

1 Review

# 2 Insights into the Diagnostic Potential of Extracellular 3 Vesicles and their miRNA Signature from Liquid 4 Biopsy as Early Biomarkers of Diabetic 5 Micro/Macrovascular Complications

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10 **Abstract:** Extracellular vesicles (EVs) represent a heterogeneous population of small vesicles, consisting of a  
11 phospholipidic bilayer surrounding a soluble interior cargo. Almost all cell types release EVs, thus they are  
12 naturally present in all body fluids. Among the several potential applications, EVs could be used as drug  
13 delivery vehicles in disease treatment, in immune therapy because of their immunomodulatory properties and  
14 in regenerative medicine. In addition to general markers, EVs are characterized by the presence of specific  
15 biomarkers (proteins, miRNAs) that allow the identification of their cell- or tissue-origin. For these features,  
16 they represent a potential powerful diagnostic tool to monitor state and progression of specific diseases. As  
17 regards, a large body of studies supports the idea that endothelial derived (EMPs) together with platelet-  
18 derived microparticles (PMPs) are deeply involved in the pathogenesis of diseases characterized by micro- and  
19 macrovascular damages, including diabetes. Existing literature suggests that the detection of circulating EMPs  
20 and PMPs and their specific miRNA profile may represent a very useful non-invasive signature to achieve  
21 informations about the onset of peculiar disease manifestations. In this Review, we discuss the possible utility  
22 of EVs in the early diagnosis of diabetes-associated microvascular complications, specifically related to kidney.

23 **Keywords:** EVs; endothelial-derived microparticles; platelet-derived microparticles; non-invasive  
24 biomarkers; miRNAs signature; diabetes associated complications; micro-macrovascular damage; diabetic  
25 nephropathy  
26

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## 27 1. Introduction

28 In the complex scenario of a cell's life cycle, scanned by differentiation, expansion, carrying out  
29 functions and programmed cell death, an incredible amount of stimuli continuously leads to the  
30 release of EVs. Physiological conditions, such as shear stress [1], cellular activation [2] or apoptosis  
31 [3] normally induce microvesiculation. In pathological conditions, as well as oncogenic  
32 transformation [4, 5, 6], inflammation [7, 8, 9] or other strong cellular stresses, this process is  
33 dramatically enhanced, with an increase in EVs production [10]. Vesicles release represents a highly  
34 conserved process in prokaryotes and eukaryotes suggesting the extent of a dynamic extracellular  
35 communication network, deeply involved in organ and tissue regulation [11]. This mechanism of  
36 cell-to-cell communication represents a necessary condition for proper coordination, both during  
37 development and among different cell types within adult tissues [12]. EVs are structures consisting of  
38 fluid surrounded by a phospholipidic bilayer, originated by mother cell membranes and contain a  
39 large variety of lipids and proteins. Membrane glycoproteins, distinctive of the parental cells, allow  
40 a fine identification of their origin (*vide infra*). In addition, EVs contain a soluble interior cargo  
41 composed by proteins and genetic material (mRNAs and micro RNAs (miRNAs)) [13]. During EVs  
42 generation, specific proteins may be included or excluded from the cell membrane, thus surface  
43 protein expression can be not identical to their parental cells. EVs were initially precipitated from

44 platelet-free plasma [rev. in 12], although for many years they were considered inert cellular debris.

45 EVs are nowadays recognized as a heterogeneous population of circulating small vesicles  
46 originating from almost all cell types: endothelial cells (EC), monocytes, lymphocytes, platelets,  
47 leukocytes and erythrocytes, but also neurons, cancer and stem cells [14]. Furthermore, they are  
48 naturally present in body fluids including blood, saliva, urine, seminal fluid, nasal secretions, tears,  
49 synovial fluid, vitreous humor, cerebrospinal fluid and breast milk [14].

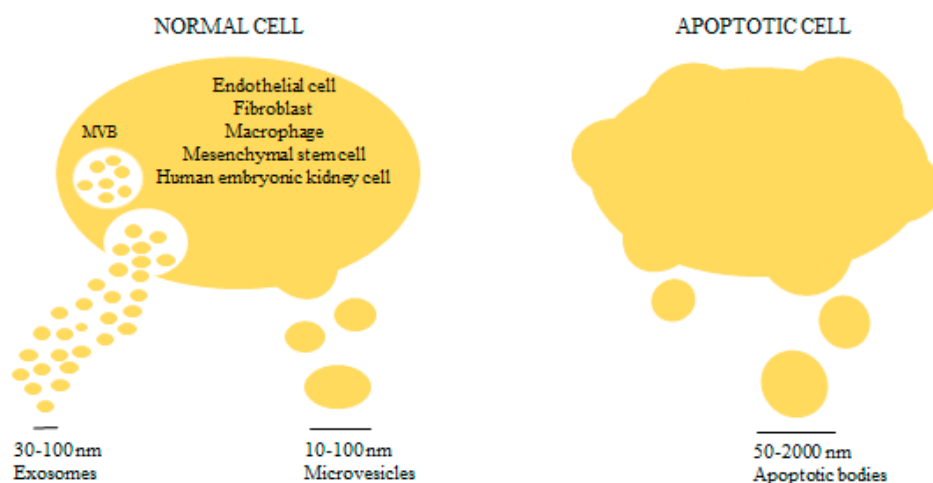
50 EVs have being largely studied for their therapeutic potential, since a system based on exosomes  
51 and microvesicles may represent a very potent tool for drug delivery, which even improves drug  
52 solubility and allows the passage across physiological barriers such as blood-brain barrier (BBB) [11,  
53 15, 16]. Interestingly, *in vitro* experiments recently demonstrated EVs efficacy in reducing  
54 glioblastoma cell proliferation and induction of tumor cell apoptosis [17]. Moreover EVs, loaded with  
55 anti-blastic drugs, exerted an enhanced anti-tumor activity *in vitro* [17].

