4±12.8	129.5±9.1	0.018*; 0.007**;0.530
Diastolic blood pressure (mmHg)	72.1±6.7	77.7±7.1	76.1±7.9	0.035*; 0.05**;0.189
Heart rate	84±8	83±6	84±6	0.175; 0.321; 0.128
HbA1C, (%)	5.3±0.4	6.2±0.8	5.8±0.4	0.010*; 0.022**; 0.050***
Blood glucose (mmol/l)	86.8±15.3	127.8±18.1	105.6±15.5	0.001*; 0.05**; 0.032***
HOMA-IR	4.1±0.25	5.1±0.69	5.0±0.72	0.045*; 0.05**
Total cholesterol, mg/dL	178±32	206±44	187±25	0.001*; 0.018**; 0.001***
HDL cholesterol, mg/dL	46.8±10.6	40.2±15.8	42.9±12.6	0.005*; 0.012**; 0.05***
LDL cholesterol, mg/dL	103.9±35.1	128.1±48.4	114.3±27.5	0.035*; 0.048**; 0.050***
Triglycerides, mg/dL	131.3±23.2	167.9±44.9	134.5±40.7	0.004*; 0.133; 0.05***
Creatinine, mg/dL	1.0±0.1	1.1±0.2	1.0±0.1	0.082; 0.141; 0.520
Plaque characteristics				
Stenosis severity, %				
Plaque morphology:				
-Fibrous, n (%)	37 (29.6)	16 (21.9)	9 (25)	0.168; 0.171; 0.180
-Fibro atheromatous, n (%)	43 (34.4)	21 (28.8)	11 (30.6)	0.208; 0.121; 0.560
-Atheromatous, n (%)	45 (36)	36 (49.3)	16 (44.4)	0.05*; 0.05**; 0.120
Active therapy (%)				
Aspirin, n (%)	107 (85.6)	65 (89)	33 (91.6)	0.120; 0.132; 0.891
Warfarin, n (%)	3 (2.4)	2 (2.7)	1 (2.8)	0.252; 0.230; 0.158
β-Blockers, n (%)	66 (52.8)	39 (53.4)	19 (52.7)	0.270; 0.260; 0.713
Calcium-channel blockers, n (%)	3 (2.4)	4 (5.5)	2 (5.5)	0.05*; 0.03**;0.301
Statins, n (%)	112 (89.6)	67 (91.8)	32 (91.6)	0.001*; 0.002**; 0.253
ACE inhibitors, n (%)	41 (32.8)	36 (49.3)	17 (47.2)	0.001*; 0.001**; 0.320
Diuretic agents, n (%)	6 (4.8)	8 (10.9)	4 (11.1)	0.05*; 0.03**;0.301
ARBs, n (%)	21 (16.8)	21 (28.8)	10 (27.7)	0.001*; 0.001**; 0.289

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Table 2. microRNAs (miRs) expression at baseline and at 2 years of follow-up in Normolgycemics (NG) vs. pre-diabetics non metformin-users (PDNMU) vs. pre-diabetics metformin-users (PDMU). Major Adverse Cardiac Events (MACE) at 2 years of follow-up in Normolgycemics (NG) vs. pre-diabetics non metformin-users (PDNMU) vs. pre-diabetics metformin-users (PDMU).

	NG (n 125)	PDNMU (n 73)	PDMU (n 36)	P value
Baseline miRs' expression				
miR 24, A.U.	182.15±57.86	1120,04±206.64	458,97±167.74	<0.05*, **, ***
miR 27, A.U.	0.91±0.04	2.06±0.21	1.54±0.30	<0.05*,**, ***
miR 100, A.U.	0.77±0.04	1.76±0.22	0.80±0.12	<0.05*, ***
miR 126, A.U.	45.64±23.49	108.65±42.71	134.22±70.58	>0.05
miR 133, A.U.	10.82±1.44	14.96±2.22	9.14±2.16	>0.05
miRs' expression at 2 years of follow up				
miR 24, A.U.	11.21±6.36	98.25±21.08	51.24±17.88	<0.05*,**,***
miR 27, A.U.	0.13±0.02	0.95±0.10	0.58±0.09	<0.05*,**,***
miR 100, A.U.	0.028±0.0004	0.089±0.006	0.059±0.001	<0.05*,**,***
miR 126, A.U.	2.79±1.98	29.55±5.89	10.43±5.84	<0.05*,**,***
miR 133, A.U.	0.30±0.06	1.74±0.28	0.94±0.28	<0.05*, **,***
Inflammatory markers at baseline				
CRP (mmol/L)	0.82±0.31	1.13± 0.49	1.10±0.46	<0.05*,**
IL6 (pg/ml)	3.49±0.38	4.41±0.52	4.36±0.43	<0.05*,**
TNFα (pg/ml)	5.56±0.92	6.96±0.56	6.92±0.54	<0.05*,**
Inflammatory markers at 2 years of follow up				
CRP (mmol/L)	0.41±0.10	0.92±0.22	0.73± 0.28	<0.05*, **, ***
IL6 (pg/ml)	3.10±0.31	4.19±0.22	3.52±0.31	<0.05*, **,***
TNFα (pg/ml)	4.72±0.71	6.52±0.49	5.54±0.38	<0.05*, **, ***
Number of MACE, n (%)	8 (6.4)	27 (36.9%)	6 (16.6)	<0.05*,**,***

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Table 3. Multivariate Cox Regression analysis to predict Major Adverse Cardiac Events (MACE) at 2 years of follow-up.

	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS		
Risk factors	**HR**	**CI 95%**	**p value**	**HR**	**CI 95%**	**p value**
Age	0.939	0.876-1.006	0.075	0.970	0.930-1.013	0.169
BMI	0.970	0.926-1.261	0.327	1.070	0.809-1.414	0.637
Hypertension	1.188	0.643-2.196	0.582	0.620	0.176-2.187	0.457
Dyslipidemia	1.707	0.921-3.163	0.089	1.425	0.560-3.629	0.457
Metformin therapy	0.185	0.085-0.401	0.001	0.386	0.053-2.823	0.348
Glucose blood values	1.014	1.009-1.020	0.001	1.020	1.001-1.038	0.029*
Cholesterol blood values	1.012	1.004-1.019	0.002	1.013	0.999-1.027	0.074
ACEi	1.325	0.650-2.702	0.439	0.583	0.159-2.137	0.416
Statins	0.883	0.450-1.730	0.716	0.501	0.166-1.503	0.217
Pre-diabetes	3.003	1.531-5.869	0.001	1.021	0.688-1.201	0.195
Atheromatous carotid plaque	6.388	2.949-13.838	0.001	5.373	1.251-11.079	0.024*
IL 6	6.246	3.195-12.210	0.001	1.283	0.020-4.003	0.350
miR 24	5.001	1.781-6.122	0.001	3.842	1.768-19.222	0.011*