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REVIEW ARTICLE

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Mass spectrometry-based "omics" technologies for the study of gestational diabetes and the discovery of new biomarkers

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Abstract

Gestational diabetes (GDM) is one of the most common complications occurring during pregnancy. Diagnosis is performed by oral glucose tolerance test, but harmonized testing methods and thresholds are still lacking worldwide. Short-term and long-term effects include obesity, type 2 diabetes, and increased risk of cardiovascular disease. The identification and validation of sensitidve, selective, and robust biomarkers for early diagnosis during the first trimester of pregnancy

Abbreviations: 2DE, two-dimensional electrophoresis; AAS, atomic absorption spectroscopy; ADA, American Diabetes Association; AFM, afamin; ANXA2, annexin A2; ANXA4, annexin A4; ANXA5, annexin A5; APMAP, adipocyte plasma membrane-associated protein; APOE, aapolipoprotein E; ARHGEF11, isoform 2 of Rho guanine nucleotide exchange factor 11; BAs, bile acids; BMI, body max index; CAMK2ß, calcium/calmodulin-dependent protein kinase II beta; CAWG, Chemical Analysis Working Group; CD36, platelet glycoprotein 4; CD59, CD59 glycoprotein; CE, capillary electrophoresis; CETP, cholesteryl ester transfer protein; CMBL, carboxymethylenebutenolidase Homolog; CNDP1, beta-ala-his dipeptidase; CRP, C-reactive protein; DD, data dependent; DIA, data independent; DPP-2, dipeptidyl peptidase-4; ELISA, enzyme-linked immunosorbent assay; F9, coagulation factor IX; FASP, filter-aided sample preparation; FCN3, Ficolin 3; FGA, fibrinogen alpha chain; FIA, flow injection analysis; FIBB, fibrinogen beta chain; FSH, follicle-stimulating hormone; FTICR, Fourier-transform ion cyclotron resonance; GC, gas-chromatography; GDM, gestational diabetes mellitus; GST, GSH-S-transferase; HAPO, hyperglycemia and adverse pregnancy outcome; HESI, heated electrospray Ionization; HILIC, hydrophilic interaction liquid chromatography; HMDB, Human metabolome Database; HR, high resolution; HSP27, heat-shock protein 27; IADPSG, International Association of the Diabetes and Pregnancy Study Groups; ICP, ion-coupled plasma; IGFBP2, insulin like growth factor-binding protein 2; IGFBP5, insulin-like growth factor-binding protein 5; IL1RA, interleukin 1 receptor antagonist; iTRAQ, isobaric tags for relative and absolute quantitation; LCAT, phosphatidylcholine-sterol acyltransferase; LGA, large for gestational; LIT, linear ion trap; LTQ, hybrid linear ion trap; LUM, Lumican (S100A9); Lys-C, C-terminal Lysine; MALDI, matrix-assisted laser desorption ionization; MIREC, Maternal Infant Research on Environmental Chemicals; MRM, multireaction monitoring; MS, mass spectrometry; MSI, Metabolomics Standard Initiative; MSTFA, N-Methyl-N-(trimethylsilyl)trifluoroacetamide; NDUFS3, NADH dehydrogenase iron-sulfur protein 3; NDUFV2, NADH dehydrogenase flavoprotein 2; NGA, normal for gestational; NGT, normal glucose tolerance; nLC-ESI-MS, nano liquid chromatography–electrospray–mass spectrometry; OGTT, oral glucose tolerance test; PABPC4, polyadenylate-binding protein 4; PAPPA, pappalysin-1; PCA, principal component analysis; PDIA3, protein disulfide-isomerase A3; PE, pre-eclampsia; PE, pre-eclampsia; PLTP, plasma phospholipid protein; PON1, serum paraoxonase/arylesterase 1; PPP3CB, calmodulin-dependent calcineurin A b; PRG2, bone marrow proteoglycan; PRX1, peroxiredoxin-1; PRX5, peroxiredoxin-5; PRX6, peroxiredoxin-6; PSG1, pregnancy-specific beta-1-glycoprotein 4; PSMA2, proteasome subunit alpha type-1; PTR, proton transfer reaction; QQQ, triple quadrupole; QTOF, quadrupole time-of-flight; RAP1A, Ras-related protein Rap-1A (0.7 fold change); RP, reverse phase; RSD, relative standard deviation; RTL, retention time locked; RT-PCR, reverse transcriptase-polymerase chain reaction; SAMP, serum amyloid P-component; SCX, strong cation exchange; SDS, sodium dodecyl sulfate; SELDI, surface-enhanced laser desorption/ionization; SHBG, Sex hormone-binding globulin; SNTA1, alpha1-syntrophin; SPE, Solid Phase extraction; SWATH, sequential windowed acquisition of all theoretical fragment ion; T2D, type 2 diabetes; TMT, tandem mass tag; TSP-4, Thombospondin-4; VEGFR-FLT1, Vascular endothelial growth factor receptor-FLT1; VTN, Vitronectin; WHO, World health organization; YWHAZ, 14-3-3 protein zeta/delta.; (PCae(40:4), choline ether phospholipid; (PC(32:1)), 1-(9Z-hexadecenoyl)−2-hexadecanoyl-Sn-glycero-3phosphocholine; (TG(48:1)), 1,2-dihexadecanoyl-3-(9Z-hexadecenoyl)-Sn-glycerol; (TG(51:1)), 1,2-dihexadecanoyl-3-9Z-nonadecenoyl-Sn-glycerol.

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are required, as well as for the prediction of possible adverse outcomes after birth. Mass spectrometry (MS)-based omics technologies are nowadays the method of choice to characterize various pathologies at a molecular level. Proteomics and metabolomics of GDM were widely investigated in the last 10 years, and various proteins and metabolites were proposed as possible biomarkers. Metallomics of GDM was also reported, but studies are limited in number. The present review focuses on the description of the different analytical methods and MS-based instrumental platforms applied to GDM-related omics studies. Preparation procedures for various biological specimens are described and results are briefly summarized. Generally, only preliminary findings are reported by current studies and further efforts are required to determine definitive GDM biomarkers.

KEYWORDS
gestational diabetes mellitus, mass spectrometry, metabolomics, metallomics, proteomics

1 | INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (Buchanan et al., 2007). Pregnancy is characterized by physiological insulin resistance of peripheral tissues caused by the presence of hormones such as progesterone and cortisol. From the 18 weeks of gestation, the effect of hormones is particularly relevant in adipose and muscle tissues causing a reduction in glucose utilization, which in turn results in physiological postprandial hyperglycemia, an increase of insulin secretion, and the development of physiological pancreatic hyperplasia (McIntyre et al., 2019; Plows et al., 2018). Inadequate insulin supply results in impaired glucose homeostasis, hyperglycemia, and GDM (Buchanan et al., 2007).

Risk factors for GDM include obesity, increased maternal age, family history of type 2 diabetes (T2D), previous history of GDM or adverse pregnancy outcome, and ethnicity (Buchanan & Xiang, 2005). Poor glycemic control in the second and third trimesters of pregnancy may lead to a condition characterized by hyperglycemia and chronic hyperinsulinemia, with a subsequent increased oxygen placental demand. A maternal uncontrolled diabetes may increase fetal growth, especially in organs sensitive to insulin activity, leading to macrosomia (McIntyre et al., 2019; Plows et al., 2018). The major neonatal complications in infants born from mothers with GDM are congenital anomalies, prematurity, perinatal asphyxia, respiratory distress, metabolic complications, hematological problems, hyperbilirubinemia, and cardiomyopathy. Long-term complications of GDM comprise T2D (Kim et al., 2002), glucose intolerance, obesity (Hedderson et al., 2010), and neurological developmental abnormalities in adult life. Moreover, the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study demonstrated that the risk of adverse maternal, fetal, and neonatal outcomes continuously increased as a function of maternal glycemia at 24–28 weeks of gestation (The HAPO Study Cooperative Research Group, 2009).

The estimation of GDM prevalence is nowadays difficult due to the lack of universally accepted diagnostic criteria (Ryan, 2011). Several different diagnostic protocols are routinary used internationally, as reported by the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) (Metzger et al., 2010), the American Diabetes Association (ADA) (2019), and the World Health Organization (WHO) (2013). Nevertheless, the harmonization of testing methods and thresholds recognized as diagnostic is still lacking.

Omics sciences are therefore of fundamental importance because through a "holistic" approach it is possible to define a disease model, starting from observations arising from biological samples collected "in vivo," which reflect the pathological condition of the patient (Robinson et al., 2009).

Regarding GDM, the search of selective, sensitive, and robust biomarkers is essential for the development of new screening strategies or diagnostic methods to be used routinely in clinical practice, and able to identify patients at risk within the first trimester of pregnancy. Moreover, biomarkers are also fundamental to evaluate the effect of GDM on both mother's and fetus's health during pregnancy and on the onset of other pathologies in childhood and aging (Singh et al., 2015; Zhou et al., 2020).

In the last decade, numerous studies were published concerning the application of mass spectrometry (MS)-based omics strategies for evaluating the physiopathology of GDM and the possibility of determining biomarkers for the diagnosis of the disease or the prediction of long-term complications. The aim of this review is the description and

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the evaluation of the different analytical methods and approaches reported in the literature concerning the study of different tissues and biological fluids collected in GDM conditions by means of MS-based proteomics, metabolomics, and metallomics. Results and clinical/biological interpretation of data are just mentioned in this study especially regarding robustness of findings, as they were extensively reported and discussed elsewhere (Singh et al., 2015; Zhou et al., 2020).

2 | FUNDAMENTALS OF OMIC WORKFLOWS IN GDM

Omic workflows generate high-value information starting from the analysis of samples by an "olistic" or nontargeted approach. Nevertheless, the accuracy and the significance of such information are strictly dependent on several preanalytical steps, to be in-depths evaluated before starting to avoid the collection of misleading and nonrepresentative data.

The first step is the experimental design. Most studies reported in the literature are based on a pairwise comparison. In particular, upregulated or downregulated chemical markers are highlighted by comparing data obtained for two groups of interest, that is, samples collected from GDM patients and samples coming from control pregnant women with normal glucose tolerance (NGT). The sample size is one of the main parameters that should be considered in this kind of study and should be correctly evaluated. The proper collection of samples by following pre-established standard operating procedure and the correct storage, usually at −80°C, are also essential for obtaining accurate and reliable data. Moreover, the addition of preservative or protease inhibitors should be accurately evaluated, but usually, such information is missing in the reviewed studies. Furthermore, sample collection should be complemented by the acquisition of other metadata necessary for the subsequent statistical evaluation including the consideration of potential confounding factors, in particular the maternal age, ethnicity, prepregnancy body max index (BMI), type of delivery, gestational age of the infant, infant sex, and birth weight. Reported studies also considered exclusion criteria, such as smoking, family history of T2D, overt diabetes, and presence of other confounding pathologies, to avoid the collection of nonsignificative samples that could affect the reliability of the analysis.

Patients stratification and selection should also be correctly evaluated. GDM is usually diagnosed by an oral glucose tolerance test (OGTT) by administering a dose of 75 g of glucose to the mother during the 24–28 weeks of gestation, with a few exceptions. Nevertheless, the criteria used for the diagnosis are usually different for many of the studies taken into consideration, depending on the country and the year of publication. This incompatibility causes difficulties in comparing reported results since considered groups of pregnant women are not homogeneous.

3 | MS-BASED PROTEOMICS IN GDM

Proteomics, that is, the comprehensive and systematic analysis of the proteins expressed in a cell, tissue, or biological fluid, has widely been applied to the study of GDM in the last 10 years (Zhou et al., 2020). In particular, high throughput MS-based proteomic approaches were applied to determine several proteins showing an altered expression in pregnant women affected by GDM in different specimens, for example, mother plasma and serum, cord plasma, placenta tissue, urine, omental adipose tissue, skeletal muscle, and colostrum whey. Findings, that is, candidate protein biomarkers closely linked to the development or progression of GDM, were considered potentially very helpful for the development of novel and early methods for diagnosis or prediction, for clarifying the physiopathology of the disease, or as markers for fetal and mother outcomes (Singh et al., 2015).

Proteomics analysis is essentially performed by two different and sometimes complementary approaches. The gel-based approach is the first method used for proteomic investigation based on mono or two-dimensional electrophoresis (2DE); proteins contained in the electrophoretic spots and exhibiting statistically significant differences in expression are subjected to the identification by matrix-assisted laser desorption ionization (MALDI)-MS, or nano-flow liquid chromatography (nLC)-electrospray (ESI)-tandem MS (MS/MS). It was demonstrated that the identification performed by nLC-ESI-MS/MS usually allowed to obtain higher sequence coverages and higher probabilities of revealing low concentrated proteins compared to MALDI-MS (Roverso et al., 2016). The second approach, defined as the gel-free approach, avoids the use of electrophoretic separation by performing digestion of the sample whole protein content and MS analysis. Differences in protein expression are highlighted by quantification or differential quantification of protein-derived peptides and statistical analysis. In general, the gel-based approach is more time-consuming but more suitable for separating and identifying protein isoforms and posttranslational modifications. The gel-free approach, on the other hand, is more comprehensive, automatable, and faster in terms of sample preparation, even if requires more sophisticated and high-speed instrumentation, such as high-resolution (HR)MS, and powerful software for data analysis (Baggerman et al., 2005;

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Lambert et al., 2005). Considering GDM proteomics, most reported studies are based on quantitative gel-free methods, while 2DE was used only in a few investigations. As the gel-free approach allows the identification of a greater number of proteins, it also improves the assessment of a greater number of upregulated or downregulated species. State-of-the-art quantitative gel-free approaches rely on isobaric labeling or label-free methods. Common labeling methods in GDM proteomics are based on isobaric tags for relative and absolute quantitation (iTRAQ) or tandem mass tag (TMT) (Chahrour et al., 2015). The alterations in protein expression due to the development of GDM were studied by both label-free and isobaric labeling, and the results obtained in terms of identified upregulated or downregulated proteins are comparable between the different methods for the same specimen. Relative quantification of proteins is also possible by tandem MS in multireaction monitoring (MRM) mode after spiking samples with a proper mix of heavy isotope-labeled standard peptides.

For all the specimens considered, the most important procedure in sample preparation is the digestion of proteins by a proper enzyme to obtain protein-derived peptides that can be easily analyzed, sequenced, and identified. In all the considered studies, proteins were digested by porcine or bovine trypsin, after reduction and alkylation of the disulfide's bridges between cysteine residues. The typical trypsin/protein ratio used is 1:30, 1:50, or the uncommon 1:100 ratio (Mavreli et al., 2020). A study also reported also the use of trypsin (1:50) in combination with Lys C (1:100) (Liu et al., 2020). Furthermore, many articles reported the use of the filter-aided sample preparation (FASP) technique for protein digestion to improve the proteome coverage. Through this method, lysis of cells and tissues, protein reduction, alkylation and digestion, desalting and removal of detergent, that is, the sodium dodecyl sulphate (SDS) used for protein solubilization, are carried out by ultra-centrifugation in common 10–30 kDa filtration units (Wiśniewski et al., 2009).

