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Empagliflozin improves the microRNA signature of endothelial dysfunction in patients with HFpEF and diabetes

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Running title: SGLT2i and miRNA profile of endothelial dysfunction

ABBREVIATIONS

Empa: empagliflozin

HFpEF: heart failure with preserved ejection fraction

Ins: insulin

Met: metformin

miRs: miRNAs (microRNAs)

mRNA: messenger RNA

SGLT2: sodium glucose cotransporter 2

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ABSTRACT

Endothelial dysfunction represents a key mechanism underlying heart failure with preserved ejection fraction (HFpEF), diabetes mellitus (DM), and frailty. However, reliable biomarkers to monitor endothelial dysfunction in these patients are lacking. In this study, we evaluated the expression of a panel of circulating microRNAs (miRNAs, miRs) involved in the regulation of endothelial function in frail older adults with HFpEF and DM that were treated for 3 months with empagliflozin, metformin, or insulin. We identified a distinctive pattern of miRs that were significantly regulated in HFpEF patients compared to healthy controls and in HFpEF patients after treatment with the SGLT2 inhibitor empagliflozin. Three miRs were significantly downregulated (miR-126, miR-342-3p, and miR-638) and two were significantly upregulated (miR-21 and miR-92) in HFpEF patients compared to healthy controls. Strikingly, two of these miRs (miR-21 and miR-92) were significantly reduced in HFpEF patients after the 3-month treatment with empagliflozin whereas no significant differences in the profile of endothelial miRs were detected in patients treated with metformin or insulin. Taken together, our findings demonstrate for the first time that specific circulating miRs implied in the regulation of endothelial function are significantly regulated in frail HFpEF patients with DM and in response to empagliflozin treatment.

Key words: Biomarker, diabetes, empagliflozin, endothelium, frailty, HFpEF, microRNA, Non-coding RNA, SGLT2i

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SIGNIFICANCE STATEMENT

We have identified a novel microRNA signature functionally involved in the regulation of endothelial function that is significantly regulated in frail patients with HFpEF and diabetes. Moreover, the treatment with the SGLT2 inhibitor empagliflozin caused a modification of some of these microRNAs in a direction that was opposite to what observed in HFpEF patients, indicating a rescue of endothelial function. Our findings are relevant for clinical practice inasmuch as novel biomarkers of disease and response to therapy have been established.

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INTRODUCTION

Endothelial dysfunction is a pathophysiologically relevant mechanism underlying heart failure with preserved ejection fraction (HFpEF) and diabetes mellitus (DM) (Hadi and Suwaidi, 2007; Giamouzis et al., 2016; Gevaert et al., 2019; Knapp et al., 2019; Premer et al., 2019) (Jankauskas et al., 2021; Mone et al., 2021a). HFpEF and DM are very common in older adults, increasing the risk of frailty, a systemic condition that leads to functional decline and adverse outcomes (Owan et al., 2006; Steinberg et al., 2012; Paulus and Tschope, 2013; Chioncel et al., 2017; McHugh et al., 2019; Jankauskas et al., 2021; Lejeune et al., 2021). The pathophysiology of frailty includes chronic inflammation which is typical of aging (inflammaging), oxidative stress, insulin resistance, loss of anabolic hormones, and reduced tolerance to physical exercise with a reduction in muscle strength (Bandeen-Roche et al., 2015; Cruz-Jentoft and Sayer, 2019; Rusanova et al., 2019). Of note, we and others have shown that endothelial dysfunction plays a fundamental role also in the pathophysiology of frailty (Alonso-Bouzon et al., 2014; Mansur et al., 2015; Amarasekera et al., 2021; Mone et al., 2021a; Mone et al., 2022a).