56 Besides their usage as drug delivery vehicles, EVs already revealed their potential application in  
57 immune therapy [18] because of their immuno-activatory or immuno-inhibitory properties. It is well  
58 known that they are responsible for the immunomodulatory effects of their mesenchymal stem cell  
59 (MSC) progenitors [19, 20] by acting through paracrine mechanisms. Infected macrophages are able  
60 to secrete EVs containing proinflammatory pathogen-derived molecules [21]. Furthermore,  
61 mycoplasma-infected cultured cells release proinflammatory exosomes stimulating B- and T-  
62 lymphocytes [22]. EVs are also able to directly present antigens and contain MHC-peptide complexes  
63 for the initiation of immune responses by antigen-presenting cells (APCs) [23]. Exosomes produced  
64 by tumor-derived membranes may exert both an immuno-stimulatory effect, by transferring tumor  
65 antigens to dendritic cells (DCs) [4] and an immuno-inhibitory response, inducing T cell apoptosis *in*  
66 *vitro* [24, 25, 26]. Furthermore, safety and efficacy of the novel anti-tumor vaccine, i.e.  $\alpha$ -type 1  
67 polarized DCs ( $\alpha$ DC1) loaded with synthetic peptides specific for glioma-associated antigen (GAA)  
68 epitopes, were demonstrated in a phase I/II vaccination trial [27]. Finally, several interesting  
69 evidences [28, 29, 30] ascribed the regenerative properties of MSCs to their EVs paracrine signals.  
70 These paracrine factors can influence both stem cell niches and tissue response on adjacent  
71 parenchymal and stromal cells, by enhancing cell survival, self-renewal and activating endogenous  
72 mechanisms for repair and regeneration [rev in 12]. As regards EVs disadvantages, their massive  
73 release by cancer cells contribute to extracellular matrix degradation, thus to invasive growth and  
74 angiogenesis, contributing to metastasis and horizontal propagation of oncogenes. Furthermore EVs  
75 can lead cancer cells to escape from immune surveillance (*vide supra*) by exposing Fas ligand, the  
76 ligand for the death receptor Fas, promoting T cell apoptosis and inhibiting T cell adaptive immune  
77 responses [rev. in 12] In recent years the scientific community has been focusing into the diagnostic  
78 and prognostic potential of EVs since they can provide a non-invasive and continuous signature to  
79 predict disease onset and monitor its progression [31]. In this review we therefore discuss the possible  
80 utility of EVs and their associated miRNAs for the early diagnosis of diabetes mellitus (DM)-  
81 associated microvascular complications, with focus on renal damages.

## 82 2. An Overview on Microvesicles Biology

83 EVs classification is based on their different sizes and biogenesis. 'Microparticles' (MPs), also  
84 known as microvesicles or ectosomes, originate from the outward budding of the plasmamembrane,  
85 'exosomes' are formed by fusion between endocytic vesicles and the plasmamembrane and

86 'apoptotic bodies' are generated by apoptotic cells [13] (Fig. 1). These latter can be more abundant  
 87 than exosomes or MPs under specific conditions and can vary in content among different biofluids  
 88 [32]. The size of EVs depends, at least in part, by their origin; a lipid bilayer has a thickness of 5 nm,  
 89 so that the smallest MP size is around 30 nm and the largest is around 1 $\mu$ m. Endosomes, whose size  
 90 ranges between 200 and 500 nm, allow release of exosomes having a 30-100 nm diameter (Figure 1).