After digestion and labeling, peptides are commonly fractionated by strong cation exchange (SCX) chromatography, hydrophilic interaction chromatography (HILIC), C18 or reverse-phase chromatography at high pH to obtain at least 10 fractions. Each fraction is usually lyophilized and further processed by nLC-ESI-MS/MS. On the contrary, samples processed by label-free methods are generally not fractionated but directly injected into the nLC-ESI-MS/MS system. The separation of peptides by nLC is performed in reverse phase mode, generally by using water and acetonitrile acidified with 0.1% formic acid. Both linear gradient or multistep gradients are used. Usually, the sample is desalted online before injection in the analytical column. Eluted peptides are subsequently analyzed by HRMS running in data-dependent (DDA) or data-independent (DIA) mode. The ionization is performed in positive n-ESI conditions, and hybrid quadrupole time-of-flight (QTOF), Orbitrap or Fourier-transform ion cyclotron resonance (FTICR) are the HR mass analyzers conventionally used.

Acquired data are further processed with dedicated software to identify proteins and to statistically highlight alteration in their expression for proposing new biomarkers. The use of proprietary software in GDM proteomics, such as Compound Discoverer, Progenis QI, and ProteinPilot, was generally more common than the use of open-source or free software, for example, MaxQuant, ProteinProspector, and so on. This is probably due to the fact that modern instruments are usually supplied together with the expensive software packages needed for a particular application. Operators are therefore less stimulated to the use of free software, which in many cases are less user-friendly and require the conversion of proprietary files into other file formats, but surely more versatile and customizable. Identification of proteins is possible thanks to the comparison of the experimental data with different databases, such as UniProt, SwissProt, International Protein Index.

Furthermore, many studies report both the validation of selected biomarkers and the evaluation of the related diagnostic accuracy by an independent analytical method in independent cohorts. Validation procedures are essential in omic sciences to confirm the robustness, reproducibility, and accuracy of findings, as well as to preliminarily prove the applicability of highlighted biomarkers in further studies. For this purpose, Enzyme-linked immunosorbent assay (ELISA), Western blot analysis and real-time polymerase chain reaction (RT-PCR) are the most used procedures. Validation by ELISA and Western blot analysis is strictly limited to the commercial availability of suitable primary antibodies to be used against the target protein in the specimen studied and to proper sample preparation (Ghosh et al., 2014). RT-PCR is also widely used, but findings are related to transcriptional changes in the gene encoding for the studied protein, which in principle are independent of protein abundance. Recently, the validation by targeted MRM-based protein analysis was also reported (Ravnsborg et al., 2016, 2019), even if applied only on very few occasions.

3.1 | Plasma and serum proteomics

Plasma and serum are the most studied specimens for the identification of novel biomarkers for GDM diagnosis (Table 1). The blood protein content is representative of the various biological processes occurring in a living being, and is associated with possible pathological states. The analysis of plasma and serum proteome is challenging due to the complexity of the matrix and the presence of analytes

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TABLE 1 Summary of MS-based proteomic studies for GDM.

Specimen	Sample population (GDM + control)	Age/BMI of GDM vs. age/BMI control	GDM diagnosis	Sample preparation	Detection system	Statistics method	Results: protein identified (upregulated, downregulated)	Validation	Bio-marker[a]	Strength/weakness	References
Plasma (16–18 gw)	22 + 22	30.7/24.1 versus 29.2/20.8	OGTT (75 g glucose)	Dilution with urea buffer; digestion by Lys-C and trypsin; desalting. Label-free	nLC Orbitrap Fusion Lumos Tribrid MS (Thermo Scientific)	*t*-test	474 (8, 16)	ELISA (15 + 15)	CRP (↑), IGFB-P2) (↓)	Single sample analysis/ Protein depletion not reported; clinical characteristics of the validation cohort are not reported; limited number of validated proteins	Liu et al. (2020)
Plasma (11–13 gw)	5 + 5	26.6/23.1 versus 26.2/22.8	OGTT (75 g glucose)	HSA and immunoglobulin depletion; digestion by trypsin; TMT labelling; fractionation by C18 reverse phase chromatography (10 fractions)	nLC-Orbitrap E-Exactive HF (Thermo Scientific)	ANOVA, Mann–Whitney *U* test	1296 (198, 40)	ELISA (25 + 25)	TSP-4 (↑), CNDP1 (↑)	State-of-the-art proteomic workflow/Very limited number of cases; limited number of validated proteins; maternal age and BMI are not harmonized between first stage and validation stage	Mavreli et al. (2020)
Plasma (16–18 gw)	20 + 20	27.6/21 versus 27.9/21	OGTT (75 g glucose)	HSA and immunoglobulin depletion; acetone + TFA precipitation; digestion with trypsin; TMT labelling; SCX fractionation (20 fractions)	NanoLC-LTQ-Orbitrap (Thermo Scientific)	One-way ANOVA	828 (25, 11)	Gel-electrophoresis—Western blot analysis (20 + 20)	CRP (↑), (FCN3) (↓), (PSG1) (↓), SHBG (↓)	First paper on GDM biomarker discovery in plasma/Western blot analysis performed on the same cohort of the MS analysis	Zhao et al. (2015)

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TABLE 1 (Continued)

Specimen	Sample population (GDM + control)	Age/BMI of GDM vs. age/BMI control	GDM diagnosis	Sample preparation	Detection system	Statistics method	Results: protein identified (upregulated, downregulated)	Validation	Bio-marker[a]	Strength/weakness	References
Plasma (16–20 gw)	12 + 12	37.0/22.4 versus 36.5/21.5	OGTT (75 g glucose)	Screening by SELDI-TOF mass spectrometry; purification of the selected proteins by monodimensional SDS-PAGE electrophoresis; identification of proteins by LC-MS/MS	SELDI-TOF (Chiphergen Biosystems), nLC-QTOF (Waters)	Mann Whitney U test	(9 altered proteins)	ELISA	APO-CIII (↑)	Interesting workflow based on fast SELDI-MS analysis of untreated plasma, useful in clinical practice/ ELISA performed on the same cohort of the MS analysis	Kim et al. (2012)
Mother plasma and cord plasma (at delivery)	4 + 4	31.0/20.0 versus 30.3/20.8	OGTT (75 g glucose)	HSA and immunoglobulin depletion; digestion by trypsin; iTRAQ labelling; fractionation by SCX chromatography (20 fractions)	nLC coupled to Triple TOF 5600 (ABSciex)	Student *t*-test	Mother plasma: 523 (19, 15) Cord plasma: 780 (29, 69)	ELISA (16 + 16)	CETP (↓), FSH (↓)	State-of-the-art proteomic workflow; analysis and comparison of both mother and newborn samples/ Extremely limited number of cases; few proteins validated by ELISA	Liao et al. (2018)
Cord plasma (at delivery)	30 + 30	27.1/23.0 versus 26.6/22.7	OGTT (75 g glucose)	HSA and immunoglobulin depletion; acetone + TFA precipitation; digestion with trypsin; TMT labelling SCX fractionation (20 fractions)	NanoLC-LTQ-Orbitrap (Thermo Scientific)	Student t-test	318 (12, 24)	Gel-electrophoresis - Western blot analysis (10 + 10)	ARHGEF11 (↓) PLTP (↑) LCAT (↑)	State-of-the-art proteomic workflow; 7 years follow-up of newborns/due to the selection of case and control groups it is not possible to discriminate variations ascribable to obesity and GDM alone in newborns	Miao et al. (2016)

(Continues)

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Specimen	Sample population (GDM + control)	Age/BMI of GDM vs. age/BMI control	GDM diagnosis	Sample preparation	Detection system	Statistics method	Results: protein identified (upregulated, downregulated)	Validation	Bio-marker[a]	Strength/weakness	References
Serum (12–16 and 24–28 g-w)	10 + 10	29.3/21.8 versus 28.8/20.7	OGTT (75 g glucose)	Enrichment of proteins with ProteoMiner Kit(Biorad); iTRAQ labeling digestion with tripsyn; fractioning by high pH reverse phase chromatography (48 fractions)	2D-nLC coupled to Triple TOF 5600 (ABSciex)	Cluster analysis, principal component analysis	31 (12–16 gw samples) 27 (24–28 gw sample) 14 (12–16 vs. 24–28, in GDM or control subjects)	ELISA	APOE (↑), FGA (↑), PON1 (↑)	Comparison between samples collected in different weeks of gestations; protein enrichment instead of immune-depletion/limited number of cases	Shen et al. (2019)
Serum (8–13 g-w)	30 + 30	31.0/32.7 versus 31.0/33.0	OGTT (75 g glucose)	HSA and immunoglobulin depletion; TMT labeling digestion by trypsin; purification by Oasis 10 mg; fractionation by HILIC chromatography	nLC-Orbitrap Q-Exactive HF (Thermo Scientific)	Mann–Whitney *U* test, Fisher's exact test	548 (25 with altered expression)	Target MRM method for 25 proteins of interest	AFM (↑), SAMP (↑), VTN (↑)	Validation of results by a targeted MS approach/limited number of validated proteins; authors did not consider that GDM patients and controls were affected by obesity (BMI > 31)	Ravnsborg et al. (2019)
Serum (12–16 gw)	10 + 10	29.4/21.7 versus 28.6/20.7	OGTT (75 g glucose)	Enrichment of proteins with ProteoMiner Kit(Biorad); iTRAQ labeling digestion with trypsin; fractioning by high pH reverse phase chromatography (48 fractions)	nLC coupled to Triple TOF 5600 (ABSciex)	NS	362 (19, 14)	ELISA	APOE (↑), F9 (↑), FGA (↑), IGFBP5 (↑)	Protein enrichment instead of immune-depletion; state-of-the-art proteomic workflow/limited number of cases	Zhao et al. (2017)
Plasma exo-somes (22–28 gw)	11 + 11	31.2/34.2 versus 34.1/34.0	OGTT (75 g glucose)	Exaction of exosomes from plasma samples; monodimensional electrophoresis; gel fractionation in 12 pieces; digestion by FASP method; label-free	nLC coupled to Triple TOF 5600 (ABSciex)	Student *t*-test	415 (78)	ELISA (13 + 23)	CAMK2ß (↑), PAPPA (↓)	Validation of results by a targeted MS approach/authors did not consider that GDM patients and controls were affected by obesity (BMI > 31)	Jayabalan et al. (2019)

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TABLE 1 (Continued)

Specimen	Sample population (GDM + control)	Age/BMI of GDM vs. age/BMI control	GDM diagnosis	Sample preparation	Detection system	Statistics method	Results: protein identified (upregulated, downregulated)	Validation	Bio-marker[a]	Strength/weakness	References
Placenta (at term)	12 GDM, 5 GDM/ LGA, 5 controls	GDM: 35.1/33.9 GDM/LGA: 34.8/38.3 Controls: 36.6/21.8	OGTT (75 g glucose)	Tissue disruption by treating with liquid nitrogen and homogenization by RIPA, label-free	nLC-Q Exactive (Thermo Scientific)	Student *t*-test	GDM versus controls: 2103 (40, 24) GDM versus GDM/LGA: 1514 (15, 22)	ELISA	DPP-4 (↑ in LGA), PRG2 (↑in LGA)	In vivo validation/ improper samples selection	Assi et al. (2020)
Placenta (at term)	13 + 12	32.8/27.5 versus 34.1/27.4	OGTT (75 g glucose)	Protein extraction and precipitation; digestion by trypsin; label-free	nLC-Synapt-MS (Waters)	Student *t*-test, principal component analysis	160 (2,1)	No	-	Analysis of single samples/validation of findings not reported; very limited number of samples analyzed	Burlina et al. (2019)
Placenta (at term)	9 (GDM) + 9 (GDM + PE)	32.7/27.8 vs 30.2/25.9	OGTT (75 g glucose)	Homogenation of syncy with SDS buffer; TMT labeling FASP digestion; Fractionation (10 fractions)	nanoLC-Q-Exactive Orbitrap (Thermo Scientific)	Student *t*-test	4140 (11, 12)	Western blotting	FLT1 (↑in GDM + PE), PABP-C4 (↑in GDM)	State-of-the-art proteomic workflow/no information on syncytiotrophoblast collection	Sun et al. (2018)
Placenta (at term)	6 + 6	37/23 versus 34/21	OGTT (75 g glucose)	Homogenization of tissue with urea buffer; Two-dimensional (2D) electrophoresis; and identification of altered spot by nLC-MS/MS and MALDI-MS	UltrafleXtreme MALDI TOF (Bruker), nLC-Synapt-MS (Waters)	ANOVA, Student *t*-test	a. 29 (13, 16) b. 159 (10, 9)	No	–	Comparison between in-gel and gel-free methods/limited number of analyzed samples; validation not reported	Roverso et al. (2016)
Placenta (at term)	8 + 8	31.3/22.1 versus 30.1/21.9	OGTT (75 g glucose)	Tissue homogenization by urea buffer; 2D electrophoresis; digestion of electrophoretic spots by trypsin	MALDI-TOF Ultraflex III (Bruker)	Student *t*-test	21 (15 + 6)	Western blot analysis, RT-PCR	ANXA2 (↑), ANXA5 (↑), YWHA-Z (↑), RAP1A (↓)	Validation by both Western blot analysis and RT-PCR/validation in an independent cohort is not reported	Liu et al. (2012)

(Continues)

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TABLE 1 (Continued)

Specimen	Sample population (GDM + control)	Age/BMI of GDM vs. age/BMI control	GDM diagnosis	Sample preparation	Detection system	Statistics method	Results: protein identified (upregulated, downregulated)	Validation	Bio-marker[a]	Strength/weakness	References
Endothelial cells from umbilical cord (at term)	44 + 55	35.0/30.3 versus 35.0/24.0	OGTT (75 g glucose)	2D electrophoresis; digestion of electrophoretic spots by trypsin	nLC-MS/MS	Student *t*-test	39 altered proteins	Quantitative RT-PCR (44, 55)	PRX1 (↓), GST (↓), PRX5 (↑), PDIA3 (↑)	Validation of results by using cell-line/ authors did not consider that GDM patients and controls were affected by obesity	Cheng et al. (2013)
Urine (15–20 gw)	16 + 16	29.9/21.8 versus 28.9/21.2	OGTT (75 g glucose)	Protein precipitation with acetone; iTRAQ labeling digestion by FASP method; fractionation by C18 reverse phase chromatography (10 fractions)	nLC- Q Exactive (Thermo Scientific)	Student *t*-test	1901 (83, 36)	ELISA (40 + 40)	IL1RA (↑), CD59 (↓)	Good sample size/ limited number of proteins validated by ELISA	Guo et al. (2018)
Omental adipose tissue (at term)	6 + 6	31.3/24.7 versus 28.3/20.1	OGTT (75 g glucose)	Protein extraction by SDT buffer; digestion by FASP method; label-free	nLC-Q-Exactive (Thermo Scientific)	NS	3528 (66)	Western blottin	APMAP (↓)	Validation of results by using cell-line/ limited number of analyzed sample; the procedure for lipid removal was not clearly reported	Ma et al. (2016)
Omental adipose tissue (at term)	6 + 6	36.0/35–3 versus 35.5/34.0	OGTT (75 g glucose)	Tissue homogenization by DIGE buffer; protein precipitation with acetone; 2D gel electrophoresis, stained by colloidal Coomassie; digestion of electrophoretic spots by trypsin	Microflex MALDI-TOF (Bruker)	Student *t*-test	61 (4, 10)	Western blot analysis (6 + 6)	FIBB (↑), LUM (↑), S100A9 (↑), ANXA5 (↓), PRX6 (↓), HSP27 (↓)	High number of validated proteins/ Questionable sample selection: GDM and controls were obese; GDM patients were treated with insulin; limited number of analyzed sample; 2DE analysis limited to proteins in the 4–7 pH range	Oliva et al. (2013)

