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Research article

Systematic review and bioinformatics analysis of plasma and serum extracellular vesicles proteome in type 2 diabetes

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ABSTRACT

Background: Type 2 diabetes (T2D) is a complex metabolic ailment marked by a global high prevalence and significant attention in primary healthcare settings due to its elevated morbidity and mortality rates. The pathophysiological mechanisms underlying the onset and progression of this disease remain subjects of ongoing investigation. Recent evidence underscores the pivotal role of the intricate intercellular communication network, wherein cell-derived vesicles, commonly referred to as extracellular vesicles (EVs), emerge as dynamic regulators of diabetes-related complications. Given that the protein cargo carried by EVs is contingent upon the metabolic conditions of the originating cells, particular proteins may serve as informative indicators for the risk of activating or inhibiting signaling pathways crucial to the progression of T2D complications.

Methods: In this study, we conducted a systematic review to analyze the published evidence on the proteome of EVs from the plasma or serum of patients with T2D, both with and without complications (PROSPERO: CRD42023431464).

Results: Nine eligible articles were systematically identified from the databases, and the proteins featured in these articles underwent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. We identified changes in the level of 426 proteins, with CST6, CD55, HBA1, S100A8, and S100A9 reported to have high levels, while FGL1 exhibited low levels.

Conclusion: These proteins are implicated in pathophysiological mechanisms such as inflammation, complement, and platelet activation, suggesting their potential as risk markers for T2D development and progression. Further studies are required to explore this topic in greater detail.

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1. Introduction

Type 2 diabetes (T2D) is a multifaceted chronic disease characterized primarily by persistent hyperglycemia, disrupting the body's ability to maintain metabolic homeostasis [1]. Its etiology involves tissue resistance to insulin action, deficiencies in insulin secretion, or a combination of both factors [2]. Various contributors to its onset include a familial predisposition, obesity, sedentary lifestyle, and a high-energy diet [3]. T2D poses a significant global public health challenge due to its widespread prevalence and insidious clinical progression, leading to progressive damage to vital organs such as the heart and kidneys, resulting in disability and, ultimately, patient mortality [4]. Despite existing treatment modalities encompassing glucose-lowering medications, exogenous insulin, and substantial dietary and lifestyle adjustments, many patients fail to achieve a reduced risk of complications. Consequently, further research into the pathophysiology of T2D is imperative [5,6]. Additionally, there is a crucial need to identify novel biomarkers for the systematic assessment of diabetes to mitigate the risk of complications [7].

Recent evidence underscores the pivotal role of the intricate intercellular communication network, where extracellular vesicles (EVs) emerge as crucial participants in the pathophysiological mechanisms of T2D and its complications [8]. EVs, a group of secreted cell-derived vesicles characterized by a lipid bilayer and a typical size ranging between 50 and 1000 nm, are primarily derived from the plasmatic membrane or endosomes. Widely distributed in extracellular biological fluids, including saliva, urine, cerebrospinal fluid, and peripheral blood, EVs encapsulate proteins, lipids, metabolites, DNA, mRNA, and non-coding RNA as cargo [9]. Leveraging these molecular components, EVs play a pivotal role in facilitating cell-to-cell communication during the progression of diabetes [10]. Moreover, EVs and their content hold promise as diagnostic and prognostic markers for identifying therapeutic targets [11].

Despite the wealth of information published on the potential roles of EVs in T2D, there has been a lack of systematic analysis of the proteins within these extracellular particles. Plasma and serum samples, chosen for EV isolation due to their minimally invasive nature, nearly painless collection, and reliability as routine clinical samples, are particularly esteemed for detecting potential biomarkers and therapeutic targets. Presently, limited studies have investigated the relevance of EV protein cargo in plasma and serum concerning human T2D. To the best of our knowledge, there has been no systematic review specifically addressing proteomic studies aimed at identifying proteins and associated signaling pathways of EVs linked to the mechanisms implicated in the advancement and progression of T2D.

The reflective nature of EV protein cargo concerning the metabolic state of their originating cells implies that specific proteins within EVs can provide valuable insights into the activation or inhibition of signaling pathways crucial to the progression of T2D and its associated complications. A thorough review of the current literature in this field is essential to propose relevant EV proteins and signaling pathways that play pivotal roles in T2D. This comprehensive understanding holds the potential to facilitate the development of targeted interventions for improved management and prevention of complications associated with T2D.

Moreover, given that each study contributes outcomes related to EV proteomics and the proteins implicated in T2D, a subsequent analysis, such as bioinformatics, conducted with combined data from multiple studies, could identify the principal signaling pathways contributing to the development and progression of T2D. Therefore, this study presents the current data from EV proteomic studies, focusing on differentially abundant proteins and the related signaling pathways closely associated with the pathophysiological mechanisms involved in the development and progression of T2D.

2. Methods

2.1. Search strategies and eligibility of studies

The protocol for this systematic review was registered on the International Prospective Register of Systematic Reviews (PROSPERO: CRD42023431464). The reporting adheres to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [12]. Eligible publications were identified through an assessment of the following databases: PubMed, Scopus, and Web of Science. The search strategy involved the following terms in title, abstract, and keywords to determine article eligibility: ('proteomic' OR 'proteomics' OR 'proteome' OR 'proteomical') AND ('extracellular AND vesicle' OR 'extracellular AND vesicles' OR 'ev' OR 'evs' OR 'exosome' OR 'exosomes' OR 'exo' OR 'exos' OR 'ectosome' OR 'ectosomes' OR 'microvesicle' OR 'microvesicles' OR 'mv' OR 'mvs') AND ('glucose' OR 'glycemic' OR 'hyperglycemia' OR 'hyperglycemic' OR 'diabetes' OR 'diabetic') AND ('retinopathy' OR 'retina' OR 'nephropathy' OR 'renal' OR 'neuropathy' OR 'peripheral arterial disease' OR 'stroke' OR 'cerebrovascular disease' OR 'cardiovascular disease'). Additionally, a manual search was conducted in the references of articles included through the systematic method and the Google Scholar database to capture eligible reports that the search strategy might not have identified.

2.2. Inclusion and exclusion criteria

Included in this systematic review were peer-reviewed longitudinal and cross-sectional studies published in journals that met specific criteria. The criteria for inclusion encompassed original research articles written in English, investigating the proteome of EVs in plasma or serum samples from human T2D patients, both with and without complications, and involving a control group. Exclusion criteria comprised articles written in languages other than English, reviews, brief reports, books, comments, erratum texts, editorials, guidelines, letters, meeting reports, preprint manuscripts, theses, articles expressing concern or retracted studies, studies conducted solely *in vitro* or with animal models, those lacking a T2D group, reports not assessing EV proteomics, or studies not analyzing plasma or serum samples.

2.3. Article selection

The process involved independent reviews by two researchers (J.G.A.-D. and J.M.M.-S.), and their analyses were subsequently verified and accepted by a third author (Referee: M.d.C·C.-P.). Initial assessments were based on titles and abstracts, with detailed scrutiny of article sections to identify eligible reports for inclusion in the review. Articles not meeting the eligibility criteria were excluded, with reasons for exclusion provided. In instances of conflicts, resolution was achieved through discussion and consultation with a third author (M.d.C·C.-P.).

2.4. Assessment of risk of bias

The quality and risk of bias in the included studies were evaluated utilizing the Appraisal Tool for Cross-Sectional Studies (AXIS) checklist [13]. Two reviewers (J.G.A.-D. and J.M.M.-S.) conducted the risk assessment.

2.5. Data extraction

Each study's collected information included the title, authors' names, year of publication, country of origin, type of sample utilized,

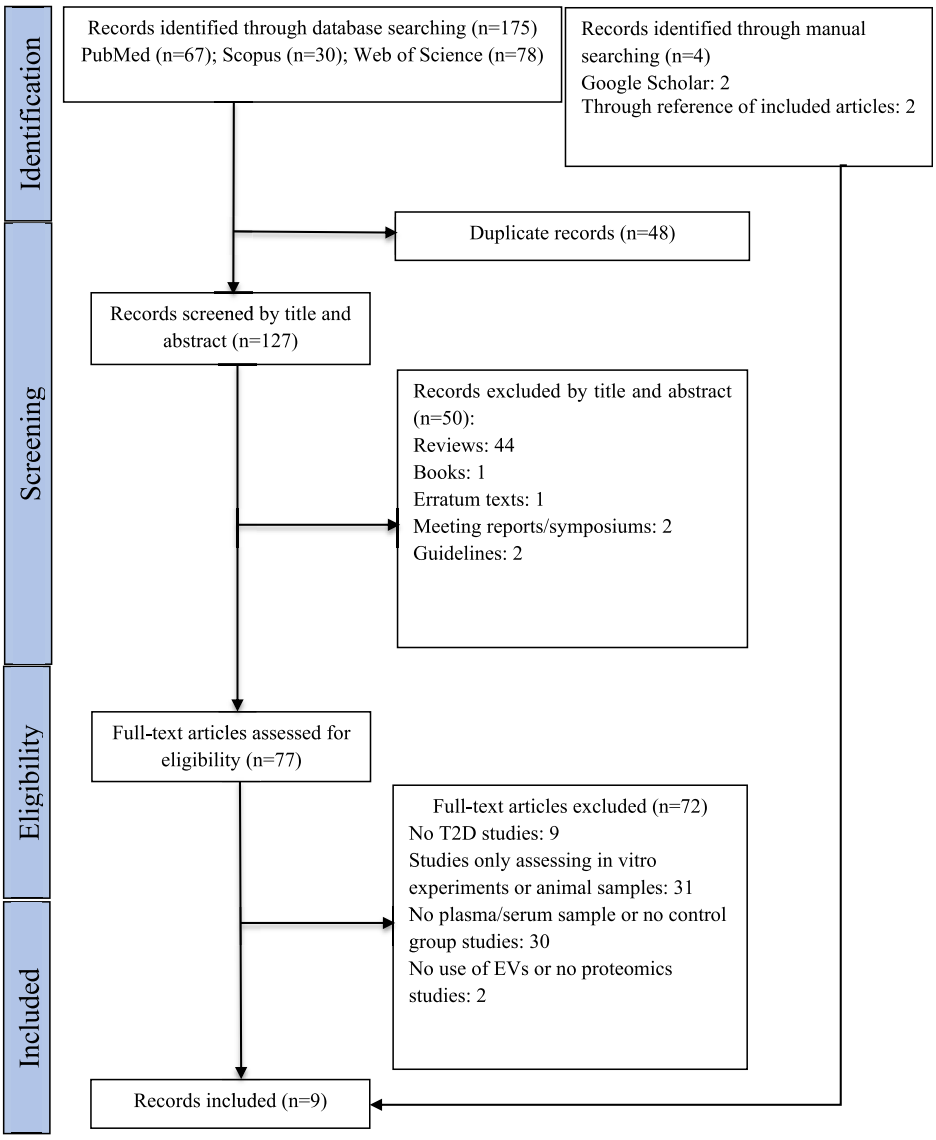

Fig. 1. PRISMA flowchart illustrating the selection of studies for systematic review of plasma and serum extracellular vesicles proteome in Type 2 diabetes. Adapted from the PRISMA guideline 2020.

