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Metabolomics and Proteomics in Type 2 Diabetes

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Abstract

The persistent increase in the worldwide burden of type 2 diabetes (T2D) and the accompanying rise of its complications, including cardiovascular disease, necessitates our understanding of the metabolic disturbances that cause diabetes. Metabolomics and proteomics, facilitated by recent advances in high-throughput technologies, have given us unprecedented insight into circulating biomarkers of T2D even over a decade prior to overt disease. These markers may be effective tools for diabetes screening, diagnosis, and prognosis. As participants of metabolic pathways, metabolite and protein markers may also highlight pathways involved in T2D development. The integration of metabolomics and proteomics with genomics in "multi-omics" strategies provide an analytical method that can begin to decipher causal associations. These methods are without their limitations, however, but with careful study design and sample handling, these methods represent powerful scientific tools that can be leveraged for the study of T2D. In this paper, we aim to give a timely overview of circulating metabolomics and proteomics findings with type 2 diabetes observed in large human population studies to provide the reader with a snapshot into these emerging fields of research.

Keywords

Metabolomics; Proteomics; Type 2 Diabetes Mellitus; Obesity; Diabetes; Type 2; Metabolism

Challenges in the Care of Individuals with Diabetes

It is projected that in 2040, 642 million adults worldwide will have diabetes—the vast majority of which will be type 2 diabetes (T2D)[1]. The unprecedented increase in the global burden of this "lifestyle disease" will be accompanied by rising mortality and disability rates, especially among adults during their most productive years. A multinational observational study that included countries in South America, North Africa, South and East Asia, the Middle East, and Russia, estimated ~50% prevalence of microvascular

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complications (e.g. retinopathy, nephropathy, and neuropathy) and ~30% prevalence of macrovascular complications, including coronary heart disease and peripheral vascular disease among individuals with T2D[2]. In regards to atherosclerotic cardiovascular disease, T2D confers a 2–4 fold increased risk of cardiovascular events and death[3], a similar increase in risk for lower extremity amputations[4], and is the leading modifiable risk factor for heart failure[5]. In turn, cardiovascular disease is estimated to cause two-thirds of deaths in individuals with T2D[6]. Together, this translates to a 2–3 fold increase in medical expenditures[7].

The complications, of diabetes, and associated health care costs, can be avoided to a significant degree by effective prevention and treatment. Changes in diet and increasing physical activity have been found to be more effective than pharmacotherapy (e.g., metformin) to delay and potentially prevent diabetes[8]. It is difficult, however, to identify individuals at risk since the metabolic dysfunction associated with diabetes development begins decades before increases in blood glucose. Also, not all individuals with elevated blood glucose will progress to diabetes. Population approaches to increase diabetes awareness, physical activity, and healthy food access while decreasing added sugar intake —all considered to be risk factors for diabetes—may be effective. Diabetes incidence in the U.S. consistently rose from 1990 to a peak incidence of 8.2 per 100 adults in 2009, but from 2011 to 2017 has remained stable with a reduction in incidence rate of 35%[9], possibly due to these population approaches. However, the casual role of these risk factors have yet to be confirmed. Furthermore, obesity, a traditional risk factor for T2D, continues to rise[10] and the prevalence of pre-diabetes remains high[11] despite the decline in frank diabetes. These findings suggest there is still much we do not understand about the pathophysiology of T2D development.

There is also evidence of significant heterogeneity of diabetes clinical presentation and course. Leveraging unsupervised clustering analyses of clinical data as well as genetics and outcomes data, Ahllqvist et al. recently identified 5 new diabetes “subgroups” among individuals traditionally considered to have T2D.[12] They identified 2 clusters of individuals, labeled as severe autoimmune diabetes (SAID) and severe insulin-deficient diabetes (SIDD), with characteristics more similar to type 1 diabetes including lower BMI, higher rates of diabetic ketoacidosis, and faster progression to insulin therapy despite one cluster having no antibody positivity. Another group that was identified, labeled as having severe insulin-resistant diabetes (SIRD), had higher risk of progression to chronic kidney disease and trended toward an increased risk for coronary events. Improving our ability to identify individuals at the highest risk for developing specific complications will help guide clinical care. Also, with the recent availability of cardiovascular and renal outcomes data for medications previously used only for glycemic control[13,14], there is now an unprecedented number of therapeutic options for specific subsets of patients with type 2 diabetes including those with established cardiovascular disease, heart failure, or kidney disease.

A Need for New Diabetes Biomarkers

Biomarkers can be effective tools for disease screening, diagnosis, and prognosis. If these biomarkers also participate in disease pathways, which is frequently the case for metabolites

and proteins, they can highlight mechanisms of disease development or therapeutic effect. Many current biomarkers for diabetes, however, are strongly correlated with dysglycemia, limiting their predictive and diagnostic value beyond a fasting blood glucose or hemoglobin A1c (HbA1c) level. This point was illustrated by Wang et al. when they simulated the stepwise addition of 100 hypothetical biomarkers to a traditional risk model for cardiovascular disease (Figure 1)[15]. The degree of correlation between biomarkers included in the clinical model was a key determinant of how much additional information each biomarker provided and how many biomarkers were needed to meaningfully change the predictive power of the risk model[16,17]. For example, more than 50 biomarkers were needed to improve the c statistic of the model by 0.05 for a set of biomarkers with mean inter-marker correlation of r = 0.4 (i.e., moderately correlated). By contrast, less than 10 biomarkers were needed if their mean inter-marker correlation was r = 0.05 (i.e., weakly correlated)[17]. High throughput technologies that allow the unbiased quantification of all circulating metabolites and proteins can help facilitate the identification of biomarkers that are from orthogonal pathways and are therefore weakly correlated.