91

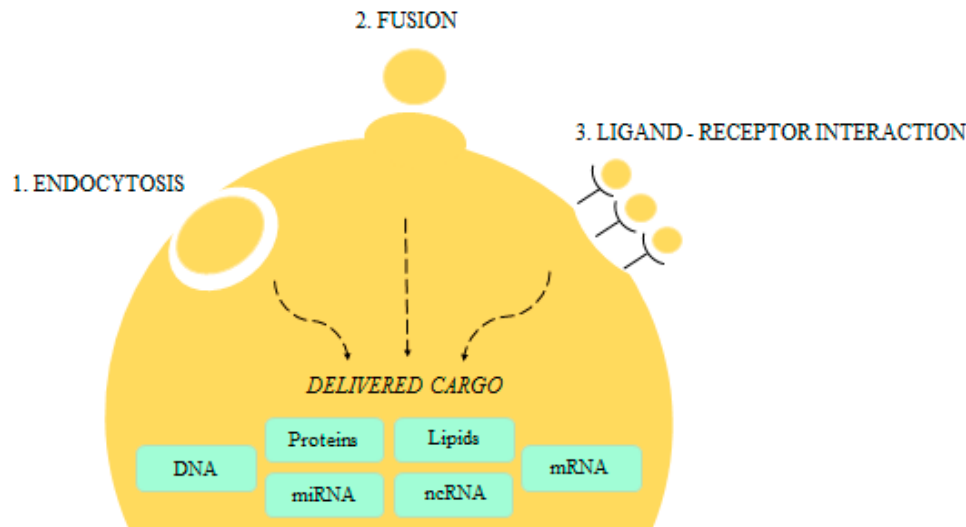
92 **Figure 1.** EVs mechanism of intercellular communication. Depending on the physical and chemical  
 93 properties of the cell compartments of biogenesis, EVs show different dimensions.

94 Among the general markers most exosomes express proteins such as tetraspannins (CD9, CD63  
 95 and CD81), Alix, flotillin, TSG101 and Rab5b [33, 34]. The enrichment in cholesterol, ceramide,  
 96 sphingolipids and raft-associated phosphoglycerides, provides an additional tracking opportunity  
 97 for exosomes characterization [35]. Cell activation and apoptosis, accompanied by an increase in  
 98 cytosolic calcium, alter the normal distribution of phospholipids in the plasmamembrane, due to  
 99 inhibition of *flippase* activity, with a consequent increased phosphatidyl serine (PS) exposure on the  
 100 outer leaflet of the membrane. PS externalization allows MVs identification, while specific protein  
 101 markers additionally define the cell origin [36]. As regards, blood cells and erythrocyte-derived MPs  
 102 are identified by the presence of CD235a on their membrane [37, 38]; CD4 and CD8 label  
 103 lymphocytes-derived MPs [39, 40, 41, 42]; PMPs are revealed by CD41 and CD42 [41, 43, 44]; CD144  
 104 and CD146 are specific for EC [45].

105 EVs shedding is highly influenced by intracellular elements such as calcium, that affects  
 106 membrane phospholipid distribution through specific enzymes, i.e. flippase, floppase and  
 107 scramblase. Calcium ions also intervene in cytoskeleton reorganization [rev in 12].

108 Interactions between microvesicles and recipient cells can occur throughout different  
 109 mechanisms (Figure 2) such as ligand-receptor binding, direct fusion with plasma membranes or  
 110 uptake by recipient cells [19]. MVs uptake can occur via endocytic pathways such as phagocytosis,  
 111 micropinocytosis, lipid-raft mediated internalization, clathrin-dependent or independent  
 112 endocytosis [19]. Interaction between specific ligands on microvesicles surface and receptors on  
 113 target cells leads to the activation of intracellular signaling pathways. Nevertheless, many EVs, once  
 114 released from a cellular element, may rapidly break down, thus releasing extracellularly their content.  
 115 EVs represent a novel mechanism through which cells exchange genetic information since nucleic  
 116 acids are protected within their membranes from plasma ribonucleases [rev in 12]. Remarkably, EVs

117 are able to induce epigenetic changes of neighboring cells by horizontal transfer of RNA.



118

119 **Figure 2.** EVs mechanisms of intercellular communication without direct cell-to-cell contact.

### 120 3. EVs Diagnostic Potential

121 The study of EVs is opening new horizons for their potential application not only as therapeutic  
 122 tools but also as clinical biomarkers for monitoring disease progression (*vide supra*) [10, 13]. Even if  
 123 most clinical data derive from studies of tumor patients, increased levels of EVs have been detected  
 124 in body fluids in a variety of cardiovascular and inflammatory pathologies, obesity, atherosclerosis,  
 125 diabetes and metabolic syndrome (*vide infra*), as well as in infectious and neurodegenerative diseases  
 126 including Alzheimer's, Parkinson's diseases and multiple sclerosis [46, 47, 48, 49, 50]. Furthermore,  
 127 in recent years, special attention was focused on miRNAs, a group of small, single-stranded, non-  
 128 coding RNAs, deeply involved in the regulation of gene expression by post-transcriptional  
 129 interference with complementary mRNAs [51]. As regards, EVs-associated specific miRNAs profiles  
 130 were found putatively correlated with peculiar pathological conditions when assayed in biological  
 131 fluids such as plasma, sera and urine [52, 53]. Indeed circulatory cell-free miRNAs are easily  
 132 detectable and very stable due to the protection from RNase degradation, being embedded in  
 133 exosomes, microvesicles or apoptotic bodies [54] or through formation of protein-miR complexes  
 134 with Argonaute 2 (Ago2) or high-density lipoprotein (HDL)-associated proteins [55, 56].