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TABLE 1 (Continued)

Specimen	Sample population (GDM + control)	Age/BMI of GDM vs. age/BMI control	GDM diagnosis	Sample preparation	Detection system	Statistics method	Results: protein identified (upregulated, downregulated)	Validation	Bio-marker[a]	Strength/weakness	References
Rectus abdominous skeletal muscle tissue (at term)	6 + 6	NS/37.5 versus NS./34.2	OGTT (100 g glucose)	Lysis of the tissue Monodimensional SDS page electrophoresis; gels cutting in 20 separate slides; digestion by trypsin	nLC LTQ FTICR (Thermo Scientific)	Nonparametric Mann–Whitney *U* test, independent *t*-test	979 (8 with altered expression)	Western blot analysis (8 + 8)	CMBL (↑), PPP3C-B (↑), CD36 (↑), NDUF-V2 (↓), NDUF-S3 (↓), ANXA4 (↓), PSMA2 (↓), SNTA1 (↓)	High number of validated proteins/ Limited number of analyzed sample; GDM patients were treated with insulin	Boyle et al. (2014)
Whey colostrum	6 + 12	34.5/31.6 versus 34.7/22.0	OGTT (50 g glucose)	Casein depletion by $CaCl_2$ addition; lipid removal and protein precipitation by Wessel and Flugge method; digestion with trypsin	nanoLC-Q-Exactive Orbitrap (Thermo Scientific)	O-PLS-DA	601 (10 with altered expression)	no	-	Individual analysis of samples/GDM patients were affected by obesity (BMI > 31)	Grapov et al. (2015)

Abbreviations: AFM, afamin; ANXA2, annexin A2; ANXA4, annexin A4; ANXA5, annexin A5; APMAP, adipocyte plasma membrane-associated protein; APOE, aapolipoprotein E; ARHGEF11, isoform 2 of Rho guanine nucleotide exchange factor 11; CAMK2ß, calcium/calmodulin-dependent protein kinase II beta; CD36, platelet glycoprotein 4; CD59, CD59 glycoprotein; CETP, cholesteryl ester transfer protein; CMBL, carboxymethylenebutenolidase homolog; CNDP1, Beta-ala-his dipeptidase; CRP, C-reactive protein; DPP-4, dipeptidyl peptidase-4; F9, coagulation factor IX; FCN3, ficolin 3; FGA, fibrinogen alpha chain; FIBB, fibrinogen beta chain; FLT1, vascular endothelial growth factor receptor 1; FSH, follicle-stimulating hormone; GST, GSH-S-transferase; Gw, gestational week; HSP27, heat-shock protein 27; IGFBP2, insulin like growth factor-binding protein 2; IGFBP5, insulin-like growth factor-binding protein 5; IL1RA, interleukin 1 receptor antagonist; LCAT, phosphatidylcholine-sterol acyltransferase; LUM, lumican (S100A9); NDUFS3, NADH dehydrogenase iron-sulfur protein 3; NDUFV2, NADH dehydrogenase flavoprotein 2; NS, not specified; PABPC4, polyadenylate-binding protein 4; PAPPA, pappalysin-1; PDIA3, protein disulfide-isomerase A3; PLTP, plasma phospholipid protein; PON1, serum paraoxonase/arylesterase 1; PPP3CB, calmodulin-dependent calcineurin A b; PRG2, bone marrow proteoglycan; PRX1, peroxiredoxin-1; PRX5, peroxiredoxin-5; PRX6, peroxiredoxin-6; PSG1: pregnancy-specific beta-1-glycoprotein 4; PSMA2, proteasome subunit alpha type-1; RAP1A, Ras-related protein Rap-1A (0.7 fold change); SAMP, serum amyloid P-component; SHBG, sex hormone-binding globulin; SNTA1, alpha1-syntrophin; TSP-4, thombospondin-4; VTN, vitronectin; YWHAZ, 14-3-3 protein zeta/delta.

[a]Validated by independent method, that is, ELISA, Western blot analysis, and so on;

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with a wide range of concentrations. GDM investigation on plasma and serum are commonly associated with the discovery of predictive biomarkers for early diagnosis during the first trimester of pregnancy.

3.1.1 | Plasma

Plasma samples, obtained from EDTA treatment of whole blood, were usually collected during the 11–20 weeks of gestation, while GDM diagnosis was performed by OGTT during the 24–28 weeks of gestation. Samples were further analyzed by both TMT labeling and label-free approaches. For TMT labeling, plasma samples were depleted for abundant proteins, for example, albumin and immunoglobulin, and after tryptic digestion, samples were fractionated by C18 reverse phase (Mavreli et al., 2020) or by SCX chromatography (Liao et al., 2018; Miao et al., 2016; Zhao et al., 2015). In both cases, the MS analysis was performed by an Orbitrap-based system and data were acquired in DDA mode. The investigation on plasma samples collected during the first trimester (11–13 weeks of gestation) (Mavreli et al., 2020) highlighted several altered proteins in GDM samples compared to controls, associated with complement and coagulation cascades. The validation by ELISA considering an independent cohort confirmed the overexpression of beta-ala-his dipeptidase (CNDP1) and thrombospondin-4 (TSP-4) in the case of the GDM group. A similar proteomic workflow applied to plasma samples collected during the early second trimester of pregnancy (16–18 weeks of gestation) reported several altered proteins involved in immune response, inflammation, transport, platelet aggregation, catalyze, and defense response. Western blot analysis, limited to samples already analyzed by the proteomic approach, confirmed the upregulation of C-reactive protein (CRP) in GDM plasma samples compared to controls, together with the downregulation of three other proteins: sex hormone-binding globulin, Ficolin 3, and pregnancy-specific beta-1-glycoprotein (Zhao et al., 2015). Plasma samples collected during the 16–18 weeks of gestation were also analyzed by a label-free approach. In this case, no information are reported regarding the use of protease inhibitors after sample collection or protein depletion, and specimens were simply diluted with urea buffer and digested by Lys-C and trypsin before the Orbitrap-based nLC-ESI-MS/MS analysis in DIA mode. Findings highlighted eight proteins showing an increased concentration and 16 decreased proteins in GDM samples, indicating that inflammation system, oxidative stress, insulin resistance, blood coagulation, and lipid homeostasis were involved in GDM onset. Overexpression of C-reactive protein (CRP) and downregulation of insulin-like growth factor-binding protein 2 (IGFBP2) were validated and alterations were confirmed by ELISA in an independent cohort (Liu et al., 2020).

Another study, focused on the same period of gestation (16–20 weeks of gestation), evaluated plasma protein distribution by surface-enhanced laser desorption/ionization (SELDI)-MS. Altered proteins were subsequently identified by monodimensional electrophoresis and nLC-ESI QTOF-MS/MS. Reported results are quite different from the previously mentioned ones, as apolipoprotein CIII (APO-CIII) was found in greater concentration in plasma samples from GDM patients compared to controls; results were also validated by ELISA (Kim et al., 2012).

Cord plasma samples, that is, plasma taken from the umbilical cord vein collected at term, were also analyzed to highlight mechanisms underlying GDM (Liao et al., 2018) and to evaluate early markers of childhood obesity (Miao et al., 2016). Both studies found dysregulation of proteins involved in lipid regulation and transport, and glucose metabolism, leading to the hypothesis that GDM is linked to the development of diabetes and obesity later in life. In particular, a decrease in the expression of cholesteryl ester transfer protein (CEPT) and increased levels of follicle-stimulating hormone (FSH) were observed in GDM cord plasma and also confirmed in GDM maternal plasma (Liao et al., 2018). In addition, phosphatidylcholine-sterol acyltransferase (LCAT) and phospholipid transfer protein (PLTP) were significantly upregulated, and Rho guanine nucleotide exchange factor 11 (ARHGEF11) was downregulated in the umbilical vein blood plasma from GDM patients with obese offspring compared to control subjects whose offspring were of normal weight (Miao et al., 2016).

A case–control study also investigated plasma exosomes, that is, plasma vesicles involved in feto-maternal communication, using a quantitative label-free DIA-MS approach (Jayabalan et al., 2019). First, altered exosomal proteins were investigated by monodimensional electrophoresis and nLC-ESI-QTOF-MS/MS to generate a local ion library for subsequent targeted analysis of individual exosome samples. Plasma samples were collected during the 24 week of gestation, from GDM and control subject with a BMI value greater than 31. Through this method, it was possible to identify several altered exosomal proteins in GDM obese patients, compared to normoglycemic obese subjects, involved in energy production, inflammation, metabolism, and transplacental lipid transport. An analogous approach was also used by James-Allan et al. (2020) to characterize the alteration of the human exosomal proteome in presence of GDM before evaluating the effect on the glucose homeostasis in human-exosomal treated mice. Results confirmed impairment of insulin signaling and attenuation in

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insulin responsiveness in mice infused with exosomes from GDM patients.

3.1.2 | Serum

Serum was generally collected during the 8–16 weeks of gestation to determine novel biomarkers for the early diagnosis of GDM by TMT and iTRAQ labeling approaches. Interestingly, different methods for samples pretreatment were reported. One study recommended the removal of abundant proteins by use of immunoaffinity depletion, even if this approach was considered a source of experimental bias, as some untargeted low abundant proteins may be removed due to unspecific co-depletion (Ravnsborg et al., 2019). The authors did not report data to properly evaluate this assumption, nor information on the proteins that may be affected by this kind of sample treatment, probably because the benefits outweigh the risks. In addition, the preconcentration of medium and low-abundant proteins by commercially available enrichment kits was also suggested to avoid the lack of specificity of immuno-affinity depletion (Shen et al., 2019; Zhao et al., 2017). Such kits are based on affinity interaction between the species of interest and a large and highly diverse bead-based library of combinatorial peptide ligands. It is worth highlighting that a comparison between the two methods is not available in the literature regarding GDM and for this reason, it is difficult to evaluate which is the best procedure to be used.

In general, findings established that the pathogenesis and onset of GDM were linked to the dysregulation of several biological pathways, often already highlighted in the case of plasma samples, that is, complement system, inflammatory and immune response, and blood coagulation. Larger concentrations of Apolipoprotein E (APOE), coagulation factor IX, fibrinogen alpha chain (FGA), and insulin-like growth factor-binding protein were measured in sera from GDM pregnant women collected during the 10–16 weeks of gestation (Zhao et al., 2017). Overexpression of FGA and APOE in GDM serum samples were also confirmed by a more recent independent study based (Shen et al., 2019), which in turn confirmed the overexpression of CRP reported in the case of plasma samples collected the 16–18 weeks of gestation. Afamin, serum amyloid P-component, and vitronectin were found upregulated in serum samples collected from GDM obese patients compared to normoglycemic obese subjects (Ravnsborg et al., 2019). Interestingly, protein alterations were further validated by a target MS-based method on a cohort composed of 210 cases. Differences in concentration between groups were minimal in this study, although statistically significant.

Serum peptides extracted from samples collected from the 24 weeks of gestation were also evaluated as possible predictor biomarkers by MALDI-MS in combination with weak cation exchange magnetic beads (Ai et al., 2015). Even in this case, an FGA-derived peptide was overexpressed in GDM sera, confirming the role of FGA in the development and progression of GDM.

3.2 | Placenta proteomics

The placenta is the most important organ related to pregnancy and its function is essential to ensure the growth of the fetus by the proper exchange of gases, nutrients, waste products, and signaling molecules between the mother and the fetus. Placenta tissue was extensively studied by different proteomics approaches with the aim of assessing the biological pathways associated with the pathogenesis of GDM and placenta remodeling. Initial works (Liu et al., 2012; Roverso et al., 2016) highlighted altered protein expression in placentas impaired by GDM using gel-based approaches. In particular, 2DE was applied for the separation of placenta proteins extracted through the homogenization of placenta villi by urea-based buffers. The electrophoretic spots differentially expressed were further characterized by MALDI-MS and nLC-ESI-MS/MS. The following works, on the contrary, were based on gel-free methods; data were acquired in DDA-MS^E mode and proteins were identified and relatively quantified by label-free approaches by nLC-ESI-MS/MS (Burlina et al., 2019; Roverso et al., 2016). The obtained results showed that several placenta proteins involved in the regulation of the insulin pathway and coagulation/fibrinolysis were altered in GDM. In particular, placenta villi showed an upregulation of Annexin A2 (ANXA2), Annexin A5 (ANXA5) and 14-3-3 protein zeta/delta (YWHAZ), and a downregulation of Ras-related protein Rap-1A (RAP1A) in GDM samples compared to controls, confirmed by both Western blot analysis and RT-PCR (Liu et al., 2012). The upregulation of these proteins was interpreted as a response to hyperglycemia to maintain the fibrinolysis balance in placenta. These proteins were not confirmed by the label-free nLC-ESI-MS^E analysis, which in turn reported the upregulation of other species in GDM placenta (i.e., galectin-1 and Collagen alpha-1 [XIV] chain), but without confirming findings by an independent method (Burlina et al., 2019; Roverso et al., 2016). It is important to highlight that the considered investigations reported the importance of a correct treatment of placenta samples during the collection and before the storage. Placenta blood content is of major concern when the protein content of placenta itself or placenta

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substructures need to be determined, as high abundant blood proteins, that is, albumin and hemoglobin chains, may cause cross-contaminations (Lapolla et al., 2013). For this reason, it is essential to properly and extensively wash the placenta specimen with a nonaggressive buffer, that is, PBS or isotonic solutions, to limit the amount of residual blood in stored samples. In addition, the washing step should be completed in a limited time period, less than 30 min, to avoid changes in the protein content of samples due to the activities of endogenous proteases. The use of specific and commercially available protease inhibitor cocktails is highly recommended, but this information for the washing step was usually not reported by the authors.