configured group names, participant count in each group, vesicle isolation method, proteomic analysis method, characterization methods (electron microscopy, nanoparticle tracking analysis, and Western blot), number and names of identified proteins, proteins exhibiting differential abundance after patient group comparisons, and the primary conclusions of the studies. The names of the identified proteins were standardized according to the Universal Protein Resource (UniProt) database (uniprot.org) [14].

2.6. Bioinformatics analysis

Proteins displaying altered abundance in plasma or serum (P/S) EVs from patients with T2D underwent Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and Over-Representation Analysis (ORA). These analyses aimed to unveil the potential biological functions of genes and were conducted using the R package clusterProfiler. For graphical representation, the circlize, enrichplot, and pathview packages were employed.

Additionally, the proteins underwent analysis for protein-protein interactions (PPI) networks using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING version 11, string-db.org) [15]. Subsequently, the PPI network generated by STRING was visualized in Cytoscape (v3.9.1, Cytoscape Consortium, CA, USA) [16]. Within Cytoscape, Reactome pathway analysis [17] and KEGG [18] pathway enrichment analysis were conducted to illustrate potential gene function at the genomic and molecular level. Initially, all analysis results were filtered with a p-value <0.01, and instances where a false discovery rate (FDR) of <0.05 was achieved led to the inclusion of Reactome pathways, GO, and KEGG terms as significantly enriched.

3. Results

3.1. Literature search and selection

The flowchart depicting the literature search is presented in Fig. 1. The initial search strategy identified a total of 175 studies from the PubMed, Scopus, and Web of Science databases. Following the elimination of 48 duplicate articles, the titles and abstracts of 127 records were assessed, resulting in the exclusion of 122 studies that were not pertinent to the objectives of this systematic review. Consequently, five studies were included in the review based on the predefined search strategy [19–23]. Additionally, an additional four studies were meticulously selected through a manual search on Google Scholar and by examining references from previously included articles [24–27].

Table 1
General characteristics of the selected studies.

Study	Country	Design	Cohort (n) (% male)	Tests for T2D status assessment	Age of groups that included individuals with T2D (Mean of years)
Vestad et al., 2021	Denmark	Cross-sectional	HIV (21) (95); HIV + T2D (16) (88); T2D (14) (71); Healthy Controls (20) (90)	FPG (mmol/L), HbA1c (mmol/mol)	57.5 (53–62)
Wu et al., 2020	USA	Longitudinal	T2D (39) (33.3); Healthy Controls (19) (26.3)	Previous report of T2D diagnosis, current medication for T2D, FPG (>125 mg/dL)	52.4 ± 8.6
Jalal et al., 2021	USA	Cross-sectional	CKD (9, half with T2D) (69); Post-transplant CKD (9) (50); Healthy Controls (9) (25)	Previous reports of controlled T2D	59 ± 12
Xiao et al., 2021	China	Cross-sectional	T2D (5) (100); PDR + T2D (5) (100); Healthy Controls (5) (100)	Previous report of T2D diagnosis, HbA1c (%), random-blood glucose (mmol/L)	64.4 ± 5.4
Chen et al., 2021	China	Cross-sectional	DK + PDR + T2D (9) (66.6); Healthy Controls (9) (NA)	Previous report of T2D diagnosis (>10 years). FPG (mmol/L), HbA1c (%)	56.11 ± 9.2
Masi et al., 2021	Brazil	Cross-sectional	T2D (7) (100); GI (7) (100); Healthy Controls (7) (100)	Newly diagnosed T2D according to American Diabetes Association guidelines (2018), FPG (≥126 mg/dL), OGTT (≥200 mg/dL), HbA1c (≥6.5 %)	44.5 ± 0.6
Nunez et al., 2022	USA	Cross-sectional	T2D (10) (50), Prediabetes (10) (50), Healthy Controls (10) (50)	American Diabetes Association guidelines (2014), HbA1C (%) FPG (mg/dL), glucose AUC (mg/dL·min)	50.7 ± 11
Xu et al., 2016	China	Cross-sectional	T2D (8) (100), Healthy Controls (8) (100)	Newly diagnosed T2D according to WHO criteria (2015), FPG (mmol/L), HbA1C (%)	53.5 ± 10.4
Marei et al., 2022	Qatar	Cross-sectional	T2D (20) (30), CCAD + T2D (20) (60), ACS + T2D (20) (75), Healthy Controls (20) (20)	Previous report of T2D diagnosis, FPG (mmol/L), HbA1c (mmol/mol)	58.7 ± 8.7

Abbreviations: Acute coronary syndrome (ACS); chronic kidney disease (CKD); chronic coronary artery disease (CCAD); diabetic keratopathy (DK); fasting plasma glucose (FPG); glucose area under the curve (AUC); glucose intolerant (GI); Hemoglobin A1c also known as glycated hemoglobin (HbA1c); Human immunodeficiency virus (HIV); Not available (NA); oral glucose tolerance test (OGTT); proliferative diabetic retinopathy (PDR); type 2 diabetes (T2D).

The nine eligible studies, spanning the years 2016–2022, all incorporated a T2D group alongside a control group. However, some studies also included participants with additional clinical conditions. Specifically, three studies encompassed individuals with pre-diabetes [22,25,26], two investigations focused on diabetic ocular complications [23,24], one study examined the Human Immunodeficiency Virus (HIV)-T2D binomial in a single group [21], another article explored chronic kidney disease, wherein nearly half of the subjects had diabetes [19], and one study featured two groups, one with individuals having T2D and cardiovascular complications and another with T2D individuals without complications [20].

The nine studies included a total of 182 participants with T2D and 107 control subjects in the proteomic analysis. In terms of the countries where the articles were published, China [23,24,27] and the USA [19,22,26] each conducted three studies, while Brazil [25], Denmark [21], and Qatar [20] each contributed one investigation. Regarding T2D definition, three of the nine studies adhered to ADA or WHO criteria [25–27], while six studies did not specify the criteria used. Notably, eight of the studies were cross-sectional in nature, with only one study employing a longitudinal design [22]. The general characteristics of each study are summarized in Table 1.

3.2. Risk of bias assessment

The quality and risk of bias in the included studies were evaluated using the AXIS tool [13], and the summarized results are presented in Table 2. In general, the primary limitations across studies that could introduce biases included the cross-sectional study design [28], observed in Refs. [19–21,23–27], a relatively small number of study participants [29], noted in Refs. [19,23–27], and the inclusion of participants not only with T2D but also with various comorbidities and stages of therapy. The latter was not intentionally planned by several studies, introducing potential confounding factors [30], as seen in Refs. [19–21,23,24]. Additionally, difficulties in correctly matching T2D patients and control participants led to observed differences in sex, age, and anthropometric measures between the control and patient groups in some records [19,20,24,27].

Other potential sources of bias included variations in the volume of blood plasma/serum obtained from each participant and the methods used to isolate EVs, both impacting the recovery and purity of these particles [31]. This often limited the study objectives to the analysis of samples with a sufficient concentration of EVs for use in proteomics and other functionalization assays. However, for the purposes of this review, all nine records were deemed reliable for further exploration.

3.3. Isolation and characterization of plasma/serum EVs

Blood plasma served as the primary source of EVs in the reviewed studies. EDTA was utilized to obtain plasma in six articles, with heparin and citrate plasma each employed in one study. Serum was collected for EV isolation in only one study (Table 3). Subsequently, EV isolation from plasma/serum was achieved through precipitation methods to enrich for EVs in three studies [20,22,25], centrifugation methods in three investigations [19,23,27], size exclusion chromatography in two studies [21,24], and non-antibody affinity beads in one article [26].

The characterization of EVs involved the typical assessment of morphology, size distribution, and protein markers [9]. Five out of the nine studies evaluated EV morphology using transmission electron microscopy (TEM) [21–25], while one study employed scanning electron microscopy (SEM) [26]. Three studies did not provide electron microscopy results [19,20,27]. Additionally, six studies utilized the nanoparticle tracking analysis (NTA) technique to confirm EV size [21–26]. The results indicated that EVs varied in size, ranging from 30 nm to 230 nm in diameter, with five studies reporting EV sizes $\leq$200 nm [21,22,24–26]. Only one study made a distinction between small extracellular vesicles (sEVs, $<$200 nm) and large extracellular vesicles (lEVs, $>$200 nm) [23].

Western blot (WB) was the preferred method for detecting EV markers, with seven articles reporting this characterization [19–25]. CD9, CD63, and TSG101 were the commonly utilized markers across studies [19,21,23–25]. Additionally, four studies assessed negative EV markers, including calnexin, GM130, and Apo-1, serving as control markers for EV contaminants. Further details can be found in Table 3.