#### 135 3.1. EVs Quantification Issues

136 EVs isolation from cell culture supernatants and from body fluids [57] has been essentially  
 137 performed by differential steps of centrifugation, aimed to recover sequentially pelleted smaller  
 138 particles [57, 58]. Nevertheless, to date, EVs quantification from liquid biopsies represents an open  
 139 challenge that requires a reliable standardization. Due to their small size, the conventional methods  
 140 used for cell quantification cannot be applied to EVs. The most utilized methods for the analysis of  
 141 EV quantity, size and features are represented by transmission electron microscopy (TEM), flow  
 142 cytometry (FACS), nanoparticle-tracking analysis (NTA), and Tunable Resistive Pulse Sensing  
 143 technology (TRPS). Total protein content, varying among different EVs subtypes, cannot be  
 144 considered an accurate method because of a possible contamination by high molecular weight  
 145 proteins [32].

146 Every single measurement method is based on different physical principles leading, therefore,  
147 to the determination of different radius values [13]. Electron microscopy uses electrons to generate  
148 an image with a resolution down to the nanometer, and allows to evaluate structure and morphology  
149 of cell-secreted vesicles [13]. TEM technique requires fixation, dehydration and staining of biological  
150 samples before imaging; these treatments may dramatically damage the vesicles and affect their size  
151 and morphology. Flow cytometry is a valid method to study EVs both in physiological and in  
152 pathological conditions, but its sensitivity is often insufficient to visualize smallest EVs [59]. NTA is  
153 another technique that measures size distribution of EVs within a 50-1000 nm range. This tool allows  
154 the direct visualization of scattering particles irradiated by a laser beam: the hydrodynamic radius of  
155 every single particle is calculated by the analysis of its Brownian motion [13, 60], with the advantage  
156 of a lower detection limit compared to flow cytometry, both in plasma and in supernatants of cultured  
157 cells [61, 62]. TRPS principle relies on ionic flow disruption at the time particles pass through a single  
158 nanopore separating two fluidic cell compartments [32].

159 The establishment of a set of EVs markers, indicative of their cell- or tissue-origin, could be  
160 useful for the quantification of specific vesicle subsets in biological samples and their potential  
161 disease correlation [12]. This issue has been especially unraveled for EMPs and PMPs.

#### 162 4. Relevance of EMPs and PMPs

163 Endothelium is a thin layer of flat epithelial cells that limits serous cavities, lymph and blood  
164 vessels, and acts as a selective barrier in the continuous exchange of molecules between blood and  
165 tissues [63, 64]. The endothelium of some tissues and organs, such as kidney or liver, is characterized  
166 by discontinuities or fenestrations between cells, large enough to allow the passage of large molecules  
167 or proteins. In other organs, EC are joined together by different types of adhesive cell-to-cell  
168 junctions, formed by transmembrane molecules linked to cytoskeletal or cytoplasmic proteins that  
169 selectively allow passage of water, macromolecules and even blood cells [65]. Vascular EC are largely  
170 involved in the regulation of normal vascular tone and permeability, homeostasis maintenance,  
171 coagulation/fibrinolysis balance, composition of subendothelial matrix, leukocytic diapedesis and  
172 thrombogenesis prevention [66]. EC are able to exert their multiple functions by releasing several  
173 regulatory mediators (nitric oxide, prostanoids, endothelin, angiotensin II, tissue-type plasminogen  
174 activator or t-PA, plasminogen activator inhibitor-1 (PAI-1)), adhesion molecules and cytokines [45].  
175 A pathological event such as dyslipidemia, hyperglycemia or inflammation occurring in several  
176 conditions (*vide supra*) may modify natural endothelial properties inducing cell activation thus  
177 endothelial dysfunction [66]. Dysfunctional EC release vasoactive substances, EMPs and chemotactic  
178 factors, that altogether contribute to the initiation of inflammatory response and to eventual  
179 atherogenic development [67, 68, 69, 70]. Beside activated EC, also apoptotic EC may release EMPs  
180 having a different surface immunophenotype [71, 72, 73]. In detail, activated cell-derived MPs  
181 express a high amount of CD62E, while apoptotic EMPs are mainly CD31+ [74, 75]. An elevated ratio  
182 of CD31+/Annexin V+ EMPs to CD62E+ EMPs reflects an impaired immune phenotype of EMPs and  
183 allows to diagnose through a specific pattern of EMPs the origin and degree of endothelial  
184 dysfunction in dysmetabolic disorders (66, 76).

185 High plasma levels of EMPs have been found in patients with hypertension [77],  
186 hypertriglyceridemia, acute coronary artery disease (CAD) [78], peripheral vascular disease [79] and  
187 DM [80]. Endothelial dysfunction cannot be considered a clear hallmark of the diabetic state, rather  
188 a key factor in the pathogenesis of athero-thrombotic complications, retinopathy, nephropathy,  
189 atherosclerosis [81, 9], micro- and macroangiopathy. Among several studies CD144 (VE cadherin)

190 positive MPs have been identified as specific EC particles; the increase of circulating CD144 EMPs  
191 represents a very specific marker of EC dysfunction and could be useful to identify DM patients with  
192 risk of CAD [9]. In a recent study, Fan et al (2016) [82] pointed out that EMPs are involved in the  
193 activation of platelet vesiculation.