Syncytiotrophoblasts from placenta samples from GDM pregnant women and GDM pregnant women affected by pre-eclampsia (PE) were also investigated to identify biomarkers for PE prediction (Sun et al., 2018). Differentially expressed proteins between groups, of which 11 upregulated and 12 downregulated, were associated with several biological processes (apoptosis, transcriptional misregulation, oxidative stress, lipid metabolism, cell infiltration and migration, and angiogenesis), indicating that GDM is potentially involved in the early onset of PE. Two proteins, that is, vascular endothelial growth factor receptor (1 FLT1) and polyadenylate-binding protein (PABPC4), were proposed as candidate biomarkers for predicting the onset of PE from women with GDM or as intervention targets for preventing GDM from developing into PE. Obviously, the analyzed cohort is extremely small to confirm this hypothesis, and future investigations are surely needed.

Placenta villi from GDM mothers with large for gestational age (LGA) fetuses and GDM mothers with normal for gestational age (NGA) fetuses were compared by a label-free proteomic study (Assi et al., 2020). It was found that placenta proteins were altered in presence of GDM and LGA newborns. Some identified proteins related to tissue differentiation and fetal growth and development, that is, bone marrow proteoglycan (PRG2) and dipeptidyl peptidase-4 (DPP-4), were proposed as therapeutic targets for future investigations. Some remarks on the selection of samples are unavoidable and for this reason, findings should be critically evaluated as other concomitant pathologies were not considered as sources of misleading information. In particular, authors compared samples from GDM patients also affected by obesity (BMI > 31) with samples from normal-weight subjects. In addition, a comparison between LGA newborns from GDM and control subjects was not reported. In conclusion, it is not clear if findings are only related to GDM and LGA newborns and which is the impact of maternal obesity.

Endothelial cells from the umbilical cord were also analyzed for assessing the link between the increased oxidative stress caused by GDM and the development of T2D and cardiovascular diseases in both mother and fetus later in life (Cheng et al., 2013). Endothelial cell proteome was investigated by 2DE and nLC-ESI-MS/MS, highlighting 39 altered proteins in the case of GDM patients. Dysregulated proteins were involved in redox homeostasis, protein oxidation, DNA damage, and diminished glutathione, confirming that GDM may contribute to the increased risk of type 2 diabetes and cardiovascular disease in offspring. Sample selection was again questionable, as GDM patients were also obese while controls were normal weight subjects. In this optic, findings are related to the combined effects of GDM and obesity and the functional alterations of the single pathologies cannot be properly and univocally determined.

3.3 | Miscellaneous matrix proteomics

Proteomics analysis of specimens collected in GDM condition was extended also to urine, omental adipose tissue, rectus abdominous skeletal muscle tissue, and colostrum.

Urine samples collected during the early second trimester (15–20 weeks of gestation) were analyzed to identify noninvasive biomarkers for GDM prediction. After protein precipitation with acetone, samples were analyzed by an iTRAQ-based approach. Four of the proteins showing an altered expression were validated by ELISA, confirming an overexpression of interleukin 1 Receptor Antagonist (IL1RA) and the downregulation of CD59 glycoprotein (CD59). The authors did not clearly describe how these species may be involved in GDM onset and progression, but obtained results paved the way for proposing IL1RA and CD59 as noninvasive and stable diagnostic predictors for GDM to be further evaluated by subsequent investigations (Guo et al., 2018).

Omental adipose tissue collected at term was processed by both gel-free and gel-based methods to identify biological processes involved in the development of insulin resistance during GDM (Ma et al., 2016) and to determine the effect of GDM on the proteome of the adipose tissue (Oliva et al., 2013), respectively. In the first study, proteins were easily extracted by treating the adipose tissue with SDT buffer, digested by FASP method, and analyzed by Orbitrap nLC-ESI-MS/MS. Sixty-six altered proteins were identified. Downregulation of adipocyte plasma membrane-associated protein (APMAP) was confirmed by Western blotting and

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immunochemistry. The role of APMAP in insulin resistance in the pathophysiology of GDM was also evaluated by inhibiting APMAP expression in 3T3-L1 adipocytes, confirming an impairment of the insulin signaling. In the second study, adipose tissue was analyzed by 2DE and MALDI-TOF-MS and 14 differentially expressed proteins involved in inflammation, lipid, and glucose metabolism, and oxidative stress were highlighted (Oliva et al., 2013). Once again, the selection of cases and controls is questionable as both GDM patients and control subjects were obese, and in addition, all the GDM patients were treated with insulin, which may be responsible for some of the changes described. Furthermore, the 2DE analysis was limited to proteins in the 2–7 pH range. Both works did not detail the protocol for tissue homogenization and lipid removal. This last procedure is fundamental in this kind of approach to limit the influence of lipids in the separation workflow, in particular for 2DE, and to maintain the reproducibility and robustness of the method. Different protocols should also have been evaluated to determine the best combination of procedures to be used.

Rectus abdominous skeletal muscle tissue collected at term from GDM and NGT obese women were analyzed by a gel-based approach to investigate the cellular mechanisms underlying a possible relationship between differences in skeletal muscle metabolism and the development of GDM. Data suggested an association between GDM and reduced skeletal muscle oxidative phosphorylation and disordered calcium homeostasis (Boyle et al., 2014).

Additionally, a label-free proteomics approach was used to examine the effect of GDM on the expression of proteins in the whey fraction of human colostrum. Samples were collected between the first and third day after delivery and, after casein depletion by treating the colostrum with $CaCl_2$, lipid removal by Wessel and Flügge method, and digestion by trypsin, were analyzed by Orbitrap-based nLC-ESI-MS/MS. Through the statistical evaluation, it was possible to identify 10 altered proteins, suggesting that GDM has consequences on human colostral proteins involved in immunity and nutrition transport. Results did not consider that GDM patients were also obese (Grapov et al., 2015).

3.4 | Comments to proteomics analysis

The general approach followed for the proteomic analysis for GDM studies is reported in Figure 1. Studies herein reviewed are also summarized in Table 1, reporting further data, for example, sample size, specific information on the analytical workflow, statistical methods used, and the proteins showing an altered expression further validated by an independent method.

Serum and plasma are the most studied matrices in GDM proteomics. Reported proteomics methods and analytical workflows were able to identify hundreds of proteins. Downregulated or upregulated proteins associated with GDM were involved in different mechanisms such as inflammation, oxidative stress, insulin resistance, blood coagulation, and lipid homeostasis. Some studies also confirmed that these proteins were also linked with maternal and perinatal short- and long-term complications. Generally, different proteomic approaches highlighted also different protein species and results are study-dependent. However, two interesting proteins, that is, CRP and FGA, were found upregulated in many of the considered investigations. CRP was upregulated in both GDM plasma and serum compared to control samples collected during the 16–20 weeks of gestation but was not dysregulated during the 11–13 weeks of gestation. Further studies are surely recommended to confirm this hypothesis and to validate CRP as an early biomarker for GDM. On the other hand, FGA showed a larger concentration in GDM serum starting from the 8 weeks of gestation and FGA-derived peptides were also detectable in serum. Even in this case, FGA is to be considered as a potential biomarker for GDM diagnosis after proper validation on a bigger and more significative cohort of patients in independent cohorts.

Generally, also considering other matrices and specimens, reported proteomic studies are based on cohorts with questionable dimensions, and reported data should be considered preliminary. It is also to highlight that proteomic investigations are very time-consuming and expensive reagents are usually required, negatively impacting the possibility of considering large cohorts. Furthermore, the proper collection and storage of a high number of samples are challenging, in particular when specimens have to be collected before the diagnosis of the disease, as in the case of the discovery of new biomarkers for GDM. This fact escalates in longitudinal or follow-up studies due to patient dropouts. The validation of results by independent methods, such as ELISA applied to independent cohorts, generally mitigates this issue, increasing the reliability of the findings. The proper selection of case and control samples is fundamental in omics approaches, in particular for pairwise comparisons as the only different variable between considered groups should be the presence or absence of the investigated pathology. Many of the reviewed studies did not consider this aspect, negatively impacting on the reliability and robustness of obtained findings. Greater importance should be dedicated to these aspects in future investigations for both obtaining relevant information for

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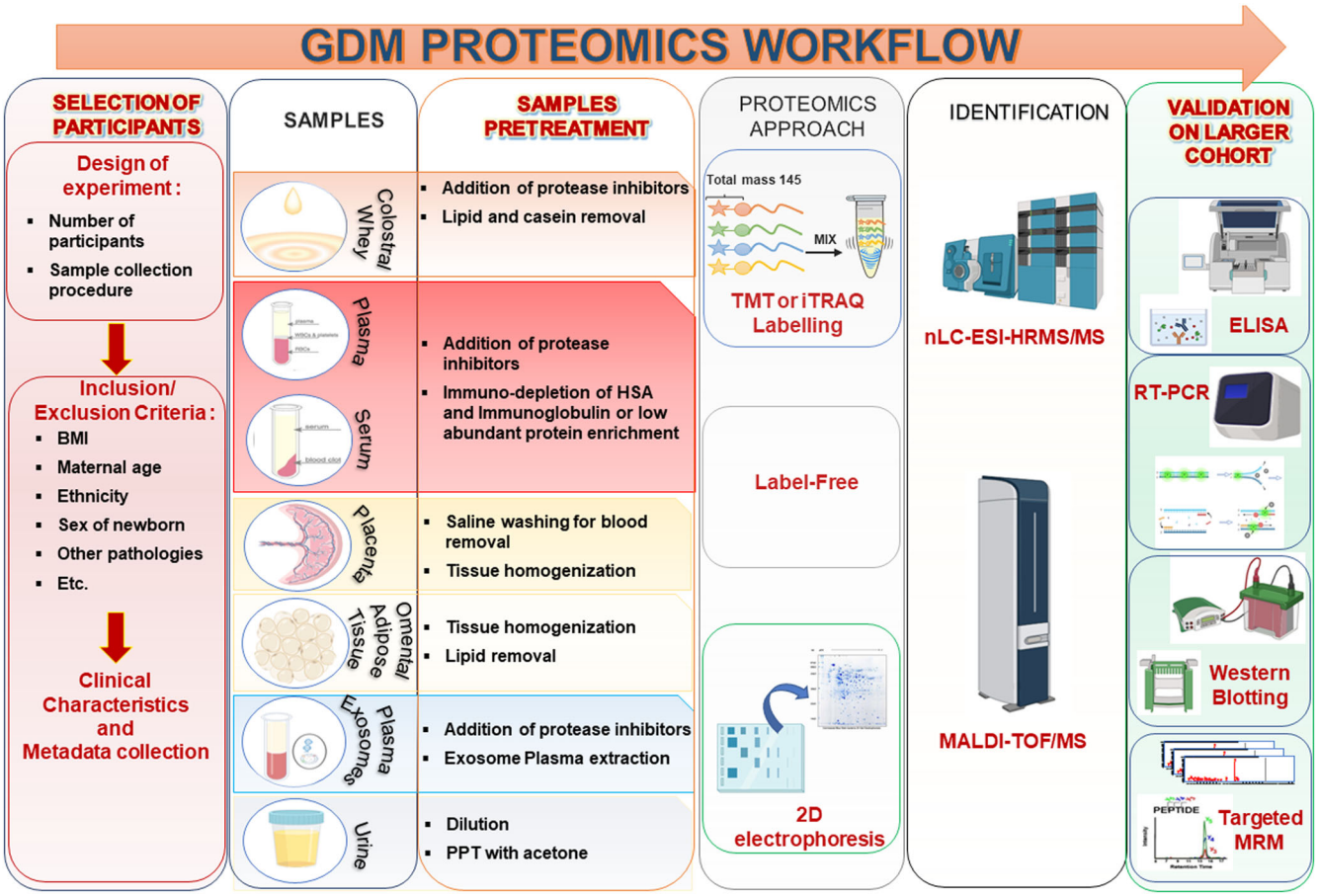

FIGURE 1 Sample preparation scheme for GDM metallomics. PPT, protein precipitation [Color figure can be viewed at wileyonlinelibrary.com]

potential clinical applications, and clearly understanding the biological processes intimately linked to GDM.

Considering the results obtained in reviewed studies, the onset and the development of GDM generally result in the alteration of several metabolic pathways, such as inflammation, oxidative stress, insulin resistance, blood coagulation, and lipid homeostasis. However, different proteins were identified in various studies, leading to low-reproducible findings. In this light, it is still difficult to propose robust protein biomarkers for GDM prediction and diagnosis, as well as for the monitoring of adverse outcomes, to be applied in clinical routine.

4 | MASS SPECTROMETRY-BASED METABOLOMICS IN GDM

Metabolomics is defined as the thorough and systematic analysis of low molecular weight products in biological fluids, cells, or tissues, originated from the various biochemical and metabolic pathways occurring in an organism at a specific time point, and under specific pathophysiological conditions. In recent years, targeted and nontargeted metabolomic approaches led to promising applications in epidemiological and stratified medicine studies for biomarker discovery, as well as to the direct investigation of thousands of metabolites in different matrices, and the enlargement of knowledge regarding the phenotypic fingerprinting (Huynh et al., 2014; McCabe & Perng, 2017; Nicholson et al., 1999). Metabolic biomarkers are directly associated with variations of the physiological state, gene expression, and other alterations of biological mechanisms caused by environmental or chemical stimuli, leading to enhanced outcomes in disease prognosis and diagnosis (Blow, 2008). Even though metabolomics is a relatively new emerging research field, extensive studies have been published concerning the monitoring of various metabolites in different specimens for therapeutic purposes and disease-related investigation (Tzoulaki et al., 2014).

As well as proteomics, GDM related metabolomics propose a comparative approach, that is, the identification of metabolites that shows an altered expression during the onset or progression of the pathology. Various

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body fluids like a serum, plasma, whole or cord blood, amniotic fluid, placenta, and urine were explored by different analytical methodologies, and various molecules were proposed as possible predictive biomarkers (Table 2).

The different possibilities of hyphenation of MS with LC, gas chromatography (GC) or with capillary electrophoresis (CE) pave the way to high throughput analysis on molecules showing a large range of chemical properties. In particular, the high selectivity and sensitivity of MS are essential for metabolite identification, slowly overshadowing proton NMR (Croft et al., 2011; Semwal et al., 2021).

Triple quadrupole (QqQ)-based GC-MS and LC-MS platforms are usually adopted in targeted studies, while QTOF or Orbitrap-based LC-MS/MS instrumentations are widely applied in untargeted investigations. The targeted analysis is often aided by commercially available kits for metabolite identification and quantification, therefore only little information about the development and the validation of analytical methods are reported in those papers. LC separation is usually based on reverse phase interaction, although HILIC is sometimes used to optimize the separation or detection of small and very polar molecules. GC analysis is usually performed after derivatization and proper sample extraction or clean-up (Lind et al., 2016).