Metabolites and Proteins are the Product of Genetic, Physiologic, and Environmental Stimuli

The aim of metabolomics is to measure metabolite concentrations in cells, tissues, organs, and biological systems to study the chemical processes involved in metabolism in a systematic fashion. Similarly, proteomics aims to quantify and characterize all proteins that participate in the biological processes of an organism. These should not be considered mutually exclusive fields of study, but rather a continuum of biochemical profiling that focuses—when included with peptidomics—on characterizing the molecular products of genetic transcription. These products could be a single compound (i.e., an amino acid such as aspartic acid), a structure composed of several constituents (i.e., a dipeptide such as aspartame), or a protein that is made up of multiple peptides (i.e., an enzyme such as aspartate transaminase); but due to differences in molecular sizes, require different technologies to identify and quantify. Metabolites and proteins are of particular interest because they are influenced by physiologic changes and environmental stimuli as well as genomic inputs. T2D is specifically suited for metabolomics and proteomics methods since it is generally a polygenic metabolic disease influenced by physiologic changes such as diet and physical exercise. Recent technological advances have allowed scientists to profile circulating metabolites and proteins rapidly and on an increasingly larger scale, several which are briefly highlighted in Table 1. This has facilitated the mapping of the complete human metabolome and proteome, analogous to how genomic advances have allowed the mapping of the human genome.

A percentage of the inter-individual variability of circulating metabolites, or proteins, can be attributed to genetics. Twin studies were initially used to study the heritability of circulating concentrations of specific blood factors[18,19]. The heritability of a wide range of metabolite concentrations in large populations can now be estimated with the availability of genome wide association study (GWAS) data. A single nucleotide polymorphism (SNP) can explain up to 16–36% of a metabolite's variance[20,21]. Metabolites, and proteins, can also be influenced by multiple genes simultaneously. General heritability estimates in a

Finnish study suggested genetics explained 23–55% of the variability in amino acid and small molecule levels. The percentages were higher, 48–76%, for lipids and lipoproteins[22]. While genetic influences are substantial, there remains a percentage that is not explained by genetics. GWAS in the Framingham Offspring cohort showed less than 20% of inter-individual variation in 34% of 217 measured plasma metabolites was attributable to genetics[23]. Some of these non-genetic contributors could be known clinical or environmental factors, but some remain unidentified.

Genetics also influence circulating protein concentrations. In a comprehensive GWAS conducted on human plasma proteins in the European INTERVAL study, genetic variants explained more than 20% of variation in only approximately 10% of the proteins measured. A higher percentage of heritability, approximately 90%, was found in the Framingham Offspring cohort but significantly fewer proteins were measured[24]. The mean heritability, however, was only 49%, suggesting again that there is a percentage of circulating protein variation that is unexplained by genetics in humans that warrants further study.

Biomarker Integration with Genomics Could Uncover Causal Relationships with Disease

Metabolite and protein biomarkers can illuminate disease pathophysiology, however, a limitation of biomarker studies are that causality is difficult to establish. Mechanistic studies in cell lines and model organ systems are usually required that are time and resource intensive. With the increasing availability of genomic data, however, advances in statistical methods allows the determination of if biomarkers are possibly causal to a disease and allows for the prioritization of specific biomarkers for further mechanistic studies.

Instrumental variable analysis exploits Mendelian randomization, or the random inheritance of genes, to make causal inferences about genetic variants and intermediate phenotypes with an outcome of interest. For example, randomized control trials have shown that the inhibition of proprotein convertase subtilisin-kexin type 9 (PCSK-9), a protein involved in low density lipoprotein (LDL) receptor degradation, successfully reduces LDL levels and likely reduces the risk for major cardiovascular events[25,26]. Ference et al. demonstrated with Mendelian randomization in a cohort of over 110,000 individuals that loss of function genetic variants of PCSK-9 were associated with lower clinical LDL cholesterol levels (e.g., the intermediate phenotype)[27]. These same variants were also associated with lower risk of cardiovascular events (e.g., the outcome of interest). This suggested that reductions in cardiovascular risk associated with these specific PCSK-9 variants were mediated by their effect on LDL levels. Interestingly, this study also found these same genetic variants, associated with the same LDL lowering effect, were also associated with increased risk for T2D in individuals with impaired fasting glucose. This is supported by clinical findings that statins, another class of LDL cholesterol lowering medications, are also associated with a small but significant increase in T2D risk. Utilizing this framework, exposure to either high or low levels of a biomarker of interest can now be substituted as the intermediate phenotype to interrogate if the biomarker is in the causal pathway for a disease of interest.

Analytical Methods in Metabolomics

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)—usually coupled to gas chromatography (GC-MS) or liquid chromatography (LC-MS)—are the most common profiling technologies used. An understanding of the strengths and weakness of the different technologies as well as appropriate sample preparation methods are imperative to generate usable data.

Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR has been used for the simultaneous detection of several metabolites in different bodily fluids for decades[28,29]. It utilizes the predictable behavior of atomic nuclei (e.g. ^{1}H is most commonly measured in metabolomics) when they are exposed to strong magnetic fields based on neighboring atoms and the frequency of the electromagnetic radiation exposure to identify molecular compound structures. The presence of protons in the vast majority of biological compounds makes this identification technique almost universally applicable. NMR is also a nondestructive analytical method that allows for *in vivo* compound identification. Also, when coupled with imaging techniques such as MRI, *in vivo* localization of metabolic activity is now possible within an ogransim[30]. A limitation of NMR, however, is that the peaks generated are often an integration of signals from several different compounds, especially when compounds of similar species are present. Also, MS is better suited for the identification of metabolites that are in higher abundance.