194 PMPs together with EMPs have also been widely investigated for their involvement in  
195 inflammation, coagulation, diseases characterized by the impairment of vascular function, such as  
196 atherosclerosis, diabetes, hypertension and in connective tissue diseases [83, 84]. Nomura et al (2004)  
197 [84] observed that PMPs are able to promote interaction between EC and monocytes in patients with  
198 Type 2 diabetes (T2D), therefore they were potentially implicated in the onset of diabetes-associated  
199 complications. As assessed by Tsimmerman et al (2011) [86] EVs from diabetic patients, especially from  
200 those with diabetic foot, show a high pro-coagulant activity. PMPs were even found significantly  
201 elevated in pediatric Type 1 diabetes (T1D) patients, particularly in association with early  
202 microvascular complications [83] (*vide infra*).

## 203 5. EVs and EVs-associated mRNAs Diagnostic Potential in Diabetes and its Complications

204 DM is the most relevant metabolic disorder, affecting about 100 million persons worldwide, with  
205 a strong trend to increase. Classically, DM is classified in Type 1 (T1D) and Type 2 (T2D). T1D, also  
206 known as insulin-dependent DM, and representing 5-10% of cases, is an autoimmune multifactorial  
207 disorder occurring in human leukocyte antigen (HLA) genetically-predisposed individuals as a  
208 consequence of organ-specific immune destruction of the insulin-producing  $\beta$  cells in the islets of  
209 Langerhans within the pancreas [87, 88, 89]. It is generally recognized that T1D derives from a  
210 breakdown in immune regulation that leads to expansion of autoreactive CD4+ and CD8+ T cells,  
211 autoantibody-producing B lymphocytes and activation of the innate immune system [88]. T2D, or  
212 non insulin-dependent DM, characterized by insulin resistance, accounts for 90-95% of cases. It is a  
213 complex metabolic disorder, of heterogeneous etiology with contributing social, behavioural and  
214 environmental risk factors [90]. The number of affected patients is expected to double during the next  
215 20 years [91].

216 DM leads to chronic complications, such as accelerated development of cardiovascular diseases,  
217 end-stage renal disease, loss of visual acuity and limb amputations, the main cause of morbidity and  
218 mortality in DM affected individuals [90]. A large amount of data supports the idea of a close  
219 connection between duration and severity of diabetes and micro/macrovascular damage [92]  
220 including coronary, cerebrovascular and peripheral arterial disease (PAD) due to complex  
221 dysfunction of main components of the vascular compartment [93, 94, 95, 96].

222 Initial studies unraveled the utility of PMPs and EMPs as diagnostic markers in diabetes.  
223 Although EVs quantification/characterization remains an open challenge within the scientific  
224 community (*vide supra*), increased plasmatic levels of PMPs and CD62P/CD63 positive platelets were  
225 found in patients with DM compared to normal controls. These novel markers correlated with  
226 hypercoagulability, suggesting the utility of antiplatelet therapy, i.e. cilostazol, to prevent the  
227 development of complications, especially nephropathy, in patients with poor blood glucose control  
228 [97].

229 Based on the use of specific markers for characterization, Tsimmerman et al (2011) [86]  
230 demonstrated that PMPs and EMPs and negatively charged phospholipid-bearing MPs were at  
231 highest levels in T2D patients with severe foot ulcers. The same result was obtained by Lakhter et al  
232 (2015) [98] in T1D patients, who exhibited higher levels of PMPs and EMPs, total Annexin V-positive

233 blood cell MP (TMP) and TMP procoagulant activity. Furthermore, the last parameter correlated with  
 234 HbA1c and dysglycemia. Instead, in T2D patients there was only an increase of TMP without the  
 235 increase in procoagulant activity [45].

236 In the study by Sun et al (2017) [99] levels of urinary CD63-positive exosomes were found  
 237 increased at early stage of renal injury in 62 early diabetic nephropathic (DN) subjects [99].  
 238 Nevertheless, CD63 expression was significantly increased in normoalbuminuric patients rather than  
 239 in the microalbuminuric group, probably due to a weak compensatory increase in GFR at an early  
 240 stage.

241 Several investigators addressed the issue of identifying a specific dysregulated plasma miRNA  
 242 signature in either T2D and obese patients or T1D affected subjects in order to depict novel  
 243 biomarkers of diagnostic utility. Table 1 focused on most frequently detected miRNAs and EV-  
 244 associated miRNAs emerging from an extensive literature review.