Empagliflozin is a relatively novel selective inhibitor of sodium glucose cotransporter 2 (SGLT2) that has been shown to reduce mortality and re-hospitalization for HF (Zinman et al., 2015; Anker et al., 2021; Varzideh et al., 2021; Braunwald, 2022). Additional benefits of SGLT2 inhibitors include improved cardiovascular energetics, reduced vascular tone, decreased renal dysfunction, increased circulating levels of ketone bodies, and overall reduced systemic inflammation (Benetti et al., 2016; Prattichizzo et al., 2018; Wan et al., 2018; Oshima et al., 2019; Verma et al., 2019; Zhang et al., 2020; Jensen et al., 2021; Li et al., 2021; Sardu et al., 2021; Varzideh et al., 2021; Huang et al., 2022; Paolisso et al., 2022; Zhang et al., 2022). We have recently demonstrated that empagliflozin significantly improves cognitive impairment in frail older diabetics with HFpEF (Mone et al., 2022c), showing also a correlation between physical and cognitive impairment (Mone et al., 2022a).

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MicroRNAs (miRs) are small non-coding RNAs molecules of 18-24 nucleotides, which typically repress messenger RNAs (mRNAs) by binding their 3′ untranslated region (Santulli, 2015; Fridrichova and Zmetakova, 2019; Stavast and Erkeland, 2019; Hu et al., 2021; Mirzaei et al., 2021; Mone et al., 2021b; Bielska et al., 2022; Karagiannopoulos et al., 2022; Mauro et al., 2022; Moisoiu et al., 2022; Qiu et al., 2022; Traber and Yu, 2022; Yaylim et al., 2022; Zeng et al., 2022). Substantial evidence has shown that miRs exert their activity in many biological processes and several miRs have been proposed as biomarkers and potential targets of novel therapeutic strategies (Creemers et al., 2012; Wronska et al., 2015; Barwari et al., 2016; Zarone et al., 2017; Chen et al., 2018; Wong et al., 2018; Morelli et al., 2019; Kawasaki et al., 2020; Wang et al., 2020; Fonseca et al., 2021; Gambardella et al., 2021; Bonnet et al., 2022; Gambardella et al., 2022a; Gambardella et al., 2022b; Kansakar et al., 2022; Varzideh et al., 2022). Several investigators have linked miRs to frailty for their involvement in inflammation, endothelial dysfunction, and senescence (Quinn and O'Neill, 2011; Olivieri et al., 2012; Geiger and Dalgaard, 2017; Rusanova et al., 2019; Bu et al., 2021).

In this study, we aimed at assessing the effect of empagliflozin on the circulating profile of miRs involved in the regulation of endothelial function in frail older adults with DM and HFpEF treated with different antidiabetic regimens.

MATERIALS and METHODS

Study design

We evaluated consecutive frail older adults with a previous confirmed diagnosis of DM and HFpEF, from October 2021 to December 2021. All subjects were recruited from the Sant'Angelo dei Lombardi Hospital, ASL (local health unit of the Italian Ministry of Health) Avellino, Italy. Inclusion criteria were: age >65 years; a previous diagnosis of T2DM, frailty, and HFpEF; Patients were excluded if they had experienced a previous stroke, acute myocardial infarction, or cardiac

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revascularization. As a control population, we enrolled age-matched subjects with no evidence of HFpEF or DM.

The patients fulfilling the above-mentioned eligibility criteria were divided into three interventional groups (empagliflozin: 10 mg; metformin: 500 mg; insulin: basal-bolus regimen) and followed-up for three months.

All patients underwent clinical evaluation. Blood samples were taken at baseline and follow-up. All patients received a transthoracic echocardiography assessment according to the American Society of Echocardiography recommendations (Lang et al., 2015). Every patient (or a legally authorized representative) signed a written informed consent. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Frailty Assessment

A physical frailty assessment was performed following the Fried Criteria, as we previously described (Mone et al., 2022b; Mone et al., 2022d). A diagnosis of frailty status was made with at least three out of the following five points: 1) Weight loss (unintentional loss of ≥4.5 kg in the past year), 2) weakness (handgrip strength in the lowest 20% quintile at baseline, adjusted for sex and body mass index, 3) exhaustion (poor endurance and energy), 4) slowness (walking speed under the lowest quintile adjusted for sex and height), 5) Low physical activity level (lowest quintile of kilocalories of physical activity during the past week).