Mass Spectrometry (MS)

MS utilizes chromatographic separation, analyte ionization, and ionic separation by mass to quantify and identify compounds from biological samples with high resolution and sensitivity. Chromatography is not a required step, but this technique reduces ion suppression and improves the quantitative accuracy of MS results especially for lower abundant analytes. It also allows the identification of isomers (compounds that have the same molecular formula and mass but different structural arrangements) which commonly occur in biological metabolites. In chromatography, there is a mobile phase that the analytes are dissolved into that interacts in a predefined manner with a stationary phase that the mobile phase is passed through, allowing for physical separation of the analytes. With GC, analytes are vaporized into a mobile gas phase and passed through a liquid layer in the chromatograph column that serves as the stationary phase. GC has superior resolution to LC and is well suited for nonpolar, low molecular weight, volatile analytes. With LC, analytes are dissolved in a liquid mobile phase that is then passed through a column filled with beads coated with different compounds with predefined chemical properties that serves as the stationary phase. LC is better suited for polar, high-molecular-mass compounds that are heat-labile. There are a variety of LC columns and techniques that facilitate the separation of a large spectrum of metabolites. Hydrophilic interaction chromatography columns (HILIC) contain hydrophilic beads with an organic solvent to water gradient are effective in separating small, polar analytes such as amino acids, nucleotides, and organic acids. Columns that contain hydrophobic beads with a gradient from water to organic solvent are effective in separating polar molecules and lipids.

There are different methods used to ionize analytes with MS, the most common being electrospray ionization (ESI). ESI is typically coupled with LC and utilizes a highly charged needle tip to create charged analyte droplets from a liquid that can be heated and ionized. There are also several different methods to achieve ionic separation in MS using different analyzers including time-of flight (TOF), quadrupole, ion trap mass, and orbitrap. The TOF instruments utilize a fixed electric field to accelerate ions through a voltage drop to impart kinetic energy and then down a flight tube of known length. The time an ion requires to reach the end detector is dependent on its charge and mass and therefore a mass to charge ratio (m/z) can be calculated with high resolution and mass accuracy. A quadrupole mass spectrometer uses oscillating electrical fields to selectively stabilize the flight path of an ion with a specific m/z of interest, serving as a mass filter. A triple quadrupole measure metabolites with high sensitivity, but can only measure a smaller subset of preselected metabolites. An ion trap mass spectrometer traps ions of a specific m/z using an electrical field and calculates the m/z based on the radio frequency required to retain the ions. The ion trap has increased sensitivity but is less accurate with quantification. An orbitrap mass analyzer monitors the frequency of oscillations of an ion once it is trapped around a central spindle-shaped electrode and calculates the m/z based on the ion oscillations around a specific axis. This analyzer provides exceptional resolution and mass accuracy. These different analyzers can also be coupled together in a single method to provide increased mass resolution and accuracy.

Metabolomics in Diabetes

Over the past decade there has been a growing body of literature describing metabolomic profiles associated with type 2 diabetes. Both targeted methods, that measure a defined group of known chemical compounds, and untargeted methods, that measure all chemicals present including those that have never been previously annotated, have been used.

Amino Acids

One of the strongest associations with diabetes and glycemic traits that has emerged from metabolomics studies is the positive association of branch chain amino acids (BCAAs)—e.g., leucine, isoleucine, and valine. Evidence relating amino acid metabolism with insulin resistance and obesity in humans was initially described decades ago[31]. Newgard et al. confirmed these associations in a cross-sectional metabolomics analysis of obese and lean individuals. BCAAs in particular were higher in individuals that were obese and, furthermore, in those that had higher insulin resistance (defined by the homeostatic model assessment of insulin resistance or HOMA-IR) even after adjustments for adiposity[32]. Wang et al. then demonstrated in a prospective analysis in the Framingham Heart Study (FHS) that individuals with BCAA concentrations in the highest quarter had a 2–3.5 fold higher odds of developing type 2 diabetes up to 12 years later compared to those in the lowest quarter[33]. These associations remained after adjustments for clinical risk factors including body mass index (BMI) and have subsequently been replicated in multiple other cohorts[34–39]. Growing experimental evidence have posited potential mechanisms[40,41] including increased BCAAs, either due to increased dietary contribution and/or defective metabolism, activating mammalian target of rapamycin (mTOR) kinase activity that uncouples insulin signaling[32]

or leading to a buildup of cytoxic metabolites that could adversely affect the pancreatic islet β-cells[42–44] or adipocytes[45].

It is still unclear, however, if BCAAs have a causal relationship with diabetes or if these associations are due to reverse causality or confounders such as obesity. Work integrating these findings with human genetics has been completed to begin to clarify these issues. Lotta et al. completed a GWAS of BCAA levels in 16,596 individuals that identified several top SNPs, some of which also imparted an increased risk for diabetes among 47,877 cases and 267,694 controls across several European cohorts[46]. Analyses in a smaller cohort utilizing Mendelian Randomization suggested a causal association of insulin resistance with BCAA levels[47], but not the reveres, which was further supported by a second analysis that included more SNPs and individuals[48]. Taken together, these findings point to elevated BCAAs as a downstream effect of adiposity and insulin resistance but that temporally precedes the development of clinical diabetes, suggesting they may be mediators to some degree in disease development.

