DIABETES AUGMENTATION ON VASCULAR DISEASE

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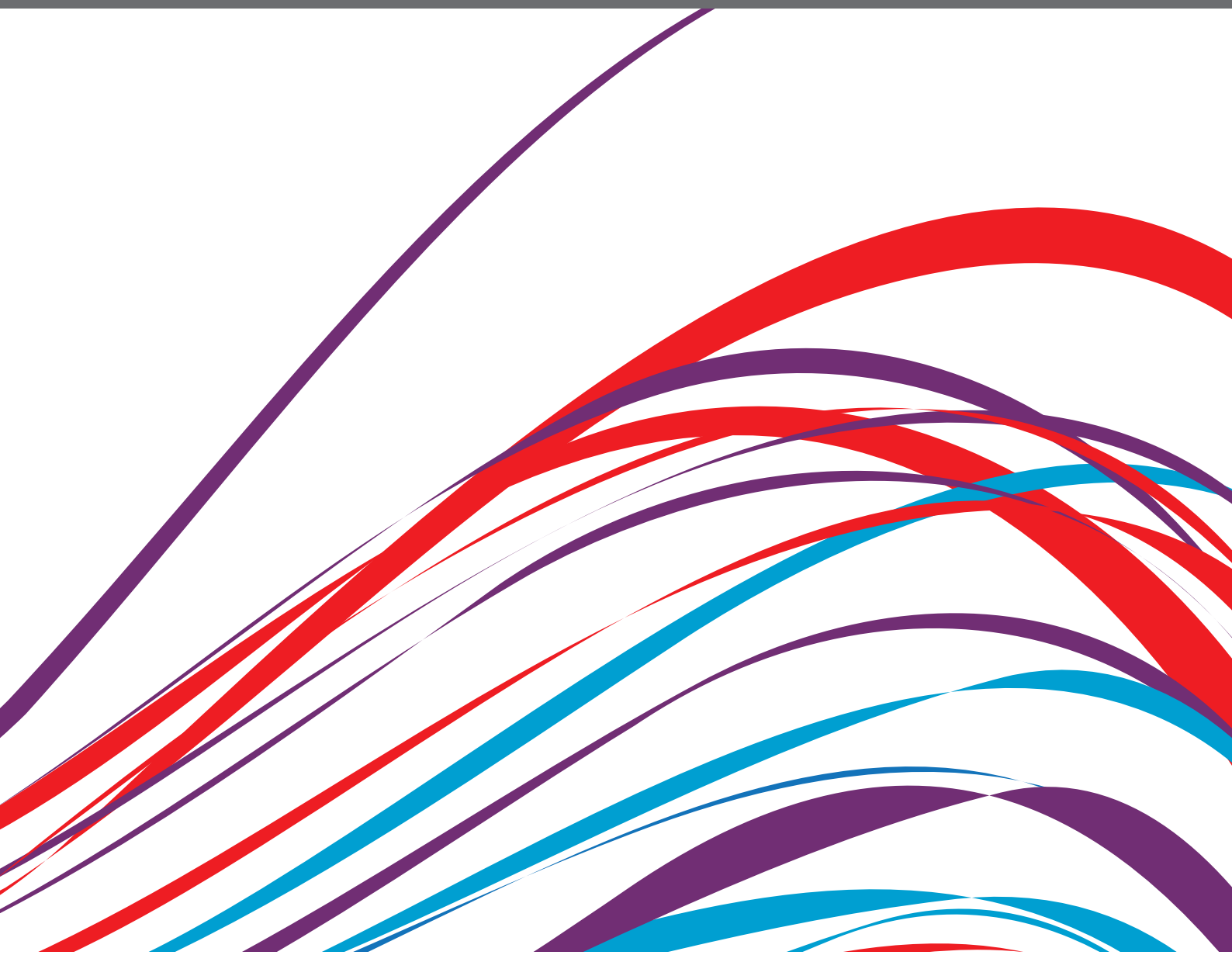

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DIABETES AUGMENTATION ON VASCULAR DISEASE

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EDITORIAL
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Editorial: Diabetes Augmentation on Vascular Disease

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Keywords: diabetes, cardiovascular disease, hyperglycemia, macrophage, RAGE (receptor for advanced glycation end products), postprandial glucose

Editorial on the Research Topic

Diabetes Augmentation on Vascular Disease

Although the collection entitled Diabetes Augmentation on Vascular Disease articulates many views on which additional research and information is needed, there is universal agreement that diabetes augments the frequency of cardiovascular disease. Diabetes is an extremely common and complex disorder with a long history, being first described as polyuria (induced by the excretion of urinary sugar) in patients more than 3,500 years ago. Diabetes is the Greek word meaning "siphon." The year 2021 is the centenary of the discovery of insulin by Banting and Best. It is anticipated that if current trends continue, by the year 2050 one in three individuals will have diabetes with the concomitant burden of vascular disease.

Diabetes enhances both macrovascular diseases, the focus of this collection, and microvascular disease (i.e., retinopathy and glomerulopathy), which is not addressed here. Diabetes attenuates the relative protective effects of cardiovascular disease in females. As pointed out by Goldberg, citing analysis of national data bases by Gregg et al. (1), the rate of vascular complications of diabetes declined significantly from 1990 to 2010 for myocardial infarction, stroke, lower limb amputation, with the greatest decline occurring in the myocardial infarction group. While myocardial infarction had the highest complication rate in 1990, by 2010 it was equivalent to stroke in the diabetic population group. This occurred in the face of increasing prevalence of diabetes over the two decades. Myocardial infarctions, stroke and amputation events reflect atherosclerosis of the coronary arteries, carotid arteries, and femoral arteries respectively. In each case the clinical complication rate is much higher for individuals with diabetes than those without diabetes. The decline in rates reflects improved treatment of hyperglycemia, dyslipidemia, and hypertension, which are common risk factors in both groups and cessation of smoking. The dyslipidemia typical of type 2 diabetes is characterized by modest if any rise in LDL cholesterol, an increase in small dense LDL, hypertriglyceridemia, and low HDL cholesterol. Even with treatment of the dyslipidemia there remains a substantial risk of atherosclerotic cardiovascular disease (ASCVD), which appears to be attributable to the plasma accumulation of triglyceride rich remnant lipoproteins containing excess quantities of apoC3 produced by the diabetic liver (2). The overproduction of apoC3 is probably a manifestation of the liver's resistance to insulin signaling. ApoC3 is an inhibitor of lipoprotein lipase leading to sustained hypertriglyceridemia when elevated. Type 2 diabetes is frequently accompanied by obesity, as an independent risk factor for the development of cardiovascular disease. Enlarged visceral adipose depots are inflamed which accounts for the insulin resistance of the metabolic syndrome that accompanies type 2 diabetes. This complex interaction between obesity, diabetes and cardiovascular disease is discussed in the comprehensive review by Chait and den Hartigh.

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Hyperglycemia is the *sine qua non* of diabetes and is thought to be a risk factor for the development of ASCVD. Both diabetes and ASCVD are chronic disorders that maintain a prolonged prodrome before the manifestation of clinically recognizable disease. The review by Nahmias et al. discusses five long term clinical trials aimed at controlling hyperglycemia on ASCVD using HbA1c as a monitor of efficacy of glucose control. Only in one study is there a suggestive reduction in ASCVD related to control of hyperglycemia. Subset analysis suggests that the early initiation of diabetes treatment is most likely to be efficacious.

HbA1c measures the average plasma glucose over a 3-month interval. But it does not account for the short-term fluctuations of plasma glucose which is an important aspect of glucose homeostasis. The postprandial spikes in blood sugar (PBG) is the topic of the review by Hanssen et al. Although hyperglycemia is associated with increased ASCVD in diabetes, as mentioned above the control of the hyperglycemia is not accompanied by a reduction in the risk of cardiovascular disease —the so called "glucose paradox." However, PBG is much better correlated with ASCVD than is HbA1c. This intermittent hyperglycemia is associated with activation of neutrophils that promotes monocytosis and thrombocytosis, both of which are related to ASCVD. In addition, hyperglycemia is associated with the increased formation of advanced glycosylation end products (AGE), which are recognized by the receptor RAGE. This receptor also recognizes non-AGE proinflammatory ligands such as S100A8/A9, S100B and HMBG1. High glucose levels promote the secretion of S100A8/A9 from neutrophils and it is postulated that its stimulation of RAGE on the common lymphoid precursor ultimately accounts for the increase in monocytes and neutrophils.

The AGE/RAGE system is involved in many inflammatory disorders as summarized by Egaña-Gorroño et al. These include atherosclerosis, peripheral vascular disease, and myocardial infarction, pathologies increased by diabetes, and obesity. RAGE is a cell surface receptor whose cytoplasmic tail interacts with diaphanous1, which is necessary for its RAGE signaling. Its extracellular portion may be released by matrix metalloproteinase and ADAM10 into the plasma. The released soluble RAGE (sRAGE) may bind RAGE ligands to attenuate RAGE signaling. That this system is significantly involved in atherosclerosis is indicated by its reduction with the expression of sRAGE or by RAGE knockout in preclinical models of atherosclerosis.

A review of the comparative composition of coronary plaques of diabetic and non-diabetic subjects reveals that the former has increased plaque burden, with increased lesional necrosis, increased macrophages and T cells and more healed plaque ruptures (3). In this collection Kanter et al. have assessed the pivotal role of monocytes and macrophages as plausible basis for the augmented ASCVD of diabetes. In diabetic cardiovascular disease, there is evidence for increased monocyte recruitment into the lesions, increased inflammatory activation of macrophages, altered macrophage lipid metabolism particularly reduced cholesterol efflux, increased cell death and decreased efferocytosis, which could contribute to the larger necrotic core. These phenotypic changes in macrophages may promote lesion progression and hinder lesion regression.

In summary, there is much yet to learn about how diabetes augments ASCVD and the development of therapeutic approaches to minimize its deleterious effects. This said, it remains essential to minimize the risk factors (hyperlipidemia, hyperglycemia, obesity, and hypertension) on an ongoing basis. To this should be added the consumption of a prudent diet.

AUTHOR CONTRIBUTIONS

GG, CR, and JB served as editors for the Research Topic. GG and CR wrote and edited the manuscript. JB edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Monocytes and Macrophages as Protagonists in Vascular Complications of Diabetes

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With the increasing prevalence of diabetes worldwide, vascular complications of diabetes are also on the rise. Diabetes results in an increased risk of macrovascular complications, with atherosclerotic cardiovascular disease (CVD) being the leading cause of death in adults with diabetes. The exact mechanisms for how diabetes promotes CVD risk are still unclear, although it is evident that monocytes and macrophages are key players in all stages of atherosclerosis both in the absence and presence of diabetes, and that phenotypes of these cells are altered by the diabetic environment. Evidence suggests that at least five pro-atherogenic mechanisms involving monocytes and macrophages contribute to the accelerated atherosclerotic lesion progression and hampered lesion regression associated with diabetes. These changes include (1) increased monocyte recruitment to lesions; (2) increased inflammatory activation; (3) altered macrophage lipid accumulation and metabolism; (4) increased macrophage cell death; and (5) reduced efferocytosis. Monocyte and macrophage phenotypes and mechanisms have been revealed mostly by different animal models of diabetes. The roles of specific changes in monocytes and macrophages in humans with diabetes remain largely unknown. There is an ongoing debate on whether the changes in monocytes and macrophages are caused by altered glucose levels, insulin deficiency or insulin resistance, lipid abnormalities, or combinations of these factors. Current research in humans and mouse models suggests that reduced clearance of triglyceride-rich lipoproteins and their remnants is one important mechanism whereby diabetes adversely affects macrophages and promotes atherosclerosis and CVD risk. Although monocytes and macrophages readily respond to the diabetic environment and can be seen as protagonists in diabetes-accelerated atherosclerosis, they are likely not instigators of the increased CVD risk.

Keywords: apolipoprotein C3, atherosclerosis, diabetes, cytokines, efferocytosis, monocytosis, necrotic core

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BOTH TYPE 1 DIABETES AND TYPE 2 DIABETES INCREASE THE RISK OF ATHEROSCLEROTIC CVD

To date, almost 10% of the US population has type 1 or type 2 diabetes mellitus (T1DM or T2DM), and the prevalence of diabetes continues to rise (1–4). The prevalence of diabetes worldwide is now approaching that of the US, with over 8% of people over the age of 18 having been diagnosed with diabetes. Diabetes is classically defined as hyperglycemia, such as fasting plasma glucose

≥7 mmol/L or glycated hemoglobin (HbA1c) ≥6.5% (5). Hyperglycemia develops largely as a result of impaired insulin production and/or insulin resistance, but dyslipidemia is often also present at varying degrees in patients with poor glycemic control. This is explained in part by insulin's non-glycemic effects, including its triglyceride-lowering actions (6). Thus, relative insulin deficiency or insulin resistance results in both elevated glucose levels and lipid levels.

Both forms of diabetes significantly increase the risk of atherosclerotic cardiovascular disease (CVD). Atherosclerotic lesion morphology appears to be similar in people with T1DM and T2DM based on post-mortem studies, and both forms of diabetes increase atherosclerosis disease burden, enlarge lesion necrotic cores and increase lesional macrophage content (based on CD68 immunoreactivity), as compared with lesions from subjects without diabetes (7).

Improved glycemic control using intensive insulin therapy reduces mortality and the risk of CVD in people with T1DM (8) even after the effect of the intense insulin therapy on glucose control has waned, and over time, a significant proportion of the beneficial effects (known as the legacy effect) of intensive insulin therapy is mediated by control of lipid risk factors (9). In T2DM, improved glucose control is often not associated with protection against incident CVD, at least not in subjects with established CVD (10, 11). However, in a recent study, participants with T2DM who had been randomly assigned to intensive glucose control for 5.6 years had a lower risk of CVD events (than those who received standard therapy) during the period in which the glycated hemoglobin curves were separated. There was no evidence of a legacy effect or a mortality benefit with intensive glucose control (12). It is not known whether the effects of intense glucose control on CVD risk are due to direct effects of glucose in the artery wall.

The observed dyslipidemia associated with diabetes is characterized, not primarily by elevated low-density lipoprotein (LDL) cholesterol levels, but rather by elevated triglyceride-rich lipoproteins (TRLs)—very low-density lipoproteins (VLDL), chylomicrons, and their remnant lipoprotein particles (RLPs)—and by lower high-density lipoprotein (HDL) cholesterol and smaller denser LDL particles. This lipid profile, commonly referred to as diabetic dyslipidemia (13), is most often seen in subjects with T2DM, less well-controlled T1DM, or in subjects with T1DM with additional traits of metabolic syndrome (3).