miR isolation, quantification, and normalization

We extracted miRs using the miRVana miRNA Isolation kit (ThermoFisher) according to the protocol provided by the manufacturer; reverse transcription was performed using the miRCURY LNA Universal RT microRNA PCR kit (Qiagen, Hilden, Germany); miR expression was analyzed by RT-qPCR. We analyzed a panel of miRs that had been previously reported to be

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implied in the regulation of endothelial dysfunction (Ni et al., 2011; Sabatel et al., 2011; Costa et al., 2013; Zhang et al., 2013; Santulli et al., 2014; Widmer et al., 2014; Kriegel et al., 2015; Ye et al., 2015; Chen et al., 2016; Santulli, 2016; Tang et al., 2017; Cheng et al., 2018; Wei et al., 2018; Gu et al., 2019; Hu and Dong, 2019; Xu et al., 2019; Du et al., 2020; Paterson et al., 2021). The RNA Spike-in kit (Qiagen) was used as an exogenous control of RNA extraction following the manufacturer´s instructions. To control yield, we used two synthetic RNA spike-ins (UniSp2 and UniSp5) in different concentrations; miR-320a and miR-423-5p were identified as the most stable miRs among all groups and were therefore used as endogenous normalizers. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

All data were analyzed using the GraphPad software (Prism, San Diego, CA, USA). Data are expressed as means ± SD or numbers and percentages. The differences in miR levels among groups were analyzed using two-tailed t-tests or one-way ANOVA followed by Bonferroni post hoc correction, as appropriate.

RESULTS

We enrolled 41 frail older adults with HFpEF and DM. 21 patients were excluded because did not meet the eligibility criteria, refused to give consent, withdraw from the study, or did not have data from blood analyses at baseline or at follow-up. Thus, 30 patients, divided into three treatment groups (empagliflozin, metformin, or insulin) successfully completed the 3-month follow-up. Baseline characteristics of our population are reported in **Table 1** whereas follow-up data are in **Table 2**.

Interestingly, the evaluation of the miR signature of endothelial dysfunction revealed a unique pattern of miRs that were significantly regulated in HFpEF patients compared to healthy

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controls and in HFpEF patients pre- and post- treatment with the SGLT2 inhibitor empagliflozin (**Figure 1**).

We were able to identify 3 circulating miRs that were significantly downregulated (miR-126, miR-342-3p, and miR-638) and 3 that were significantly upregulated (miR-21 and miR-92) in HFpEF patients compared to healthy controls ($p<0.001$) (**Figure 2A**). Intriguingly, circulating levels of two of these miRs (namely miR-21 and miR-92) were significantly ($p<0.001$) reduced in HFpEF patients after the 3-month treatment with empagliflozin (**Figure 2B**). Instead, no significant differences in the profile of endothelial miRs were detected in patients treated with metformin (**Figure 2C**) or insulin (**Figure 2D**).

DISCUSSION

To the best of our knowledge, this is the first study investigating the effects of SGLT2 inhibitors on circulating miRs, with a significant relevance both in terms of mechanisms of action and clinical practice. Empagliflozin has been shown to have beneficial effects on cardiovascular outcomes, particularly on the re-hospitalization rate for HF (Dave et al., 2020). Nevertheless, there are limited reports investigating the functional role of potential biomarkers to monitor the effects of SGLT2 inhibitors. In this sense, miRs have been widely used as biomarkers; however, limited data are available on the miR profile in frailty (Ipson et al., 2018; Carini et al., 2021). Besides, there are no studies investigating miRs in terms of endothelial dysfunction in HFpEF or frailty.