Complex and time-consuming sample preparation procedures are usually avoided in metabolomics. Protein precipitation is a usual pretreatment performed with different combinations of solvents, that is, methanol or acetonitrile, in basic or acidic conditions, followed by ultracentrifugation and solvent evaporation at controlled temperature or under vacuum conditions. Sample concentration and clean-up procedures are generally reported, to determine analytes at a low concentration range and to reduce the matrix effect, respectively. Solid-phase extraction is rarely reported and applied only to the analysis of target compounds, that is, bile acids (Eslami et al., 2016; Lorenzo et al., 2015). The selection of the proper method is however analyte- and specimen-dependent. In general, urine, serum, plasma, and amniotic fluid are simply treated by protein precipitation and dilution, while more complex matrices such as placenta or mother hair (He et al., 2016) require more complex preparation steps. The correct storage of samples is also a major concern and must be thoroughly considered for the integrity and stability of specimens before the analysis (Dudzik et al., 2018). In general, samples should be stored at −80°C and for short-term periods. As for proteomics, large amount of data must be statistically evaluated to extract information concerning the possible alteration of metabolites in the studied specimen. Various software packages are commercially available, that is, Compound Discoverer, Progenesis QI, and so on, together with many free or open-source tools such as the XCMS platform (Tautenhahn et al., 2012). Multivariate statistical techniques and several classification methods, i.e. PCA, PLS, PLS-DA and OPLS-DA, are widely used to shrink the number of variables and highlight potential biomarkers, as well as for validation of findings.

Another critical and challenging task in untargeted metabolomics is the annotation of metabolites. The Chemical Analysis Working Group (CAWG) of the Metabolomics Standard Initiative (MSI) identified four different levels of metabolite identification confidence: confidently identified compounds (Level 1), putatively annotated compounds (Level 2), putatively annotated compound classes (Level 3), and unknown compounds (Level 4) (Goodacre et al., 2007; Sumner et al., 2007). Guidelines assess that the level of identification should be clearly reported for all the metabolites described by the investigation.

Most studies report metabolites already identified and well-characterized, ascribable to Level 1. Thus, nonnovel metabolites are identified by comparing singular chemical parameters with authentic samples or chemical reference standards, but sufficient information, that is, minimum standards for Level 1, must be provided. Validation of nonnovel metabolites required at least two independent orthogonal data obtained by identical experimental conditions, which is for LC or GC-MS analysis either retention time or retention index with MS spectrum, accurate mass with MS/MS spectrum, or accurate mass with the isotopic pattern. Additional confidence requires anyway spectral matching with a public or proprietary library, and additional orthogonal data for unambiguous metabolite identification, such as information on stereo configuration, selective extraction process, diode array spectra, selective derivatization, and isotope labeling. Lower levels of identification are based on the comparison of the experimental physicochemical properties or spectral similarities with commercially available or open-access spectral libraries without utilizing chemical reference standards (Level 2), or on spectral similarity to known molecules of a particular chemical class (Level 3). Unidentified or unclassified metabolites that can be distinguished by spectral data are classified at Level 4.

4.1 | Plasma and serum metabolomics

Plasma and serum are the most common matrices investigated by different targeted and untargeted

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TABLE 2 Summary of MS-based metabolomic studies for GDM

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
Plasma	38 nonpregnant women with a history of GDM	37.9/29.3 versus 33.9/27.3	OGTT (75 g glucose)	Derivatization by "EZ:faast" kit for amino acids analysis	GC-MS	Student paired *t*-tests	Targeted, Level 1	20 Amino acids (↓); cystine (↑)	Follow-up of women with a history of GDM; several metadata and other biochemical parameters considered/small sample size; analytical method not completely reported	Bentley-Lewis et al. (2014)
Plasma (second trimester)	20 GDM, 20 Controls	28.1/27.4 versus 28.5/23.8	OGTT (75 g glucose)	Protein precipitation with MeOH/EtOH (1/1); separation by C18 column	LC-QTOF-MS/MS	PCA, PLS-DA, OPLS-D, Welch's *t*-test	Untargeted, Level 2	LPE(20:1), LPE(20:2), LPE (22:4), LPC(20:5), LPC (18:2), LPC(18:1), LPI (20:4), LPS(20:0), lysophosphatidic acid LPA(18:2), lipoxin C4, taurine-conjugates bile acids, trihydroxy-cholestanoyl taurine, taurolythocholic acid glucuronide (↓)	Multi-platform approach; normalization by creatinine for urine and C18:0 methyl ester for plasma/samples collected during the second trimester; quite small sample size	Dudzik et al. (2014)
				Protein precipitation with cold acetonitrile; derivatization with O-methoxyamine hydrochloride in pyridine, and silylation by N,O-bis (trimethylsilyl) trifluoroacetamide in 1% trimethylchlorosilane	GC-Q-MS		Untargeted, Level 2	2-hydroxybutyrate, 3-hydroxybutyrate, glycerol, linoleic acid, fumaric acid (↑); creatinine, pyruvic acid, L-tryptophan, glycine, L-glutamic acid, lauric acid (↓)		
Urine (second trimester)				Incubation with 4 volumes of 0.125 M HCOOH	CE-TOF-MS		Untargeted, Level 1	Histidine, glutamine, phenylalanine, tryptophan, cysteine (↑) carnitine (↓)		
Plasma (second trimester, third trimester, and 1 month and 3 months after delivery)	24 GDM, 24 controls	28.3/27.7 versus 29.2/25.4	OGTT (75 g glucose)	Protein precipitation with cold acetonitrile; derivatization with O-methoxyamine hydrochloride in pyridine, and silylation by N,O-bis (trimethylsilyl)	GC–QQQ-MS	PCA, PLS-DA, OPLS-D	Untargeted, Level 2	2-hydroxybutyrate, 3-hydroxybutyrate, stearic acid and sorbitol (↑)	Follow-up of patients until 3 months after delivery; method is well described/quite small sample size; highlighted biomarkers should be identified at Level 1	Dudzik et al. (2018)

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
				trifluoroacetamide in 1% trimethylchlorosilane						
Plasma (24–28 gw: 30 min and 120 min after OGTT)	9 GDM, 15 controls	32/29.8 versus 30/25.6	OGTT (75 g glucose)	Samples were prepared by AbsoluteIDQ® p180 kit for FIA and LC analysis; fatty acids were analyzed by GC-MS after methanol transesterification in acidic condition	LC-QTrap-MS/MS; FIA-QTrap-MS/MS; GC-MS	*F*-test	Targeted, Level 1	C18:0 acylcarnitine (↑); diacyl phosphatidylcholines (PC aa C34:4, PC aa C36:4, PC aa C38:5, PC ae C36:4, PC ae C36:5, Lyso PC C20:4), arachidonic acid (↓)	Target approach for 231 metabolites/small sample size; BMI is statistically different between controls and GDM patients	Lehmann et al. (2015)
Plasma (24–28 gw)	18 GDM, 13 controls	31.5/26.8 versus 31.2/24.6	OGTT (75 g glucose)	Free amino acids were analyzed by aTRAQ™ kit and C18-based chromatography; peptides were analyzed by MALDI-MS	LC-QTrap-MS/MS	*t*-tests and Man–Whitney U tests, PLS-DA, Discriminant Function Analysis (DFA)	Targeted, Level 1	L-citrulline (↑); L-asparagine, L-valine, and L-ornithine (↓)	Circulating peptides were considered in the analysis/limited number of metabolites considered; small sample size	Hajduk et al. (2015)
Plasma (10–14 gw, 23–27 gw, 29–33 gw)	27 GDM, 34 controls	29.0/20.9 versus 26.9/22	OGTT (75 g glucose)	Phospholipid and protein depletion by Ostro pass-through 96-well sample preparation *p* plate (C18 sorbent); freeze-drying; analysis by HILIC and reverse-phase chromatography	UPLC-QTOF-MS	PCA, PLS-DA	Untargeted, Level 2	*Plasma phospholipids:*polyunsaturated glycerophosphoethanolamine (↓ first two trimesters), N-acyl lysophosphatidylethanolamine, N-stearoyl-1-oleoyl *sn*-glycero-3-phosphoethanolamine (↑ in third trimester). *Mevalonate pathway metabolite:*isopentenyl phosphate (↓) *plasma Free Fatty Acid*: (2E)−14-hydroxytetradec-2-enoic acid, (2E,13 R)−13-hydroxytetradec-2-	Metabolomics and lipidomics using the same sample preparation; metadata and clinical and behavioral characteristincs of enrolled subjects were considered in the statistical analysis/small sample size	Law, Mao et al. (2017)

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
								enoic acid, (7 R,8 S,9Z,12Z,15Z) −7,8-dihydroxy-9,12,15-octadecatrienoic acid (↓) Steroids: 4-hydroxyestrone sulfate (↑) *Flavonoids:*sesaminol 2-O-triglucoside and tricin 7-neohesperidoside are flavonoid, dihydro-12-oxo-15-phytoenoic acid, 11α,20,26-trihydroxyecdysone, prostanoic acid (↑)		
Plasma (15 gw)	54 GDM, 563 Controls	Not reported for each group	OGTT (75 g glucose)	Not reported	FIA-MS; identification by UHPL-MS	PLS, PLS-DA	Not reported	TG (51.1), TG (48:1), PC (32:1), PCae (40:3), and PCae (40:4)	Findings were validated in an independent cohort; identified lipid biomarkers are independent of age and BMI/GDM group is numerically inconsistent with controls; methods are not clearly reported	Lu et al. (2016)
Plasma (6–9 weeks postpartum after 2 h from the OGTT)	130 T2D women (with a history of GDM) and 130 controls (with a history of GDM)	35.4/33.1 versus 35.4/32.6	OGTT (75 g glucose)	163 metabolites were assayed by the p150 AbsoluteIDQ plate; amino acids were analyzed in HILIC condition; fatty acid by GC-MS after derivatization by 1% pentafluorobenzyl bromide and 1% diisopropylamine	LC-QTrap-MS/MS; GC-MS/MS	*t*-test	Targeted, Levels 1 and 2	2-aminoadipic acid, Isoleucine, Leucine threonine, tryptophan, tyrosine, valine, xleucine, Hexoses, and AC3 (↑) Glycine, SM (OH) C16:1, SM (OH) C22:2, SM C18:0, SM C18:1, SM C20:2, SM C24:2, PC ae C40:5, PC ae C42:5, PC ae C44:5, AC10, and palmitoleic acid (↓)	Two years follow-up of GDM patients to assess T2D development; good sample size/considered subjects were obese (BMI > 31)	Allalou et al. (2016)

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
Serum (20 gw)	22 GDM, 26 controls	Not reported	Not reported	Samples were freeze-dried, extracted with MeOH, and derivatized by methyl chloroformate	GC-MS	*t*-test	Untargeted, Level 2	Itaconic acid (↑)	/Small sample size; limited number of metabolites considered; clinical characteristics of the participants were not reported	Seymour et al. (2014)
Serum (16 gw)	178 GDM, 180 controls	34.0/26.7 versus 33.1/23.4	OGTT (50 g glucose)	Protein precipitation by MeOH, isopropanol, and water (3:3:2); derivatization by MSTFA	GC-TOF-MS	lasso regression; multivariable regression analysis	Untargeted, Level 2	*Fatty Acid*: linoleic acid, oleic acid, myristic acid (↑) *Sugars and alcohols*: D-galactose, D-sorbitol (↑), o-phosphocolamine (↓), *Amino acids*: L-alanine, L-valine (↑), 5-hydroxy-L-tryptophan, L-phenylalanine-phenyl, L-serine, sarcosine, L-pyroglutamic acid, and L-mimosine (↓) *organic acids*: L-lactic acid, fumaric acid (↑), glycolic acid, and urea (↓)	Good sample size/ diagnostic metabolites should be identified at Level 1	Enquobahrie et al. (2015
Serum (28–36 gw; 8–14 months afterbirth)	11 GDM, 24 controls, 21 NGT (increased risk)	34.3/26 versus 33/22	OGTT (75 g glucose)	Not reported	GC-TOF-MS	PCA, OPLS	Not reported	Tryptophan, ornithine, proline, lactose, dimethylarginine and citrulline, leucine, isoleucine, and Valine (↑) Chorell et al.,	Follow-up of patients afterbirth /Small samples size; limited number of investigated metabolites	Chorell et al. (2017)
Serum (first trimester)	96 GDM, 96 Controls	33.3/28.2 versus 32.4/3.7	OGTT (75 g glucose)	Not reported	LC-MS	*t*-test	Targeted, Level 1	Anthranilic acid, alanine, glutamate, allantoin, and serine (↑); creatinine (↓)	Good sample size/Method was not reported	Bentley-Lewis et al. (2015)

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
Serum (third trimester, postpartum within 3 days)	12 GDM 10 Controls	30.75/27.62 versus 29.50/24.31	OGTT (75 g glucose)	Extraction with CAN; separation by C18 column	UPLC-QTOF-MS	PCA, PLS-DA, OPLS-DA	Untargeted, Level 2	3-methylthiopropionic acid, homovanillic acid, 1-methyladenosine, arabinonic acid, glycinea, glucosamine, *trans*−3-octenedioic acid, indole acetaldehyde, pyridoxal, 3-oxooctadecanoic acid, capric acid, stearic acid, LysoPC(20:4), LysoPC (20:2), LysoPC(16:1), PE (14:1/20:0), SM(d18:0/18:0), L-Carnitine (↑) 2-oxo-4-methyl-thio-butanoic acid, phosphorylcholine, L−2-hydroxyglutaric acid, L-lactic acid, L-tyrosine, gluconolactone, 3-methylhistidine, L-lysine, vitamin A, ribothymidine, corticosterone, LysoPE (22:6/0:0), 17-hydroxyprogesterone, and 1, 4-beta-D-glucan (↓)	Simplified sample preparation/small sample size; BMI is statistically different in GDM patients compared to controls	Liu et al. (2016)
Serum (26–31 gw)	38 GDM 21 ICP 27 controls	30.1/23.1 versus 27.8/23.2	OGTT (75 g glucose)	Acidified with HCOOH; preconcentration on SPE Bond Elut-C18 cartridge; separation by C18-based column	UPLC-QTOF-MS	Student-Newman-Keuls (S-N-K), PLS-DA, OPLS-DA	Targeted (Level 1) untargeted, (Level 2)	Trihydroxy conjugated Bile acid: β-MCA, dihydroxy conjugated BILE acid: GHDCA, THDCA, Sulfate Bile acid: dehydro-1S, mono-2S + mono-3S, mono-4S, and mono-5S.	Combined targeted and untargeted analysis/small sample size	Gao et al. (2016)

