Type 2 Diabetes Mellitus and COVID-19

Subjects: Biology

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Type 2 diabetes mellitus (T2DM) is one of the world's leading causes of death and life-threatening conditions. Researchers shed light on the single-cell-based technologies and multi-omics approaches that have reached breakthroughs in the understanding of the pathomechanism of T2DM. Hyperglycemia initiates a pathobiochemical cascade that results in increased mortality in SARS-CoV-2-infected diabetic patients. The underlying molecular mechanisms are responsible for the worsening of both metabolic and hemodynamic conditions.

type 2 diabetes mellitus COVID-19 insulin resistance

1. Introduction

Diabetes Mellitus (DM) is classified into three categories: type 1 diabetes mellitus (T1DM, juvenile diabetes), type 2 diabetes mellitus (T2DM, adult-onset diabetes), and other special types such as gestational diabetes (GDM), endocrinopathies, drugs, and chemical-induced forms, among which T2DM represents nearly 90% of cases. The main hallmark of DM is hyperglycemia ≥126 mg/dL (7.0 mmol/L) and a normal fasting blood sugar of between 70 and 99 mg/dL (from 3.9 to 5.5 mmol/L) [1]. Here, T1DM , GDM and T2DM are discussed. T1DM is characterized by a lack of insulin production with pancreatic β-cell destruction through an idiopathic autoimmune mechanism. GDM is a frequent pregnancy complication in which spontaneous hyperglycemia appears, even in non-obese women, affecting approximately 10–14% of pregnancies worldwide [2]. GDM can be controlled with a low carbohydrate diet and/or insulin administration during pregnancy, and glucose metabolism should be regularly monitored after delivery because it can develop into persistent T2DM and even cardiovascular disease (CVD) in the mother and/or in the descendant [2]. Human placental lactogen is one of the most important factors during the development of gestational diabetes, and it is characterized by low insulin sensitivity or insulin resistance (IR) that leads to chronic hyperglycemia during pregnancy. This hormone is capable of provoking alterations and modifications in the insulin receptors [3]. T2DM also develops because of IR and/or faulty insulin secretion. T2DM is now being diagnosed more often in children and adolescents with obesity due to β-cell malfunction or unresponsiveness to insulin in the organs; in this vicious circle, the insulin secretion is insufficient to compensate for IR [4][5]. IR is linked to environmental factors, low physical activity, high-fat diet, obesity, aging in western society, and genetic background [4][6][7][8]. Genetic predisposition has been described for the development of T2DM via the dysfunction of several genes. Early genome-wide association studies (GWAS) identified approximately 70 genes with mutations or with single-nucleotide polymorphisms (SNPs), and recent multi-ancestry genetic studies found more than 500 risk loci associated with a higher risk for the manifestation of T2DM; the full list of these loci has been reviewed elsewhere [9][10][11]. T2DM represents the disturbance of the metabolomic homeostasis via a low insulin:glucagon ratio, with

decreased insulin and increased glucagon production pushing the balance toward hyperglycemia. While insulin supports anabolic processes, the deposition of glucose, the production of proteins, and reductions in free fatty acids, glucagon supports catabolic processes such as the mobilization of glucose and the release of free fatty acids from adipose tissue [12][13]. The conditions of elevated blood glucose and free fatty acid level influences the composition of the microbiota in the gut and the release of pro-inflammatory mediators, and the generation of reactive oxygen species leads to mitochondrial dysfunction and endoplasmic reticulum stress at a sub-cellular level [14][15].