In our study, we identified 5 miRs as significantly regulated in HFpEF patients *vs* healthy control subjects, namely miR-21, miR-92 (upregulated), miR-126, miR-342-3p, and miR-638 (downregulated). Our findings are fully in agreement with previous reports. Indeed, miR-21 had been previously linked to inflammaging and age-related diseases: miR-21 had been proposed as biomarker of systolic heart failure (Ben-Zvi et al., 2020) and its plasma levels been linked to aging (Olivieri et al., 2012; Rusanova et al., 2019). Additionally, an increased expression of miR-21 in

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older adults has been shown to diminish the induction of transcription factor networks involved in memory cell generation (Kim et al., 2018).

Equally important, miR-92 is upregulated after vascular injury both *in vitro* and *in vivo* (Deng et al., 2019), has been previously advocated as a biomarker of HF (Napoli et al., 2020), and its inhibition has been shown to have favorable effects in preventing detrimental cardiac remodeling (Bellera et al., 2014). Strikingly, both these miRs were downregulated after empagliflozin treatment, strongly suggesting a rescue of the endothelial dysfunction in HFpEF patients after a 3-month treatment with this SGLT2 inhibitor.

Consistent with our data, Cheng and collaborators had demonstrated that miR-342-3p is an indispensable modulator of angiogenic activation in endothelial cells, and deregulation of its expression mediates the vascular dysfunction caused by hyperinsulinemia (Cheng et al., 2018). Further studies are needed to determine the exact clinical relevance of miR-638 downregulation in HFpEF, which could also be compensatory, since previous studies, performed in the setting of hepatocellular carcinoma, suggested that this miR is promoting angiogenesis (Cheng et al., 2016; Yokota et al., 2021).

We observed decreased circulating levels of the master regulator of endothelial function, miR-126 (Liu and Olson, 2010; Santulli et al., 2014; Pei et al., 2020), in HFpEF patients, corroborating the view that endothelial dysfunction is playing an instrumental role in HFpEF. Consistently, previous analyses had evidenced lower levels of miR-126 in diabetic patients (Zampetaki et al., 2010).

Another miR that was found to be significantly downregulated after empagliflozin treatment is miR-221, which had been linked to muscle proliferation and sarcopenia both in elderly patients and aged mice (Hamrick et al., 2010; He et al., 2020; Roldan Gallardo and Quintar, 2021); the same miR had been also associated with DM and obesity (Lustig et al., 2014). Notably, we did not evidence any significant result in terms of endothelial miR network in patients treated with metformin and insulin.

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In line with the present findings, most recently we demonstrated that empagliflozin improves endothelial function by reducing mitochondrial calcium overload and generation of reactive oxygen species (Mone et al., 2022e), and that SGLT2 inhibition has a beneficial impact on quality of life.

In conclusion, our findings demonstrate for the first time that a specific profile of circulating miRs implied in the regulation of endothelial function are significantly regulated in frail HFpEF patients with DM and in response to empagliflozin treatment.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Participated in research design: Mone, Lombardi, Frullone, and Santulli

Conducted experiments and *contributed new reagents or analytic tools:* Mone, Kansakar, Varzideh, Jankauskas, Pansini, De Gennaro, Famiglietti, Macina, Frullone, and Marzocco.

Performed data analysis: Mone, Santulli.

Wrote or contributed to the writing of the manuscript: Mone, Lombardi, Santulli.

All authors contributed to the article and approved the submitted version.

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Figure Legends

Figure 1.

Heat-map of the expression of circulating miRNAs in the indicated groups of patients.

HFpEF: heart failure with preserved ejection fraction; Healthy: healthy control subjects; Empa: patients receiving empagliflozin; Met: patients receiving metformin; Ins: patients receiving insulin.

Figure 2.

Volcano plots depicting the miRNA analyses in the different groups.

A: HFpEF vs healthy controls, **B**: Effects of empagliflozin treatment in HFpEF patients, **C**: Effects of metformin treatment in HFpEF patients, and **D**) Effects of insulin treatment in HFpEF patients. The horizontal dotted line represents a P value of 0.001; thus, the points in the plot above that line represent the differentially expressed miRNAs with statistical significance.