MiRNA	Level	Confirmed EVs association	Complications	Ref.	Type of Diabetes
126	↓		VEGF resistance, endothelial dysfunction, inflammation	Zampetaki et al. 2010 [101] Barutta et al. 2016 [100] Osipova et al. 2014 [104] Jansen et al. 2016 [103] Ollmeri et al. 2015 [102]	T2D T2D T1D T2D T2D
21	↑		Kidney inflammation Cardiovascular damages	Osipova et al. 2014 [104] Ollmeri et al. 2015 [102]	T1D T2D
29 (29a, 29b, 29c)	↑			Nielsen et al. 2012 [112] Kong et al. 2011 [113]	T1D T2D
27a	↑			Karolina et al. 2012 [116]	T2D
27b, 320	↑	Present	Retinopathy	Karolina et al. 2012 [116], Zampetaki et al. 2016 [117]	T2D
24	↓			Zampetaki et al. 2010 [101] Deng et al. 2017 [123]	T2D T2D

245

246 **Table 1.** MiRNAs and EV-associated miRNAs in T1D and T2D. Most frequently detected  
 247 dysregulated miRNAs in T1D and T2D patients with disease-associated complications.

miRNA	Level	Confirmed EVs association	Complications	Ref.	Type of Diabetes

248 MiR-126, highly enriched in EC and in platelets is one of the miRNAs more frequently  
 249 investigated for its relevance in endothelial homeostasis, in maintaining vascular integrity, in  
 250 angiogenesis and in wound repair. When released by EC, miR-126 modulates VEGF (vascular-  
 251 endothelial growth factor) responsiveness, thus contributing to vascular protection in a paracrine  
 252 manner. As endothelial activation and inflammation are hallmarks of micro- and macrovascular  
 253 complications in diabetes, loss of miR-126 was considered predictor as well as risk

254 estimation/classification marker not only for early diabetes but also for endothelial dysfunctions due  
255 to diabetes [100]. Being VEGF a crucial mediator in DN, miR-126 could be helpful also in predicting  
256 this type of complication (*vide infra*). Furthermore, miR-126 could represent a candidate marker for  
257 monitoring the efficacy of miRNA-based therapeutic intervention of vascular complications related  
258 to the disease [101]. Coming to the analysis of relevant manuscripts, Zampetaki et al (2010) [101]  
259 provided a detailed plasmatic mi-RNA signature in a large population-based cohort, the Bruneck  
260 study. This was initially designed to investigate the epidemiology and pathogenesis of  
261 atherosclerosis and later extended to all major human diseases, including T2D. Reduced levels of  
262 miR-126 were observed, and correlated to peripheral artery disease in T2D. The same aberrant  
263 miRNA expression was observed by Barutta et al (2016) [100] in an extensive analysis of more than  
264 400 serum samples of T2D patients and healthy subjects. In other studies, Olivieri (2015) [102] and  
265 Jansen (2016) [103] confirmed a reduction of miR-126 in T2D patients. In particular, Jansen et al [103]  
266 found that loss of miR-126 is related to CAD risk.

267 Unlike T2D, the role of miR-126 in T1D is not fully clarified as yet. Osipova et al (2014) [104] for  
268 the first time analysed blood and urine samples of T1D pediatric patients, focusing on miRNAs  
269 known to have relevance in diabetes and cardiovascular/renal damages. Regarding miR-126, no  
270 differences emerged in plasmatic T1D samples, while lower miR-126 levels were confirmed in urine  
271 T1D samples compared to controls (*vide infra*).

272 The same authors focused their studies also on miR-21, a profibrotic miRNA in cardiovascular  
273 diseases [105], known to induce fibrosis in many organs including heart and kidney [105, 106] and  
274 involved in endothelial-to-mesenchymal transition [107]. MiR-21 was upregulated both in plasma  
275 and urine samples of pediatric T1D patients [104]. MiR-21 upregulation was also proposed as useful  
276 biomarker for already existent fibrotic remodelling. Furthermore, the positive correlation emerged in  
277 urine samples between miR-21 and the inflammatory C-reactive protein (CRP) suggesting the  
278 presence of ongoing inflammatory events in the kidney of T1D patients [104]. Regarding T2D, Olivieri  
279 et al (2015) [102] confirmed higher plasma levels of miR-21 in diabetic patients with cardiovascular  
280 complications.

281 MiR-29 is another relevant miRNA involved in diabetes and its complications. MiR-29 family is  
282 composed of miR-29a, miR-29b and miR-29c, sharing the same seed sequence. The most important  
283 function of miR-29 consists in its protective role in fibrotic disease, including kidney fibrosis [108].  
284 MiR-29 is also involved in the pathogenesis of DN in diabetic mice [109, 110]. Furthermore, miR-29  
285 is upregulated in muscle, fat and liver in type 2 diabetic rats and caused insulin resistance in  
286 adipocytes [111]. An increase in miR-29 levels in the serum of T1D children [112] and adult patients  
287 with T2DM [113]. Both hyperglycemia and proinflammatory cytokines, the hallmarks of DM,  
288 upregulated the expression of miR-29 family miRNAs [114, 109], and the suppression of miR-29 with  
289 anti miR-29 oligomers protected against DN [109]. Additional studies highlighted the presence of a  
290 wide spectrum of putative miRNAs useful as DM biomarkers. The Bruneck study [101] revealed  
291 lower plasma levels of miR-20b, miR-15a, miR-191, miR-197, mi-223, miR-320 and miR-486 while a



