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ROLE OF RELEASED AND EXOSOMIAL microRNAS, INFLAMMATORY AND DIABETES ASSOCIATED (REx-MIDAS), IN ENDOTHELIAL METABOLIC MEMORY

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1. Project summary

The harmful cardiovascular (CV) outcomes of type 2 diabetes mellitus (T2DM) still represent a major health and socioeconomic issue. An intensive, early control of glycaemia, lipids, and blood pressure attenuates but does not suppress the incidence of late CV complications (1,2). An early glycaemic control needs >10 years to observe a diverse incidence of CV endpoints, a phenomenon known as "metabolic memory" or "legacy effect" (3), while improving glycaemia and promoting weight loss through lifestyle measures had multiple metabolic benefits but did not translate into reduced incidence of CV diseases (4). In addition, different glucose-lowering drugs are accompanied by a diverse incidence of CV events, despite a comparable effect on glycaemic control (5). Similarly, diabetic cohorts of trials targeting lipids still present a strikingly high incidence of CV events, despite the efficacy of statins and other LDL-targeting interventions (6). Collectively, these data strongly suggest the existence of a residual CV risk in T2DM patients and that glucose, lipids, and blood pressure are not the only mediators of CV diseases in T2DM (7,8).

Recent data suggest that a plethora of additional imbalances are experienced by T2DM patients and could contribute to the observed residual CV risk. Among the various mechanisms proposed, endothelial dysfunction, senescent-cell accumulation, and a pervasive status of chronic, low-grade inflammation are emerging as key players in development of T2DM complications (8). These phenomena are all modulated by long-lasting epigenetic changes, including microRNA (miRNA) alterations (9).

miRNAs are small (22 nucleotides), highly conserved non-coding RNAs, processed from longer transcripts by the coordinated action of specific RNA endonucleases. miRNAs usually pair to sites within the 3' untranslated region of messenger RNAs (mRNAs), causing mRNA decay and block of protein translation (10). Increasing evidence suggests that miRNAs are actively secreted outside the cell and circulate in human blood associated with either vesicles or protein complexes that protect them from degradation (11). In particular, extracellular vesicles (EVs) carried miRNAs are emerging as key endocrine/paracrine mediators of metabolic and CV homeostasis (12). Indeed, three seminal papers suggest that:

- adipose tissue is a major source of circulating EVs-miRNAs that can regulate gene expression in other tissues (13);
- pro-inflammatory macrophages from adipose tissue of obese mice secrete EVs with an altered miRNA repertoire capable of spreading inflammatory signals and inducing insulin resistance (14);
- transfer of circulating EVs from high-fat-fed to naïve mice is sufficient to promote dysglycaemia, an effect mediated by specific miRNAs (15).

An altered miRNA content in EVs has also been suggested to underpin the development of CV complications of T2DM. For instance, a breakthrough manuscript showed that EVs-mediated exchange of miR-126 supports endothelial growth, a mechanism that is blunted by T2DM (16). Similarly, EVs derived from cardiomyocytes of diabetic rats have an increased miR-320 content which inhibits target genes in recipient endothelial cells (EC)s, blunting endothelial migration and tube formation (17). Another study showed that high glucose promotes the shedding of endothelial EVs carrying miR-503, inducing its transfer from ECs to vascular pericytes, thus fostering their impaired migration and proliferation (18).

Based on this background, we hypothesized that part of the residual CV risk observed in T2DM patients may be attributable to alterations in the miRNAs' payload of circulating EVs (9). Thus, the main goals of the project were:

1- To dose the expression of miRNAs associated with inflammation and vascular performance within EVs from patients with T2DM, with or without CV complications;
2- To gain preliminary insights regarding the ability of such EVs in inducing a pro-inflammatory phenotype in endothelial cells when administered *in vitro*.

Bibliography for the summary

1- Rawshani A, Rawshani A, Franzén S, et al. Mortality and Cardiovascular Disease in Type 1 and Type 2 Diabetes. N Engl J Med. 2017 Apr 13;376(15):1407-1418.
2- Gaede P, Lund-Andersen H, Parving HH, Pedersen O. Effect of a multifactorial intervention on mortality in type 2 diabetes. N Engl J Med. 2008 Feb 7;358(6):580-91
3- Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-year follow-up of intensive glucose control in type 2 diabetes. N Engl J Med. 2008 Oct 9;359(15):1577-89.