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
Serum (first trimester)	243 GDM, 243 Controls	29.5/24.1 versus 28.3/22.1	OGTT (75 g glucose)	Protein precipitation by cold ACN with ammonia	LC-QTOF-MS/MS	paired t-test, Wilcoxon signed-rank test	Targeted, level 1	Glycoursodeoxycholic acid, deoxycholic acid (↓)	Good sample size; risk factors for GDM were considered in the statistical analysis/ lifestyle was not considered	Li et al. (2018)
Serum (24–28 gw)	131 GDM, 138 Controls	31.4/22.3 versus 30.4/20.9	OGTT (75 g glucose)	The reported procedure is not well described	UPLC-QTOF/MS	Student's t-test, Mann-Whitney U-test, OPLS-DA	Target, level 1	Free fatty acids (↑): C14:1(trans-9), C16:1 (cis-7), C17:1 (cis-10), C18:1, C19:1, C20:1, C12:0, C16:0, C17:0, C20:0, C16:2 (cis-9_12), C18:2 (9_11&10_12-cis&trans-conjugated-99%), C18:3 (cis-9_12_15), C22:3 (cis-13_16_19), C18:3 (cis-6_9_12), C18:2 (cis-9_12), C20:4 (cis-5_8_11_14), C20:3 (cis-8_11_14), C20:2 (cis-11_14), C22:5 (cis-7_10_13_16_19)	Multiplatform analysis; good sample size/details on analytical methods are not reported	Hou et al. (2018)
					UPLC-QQQ-MS			Bile acids: CA, dehydro_LCA (↓); THDCA, LCA, HDCA, isoDCA, 6_7_diketoLCA (↑)		
					GC-TOF/MS		Untarget, Level 2	Leucine, Valine, Acetylaspartic acid, Alanine, Glutamic acid 2-aminobutanoic acid, 2-oxo-4-methylvaleric acid, gamma-aminobutanoic acid, aminomalonic acid, pyruvic acid, 1-monooleoylglycerol, 2-ethylhexanoic acid, Mannose, Maltose, Threitol (↓); Cysteine, 2,3,4-trihydroxybutyric acid, Threonic acid (↓)		

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
Mother and cord serum (afterbirth)	31 GDM, 381 Controls	32.6/24.6 versus 30.3/22.5	OGTT (75 g glucose)	163 metabolites were assayed by the p150 AbsoluteIDQ plate	FIA-ESI-MS/MS	Unpaired *t*-test	Targeted, Level 1	Cord blood: phosphatidylcholine acyl-alkyl C 32:1, proline (↑)	Simple and fast analytical methods for targeted metabolomics/GDM group is numerically inconsistent with controls	Lu et al. (2018)
Meconium (afterbirth)	142 GDM, 197 Controls	28.07/21.98 versus 26.14/20.51	OGTT (75 g glucose)	Freeze-drying; extraction by MeOH, sonication, centrigugation; separation by C18-based chromatograohy	UPLC-QTOF-HRMS	PCA, PLS-DA	Untargeted, Level 2	Argininosuccinic acid, tetrahydrodipicolinate, methyladenosine, taurodeoxycholic acid, DHAP(8:0), methylepicatechin, methylxanthine, dimethyluric acid (↑), hydroxyindoleacetylglycine, methylguanosine, uric acid, glycocholic acid, oxotrihydroxyleukotriene B4, vanilloylglycin (↓)	Good sample size; analysis of both urine and meconium; simple methods to prepare samples/Diet and other behavioral characteristics of participants should be considered	Peng, Zhang et al. (2015)
Urine (afterbirth)				Centrifugatio; Dilution with deionized water (1:1); separation by C18-based chromatograohy	UPLC-Orbitrap-HRMS			uric acid, uridine (↑) estrone (↓)		
Urine (16–17 gw)	25 GDM, 25 Controls	34.2/27.0 versus 34.2/25.0	50 g OGCT failure followed by 100 g OGTT	Not reported	LC-MS/MS	Mann–Whitney test. Spearman correlation coefficients (CC), logistic regression	Targeted, Level 1	Ethylmalonate, pyruvate (↑); adipate (↓)	First to examine pre-diagnostic samples/ Information on the analytical method are not reported; small sample size	Qiu et al. (2014)

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
Urine (6–19 gw)	46 GDM, 46 Controls	32.2/31.5 versus 31.8/30	OGTT (75 g glucose)	Not reported	UPLC-MS/MS, GC-MS	Wilcoxon rank-sum test, chi-square test, gradient-boosted logistic regression	Not clear	Dihydroorotate, phenol glucuronide, 7,8-dihydroneopterin, nicotinate ribonucleoside, lanthionine, and dopamine	Multiplatform analysis; multivariate statical operation/method is not reported; metabolite ID not defined; cases and controls were obese (BMI > 31)	Koos et al. (2021)
Urine (first, second, and third trimester)	27 GDM, 34 Controls	18-35 versus 18-30	OGTT (75 g glucose)	Dilution with 0.2% HCOOH (1:1); centrifugation at 4°C for 15 min	UPLC-QTOF-MS	PCA, PLS-DA	Untargeted, Level 2	*Tryptophan Metabolites*:indole acetaldehyde, indoleacetic acid, indole-3-acetamide, serotonin, oxitriptan, 5-hydroxykynurenamine (↑) *Nucleoside or Purine Metabolites*:xanthine. xanthosine, 1-methylhypoxanthine, N4-acetylcytidine, succinyladenosine, aminomethyl-7-carbaguanine (All 3 trimesters), 1,7-dimethylguanosine, N6-carbamoyl-L-threonyladenosine, O6-ethyl-2′-deoxyguanosine, uridine, Hypoxanthine, 7-Methylguanine, 1-methyladenosine, N6-methyladenosine, 3-(3-amino-3 carboxypropyl) uridine, 9-riburonosyladenine (↑)	Samples were collected at three time-points during pregnancy; simple method for sample preparation/quite small sample size	Law, Han et al. (2017)

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TABLE 2 (Continued)

Specimen	Sample population	Age/BMI GDM versus Age/BMI control	Criterion of GDM diagnosis	Sample preparation	Analytical tool	Statistical operation	Type of analysis and level of annotation	Significantly altered metabolites in GDM	Strength/Weakness	Ref.
Urine (22–28 gw)	20 GDM, 20 Controls	28.1/27.4 versus 28.5/23.8	OGTT (75 g glucose)	Preconcentration on SCX SPE; derivatization by PFPA	Chiral GC-MS	Not reported	Targeted, Level 1	D-phenylalanine (↑)	Small sample size	Lorenzo et al. (2015)
Placenta (at term)	6 GDM nonobese, 10 obese, 17 controls	32/23.3 versus 31/22.5	OGTT (75 g glucose)	Extraction by MeOH/$CHCl_3$ (1:2); separation by C18 column	UPLC-QQQ-MS	Mann–Whitney-U tests	Targeted, level 1	Arachidonic acid, docosahexaenoic acid and species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), especially PE(16:0/22:6), PE(18:0/20:4) (↑), PC(18:0/20:3), PC(18:1/20:3) and PS(18:0/18:2) (↓)	Comparison between obese and GDM patients/the comparison between normal-weight GDM and obese GDM subjects was not considered; quite small sample size	Uhl et al. (2015)
Amniotic fluid (16–18 gw)	20 GDM, 20 controls	37.4 versus 37.3	Not reported	Protein precipitation by methanol. LC analysis was performed by C18 and Amide-based chromatography	GC-MS, LC-Orbitrap-HRMS	PCA	Targeted, Level 1	Glucose, amino acid, glutathione, fatty acid, sphingolipid, bile acid docosahexaenoic acid, and arachidonic acid	Samples collected during the early second semester/small sample size	O'Neill et al. (2018)
Breath (24–28 gw)	8 GDM, 32 NGT, 12 IGT	22.8 versus 23	OGTT (75 g glucose)	-	PTR-MS	MANOVA	Targeted, level 2	Acetone, aldehydes, thiols, sulfide	Fast analysis for GDM screening/small sample size	Halbritter et al. (2014)

Abbreviations: CAN, acetonitrile; Gw, gestational week; MeOH, methanol; OPLS-DA, orthogonal PLS discriminant analysis; PFPA, pentafluoropropionic anhydride; PLS-DA, partial least squares discriminant analysis; SPE, solid-phase extraction.

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metabolomic approaches, based on both GC and LC coupled to MS. The focus was the determination of biomarkers for GDM diagnosis and to evaluate future outcomes. Details on used methods and instrumental platforms, as well as the applied statistical evaluation and levels of metabolite identification are reported in Table 2.

4.1.1 | Plasma

Polar and nonpolar analytes were identified and quantified in plasma, considering samples collected during the various trimester of pregnancy, but also after birth. In Hajduk et al. (2015) amino acids were evaluated by a targeted approach, combined with a nontargeted screening of circulating peptides, applied to plasma samples collected during the 24–28 weeks of gestation to determine specific GDM biomarkers. Ethanolamine, L-citrulline, L-asparagine, together with several peptides, were overexpressed in GDM samples and were implemented in the final prediction model. A targeted analysis was also carried out to assess possible modification of the metabolite pattern before and after a 75 g OGTT performed during the 24–27 weeks of gestation in healthy and GDM women. The analysis was focused on 231 plasma metabolites, including amino acids, biogenic amines, acylcarnitines, glycerophospho-lipids, sphingomyelins, hexoses and free fatty acids. The study identified eight insulin resistance-associated metabolites showing an alteration in the expression during the first 30 min from the starting of the OGTT. It is also to report that selected GDM patients showed a higher BMI than the controls, therefore possible wrong data interpretation should be considered (Lehmann et al., 2015).

In another study, the postpartum T2D transition due to metabolic modifications occurring in GDM was examined in a follow-up manner by Allalou et al. (2016). Plasma samples were collected 6–9 weeks after postpartum, and results revealed two novel metabolites, phosphatidylcholine ae C40:5 and sphingomyelin (OH) C14:1, as being predictive for incidence of T2D. Amino acids were also analyzed by a target GC-MS method in plasma samples collected before and after 2 hours from a 75 g OGTT administered to women with a previous history of GDM in the following 3 years after birth to highlight possible correlations with risk factors for T2M. Interestingly, most of the observed changes in the amino acid profile were associated with a longer duration of breastfeeding and higher BMI (Bentley-Lewis et al., 2014).

Considering untargeted metabolomics, early biomarkers for the diagnosis and prognosis of GDM were evaluated by a multiplatform approach on plasma and urine samples collected during the 22–24 weeks of gestations (Dudzik et al., 2014). Altered metabolites were associated with impaired glucose homeostasis, low-grade inflammation and altered redox balance. Among the various highlighted species, plasma lysophospholipids were the most altered metabolites in the case of GDM patients compared to controls, with the highest discriminant power.

Plasma metabolome was also investigated by an untargeted longitudinal study to assess differences in pregnancy progression between GDM and NGT women (Law, Mao, et al., 2017). A large range of plasma metabolites was covered by using both HILIC and RP-based chromatography. Polyunsaturated and chemically modified phospholipids were significantly lowered in plasma from GDM patients compared to healthy controls, independently on the stage of gestation. The alteration of these metabolites was explained by hypothesizing an alteration of the enzymatic activity in the case of GDM, but also to the dietary intake. As for other omic approaches, it is essential in metabolomics to collect proper data regarding the clinical characteristics and lifestyle behaviors of individuals, by administering well-organized and validated questionnaires to participants. Such information should be implemented in the statistical analysis to reduce the possibility of obtaining wrong interpretations due to confounding factors. In this light, lipidomics was applied to the identification of predictive lipid biomarkers in samples collected during the early second trimester. Identified triglycerides, phosphatidylcholines and choline ether phospholipids were able to predict GDM independently on maternal age and BMI, enhancing the predictive performance of existing risk factor-based approaches (Lu et al., 2016). It is worth noting that such biomarkers were discriminated considering patients' characteristics as well as GDM status.

Untargeted metabolomics was also applied to a longitudinal study, from the second trimester of pregnancy until 3 months after delivery, to evaluate how the glycemic control of patients was associated with alterations of the metabolism. Interestingly, many metabolites remain altered in GDM patients compared to healthy subjects, also in subjects with an effective glycemic control. In addition, 2-hydroxybutyrate and 3-hydroxybutyrate were considered as prognostic biomarkers to predict the onset of postpartum complications, in particular T2M (Bentley-Lewis et al., 2015).

4.1.2 | Serum

Metabolomics studies on serum used similar approaches than those on plasma.

Chorell et al. (2017) investigated the modifications of the serum metabolomic profile, from the third trimester of pregnancy until 1 year postpartum, were evaluated by GC-MS to highlight branched-chain amino acids as markers

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of progression from GDM to T2D. Leucine, isoleucine, and valine resulted upregulated in serum samples collected postpartum from GDM patients compared to NGT subjects, although no significant differences were found in the two groups in plasma samples collected during the late third trimester. Furthermore, possible correlations with T2D were not possible, as patients were not followed-up until the development of the pathology. Branch chain amino acids and other metabolites were also evaluated as possible markers for GDM prediction in serum samples collected during the first trimester of pregnancy, but differences between groups were not significative (Bentley-Lewis et al., 2015).

Serum bile acids were investigated in samples collected during the first trimester (Li et al., 2018) and during the third trimester (Gao et al., 2016). Decreased levels of glycoursodeoxycholic acid and deoxycholic acid were associated with increased risk of GDM in Chinese pregnant women and evaluated as possible biomarkers for GDM diagnosis during the first trimester of pregnancy.

Multianalytes targeted metabolomics was also applied to the discovery of biomarkers for GDM diagnosis in samples collected during the 24–28 weeks of gestation (Hou et al., 2018). Significant changes were observed in free fatty acids, bile acids, branched chain amino acids, organic acids, lipids and organo-oxygen compounds and different combinations of these analytes showed good discriminative power for GDM. Although the study was well-designed and the number of participants was appropriate, highlighted biomarkers should be further validated in the early first trimester, as the diagnosis of GDM in the second trimester is already possible by OGTT.

Interestingly, GDM associated metabolites were investigated also in serum cord blood by a targeted FIA-MS/MS analysis (Lu et al., 2018). Fetal phosphatidylcholine acyl-alkyl C 32:1 and proline showed an independent association with GDM after a proper statistical evaluation, suggesting a potential effect of GDM on the fetal metabolic profile. Furthermore, the used method is to be highlighted, as FIA is faster and cheaper (no columns are used and only some milliliters of eluents are wasted) compared to LC, allowing short-response time and the analysis of a greater number of samples.

An untargeted GC/TOF-MS approach analyzed serum samples collected during the 16th week of gestation from 178 GDM and 180 pregnant women. The statistical analysis highlighted several metabolites, including amino acids, organic acid, sugars, and free fatty acids as early predictor markers for GDM (Enquobahrie et al., 2015). Although findings are promising for future application in clinical routine, the identification of metabolites analyzed by derivatization and GC-MS should be reported at level 1, as for some classes of molecules, that is, sugars and organic acids, the assessment of the right isomer present in samples may be affected by the derivatization procedure (Black et al., 1996). Another research identified 48 altered biomarkers of the same chemical classes in serum samples collected during the 20 weeks of gestation and analyzed by methyl chloroformate alkylation followed by single quadrupole GC-MS (Seymor et al., 2014). Itaconic acid was highlighted as a potential biomarker to be validated in a larger cohort.