Furthermore, recent studies suggest that apolipoprotein C3 (APOC3), an apolipoprotein that prevents clearance of TRLs and their RLPs (14–18), predicts incident CVD in subjects with T1DM even when triglyceride levels are in the normal range or close to normal range (19, 20). The effect of APOC3 as a CVD risk factor was independent of glycemic control (HbA1c levels). Therefore, changes in lipids are likely to be critical in promoting CVD risk in both T1DM and T2DM. It is, however, possible that hyperglycemia acts in concert with the lipid changes. For example, it has been suggested that hyperglycemia, due to increased formation of advanced glycation end products, leads to cross-linking of extracellular matrix molecules and arterial stiffening (21, 22), which could, in turn, exacerbate the atherogenic process driven by other factors, such as lipids. Furthermore, when present, risk factors for CVD identified in the general population, such as smoking and elevated blood pressure, exacerbate CVD risk also in people with diabetes.

The metabolic hallmarks of diabetes discussed above affect multiple cell types in the artery wall, promoting pro-atherogenic processes and progression of atherosclerosis. In particular, diabetes results in changes in the myeloid cell compartment, which includes monocytes and macrophages. In this review, we will discuss diabetes-induced alterations in monocyte and macrophage phenotypes and how these alterations might contribute to the increased CVD burden in diabetes.

MONOCYTES AND MACROPHAGES ARE KEY PLAYERS IN DIABETES-ACCELERATED ATHEROSCLEROSIS

Monocytes and macrophages play essential roles in all stages of atherosclerosis (23), both in the presence and absence of diabetes (24). Initially, monocytes enter the subendothelial space in susceptible arteries in response to the retention of apolipoprotein B (APOB)-containing lipoproteins (25), which bind through positive charges in APOB to negatively charged extracellular matrix molecules, primarily glycosaminoglycans (26). The accumulation of monocytes in the artery wall and subsequent maturation of these cells to macrophages propagate a chronic, non-resolving low-grade arterial inflammation (27, 28). Macrophages also contribute to advanced lesions; macrophage death is largely responsible for formation and expansion of necrotic cores in advanced lesions (29). Lesions of atherosclerosis can regress in response to aggressive lipid lowering. Lesion regression is accompanied by a reduced abundance of lesional macrophages, rendering the lesion more fibrotic and likely more stable, and a pro-resolving "M2-like" macrophage phenotype (30, 31).

Early studies of the effects of diabetes on macrophages and other immune cells were pursued in part because of the interest in pancreatic immune cells contributing to diabetes development. Some of these studies suggested that diabetes suppresses macrophage functions, such as phagocytosis (32) and migration (33) in animal models of diabetes and in humans. Later studies indicated an increased activation of macrophages in diabetes (34). Different macrophage isolation methods and differences in macrophage populations might explain these discrepant findings.

Both mouse and human studies implicate macrophages as a key cell type in atherosclerosis associated with diabetes (35–37). Thus, autopsy and coronary atherectomy samples from humans have shown that lesions from people with diabetes are enriched in macrophages, as compared with specimens from patients without diabetes (38). Studies of mouse and porcine models of diabetes have revealed that arterial accumulation of glycosaminoglycans and accumulation of macrophages in early fatty streak lesions are accelerated by diabetes (39–42), consistent with an acceleration of monocyte recruitment and macrophage accumulation according to the response-to-retention hypothesis

(43). Other mouse models have shown that diabetes hinders the regression of lesions through a process dependent on monocyte recruitment and skewing of the phenotype toward a more inflammatory lesional macrophage (36, 44–47). Diabetes also promotes progression of more advanced lesions in mouse and porcine models through mechanisms related to changes in macrophage abundance and phenotypes (19, 45, 46, 48–51). Thus, monocytes and macrophage are altered by the diabetic environment, and may be critical mediators of the proatherogenic effects of diabetes both in progressing and regressing lesions of atherosclerosis.

WHAT IS THE ROLE OF MONOCYTOSIS IN DIABETES-ACCELERATED ATHEROSCLEROSIS?

Elevated levels of circulating blood monocytes (monocytosis) can result in increased monocyte recruitment to lesions, thereby accelerating lesion progression, and can also impede atherosclerosis regression. Numerous animal studies support the idea that increased monocyte levels in circulation can result in increased monocyte recruitment to the artery wall (52–54). Hypercholesterolemia is a strong driver of hematopoiesis in mice, and the early myeloid precursors in bone marrow are especially sensitive to alterations in cholesterol homeostasis (55). Recently, Nagareddy et al. in the Goldberg, Fisher and Tall laboratories suggested that these bone marrow progenitor cells also respond to signals associated with hyperglycemia (44). Previous work from this group has shown that diabetes impairs atherosclerotic lesion regression in response to dramatic lipid-lowering (36). Nagareddy demonstrated that lowering blood glucose by using a sodium-glucose cotransporter 2 (SGLT2) inhibitor in mouse models of T1DM prevented diabetes-induced monocytosis, which in turn reduced monocyte recruitment to the artery wall and improved lesion regression in diabetic mice (44). Furthermore, this interesting study suggested that rather than glucose acting directly on the bone marrow hematopoietic progenitor cell compartment, neutrophil-derived S100A8/S100A9 stimulated myelopoiesis by activating the receptor for advanced glycation end products (RAGE) on bone marrow progenitor cells (44). S100A8 and S100A9 are damage-associated molecular pattern proteins released from neutrophils and monocytes (56). S100A8 and S100A9 form dimers and exhibit both intracellular and extracellular functions. Extracellularly, S100A8/S100A9 is believed to activate RAGE or the lipopolysaccharide (LPS) receptor toll-like receptor 4 (TLR4). Subsequent studies demonstrated that the increase in neutrophil S100A8/S100A9 release in diabetic mice also causes reticulated thrombocytosis by activating RAGE on hepatic Kupffer cells, resulting in increased interleukin-6 (IL-6) production and, through increased thrombopoietin production, thrombocytosis (57). This pathway was shown to contribute to diabetes-accelerated atherosclerosis. The stimulatory effect of diabetes on thrombocytosis was also prevented by SGLT2 inhibition, suggesting that SGLT2 inhibitors prevent both diabetes-induced monocytosis and thrombocytosis in these mouse models. The same group inferred a non-glucose mediated mechanism whereby obesity/T2DM could stimulate monocytosis (58). In the obese mouse models investigated, inflamed adipose tissue rather than neutrophils appears to be the primary source of S100A8/S100A9. S100A8/S100A9 prompts adipose tissue macrophages to release interleukin-1β (IL-1β) through a TLR4-dependent mechanism, which in turn stimulates myelopoiesis by acting on bone marrow progenitors (58).

Together, these studies demonstrate that monocytosis is associated with exacerbated atherosclerosis in diabetic mouse models and that the damage-associated molecular pattern molecules S100A8 and S100A9 play a critical role in this process. Similar mechanisms might be relevant in humans because plasma S100A8/S100A9 levels were shown to correlate with leukocyte counts and coronary artery disease in patients with T1DM (44) and to be increased in patients with T2DM (57). Furthermore, in a mouse model of T1DM, increased intraplaque hemorrhage in advanced lesions was shown to correlate with increased lesional S100A9 immunoreactivity (48). Very recently, diabetes-induced myelopoiesis, monocytosis, neutrophil production of S100A8/S100A9 and impaired lesion regression were found to be prevented by the increased levels of HDL in a human APOA1 transgenic mouse model (47), suggesting that diabetes-induced monocytosis and hampered lesion regression can be prevented by increasing cholesterol efflux from myeloid cells and their bone marrow progenitors.

However, diabetes is not consistently associated with monocytosis in mouse models, and monocytosis is not routinely shown to be present in people with diabetes. Furthermore, in a recent study, SGLT2 inhibition accelerated atherosclerosis regression in diabetic mice without altering circulating monocyte levels (59), demonstrating that SGLT2 inhibition does not act solely by reducing monocytosis in mouse models. In this study, SGLT2 inhibition prevented leukocyte adhesion to the artery wall as well as lesional macrophage proliferation, rather than affecting monocytosis. In mouse models, the ability of diabetes to induce monocytosis appears to be dependent on the diet. Thus, our studies have shown that hyperglycemic mouse models of T1DM fed a low-fat semi-purified diet do not exhibit monocytosis, yet show accelerated atherosclerosis (19, 60). In obese hyperlipidemic mouse models of T2DM or metabolic syndrome (pre-diabetes), on the other hand, monocytosis is consistently present and is likely to contribute to the accelerated atherosclerosis in these mice (61, 62). These findings are consistent with the observations that cholesterol promotes monocytosis by acting on bone marrow myeloid cell precursors (55) and that diabetes-induced myelopoiesis can be prevented by increasing cholesterol efflux through elevated HDL levels (47).

Together, these studies suggest that diabetes-induced monocytosis can contribute to, but is not necessary for, diabetes-accelerated atherosclerosis in mouse models. Thus, additional mechanisms are important in driving diabetes-accelerated atherosclerosis both in mouse models and in humans.

DOES INCREASED INFLAMMATORY ACTIVATION OF MONOCYTES AND MACROPHAGES CONTRIBUTE TO DIABETES-ACCELERATED ATHEROSCLEROSIS?

Monocytes enter peripheral tissues in response to tissue changes the body perceives as an injury, such as atherosclerosis. Once in tissues, these cells differentiate into macrophages. Macrophages are highly plastic cells that take on different phenotypes depending on their surrounding micro-environment. Their primary roles are to phagocytose pathogens and dead and dying cells, which contributes to healing of the perceived injury, and to produce chemokines and cytokines to call in additional leukocytes to aid in the protection of the organism when needed. Macrophages thereby contribute to both the pro-inflammatory and the pro-resolution phases of inflammation.

Many studies have demonstrated that diabetes causes monocytes to take on a more inflammatory phenotype, both in mouse models and in humans. For example, isolated CD14-positive human peripheral blood monocytes from subjects with T1DM or T2DM express and produce elevated levels of the pro-inflammatory cytokines IL-1β, tumor necrosis factor-α (TNF-α), and IL-6, as compared to monocytes isolated from control subjects (63–66). Likewise, blood monocytes from a mouse model of T1DM exhibit elevated levels of *Tnfa* mRNA (37). In addition to increased cytokine production under basal conditions, several groups have suggested that monocytes isolated from subjects with diabetes are "primed" to respond more robustly, as compared with monocytes isolated from controls, to inflammatory stimuli, such as LPS or interferon-γ. The increased expression of TLRs, such as TLR2 and TLR4, on the cell surface of monocytes from people with diabetes supports this hypothesis (63–65, 67). It is possible that the primed state of monocytes results in a heightened inflammatory activation once the monocytes encounter TLR ligands in the lesion (**Figure 1**).

Post-mortem studies of atherosclerotic lesions from both subjects with T1DM and T2DM suggest that diabetes increases the abundance of CD68-positive lesion cells likely to be macrophages (7, 38). To date, there are no studies on macrophage function or phenotype in response to diabetes in human atherosclerotic lesions. However, isolated CD68-positive macrophages from chronic wounds suggest that diabetes results in a less anti-inflammatory and a more pro-inflammatory macrophage phenotype, characterized by reduced expression of CD206 (a pattern recognition receptor that can recognize microbial carbohydrates) and CD163 (a scavenger receptor for the hemoglobin-haptoglobin complex), which are often used as markers of the alternatively activated "M2" macrophage phenotype, and increased expression of the pro-inflammatory cytokine IL-12 upon *ex vivo* stimulation (68). Whether similar changes also occur in human lesional macrophages in response to diabetes is unknown.

Studies in obese hyperglycemic leptin receptor-deficient mice (a model of T2DM) imply that diabetes impairs the resolution of the inflammatory response during wound healing, with macrophages from diabetic mice continuing to produce pro-inflammatory markers even during the resolution phase (68, 69). At least in mice, similar processes transpire in atherosclerotic lesions in response to aggressive lipid-lowering, in which CD68-positive cells in diabetic mice fail to undergo a switch to a less inflammatory and more "M2-like" pro-resolution macrophage (36, 44). Under conditions of atherosclerotic lesion regression in response to aggressive lipid-lowering, a large proportion of the reduction in lesional macrophage accumulation could be attributed to reduced monocyte recruitment together with a stable rate of apoptosis of plaque macrophages [(70); **Figure 1**]. Interestingly, work from the Fisher laboratory demonstrated that lesion regression appears to require recruitment of the Ly6C^{hi} monocyte population, and the conversion of these monocytes to "M2-like" alternatively activated macrophages within the lesion (30). This pathway appears to be impaired by diabetes. The mechanism whereby alternatively activated or pro-resolving macrophages contribute to lesion regression is still unclear. One possibility is that these macrophages are more effective in clearing dying or dead cells through efferocytosis than are inflammatory macrophages (71).

Interestingly, at least in mice, the changes observed in macrophages during wound healing are driven by epigenetic modifications that occur in the hematopoietic compartment (68, 69). The notion that diabetes results in lasting effects on the myeloid cell compartment are further highlighted by the fact that if blood monocytes are isolated from T2DM subjects or matched controls, and then differentiated into macrophages for 5 days *in vitro*, monocyte-derived macrophages from subjects with diabetes express elevated levels of numerous pro-inflammatory cytokines, including *IL1B*, *CCL2,* and *IL6*, as compared with monocyte-derived macrophages from controls (72). Using a similar model, it was shown that monocyte-derived macrophages from patients with T2DM exhibit increased expression of NLRP3 and inflammasome activation [(72); **Figure 1**].

The factors responsible for monocyte priming in diabetes are largely unknown. One study showed that the priming can be mimicked *in vitro* by a combination of LDL and high glucose and appears to be mediated by the redox-sensitive MAPK phosphatase MKP-1 (73). Furthermore, epigenetic changes have recently been shown to be responsible for a phenomenon called "trained immunity," which allows innate immune cells to mount a response to reinfection (74). It has been suggested that diabetes might induce epigenetic changes in monocytes through trained immunity [(75); **Figure 1**], although research is needed to provide evidence for this hypothesis.