2. Pathophysiology of Type 2 Diabetes Mellitus and COVID-19

A chronic glucose level leads to the hyperglycosylation of the ACE2 receptor and increased viral cell proliferation [18]. It has long been known that ACE2 is responsible for the conversion of angiotensin I into angiotensin II during the physiological state, and it has been identified as the receptor for SARS-CoV-2 viral entry into cells. ACE2, which directly interacts with the spike glycoprotein [19], is expressed in many cell types and is also present in epithelium of the lung at a high density. It has been shown that ACE2 is highly expressed in patients with hypertension, diabetes and coronary heart disease, thus leading to higher viral entry during SARS-CoV-2 infection. It is also well-known that T2DM is associated with both macrovascular and microvascular complications that lead to multiorgan failure, which worsens the outcome of COVID-19 in diabetic patients and increases mortality rates. The dysregulation of glucose metabolism and insulin resistance contribute to vasculopathy in both large and small vessels through various mechanisms [20]. Diabetes, mainly T2DM, is featured by chronic systemic inflammation and insulin resistance, which can result in endothelial dysfunction, oxidative damage, changes in the mitochondrial expression of superoxides, the increased formation of AGEs, and the activation of the receptors for advanced glycation end products (RAGE). The AGE–RAGE axis increases the progression of atherosclerotic legion formation in the arteries and thus accelerates vascular-damage-related conditions called diabetic vasculopathies [21]. However, the direct relationship between T2DM and COVID-19 remains complex; it is well-known that chronic hyperglycemia induces a dysregulated immune response in innate and adaptive immunity, including abnormal cytokine responses, the inhibition of leukocyte recruitment, the attenuation of macrophage and other leukocyte activity in eliminating pathogens, and defects in pathogen recognition and neutrophil functions [22]. Several other immune mechanisms, such as the decreased production of interleukins in response to an infection, reduced chemotaxis and phagocytic activity, and the immobilization of polymorphonuclear leukocytes, are affected in obesity. IFN-gamma released after virus infection downregulates the insulin-receptor expression of skeletal muscle, and viral infection enhances the progression of T2DM in obesity, thus worsening hyperglycemia [23]. Patients with T2DM tend to develop more severe forms of SARS-CoV-2 infection and have significant increases in acute phase proteins and inflammatory markers compared to non-diabetics. This may also enhance tissue tropism and viral penetration into the cells, leading to increased virulence, pathogenicity, and susceptibility to severe infections [24]. In patients suffering from COVID-19, DM was found to be the third most common comorbidity, with a 33.8% prevalence, after hypertension and obesity [25]. Several mechanisms have been suggested as an underlying additional explanation for the more severe course of COVID-19 in patients with diabetes. Behind the impaired

immune system, hyperglycemia and hyperinsulinemia diabetes are also associated with a hypercoagulable state. The metabolic disturbances associated with oxidative stress and impaired immunity may accelerate the occurrence of thrombotic and ischemic events.

Patients with diabetes generally have an increased risk of thrombosis, which, in the case of COVID-19, can add to a high risk of death. Endothelial cell dysfunction plays a key role in the initiation and precipitation of thrombosis. The initiation of this process when the nitric oxide synthesis is decreased in endothelial cells via several mechanisms including the activation of NF-κB and protein kinase C (PKC) leads to the impairment of vasodilation, the expression of adhesion molecules, and the worsening of vascular inflammation. This results in increased platelet activation and a prothrombotic/hypofibrinolytic environment that facilitates thromboembolic events [26]. It is still unclear whether the dysregulation of glucose metabolism, the severe COVID-19 effects, or the SARS-CoV-2 infection itself is responsible for the worsening of carbohydrate metabolism in diabetic patients. The associations between glycemic control and short- to long-term outcomes were examined in a multi-center prospective cohort study including 574 COVID-19 patients; a one year follow up showed that the glycemic control was significantly associated with short-term outcomes in COVID-19 patients with T2DM and decreased the risk of respiratory sequelae [27]. In a German study of about 8.8 million people, 35,865 were infected by COVID-19, and 15.8 per 1000 person-years versus 12.3 per 1000 person-years of these patients developed T2DM versus other upper respiratory infections, respectively [28]. The results of that study suggest that SARS-CoV-2 infection may also increase the risk of developing T2DM. Future studies will answer the questions of whether SARS-CoV-2 really can induce T1DM, T2DM, or even a new type of diabetes. Long-term follow up studies are needed to evaluate whether the virus has a diabetogenic impact on patients with a higher risk for DM or it can stimulate a new type of DM [28].