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	Control	Empagliflozin	Metformin	Insulin
N	10	10	10	10
Age (years)	79.8±8.9	81.6±6.8	80.8±6.9	81.8±6.5
Female Sex, n (%)	5 (50.0)	6 (60.0)	6 (60.0)	5 (50.0)
BMI (kg/m^2)	25.6±1.8	27.7±1.4*	27.6±1.7*	28.1±1.5*
SBP (mmHg)	118.8±7.8	119.4±7.2	119.8±7.4	120.1±7.3
DBP (mmHg)	76.3±8.8	79.0±7.0	79.3±6.8	79.2±6.9
Heart rate (bpm)	78.8±11.1	87.3±8.2	86.8±8.5	87.3±8.6
EF (%)	65.8±7.3	55.4±5.2*	55.8±5.4*	55.2±5.1*
Comorbidities				
Hypertension, n (%)	4 (40.0)	7 (70.0)	6 (60.0)	8 (80.0)
Dyslipidemia, n (%)	7 (70.0)	8 (80.0)	8 (80.0)	7 (70.0)
COPD, n (%)	4 (40.0)	4 (40.0)	5 (50.0)	6 (60.0)
CKD, n (%)	3 (30.0)	5 (50.0)	6 (60.0)	7 (70.0)
Laboratory parameters				
Plasma glucose (mg/dl)	103.5±30.6	161.8±39.1*	163.7±39.2*	164.1±39.0*
Cholesterol (mg/dl)	202.9±22.1	206.1±20.2	205.9±20.1	206.0±19.8
LDL-cholesterol (mg/dl)	133.1±16.1	132.3±19.7	132.4±19.5	132.5±19.8
HDL-cholesterol (mg/dl)	35.1±3.5	37.5±3.4	36.9±3.7	37.1±3.4
Creatinine (mg/dl)	0.9±0.3	1.2±0.3*	1.2±0.4*	1.3±0.3*
HbA1c (mmol/mol)	-	56±6.4	55±7.5	57±5.3
BNP (pg/mL)	-	443.8±24.7	445.1±24.5	446.2±25.0

Table 1.

Baseline characteristics of the patients.

Data are means ± SD or n (%). "Control" refers to subjects who did not have any evidence of HFpEF or DM. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; EF: ejection fraction; COPD: chronic obstructive pulmonary disease; CKD: chronic kidney disease; HbA1c: glycated hemoglobin; BNP: brain natriuretic peptide. *: $p<0.05$ vs control.

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	Control	Empagliflozin	Metformin	Insulin
N	10	10	10	10
BMI (kg/m^2)	25.4±1.7	27.1±1.1*	27.3±1.2*	28.0±1.3*
SBP (mmHg)	117.9±7.9	118.7±6.8	118.6±6.9	120.0±7.1
DBP (mmHg)	76.2±8.7	78.9±6.4	79.0±6.5	79.3±6.8
Heart rate (bpm)	77.6±10.3	87.0±7.8*	86.9±8.1*	87.2±8.2*
EF (%)	65.6±7.4	56.2±5.0*	55.9±5.2*	55.1±5.0*
Laboratory parameters				
Plasma glucose (mg/dl)	100.2±28.8	159.8±37.8*	162.9±38.6*	163.3±38.8*
Cholesterol (mg/dl)	201.5±22.4	205.6±20.0	205.5±20.3	205.9±19.9
LDL-cholesterol (mg/dl)	130.1±16.5	131.8±19.4	132.1±19.3	132.3±19.4
HDL-cholesterol (mg/dl)	36.1±3.6	37.2±3.2	36.8±3.6	37.0±3.3
Creatinine (mg/dl)	0.9±0.3	1.0±0.2	1.0±0.2	1.0±0.2
BNP (pg/mL)	-	439.7±23.8	444.5±24.1	444.8±24.6

Table 2.

Follow-up characteristics of the patients 3 months after starting the study.

Data are means ± SD or n (%). “Control” refers to subjects who did not have any evidence of HFpEF or DM. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; EF: ejection fraction; BNP: brain natriuretic peptide. *: $p<0.05$ vs control.