Several studies have also found positive associations of aromatic amino acids (AAAs), including tyrosine and phenylalanine, with future development of diabetes[34–37,48–50]. AAAs in solution with BCAA also alter cellular insulin signaling through the mTOR pathway[51] and they compete with BCAA for the same intracellular transporter[52] but further data on AAA causing diabetes or insulin resistance is limited. Glutamate and glutamine, two amino acids central to both nitrogen and carbon cycling and linked with BCAA metabolism, have also been associated with the development of diabetes in several cohorts.

Glutamate, synthesized from the citric acid cycle product α-ketogluteric acid and an intermediate in the generation of the antioxidant glutathione[53], has been consistently found to be positively associated with diabetes[39,54]. Glutamine, a transamination product of glutamate[55], has been found to be inversely associated with the development of diabetes as well as the ratio of glutamine/glutamate[35,37,49,54,56,57]. Glycine[58], an amino acid synthesized from serine, has also been consistently found to be inversely associated with development of T2D[34–37,57] and impaired glucose tolerance[34]. The roles these metabolites could potentially have in diabetes development have yet to be clarified; however, each have central roles in several cellular metabolism pathways. An interesting proposed common pathway could be through their interaction with NMDA glutamate receptors that may regulate insulin secretion in the β-cell[59]. Serine, which has also been found to have an inverse association with incident diabetes[35,36,57], could also participate in this pathway.

Higher levels of 2-aminoadipic acid (2-AAA), a lysine degradation product, was also found to be associated with increased risk for incident diabetes in FHS and MDC[60]. Individuals with concentrations in the highest quarter had more than a 4-fold increased odds of developing diabetes over 12 years. These results mirrored findings of increased concentrations of 2-AAA found in obese mice, hyperinsulinemic mice fed a high fat diet, and diabetic rats. Also, augmented insulin secretion was demonstrated in both murine and human islet cells that were acutely or chronically exposed to 2-AAA. While it remains unclear if 2-AAA levels rise prior to the development of insulin resistance, it may have a role in compensatory mechanisms afterward.

Organic Acids

Alpha-hydroxybutyrate, a product of amino acid catabolism that is derived from α-ketobutyrate, a participant in the glutathione production pathway, has been positively associated with incident diabetes[39,57]. In a group of individuals free of diabetes, α-hydroxybutyrate levels have also been found to be inversely associated with insulin sensitivity[61]. Acetoacetate, a ketone body synthesized from fatty acids as an energy source when glucose is low, has also been positively associated with the risk for diabetes in a group of Finnish men[62] and in a smaller Bavarian study[63]. Alpha-keto acids, specifically branched chain α-keto acids, are of interest because they are formed in the first irreversible step of BCAA catabolism[64].

Bile Acids

In a European study that utilized a non-targeted approach, three bile acids—e.g., deoxycholic, glycocholic, and glycodeoxycholic acid—were positively associated with incident disease in age- and sex-adjusted models[50]. Only the association of deoxycholic acid remained significant after additional adjustments for clinical risk factors[50]. This study also identified a SNP in the CYP7A1 coding region associated with deoxycholic acid levels that was also associated with type 2 diabetes in published GWAS meta-analyses. While the genetic findings were not a formal Mendelian randomization analysis and thus cannot prove causality, these findings support emerging experimental evidence of both receptor-mediated and non-receptor-mediated mechanisms (that involve incretin stimulation) for circulating bile acids to effect glycemia[65].

Carbohydrates

Hexose sugars—typically measured as a composite of multiple different isomers of 6 carbon monosaccharides including glucose and fructose—are the most frequently analyzed carbohydrate in metabolomics studies of incident diabetes[35,39,66,67]. These composite measures consistently have a positive association with disease even after adjustments for clinical measures of glucose. This reflects the high degree of sensitivity of the analytical technologies to detect the hexose sugars present in the samples that are not measured by clinical glucose assays. Circulating levels of trehalose—a non-endogenous sugar obtained from the diet in humans—has also been positively associated with diabetes[39,66]. Using untargeted methods, a species of mannitol and several deoxy-hexose sugars were found to be inversely associated with diabetes risk in a nested case-control study of the EPIC-Potsdam Germany cohort[68].

Lipids and Acylcarnitines

Lipids are an integral part of cellular energy homeostasis, serving in multiple roles including as metabolic substrates, signaling hormones, or cellular membrane building blocks. Elevated clinical measures of lipids, specifically of bulk triglycerides, is considered a tradition risk factor for T2D. In the 1960s, Randle et al. described the competitive relationship of fatty acids and glucose for oxidative cycling and proposed that excessive fatty acid oxidation contributed to impaired glucose homeostasis and insulin resistance[69]. The intracellular accumulation of fatty acid oxidation products such as diacylglycerols (DAGs),

triacylglycerols (TAGs), and ceramides have also been linked with insulin resistance[70]. Extensive experimental work is being conducted to understand the mechanisms causing these associations and whether these oxidation products—as well as other lipid species—are causative or consequences of insulin resistance[67].