3.4. Plasma or serum EV proteins of patients with T2D

The results of the proteomic analysis of T2D P/S EVs conducted in the nine selected studies are summarized in Table 4. All nine studies undertook proteomic analyses, with liquid chromatography-tandem mass spectrometry (LC-MS/MS) being the predominant method. Following a thorough examination of the selected records, we consolidated the key findings related to EV proteomics, including the number of proteins reported in each article. Five of the nine articles validated their reported proteins by cross-referencing with public databases and repositories of proteins found in EVs. Three of these articles compared their findings with the ExoCarta database (http://www.exocarta.org/) [23–25], while two studies reviewed their data using the Vesiclepedia database (http://microvesicles.org/) [21,26].

We also investigated proteins reported in different articles with variable concentrations; whenever this information was available, we gathered the number and names of the proteins identified in the studies. All nine studies considered a fold change estimate, and although the full results of this analysis were not always reported, we reviewed the EV proteins studied in the articles (see Table 4) and compiled the differentially abundant proteins in EVs of patients with T2D, compared to controls in Table S1. A total of 426 proteins were reported, counting the full-length research articles and their supplementary information. This number includes some proteins mentioned in two or more selected studies, as described below. In addition to the 426 proteins, only one of the nine articles identified 191 phosphorylated proteins (Table S1). Therefore, no inter-article comparisons regarding phosphorylated proteins were relevant to this systematic review.

Table 2
Appraisal tool for Cross-Sectional Studies (AXIS) checklist.

Study	Was the research question or objective in this paper clearly stated?	Was the study population clearly specified and defined?	Was the participation rate of eligible persons at least 50 %?	Were all the subjects selected or recruited from the same or similar populations?	Was a sample size justification, power description, or variance and effect estimates provided?	For the analyses in this paper, were the exposure(s) of interest measured prior to the outcome (s) being measured?	Was the timeframe sufficient so that one could reasonably expect to see an association between exposure and outcome if it existed?
Vestad et al., 2021	✔	✔	✔	✔	×	✔	×
Wu et al., 2020	✔	✔	✔	✔	×	✔	×
Jalal et al., 2021	✔	✔	✔	✔	×	✔	×
Xiao et al., 2021	✔	✔	✔	✔	×	✔	×
Chen et al., 2021	✔	✔	✔	✔	×	✔	×
Masi et al., 2021	✔	✔	✔	✔	×	✔	×
Nunez et al., 2022	✔	✔	✔	✔	×	✔	×
Xu et al., 2016	✔	✔	✔	✔	×	✔	×
Marei et al., 2022	✔	✔	✔	✔	×	✔	×

Quality was rated as 0 for poor (0–4 out of 14 questions), i for fair (5–10 out of 14 questions), or ii for good (11–14 out of 14 questions); NA: not applicable, NR: not reported.

The key findings from the nine selected articles for this study are presented in Table 4, highlighting the differences observed in the levels of proteins contained in the EVs of T2D patients compared to healthy controls. In certain cases, the protein content of EVs was correlated with T2D status, as indicated by markers such as fasting plasma glucose (FPG), A1c, HOMA-B, as well as indicators of the development and progression of diabetes complications, including ocular, liver, and nephrotic complications. Inflammation, platelet activation, coagulation, endothelial dysfunction, and angiogenesis emerged as pathophysiological processes highly related to the P/S EV proteins of T2D patients. Based on this information, the authors proposed that specific proteins in P/S EVs could hold pathophysiological relevance in diabetes and its complications.

3.5. Differentially abundant proteins were reported more than once in the selected articles

All identified P/S EV proteins reported more than once in the selected articles are listed in Table S2. The change (increase or decrease in level) of proteins within the EVs of T2D subjects compared to those from healthy controls is also detailed. Specifically, 18 proteins were reported in two studies: Cystatin M/E/6 (CST6), Complement decay-accelerating factor (CD55), Insulin-like growth factor-binding protein 4 (IGFBP4), Pro-cathepsin H (CTSH), Protein S100-A8 (S100A8), Fibrinogen-like protein 1 (FGL1), Hemoglobin subunit alpha (HBA1), Ras suppressor protein 1 (RSU1), Thymidine phosphorylase (TYMP, also named Platelet-derived endothelial cell growth factor, PD-ECGF), Integrin alpha-IIb (ITGA2B, also called Platelet Membrane Glycoprotein IIb, with antigen identification CD41), Alpha-2-antiplasmin (SERPINF2), Triosephosphate isomerase (TPI1), Inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2), Clusterin (CLU), Dual specificity mitogen-activated protein kinase 2 (MAP2K2), Receptor-type tyrosine-protein phosphatase C (PTPRC), Ceruloplasmin (CP), and Fermitin family homolog 3 (FERMT3). The S100-A9 (S100A9) protein was reported on three occasions.

Among the mentioned proteins, three proteins (CST6, CD55, and HBA1) in P/S EVs were significantly upregulated in T2D patients compared to a healthy control group (see Table S2). FGL1 was the only protein reported in two studies with significant downregulation in P/S EVs of T2D patients compared to controls without diabetes [24,26], one of which included patients with diabetic keratopathy and proliferative diabetic retinopathy [24]. S100A8 and S10A9 proteins exhibited upregulation in P/S EVs from individuals with T2D compared to control participants without diabetes [27]. Although other authors observed differences compared to healthy controls, S100A8 and S10A9 appear to decrease initially and then significantly increase their levels as diabetes progresses to proliferative diabetic retinopathy [23]. Furthermore, an increase in phosphorylated S100A9 protein was detected in the P/S EVs of T2D patients compared to healthy controls well matched for age, sex, and body mass index [26].

In one of the nine articles, Nunez et al. observed a slight upregulation in the levels of the RSU1 protein in the EVs of patients with T2D compared to individuals without diabetes [supplementary data of [26]]. However, Xu and his colleagues only detected this protein in the P/S EVs of T2D patients but not in the P/S EVs of the control group [27]. Notably, Nunez et al. included patients with T2D who were either not receiving medication or were on early treatment with a medication that could include insulin [ClinicalTrials.gov, ID: NCT02226640 [26]], whereas Xu et al. only included newly diagnosed T2D subjects [27].

Regarding the PD-ECGF protein, Nunez and colleagues observed that PD-ECGF was downregulated in the P/S EVs of T2D patients without apparent macrovascular or microvascular disease compared to subjects with normal glucose tolerance [26]. This finding contrasts with Marei et al. who initially reported no changes in the abundance of PD-ECGF in T2D patients without complications but later disclosed a significant decrease in its concentration between patients with T2D and acute coronary syndrome [20]. Furthermore, Jalal et al. investigated T2D patients with chronic kidney disease (CKD) and found that IGFBP4 and CTSH were upregulated [19]. In

For exposures that can vary in amount or level, did the study examine different levels of the exposure?	Were the exposure measures (independent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?	Was the exposure(s) assessed more than once over time?	Were the outcome measures (dependent variables) clearly defined, valid, reliable, and implemented? consistently across all study participants?	Were the outcome assessors blinded to the exposure status of participants?	Was loss to follow-up after baseline 20 % or less?	Were key potential confounding variables measured and adjusted statistically for their impact on the relationship? between exposure(s) and outcome(s)?	Summary Quality
✔	✔	×	✔	NR	NA	✔	i
✔	✔	✔	✔	✔	NA	✔	i
✔	✔	×	✔	NR	NA	✔	i
✔	✔	×	✔	NR	NA	✔	i
×	✔	×	✔	NR	NA	✔	i
✔	✔	×	✔	NR	NA	✔	i
✔	✔	×	✔	NR	NA	✔	i
×	✔	×	✔	NR	NA	✔	i
✔	✔	×	✔	NR	NA	✔	i

contrast, Nunez and colleagues, who studied T2D patients either receiving no medication or undergoing early treatment, observed downregulation of both proteins in T2D patients compared to subjects with normal glucose tolerance [26].

Lastly, ITGA2B, SERPINF2, TPI1, ITIH2, CLU, MAP2K2, PTPRC, CP, and FERMT3 were detected through proteomic and phosphoproteomic approaches. The total protein level of ITGA2B, SERPINF2, TPI1, MAP2K2, and FERMT3 was increased, while ITIH2, CLU, and CP were decreased in the P/S EVs of T2D subjects compared to subjects without diabetes (see Table S2). Total PTPRC (CD45 antigen) protein was identified only in P/S EVs of T2D patients in Xu et al. [27]. Furthermore, in Nunez et al. [26], phosphorylation of ITGA2B, SERPINF2, TPI1, ITIH2, CLU, PTPRC, CP, and FERMT3 was decreased, whereas MAP2K2 phosphorylated protein was increased in P/S EVs of T2D patients compared to subjects with normal glucose tolerance (Table S2).

The proteomic and phosphoproteomic results revealing differentially abundant P/S EVs proteins in T2D patients compared to controls suggest that these proteins may serve as potential biomarkers. However, further investigations are necessary to demonstrate

Table 3
EV isolation and characterization methodology in the included studies.

Study	Sample for EV isolation	EV isolation method	Characterization of EV		
			Electron microscopy	Nanoparticle tracking analysis (nm)	Western blot
Vestad et al., 2021	EDTA plasma	Size exclusion chromatography (Izon Science)	TEM	140–150	CD9, CD63, Hsc70/Hsp70, Calnexin
Wu et al., 2020	EDTA plasma	ExoQuick Exosome precipitation solution (System Biosciences) for proteomic and differential ultracentrifugation (2 times at 120,000 g for 2 h using a Beckman Coulter ultracentrifuge with a sw55ti rotor) for EVs characterization	TEM	200	Alix, FLOT1, CD81, GM130
Jalal et al., 2021	EDTA plasma	Ultracentrifugation (20,000 g for 2.5 h using a Beckman XL-80 ultracentrifuge with a sw55ti rotor)	N/A	N/A	CD63, CD83, TSG101, Calnexin
Xiao et al., 2021	EDTA plasma	Ultracentrifugation (2 times at 110,000 g for 2 h using a Beckman Coulter ultracentrifuge with a sw41ti rotor)	TEM	sEVs 127.2; lEVs 230.1	CD9, CD63, TSG101, Apo-A1
Chen et al., 2021	EDTA plasma	Size exclusion chromatography (Izon Science)	TEM	75–100	CD63, TSG101
Masi et al., 2021	EDTA plasma	miRCURY Exosome Isolation Kit-Serum and Plasma (Exiqon)	TEM	30–150	CD9, CD63, CD81, HSP70
Nunez et al., 2022	Serum	EVTrap, non-antibody-based affinity technology (Tymora Analytical)	SEM	100–200	N/A
Xu et al., 2016	Citrate plasma	Centrifugation (2 times at 20,000 g for 0.5 h)	N/A	N/A	N/A
Marei et al., 2022	Heparin plasma	ExoQuick-LP. Lipoprotein pre-clear and Exosome isolation (System Biosciences)	N/A	N/A	CD14, CD16, CD41

Abbreviations: Large extracellular vesicles (lEVs); not available (N/A); scanning electron microscope (SEM); small extracellular vesicles (sEVs); transmission electron microscopy (TEM).