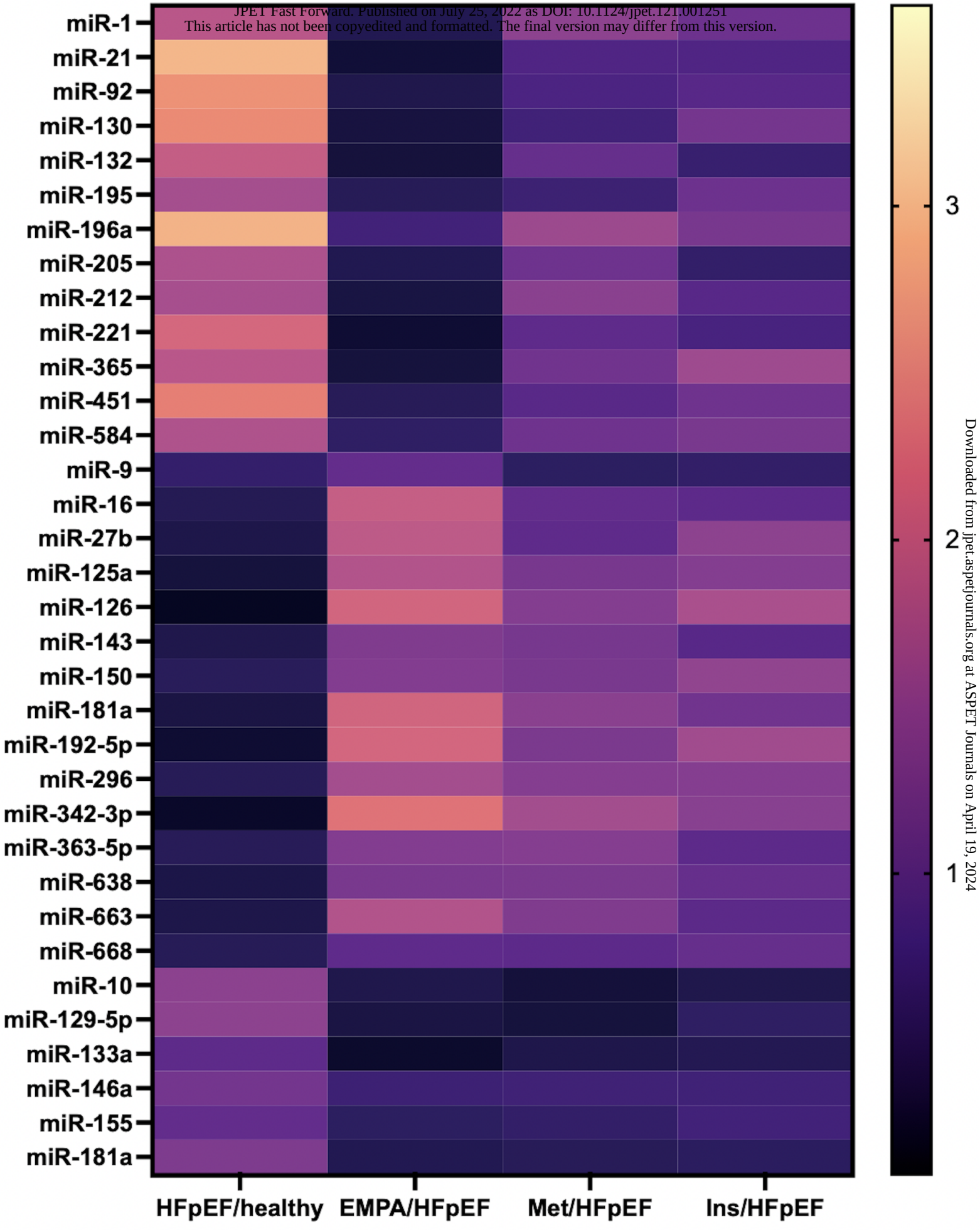

Figure 1.