Traditional clinical measurements of lipids often lacked specificity, but with GC and LC-MS techniques, unique lipid species can now be identified by total acyl chain carbon number and double bond content. In FHS, individuals with higher levels of TAGs with shorter acyl carbon chains and fewer double bonds were at increased risk for the development of diabetes even after adjustments for clinical risk factors[71]. A similar trend was also observed for cholesterol esters (CEs) and specific phospholipids including lysophosphatidylcholines (LPCs), phosphatidylcholines (PCs), and lysophosphatidylethanolamines (LPEs). A study in Finnish men found a positive association of the ratio of monounsaturated fatty acids (FAs) to total FAs with increased risk for future T2D. The ratio of saturated and n-7 and n-9 FAs to total FAs along with glycerol, total FAs, and total TAGs levels were also found to be positively associated with T2D risk while the ratios of docosahexaenoic acid (DHA), an omega-3 FA, and linoleic acid, a n-6 FA, with total FAs were inversely associated[72]. Lipoprotein lipid subclasses can also be profiled with NMR techniques, and in the Metabolic Syndrome in Men (METSIM) study of Finnish men, the ratio of apolipoprotein A1 to HLD was the strongest predictor of future T2D risk[73]. In a more recent study of young Fins, lipid subfractions of lipoproteins were measured and higher cholesterol concentrations in very large LDL particles was positively associated with T2D risk while higher concentrations in very large and large HDL particles—especially of non-esterified cholesterols—were inversely associated[74]. Higher relative TAG content in all lipoprotein subclasses was positively associated with T2D risk.

Specific phospholipid species have also been studied. Wang-Sattler et al. found an inverse association of C18:2 (denoting acyl chain carbon length:number of double bonds) LPC, along with glycine, with future development of both impaired glucose tolerance and incident T2D in the Cooperative Health Research in the Region of Augsburg (KORA) cohort and in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort[34]. In a separate analysis that used an EPIC-Potsdam subcohort for discovery and KORA for validation, specific diacyl-phosphatidylcholines (C32:1, C36:1, C38:3, and C40:5) were positively associated with T2D risk. They also confirmed the inverse association of C18:2 LPC and found C16:1 sphingomyelin, and specific acyl-alkyl-phosphatidylcholines or plasmalogen PCs (C34:3, C40:6, C42:5, C44:4, and C44:5) were inversely associated with T2D risk[35]. Further untargeted work in the EPIC-Potsdam cohort has confirmed the inverse association of specific LPCs and PCs with T2D[68], while work in a small American Indian cohort has also confirmed the findings in PCs, albeit with different subspecies[75]. Phospholipid linoleoyl-glycerophosphocholine (L-GPC)[57] has also been inversely associated with future diabetes.

The transport of fatty acids into the mitochondria for cellular β-oxidation is facilitated by the formation of acylcarnitines, especially medium-length acylcarnitines. BCAA catabolism also leads to the production of specific acylcarnitine species. This class has been studied in several metabolomics cohorts given their position at the intersection of BCAA and fatty acid

metabolism. Elevated C3 and C5 acylcarnitines, products of BCAA catabolism, were found by Newgard et al. to be strongly associated with insulin resistance[32]. C2 acetylcarnitine was also found to be positively associated with incident diabetes in KORA[34].

Proteomics

The study of proteins, as the final product of genetic transcription and post-transcriptional modifications, has also played a pivotal role in the understanding of disease. Mass spectrometry and immuno-assays of single proteins were utilized to identify and uncover the association of circulating levels of adiponectin[76], leptin[77], sex-hormone binding globulin[78], and the vitamin E binding, afamin[79], with type 2 diabetes risk. Recent developments, however, in protein profiling techniques have increased the efficiency and numbers of circulating proteins that can now be measured. While MS remains a powerful tool for the detection and quantification of proteins, the process is labor and time-intensive and this technique remains limited in the number of proteins it can measure simultaneously. The development of affinity-based methods utilizing multiplexing antibodies and/or novel affinity reagents has drastically expanded the number of proteins that can be quantified. Two high-throughput technologies now commonly used include the use of nucleic acid affinity reagents (aptamers) or nucleotide-labeled antibodies. With aptamers, the diverse structural confirmations that can be achieved with oligonucleotides are utilized to bind to target protein epitopes to facilitate protein detection and quantification. Nucleic acid labeling of antibodies has also allowed the use of polymerase chain reaction (PCR) technology to amplify, detect, and quantify proteins. The natural tendency of complimentary DNA oligonucleotide sequences to anneal and serve as PCR templates has been leveraged in proximity extension assays (PEAs) to improve the specificity of antibody mediated protein identification, especially in high-throughput methods[80,81]. Binding specificity of either the aptamer or nucleic acid labeled antibodies remains one of the greatest limitations of these techniques. Confirmation with traditional immunoassays, MS, and integrative genomics, however, can help confirm the specificity of protein identification[82,83]. Figure 2 depicts the workflow for these methods.

A Swedish study utilizing nucleic acid labeled antibodies and PEA identified 7 circulating proteins associated with HOMA-IR—including the novel association of cathepsin D as well as previously reported proteins leptin, renin, interleukin-1 receptor antagonist (IL-1ra), hepatocyte growth factor, fatty acid-binding protein 4 (FABP4), and tissue plasminogen activator (t-PA). Of these, IL-1ra and t-PA were also positively associated with incident diabetes, however, these associations were completely attenuated after adjustments for fasting glucose[84]. Mendelian randomization analyses also suggested insulin resistance had a casual effect on t-PA antigen levels. In a more recent, and larger, cross-sectional Swedish study, 29 proteins were found to be associated with prevalent diabetes at a false discovery rate < 5%. Of these, 14 were reportedly novel associations[85]. However, none of these were found to be causally associated with diabetes in Mendelian randomization analyses. A recent example of MS proteomics analysis, paired with 2-dimentional gel electrophoresis, was conducted in a small cross-sectional cohort of normoglycemic lean, normoglycemic abdominally obese, prediabetic, and diabetic Koreans showed higher levels of serpin peptidase inhibitor A1 (AAT/SERPINA1), haptoglobin protein (HP), zinc-alpha2-

glycoprotein (ZAG), apolipoprotein A-1 (APOA1), and retinol binding protein 4 (RBP4) and lower levels of growth-inhibiting protein 25 (GIG25/AACT/SERPINA3), albumin (ALB), and transthyretin (TTR) in those with abdominal adiposity or insulin resistance compared to normal individuals[86].