The recent Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) indicates a causative role for IL-1β in atherosclerotic cardiovascular disease in humans (76). Subjects who had already had a myocardial infarction and had elevated levels of high-sensitivity C-reactive protein were treated with a blocking antibody to IL-1β, and cardiovascular death, myocardial infarction, or stroke were evaluated. There was a significant reduction in the rate of CVD in those treated with the IL-1β blocking antibody, demonstrating for the first time a role for inflammation in CVD in humans in these high-risk subjects (76). Interestingly, subjects with and without diabetes within

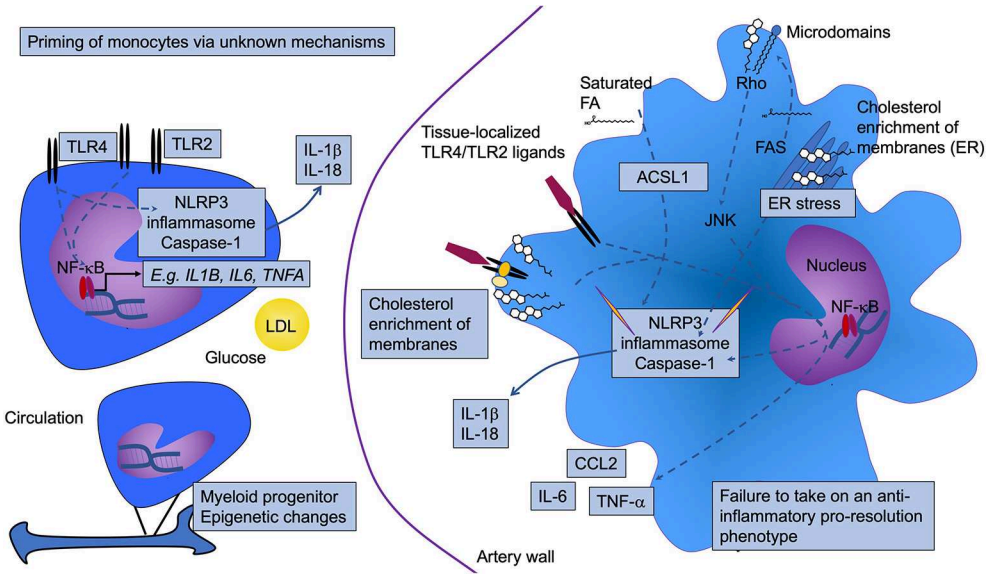

FIGURE 1 | Diabetes is associated with increased monocyte and macrophage inflammatory status. Diabetes results in activation and priming of monocytes to take on a more inflammatory phenotype. Although the mechanism whereby the priming occurs is incompletely understood, glucose, low-density lipoprotein (LDL), and epigenetic modifications have been implicated. This phenotype includes increased gene expression of prototypical pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6 (likely through activation of NF-κB), as well as increased expression of toll-like receptors (TLR) 2 and TLR4. Once the monocytes enter the artery wall and differentiate into macrophages, they also display a heightened inflammatory status. The elevated TLR2/4 levels may interact with tissue-localized TLR ligands. Alternatively, or in addition, the aberrant lipid loading observed in diabetes might result in changes in membrane cholesterol accumulation, resulting in increased proximity of TLRs with other proteins that enhance TLR signaling. Increased cholesterol and saturated fatty acids (FA), either through altered cellular membrane properties, as crystals, and/or as inducers of ER stress, can activate the NLRP3 inflammasome and caspase 1, thereby contributing to increased inflammation through release of IL-1β and IL-18. In human monocytes, TLR4 activation is sufficient for inflammasome and caspase 1 activation, as indicated by a direct arrow from TLR4 to the NLRP3 inflammasome and caspase 1. ACSL1 (long-chain acyl-CoA synthetase 1) is induced in inflammatory macrophages. This enzyme acts on free fatty acids to produce acyl-CoA species, which in the case of saturated acyl-CoAs have been shown to contribute to lipotoxicity and inflammasome activation. Fatty acid synthase (FAS) might also contribute by generating fatty acid substrates that are then incorporated into glycerolipids in the membrane where membrane microdomains regulate signaling via Rho activation of JNK. Pathways that have been shown to be altered by diabetes are highlighted by boxes. NLRP3, NOD-like receptor protein 3.

the trial had a similar reduction in major cardiovascular events (77). This might suggest that inflammation is not the culprit in the diabetes-acceleration of atherosclerosis. Alternatively, it might merely indicate that when comparing diabetes to other states of heightened inflammation (per inclusion criteria for this trial), there is no further role for inflammation associated with diabetes.

A very recent single-cell RNA-sequencing study describes the transcriptome of leukocytes from asymptomatic and symptomatic human carotid atherosclerotic lesions. Strikingly, the authors found that a TLR4 and IL-1β signaling signature was associated with macrophages from asymptomatic lesions, rather than symptomatic lesions (78). Instead, macrophages in symptomatic lesions were characterized by an alternatively activated signature, including IL-4-mediated signaling, suggesting a healing function. This finding indicates that macrophage phenotypes in symptomatic lesions are not geared to inflammation but rather to healing, whereas macrophages in lesions that have not yet caused symptoms harbor more active inflammation. These types of studies have not yet been done in people with diabetes.

In summary, there is evidence that diabetes promotes a more inflammatory monocyte and macrophage phenotype, and that the heightened inflammatory capacity of macrophages might be facilitated by priming of blood monocytes or potentially of myeloid progenitor cells by the diabetic environment (**Figure 1**). It is not known to what extent the increased inflammatory capacity of monocytes and macrophages directly contributes to atherosclerosis progression in diabetes.

ABERRANT LIPID METABOLISM IN MONOCYTES AND MACROPHAGES IS A CRITICAL COMPONENT OF DIABETES-ACCELERATED ATHEROSCLEROSIS

Macrophages in lesions of atherosclerosis will engulf APOB-containing lipoprotein particles (LDL, VLDL, and RLPs), resulting in the formation of the hallmark cell type of atherosclerosis—foam cells. Large TRLs, such as chylomicrons derived from the gut after a meal, are believed to be too large to enter the artery wall without first being partly hydrolyzed to RLPs by lipoprotein lipase. While LDL needs to be modified, e.g., by oxidation, to be effectively ingested by macrophages, VLDLs and RLPs are readily taken up by

macrophages without modification (79, 80). Early studies showed that VLDL isolated from patients with T2DM is taken up more effectively by mouse peritoneal macrophages, and results in increased lipid accumulation, as compared with VLDL from subjects without diabetes (81). In addition to macrophages, other cell types significantly contribute to foam cell formation in lesions, such as smooth muscle cells, which are known to transdifferentiate into macrophage-like cells after they become lipid-laden (82). Although the effect of diabetes on smooth muscle cell lipid loading and subsequent transdifferentiation to macrophage-like cells is unknown, it has been shown that macrophages become more loaded with cholesteryl esters in the presence of diabetes (19). This might be due in part to increased levels of RLPs in the interstitial fluid surrounding macrophages in the setting of diabetes [(19); **Figure 2**]. Diabetes has also been shown to lead to reduced expression of the cholesterol transport proteins ABCA1 and ABCG1 in macrophages (83–85), which would reduce cholesterol efflux and further increase their cellular cholesterol content. Furthermore, high glucose and reduced insulin signaling have been suggested to increase the expression of CD36, a lipid scavenger receptor, in macrophages and monocytes through post-transcriptional mechanisms (86, 87). Together, these observations demonstrate that macrophages carry a larger load of lipid in the presence of diabetes (**Figure 2**).

It was long believed that the uptake of lipoproteins invariably results in an increased pro-inflammatory state of macrophages (88–90). However, recent studies indicate the opposite is true, at least under some circumstances, perhaps when the macrophage's ability to handle the increased lipid load is unharmed (91, 92). Accordingly, Kim et al. recently demonstrated that foamy macrophages isolated from atherosclerotic lesions had an attenuated inflammatory repertoire. In contrast, the non-foamy macrophages expressed elevated levels of pro-inflammatory markers and overlapped with the inflammatory macrophage cluster identified by single-cell RNA sequencing (92). Along the same lines, we recently demonstrated that macrophage lipid loading and their inflammatory capacity do not go hand-in-hand in the setting of diabetes (19). We observed, by using a mouse model of T1DM, that diabetes results in both heightened inflammation and increased cholesteryl ester loading in cells isolated from the peritoneal cavity. However, when the diabetic mice were treated with an antisense oligonucleotide (ASO) to APOC3 *in vivo*, the increased macrophage cholesteryl ester loading associated with diabetes was prevented while the heightened *Il1b* and *Tnfa* gene expression was unaffected (19). Moreover, diabetes-accelerated atherosclerosis closely associated with the prevention of macrophage cholesteryl ester accumulation and was dramatically reduced by the APOC3 ASO, suggesting that reduced lipid loading of macrophages might be

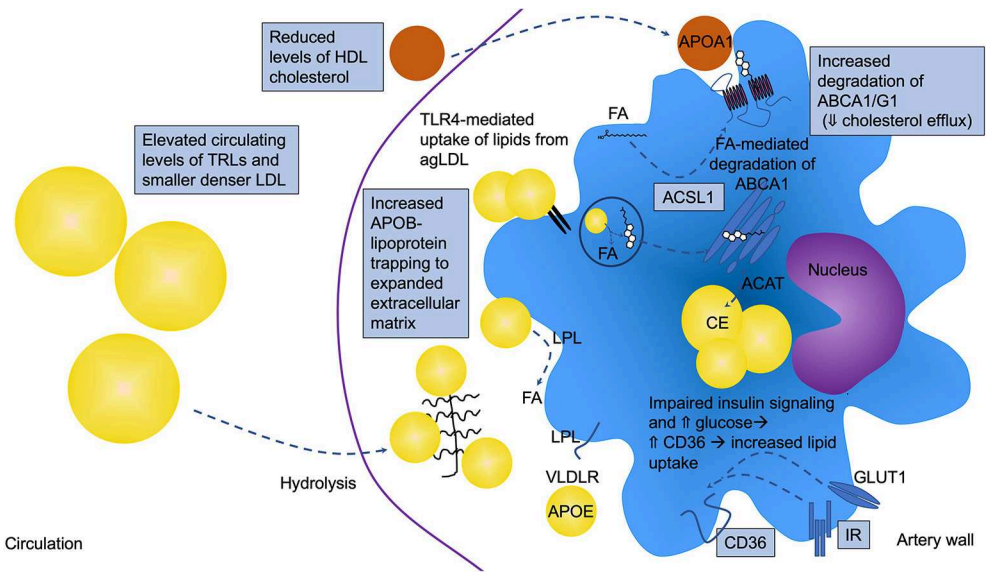

FIGURE 2 | Altered macrophage lipid handling is critically involved in diabetes-accelerated atherosclerosis. Under diabetic conditions, macrophages are often lipid loaded and contain increased levels of cholesteryl ester (CE) in lipid droplets. T2DM and poorly controlled T1DM are associated with elevated circulating levels of triglyceride-rich lipoproteins (TRLs), which contribute to macrophage lipid loading, but the increased lipid loading can also be in part due to increased retention of atherogenic lipoproteins in the artery wall, such as LDL, VLDL, and remnant lipoprotein particles (RLPs). These APOB-containing lipoproteins can bind the expanded/altered extracellular matrix present in lesions in diabetes. Diabetes also alters the balance between uptake and efflux of lipids by modulating the levels of receptors and transporters involved in cholesterol and fatty acid uptake and efflux. CD36 and very low-density lipoprotein receptor (VLDLR), in addition to other receptors and mechanisms, mediate lipid uptake while ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1) are critical mediators of cholesterol efflux to HDL and APOA1. ABCA1 and CD36 have been shown to be reduced and elevated, respectively, under diabetic conditions. Fatty acids have been demonstrated to mediate increased degradation of ABCA1 through a mechanism dependent on ACSL1. Impaired insulin signaling via the insulin receptor (IR) and elevated glucose can lead to increased CD36 levels. Furthermore, the elevated levels of TLR4 might result in increased uptake of lipids from aggregated LDL (agLDL), further contributing to the increased lipid loading. Lipoprotein lipase (LPL) aids in the hydrolysis of TRLs, generating free fatty acids (FA) that are easily taken up by cells. All of these pathways can be altered by the diabetic environment as indicated in the schematic by boxes, resulting in lipid accumulation and a pro-atherogenic macrophage phenotype.

more important for atherosclerosis than inflammatory changes in these cells in diabetes.

Macrophage lipid loading results in an impaired migratory capacity, which could contribute to trapping of the macrophage within the lesion [(93, 94); **Figure 3**]. Work from the Randolph laboratory suggests that macrophages within the atherosclerotic lesion have a limited migratory capacity (95) and that macrophages are stagnant cells with limited mobility once recruited to lesions, especially if the cells display a morphology consistent with lipid-laden cells. Smaller cells nearer the shoulder region and surface of the plaque exhibit increased movement; however, when monocytes were labeled at different time-points, there was no overlap in the macrophages being labeled, suggesting little movement of the macrophages once they were located deeper in the lesion. This important experiment also showed that monocytes accumulate in waves and implied that there is little phagocytosis of older macrophages by newly infiltrating cells (95). Lipid loading extends beyond macrophages; monocytes have been shown to accumulate lipid droplets, especially if they encounter TRLs, which in turn impair their migration (96). Our recent findings suggest that diabetes increases the ensnaring of pro-atherogenic particles containing APOB, APOC3, and APOE (indicating that they might be TRLs or RLPs derived from TRLs) in the artery wall and that this contributes to accelerated atherosclerosis (19). The extracellular matrix, which is responsible for retention of these lipoprotein particles in the artery wall, is expanded and altered under diabetic conditions (39, 42), and VLDL and LDL particles enriched in APOC3 bind better to matrix proteins (97), potentially indicating that under diabetic conditions, macrophages encounter more lipids within the artery wall (**Figure 2**).

There are, however, several circumstances in which lipid handling goes awry and lipids promote inflammatory activation of macrophages (**Figure 1**). One such mechanism is NLRP3 inflammasome activation by cholesterol crystals, which are present in lesions of atherosclerosis (98). A similar mechanism has been proposed for crystals generated by saturated fatty acids (99). The extent to which cholesterol- or saturated fatty acid crystals activate macrophages *in vivo* still needs to be investigated, as some have cautioned that the crystalline material may have been formed during sample processing due to the crystallization of lipids in refrigerated samples (100). The NLRP3 inflammasome is a multimeric complex that processes pro-IL-1β and pro-IL-18 into their mature forms. The NLRP3 inflammasome typically requires two signals—a priming step depending on TLR activation that induces the transcription of *Nlrp3* and *Casp1* (caspase 1)—and an activating step resulting in the assembly and activation of caspase 1 and subsequent processing of pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18 (101). The activation step can be achieved by crystalline materials, ion flux induced e.g., by ATP, and ER stressors. Interestingly, human monocytes, as opposed to human macrophages, release processed IL-1β after a one-time stimulation of TLRs (102). This was explained by the finding that these cells also release endogenous ATP (102), or that an alternative inflammasome pathway exists in these cells (103). The ATP released from newly recruited monocytes might therefore contribute to inflammasome activation in human lesional macrophages by providing ATP as an activating signal.