Possible mechanisms underlying the development and progression of GDM were investigated by Liu et al. (2016) through a longitudinal untargeted study on blood serum. Samples were collected in the first trimester of pregnancy and within 3 days postpartum from 12 GDM and 10 NGT volunteers. GDM patients showed an alteration of 35 metabolites compared to controls, involved in lipid, carbohydrate, and amino acid metabolism. It is to highlight that the average BMI was higher in the GDM cohort compared to controls and therefore results should be properly validated in a larger and well-selected pool of patients.

4.2 | Miscellaneous matrix metabolomics

GDM metabolomics is not confined to blood, but also urine and other uncommon matrices, that is, amniotic fluid, placenta, cord blood, meconium, maternal hairs, and breath were considered.

Meconium and urine were collected afterbirth from newborns delivered by GDM and healthy mothers to identify biomarkers for the monitoring of historic metabolic alterations due to prenatal disorders. Samples were analyzed by an untargeted metabolomic approach revealing several endogenous biomarkers associated with maternal GDM risk and involved in lipid, amino acid and purine metabolism (Peng, Zhang, et al., 2015; Peng, Liu, et al., 2015).

Urine samples investigated for GDM-metabolomic profiling were collected during different periods of gestation, either in the first trimester (Koos et al., 2021; Qiu et al., 2014), second trimester (Lorenzo et al., 2015), or throughout the gestations (Dudzik et al., 2014; Law, Han et al., 2017).

Qiu et al. (2014) analyzed urine samples collected during the 16–17 weeks of gestation by a not-well detailed target LC-MS/MS method for assessing the concentration of molecules involved in fatty acid and carbohydrate metabolism. It was found that a temporal relationship exists between the development of GDM and the urinary excretion profile of organic acid. In addition, three metabolites (two related to the metabolism of fatty acid and one related to carbohydrate metabolism) were proposed as early diagnostic markers for GDM. Urine samples collected in the same period of gestation were also analyzed by LC-MS/MS in dual ionization mode and

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GC-MS analysis, but it is not clear which was the analytical approach used and the identification level for the 626 reported metabolites (Koos et al., 2021). Findings highlighted seven molecules independently associated with GDM (patients and controls were obese) and involved in glucose oxidation, fatty acid oxidation, and amino acid catabolism. Maternal urine was also investigated in a longitudinal cohort by an untargeted approach to provide new insight into the pathophysiology of GDM. Tryptophan metabolites and purine nucleosides were upregulated in GDM subjects compared to controls in each stage of gestation (Law, Han, et al., 2017).

D-amino acids were evaluated in urine samples collected during the second trimester and a higher concentration of D-phenylalanine was found in the case of GDM samples compared to controls (Lorenzo et al., 2015).

The placenta lipid profile was evaluated by a targeted LC-ESI-MS/MS method after extraction of samples by chloroform-methanol (2:1), considering GDM and obese cohorts compared to NGT subjects (Uhl et al., 2015). Different glycerophospholipids were similarly altered in both GDM and obese groups, probably due to a change in the placental transfer of fatty acids. Authors hypothesize that the impairment of this mechanism may be involved in long-term outcomes, such as insulin resistance.

O'Neil et al. (2018) evaluated the metabolome of second-trimester amniotic fluid using a targeted multiplatform metabolomic approach. GDM exposure was linked to the alteration of 69 metabolites in a sex-specific manner, related to glucose, amino acid, glutathione, fatty acid, sphingolipid, and bile acid metabolism.

Investigation of noninvasive GDM biomarkers in the maternal breath gas was also evaluated by proton transfer reaction—quadrupole mass spectrometry (PTR-MS). Volatile organic compounds were constantly monitored for 6–10 min in the breathing gases of 24 pregnant women after administration of a 75 g OGTT test. The end-tidal breath gas analysis reported an increment in oxidation products of glucose and lipids, acetone metabolites, and thiols, marking the advantage of this online technique for future application in GDM screening (Halbritter et al., 2014).

4.3 | Comments to metabolomic analysis

Various metabolomic investigations were reported regarding GDM and the most altered metabolites in different matrices are presented in Figure 2. Plasma, serum, and urine were analyzed by both targeted and nontargeted analyses, generally to assess early biomarkers for diagnosis, but also to identify the alteration of metabolic pathways involved in the physiopathology of GDM. Interestingly, some of the reported studies are longitudinal investigations evaluating the long-term adverse outcomes of GDM, although generally focused on the mother instead of the newborn. Placenta and

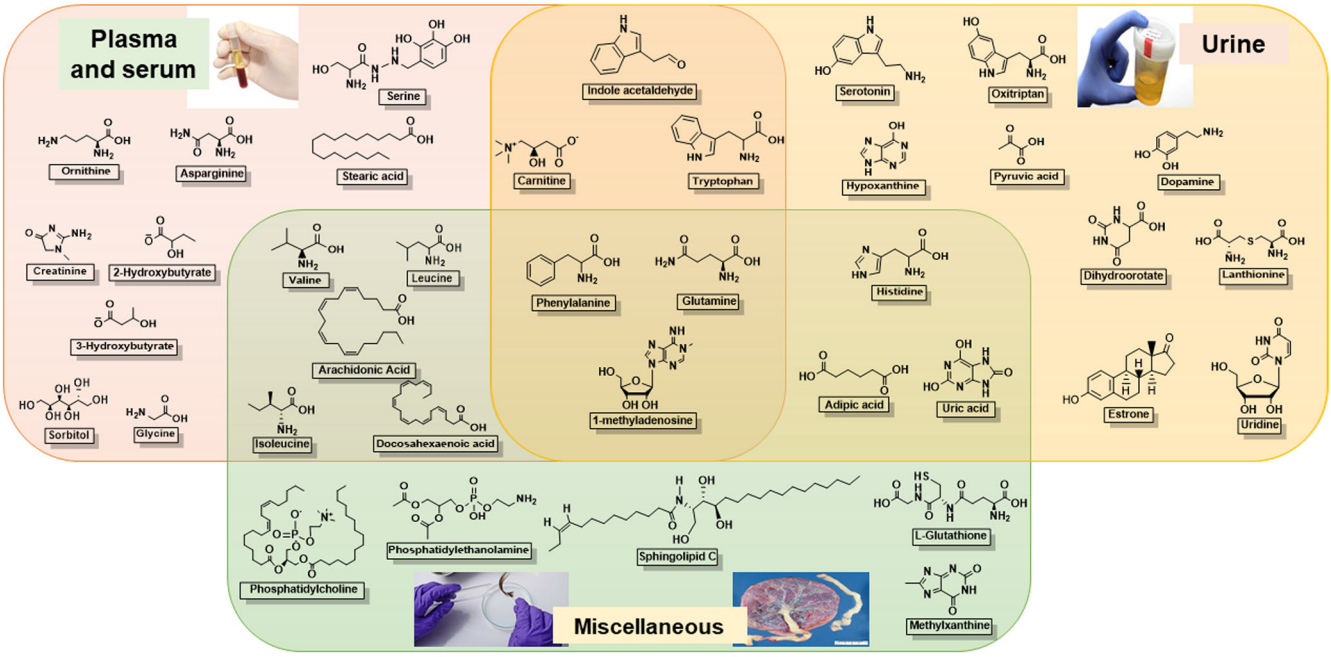

FIGURE 2 Common altered metabolites in gestational diabetes [Color figure can be viewed at wileyonlinelibrary.com]

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amniotic fluid were investigated by targeted analytical methods. Few details about sample preparation procedures are reported and often studies adopted analytical methods previously developed for other purposes. The number of samples considered in the various studies is generally slightly higher compared to proteomics, probably because sample preparation procedures are less demanding in time and costs. On the other hand, very few studies proposed cross-validation of results in independent cohorts. Furthermore, the evaluation of the reproducibility and robustness among studies remains critical, as it is difficult since each matrix was evaluated for different analytes by different techniques. Amino acids and some lipid classes were the molecules mostly altered in GDM patients, but final conclusions are still premature, and findings should be further evaluated and properly validated. These are probably the main reasons for which findings have never been included or tested in clinical routine.

The identification of analytes is another point to critically discuss. Considering the studies reported in the present review, analytes highlighted by untargeted approaches are classified at Level 2, and this is generally considered sufficient and globally accepted, in particular when the identification is based on high-resolution data together with information on the pattern of fragmentation. However, some classes of molecules require dedicated procedures to be identified, also limiting the investigation to Level 2. For example, different monosaccharides were identified in serum by GC-MS (Seymor et al., 2014), but details on the sample preparation were not fully reported. It is also well-known that monosaccharides treated by trimethylsilyl derivatization, one of the most used techniques for derivatization, produce up to four different peaks when analyzed by GC (Black et al., 1996), making the analysis of different isomers very challenging. Through these considerations, it is quite difficult to evaluate if analytes were correctly identified in the previously reported work and to confirm the alteration in the expression of different monosaccharides and further biological interpretations. For these reasons, when challenging analytes are reported and identified, a full description of methods should also be reported, together with the analysis of standards. Analogous considerations are possible for LC-MS/MS analysis, for example, considering isomeric metabolites with superimposable fragmentation patterns, that is, low molecular organic acids. In this case, the identification level should be limited to three until proper confirmation by using certified analytical standards is provided.

It is also to highlight that in the case of metabolomics applied to the study of GDM, reported investigations are more focused on the identification of diagnostic biomarkers instead of describing the pathogenesis of the disease, as the interpretation of the role of altered metabolites is generally only slightly mentioned, while the evaluation of their specificity and sensitivity in the discrimination of patients and healthy subjects is always reported.

5 | MASS SPECTROMETRY-BASED METALLOMICS IN GDM

While metabolomics and proteomics are based on the profiling analysis of the organic molecules content, metallomics is focused on the analysis of the inorganic composition of cells, tissues, or biological fluids, and in the determination of possible alterations due to the onset of a disease or as a result of the exposure to inorganic species via food or environment.

Exposure to certain elements is already known to significantly be involved in the modification of the physiological processes occurring in the human body (Beal et al., 2015; Kessler et al., 2013; Peng, Zhang, et al., 2015), and GDM onset and progression were already associated with the exposure to high metal levels (Wang et al., 2019). Other studies highlighted the association between metals and GDM risk factors (Peng, Zhang et al., 2015; Shapiro et al., 2015). Most of the studies are limited to a few targeted elements as analyses were performed by Atomic Absorption Spectroscopy (AAS) and thus the whole inorganic content of the specimens considered is not available. Recent studies are based on inductive coupled plasma-mass spectrometry (ICP-MS), which allows the acquisition of the elemental fingerprint of the sample under study and improves the sensitivity and detection limits, thus paving the way to metallomics (Roverso et al., 2015, 2019; Wang et al., 2019). However, metallomic investigations related to GDM are limited in literature and usually focused on targeted elements in blood, placenta, and meconium.

Although elements are certainly known and their number limited compared to organic molecules, sample preparation is anyway a crucial step in metallomics, particularly for the determination of trace analytes and to prevent contaminations. The generic sample preparation protocol usually adopted can be schematized as in Figure 3. In general, samples are mineralized by the addition of concentrated nitric acid to dissolve most of the matrix interferences, followed by the addition of hydrogen peroxide and digestion at different temperatures. Microwave-assisted digestion is generally preferred to decrease digestion time and increase the temperature of the process. In the end, it is commonly necessary to adjust

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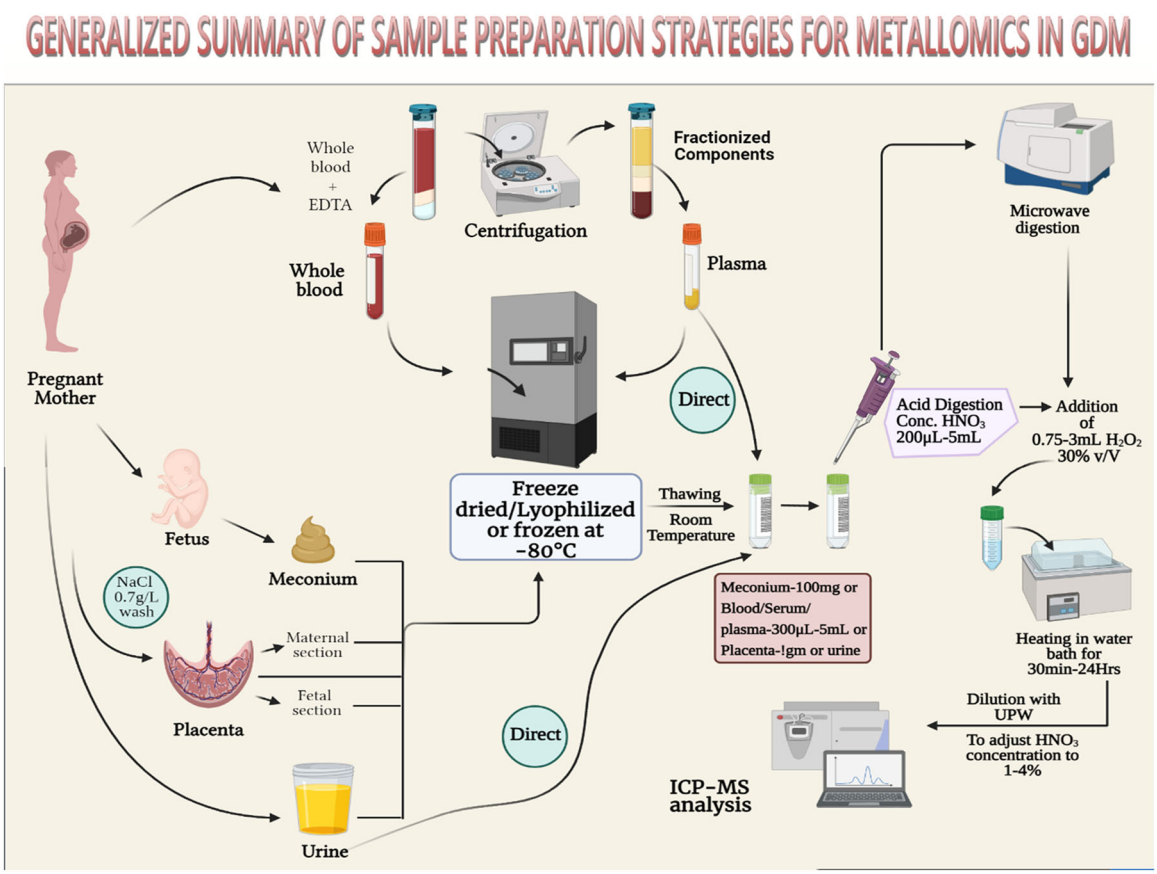

FIGURE 3 Sample preparation scheme for gestational diabetes metallomics. UPW, ultra-pure water [Color figure can be viewed at wileyonlinelibrary.com]

the nitric acid concentration by dilution. Further details on the reviewed studies are reported in Table 3.