Table 4
Proteomic results of plasma/serum EVs in the nine included studies.

Study	Techniques involved in proteomics	Total number of proteins detected by proteomics	Proteins in ExoCarta (% of the total proteins they detected)	Top 100 exosome proteins in Vesiclepedia (% of the total proteins they detected)	Number of differentially abundant proteins in plasma/serum EVs of groups with T2D patients	Highlighted proteins in results and/or discussion sections of articles	Results regarding highlighted proteins	Main results and conclusions of the articles
Vestad et al., 2021	Liquid chromatography-tandem mass spectrometry (LC-MS/MS)	558	N/A	60 (10.75)	161	O43866 (CD5L) Q08380 (LGALS3BP)	In HIV-positive patients with type 2 diabetes (T2D), CD5L and LGALS3BP levels were elevated compared to controls. Additionally, CD5L was higher in T2D patients without HIV compared to controls. The authors propose that CD5L may play a crucial role in recognizing microbial components and regulating inflammatory responses associated with infection, atherosclerosis, and cancer. Moreover, they referenced evidence linking elevated LGALS3BP levels to long-term mortality in coronary artery disease.	In patients with both HIV and type 2 diabetes (HIV + T2D), higher plasma concentrations of EVs were observed compared to the control group without diabetes. This finding was correlated with elevated levels of plasma lipopolysaccharides, triglycerides, and the Framingham score, although not associated with alterations in gut microbiota. Proteomic analysis identified 558 human proteins, predominantly linked to genes associated with cardiometabolic diseases. Notably, there was a downregulation of inflammatory pathways, including IL-6 and IL-1β, along with 30 bacterial proteins, mainly originating from lipopolysaccharide-producing Proteobacteria. The study supports the notion that EVs are implicated in microbial translocation processes in HIV + T2D patients. The proteomic content suggests a potential contribution to low-grade inflammation and the development of cardiovascular risk.
Wu et al., 2020	Olink proteomics biomarker inflammation panel using proximity extension assay technology (Olink Proteomics)	33	N/A	N/A	7	P25942 (CD40) P15692 (VEGFA)	In a longitudinal study, EV CD40 levels were found to be significantly associated with the transition from an euglycemic state to a diabetic diagnosis in individuals. The authors suggested that the content of CD40 in EVs may serve as a predictor for vascular disease in diabetes. Additionally, EV VEGFA levels showed associations with T2D	In patients with T2D, elevated levels of inflammatory proteins were observed in plasma EVs compared to the control group without diabetes. The authors presented preliminary data suggesting that the inflammatory protein content of EVs from T2D patients may have functional effects on endothelial cells, including alterations in cell morphology and migratory behavior. These findings imply

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							status, as well as with HOMA-B and HOMA-IR.	that plasma EVs play a significant role in the peripheral vascular disease associated with T2D and propose that plasma EVs could be a valuable diagnostic tool for the disease.
Jalal et al., 2021	SOMAscan assay (SomaLogic Operating Co.)	60	N/A	N/A	42	P00746 (CFD) P00797 (REN) P61769 (B2M) P07478 (PRSS2) P01034 (CST3) P20333 (TNFRSF1B) O43915 (VEGFD) P52799 (EFNB2) P39060 (COL18A1)	In patients with chronic kidney disease (CKD), the levels of CFD, REN, B2M, PRSS2, CST3, TNFRSF1B, VEGFD, EFNB2, and COL18A1 were significantly elevated in extracellular vesicles (EVs) compared to healthy controls. Among these, CST3 and B2M demonstrated correlations with the ratio uACR, a surrogate marker of endothelial dysfunction, which serves as a clinical indicator of vascular disease and CKD progression. Additionally, both CST3 and B2M showed negative correlations with the estimated glomerular filtration rate (eGFR).	Angiogenic proteins exhibited elevated levels in both native and post-transplant CKD patients compared to healthy controls. The Ingenuity Pathway Analysis (IPA) identified Ephrin receptor signaling, serine biosynthesis, and transforming growth factor-β as the main activated pathways in both CKD groups. Notably, pro-inflammatory proteins were higher only in the EVs of native CKD patients, a group comprising 47 % of individuals with T2D). IPA analysis in this case revealed activation of acute phase response signaling, insulin-like growth factor-1, tumor necrosis factor-α, and interleukin-6 pathways. These findings suggest the activation of angiogenesis and inflammation pathways in the plasma and EVs of individuals with CKD, respectively. Common pathways observed in both native and post-transplant CKD may indicate shared cardiovascular disease mechanisms.
Xiao et al., 2021	Liquid chromatography-tandem mass spectrometry (LC-MS/MS)	901	744 (82.57)	N/A	90	P05109 (S100A8) P06702 (S100A9) O95379 (TNFAIP8)	As proliferative diabetic retinopathy (PDR) progresses, the levels of S100A8, S100A9, and TNFAIP8 in small plasma EVs increase compared to healthy controls. Notably, these proteins were lower in the T2D without PDR group than in controls. The authors suggest that the reduced protein secretion in sEVs may	In diabetic retinopathy (DR), higher levels of TNFAIP8 were observed in both plasma sEVs and vitreous compared to healthy controls. TNFAIP8 was found to stimulate cell migration, tube formation, and cell viability in HRMEC, while depletion of TNFAIP8 resulted in reduced HRMEC proliferation. Functional evaluations suggested that

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							result from microvascular cell apoptosis in the early stages of T2D. The transition from no diabetic retinopathy (no-DR) to PDR is driven by chronic hypoxia and the expression of proangiogenic growth factors, stimulating abnormal proliferation in retinal microvasculature and leading to active exosomal protein secretion.	TNFAIP8 could serve as a crucial angiogenic factor in DR. The study demonstrated that TNFAIP8, present in plasma EVs, could potentially act as a biomarker for diabetic retinopathy.
Chen et al., 2021	High-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS)	952	245 (25.73)	N/A	28	Q14254 (FLOT2) P80108 (GPLD1) P10909 (CLU) P00450 (CP) Q06033 (ITIH3) O14646 (CHD1) P20742 (PZP) Q6UX06 (OLFM4) P08697 (SERPINF2) P0C0L5 (C4B) P55058 (PLTP) P19823 (ITIH2) Q14573 (ITPR3) Q9UPT5 (EXOC7)	FLOT2, GPLD1, CLU, CP, ITIH3, CHD1, PZP, OLFM4, SERPINF2, C4B, PLTP, ITIH2, ITPR3 are significantly decreased in patients with T2D-related DK compared with non-diabetic controls. Regarding FLOT2, the authors reasoned that FLOT2, as the initial protein of the insulin signaling pathway, is closely linked to the mechanism of T2D + DK. Hence, FLOT2 was selected as the target protein. They confirmed the decrease in FLOT2 and the increase in EXOC7 (a downstream protein of GLUT4) in EVs through Western blot analysis. FLOT2 plays a crucial role in maintaining normal blood glucose conditions, and the upregulation of exosomal FLOT2 protein in T2D + DK potentially has a protective effect on corneal epithelial damage caused by diabetes.	Patients with DK + T2D exhibited lower levels of FLOT2 in plasma EVs ranging from 75 to 100 ηm compared to healthy controls. The authors propose that FLOT2 levels in the plasma EVs of T2D patients could serve as a biomarker for diagnosing and monitoring diabetic keratopathy. Consequently, the normalization of EVs to physiological conditions could be considered a potential approach for the treatment of DK.
Masi et al., 2021	Ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF MS)	48	39 (81.25)	N/A	1	P02787 (TF)	Transferrin (TF) showed elevated levels in T2D patients compared to normal glucose subjects and those with glucose intolerance. The authors suggest that TF,	Individuals with glucose intolerance (GI) exhibited lower levels of IGHG1 and higher levels of ITIH2 in plasma EVs compared to controls. Additionally, individuals with T2D had higher

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							responsible for delivering iron to all cells, experiences increased turnover in T2D due to heightened systemic oxidative stress. The association of increased iron stores with an elevated risk of developing T2D is noteworthy. The liver, a major iron reservoir, exhibits an augmented TF protein in EVs from T2D patients, suggesting its role as the primary organ initiating intercellular communication through EVs at the onset of the disease.	TF in plasma EVs than controls. The authors proposed a potential diagnostic and prognostic biomarker panel, comprising molecules present in plasma EVs, including five miRNAs (miR-141–3p, miR-324–5p, miR-376c-3p, miR-26b-5p, and miR-374b-5p) and three proteins (IGHG1, ITIH2, and TF), for diabetic complications.
Nunez et al., 2022	Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)	2372 proteins; 716 phosphoproteins	N/A	(91)	308 proteins; 191 phosphoproteins	P31749 (AKT1) P49841 (GSK3B) P07948 (LYN) P36507 (MP2K2) Q15746 (MYLK) Q05655 (PRKCD) P07359 (GP1BA) P05556 (ITGB1) P17301 (ITGA2) P23229 (ITGA6) P01903 (HLA-DRA) P08514 (ITGA2B) Q13043 (STK4)	In individuals with T2D, the total protein of the platelet surface marker GP1BA is elevated compared to healthy controls. Phosphoproteins such as AKT1, GSK3B, LYN, MAP2K2, MYLK, and PRKCD also increase in T2D compared to healthy controls. Regarding integrins, phosphorylated ITGA2 and total ITGA6 protein levels increase, while phosphorylated ITGB1 and ITGA2B decrease in T2D compared to healthy controls. Notably, AKT1 phosphoprotein exhibits the largest increase in the phosphoprotein/protein ratio in T2D. The change in phosphorylated AKT1 in circulating EVs was negatively correlated with AIRg, DI, HOMA-B, and Sg, whereas it showed a positive correlation with FPG, glucose AUC, and HbA1c. Conversely, the change in total AKT1 protein was, as	Individuals with T2D showed elevated levels of phosphorylated kinases (AKT1, GSK3B, LYN, MAP2K2, MYLK, and PRKCD) in their serum EVs compared to controls without diabetes, indicating potential dissemination of activated kinases in T2D. The study also revealed decreased total protein levels but an increased phosphorylation status of AKT1 in serum EVs from T2D patients compared to controls without diabetes, possibly preceding activation of CDK1 and PKCδ kinases since prediabetes, contributing to T2D pathogenesis. Additionally, "integrin switching" changes in serum EVs of prediabetes and T2D were identified, a crucial element potentially impacting disease development and complications. The study identified downregulated liver-specific EV proteins in the EV proteome and phosphoproteome in prediabetes