Many groups have reported that diabetes results in increased expression of genes that would be consistent with increased inflammation and with priming/and or activation of the NLRP3 inflammasome in both T1DM and T2DM (36, 37, 44, 58, 66, 104–109) (see the above section), but it is not yet clear what the diabetes signal that drives augmented NLRP3 activation might be. It is possible that increased intracellular cholesterol crystals, crystals formed by saturated fatty acids, or altered membrane levels of cholesterol or saturated fatty acids might explain this phenomenon. Aberrant intracellular cholesterol handling can also trigger the inflammasome via increased ER cholesterol accumulation [(100, 110, 111); **Figure 1**]. In diabetes, the aberrant cholesterol accumulation in membranes might

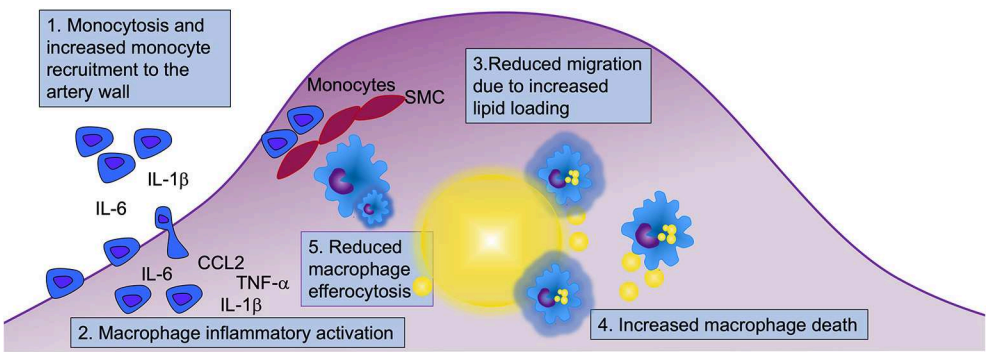

FIGURE 3 | Myeloid cells contribute to diabetes-accelerated atherosclerosis and hindered lesion regression through at least five mechanisms. Diabetes alters many aspects of myeloid cell biology that can contribute to accelerated atherosclerosis and hampered lesion regression. Both monocytes and macrophages display an augmented inflammatory potential, and under certain conditions, diabetes results in elevated levels of monocytes in circulation, which when present contribute to increased recruitment of monocytes to the artery wall. Diabetes increases macrophage lipid loading (indicated by yellow lipid droplets in blue macrophages), which in turn might reduce their ability to migrate. Lipid overload may drive macrophage cell death, and together with reduced efferocytosis, this is likely to contribute to the acceleration of necrotic core formation and progression of atherosclerosis in diabetes. Five mechanisms involving monocytes and macrophages that are likely to contribute to diabetes-accelerated atherosclerosis are highlighted (numbered in boxes). SMC, smooth muscle cells.

be due to reduced expression of the cholesterol transport proteins ABCA1 and ABCG1 in macrophages (83–85). These transporters efflux cholesterol to APOA1 and HDL, thereby reducing cellular and membrane cholesterol levels (**Figure 2**). The ER membrane appears to serve as an important sensor of intracellular cholesterol homeostasis (112). Some, but not all studies, have found that deletion of the inflammasome reduces atherosclerosis under non-diabetic conditions (63, 98, 100, 113, 114). Further studies are needed to investigate the role of the NLRP3 inflammasome in macrophages in the setting of diabetes.

Based primarily on studies of macrophages deficient in ABCA1 and ABCG1, in which cholesterol loading is particularly high due to the defective cholesterol efflux, it has been shown that TLR4 signaling is enhanced by cellular cholesterol accumulation. The mechanism is believed to be due to increased activation of TLR4 due to increased proximity of TLR4 with its binding proteins MD2 and CD14 in lipid rafts [(115); **Figure 1**]. Furthermore, a recent study suggested a different role of TLR4 in relation to macrophage lipid loading. This study demonstrated that TLR4 is required for macrophage lipid uptake from aggregated LDL, by promoting the release of exosomal enzymes for degradation of lipids in these aggregates (116). Accordingly, TLR4-deficiency reduced lipid loading of macrophages through a pathway involving PI3K and Akt (**Figure 2**). Direct evidence for these pathways in macrophages in diabetes is missing, but the models would fit with the increased inflammatory activation and lipid loading of these cells in diabetes.

In addition, increased fatty acid load is likely to contribute to macrophage activation through several mechanisms. Recent studies have revealed that fatty acid synthesis through fatty acid synthase (FAS) is required for macrophage inflammatory activation (117). Loss of FAS altered membrane order and composition, impairing the retention of plasma membrane cholesterol and disrupting Rho GTPase trafficking, resulting in impaired cell adhesion, migration, and activation (117). Interestingly, the effect of FAS-deficiency could be reversed by the addition of cholesterol, suggesting that endogenous fatty acids are critical for correct membrane cholesterol localization (**Figure 1**).

Disruption of fatty acid storage in inert triacylglycerol is associated with saturated fatty acid-induced inflammatory changes in macrophages (118). However, lipid uptake via the VLDL receptor (VLDLR) results in pro-inflammatory effects in macrophages concomitant with both increased triacylglycerol and C16:0 ceramide levels (119). VLDLR binds APOE, rather than APOB, and acts in concert with LPL to promote uptake of lipids from APOE-containing VLDL, RLPs and chylomicrons (120). Furthermore, we and others have demonstrated that deletion of acyl-CoA synthetase 1 (ACSL1), an enzyme that esterifies fatty acids into their acyl-CoA derivatives, a step necessary for most downstream fatty acid metabolism, reduces inflammation and lipotoxicity in response to saturated fatty acids and diabetes (37, 121, 122). Moreover, high levels of unsaturated fatty acids (C18:1 and C18:2) induce degradation of ABCA1, reducing cholesterol efflux capacity through a mechanism mediated by ACSL1 in macrophages (123). Myeloid cell-targeted deletion of ACSL1 also prevented diabetes-accelerated atherosclerosis (37), demonstrating the important role of fatty acid metabolism in atherosclerosis in the setting of diabetes (**Figures 1**, **2**).

Together, these studies indicate that aberrant lipid accumulation and handling in macrophages can cause, or is associated with, increased inflammation. Prevention of macrophage lipid loading in diabetic mice associates with prevention of accelerated atherosclerosis (19). Further studies are needed in order to clarify the effects of diabetes on macrophage cholesterol and fatty acid handling, the relationships among lipids and inflammation, and the relative impacts of these pathways on atherosclerosis.

DIABETES EXACERBATES LESION PROGRESSION AND NECROTIC CORE EXPANSION

Human imaging studies suggest that diabetes accelerates lesion progression. Thus, Won et al. used coronary computed tomography angiography to demonstrate that atherosclerotic lesion progression is faster in people with diabetes than in those without, despite having lower LDL cholesterol at baseline (124). In a similar study, Kim et al. demonstrated that plaque progression (necrotic core expansion), was accelerated in diabetes—again in the absence of elevated LDL cholesterol (125). These studies highlight the concept that diabetes accelerates atherosclerotic lesion progression even in settings in which LDL cholesterol levels are lower or similar as compared with the non-diabetic control cohort. In both the aforementioned studies, mean LDL cholesterol levels were ∼100 mg/dL, which is in the normal range. Mouse studies of diabetes-accelerated atherosclerosis also support this concept (39).

As the atherosclerotic lesion progresses, a necrotic core is formed. Necrotic core expansion is thought to be due, at least in part, to the death and reduced clearance of dead and dying macrophages (**Figure 3**). Both human pathology studies and imaging studies have demonstrated that diabetes results in an expansion of lesional necrotic cores associated with lesion progression (126–129). Necrotic core expansion can also be observed in mouse models of T1DM (19) and of T2DM/metabolic syndrome (61, 130). It is unclear to what extent the expanded necrotic cores observed in diabetes are due to a selective modulation of the myeloid cell compartment, resulting in increased macrophage death or reduced clearance of dead and dying macrophages, or if the expanded necrotic core is a marker of a faster-progressing lesion, which might be due to increased recruitment and turnover of macrophages (**Figure 3**). Alternatively, all three scenarios could be occurring. For example, deletion of insulin receptors in myeloid cells results in increased susceptibility of macrophages to apoptosis in response to altered cholesterol metabolism, causing larger necrotic cores in mouse models [(131); **Figure 3**]. Thus, insufficient insulin signaling might contribute directly to increased macrophage death and necrotic core expansion. Furthermore, if there indeed is increased inflammasome activation in diabetes, this can result in pyroptosis, an inflammatory type of cell death linked to

the NLRP3 inflammasome that has been proposed to play a role in atherosclerosis (132). Future research will reveal whether pyroptosis or other cell death pathways are increased in macrophages in diabetes and if such mechanisms contribute to diabetes-accelerated lesion progression.

The clearance of dying or dead cells by phagocytes is termed efferocytosis. Efferocytosis has been shown to be impaired in mouse models of T2DM, an effect ascribed to the increased membrane content of saturated fatty acids (130). Reduced efferocytosis can contribute to the expansion of necrotic cores and the progression of the atherosclerotic lesion [(133); **Figure 3**].

Efferocytosis requires metabolic reprogramming to increase glucose uptake and aerobic glycolysis (134). A prevailing hypothesis in the field of diabetes is that if a cell is bathed in excessive glucose, it ought to take up more glucose, especially if the cell expresses non-insulin dependent glucose transporters, such GLUT1. Macrophages express relatively high levels of GLUT1 and lower levels of the insulin-sensitive GLUT4. However, because GLUT1 exhibits a K_m for glucose of 1–7 mM, it is generally believed to be nearly saturated at physiological ranges of glucose (135). Instead, increased glucose influx through GLUT1 might be a result of inflammatory activation of macrophages (136). To investigate if increased glucose flux into myeloid cells *per se* could mimic the effects of diabetes in non-diabetic mice, we overexpressed GLUT1 under control of the CD68 promoter in hematopoietic cells, which results in expression primarily in myeloid cells (136). Forcing macrophage to take up more glucose through GLUT1 overexpression did not induce inflammatory changes in primary macrophages *ex vivo* or accelerate atherosclerosis or alter necrotic core size (136). However, deletion of GLUT1 prevented normal inflammatory activation of these cells by LPS or LPS and interferon-γ (136, 137). Thus, GLUT1 is required for normal inflammatory activation of macrophages, consistent with previous studies (138), but increased glucose flux through GLUT1 is not sufficient to promote lesion progression in non-diabetic mice. However, if GLUT1 is deleted selectively in myeloid cells in non-diabetic mice, efferocytosis is reduced, and the necrotic core area is expanded, because of the reliance on glucose metabolism to carry out phagocytosis and efferocytosis (134, 137). Conversely, GLUT1 deletion from the entire hematopoietic compartment results in reduced myelopoiesis in response to dyslipidemia, reduced monocyte recruitment to the artery wall, and reduced atherosclerosis (139). Together, these three studies highlight the complexity of glucose metabolism within different closely related cell types. Increased glucose uptake through GLUT1 in myeloid cells does not appear to be sufficient for inflammatory activation or lesion progression, but GLUT1 is required for several functions of myeloid cells and their precursors.

The findings to date suggest that diabetes enhances necrotic core formation in lesions, perhaps because it promotes lesion progression to more advanced lesions, and possibly in part because it has direct effects on macrophage death and adverse effects on efferocytosis. The effects of diabetes on necrotic core expansion do not appear to be due to increased glucose uptake in macrophages, but rather to be dependent on a reduction in efferocytosis due to increased macrophage membrane saturation (130), and perhaps to increased macrophage death due to insufficient insulin signaling or other mechanisms. Lesions with large necrotic cores are believed to be responsible for a significant proportion of the clinical events of CVD, being less stable and more susceptible to fissuring or rupturing.

ARE KIDNEY MACROPHAGES IN DIABETIC KIDNEY DISEASE ALTERED IN WAYS SIMILAR TO LESIONAL MACROPHAGES IN ATHEROSCLEROSIS?

Since monocytes are circulating cells that enter all injured tissues, do some of the changes observed in lesional macrophages also occur in other tissues affected by diabetes complications? In addition to macrovascular disease, diabetes increases the risk of microvascular complications, such as diabetic retinopathy and diabetic kidney disease (DKD). Several lines of evidence suggest that the presence of microvascular disease dramatically increases the risk of macrovascular complications (140–142). Whether this is due to a shared underlying mechanism or if one complication accelerates the other is unknown. If diabetes influences the myeloid cell compartment and alters monocyte and macrophage function as it relates to atherogenesis, perhaps changes in monocytes and macrophages also contribute to other complications of diabetes, such as DKD. Such changes in monocytes and macrophages might, in part, explain why DKD and atherogenesis are tightly linked.

Do monocytes and macrophages play a causative role in DKD? Macrophages are known to accumulate in the glomerulus and renal interstitium in kidney disease (143–145). Monocyte depletion studies in preclinical models suggest that monocytes and macrophages play a direct pathological role in DKD, perhaps by affecting podocyte barrier function (146). Similarly, deletion or reduction of CCL2, a key chemokine involved in monocyte recruitment, reduces diabetic kidney disease in mice (147–149). More recently, two clinical trials targeting CCL2 have been completed with promising improvements of albuminuria in humans with T2DM and kidney disease (150, 151). Very recently, Niewczas et al. highlighted the importance of chronic inflammation in the progression of kidney disease in diabetes (152). A cluster of 17 kidney disease risk signature proteins that include TNF family proteins and several cytokines predicted the risk of end-stage renal disease, with a least some of these proteins being produced in the glomerulus, and some of them correlating with histopathological changes associated with diabetes, such as glomerulosclerosis (152). This network included the IL-1 receptor, which might suggest activation of the inflammasome pathway.

Lipids accumulate in glomeruli in DKD (153), consistent with the reduced ABCA1 expression in kidneys from diabetic mice (83). TNF-α induces cholesterol accumulation in cultured podocytes, which in turn exacerbates apoptosis in these cells (154), suggesting links among lipid accumulation, lipotoxicity, and inflammatory stimuli. Consistent with this

notion, dyslipidemia associated with atherosclerosis augmented albuminuria in a mouse model of DKD (155). Interestingly, the pro-atherogenic dyslipidemia was associated with increased glomerular macrophage accumulation and a dramatic increase in renal cortex inflammation, suggesting that dyslipidemia contributes to DKD, perhaps via augmented macrophage activation analogous to that in the artery wall. Further research is needed to address the links among diabetes-induced macrophage phenotypic changes and DKD and other diabetes complications.

DO ANTI-DIABETIC MEDICATIONS SHOWN TO BE EFFECTIVE IN PREVENTING CVD RISK ALTER MONOCYTE AND MACROPHAGE PHENOTYPES?