3. Prognosis of Type 2 Diabetes Mellitus Patients with COVID-19

The novel coronavirus, SARS-CoV-2, infected more than 500 million and caused the coronavirus disease 2019 (COVID-19) with the death of more than 6 million people worldwide by July 2022 (online COVID-19 Data Repository at Johns Hopkins University). Metabolic diseases such as DM are associated with an increased risk of a severe COVID-19 illness and death because of their associated hypercoagulation state and uncontrolled inflammation [29], although it seems that T1DM patients have higher risk than T2DM patients. Epidemiological studies have shown that hospitalization with diabetes and SARS-CoV-2 infection represents as a comorbidity with poor outcome during hospital stay [30]. A recent study reported that 15% of T2DM patients died from COVID-19, with the poor prognoses for those of elder age and elevated glucose and serum amyloid A levels [31]. In a Swedish study of 385,021 T2DM patients, an elevated glycemic hemoglobin level was shown as a bad prognostic factor and the risks for hospitalization, admission to intensive care, and fatal outcome of T2DM patients with COVID-19 were twice those of a control group [32]. On the contrary, the control of the glycemic index was significantly associated with less mortality and hospital stay in an analysis of 574 T2DM patients with COVID-19 in China [27]. Upon SARS-CoV-2 infection, T2DM patients had adjusted odds ratios (ORs): 3.36 for hospitalization, 3.42 for disease severity, and 2.02 for death [33][34]. In a national cohort study of 19,256 subjects in England conducted between March and

July 2020, 18.3% of hospitalized COVID-19 patients also had T2DM [17]. In a Spanish study, 30.05% versus 19.57% was the ratio of deceased versus surviving diabetes patients, respectively [35]. Taken together, the risks of SARS-CoV-2 infection in patients with T2DM are well-documented and urge the prioritization for vaccination [29][36]. In an Italian study of 277 T2DM subjects (83.4% received an mRNA-based vaccine of mRNA-BNT162b2 or mRNA-1273 and 16.6% received a viral vector-based vaccine of ChAdOx1-S), the neutralizing antibody level and the number of SARS-CoV-2-reactive T-cells (CD4+/TNF-α+, CD4+/IL-2+, CD4+/IFN-γ+) were higher in patients with good glycemic control (HbA1c < 7%) at 52 days after the second vaccine [37]. In a retrospective clinical study of 1356 T2DM patients hospitalized with COVID-19, it was shown that the metformin-treated group showed less mortality and shorter stays in hospital, probably due to the anti-inflammatory effect of metformin [38]. However, T2DM therapy should be designed in accordance with local guidelines while taking personal parameters and comorbidities into account; therefore, current therapeutic regimens for the management of T2DM are not discussed here. Recent review articles about the management of COVID-19 in patients with T2DM have been published elsewhere [39][40][41].

Angiotensin-converting enzyme 2 (ACE2) is one of the best-characterized proteolytic enzymes and a functional receptor on cell surfaces through which SARS-CoV-2 enters the cells. ACE2 is abundantly found in the lung alveolar epithelial cells, lung vascular endothelial cells, heart, kidneys, and pancreas [42]. However, controversial results have been found regarding the expression profile of the ACE2 protease enzyme and receptor. Some studies have suggested that it is more preferably expressed in the exocrine duct cells than in the islets, whereas other studies have shown that ACE2 is expressed in beta-cells; moreover, ACE2 was detected in the microvasculature of both the exocrine and endocrine pancreas. These discrepancies were clarified by Stellenbock et al., who examined the expression of ACE2 in pancreatic autopsy tissues from eleven patients that died of COVID-19. They found that the pancreata were infiltrated with CD45-positive immune cells and that mainly beta cells were infected by SARS-CoV-2 virus. They speculated that other receptors/entry-points may be involved in facilitating the uptake of SARS-CoV-2 into beta-cells because the ACE2 positivity of beta cells was only detected in some the human subjects [43].

Among other risk factors for COVID-19-related death, DM has been shown one of the main predictors of the SARS-CoV-2 infection-associated mortality rate. Therefore, research reviewed the most relevant pathobiochemical aspects, summarized the known molecular background of SARS-CoV-2-induced pathomechanical abnormalities, and dissected the current prognosis of COVID-19 patients in T2DM.