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							expected, negatively correlated with fasting plasma glucose (FPG), glucose area under the curve (AUC), and HbA1c. Similar to phosphorylated AKT1, the change in phosphorylated LYN and PRKCD kinases was also positively correlated with the change in FPG, glucose AUC, HbA1c, and the acute insulin response to glucose (AIRg). Notably, the upstream activation of STK4, a major signaling kinase of the Hippo pathway, appears to be the sole kinase responsible for the increased phosphorylation of MYLK, a critical kinase in the upregulated phosphoprotein network observed in individuals with T2D compared to healthy controls.	compared to controls without diabetes, with similar observations in established T2D patients, suggesting a reduction in EV output from the liver. This reduction could stem from a disrupted endocytic secretory pathway in the early stages of disease development. Additionally, the study noted upregulated EV proteins and phosphoproteins associated with platelet activation, coagulation, chemokine signaling, and oxidative phosphorylation pathways in the initial phases of disease progression.
Xu et al., 2016	Liquid chromatography-tandem mass spectrometry (LC-MS/MS)	496	N/A	60 (10.75)	40	P61224 (RAP1B) P21926 (CD9) P08514 (ITGA2B) P02452 (COL1A1) P05109 (S100A8) P06702 (S100A9)	RAP1B, CD9, and ITGA2B (also known as CD41) exhibited increased levels, while COL1A1 showed a decrease in the EVs of patients with T2D compared to controls without diabetes. The notable 3.5-fold rise in CD41 abundance in T2D EVs was suggested to contribute to arteriosclerosis and the induction of a hypercoagulable state associated with diabetes. Additionally, elevated levels of S100A8 and S100A9 in T2D EVs were proposed to play a crucial role in endothelial dysfunction and other complications in individuals with T2D.	Plasma EV proteins, including S100A8, S100A9, and CD41, in individuals with T2D participate in platelet activation, cell adhesion, and inflammation pathways. This suggests that plasma EVs from T2D patients may be associated with hypercoagulation in T2D subjects and contribute to the progression of diabetic complications.

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Marei et al., 2022	Human angiogenesis proteome profiler array kit (R&D Systems)	55	N/A	N/A	6	P01137 (TGFB1) P02776 (PF4) P05121 (SERPINE1) P07996 (THBS1) P19971 (TYMP) O15123 (ANGPT2) P41159 (LEP) P36955 (SERPINF1)	ANGPT2, TGFB1, PF4, SERPINE1, LEP, SERPINF1, and THBS1 were reduced in the EVs of T2D patients compared to controls without diabetes. Additionally, TYMP (PD-ECGF) exhibited increased levels in T2D patients and those with T2D and chronic coronary artery disease (CAD), while it was decreased in T2D with acute coronary syndrome (ACS). These findings suggest alterations in the angiogenic and antiangiogenic balance, impacting endothelial function, where molecules like PF4 and SERPINE1 inhibit angiogenesis, while PD-ECGF is an activator of angiogenesis. Their findings suggest an imbalance in the abundance of angiogenic factors in the diseased EVs, where all disease groups involved patients with T2D, indicating a potential shift towards a proangiogenic phenotype. Additionally, they emphasized that PD-ECGF, PF4, SERPINE1, THBS1, and primarily TGFB1 inhibit key pathways crucial for normal endothelial function.	Plasma EVs from patients with ACS and diabetes exhibited distinct abundance patterns of angiogenic-related proteins, such as TGFB1, PD-ECGF, PF4, SERPINE1, and THBS1. Ingenuity Pathway Analysis indicated that these angiogenic factors, particularly TGFB1, hinder essential pathways associated with normal endothelial function. Network analysis further confirmed the inhibition of normal endothelial cell function. Notably, DDP-IV was the sole differentially abundant protein in plasma EVs distinguishing ACS from chronic CAD in individuals with T2D.

Abbreviations: acute coronary syndrome (ACS); acute insulin response to glucose (AIRg); glucose area under the curve (AUC); cardiovascular disease (CVD); chronic kidney disease (CKD); coronary artery disease (CAD); diabetic keratopathy (DK); diabetic retinopathy (DR); disposition index (DI); CKD-EPI estimated glomerular filtration rate (eGFR); extracellular vesicles (EVs); fasting plasma glucose (FPG); glucose intolerant (GI); hemoglobin A1c or glycated hemoglobin (HbA1c); homeostasis model assessment of β-cell function (HOMA-β); human immunodeficiency viruses (HIV); human retinal microvascular endothelial cell (HRMEC); ingenuity pathway analysis (IPA); not available (N/A); proliferative diabetic retinopathy (PDR); glucose effectiveness (Sg); type 2 diabetes (T2D); ratio of urinary albumin/creatinine (uACR).

their clinical relevance in a diagnostic or prognostic context.

3.6. Gene Set Enrichment Analysis and Over-Representation Analysis

The 426 differentially abundant P/S EV proteins (Fig. 2A and Table S1), identified through proteomics in this systematic review, underwent Gene Set Enrichment Analysis (GSEA) using the ClusterProfiler R v4.3.1 package. This analysis aimed to identify enriched terms in Gene Ontology (GO) Biological Process (BP), Cellular Components (CC), and Molecular Function (MF) (Fig. 2B), as well as

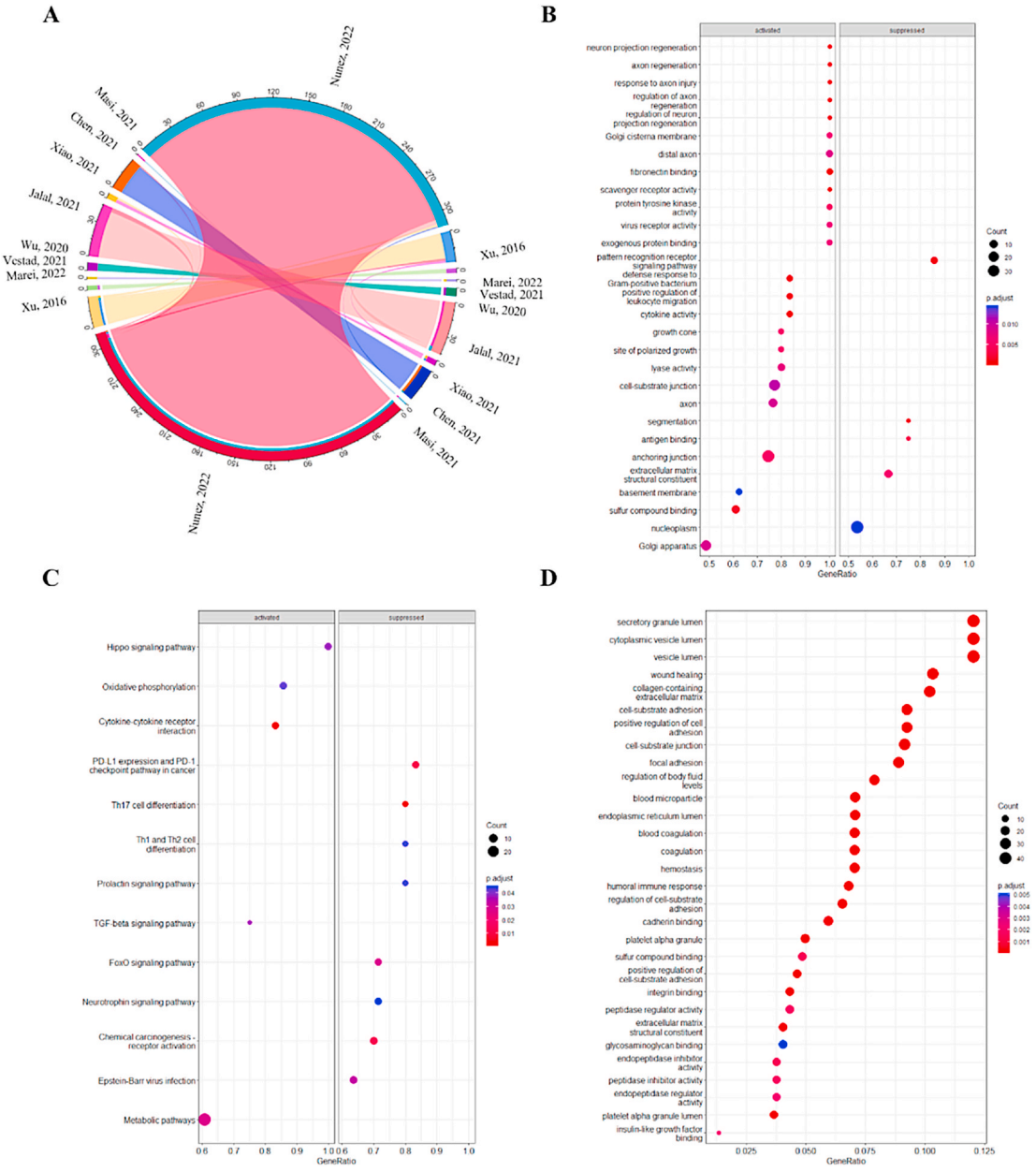

Fig. 2. Gene Set Enrichment Analysis conducted on differentially abundant plasma/serum EV proteins in patients with T2D compared to controls. (A) Chord diagram of the 426 differentially abundant proteins stratified by article. (B) GSEA shows the top 10 enriched pathways in GO-sub ontologies: BP, MF, and CC. The circle size represents the number of proteins participating in each pathway and the graph reflects the gene ratio. The color scale indicates the adjusted p-value, while the representation of the activated or suppressed state signifies pathways enriched by up or down-regulated proteins in the P/S EVs from patients with T2D compared to control subjects without diabetes, respectively. (C) Enriched KEGG pathways. (D) Over-Representation Analysis shows the top 10 enriched pathways in GO-sub ontologies BP, MF, and CC. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Kyoto Encyclopedia of Genes and Genomes (KEGG) terms (Fig. 2C and Figs. S1A–C). The Over-Representation Analysis (ORA) highlighted the top 10 enriched terms for GO sub-ontologies BP, MF, and CC (Figs. 2D and 3A–D). Dot plot graphs stratified the pathways enriched by up or down-regulated proteins identified in T2D patients compared to control subjects without diabetes, represented as activated or suppressed, respectively, and ranked by Normalized Enriched Score (NES) and p-value.