In 2008, the Food and Drug Administration recommended to include cardiovascular endpoints in all phase 2 and phase 3 clinical trials of new anti-diabetic therapies. As a result of this recommendation, new anti-diabetic therapeutics were revealed to have unexpected protective effects on cardiovascular outcomes. Glucagon-like peptide 1 (GLP-1) receptor agonists, which increase insulin secretion, and SGLT2 inhibitors, which lower blood glucose levels by preventing glucose reabsorption in the kidney, both were shown to reduce cardiovascular events in patients with T2DM (156–160). GLP-1 receptor agonists have multiple beneficial effects in several organs in humans, resulting in reduced risk of atherosclerotic CVD events and CVD death. Among the many beneficial effects of GLP-1 receptor activation are anti-inflammatory effects, direct effects in the heart, increased natriuresis and diuresis, reduced body weight, reduced coagulation, and a reduction in post-prandial lipids, as reviewed extensively elsewhere (161). SGLT2 inhibitors display the greatest beneficial effect on heart failure and progression of renal disease, and have only modest beneficial effects on reducing atherosclerotic CVD, and primarily are effective in patients with established atherosclerotic disease (162). Furthermore, SGLT2 inhibitors exert beneficial actions independent of glucose control, consistent with results from the recent DAPA-HF (Dapagliflozin and Prevention of Adverse Outcomes in Heart Failure) trial, which indicated beneficial effects of this SGLT2 inhibitor (dapagliflozin) in patients with heart failure and a reduced ejection fraction even when diabetes was not present (163). Suggested beneficial mechanisms not directly related to blood glucose-lowering effects include natriuresis and osmotic diuresis, reduced inflammation, reduced oxidative stress, reduced arterial stiffness, reductions in blood pressure and body weight, and renoprotective effects (164). According to the safety profiles, SGLT2 inhibitors do not appear to reduce monocyte counts in humans with T2DM, suggesting that the effects of SGLT2 inhibitors on monocytosis in diabetic mice might not be translated to humans. Other clinical trials have demonstrated beneficial effects on CVD outcomes of icosapent ethyl ester, a highly pure fish oil ester, in cohorts with and without T2DM (165, 166).

Do these drugs have direct effects on monocytes and macrophages and is it possible that some of the beneficial effects are due to alterations of lesion macrophages? SGLT2 inhibitors have been shown to prevent macrophage foam cell formation in diabetic mice concomitant with reduced atherosclerosis (46). The mechanism was suggested to be due to downregulation of the increased LOX1 and ACAT and upregulation of the decreased ABCA1 in macrophages from diabetic mice treated with the SGLT2 inhibitors. Overall however, current research suggests that the main therapeutic effects of SGLT2 inhibitors are unlikely to be mediated by effects in myeloid cells, at least in humans.

GLP-1 receptor agonists prevent incident atherosclerotic CVD in people with diabetes. Some of the effects might be mediated by direct effects on macrophages, although GLP-1 receptor agonists exert effects in a plethora of cell types, and macrophages are unlikely to be a major point of action. Nevertheless, exendin-4 (a GLP-1 receptor agonist) has been shown to reduce atherosclerosis and monocyte adhesion to aortas as well as inflammatory gene expression in macrophages in mice, likely by increasing cAMP levels in these cells (167).

In relation to the beneficial effects of icosapent ethyl ester on incident CVD, fish oils have been shown to have beneficial effects in macrophages, including reversal of the defective efferocytosis associated with diabetes (130). Although the mechanism of icosapent ethyl ester might be distinct from that of natural mixed fish oils, it is relevant that fish oils can be converted to bioactive lipid mediators involved in the resolution of inflammation (168).

More research is needed to understand the beneficial effects of these therapies in patients with diabetes. Such studies could also shed additional light on the mechanisms whereby diabetes increases CVD risk.

SUMMARY AND FUTURE QUESTIONS

Diabetes creates an environment that fosters the initiation and progression of the atherosclerotic lesion as well as a hampered lesion regression through at least five likely mechanisms in monocytes and macrophages (**Figure 3**). First, diabetes increases monocyte recruitment to the lesion, especially in the setting of monocytosis. However, while monocytosis can exacerbate monocyte recruitment to lesions, it is not required for diabetes-accelerated atherosclerosis. Second, monocytes appear to be primed in circulation to have an increased inflammatory potential, and once in the artery wall, their boosted cytokine/chemokine production can contribute to increased recruitment of new monocytes and activation of macrophages and other lesional cells. Third, diabetes increases macrophage lipid loading, perhaps mainly due to trapping of TRLs and RLPs or modified LDL within the artery wall via increased retention of these lipoprotein particles through binding to glycosaminoglycans. Once loaded with lipids, macrophages struggle to migrate and egress out of the lesion, contributing to necrotic core expansion. Fourth, due to increased sensitivity to cell death induced by the elevated lipid environment and/or insufficient insulin signaling, and, fifth, reduced efferocytosis

capacity, lesions progress faster under diabetic conditions than under non-diabetic conditions and develop expanded necrotic cores. Similar mechanisms are involved in mediating the detrimental effects of diabetes on lesion regression.

Many pieces of this puzzle remain to be found. Although monocytes and macrophages are present in all stages of atherosclerosis and their function is altered by diabetes, a major question is whether the myeloid cell compartment could be successfully targeted for prevention of diabetes-accelerated atherosclerosis and CVD. The CANTOS trial was the first trial to target inflammation and produce a protective effect on CVD. Such an approach might be feasible and successful in people with diabetes and heightened inflammation. Furthermore, based on the research available to date, new therapies that target apolipoproteins, such as APOC3, would be predicted to be effective in preventing CVD in patients with diabetes and elevated levels of APOC3. Other important questions for future research include the mechanisms whereby diabetes induces adverse effects in myeloid cells. For example, what exactly goes awry in myeloid cell lipid handling in the setting of diabetes, and what is the mechanism whereby lipids accumulate in these cells? What is the role of inflammatory changes vis-à-vis lipid over-accumulation? What changes are critical for necrotic core expansion and CVD events? Do different myeloid cell populations respond differently to the diabetic environment? Some of these important answers are likely to be revealed by new methodology, such as single-cell RNA-sequencing, proteomics, metabolomics and lipidomics.

AUTHOR CONTRIBUTIONS

JK and C-CH wrote the manuscript. KB wrote parts of the manuscript and edited the full manuscript.

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REVIEW
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Adipose Tissue Distribution, Inflammation and Its Metabolic Consequences, Including Diabetes and Cardiovascular Disease

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Adipose tissue plays essential roles in maintaining lipid and glucose homeostasis. To date several types of adipose tissue have been identified, namely white, brown, and beige, that reside in various specific anatomical locations throughout the body. The cellular composition, secretome, and location of these adipose depots define their function in health and metabolic disease. In obesity, adipose tissue becomes dysfunctional, promoting a pro-inflammatory, hyperlipidemic and insulin resistant environment that contributes to type 2 diabetes mellitus (T2DM). Concurrently, similar features that result from adipose tissue dysfunction also promote cardiovascular disease (CVD) by mechanisms that can be augmented by T2DM. The mechanisms by which dysfunctional adipose tissue simultaneously promote T2DM and CVD, focusing on adipose tissue depot-specific adipokines, inflammatory profiles, and metabolism, will be the focus of this review. The impact that various T2DM and CVD treatment strategies have on adipose tissue function and body weight also will be discussed.

Keywords: adipokines, subcutaneous white adipose tissue, visceral white adipose tissue, brown adipose tissue, beige adipose tissue, metabolic syndrome, insulin resistance

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INTRODUCTION

Obesity has now reached epidemic proportions, with over 60% of the US population classified as overweight or obese (defined by a body mass index $\geq$ 25 or 30 kg/m^2, respectively) (1). The incidence of type 2 diabetes mellitus (T2DM) has also risen in parallel to the obesity epidemic, and thus is considered a major co-morbidity associated with obesity (2, 3). Recent epidemiological evidence has shown that 85% of type 2 diabetic adults are also obese (4), and it has been projected that more than 300 million people worldwide will have T2D as a consequence of obesity by 2025 (5). While much recent research has aimed to delineate the precise cause(s) of obesity-associated T2DM, the primary mechanism is believed to be insulin resistance that derives from white adipose tissue, liver, and/or skeletal muscle, accompanied by impaired insulin secretion by pancreatic β-cells (6). Furthermore, both obesity and T2DM increase the risk of cardiovascular disease (CVD), increasing morbidity and mortality by greater than 2-fold (7–10). The distribution of adipose tissue is of great importance with regards to these co-morbidities. Insulin resistance often occurs when fat accumulates in intra-abdominal depots and is associated with a constellation of CVD risk factors, in what is known as the metabolic syndrome (11). Simply measuring body weight, waist circumference, or calculating BMI does not portray a clear picture of body composition nor fat

distribution. Thus, other indices have become more useful for assessing body fat distribution, such as waist-to-hip ratios, as well as methods for assessing body composition, including anthropometry, dual-energy X-ray absorptiometry (DEXA), and computed tomography (CT) scanning. A clear picture of body fat distribution in obese subjects is critical for determining how susceptible they are or will be to developing diabetes and/or cardiovascular disease. In this comprehensive review, the complex and interrelated associations between obesity, diabetes, and CVD will be explored in greater detail.

TYPES OF ADIPOSE TISSUE

Adipose tissue can be classified by *morphology* into white, brown, or beige subsets. In addition, white adipose tissue (WAT) can be broadly classified by *location*, largely defined as subcutaneous (located under the skin) and visceral/omental (located intra-abdominally, adjacent to internal organs). Adipose tissue is comprised of many different cell types, which coordinately secrete numerous cytokines, chemokines, and hormones. Approximately one third of the cells within adipose tissue are adipocytes, with the rest represented by fibroblasts, endothelial cells, macrophages, stromal cells, immune cells, and pre-adipocytes. In most lean, healthy individuals, WAT is confined to defined depots. But in certain conditions such as obesity and lipodystrophy, WAT mass can increase ectopically in areas that may influence the susceptibility to comorbidities such as diabetes and atherosclerosis. Such ectopic WAT areas are mostly located within the visceral cavity, and include intrahepatic (discussed in the section on Ectopic Fat below), epicardial (epiWAT, between the heart and the pericardium), perivascular (PVAT, surrounding major blood vessels), mesenteric fat (MWAT, contiguous with digestive organs in the viscera), omental fat (OWAT, an apron of fat that stretches over the intestines, liver, and stomach), and retroperitoneal fat (RWAT, surrounding the kidneys). The latter three depots (MWAT, OWAT, and RWAT) will be classified together herein as "visceral fat" (12). In addition to WAT depots, brown adipose tissue (BAT) represents a distinct type of adipose tissue that is characterized by its morphology and function, with concentrated mitochondria giving it a characteristic brown appearance. Beige fat represents a third new classification of adipose tissue, in which brown adipocytes appear within classical WAT depots. Each of these adipose depots will be discussed in more detail below.

Abbreviations: ALT, Alanine transaminase; M2, Alternatively activated macrophages; AMPK, AMP-activated protein kinase; ARG1, arginase-1; AST, aspartate transaminase; β-AR, beta-adrenergic receptor; BMI, body mass index; BAT, brown adipose tissue; CRP, C-reactive protein; CVD, cardiovascular disease; CT, computed tomography; DAGs, diacylglycerols; DEXA, dual-energy X-ray absorptiometry; eNOS, endothelial nitric oxide synthase; epiWAT, epicardial white adipose tissue; ECM, extracellular matrix; FXR, farnesoid X receptor; FGFs, fibroblast growth factors; FGF21, fibroblast growth factor 21; FDG, 2-[^{18}F]fluoro-2-deoxyglucose; FFA, free fatty acid; TGR5, G-protein-coupled bile acid receptor; GLP-1, glucagon-like peptide-1; HDL, high-density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HOMA-IR, homeostatic model assessment of insulin resistance; HSL, hormone sensitive lipase; IKK, IkB kinase; IRS-1, insulin receptor substrate-1; ICAM-1, intercellular adhesion molecule-1; IFNγ, interferon γ; IRF3, interferon regulatory factor 3; IL-1Ra, interleukin-1 receptor antagonist; IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; LEPR, leptin receptor; LXR, liver X receptor; LDL, low-density lipoprotein; MGL1, macrophage galactose type C-type Lectin/CD301a/CLEC10A; MHC, major histocompatibility complex; CD206, mannose receptor; MWAT, mesenteric white adipose tissue; MMe, metabolically activated macrophage; MHO, metabolically healthy obesity; MUHO, metabolically unhealthy obesity; MCP-1, monocyte chemotactic protein-1; NKT cells, natural killer T cells; NAFLD, non-alcoholic fatty liver disease; NE, norepinephrine; NFκB, nuclear factor kappa-B; OWAT, omental white adipose tissue; PVAT, perivascular adipose tissue; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; RWAT, retroperitoneal white adipose tissue; SAA, serum amyloid A; SCFA, short-chain fatty acids; SGLT-2, sodium glucose co-transporter-2; SCD1, steroyl-CoA desaturase-1; SNS, sympathetic nervous system; TZDs, thiazolidinediones; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor alpha; T2DM, type 2 diabetes mellitus; UCP-1, uncoupling protein-1; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VLDL, very low-density lipoprotein; WAT, white adipose tissue.

ADIPOSE TISSUE DISTRIBUTION

Subcutaneous Fat

Primarily localized to upper and lower body depots in humans, subcutaneous WAT is the most prominent WAT depot in lean, healthy subjects, making up ∼80% of all adipose tissue (13). Thus, more than any other depot, subcutaneous WAT represents a physiological buffer for excess energy intake during times of limited energy expenditure. Subcutaneous WAT acts as a metabolic "sink" for excess lipid storage (14). When this storage capacity is exceeded, either due to an inability to generate sufficient new adipocytes (limited hyperplasia) or an inability to further expand existing adipocytes (limited hypertrophy), fat begins to accumulate ectopically in areas outside the subcutaneous WAT (see sections on Ectopic and Visceral Fat below). Additionally, subcutaneous WAT functions as an insulator to prevent heat loss, as a barrier against dermal infection, and as a protective cushion against physical external stress (15).

Subcutaneous WAT likely arises from adipocyte precursor cells that are distinct from adipocytes that arise ectopically, for example in visceral fat (16). Elegant work by Kahn et al. has demonstrated that pre-adipocytes isolated from mouse and human subcutaneous WAT expresses developmental genes that are present prior to the development of WAT in a pattern that is maintained throughout adulthood, suggesting a cell-autonomous function (16). Thus, WAT distribution has a strong heritable component (17).