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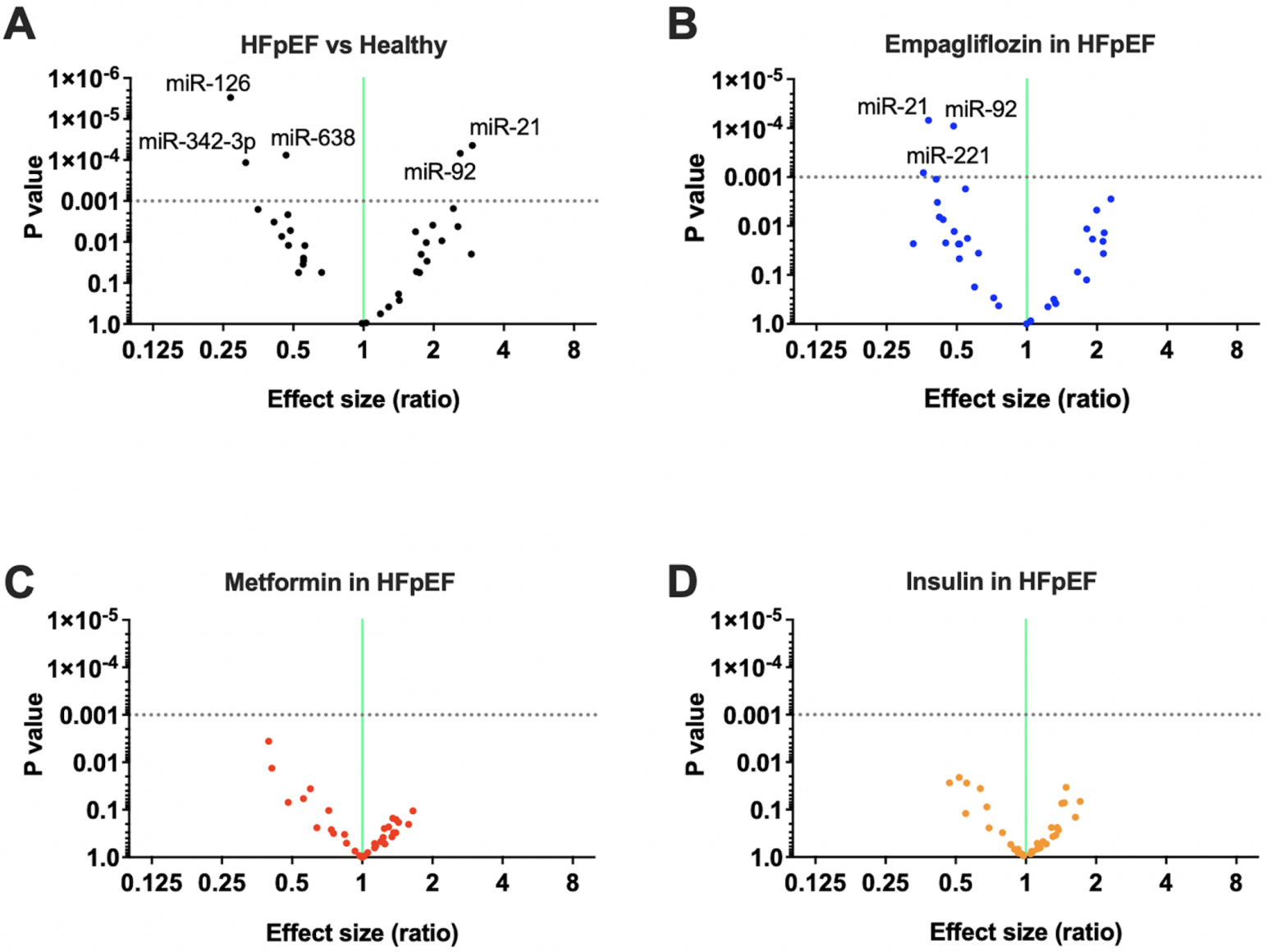

Figure 2.