4. The Potential Role of Multi-Omics and Single Cell-Based Technologies in the Current Research of Type 2 Diabetes Mellitus

In the last decade, the “multi-omics” approaches reached a breakthrough in understanding the pathomechanism and clinical complications of T2DM. Different next-generation sequencing (NGS) and mass-spectrometry-based genomics and metagenomic approaches have emerged and are used to identify possible disease-associated diagnostic or therapeutic targets from affected tissues and blood based on specific gene expression changes

including those of diabetes (**Table 1**) [44][45][46][47][48]. The NGS analysis of 16S rRNA genes showed that comorbidity of T2DM with HIV led to a lower microbiome diversity, which was negatively impacted by smoking and normalized by metformin treatment [49]. In the study of Tong et al., the sequencing of 16S rRNA by NGS revealed that metformin treatment increased the proliferation of *Blautia* spp. in the gut in correlation with the normalization of hyperglycemia and hyperlipidemia [50]. An analysis of 40 single-nucleotide polymorphisms (SNPs) in 40 genes of 503 T2DM patients vs. 580 healthy controls on a Sequenom platform identified SNPs in the CAT, FTO and UCP1 genes associated with the retinopathy and nephropathy complications of T2DM [51]. Although early GWAS studies identified approximately 75 genetic loci associated with the development of T2DM, recent multi-ancestry genetic studies found more than 500 risk loci, and the heritability of T2DM via these genes has been shown in only 10–15% of cases, so it is more likely that lifestyle and environmental factors have a significant additional effect that contributes to the manifestation of T2DM [9][10][11][52].

Many epigenetic studies, including the investigation of DNA methylation patterns and accessible chromatin profiles in different tissues, have also contributed to researcher's current knowledge of T2DM [53][54]. One of the most extensive epigenome-wide association studies (EWAS) revealed the CpGs methylation pattern of 52 genes in the blood of European T2DM subjects with the Illumina 450 K methylation array and identified five genes with altered CpG methylation patterns—ABCG1, LOXL2, TXNIP, SLC1A5 and SREBF1—that were significantly associated with the disease [55]. Using “Assay for Transposase-Accessible Chromatin with high throughput sequencing” (ATAC-seq method), Ackermann et al. determined the human pancreatic alpha or beta cell-specific open chromatin landscape and found that alpha or beta cell-specific ATAC-seq peaks overlapped with known binding motifs for various transcription factors, including alpha cell-specific ISL1 and MAFB or beta cell-specific SMAD2, as well as previously identified T2DM-risk-associated SNPs [56]. Greenwald and colleagues combined a high-throughput chromosome conformation capture technique (Hi-C) assay-based high-resolution map of islet chromatin loops with the ATAC-seq and publicly available chromatin immunoprecipitation sequencing (ChIP-seq) data-defined enhancers. They identified thousands of pancreatic islet-specific enhancer–target gene pairs. The T2DM-risk-linked SNPs were significantly enriched at the active enhancers of the protein transport and secretion pathway-associated genes. In the case of the IGF2BP2 gene, the identified T2DM-specific SNP could attenuate both islet enhancer activity and IGF2BP2 expression, and the islet-specific conditional deficiency of Igf2bp2 gene led to impaired glucose-induced insulin secretion in mice [57].

Recently, several research groups started to study the development and progression of T2DM in human patients by applying state-of-the-art single-cell RNA-sequencing (scRNA-seq) and single-sell ATAC-sequencing (scATAC-seq) methods focusing on the pathological changes in pancreatic islets. Lawlor and colleagues investigated the cellular heterogeneity in nondiabetic and T2DM human islet samples, and they were able to detect T2DM-specific gene expression signatures in alpha, beta, and delta cells using scRNA-seq that remained invisible in paired whole-islet analyses [58]. Additionally, scRNA-seq and complex computational tools revealed an altered regulatory network in the pancreas of T2DM patients with disease-related transcriptomic changes, showing increased PageRank centrality in 162 genes. After analyzing five centralities driving the regulatory changes in diabetes, they found six markers with increased levels (OTUD7B, PPRC1, ARRB2, C17orf96, NME2, and E2F1) and four markers with decreased centrality (FBXW7, CXCL8, FHL1, and CELF4) [59]. By applying scATAC-seq and deep learning

approaches, Rai et al. found that T2DM-associated SNPs were significantly enriched in beta cell-specific and common islet-specific open chromatin but not in alpha or delta cell-specific open chromatin signatures [60]. Marques et al. performed a meta-analysis of the scRNA-seq data of human α- and β-cells of T2DM patients and identified disease-associated genes responsible for energy metabolism, immune homeostasis, autophagy, and especially nuclear factor erythroid 2-related factor 2 (NFE2L2) in β-cell maturation and dysfunction [61].