The beneficial effects of subcutaneous WAT to glucose metabolism have been demonstrated in numerous ways. However, subcutaneous WAT can be further subdivided into "upper" and "lower" regions, located primarily in the trunk and gluteo-femoral regions, respectively. Upper subcutaneous WAT is often lumped together with visceral WAT, classified together as "abdominal fat." The distinction between upper and lower subcutaneous WAT and how they contribute to metabolic health will be discussed in later sections.

Epicardial Fat

Epicardial adipocytes share embryonic origins with mesenteric and omental adipocytes (18). epiWAT (also termed pericardial WAT) is in close proximity to the myocardium, enabling a shared

microcirculation between epiWAT and certain areas of the heart (19). Due to its proximity to the heart, epiWAT is thought to be approximately twice as metabolically active as other WAT depots, with higher levels of fatty acid uptake and fatty acid release due to lipolysis (20). As a metabolically active WAT depot, epiWAT secretes several adipokines and vasoactive substances such as adiponectin, resistin, vascular endothelial growth factor (VEGF), and inflammatory cytokines and chemokines that impact the adjacent myocardium (21). In fact, due to the complete lack of a fibrous fascial layer between epiWAT and the myocardium, diffusion of fatty acids and other bioactive hormones from epiWAT to myocytes and coronary vessels is easily facilitated (22). Most humans possess a small amount of epiWAT, which provides fatty acids through lipolysis of its triglyceride stores for energy use by the heart. However, obese humans possess an enlarged epiWAT depot, which is clinically related to features of the metabolic syndrome (discussed in later sections).

Perivascular Fat

Fat that surrounds blood vessels is termed perivascular fat (PVAT). It has now been recognized that PVAT has characteristics that resemble both BAT and WAT, and is considered to be an active participant in vascular homeostasis (23). PVAT produces many bioactive molecules that influence vascular reactivity, including adipokines (e.g., leptin, adiponectin, omentin, visfatin, resistin, and apelin), cytokines/chemokines [e.g., interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and monocyte chemotactic protein-1 (MCP-1)], and vasoactive molecules (e.g., nitric oxide, prostacyclin, and angiotensin II) (24). Thus, PVAT can directly contribute to vascular tone, in addition to playing a supportive role in maintaining vessel structure. It has been suggested that PVAT in the thoracic aorta resembles BAT, while PVAT in the abdominal aorta exhibits properties of both BAT and WAT (24). Thus, if PVAT becomes dysfunctional in the setting of obesity, it can pivot from providing an atheroprotective role to promoting atherosclerosis. This concept will be evaluated further in later sections.

Visceral Fat

Fat localized within the visceral compartment has been classified as omental, mesenteric, and retroperitoneal. Lean, healthy individuals do not have large amounts of visceral fat, which largely falls into the category of ectopic fat. Visceral fat is highly metabolically active and is constantly releasing free fatty acids (FFA) into the portal circulation. As such, visceral fat content contributes to various features of the metabolic syndrome, such as hyperinsulinemia, systemic inflammation, dyslipidemia, and atherosclerosis (25), to be discussed in more detail in later sections pertaining to obesity.

Brown Fat

BAT is localized to distinct anatomical regions that have been well-characterized in rodents (26). By taking up circulating fatty acids, BAT functions to generate heat by uncoupling chemical energy production (ATP) via oxidative phosphorylation into heat production (non-shivering thermogenesis), thereby contributing to the clearance of plasma triglycerides and the mitigation of ectopic lipid storage (27). While originally believed to be a depot exclusive to hibernating and small mammals, and present to some degree in human infants, adult humans have recently been shown to have functional and inducible levels of BAT that respond to cold and sympathetic nervous system activation (28–30). Such BAT represents between 1 and 2% of total fat stores in humans, and is localized primarily in the cervical, axillary, and paraspinal regions (26, 31, 32). Similarly to WAT, BAT synthesizes and secretes "batokines" such as fibroblast growth factors (FGFs) including FGF21, neuregulin 4, VEGF, and cytokines such as IL-6 (33). Given the relatively small amount of BAT present in humans, the endocrine potential of batokines is relatively unknown, but it is clear that factors secreted from BAT exert paracrine and autocrine functions. While the relative BAT mass in humans and rodents is small compared to other adipose depots, its relative contribution to metabolic health may be higher.

In rodents and other small mammals, the primary BAT depots are located in the interscapular space and supraclavicular regions, among many others (26, 34). With prolonged stimulation, i.e., cold exposure, the size and activity of these BAT depots will increase, a term called BAT recruitment. BAT recruitment is associated with enhanced proliferation and differentiation of BAT precursor cells.

Beige Fat

In addition to WAT and BAT, a third fat type has been described, termed "browned," "beige," or "brite" (brown-in-white) fat. As the name suggests, beige fat has been described as the presence of brown adipocytes within classic WAT depots. While beige fat shares some features of classical BAT such as systemic triglyceride-lowering, beige fat is thought to be physiologically distinct from BAT, with differential expression of certain genes involved in metabolism, inflammation, and transcription (35, 36). Moreover, human BAT exhibits similar morphology and function as both rodent BAT and beige tissue (30, 37–39), complicating comparisons between the two species. In rodents, subcutaneous WAT is the most susceptible depot to browning, while in humans it is visceral WAT (40). It is generally believed that the majority of WAT depots can develop browning under particular conditions, but more work is needed in this area. There is a growing list of physiological stressors that can promote the browning of WAT, including cold exposure, exercise, bariatric surgery, cancer cachexia, severe burns, as well as pharmacological and dietary components such as conjugated linoleic acid, short-chain fatty acids, capsaicin, non-caffeinated green tea extract, thiazolidinediones (TZDs), and β-adrenergic receptors (41–52).

There is some debate regarding the origins of beige adipocytes, as well as their impact on energy homeostasis. Beige adipocytes may arise from *de novo* adipogenesis from specific progenitor cells when initially stimulated by cold exposure (36, 53), but then may lie "dormant" until stimulated again (54). This theory suggests that dormant beige adipocytes can become quickly and readily activated when needed, reminiscent of an immune response. This newly defined relative flux between "dormant" and "active" beige cells may be what has been previously termed "transdifferentiation" of white-to-beige

adipocytes (54). Beige adipocytes were initially thought to arise from transdifferentiation from white adipocytes, with the ability to de-differentiate back into white adipocytes (55, 56). Additional studies *in vitro* suggest that this is likely not the case (57). The identity of committed beige adipocyte precursors has not been fully elucidated, but there is evidence from isolated WAT stromal cells that beige adipocyte precursors are distinct from white adipocyte precursors (36, 39, 58). It has been suggested that strategies that increase the number of beige adipocytes in mouse WAT also protect them from diet-induced obesity (59–63).

NORMAL ADIPOSE TISSUE FUNCTION

White Adipose Tissue: Energy Storage and Distribution

Adipose tissue is an essential organ for the regulation of energy homeostasis. Primarily tasked with storing excess energy as triglycerides, adipocytes undergo hyperplasia to increase the number of adipocytes and hypertrophy to increase the size of each adipocyte, allowing adipose tissue to expand in times of nutrient excess. As needed, i.e., during fasting and exercise, triglycerides stored in adipose tissue are mobilized to provide fatty acids for energy utilization by the rest of the body. Stored triglycerides are therefore in a constant state of flux, whereby energy storage and energy mobilization are determined largely by hormonal fluctuations. Thus, adipose tissue functions as an energy balance "hub" that integrates and services the energy requirements of diverse organ systems, such as the liver, skeletal and heart muscle, pancreas, and brain (64).

In healthy lean individuals, the majority of adipose tissue resides in subcutaneous depots, where it serves a thermoregulatory function, and from which stored triglycerides can be readily mobilized when needed (65). Conditions that favor adipose tissue expansion, if endured chronically, will eventually exceed the storage capacity of defined adipose tissue depots, leading to the ectopic deposition of triglycerides in other tissues, including intra-abdominal depots (discussed in more detail in later sections).

Non-shivering Thermogenesis

BAT plays an important role in thermoregulation in mammals, including adult humans (66). BAT tissue is rich in mitochondria and uniquely expresses uncoupling protein-1 (UCP-1), which enables heat production by uncoupling ATP synthesis. BAT-mediated thermogenesis has garnered substantial attention recently, as increasing BAT mass or activity could be an effective strategy to combat obesity. While the primary function of WAT is to manage energy storage, brown adipocytes efficiently burn fatty acids released from WAT during adaptive thermogenesis (67). BAT plays an active role in metabolism in animals and humans (28); therefore, strategies that increase BAT mass and/or activity could promote fat loss in obese populations. In addition, beige fat could also contribute to fat catabolism, potentially reducing WAT stores. Human brown adipogenesis occurs in response to chronic or repeated cold stimulation, or in response to pharmacologic compounds such as beta adrenergic receptor (β-AR) agonists (68, 69). However, these browning-inducing methods mediated by the sympathetic nervous system are not practical as a weight loss strategy for several reasons: (1) the browning effects of cold exposure are rapidly reversible, (2) repeated cold exposure is too time- and energy-consuming to be a practical therapeutic, and (3) β-ARs promote adverse cardiometabolic events. Therefore, mechanisms of WAT browning that are long lasting and act independently from the sympathetic nervous system are highly sought after. A new mechanism of WAT browning that does not involve the sympathetic nervous system (SNS) has recently been described. Adipose tissue resident macrophages can secrete norepinephrine (NE), the neurotransmitter that is also secreted by sympathetic neurons to activate BAT and WAT browning (70). Several follow up studies have suggested that eosinophils, type 2 cytokines, and alternatively activated macrophages play critical roles in supporting WAT browning with concomitant increased energy expenditure and weight loss (71–79). However, the notion that immune cells can influence WAT browning has recently been challenged, using different murine and *in vitro* approaches (80). As such, there is some discordance regarding the role of macrophages in WAT browning, necessitating further studies.

Secretion of Hormones and Adipokines

Originally classified as a simple energy storage organ, adipose tissue is now known to function as a major endocrine system that secretes adipokines, growth factors, cytokines, and chemokines (81). The secretion pattern of adipokines appears to vary by adipose tissue depot and is dependent on the energy status of the adipose depot, leading to variable paracrine/autocrine effects of adipokines within particular depots. Adipokines are important mediators of various metabolic processes such as fatty acid oxidation, *de-novo* lipogenesis, gluconeogenesis, glucose uptake, insulin signaling, and energy expenditure in metabolically active tissues such as the liver, skeletal muscle, and brain (81). The various adipokines secreted from adipose tissue and their functions will be described in more detail below. The discussion will be limited to adipokines that are known to be produced to a large extent by adipocytes, in addition to other cell types within adipose tissue such as immune cells.

Leptin

Discovered in 1994, leptin is a peptide hormone that is expressed exclusively by adipocytes and is essential for body weight regulation. Leptin, adiponectin, and omentin (the latter two will be described below) are the only generally accepted adipokines with true endocrine function, meaning they are released from adipose tissue and exert effects on distant target organs. Leptin is encoded by the obesity gene (*ob*). Leptin-deficient (*ob*/*ob*) mice become spontaneously obese due to unrestricted food intake, highlighting the importance of this adipokine in suppressing appetite through the central nervous system (82). Rodents and humans that lack either leptin or the leptin receptor (LEPR) are not only extremely obese, but are also hyperglycemic and extremely insulin resistant (83). In lean and obese animals and humans, circulating leptin levels positively correlate with adiposity (84). Prolonged fasting is associated with a sharp drop in plasma leptin levels, which drives food intake (85). While

leptin is expressed in all adipose depots, including BAT, its expression is highest in subcutaneous WAT (86).

Adiponectin

As one of the first adipokines discovered in the mid-1990s (87–90), adiponectin is a well-described insulin-sensitizing hormone that impacts a wide range of tissues. Adiponectin is a distinctly unique adipokine, as its expression and circulating levels are inversely proportional to adiposity levels, in stark contrast to leptin. Adiponectin expression levels vary between sexes, with higher levels observed in females than males (91–93), and between adipose tissue depots, with higher expression in subcutaneous than visceral WAT (94, 95). The insulin sensitivity-promoting properties of adiponectin are well-known, and are exemplified by the development of insulin resistance in adiponectin-deficient mice (96), and the preservation of insulin sensitivity in adiponectin-overexpressing mice (97). Adiponectin signals through two related receptors, ADIPOR1 and ADIPOR2, followed by docking of the adaptor protein APPL1 (98). The resulting signaling pathway, mediated through peroxisome proliferator-activated receptor alpha (PPARα), leads to metabolic improvements involving decreased hepatic gluconeogenesis, increased liver and skeletal muscle fatty acid oxidation, increased glucose uptake in skeletal muscle and WAT, and decreased WAT inflammation (99). Thus, adiponectin receptors are highly expressed in skeletal muscle, liver, and adipose tissue. In addition, adiponectin receptors are expressed in the pancreas, where adiponectin functions to mitigate β-cell loss by neutralizing inflammatory and lipotoxic ceramides and diacylglycerols (100). In addition to β-cells, adiponectin has also been shown to exhibit strong anti-inflammatory effects on other cell types such as macrophages and fibrogenic cells (99, 101, 102). Taken together, adiponectin plays a protective role in mitigating features of the metabolic syndrome.

Resistin

Resistin is a polypeptide that is secreted by obese adipose tissue. It was originally described as an adipocyte-specific hormone, but it is now thought to originate from macrophages residing in inflamed adipose tissue in mice (103) and from circulating monocytes and tissue macrophages in humans (104, 105). Human resistin is only 59% homologous to mouse resistin (106), which has raised some controversy over the pathogenic role of resistin, and limits comparisons between animal models and human disease (107). Resistin is so named due to its ability to "resist," or interfere with insulin action (108), based on initial studies in mouse models. Evidence for this comes from an initial study in which it was observed that plasma resistin levels are elevated in a diet-induced obese mouse model, that blocking resistin action using a neutralizing antibody improves insulin sensitivity, and that recombinant resistin administration to healthy mice promotes insulin resistance (108). These initial studies led to the suggestion that resistin plays an important role in modulating insulin resistance in the context of obesity, and it has been shown to correlate with insulin resistance in mice and humans (109). Plasma resistin levels have been shown to be increased in obese animal models and humans (110–113) and to decrease with weight loss in humans (114). Conversely, some studies have shown that adipose tissue-derived resistin is suppressed in obesity (115–117), inciting the controversy over what role resistin plays in obesity that persists today. Evidence suggests that visceral fat is the largest contributor to circulating resistin levels (113), supporting the case for an association between resistin and insulin resistance. Moreover, resistin is believed to be an active participant in propagating inflammatory responses. Resistin can upregulate inflammatory cytokines such as TNFα and IL-6 in monocytes and macrophages in a nuclear factor kappa-B (NFκB)-dependent manner (118), and is positively associated with circulating inflammatory markers such as C-reactive protein (CRP) and TNFα (107). Thus, while resistin is an established adipokine and has been shown in some cases to be associated with adverse health conditions such as obesity and insulin resistance, a clear role for resistin is still under active investigation.