4- Look ARG, Wing RR, Bolin P, et al. Cardiovascular effects of intensive lifestyle intervention in type 2 diabetes. N Engl J Med. 2013;369(2):145-154.
5- Cefalu WT, Kaul S, Gerstein HC, et al. Cardiovascular Outcomes Trials in Type 2 Diabetes: Where Do We Go From Here? Reflections From a Diabetes Care Editors' Expert Forum. Diabetes Care. 2018 Jan;41(1):14-31.
6- Dash S, Leiter LA. Residual cardiovascular risk among people with diabetes. Diabetes Obes Metab. 2019 Apr;21 Suppl 1:28-38.
7- Gæde P, Oellgaard J, Carstensen B, et al. Years of life gained by multifactorial intervention in patients with type 2 diabetes mellitus and microalbuminuria: 21 years follow-up on the Steno-2 randomised trial. Diabetologia. 2016;59(11):2298-2307.
8- Prattichizzo F, De Nigris V, Spiga R, Mancuso E, La Sala L, Antonicelli R, Testa R, Procopio AD, Olivieri F, Ceriello A. Inflammageing and metaflammation: The yin and yang of type 2 diabetes. Ageing Res Rev. 2017Oct31;41:1-17.
9- Prattichizzo F, Giuliani A, De Nigris V, et al. Extracellular microRNAs and endothelial hyperglycaemic memory: a therapeutic opportunity? Diabetes Obes Metab. 2016 Sep;18(9):855-67.
10- Bartel DP. Metazoan MicroRNAs. Cell. 2018;173(1):20-51. https://www.cell.com/cell/fulltext/S0092-8674(18)30286-1
11- Turchinovich A, Tonevitsky AG, Burwinkel B. Extracellular miRNA: A Collision of Two Paradigms. Trends Biochem Sci. 2016 Oct;41(10):883-892.
12- Xiao Y, Zheng L, Zou X, Wang J, Zhong J, Zhong T. Extracellular vesicles in type 2 diabetes mellitus: key roles in pathogenesis, complications, and therapy. J Extracell Vesicles. 2019 Jun 14;8(1):1625677
13- Thomou T, Mori MA, Dreyfuss JM, et al. Adipose-derived circulating miRNAs regulate gene expression in other tissues. Nature. 2017 Feb 23;542(7642):450-455.
14- Ying W, Riopel M, Bandyopadhyay G, et al. Adipose Tissue Macrophage-Derived Exosomal miRNAs Can Modulate In Vivo and In Vitro Insulin Sensitivity. Cell. 2017 Oct 5;171(2):372-384.e12.
15- Castaño C, Kalko S, Novials A, Párrizas M. Obesity-associated exosomal miRNAs modulate glucose and lipid metabolism in mice. Proc Natl Acad Sci U S A. 2018 Nov 27;115(48):12158-12163.
16- Jansen F, Yang X, Hoelscher M, et al. Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles. Circulation. 2013 Oct 29;128(18):2026-38.

17- Wang X, Huang W, Liu G, Cai W, Millard RW, Wang Y, Chang J, Peng T, Fan GC. Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells. J Mol Cell Cardiol. 2014 Sep;74:139-50.
18- Caporali A, Meloni M, Nailor A, et al. p75(NTR)-dependent activation of NF-κB regulates microRNA-503 transcription and pericyte-endothelial crosstalk in diabetes after limb ischaemia. Nat Commun. 2015 Aug 13;6:8024

2. Results

In the main publication produced during the development of the project (Prattichizzo et al., CD31 Positive-Extracellular Vesicles from Patients with Type 2 Diabetes Shuttle a miRNA Signature Associated with Cardiovascular Complications. *Diabetes, 2021*), we adapted an immunomagnetic bead-based method to isolate plasma CD31 positive (+) EVs to harvest vesicles deriving from tissues relevant for T2DM complications. We selected this approach due to the marked and specific expression of CD31, i.e. platelet endothelial cell adhesion molecule (PECAM-1), in platelets, immune cells and endothelial cells, three cell types playing a prominent role in the development of T2DM-related complications.