The manifestation of T2DM in Asian Indians is more frequent, even in the case of normal BMI, a situation known as the “thin fat” phenotype in which the peripheral fat is thin but the visceral fat accumulates [62]. Microarray data of T2DM-derived peripheral fat of Asian Indians were sued to highlight the top 20 differentially expressed genes (DEGs) and pathways associated with adiposopathy in T2DM [63]. Using the whole transcriptome RNAseq, the same group further investigated the peripheral subcutaneous adipose tissue of Asian Indians and found altered lipid, glucose, and protein metabolisms; adipogenesis defects; and inflammation associated with T2DM [64]. Using the AGENA MassARRAYiPLEX™ platform, Irgam et al. recently identified seven significant SNPs (s2241766-G (ADIPOQ), rs6494730-T (FEM1B), rs1799817-A, rs2059806-T (INSR), rs11745088-C (FST), rs9939609-A, and rs9940128-A (FTO)) associated with T2DM in a southern Asian Indian population of 500 cases [65].

Besides genomics studies, multiplex proteomic investigations have revealed markers associated with disease severity or complications in T2DM. Using the Milliplex Luminex assay, Barchetta et al. showed that blood levels of osteopontin and osteoprotegerin were significantly higher in 83 T2DM patients versus 71 healthy controls and that these proteins were positively correlated with higher systolic blood pressure [66]. Using the same multiplex Luminex technology, Colombo et al. showed that the elevated serum concentrations of kidney injury molecule 1 (KIM-1) and β2-microglobulin (B2M) were correlated with renal failure and a decreased glomerular filtration rate in T2DM [67]. The study of Heinzel also based on the Luminex quantitation of plasma biomarkers identified KIM-1 among 12 proteins of 17 measured markers that predicted declines in the glomerular filtration rate [68]. Although T2DM is not autoimmune-mediated, using single-cell imaging mass cytometry, Wu et al. showed increased percentages of HLA-DR+ macrophages and HLA-DR+ CD8+ T-cells in the islets of the pancreata of T2DM patients, thus suggesting their role in local inflammation [69]. Novel experimental models can also be used to understand better the pathomechanisms of different diabetic syndromes. Researchers' group was to first to optimize a special three-dimensional organoid, the Real Architecture For 3D Tissue (RAFT™) culture system, for the ex vivo maintenance of functional murine pancreatic islets [70].

A lipidomics study of 250 T2DM patients and 639 non-cases showed that the plasma lipid profiles of elevated TAGs (triacylglycerols), DAGs (diacylglycerols), and PEs (phosphatidylethanolamines) with a high risk of T2DM and lipid constituents such as LPs (lysophospholipids), PC–PLs (phosphatidylcholine–plasmalogens), SMs (sphingomyelins), and CEs (cholesterol esters) were associated with lower risks of T2DM [71]. In a Finnish lipidomics study analyzing 277 plasma lipids with ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry of 955 subjects with a 5-year follow-up also found increases in TAGs and DAGs and decreases in PC–PLs associated with risk of T2DM [72]. Taken together, the disbalance of fatty acids (FAs) may not only be considered as a consequence of altered metabolism; rather, FAs may be involved in the translocation of glucose transporters and influence insulin receptor binding as causative agents in the development of T2DM [73].

Table 1. Recent multi-omics approaches that revealed T2DM-associated factors.