Omentin

Initially described as an adipokine secreted from omental WAT (119), it is now generally accepted that omentin is also expressed in other WAT depots such as epicardial fat, and that it derives specifically from the stromal vascular fraction of WAT (119, 120). Omentin is a true endocrine hormone that circulates in the blood (121, 122). Omentin levels are reduced in subjects with obesity (123) and T2DM (124, 125), leading investigators to speculate that omentin may be involved in glucose homeostasis. Indeed, studies using *in vitro* models showed that omentin enhances insulin-stimulated glucose uptake in human adipocytes by activating Akt signaling pathways (119), and studies in humans show a significant negative correlation between serum omentin levels as well as adipose omentin mRNA levels with insulin resistance (124, 126, 127). Omentin levels have been shown to gradually increase in response to weight loss (128, 129). Additional studies suggest that omentin has anti-inflammatory properties. Omentin blunts cytokine expression in endothelial cells (130), vascular smooth muscle cells (131, 132), macrophages (133), cardiomyocytes (134), and adipose tissue itself (135), and is negatively associated with systemic inflammatory markers such as TNF and IL-6 (136). Thus, omentin is considered to be a biomarker for metabolic health that may function to blunt obesity-related cytokine effects (137).

Fibroblast Growth Factor 21 (FGF21)

FGF21 is an endocrine hormone that is involved in the regulation of lipid, glucose, and energy homeostasis (138). FGF21 has received a lot of attention for its insulin-sensitizing and weight loss-inducing effects when administered pharmacologically (139). The liver is the primary source of circulating FGF21, induced by metabolically stressful conditions such as fasting, a ketogenic diet, protein restriction, and bariatric surgery (140), while the brain and adipose tissue are primary FGF21 targets (141, 142). Other tissues are known to also secrete FGF21, including the pancreas and skeletal muscle (143, 144). However, under certain metabolic conditions such as obesity, WAT and BAT may also produce FGF21 (145). This is supported by several studies showing that BMI and adiposity positively correlate with

circulating FGF21 levels in mice and humans (145–149). It is clear that FGF21 levels become elevated as obesity develops in mice and humans, and are positively correlated with BMI, adiposity, and FGF21 expression levels in adipose tissue (145–149). While many studies have shown that adipose tissue expresses FGF21 in rodents (145, 150–154), there is still some debate about whether FGF21 is readily expressed in human adipose tissue. There are a handful of studies that suggest that adipose tissue *FGF21* mRNA expression is below detection levels (155) or not expressed by adipose tissue (156). However, numerous additional studies have found detectable *FGF21* mRNA expression in visceral WAT (157, 158), subcutaneous WAT (145, 157, 158), epicardial WAT (159), cervical adipose tissue (160, 161), and PVAT (162, 163), with the latter two depots containing both WAT and BAT. FGF21 protein has also been detected in adipose tissue by Western blot and immunohistochemistry (162). It is not clear why some but not all groups have been able to detect *FGF21* expression in human adipose tissue, but could depend on the metabolic and/or nutritional status of the subjects sampled (e.g., whether subjects were fasting or fed).

Some studies suggest that adipose-derived FGF21 is a marker of metabolic stress, as it has been shown to correlate with features of the metabolic syndrome (145, 164, 165). Regardless, a clearly-defined function of adipose-derived FGF21 has not yet been established, nor whether adipose-derived FGF21 promotes primarily local effects or contributes to the circulating FGF21 pool under particular metabolic conditions. Elegant studies using tissue-specific Fgf21 KO mice show that adipocyte-derived Fgf21 is not involved in obesity-associated insulin resistance, and that adipose-derived Fgf21 doesn't circulate, instead acting in a paracrine fashion (140). However, the mice used in that study were fasted for 24 h, introducing a metabolic stress that would likely only induce liver-derived Fgf21 that may have masked any contribution from adipose-derived Fgf21. In later studies, a thermogenic role for adipose-derived Fgf21 has been described, in which the browning of WAT was shown to require adipocyte-Fgf21 (141, 166). Thus, it is possible that hepatic- and adipose-derived FGF21 are induced by different stimuli, and that more studies are required to conclusively define a role for adipose-derived FGF21.

OBESITY

Obesity results when energy intake chronically exceeds energy expenditure. Many factors are involved, including genetic, epigenetic, hormonal, and lifestyle factors that are beyond the scope of this review. Adipocyte number is believed to be tightly regulated and determined during childhood (167). However, during the development of obesity, adipose tissue can expand by either hypertrophy (an increase in adipocyte size) or hyperplasia (an increase in adipocyte number due to the recruitment of new adipocytes). Obesity is characterized by dysfunctional adipose tissue, in which adipocytes initially become hypertrophic during periods of caloric excess and secrete adipokines that result in the recruitment of additional pre-adipocytes, which differentiate into mature adipocytes as compensatory protection against some of the adverse metabolic consequences of obesity (168). This concept is supported by observations in AdipoChaser mice, a model for tracking adipogenesis (169). AdipoChaser mice fed a high fat diet display evidence of hypertrophy of visceral WAT within 1 month, while hyperplasia occurs after 2 months. Importantly, subcutaneous WAT does not undergo hyperplasia, and hypertrophy lags behind the visceral compartment, with evidence of subcutaneous WAT hypertrophy after 2 months of high fat feeding (170). However, when the capacity for adipocyte recruitment and hypertrophy is overwhelmed, fat accumulates in ectopic sites such as visceral depots, the liver, skeletal muscle, and pancreatic beta cells. These changes are accompanied by inflammation, insulin resistance and other features of the metabolic syndrome, and have been termed metabolically unhealthy obesity (MUHO) (171, 172). In contrast to MUHO, some people accumulate fat mainly in subcutaneous depots, a condition that has been termed metabolically healthy obesity (MHO). MHO is not accompanied to any great extent by insulin resistance, adipose tissue and systemic inflammation, and other features of the metabolic syndrome such as dyslipidemia and hypertension (173–176). Thus, the distribution of fat accumulation is a major determinant of metabolic complications associated with obesity, which can increase the risk of CVD. Various features that contribute to dysfunctional WAT in obesity will be discussed in the sections that follow.

Metabolically Healthy Obesity (MHO)

A sub-group of obese individuals remain insulin-sensitive, and exhibit normal metabolic and hormonal profiles despite having a BMI that would characterize them as obese (177, 178). Such individuals have been classified as having "metabolically healthy obesity" (MHO), and appear to be distinct from those with "metabolically unhealthy obesity" (MUHO) in that they remain insulin sensitive and do not have much adipose tissue inflammation or other features of the metabolic syndrome (179, 180). Therefore, MHO individuals have a lower risk for developing T2DM and cardiovascular disease (174). MHO is sometimes defined as having 2 or less features of the metabolic syndrome or based on homeostatic model assessment of insulin resistance (HOMA-IR) measures, but consensus on a precise definition does not exist (176). Thus, some individuals classified as having MHO rather fall somewhere between metabolically healthy and unhealthy. Moreover, individuals with so-called MHO can progress to develop features of the metabolic syndrome with time (181–184). Because CVD outcomes in general relate to the number of metabolic abnormalities present in individuals with MUHO (185–188), there is less CVD in individuals with MHO than those with the metabolic syndrome. In addition, while MHO individuals are so defined due to a healthier cardiometabolic profile than those with MUHO, the true clinical benefits of MHO remain in question, as the cardiometabolic profile and insulin sensitivity of MHO individuals typically does not improve significantly with weight loss (179, 189–192). Nevertheless, evidence from animal models and cultured adipocytes do suggest that the preservation of the capacity for subcutaneous WAT expansion mitigates extensive

visceral and hepatic fat accumulation, potentially driving the MHO phenotype (76, 97, 193).

Metabolically Unhealthy Obesity (MUHO)

Visceral Adiposity and the Metabolic Syndrome

Other obese individuals tend to accumulate fat mainly intra-abdominally in visceral depots, which is also known as central obesity. Visceral adiposity is associated with insulin resistance, a predisposition to diabetes, local and systemic inflammation, dyslipidemia [characterized by hypertriglyceridemia, a preponderance of small, dense low-density lipoprotein (LDL) particles and reduced high-density lipoprotein (HDL)-cholesterol levels], insulin resistance, dysglycemia [a broad term that refers to an abnormality in blood sugar stability], adipose tissue and systemic inflammation, hypertension, a thrombogenic profile and non-alcoholic fatty liver disease (NAFLD) (194). This constellation of CVD risk factors associated with visceral obesity is widely known as the metabolic syndrome and is a hallmark of MUHO, illustrated in **Figure 1**. Visceral obesity and the metabolic syndrome are associated with an increased risk of developing CVD, which is exacerbated when overt diabetes develops as a result of insulin secretion failing to adequately compensate for insulin resistance. Interestingly, even normal weight individuals who accumulate fat intra-abdominally have these metabolic abnormalities (195, 196), including an increased risk of CVD. Asians and Asian-Americans are particularly prone to accumulate intra-abdominal fat and have features of the metabolic syndrome despite having normal weights and BMI values by Western standards (196), raising the question of whether different normal values should apply to individuals of Asian ancestry. Moreover, this raises the question of the validity of body weight or body mass index (BMI -weight in kg/height in m^2) as an index of obesity or adiposity, since these measures do not differentiate the 2 major types of obesity. Measures such as waist circumference, waist/hip ratio and weight to height ratio have been used. These indexes are notable for their inclusion of upper subcutaneous WAT, which some consider to contribute as much, if not more, to metabolic syndrome than visceral WAT alone (197). CT scanning at the level of the umbilicus has been found to be useful but is expensive and not practical other than for research purposes at present. Lower body subcutaneous WAT does not correlate with risk factors for the metabolic syndrome, potentially due to a slower FFA turnover, higher levels of adipocyte hyperplasia, and lower levels of inflammation (198–201).

Notable differences in the adipokine profile between MHO and MUHO subjects have been reported, which could contribute to their respective risks for T2DM and CVD. Leptin has been shown to be higher in MUHO than MHO obese Chinese children in one study (202), but was not found to differ between adult groups in several other studies (203–205). By contrast, adiponectin has consistently been shown to be higher in subjects with MHO than in those with MUHO, despite both populations having lower adiponectin than metabolically healthy lean controls (203, 205–209). Resistin and FGF21 levels tend to be highest in the MUHO population (148, 208). Data on whether omentin levels differ between MHO and MUHO has been inconsistent, with one study suggesting that MUHO subjects have higher omentin levels than MHO subjects (210), and other suggesting the opposite, that omentin levels are negatively correlated with the metabolic syndrome (122, 211). Cytokines such as TNFα and IL-6 as well as the chemokines SAA and MCP-1 have been shown to be elevated in MUHO (208). These adipokine differences between subjects with MHO and MUHO are depicted in **Figure 1**.

White Adipose Tissue Inflammation

Macrophages and inflammation

Adipose tissue expansion in obesity is accompanied by inflammatory changes within adipose tissue, contributing to chronic low-grade systemic inflammation that is characterized as mildly elevated levels of circulating cytokines, chemokines, and acute phase reactants. In mice fed a high fat diet, obesity is associated with the induction of a large number of inflammatory pathways, constituting as many as 59% of total pathways that are differentially regulated (212). Expansion of adipose tissue depots during weight gain is accompanied by an infiltration of new inflammatory cells, the major one initially being macrophages. Reported to represent ∼5–10% of total cells within lean adipose tissue, macrophages in obese adipose tissue represent up to 60% of all cells present (213). These pro-inflammatory cells are recruited in response to chemokines such as monocyte chemotactic protein-1 (MCP-1) produced by hypertrophic adipocytes (213, 214). Studies in mice have demonstrated that most macrophages in obese adipose tissue are derived from circulating monocytes (213), although a small percentage appear to derive from proliferation of resident tissue macrophages (215). Resident macrophages that are present in normal adipose tissue express markers of "alternatively activated," or M2 macrophages such as the mannose receptor (CD206), macrophage galactose type C-type Lectin/CD301a/CLEC10A (MGL1), and arginase-1 (ARG1). These anti-inflammatory macrophages are believed to be responsible for maintaining tissue homeostasis (216). It remains unclear whether the derivation of adipose tissue macrophages is the same in human obesity.

Macrophage accumulation occurs to a greater extent in visceral than in subcutaneous adipose depots in both rodents and humans (217–220). Macrophages are seen in crown-like clusters, where they are thought to represent an immune response to dead and dying adipocytes (219). These recruited macrophages demonstrate a phenotypic switch from being anti- to pro-inflammatory, and develop some features similar to "classically activated," or "metabolically-activated" macrophages (MMe) (221–223). However, use of genetic markers show that these cells have significant differences from classical M1 macrophages and alternate nomenclatures have been suggested for these pro-inflammatory cells. Morris and Lumeng have divided adipose tissue macrophages into several populations based on cell surface markers and expression profiling (224). Using a proteomics approach, Kratz et al. showed that markers of classical activation were absent on ATMs from obese humans. Stimulation of macrophages with

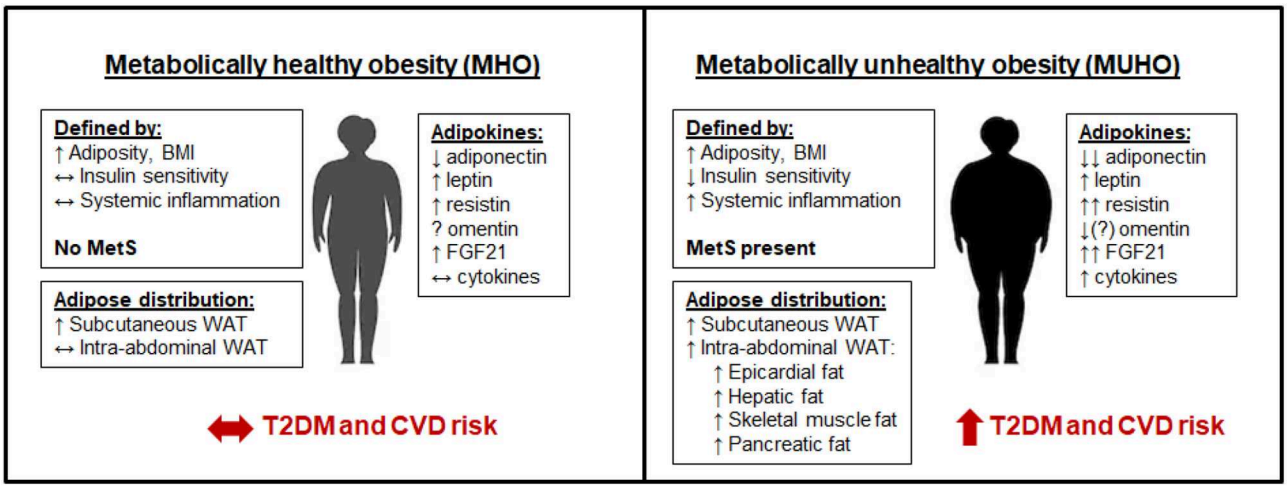

FIGURE 1 | Metabolically healthy obesity (MHO) vs. metabolically unhealthy obesity (MUHO). In comparison with lean metabolically healthy subjects, those with MHO have increased adiposity and BMI, but with reduced systemic inflammation and retained insulin sensitivity, thus defining them as not having metabolic syndrome (MetS). MHO subjects have elevated subcutaneous white adipose tissue (WAT) levels, without excessive accumulation of visceral fat. Their adipokine profile is similar to lean subjects, but with increased leptin, resistin, and FGF21, and decreased adiponectin, which limits their risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) in the short term. By contrast, those with MUHO exhibit elevated insulin resistance and systemic inflammation in addition to increased adiposity and BMI over lean controls, contributing to MetS. MUHO individuals have excess subcutaneous and intra-abdominal adipose tissue, with increased hepatic fat and fat distributed amongst other visceral organs. This leads to a dysfunctional adipokine profile, characterized by reduced adiponectin and omentin, with further elevated leptin, resistin, FGF21, and cytokines when compared to lean controls. Thus, MUHO subjects are at risk for developing T2DM and CVD.

glucose, insulin, and palmitate resulted in the production of a "metabolically activated" MMe phenotype distinct from classical activation. Such markers of metabolic activation were expressed by pro-inflammatory macrophages in adipose tissue from obese humans and mice and correlated with the extent of adiposity (225).