Associations with Cardiovascular Disease

Metabolomics and proteomics have also been leveraged to study biomarkers of cardiovascular disease. These findings have revealed some potential common metabolic pathways involved in insulin resistance, T2D, and cardiovascular disease. To list a few, BCAA and BCAA related metabolites have been positively associated with coronary artery disease (CAD)[87–89]. Levels of glutamate/glutamine and several acylcarnitines were also shown to differentiate between individual with CAD and controls even after adjustments for traditional clinical factors including BMI and diabetes[87]. For lipids, specific LPC and sphingomyelin species have been associated with incident CAD[90] while higher levels of LPC and LPC plasmalogens containing unsaturated fatty acids—as well as PCs containing DAGs, sphingomyelins, and ceramides—and decreased levels of LPC and LPC plasmalogens containing saturated fatty acids—were associated with increased prevalent CAD[91]. For proteins, in a study conducted in Americans from the San Francisco Bay Area with stable coronary heart disease, 200 proteins were associated with cardiovascular events including several families also associated with T2D (e.g., interleukins, cathepsins, and fatty acid-binding proteins). The protease SERPINA3 was one of nine proteins included in a clinical risk prediction model that improved on previously established clinical risk factors[92]. These findings could begin to highlight important metabolic pathways that may be used to untangle the mechanism for T2D associated cardiovascular disease.

Future Directions

Metabolomic and proteomic studies provide a wealth of information, especially when combined with genomic, transcriptomic, epigenomic, and microbiome information. A current challenge, however, is how to organize this data into meaningful information and successfully prioritize relevant associations for further scientific discovery. A common approach, which has been described extensively above, is to leverage genomic data to identify biomarkers that are disease causal[46,47,85]. Pathway analysis is also an emerging statistical methodology used to cluster disparate biomarkers together into hypothetical pathways. Further work including statistical innovation, however, is needed to overcome these hurdles.

The vast majority of metabolomic and proteomic analyses have also been focused on known metabolites or proteins included on high-throughput platforms due to *a priori* knowledge of their associations with specific biological pathways. Still, there are hundreds to potentially thousands of circulating low abundance biomarkers that have yet to be described. Untargeted metabolomics can be utilized to study these molecules in an unbiased fashion. Dimethylguanidino valeric acid (DMGV), a novel circulating biomarker of nonalcoholic fatty liver disease also associated with the future development of T2D[93] and reduced cardiometabolic fitness[94], was identified through the integration of untargeted MS data with genomic and phenotypic data in large human cohorts. The availability of a growing amount

of genomic data will be instrumental in facilitating the identification of these unknown compounds.

The majority of studies described thus far in this review have been population studies focused on assessing biomarker associations with T2D and glycemic traits. These technologies can also be leveraged to study treatment effect. Individual responses to treatment and prevention of T2D can be markedly different[8,95]. Genetic variants in enzymes involved in drug metabolism (e.g. SLC22A1 genetic variant effects on metformin pharmacokinetics[96]) as well as differences in clinical factors[8,95] can explain some of these differences. In the Diabetes Prevention Program, metabolomics has demonstrated that the baseline concentration of specific metabolites are associated with difference in lifestyle modification verses metformin effects on the prevention of T2D[97]. Changes in metabolites including betaine, due to changes in physical activity and diet[98], and arginine and arginine metabolites, due to changes in diet alone[99], have also been associated with decreased risk for development of T2D with these interventions. For proteomics, Williams et al. utilized the previously generated nine protein risk score for cardiovascular events in individuals with stable coronary heart disease[92] to demonstrate they could predict that Torcetrapib, a novel cholesterol medicine found in clinical trials to be associated with increased cardiovascular events, would be associated with adverse cardiovascular effects within 3 months of clinical trial initiation compared to the median 550 days of follow up that occurred before trial termination[100]. These findings, taken in total, suggest that metabolomics and proteomic biomarkers could have a role in therapeutic prognosis as well as elucidate therapeutic mechanisms.

Conclusion

In conclusion, metabolomics and proteomics are powerful technologies that can be leveraged to study biomarkers of T2D. The integration of data from these platforms with genomics and other omics information could help elucidate pathways of disease development as well as therapeutic response. Overlapping findings with cardiovascular disease also highlight common pathways that may explain T2D associated cardiovascular morbidity and mortality. Significant care, however, must be put into technology choice, study design, sample preparation, and data analysis to obtain informative results. In the future, untargeted methods can drastically expand the pool of circulating biomarkers that can be studied while use of these technologies in therapeutic trials could also identify markers of individual response to therapies. In this way, these technologies can further the clinical treatment as well as scientific understanding of T2D.