292 modest upregulation of miR-28-3p even at an early stage [101]. A downregulation of miR-191, parallel  
293 to miR-200b, was shown by Dangwal et al (2015) [115]. Plasmatic miR-150, -192, -27a and -320a were  
294 found specifically upregulated by Karolina et al (2012) [116] both in metabolic syndrome and T2D.  
295 MiR-320a, together with miR-27b, was found upregulated and associated to diabetic retinopathy also  
296 by Zampetaki et al (2016) [117]. Furthermore, the authors detected miR-17, -197, -509-5p, -92a and -  
297 320a in plasmatic exosomes, with a similar expression pattern as in whole blood, supporting the  
298 hypothesis that circulating cell-free miRNAs are packaged into exosomes [117]. Pescador et al (2013)  
299 [118] demonstrated that miR-138 or miR-376a could be a useful predictive tool for distinguishing  
300 obese patients from healthy controls, diabetics and obese diabetics. In particular the combination of  
301 miR-503 and miR-138 could discriminate diabetics from obese diabetics. A decreased serum level of  
302 miR-146a was indicated as a potential marker of chronic inflammation in T2D patients by Baldeon et  
303 al (2014) [119]. Santovito et al (2014) [120], discovered a significant upregulation of miR-326, -186, -  
304 532-5p, -127-3p and a significant downregulation of let-7a and let-7f in plasma of T2D patients  
305 compared to controls. Other miRNAs candidates as diabetes and diabetic complications biomarkers  
306 are represented by miR-103 [121], miR-18a and miR-34c [122], miR-222, miR-let7d, miR-139 miR-199  
307 and miR-26a [103], miR-24 [101, 123], miR-454-3p, miR-222-3p, miR-144-5p and miR-345-3p [124].

## 308 **6. Potential Role of EVs and their MiRNAs Profiles in the Prediction of Diabetic Renal** 309 **Complications**

310 DN represents one of the most relevant chronic complications of DM [125] and the major cause  
311 of end-stage renal failure [126]. The number of patients with chronic renal damage due to DN is  
312 dramatically increased over the past decades [97] mostly due to the incidence of obesity and T2D in  
313 developed countries [127]. Metabolic and hemodynamic alterations as well as inflammation underlie  
314 DN development. Early blood pressure changes within the kidney and impairment of glomerular  
315 microcirculation, leading to glomerular hypertrophy and sclerosis, are critical in DN progression  
316 [127]. At present, clinical biomarkers including glomerular filtration rate (GFR), proteinuria and  
317 urinary sediment evaluation can help to identify etiology of chronic kidney disease but do not allow  
318 a specific diagnosis neither clarify disease staging [128]. Therefore, the finding of non-invasive  
319 biomarkers could obviate the use of kidney biopsy, a procedure implying complication risks, and  
320 could improve diagnostic accuracy. To this extent the best source of biomarkers to unravel renal  
321 damage in diabetes is represented by urine. An easy and non-invasive analysis of miRNAs contained  
322 in urinary exosomes has recently been proposed in several studies in order to monitor early renal  
323 complications, since their dysregulated levels have been detected in urine of human diabetic patients  
324 [rev in 128]. Table 2 focuses on most frequently detected miRNAs and EV-associated miRNAs in  
325 diabetic patients affected by renal complications following an extensive literature review.

MiRNA	Level	Confirmed EVs association	Renal Complications	Ref.
15 17 21, 216a	↓ ↑ ↓		Diabetic glomerulosclerosis IgA nephropathy Renal functions decline	Szeto et al. 2012 [129]
638 152 200c	↓ ↓ ↑		Diabetic nephropathy Diabetic nephropathy, glomerulosclerosis Minimal change nephropathy, focal glomerulosclerosis	Wang et al. 2013 [131]
130a, 145 155, 424	↑ ↓	Present Present	Microalbuminuria	Barutta et al. 2013 [132]
25a 29c	↑ ↑		Diabetic nephropathy, albuminuria Diabetic nephropathy	Peng et al. 2013 [133]
126 21, 210	↓ ↑		Preclinical kidney disease, renal fibrosis	Osipova et al. 2014 [104]

326

327 **Table 2.** MiRNAs and EV-associated miRNAs in patients with diabetic renal involvement. Most  
328 frequently detected miRNAs signature detectable in urine of patients with DN at different stages of  
329 disease

miRNA	Level	Confirmed EVs association	Renal Complications	Ref.