Omics/Field	Measures	Results	Assay	References
Genomics	16S rRNA on microbiome analysis	Smoking and/or HIV lowers microbiome diversity in T2DM	NGS	[49]
Genomics	16S rRNA on microbiome analysis	Metformin helps to normalize microbiome with the support of *Blautia* spp.	NGS	[50]
Genomics	Analysis of SNPs	SNPs in the CAT, FTO and UCP1 genes associated with retinopathy and nephropathy	Sequenom platform	[51]
Genomics	Genome sequencing	Heritability of T2DM is approximately 10–15%	GWAS	[9][10][11][52]
Epigenomics	CpGs methylation pattern	CpG methylation of ABCG1, LOXL2, TXNIP, SLC1A5 and SREBF1 is associated with T2DM	EWAS, Illumina 450K methylation array	[55]
Epigenomics	Alpha or beta cell-specific open chromatin landscape	Alpha cell-specific ATAC-seq peaks: ISL1 and MAFB; beta cell-specific: SMAD2	ATAC-seq	[56]
Epigenomics Genomics	Open chromatin regions/SNPs	Thousands of pancreatic islet-specific enhancer–target gene pairs	Hi-C, ATAC-seq, ChIP-seq	[57]
Transcriptomics	Gene expression	T2DM-specific gene expression signatures in alpha, beta and delta cells	scRNA-seq	[58]
Transcriptomics	Gene expression, regulatory networks	Increased OTUD7B, PPRC1, ARRB2, C17orf96, NME2, and E2F1 or four markers with decreased PageRank centrality (FBXW7, CXCL8, FHL1, and CELF4)	scRNA-seq	[59]
Epigenomics Genomics	scRNA-seq and deep learning approaches	T2DM-associated SNPs were significantly enriched in beta cell-specific and common islet-specific open chromatin	scRNA-seq and deep learning approaches	[60]
Transcriptomics	Gene expression, pathway analysis	T2DM-associated genes responsible for energy metabolism, immune homeostasis, and autophagy	Meta-analysis of scRNA-seq data	[61]
Transcriptomics	Whole transcriptome analysis	Top DEGs in peripheral fat of Asian Indians associated with T2DM: *HOXB3*, *RSPO3*, *HOXA5*, *GREM1*, *ORMDL1*, *C7*, *TRIM23*, *CLDN11*, *ABCA10*, *ETV5*, *TRIM2*, *TP53INP1*, *ST6GAL1*, *THBS2*, *ERAP1*, *OGT*, *RARRES1*, *CTDSPL* and *TBCC*	Affymetrix GeneChip PrimeView Human	[63]

Taken together, the above-discussed studies illustrate the relevance of multi-omics and single cell-based technologies in the study of T2DM pathomechanisms. However, many questions remain unanswered, including (i) which islet-specific enhancers/open chromatin regions are associated with the different therapeutic responsiveness levels, (ii) how non-pharmacological and pharmacological treatments can modulate the cellular heterogeneity in the pancreas, and (iii) which gene expression and epigenetic signatures or plasma biomarkers may be helpful to predict the therapeutic responsiveness in T2DM patients. The limitations of genome-based or transcriptome-based investigations, such as lack of functional tests, a lack of functional evaluation of metabolic traits, and protein circuits in cell-to-cell communication, should be considered. Proteomics and lipidomics approaches also share limitations with the aforementioned technologies, with questions of assay sensitivity, sample preparation, and throughput, among others. No single omics technology can identify and quantify the T2DM-related factors responsible for both disease heredity, manifestation, and severity or serve as a therapeutic target or prognostic/diagnostic marker. Rather, the combination of the presented technologies may accelerate the understanding of the molecular

Omics/Field	Measures	Results	Assay	References
			Gene Expression Array	
Transcriptomics	Whole transcriptome analysis	Altered lipid, glucose, and protein metabolism; adipogenesis defect; and inflammation in peripheral fat of Asian Indians associated with T2DM	Bulk RNAseq	[64]
Genomics	Analysis of SNPs	s2241766-G (ADIPOQ), rs6494730-T (FEM1B), rs1799817-A, rs2059806-T (INSR), rs11745088-C (FST), rs9939609-A, and rs9940128-A (FTO) were associated with T2DM in southern Asian Indians	AGENA MassARRAYiPLEX™ platform	[65]
Proteomics	Protein concentrations	Osteopontin and osteoprotegerin are elevated in T2DM	Milliplex Luminex assay	[66]
Proteomics	Protein concentrations	High KIM-1 and β2-B2M are associated with renal failure	Luminex Multiplex ELISA Luminex assay	[67]
Proteomics	Protein concentration	High KIM-1 is associated with low GFR	Multiplex Luminex Panel	[68]
Proteomics	Immune cell infiltration	High HLA-DR+ macrophages and HLA-DR+ CD8+ T-cells in the islets of pancreata of T2DM patients	Single-cell imaging mass cytometry	[69]
Lipidomics	Lipid composition	High TAGs, DAGs, PEs: high risk for T2DM High LPs, PC–PLs, SMs, CEs: low risk for T2DM	Mass spectrometry (MS)	[71]
Lipidomics	Lipid composition	High TAGs, DAGs and Low PC–PLs: high risk for T2DM	Ultra-performance liquid chromatography and MS	[72]

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