Furthermore, in ORA describing the top enriched pathways of GO sub-ontologies BP, MF, and CC, the included studies revealed significant associations with terms such as secretory granule lumen, cytoplasmic vesicle lumen, focal adhesion, vesicle lumen, blood microparticle, wound healing, blood coagulation, coagulation, humoral immune response, hemostasis, cadherin binding, integrin binding, and extracellular matrix structural constituent (Figs. 2D and 3A–D). These enriched terms provide valuable insights into the potential functional roles and biological processes associated with the identified P/S EV proteins in the context of T2D pathophysiology. The systematic review underscores the importance of further investigations to validate these proteins as potential biomarkers and unravel their clinical relevance in the diagnosis, prognosis, and management of T2D and its complications.

The ORA of GO sub-ontologies Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) reveals potential functional roles of P/S EV proteins in T2D pathophysiology. Enriched CC terms include components of vesicles, focal adhesion, and blood microparticles, while BP terms highlight involvement in wound healing, blood coagulation, immune response, and hemostasis. Enriched MF terms suggest roles in cadherin and integrin binding, and extracellular matrix structural constitution. These findings underscore the complex intercellular communication network mediated by P/S EV proteins and emphasize the need for further research to validate their potential as biomarkers and understand their functional significance in T2D and associated complications.

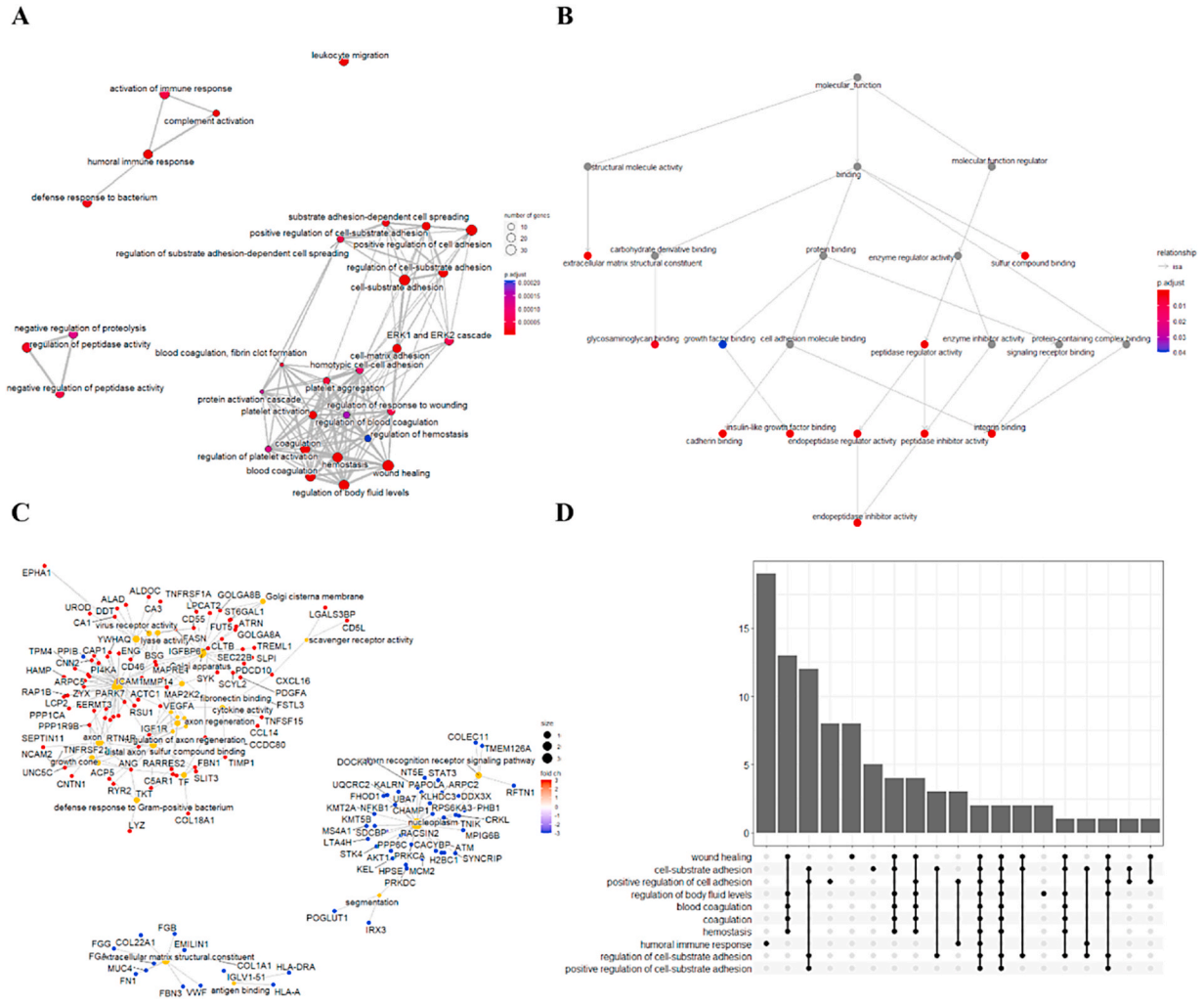

Fig. 3. Over-Representation Analysis of differentially abundant plasma/serum EV proteins in patients with T2D compared to controls. (A) Emapplot shows the association between the top 10 enriched pathways of GO sub-ontologies BP, MF, and CC. (B) Goplot represents the significant GO MF terms and their distribution across the GO graph. (C) Cnetplot illustrates the up-regulated and down-regulated proteins in the P/S EVs from patients with T2D compared to control subjects without diabetes belonging to the enriched pathways in A. (D) UpSet plot displays common elements of the top 10 pathways over-represented in GO-ORA.

3.7. Protein-protein interaction (PPI) network analysis

We utilized STRING to perform a PPI analysis on 200 upregulated P/S EV proteins identified in T2D patients compared to control subjects without diabetes from the nine selected records. Setting a high interaction score threshold of 0.900 resulted in a PPI network with 200 nodes and 54 edges. The average node degree was 0.54, the average local clustering coefficient was 0.244, and the p-value for PPI enrichment was <6.29e-09 (Fig. 4A). Furthermore, the PPI-KEGG pathways enrichment analysis identified seven significantly enriched pathways, including hypertrophic cardiomyopathy (hsa05410), platelet activation (hsa04611), cardiac muscle contraction (hsa04260), Fc epsilon RI signaling pathway (hsa04664), dilated cardiomyopathy (hsa05414), hematopoietic cell lineage (hsa04640), and adrenergic signaling in cardiomyocytes (hsa04261), as illustrated in Fig. 4B. As a result, the Reactome pathways analysis highlighted the seven most significant pathways associated with the upregulated P/S EV proteins in T2D patients compared to control subjects without diabetes. These pathways include metal sequestration by antimicrobial proteins (HSA-6799990), activation of matrix metalloproteinases (HSA-1592389), regulation of complement cascade (HSA-977606), platelet degranulation (HSA-114608), ER-phagosome pathway (HSA-1236974), oncogenic MAPK signaling (HSA-6802957), and integrin cell surface interactions (HSA-216083), as depicted in Fig. 4C.