Sources of funding

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Nonstandard Abbreviations and Acronyms:

T2D	type 2 diabetes

ASCVD	atherosclerotic cardiovascular disease
HbA1c	hemoglobin a1C
NMR	nuclear magnetic resonance
MS	mass spectrometry
GC	gas chromatography
LC	liquid chromatography
PEA	proximit extension assay
ESI	Electrospray ionization
TOF	time-of flight
VLDL	very low density liopoprotein cholesterol
IDL	intermediate density lipoprotein cholesterol
HDL	high density lipoprotein cholesterol
BCAA	branch chain amino acids
mTOR	mammalian target of rapamycin
GWAS	genome wide association study
SNP	single nucleotide polymorphism
AAA	aromatic amino acid
2-AAA	2-aminoadipic acid
DAG	diacylglycerol
TAG	triacylglycerol
LPC	lysophosphatidylcholines
PC	phosphatidylcholine
LPE	lysophosphatidylethanolamine
FA	fatty acid
DHA	docosahexaenoic acid
L-GPC	linoleoyl-glycerophosphocholine
p,p’-DDE	or trans-nonachlo, or ordichlorodiphenyltrichloroethane
IL-1ra	interleukin-1 receptor antagonist
FADBP4	fatty acid-binding protein 4

t-PA	tissue plaminogen activator
AAT/SERPINA1	serpin peptidase inhibitor A1
HP	haptoglobin protein
ZAG	zinc-alpha2-glycoprotein (ZAG)
APOA1	apolipoprotein A-1
RBP4	retinol binding protein 4
GIG25/AACT/SERPINA3	growth-inhibiting protein 25
ALB	albumin
TTR	transthyretin
CAD	coronary artery disease
PCSK-9	proprotein convertase subtilisin-kexin type 9
DMGV	dimethylguanidino valeric acid
NAFLD	nonalcoholic fatty liver disease

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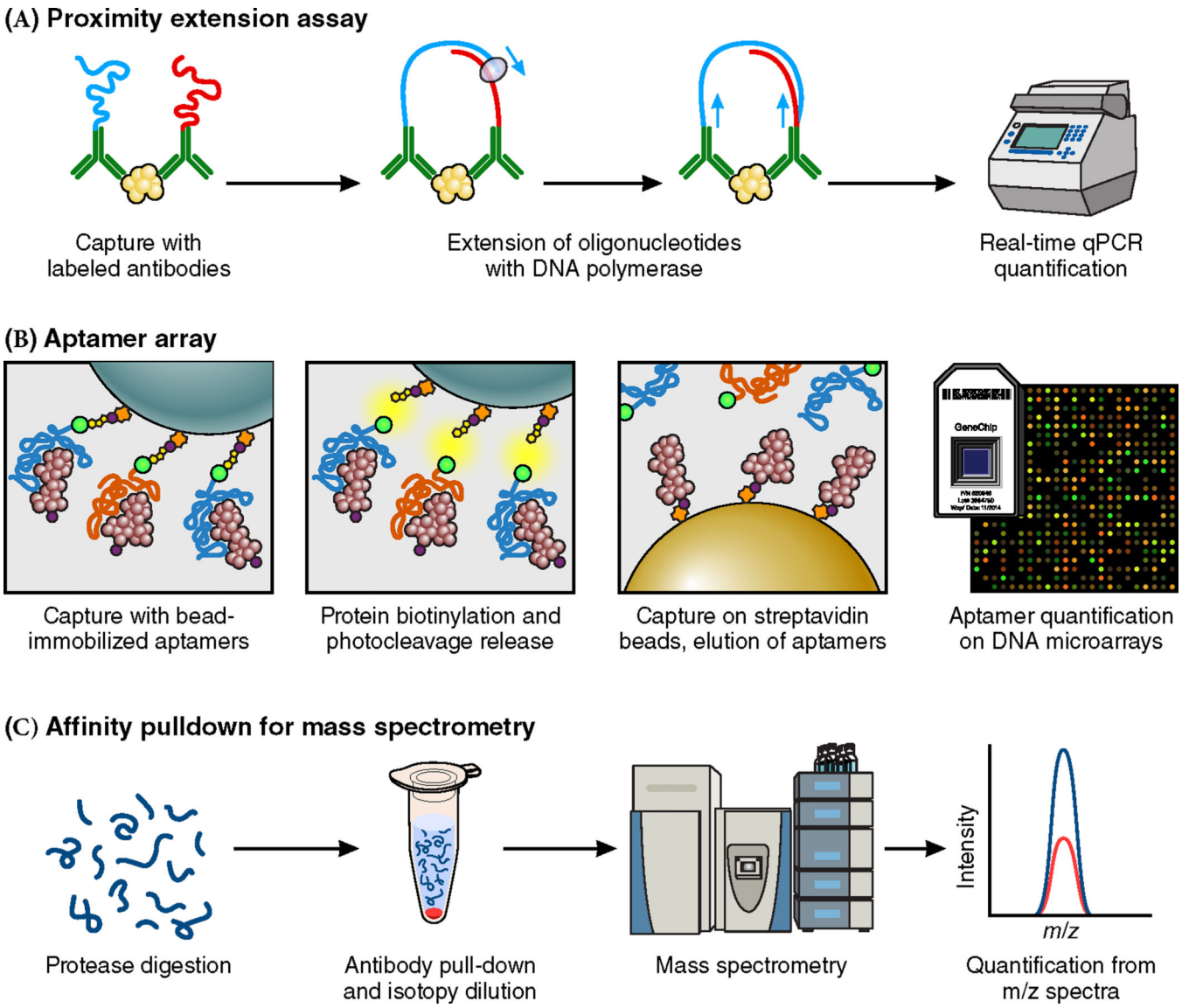

Figure 1.

Incremental improvements in discrimination of hypothetical biomarkers based on a simulation of the predicted hazards ratio per 1 SD increase in a variable number of biomarkers with different marker-marker correlation (r). This figure was generated by Thomas Wang M.D., and Michael Pencina Ph.D. (Wang, Circulation. 2011.)