We then performed a deep characterization of isolated EVs according to MISEV guidelines. We showed that we were able to isolate CD31+ EVs, representing a fraction of total plasma EVs with a heterogeneous origin but compatible with the hypothesis that platelets and endothelial cells are major contributors to this specific EV pool.

To explore the possible association of CD31+EV-shuttled miRNAs with T2DM status and T2DM complications, we selected 11 miRNAs for two characteristics:

1. being proposed to play a role in the development of CV complications of T2DM or previously suggested to have diagnostic potential in CV studies;
2. being robustly expressed in our setting of isolated CD31+ EVs.

This selected panel was composed of miR-126-3p, miR-343 146a-5p, miR-155, miR-195-5p, miR-21-5p, miR-24-3p, miR-320a, miR-342-3p, miR-376a, miR-422, and miR-451a. We quantified single miRNAs by qPCR in CD31+ EVs isolated from plasma of a

cross-sectional cohort of 218 individuals, including 60 healthy (Ctrl), 57 with uncomplicated T2DM (T2DM-NC), and 101 with T2DM and complications (T2DM-C). We found that 10 of the tested miRNAs are affected by T2DM, while the signature composed by miR-146a, -320a, -422a, -451a efficiently identified T2DM patients with complications. Furthermore, another CD31+EV-shuttled miRNA signature, *i.e*. miR-155, -320a, -342-3p, -376, and -422a, detected T2DM patients with a previous major adverse cardiovascular event. Many of these miRNAs significantly correlate with clinical variables held to play a key role in the development of complications.

Finally, since one of the hypotheses of the project was to explore if EVs harvested from patients with diabetes are able to induce a deleterious effect in receiving cells, we treated endothelial cells in vitro with EVs collected from patients' plasma. EVs from both T2DM-NC and T2DM-C significantly increased the expression of chemokine (C-C motif) ligand 2 (CCL2,) and interleukin 1 alpha (IL-1α) when compared to EVs from control subjects, while only EVs from T2DM-C induced the expression of TNFα in recipient endothelial cells when compared to both T2DM-NC and controls. Finally, the expression of IL-6, chemokine (C-X-C motif) ligand (CXCL)-1, and CXCL-8 was not affected by any of the treatments, possibly suggesting a peculiar pro-inflammatory effect of EVs, rather than a non-specific inflammatory response.

3. Relevance and possible future implications

The results of the study support the notion that EV-shuttled miRNAs might be harnessed as a potential tool to foresee the development of complications in patients with T2DM. Indeed, we showed that harvesting CD31+ EVs, compared to whole plasma, improves the ability of miR-21-5p and miR-146a-5p to detect T2DM and its complications. In addition, these data corroborate the hypothesis that CD31+ EVs from T2DM patients are endowed with pro-inflammatory properties, possibly contributing to the observed residual CV risk of patients with this condition. Overall, results of the project encourage further research to explore both the diagnostic potential and the functional role of T2DM-driven EV alterations.

In a collateral publication, we largely discussed the state-of-the art and the possible future directions of this very specific field of research (Prattichizzo et al., *Theranostics,*

2021). EV biology is a rapidly growing field of research, with the potential to revolutionize the diagnosis and the therapy of a wide range of complex, multi-factorial diseases, including T2DM and its CV complications. EV dysregulation has been involved in virtually all stages of the T2DM trajectory, affecting the development of insulin resistance, β-cell dysfunction, dyslipidaemia, and atherosclerosis, as well as of other CV complications. However, additional research is needed to clarify the effective contribution of EVs to pathological processes relevant for T2DM development and progression in humans. Indeed, human findings collected thus far are associative rather than causative. Also, standardized methods for isolation, preparation, characterization, and quantitation of EVs are mandatory to progress to clinical stages, for both diagnostic and therapeutic purposes. EV number and molecular cargo have also been shown to be affected by diet and exercise, two of the most powerful strategies to prevent T2DM and CVDs. This picture is further puzzled by the observations that food itself might contain biologically active EVs. Given the progressively increasing ease of their manipulation, EVs from multiple sources may represent the ideal candidates to eventually improve a number of aspects of T2DM treatment and management, such as attenuation of insulin resistance in initial stages, amelioration of β-cell failure, improved risk stratification for CVD development, and modulation of pathways promoting the development of complications. Considering the complex package of information shuttled, EVs may eventually affect these pathological components in a long-term manner, providing an additional and different strategy to conventional therapies. On the other hand, it is unlikely that such a powerful tool would not be accompanied by side effects, bearing in mind that EV-shuttled molecules, including miRNAs, are known to display a pleiotropic activity. Thus, while pre-clinical research is still in its infancy, we can foresee that the translation to a clinical stage will require tailored and specific settings. However, available evidence encourages further research to explore the potential of EVs as future nanodiagnostic and nanotherapeutic tools for T2DM management.