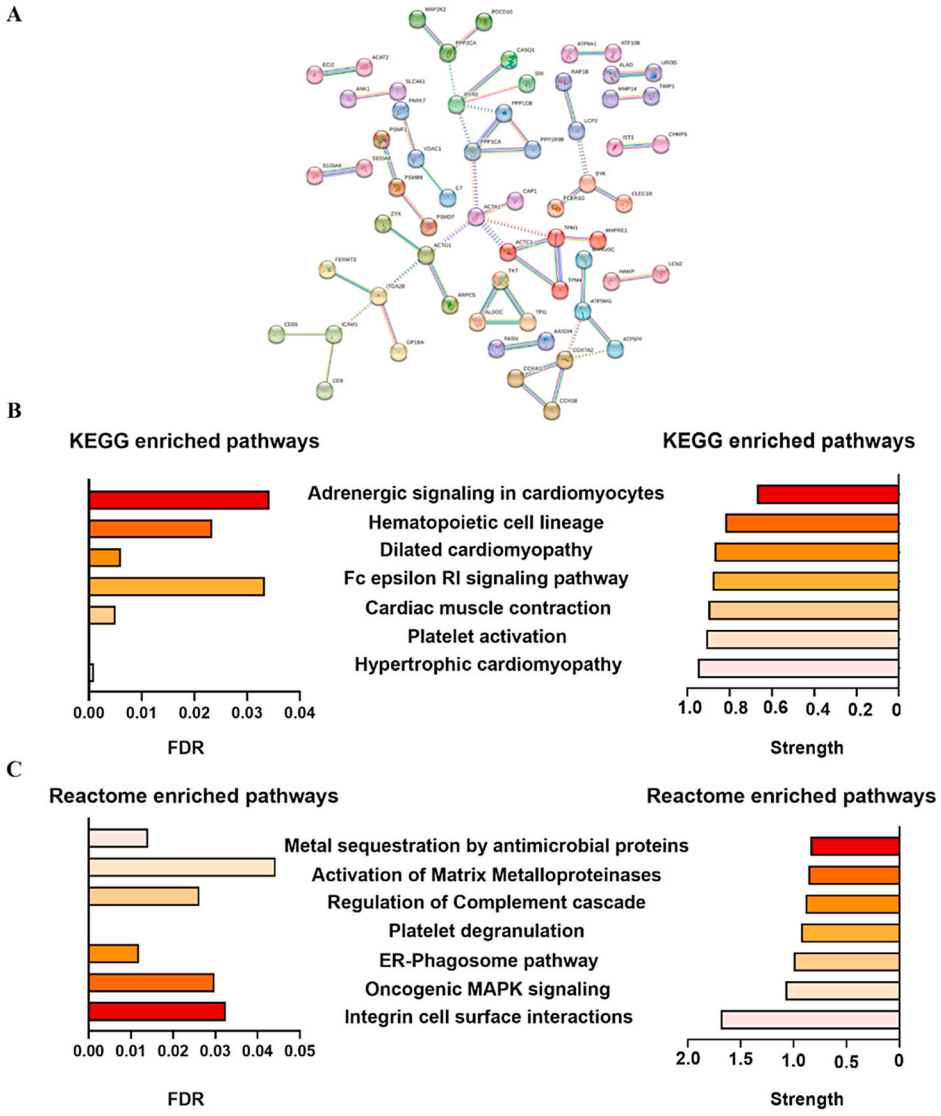

Fig. 4. Protein-Protein Interaction (PPI) Analysis focusing on up-regulated plasma/serum EV proteins in patients with T2D compared to controls. (A) PPI network is displayed for the up-regulated proteins (node number = 200) derived from the differentially abundant proteins of P/S EVs from patients with T2D compared to control subjects without diabetes. The network was established using STRING with MCL clustering and an inflation parameter of 2.4. (B) Top seven enriched KEGG pathways represented by FDR and Strength. (C) Top seven enriched Reactome pathways represented by FDR and Strength.

3.8. Comparison of the results of the GO and KEGG enrichment analysis of the present review with the selected articles

In assessing the cellular compartments and molecular functions of differentially abundant P/S EV proteins in T2D patients, we compiled results from GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses reported in the selected articles. Out of the nine studies, six conducted GO and/or KEGG pathway analyses, with four specifically evaluating cellular component (CC) analysis. Notably, terms such as blood microparticle (GO:0072,562) and focal adhesion (GO:0005925) were consistently enriched across multiple studies, reflecting the expected characteristics of P/S EVs. These findings suggest standardized practices in EV characterization. Furthermore, specific CC terms like cell-substrate junction (GO:0030,055), secretory granule lumen (GO:0034,774), cytoplasmic vesicle lumen (GO:0060,205), vesicle lumen (GO:0031,983), and collagen-containing extracellular matrix (GO:0062,023) were reported in studies evaluating significant enriched CC terms. This comprehensive analysis provides insights into the cellular and molecular context of differentially abundant proteins in P/S EVs of T2D patients. Regarding the molecular function (MF) terms obtained in our analysis (Fig. 2B–D), several terms were identified across the selected studies. Integrin binding (GO:0005178) [23], peptidase regulator activity (GO:0061,134) [23], endopeptidase inhibitor activity (GO:0004866) [23,24], peptidase inhibitor activity (GO:0030,414) [23,24], endopeptidase regulator activity (GO:0061,135) [23,24], and antigen-binding (GO:0003823) [27] were consistently reported in our list and found in at least one of the three articles that performed MF term evaluations. These findings highlight specific molecular functions associated with differentially abundant proteins in plasma/serum extracellular vesicles of T2D patients, providing valuable insights into potential mechanisms and roles of these proteins in T2D pathophysiology. BP term enrichment analysis was conducted as part of our gene ontology assessment of the collected data. Our analysis disclosed the enrichment of biological process (BP) terms, specifically noting the recurrence of "blood coagulation" (GO:0007596) [27] and "humoral immune response" (GO:0006959) [23] among the included studies.

The primary aim of the KEGG database is to attribute functional implications to genes, encompassing both molecular and higher-level associations [18]. In our investigation, seven enriched KEGG pathways (Figs. 2C and 4B, and Fig. S1) were identified in at least one of the five articles included in our study. Notably, "platelet activation" (hsa04611) [23,26,27] and "complement and coagulation cascades" (hsa04610) [23,24,26] were recurrent, mentioned three times each. Furthermore, pathways like "AGE-RAGE signaling pathway in diabetic complications" (hsa04933) [25], "hypertrophic cardiomyopathy" (hsa05410) [26], "hematopoietic cell lineage" (hsa04640) [27], "dilated cardiomyopathy" (hsa05414) [26], and "hippo signaling pathway" (hsa04390) [25] were also documented.

4. Discussion

Diabetes presents a substantial global health challenge characterized by chronic hyperglycemia [32]. This condition carries the potential for severe vessel and nerve damage, resulting in disabling complications [33], and may even elevate the risk of cancer [34]. Type 2 Diabetes (T2D), the most prevalent form, still lacks a comprehensive understanding of its intricate mechanisms that contribute to systemic complications. Although factors such as insulin resistance and hyperglycemia are recognized, alterations in cellular communication may also play a significant role [35,36].

4.1. Circulating EV proteomic studies in T2D

Emerging evidence underscores the pivotal role of EVs in the progression of T2D, with a specific focus on their protein content as scrutinized through proteomic studies [19–27,35–38]. In this context, we systematically compiled and analyzed these studies, elucidating EV isolation and characterization methods, proteomic analyses, and their implications for T2D pathophysiology. By identifying EV proteins altered in diabetes and its complications, we illuminate potential biomarkers and therapeutic targets. Furthermore, leveraging bioinformatics, we investigated the pathways implicated in T2D that are targeted by EV proteins. Notably, we observed that, alongside the methodology employed in proteomic analysis, the isolation of EV samples represented a critical challenge essential for achieving robust research practices and objectives.

In our analysis, we identified diverse methodologies employed in proteomics, with MS-based methods emerging as the most prevalent for proposing new biomarkers and yielding the highest number of proteins. However, within the domain of proteomic studies, EV samples often encounter challenges, as the isolation method may introduce biases into experiments [31]. Specific isolation techniques, such as size exclusion chromatography and immune/nonimmune affinity-based methods, are noted for providing higher purity of recovered proteins compared to others, thus becoming favored methods for proteomic biomarker discovery due to low protein contamination and minimal co-purified material [39–41]. Studies by Vestad et al. [21] and Chen et al. [24] employed size exclusion isolation, Nunez and colleagues utilized magnetic beads for EV purification [26], employing a non-immuno-affinity-based method [42], while Marei et al. incorporated a clean-up step to deplete plasma lipoprotein particles before precipitating EVs [20].

However, there is a clear need for further studies to explore more effective EV isolation methods for proteomic purposes. These efforts should not only prioritize performance in terms of yield or purity but should also, when necessary, consider the functional activity of proteins and EVs. In this context, Li et al. have provided a comprehensive review on isolation methods for proteomic studies of EVs [43], offering valuable insights for future investigations aiming to mitigate technical and methodological biases in the biomarker discovery process.

Despite considerable research progress, the heterogeneity of EVs poses a challenge in EV research. Due to the absence of gold-standard EV research methods, the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines were introduced to enhance the reliability and reproducibility of EV studies [9]. Following the ISEV guidelines, EVs should be characterized by assessing size, morphology, marker expression, among other factors. There has been a growing trend, especially after 2018, to adhere

to the ISEV guidelines for EV characterization [44]. Therefore, strict adherence to these characterization methods and reporting results according to the ISEV guidelines is strongly recommended to ensure repeatability and comparability between studies. In the current review, we have compiled a wealth of information associated with these recommendations (see Table 3). However, there remains a significant need for further studies to strive for normalized EV characterization, following the established standardization guidelines for EV studies. While some analyzed studies did not disclose the total number of detected proteins, reporting various differentially abundant proteins in EVs offers specific and detailed insights, significantly contributing to our understanding of the association between EVs and T2D. These results indicate that using plasma/serum samples for EV isolation could be a viable approach for detecting protein biomarkers.

Considering the points mentioned, our study has some limitations, particularly concerning the divergence in proteomics results. The distinct study designs, the studied populations, the variety of EV isolation techniques, and the heterogeneity in proteomic assays impede direct comparisons and contribute to divergent results. Each study focused on and analyzed a specific subset of proteins, potentially omitting crucial proteins due to measurement biases. While recognizing the specific research questions addressed in each article, the absence of information on the total proteins found in some studies hampers the identification of a more realistic protein profile. Furthermore, insufficient data and variations in diabetic states and complications among patients hinder more in-depth analyses, such as meta-analyses.

4.2. P/S EV levels of CST6, CD55, HBA1, S100A8, S100A9, and FGL1 proteins in T2D patients

Our analysis revealed recurrently increased levels of P/S EV Cystatin (CST6), CD55, and HBA1 proteins in T2D patients compared to the control group without diabetes (Table S2).

Cystatin M/E/6 (CST6) is a glycoprotein known for its inhibitory activity against cysteine proteases [45,46]. Studies conducted by Jalal et al. and Nunez et al. consistently observed elevated levels of CST6 in plasma/serum EVs from T2D patients compared to individuals without diabetes [19,26]. The first report specifically investigated a group comprising both CKD and diabetic patients. The analysis by Matias-Garcia et al. associated CST6 with glomerular filtration rate (eGFR) in cohorts containing varying diabetes prevalence [47]. CST6 was implicated in eGFR possibly due to its inhibitory function on cysteine proteases like Legumain, which are involved in kidney matrix remodeling [47]. Despite these associations, the specific mechanisms through which CST6 impacts eGFR in T2D remain elusive.