330 In initial investigations Szeto et al (2012) [129] found lower miR-15 levels in association with  
331 diabetic glomerulosclerosis, and an increased level of miR-17 in patients with IgA nephropathy.  
332 Furthermore, lower levels of miR-21 and miR-216a in urinary sediments correlated with a faster  
333 decline of renal functions [129]. Conversely increased levels of miR-21 and miR-210 in plasma and  
334 urine samples of T1D pediatric patients were reported by Osipova et al (2014) [104]. In the last report  
335 urinary miR-126 levels were significantly lower in diabetic patients than in age- and gender-matched  
336 controls (*vide supra*). This miRNA concentration negatively correlated with HbA1c levels, suggesting  
337 a damaging effect driven by long-term high plasma glucose. It was demonstrated that miR-126 is  
338 expressed in glomerular and peritubular EC targeting SPRED1 (Sprouty-related, EVH1 domain  
339 containing protein) and PIK3R2 (phosphoinositol-3 kinase regulatory subunit 2), i.e. negative  
340 repressors of VEGF pathway [130] (*vide supra*). These phenomena envisage that decreased levels of  
341 miR-126 are associated with reduced response to VEGF and endothelial dysfunction.

342 As detailed in Table 2 several other miRNAs were highlighted in other investigations carried  
343 out with the aim to precisely characterize and quantify EVs and EV-associated miRNA profile  
344 predictive of diabetic complications. As stated before, an easily available biological sample such as  
345 urine represents a major advantage. A close association between single miRNA variation and renal  
346 diabetic complications was always depicted in spite of variability on reported miRNAs specificities.  
347 As yet a unique urinary minimum signature related to diabetes complications remains to be fully  
348 validated.

349 Reduced urinary levels of miR-192 were found in nephropathic patients characterized by  
350 diabetic glomerulosclerosis, and increased levels of miR-200c were detected in patients with minimal  
351 change nephropathy and with focal glomerulosclerosis [131]. By the analysis of Barutta et al (2013)  
352 [132], miR-130a and miR-145 were found enriched in diabetic patients with microalbuminuria while  
353 miR-155 and miR-424 were decreased compared to normoalbuminurics and non-diabetic controls.

354 Peng et al (2013) [133] focused their studies on miR-29 family (consisting of miR-29a, miR-29b,  
355 miR-29c, *vide supra*) involved in DN pathogenesis, and proposed miR-29 as biomarker for DN and  
356 atherosclerosis in T2D patients. By analyzing 83 T2D patients, urinary miR-29a and miR-29c were  
357 significantly higher compared to miR29-b. Furthermore, urinary miR-29a was significantly increased  
358 in patients with albuminuria [133] than in normoalbuminurics. MiR-29b correlated with carotid  
359 intima-media thickness in T2D patients. Other putative DN biomarkers were identified in other  
360 studies. As regards miR-619, -486-3p, -335-5p, -552, -1912, -1224-3p, -424-5p and -141-3p [134], miR-  
361 320c and miR-6068 [135] were found upregulated, while miR-2861, miR-1915-3p and miR-4532 were  
362 downregulated in DN patients [128]. Increased levels of serum miR-217 were correlated with the  
363 development of proteinuria in T2DN patients [136]. Urinary exosomal miR-133b, miR-342, and miR-  
364 30a [137] and miR-192 [138] were expressed at significantly higher levels in T2DN patients compared  
365 to normal.

## 366 7. Conclusions and future perspectives

367 In recent years, the scientific community has been debating methods for EVs isolation,  
368 characterization and quantification. The expensive and complex procedures being used so far need  
369 to be further improved in order to feasibly distinguish different EVs subpopulations in highly pure  
370 EVs preparations. A more defined standardization of these technological tools could lead to easier  
371 downstream characterization of EVs by transcriptomic, miRnomic and proteomic platforms in order  
372 to accurately define 'selective diagnostic panels of markers' for disease prediction, staging and  
373 progression. . Nevertheless, it needs to be pointed out that the translational significance of results  
374 that could be obtained by novel technologies always strictly relies on the appropriate selection of  
375 biological material from a homogeneous cohort of patients with same clinical characteristics, stage of  
376 disease and ethnic origin.

377 In the light of the foregoing extensive discussion of the existing literature, we can easily envisage  
378 that EVs and their miRNA cargo from liquid biopsies represent, nowadays, non-invasive biomarkers  
379 with great potential in longitudinal investigations related to several disease conditions including DM.  
380 In particular, EVs detection in urine could especially improve prediction by introducing non-invasive  
381 renal signatures of early onset and progression of microvascular renal damage in DM without the  
382 need for invasive diagnostic or radiological procedures. Nevertheless, future studies will clarify the  
383 precise cause-effect link between dysregulation of EV-related miRNAs and DN, and the precise role  
384 of these small non-coding RNA in the progression of diabetic complications [127].

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389 of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the  
390 decision to publish the results.

391 **Abbreviations**

392	EVs	extracellular vesicles
393	MPs	microparticles
394	EMPs	endothelial-derived microparticles
395	PMPs	platelet-derived microparticles
396	EC	endothelial cells
397	DM	diabetes mellitus
398	T1D	type 1 diabetes
399	T2D	type 2 diabetes
400	miRNA	microRNA
401	DN	diabetic nephropathy
402	CAD	coronary arterial disease
403	CHD	coronary heart disease
404	PAD	peripheral arterial disease
405	VEGF	vascular endothelial grow factor
406	GFR	glomerular filtration rate
407	MVs	microvesicles
408	PS	phosphatidyl serine
409	CRP	C-reactive protein

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