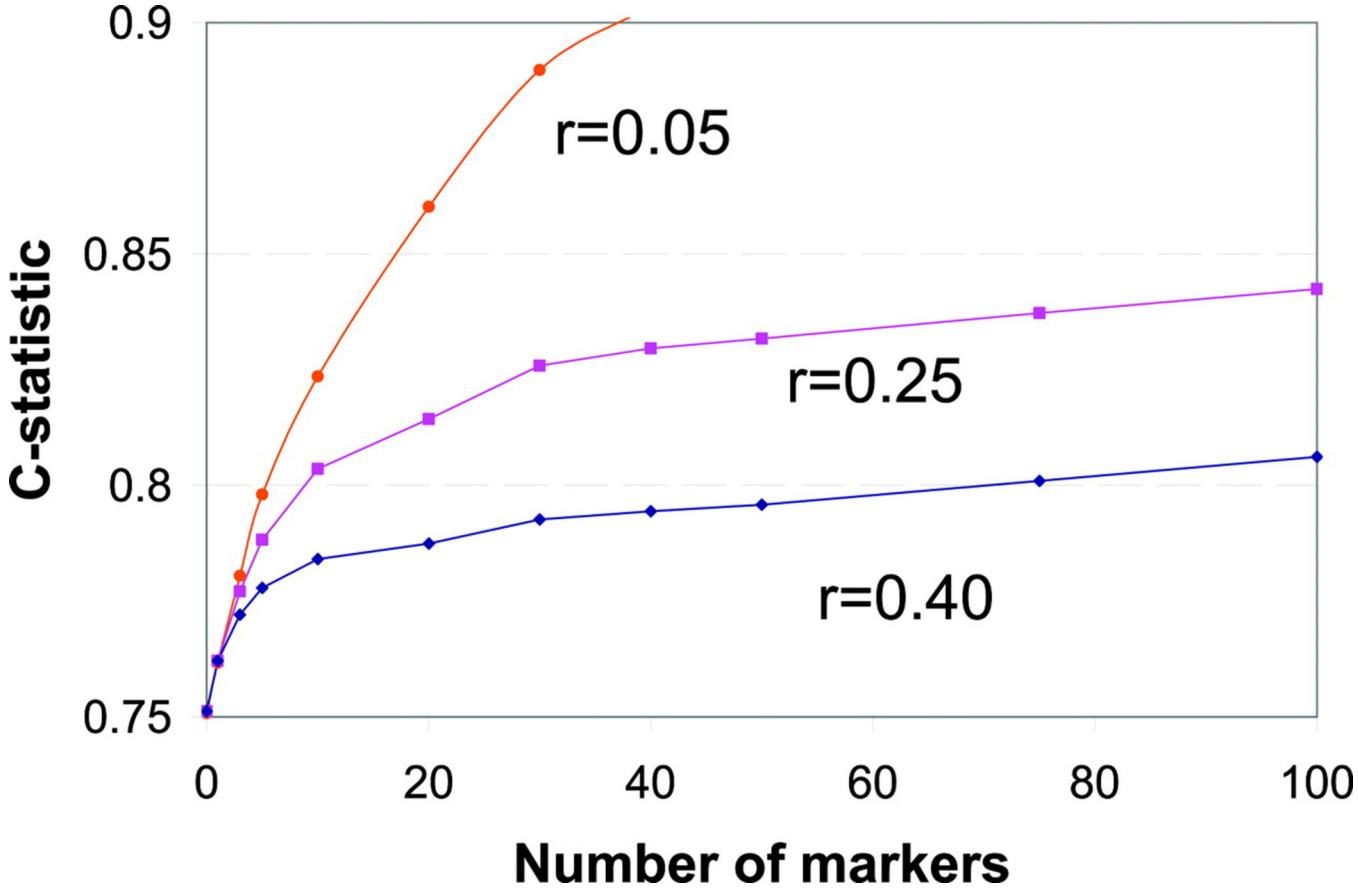

Figure 2.

Workflow for different high-throughput proteomics technologies.

DNA: deoxyribonucleic acid. m/z: mass to charge ratio. qPCR: quantitative polymerase chain reaction. Adapted from a figure by J. Gustav Smith, M.D., Ph.D., and Robert Gerszten, M.D. (Smith and Gerszten. Circulation. 2017).

Table 1.

Comparison of metabolomics and proteomics technologies for circulating biomarker discovery

	Metabolomics	Proteomics
Analytes Measured	Circulating small molecules	Circulating proteins
Technologies	1. NMR 2. MS a. GC-MS b. LC-MS	1. MS 2. Multiplex-nucleic acid affinity reagents (aptamers) 3. PEA with nucleotide-labeled antibodies
Strengths	1. Technologies are high-throughput 2. High sensitivity and specificity 3. NMR is sample non-destructive 4. Small sample amount needed 5. Unbiased measurement of unknown compounds are possible	1. Most technologies are high-throughput 2. Relatively good sensitivity and specificity 3. Small sample amount needed 4. Proteins are direct products of transcription, facilitating integration with genomic data
Limitations	1. Identification of unknown compounds is labor intensive 2. Metabolite pathway analysis is complex	1. High-throughput methods may have slightly lower specificity 2. Limited ability to identify unknown proteins

NMR: nuclear magnetic resonance, MS: mass spectrometry, GC: gas chromatography, LC: liquid chromatography, PEA: proximity extension assay