CST6 likely plays a role in the progression of CKD in T2D. Further investigations are needed to clarify its specific role in both CKD and T2D. Functional assays conducted in *in vitro* and *in vivo* models aim to understand the biological function of the protein and its influence on the disease's pathophysiology, and human studies confirming the association between CST6 and eGFR could position this protein as a potential biomarker.

The complement decay-accelerating factor (CD55) is associated with regulating the complement system. CD55 functions by inactivating C3 convertases, breaking them down into their constituent proteins and preventing their assembly [48], thereby averting the formation of the membrane attack complex [49].

In situations such as inflammation or aging of erythrocytes, it has been suggested that the secretion of CD55 in EVs could increase complement-mediated lysis of the EV-secreting cells, potentially leading to cell death [50]. Recent reports have indicated significantly lower CD55 levels in the erythrocytes and leukocytes of T2D patients with nephropathy, retinopathy, and cardiovascular disease compared to controls without diabetes, likely linked to complications [51–53]. Interestingly, the studies by Jalal et al. and Nunez et al. showed elevated CD55 levels in circulating EVs of T2D and CKD patients compared to control subjects without diabetes [19,26]. Subsequent investigations should explore the roles of this complement regulatory protein found on the surface of EVs and determine whether the presence of these proteins in the EVs could be related to the decreased protein levels in the circulating cell membranes of these patients.

Additionally, HBA1, an alpha subunit of hemoglobin present in red blood cells (RBCs), exhibited increased levels in EVs from individuals with T2D compared to controls without diabetes in studies by Nunez et al. and Xu et al. [26,27]. RBCs release EVs during erythropoiesis, cellular aging, and under certain pathological conditions [54,55]. These RBC-derived EVs (RBCEVs) are implicated in the pathophysiological progression of diabetes and genetic hematologic disorders, contributing to coagulopathy [55]. Studies have linked elevated plasma Hb and Hb levels within EVs in diseases like sickle cell anemia and thalassemia intermedia, where increased EV levels correlated with plasma Hb [56]. RBCEVs from T2D patients possess nitric oxide (NO) scavenging properties, potentially contributing to heme-mediated endothelial dysfunction by reducing NO bioavailability [57,58].

T2D patients exhibit low-grade intravascular hemolysis (IVH), potentially linked to increased release of heme-loaded EVs by T2D RBCs [59]. These RBCEVs induce reactive oxygen species (ROS) production and activate thrombin in human endothelial cells, suggesting alterations in volume, cargo, and membrane surface properties [59]. Analogous to hemolytic diseases [56,60,61], RBCEVs in T2D might exacerbate vascular damage and inflammation [59]. The increased HBA1 levels in our review are likely associated with elevated Hb levels in RBCEVs from T2D patients compared to control subjects without diabetes. As the authors have suggested, RBCEVs may promote coagulopathy in T2D. Therefore, HBA1 and Hb in EVs from T2D patients could potentially serve as coagulopathy biomarkers. Studies employing a longitudinal design are needed to assess the levels of these EV proteins over time in patients, aiming to understand their association with the presence and progression of the condition.

Fibrinogen-like protein 1 (FGL1) exhibited significantly lower levels in EVs from patients with uncomplicated T2D, diabetic keratopathy, and proliferative diabetic retinopathy compared to those without diabetes in the control group [24,26]. Notably, the downregulation of FGL1 in both the studies by Chen et al. and Nunez et al. was strikingly similar, with nearly a −1.2-fold decrease. FGL1, a 68 kD protein, is primarily produced by hepatocytes in the liver [62], contributing to hepatocyte mitosis and energy

regulation, including blood glucose levels [63]. This protein is detected as an acute reactant in plasma, influenced by metabolic factors like hyperglycemia and hyperlipidemia, suggesting its potential involvement in other tissues [64,65]. Furthermore, plasma levels of FGL1 are regarded as potential biomarkers for several acute inflammatory, infectious, and autoimmune diseases [66,67].

Delving into the specific roles of FGL1 in EVs is likely to establish this protein as a potential inflammatory biomarker for T2D. Longitudinal studies could analyze FGL1 levels in EVs over time in a cohort of T2D patients, observing how they vary at different stages of the disease and in relation to the progression of complications. It would also be valuable to conduct comparative analyses, contrasting FGL1 levels in EVs with inflammatory biomarkers in T2D patients and those affected by other inflammatory conditions or metabolic disorders.

S100A8 and S100A9 proteins, forming the calprotectin heterodimer, exhibited significant elevation in circulating EVs among individuals with diabetic retinopathy and newly diagnosed T2D patients compared to control subjects without diabetes [23,27]. These proteins have close associations with inflammation, atherosclerosis, angiogenesis, and various diabetic complications, including retinopathy, nephropathy, and myocardial infarction [68,69]. Importantly, *in vitro* studies indicate that hyperglycemia and insulinemia induce S100A8 secretion [70]. Both S100A8 and S100A9 proteins hold potential as biomarkers for prediabetes, T2D, and associated complications [68,69].

In pathological conditions, the mechanisms of S100A8 and S100A9 likely involve antimicrobial, proinflammatory, and prothrombotic properties [71]. Studies suggest their potential therapeutic relevance, including strategies for treating diabetic nephropathy [72]. However, understanding the connection between elevated S100A8, S100A9, and calprotectin levels, persistent hyperglycemia, and their impact on complication development necessitates further investigation. Only a few articles demonstrate the presence of S100A8 and S100A9 in EVs, indicating that these proteins are highly abundant in EVs under inflammatory conditions [73,74]. Xu and colleagues propose that future research could explore strategies to reduce the abundance of S100 EV-enriched proteins as a potential approach to treat various disorders, including diabetic complications [27].

4.3. Bioinformatics analysis of P/S EV proteome in T2D

Differentially abundant proteins underwent GSEA and ORA bioinformatic analysis to identify enriched GO and KEGG terms elucidating their functions in P/S EVs of T2D. Notably, enriched cellular component (CC) terms, such as vesicle lumen, cytoplasmic vesicle lumen, focal adhesion, and blood microparticle, were identified for the compiled proteins from P/S EV samples. This supports the conclusion that high-purity EVs were successfully isolated [21,24,27,75,76]. Additionally, the proteins contributed to enriched molecular function terms, including cadherin binding, antigen binding, and integrin binding, aligning with existing reports [76] on their involvement in EV biogenesis and/or functions [77].

EVs can be released by nearly all cell types, encompassing prokaryotic cells within the human host [21]. They possess the ability to target and elicit responses in various cell types through cell surface receptors or intracellular delivery of inflammatory mediators, receptors, enzymes, antigens, mRNA, and noncoding RNAs [78], thereby stimulating or inhibiting pathways [79]. Notably, our analysis highlighted highly enriched biological processes, such as blood coagulation [27], and terms associated with the humoral immune response [23], which are emphasized in the articles reviewed. The significantly enriched KEGG pathways, consistently reported in the reviewed articles, include platelet activation [23,26,27] and complement and coagulation cascades [23,24,26].

EVs exert functional effects by binding through protein-protein interactions, initiating signaling cascades within the target cell, or being taken up by the target cell [80]. Recognizing that EVs act as disease-specific messengers [81], various research groups are endeavoring to discern the functions of EVs in diabetes [36]. Various factors characterizing T2D, such as hyperglycemia, dyslipidemia, hypertension, obesity, and insulin resistance, likely impact the cargo of EVs [82]. To date, EVs in T2D have been linked to the modulation of diverse pathophysiological mechanisms, including vascular damage [22,83], angiogenesis [84,85], immune responses [10,86], platelet activation [87], complement and coagulation cascades [88], inflammation [11,35], and cancer progression [11,89]. The mechanisms associated with T2D conditions, as indicated by the enriched processes and pathways identified in our analysis (GO: BP, Reactome, and KEGG), are undeniably linked to altered EV cargo and the capacity of these structures to modulate cells [90]. Future research aiming to pinpoint specific target molecules and biomarkers, potentially among the proteins compiled in this review, holds significant promise for the development of new strategies in the care of T2D patients.

5. Conclusions

In this review, a handful of proteins consistently exhibited altered levels in the P/S EVs isolates from T2D patients compared to control subjects without diabetes. Notably, CST6, CD55, HBA1, S100A8, and S100A9 showed increased levels, while FGL1 exhibited decreased levels in the P/S EVs of T2D patients. Bioinformatic analysis unveiled that these differentially abundant proteins in circulating EVs of T2D patients could be implicated in the disease through various mechanisms, primarily involving inflammation, complement, and platelet activation. Further studies are essential to elucidate the pivotal role of the identified pathways. Additionally, employing proteomic approaches on circulating EVs is warranted to compare well-identified and matched groups, encompassing individuals with and without T2D (newly diagnosed T2D, controlled and uncontrolled T2D, long history of T2D with or without complications) in a prospective longitudinal design. These findings may contribute to comprehending the proteomic content of plasma/serum EVs, offering new insights into the mechanisms linking EVs to diabetic pathophysiology, and suggesting potential biomarkers and therapeutic targets for diabetes and its complications.

6. Ethics declarations

Approval by an ethics committee was not required. The authors declare their commitment to the ethical utilization of information obtained from the reviewed articles through this systematic review. All information presented in this review has been handled responsibly, crediting the authors appropriately, and in compliance with ethical guidelines governing the utilization of academic and scientific content.

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Data availability statement

The authors confirm that the data supporting the conclusions of this study are available within the article/supp. material/referenced in the article.

CRediT authorship contribution statement

Jeanette Guadalupe Arredondo-Damián: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Juan Manuel Martínez-Soto:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Francisco A. Molina-Pelayo:** Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jesús Adriana Soto-Guzmán:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Luis Castro-Sánchez:** Writing – review & editing, Resources, Methodology. **Luis Fernando López-Soto:** Writing – review & editing, Resources, Methodology. **Maria del Carmen Candia-Plata:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25537.

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