One of the earliest studies establishing a relation between GDM development and the elemental composition of nonheparinized blood samples collected during 24–28 weeks of pregnancy in GDM and NGT pregnant women was carried out by Caglar et al. (2012) by ICP-MS for evaluating the effect of boron. This element is involved in triglycerides and glucose homeostasis and may act as a metabolic regulator in several enzymatic systems. Results were compared with previous data obtained by a colorimetric assay, and great improvements in sensitivity, limits of quantification, and inter and intra-assay variability (RSDs of 3.22% and 1.66%, respectively) were reported for MS measurements. A recent study also reported the positive correlation between serum thallium concentration in early pregnancy and the risk of GDM, but also with higher pre-pregnancy BMI in an age-dependent manner (Zhu et al., 2019). The study enrolled 3013 women during the first trimester of pregnancy, of which 383 were subsequently diagnosed with GDM. Serum analysis was performed by ICP-MS but other details on sample preparation are not clearly reported. In this context, it is not clear which is the correct relationship among the variables, as GDM incidence is known to be higher for subjects with higher BMI and age, but the authors did not report information regarding the relationship with thallium blood concentration, thus it is not possible to conclude that GDM is the discriminating factor.

The Maternal Infant Research on Environmental Chemicals (MIREC) study reported data regarding As, Pb, Cd and Hg (Shapiro et al., 2015). The investigation was carried out on maternal whole blood collected during the first trimester of pregnancy, to assess the role of these elements as risk factors for GDM. Also in this case, details on the method used for sample preparation were not reported. The logistic regression of data evidenced that the associated GDM risk increases significantly after exposure to As, showing dose-dependent response. Results were still significant even after the general adjustment for several GDM influencing factors.

Maternal blood was also analyzed by another targeted approach in a case–control study enrolling 776 subjects

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TABLE 3 Summary of metallomic studies in GDM performed by ICP-MS

Specimen	Sample population	Sample preparation	Significantly altered elemental component	Strength/weakness	References
Maternal blood (24–28 gw)	19-GDM 15-Control	Digestion with Conc. HNO_3 by microwave oven (600 W); digestion with H_2O_2; filtration and dilution with deionized water	B (not confirmed)	Very simple protocol for sample digestion/ limited number of analyzed samples; only one element analyzed	Caglar et al. (2012)
Maternal blood (14 gw)	383-GDM 2630-Controls	Samples were analyzed by ICP-MS, but details are not reported	Tl	Good sample size/GDM group is numerically inconsistent with controls; conclusions are not completely justified	Zhu et al. (2019)
Maternal blood (6–14 gw)	27-GDM 59-IGT 160-Controls	Samples were analyzed by ICP-MS, but details are not reported	As (High)	GDM and IGT groups are numerically inconsistent with controls	Shapiro et al. (2015)
Maternal blood (at term?)	776-GDM 776-Controls	Microwave-assisted digestion with Conc. HNO_3 and H_2O_2; dilution with deionized water to 10% HNO_3	Hg (most significant), Ni, Pb, and As	Excellent sample size/gw of sample collection not reported	Wang et al. (2019)
Plasma (8–13 gw)	264- GDM 1593-controls	Samples were analyzed by ICP-MS, but details are not reported	Cu (High), Mo (Low)	Excellent sample size/GDM group is numerically inconsistent with controls; sample preparation protocol is not reported	Zheng et al. (2019)
Serum (24–28 gw)	60-GDM 52-Controls	Dilution and microwave-assisted digestion with deionized water and conc. HNO_3 (180 °C); dilution with deionized water	Cd, Cu, Pb, Sb (high) CrIII, Zn, Se (low)	Good sample size for a preliminary investigation	Onat et al. (2021)
Human meconium (at term)	137-newborns (GDM mothers) 190-Controls	Digestion with equal volume of Conc. HNO_3 and H_2O_2 at 100°C; dilution with deionized water	As, Cd, and Cr (high)	Good sample selection and size/conclusions are not completely justified	Peng et al. (2015)
Maternal urine (20 gw)	241-GDM 1849-Controls	Dilution with diluted HNO_3	As, Cd, Ni, Sb, Co, Va	Very simple protocol for sample preparation/ GDM group is numerically inconsistent with controls; conclusions are not completely justified	Wang et al. (2020)
Placenta (at term)	28-GDM 19-Controls	Microwave-assisted digestion with Conc. HNO_3; dilution with deionized water to 4% HNO_3	Cd, Se	First metallomics study reported for GDM/ Limited number of analyzed samples	Roverso et al. (2015)

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TABLE 3 (Continued)

Specimen	Sample population	Sample preparation	Significantly altered elemental component	Strength/weakness	References
Cord blood (at term)	38-GDM 39-Controls	Digestion with Conc. HNO_3 at 100°C; dilution with deionized water to 3.45% HNO_3	Ca, Cu, Na, Zn (high) Fe, K Mn, Rb, P, S, Si (low)	Optimization of sample preparation protocols; inclusion and statistical evaluation of metadata/limited sample size	Roverso et al. (2019)
Fetal blood (at term)			Al, Fe, P, Mn, Rb, PS, S (high) Ca, Cu, Na, Zn, Co (low)		

Abbreviations: GDM, gestational diabetes; gw, gestational week; ICP-MS, inductive coupled plasma-mass spectrometry.

to determine the level of Ni, As, Cd, Sb, Tl, Hg, and Pb and highlight possible correlations with GDM risk (Wang et al., 2019). The mother exposure to Ni, Pb, As, and Hg was found as a possible risk for the subsequent onset of GDM, with different statistical significance depending on the metal considered. However, the selection of the cohort is not clear as the authors did not report the exact week of gestation in which samples were collected. This is fundamental to assess the role of the metals as risk factors for GDM or as probes to evaluate the effect of GDM at term.

Zheng et al. (2019) focused on Zn, Se, Cu, and Mo in plasma collected during the late first trimester of pregnancy to highlight a possible correlation with blood glucose level. A positive correlation was reported between Cu and glucose blood levels, whereas plasma levels of Mo were inversely correlated. It is to highlight the number of GDM patients considered was not matching the number of controls, negatively affecting the statistical analysis and the robustness of final considerations. A similar investigation was recently reported by Onat et al. (2021), where heavy metal and trace element composition of serum obtained from 112 subjects enrolled in a case-control GDM study were quantified by a validated ICP-MS targeted approach. The authors demonstrated that insulin resistance correlated with higher levels of Cd, Pd, Sb, Hg, As, Cr, Zn, Cu, and Se in serum. The possible involvement of altered metals in inflammation and oxidative stress was also hypothesized.

A study by Peng, Zhang et al. (2015) investigated the possible risk associated with exposure to heavy metals in triggering GDM during pregnancy, analyzing targeted metal species in newborns' meconium as a novel, noninvasive matrix. The case-control study was performed on an interesting number of subjects, that is, 137 GDM pregnant women and 190 controls. Meconium samples were freeze-dried and digested by concentrated nitric acid and 30% hydrogen peroxide at 100°C for 5 h. Even if the relationship between the metal composition of meconium and the exposure of the mother to metal residues during pregnancy was not clearly described, the performance of the reported method in term of sensitivity and recoveries were tempting for Cr, As, Cd, Hg, and Pb, also considering the complexity of the matrix. Authors summarized that higher levels of three heavy metals (As, Cd, and Cr) were associated with the prevalence of GDM in a binary logistic regression, post adjustment to six influencing factors, that is, maternal age, pregnancy BMI, gravidity, parity, hepatitis B infection, and newborn sex.

Notably, urine samples were simply centrifuged (15 min, 5000 rpm) and diluted 10 times with a 2.0% v/

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v nitric acid before injecting in the ICP-MS system in a study on urine samples collected during the 20th week of gestation (Wang et al., 2020). The investigation, carried out on 2090 subjects, of which 241 diagnosed as GDM, revealed a positive relation of Ni, As, Co, Sb, and V with the disease, which may suggest the correlation of metal exposure in early pregnancy with the risk of GDM.

Aside from target studies on plasma, serum, and urine described above, untargeted metallomics approaches were carried out for the identification of metals or other elements in many biofluids and tissues. The main difference between the above analyses was related to the choice of placenta instead of blood as a more informative and exhaustive matrix. Considering the first of these studies, (Roverso et al., 2015), placenta samples collected from a limited number of GDM and NTG pregnant women were dissected to separate the maternal and fetal side of the tissue and treated with concentrated nitric acid. Instrumental sensitivity and accuracy were monitored by using metallic isotopes as internal standards to prevent instrumental drift. Interestingly, the data analysis by means of PCA methods identified an anticorrelation between the levels of Se and Cd and the GDM onset. Another study was performed on placenta, whole blood, and cord blood aiming to identify elements associated with the development and prevalence of GDM (Roverso et al., 2019). In this case, an alternative approach for placenta samples collection was proposed to remove residual blood before storage and digestion. In particular, the placenta samples were extensively washed with a NaCl solution suddenly after delivery, followed by separation of the placenta into two different anatomic components, that is, maternal and fetal, freeze-drying and mineralization by nitric acid. Whole blood samples were simply digested by nitric acid. Statistical analysis was carried out also taking into consideration the diet adopted before and during pregnancy by the subjects enrolled, also considering the consumption of dietary supplements containing minerals for evaluating the presence of confounding factors in the correlation between GDM and elemental composition of samples. The results for placenta samples and for mother blood showed a large interindividual variability of mother blood and placenta elemental composition, making it difficult in discriminating the presence of GDM. On the contrary, the present study showed the key role of whole cord blood in differentiating fetuses from GDM mothers and from healthy subjects. The concentrations of Ca, Cu, Fe, K, Mn, Na, P, Rb, S, Si, and Zn in fetal blood were strictly correlated with the pathology, suggesting an impairment in the transport mechanisms of these elements across the placenta due to the development of GDM (Roverso et al., 2019).

5.1 | Comment to metallomic analysis

Metallomics of GDM is still an unexplored research field. Some targeted studies on heavy metal distribution in blood and urine were reported, but "omics" approaches are limited to two studies on whole blood and placenta collected at term. It is to highlight that the term "untargeted" is not completely ascribable to metallomics as the number of elements in the periodic table is finite, but the general approach is comparable with other omic investigations. Furthermore, considering the large availability of multicomponent standards for ICP-MS and the possibility of obtaining quantitative or semiquantitative information on mostly the known elements by a unique sample preparation procedure that depends only on the matrix considered, an untargeted approach is highly recommended.

Sample preparation procedures are generally easy and fast, in particular when specimens are digested by microwave-based methods. As a consequence, metallomic studies generally report a more robust statistic, as a relevant number of samples can be managed within a single study. Instrumental drifts should be accurately and carefully monitored during the analysis by injecting blank samples and quality controls, as for other omic approaches. It was demonstrated that the final statistical analysis needs to be implemented with data concerning the lifestyle of the participants, that is, diet, type of work, consumption of dietary supplements, smoking, presence of concomitant pathologies, and so on. This information is fundamental to highlight cofounding factors and to prevent failure in data interpretation.

As metals content can be a valuable indicator for assessing mother's exposure from environment and food chain, it is also important to properly design the experiment and the sample collections to exactly demonstrate this relationship. For example, considering the work by Peng, Zhang et al. (2015), meconium samples were collected at term, but authors claimed that the altered species were associated with an increased risk of developing GDM due to early environmental exposure to these metals. It is well-known that meconium is produced during the pregnancy but is not clear which is the assumption for which this specimen, collected at term, can reflect the maternal exposure to metals during the pregnancy. In fact, the impact of GDM on the transplacental transport of metals and other nutrients is still unknown and for this particular reason, it is not possible to unequivocally assess that the metal concentrations in meconium are the result of maternal exposure instead of a different translocation of metals in the mother–fetus system in presence of GDM. This consideration is even more important considering that the differences in

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concentration measured between cases and control are generally not so marked and thus probably not fully ascribable to environmental exposure. To confirm their potential role as risk factors, the highlighted metal species should also be confirmed in the mother body, that is, by analyzing maternal plasma or serum collected at the correct weeks of gestation. The same consideration can be made for urine samples as they are representative of the excretion of metals from the body, but not unequivocally representative of the initial exposure when the scenario is complicated by a certain pathology until a proper demonstration is not reported. Therefore, investigations on urine should be confirmed by blood analysis. On the other hand, metals with a different concentration in urine from potential GDM patients and normal subjects collected during the first trimester are prone to be validated as potential biomarkers for GDM prediction or diagnosis. Finally, information on altered concentrations of the various elements in a target organ or biological fluid ascribable to a well-defined pathology are the starting point to hypothesize the impairment of target biological mechanisms to be further evaluated by other approaches.

6 | CONCLUSION

In last decade, various omics-based investigations were reported regarding the identification of biological mechanisms involved in GDM onset and progression. In this context, MS enlightened the exploration of the entire chemical fingerprint involved in the different biological mechanisms and various metabolic pathways, by untangling the qualitative and quantitative role of the molecules acting in this complex but well-organized system, from big proteins to little metabolites and elements. A list of the major strength and/or weakness for each approach is reported below:

- Proteomic studies, based on gel-based or gel-free approaches, both label-free or labeling-based, were generally performed on a limited number of samples, though validation of results by independent methods was reported. Patient selection should be improved in future studies, as in many occasions other pathologies, that is, obesity, were not taken into consideration.
- Targeted and untargeted metabolomics was performed on various biological specimens collected at different timeframes of pregnancy, without testing the reproducibility of the findings in independent cohorts. Amino acids, free fatty acids and phosphatidylcholines are the main altered metabolites, but results are still very preliminary, as well as their reliability. Although some classes of molecules were highlighted, it is not still possible to propose a set of molecules to be used as biomarkers, nor for GDM diagnosis nor to evaluate potential negative long term effects and negative outcomes.
- Metallomics is still an unexplored field in GDM. Many targeted studies were reported, but conclusions are not consistent with the selection of participants. Untargeted studies are limited to placenta and whole blood, but some points to be confirmed were highlighted, such as the impairment of transport mechanisms in the placental tissue.

Many biomarkers for early diagnosis of GDM and for the prediction of possible adverse outcomes afterbirth were proposed. Although many efforts were made, markers with high sensitivity, selectivity, and robustness to be used in clinical routine remain undiscovered or at least not fully validated and properly tested.

At the state of the art, further progression on this field is surely possible, in particular by setting new investigations based on a well-defined and organized experimental design. Key parameters, such as the number of samples to be analyzed, biological matrix to be used, selection criteria and clinical characteristics of the participants, sample collection and storage procedures, sample processing methodologies, methods of analysis, and for data elaboration and evaluation, need to be evaluated and harmonized among studies, to collect significant, reproducible and comparable data. Standard operating procedures are urgently needed for this purpose. In addition, proposed biomarkers should be deeply validated in larger cohorts to reach clinical significance. The proposal of multicenter studies may be the turning point.

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