Finally, we would like to mention that a number of collateral ideas were generated during the progression of the project, so a number of additional publications were produced thank to the funding provided by La Marató. Albeit not strictly related to the main goal, these publications cover the use of miRNA as tools:

1- to monitor the response to pharmacological intervention;

2- to assess the effect of T2DM-related stressors in various relevant pre-clinical models.

The list of all the publications generated (main and secondaries) is provided below.

4. Scientific bibliography generated during project's development (main, strictly relevant, are papers underlined)

1: Prattichizzo F, Matacchione G, Giuliani A, Sabbatinelli J, Olivieri F, de Candia P, De Nigris V, Ceriello A. Extracellular vesicle-shuttled miRNAs: a critical appraisal of their potential as nano-diagnostics and nano-therapeutics in type 2 diabetes mellitus and its cardiovascular complications. Theranostics. 2021 Jan 1;11(3):1031-1045.

2: Prattichizzo F, De Nigris V, Sabbatinelli J, et al. CD31+ Extracellular Vesicles From Patients With Type 2 Diabetes Shuttle a miRNA Signature Associated With Cardiovascular Complications. Diabetes. 2021 Jan;70(1):240-254.

3: Mensà E, Guescini M, Giuliani A, et al. Small extracellular vesicles deliver miR-21 and miR-217 as pro-senescence effectors to endothelial cells. J Extracell Vesicles. 2020 Feb 18;9(1):1725285

4: Prattichizzo F, Giuliani A, Sabbatinelli J, Mensà E, De Nigris V, La Sala L, de Candia P, Olivieri F, Ceriello A. Extracellular vesicles circulating in young organisms promote healthy longevity. J Extracell Vesicles. 2019 Aug 15;8(1):1656044.

5: Prattichizzo F, Giuliani A, Mensà E, Sabbatinelli J, De Nigris V, Rippo MR, La Sala L, Procopio AD, Olivieri F, Ceriello A. Pleiotropic effects of metformin: Shaping the microbiome to manage type 2 diabetes and postpone ageing. Ageing Res Rev. 2018 Dec;48:87-98.

6: Prattichizzo F, De Nigris V, Micheloni S, La Sala L, Ceriello A. Increases in circulating levels of ketone bodies and cardiovascular protection with SGLT2 inhibitors: Is low-grade inflammation the neglected component? Diabetes Obes Metab. 2018 Nov;20(11):2515-2522.

7: La Sala L, Micheloni S, De Nigris V, Prattichizzo F, Ceriello A. Novel insights into the regulation of miRNA transcriptional control: implications for T2D and related complications. Acta Diabetol. 2018 Oct;55(10):989-998.

8: Prattichizzo F, De Nigris V, Mancuso E, et al. Short-term sustained hyperglycaemia fosters an archetypal senescence-associated secretory phenotype in endothelial cells and macrophages. Redox Biol. 2018 May;15:170-181.

9: Prattichizzo F, De Nigris V, Spiga R, Mancuso E, La Sala L, Antonicelli R, Testa R, Procopio AD, Olivieri F, Ceriello A. Inflammageing and metaflammation: The yin and yang of type 2 diabetes. Ageing Res Rev. 2018 Jan;41:1-17.

10: Testa R, Bonfigli AR, Prattichizzo F, La Sala L, De Nigris V, Ceriello A. The "Metabolic Memory" Theory and the Early Treatment of Hyperglycemia in Prevention of Diabetic Complications. Nutrients. 2017 Apr 28;9(5):437.