Other immune cells

In addition to macrophages, T-cells also are present in normal adipose tissue and demonstrate phenotypic change during weight gain. Both CD4+ and CD8+ T cells are found in adipose tissue and are increased in the obese state. Th2 cytokines (e.g., IL-4 and IL-13) are responsible for generating "alternatively activated" (M2) macrophages in lean adipose tissue. With weight gain in mice there is a shift away from a predominance of TH2 T cells present in lean adipose tissue and toward more TH1 and cytotoxic T cells as well as a reduction in regulatory T cells (Tregs) (226). Interferon γ (IFNγ)–expressing Th1 polarized T cells appear to promote adipose tissue inflammation and increased IFN-γ activity has been reported in adipose tissue in both mice and humans (227, 228). T-cell activation involves peptide antigen presentation via major histocompatibility complex (MHC) class II (CD4+) or MHC class I (CD8+). A subset of T cells called natural killer T (NKT) cells respond to lipid or glycolipid antigens (229–231). The number of invariant NKT (iNKT) numbers has been observed to be reduced in adipose tissue and livers from obese mice and humans (232–235). B-cells and mast cells also are increased in adipose tissue in the obese state (227, 236, 237). Use of specific cell surface markers has also demonstrated the presence of dendritic cells in adipose tissue, and studies indicate that dendritic cells are independent contributors to adipose tissue inflammation during obesity (238, 239).

Chronic inflammation in obesity

Adipose tissue inflammation in obesity differs from typical inflammatory responses employed in host defense in that it is chronic, sterile, low grade, and affects the metabolic control of nutrient flow in adipose tissue, liver, muscle and pancreas, and has been termed "meta-inflammation." One way it affects nutrient flow is by causing insulin resistance. There is good evidence to support the notion that the systemic inflammation that is associated with obesity and contributes to insulin resistance begins with adipose tissue inflammation. The regulation of hepatic C-reactive protein (CRP) and serum amyloid A (SAA) is likely in response to IL-6 secretion from visceral adipose tissue that directly targets the liver via the portal circulation (240–244). CRP is a prominent biomarker for insulin resistance and CVD (245–247), and SAA antagonizes insulin action in adipocytes, thus contributing to systemic insulin resistance (248). SAA also has been associated with CVD in some rodent and human models (218, 249–253). In summary, the discovery of elevated secretion of inflammatory cytokines by obese adipose tissue provides evidence that obesity directly mediates systemic inflammation, which contributes to insulin resistance and CVD (discussed further in later sections).

Cytokines and Chemokines

Obesity is associated with elevated circulating levels of IL-6 and TNFα, which are subsequently decreased with weight loss (254, 255). Adipose tissue is a major source of these cytokines (256) as well as the chemokine MCP-1, which is important

for recruitment of inflammatory cells such as macrophages to expanding adipose tissue (257). While such inflammatory mediators that originate from adipose tissue could technically be classified as adipokines, they are also produced by the majority of cell types in the body and will therefore be described in further detail in this section. It should be noted that cytokine and chemokine production is limited in lean adipose tissue and in subjects with MHO. Many cell types synthesize and secrete these cytokines and chemokines, including several that make up the adipose tissue milieu such as monocytes, macrophages, dendritic cells, B cells, and T cells. As such, they play a prominent role in adipose tissue pathophysiology associated with obesity.

IL-6

Much research has been devoted to the role that adipose-derived IL-6 plays in the etiology of obesity. The expansion of adipose tissue is accompanied by excessive adipocyte lipolysis and subsequently elevated FFA levels, which promotes adipocyte IL-6 secretion (258, 259). Omental fat produces 2 to 3-fold higher levels of IL-6 than subcutaneous fat (260), providing a potential mechanism for the higher contribution of omental WAT to insulin resistance (261). Most studies *in vitro* and in mice suggest that adipose-derived IL-6 promotes hepatic insulin resistance and glucose intolerance (259, 262, 263), while some indicate that in certain contexts IL-6 signaling in WAT and liver may be protective against metabolic disease (264, 265). For example, mice with genetic disruption of the IL-6 receptor specifically in the liver exhibit exacerbated hepatic inflammation and impaired glucose tolerance (264), suggesting that IL-6 may also function to limit hepatic inflammation. Thus, the context in which IL-6 signaling is studied is critically important for the interpretation of its function.

TNFα

In addition to its secretion from inflammatory cells such as monocytes and macrophages, TNFα was first described as an adipokine in 1993 (266). As with IL-6, TNFα levels positively correlate with adiposity, BMI, insulin levels, and insulin resistance (267, 268). While adipocytes themselves can secrete TNFα, the majority of TNFα secreted from adipose tissue is derived from immune cells in the stromal vascular fraction, and that obesity-associated increases in TNFα largely reflect the infiltration of pro-inflammatory macrophages within expending adipose tissue (213). One mechanism by which adipose-derived TNFα may promote insulin resistance is by directly activating hormone sensitive lipase (HSL), thereby increasing FFA release from adipocytes which promotes insulin resistance in the liver and skeletal muscle (269). Another mechanism is via autocrine activation of insulin receptor substrate-1 (IRS-1), which prevents insulin from interacting with its receptor (270).

MCP-1

Monocyte chemotactic protein-1 (MCP-1) is a potent chemotactic factor that promotes monocyte and macrophage recruitment into sites of inflammation during tissue injury and infection. It is secreted by adipocytes during the development of obesity and leads to infiltration of monocytes, which differentiate to become adipose tissue macrophages. The macrophages in turn secrete additional MCP-1 leading to further recruitment of inflammatory cells (271, 272). Body mass index and adiposity strongly correlate with adipose *CCL2* (the gene encoding MCP-1) expression levels, and MCP-1 decreases following weight loss in humans (273). *Ob*/*ob* mice, a commonly used mouse strain that spontaneously develops obesity due to leptin deficiency-induced hyperphasia, as well as diet-induced obese mice, display elevated levels of plasma Mcp-1 and *Ccl2* adipose tissue expression (213, 274, 275). In addition, mice engineered to express elevated levels of *Ccl2* specifically from adipocytes exhibit increased macrophage recruitment into adipose tissue, and subsequently increased insulin resistance, effects that were not observed in diet-induced obese mice that were deficient in *Ccl2* (274). Potential mechanisms by which adipose-derived MCP-1 could increase insulin resistance include changes in liver mRNA expression of genes involved in lipid and glucose metabolism in response to elevated FFA (274), or more likely due to increased recruitment of macrophages into adipose tissue (described in the section on "Obesity and insulin resistance"). Evidence suggests that human visceral WAT secretes higher levels of MCP-1 than subcutaneous WAT (276). These studies and others have prompted the suggestion that MCP-1 could be a viable therapeutic target for the treatment of obesity and associated insulin resistance.

Serum amyloid A (SAA)

While well-described as an acute phase protein secreted by the liver in response to pro-inflammatory cytokines, SAA is also expressed in adipocytes and macrophages and correlates with adiposity (244, 277–281). There are 4 subtypes of SAA: SAA1–4. SAA1 and SAA2 are highly upregulated in response to inflammation, while SAA4 is largely constitutively expressed. SAA3 is a pseudogene in humans, replaced by SAA1 and SAA2 in extra-hepatic tissues. While the best defined cell source of SAA1 and SAA2 is hepatocytes, SAA1 and SAA2 are also expressed from adipocytes and macrophages under inflammatory conditions in metabolic diseases such as obesity, insulin resistance, and cardiovascular disease (250). SAA3 expression is increased during hypertrophy of cultured mouse adipocytes (214) and in gonadal fat in obese mice (218, 282). Inducible forms of SAA also are expressed in both subcutaneous (277) and omental WAT (283) from obese humans. Thus, the increased adipocyte size and number that accompanies obesity is also associated with elevated adipose tissue-derived SAA levels, likely in part due to increased hepatic secretion in response to cytokines produced in adipose tissue.

Ectopic Fat

In obesity, white adipose tissue may become dysfunctional and unable to properly expand to store excess ingested energy, triggering storage of triglycerides in sites where the primary function is not fat storage. Ectopic fat that is localized to major glucose regulatory organs such as the liver, skeletal muscle, and pancreas is commonly regarded as being "lipotoxic," since this ectopic fat can interfere with normal insulin signaling and promote insulin resistance and increase the risk for T2D (284,

285). Excessive amounts of visceral fat also is considered to be a form of ectopic fat, and as noted earlier, is associated with features of the metabolic syndrome and an increased risk of T2DM and cardiovascular complications (286). In animal models as well as in humans, it has been shown that the accumulation of lipotoxic diacylglycerols (DAGs) and ceramide, as occurs with visceral obesity, leads to impaired insulin signaling and reduced glucose uptake in skeletal muscle and liver (287–290). More specific mechanisms by which ectopic fat accumulation in particular tissues promotes insulin resistance will be explained in the following sections.

Hepatic lipid accumulation and inflammation

Several studies have reported an inverse relationship between hepatic lipid content and whole-body insulin sensitivity (291–293). The liver is a major target for the excessively produced inflammatory cytokines and FFAs released from obese WAT (294) (see later). It has been estimated that nearly 60% of ectopic hepatic triglycerides in obese NAFLD patients derive from FFA released from adipose tissue (295). FFA-derived triglycerides accumulate in the cytoplasm of hepatocytes in the form of lipid droplets. While the lipid droplets may not be lipotoxic *per se*, various intermediate lipid moieties generated during triglyceride synthesis (e.g., DAGs and ceramide) have been shown to promote lipotoxicity and enhance hepatic insulin resistance (296), likely by inhibiting insulin signaling pathways (297, 298). Selective upregulation of ceramide degradation pathways in the liver has been shown to reverse hepatic lipid accumulation and improve glucose tolerance in diet-induced obese mice (299). Moreover, obesity-associated reductions in adiponectin have also been shown to contribute to hepatic steatosis, presumably by blunting hepatic fatty acid oxidation, a process regulated by adiponectin (300–302).

It also has been suggested that adipose tissue inflammation contributes to hepatic lipid accumulation. Kanda et al. showed that overexpressing *Ccl2* from adipocytes in mice led to macrophage accumulation in adipose tissue and subsequent hepatic steatosis and hepatic insulin resistance, without an obese phenotype (274). Similarly, mice in which *Ccl2* had been deleted showed resistance to high fat diet-induced insulin resistance and hepatic steatosis, an effect that was accompanied by reduced expression of TNFα in adipose tissue (274). Additional evidence to support the notion that adipose tissue inflammation promotes hepatic steatosis derives from studies showing that adipose-derived cytokines promote lipolysis of WAT stores (303, 304), thus increasing circulating FFA levels.

Kupffer cells are liver-resident macrophages, and reportedly comprise 80–90% of all tissue-resident macrophages in the body (305). In the healthy liver, the role of Kupffer cells is to phagocytose pathogens and toxins and to maintain tissue homeostasis and repair, akin to an M2 macrophage (306, 307). In contrast with adipose tissue macrophages, hepatic Kupffer cell numbers do not increase with adiposity, but instead become "activated," akin to M1 or MMe macrophages (308). The primary stimuli for Kupffer cell activation likely derive from dysfunctional adipose tissue, including FFA, cytokines, and adipokines (309). Adipokine imbalance such as the hypoadiponectinemia that results from visceral adipose tissue expansion fails to suppress hepatic inflammation and oxidative stress, contributing to Kupffer cell activation. Thus, signals from dysfunctional obese adipose tissue propagate hepatic inflammation by activating resident Kupffer cells, which then themselves secrete pro-inflammatory cytokines, further amplifying systemic inflammation (310).

Ectopic fat in skeletal muscle

Lipids also can be stored within skeletal muscle when the capacity for fat storage by WAT is exceeded (311). Lipids can be stored either between muscle fibers (as adipocytes, or extramyocellular lipids), or within muscle cells (cytosolic triglycerides, or intramyocellular lipids) (312). Pre-adipocytes have been identified within skeletal muscle, providing evidence that distinct adipocyte cells may reside between skeletal muscle fibers (313). There is an association between ectopic skeletal muscle fat and insulin resistance that is largely dependent on BMI, but this association persists when BMI is statistically accounted for (314–316). It remains to be determined whether skeletal muscle fat is simply a marker of metabolic dysfunction or if it plays an active role in mediating insulin resistance. Ectopic skeletal muscle fat, as with ectopic fat in other areas, has the potential to impair insulin action in skeletal muscle through the inhibition of insulin signaling by lipotoxic DAGs and ceramide (317, 318). Several large clinical trials including SECRET and CARDIA have recently suggested that skeletal muscle fat could play a direct role in increasing cardiometabolic risk (319–322). However, while ectopic fat in skeletal muscles is often associated with metabolic disease, highly trained athletes have been reported to have comparable amounts of skeletal muscle fat as subjects with T2DM, yet their tissue remains highly insulin sensitive (323). This phenomenon has been called "the athlete's paradox" and is likely due to the high energy demands of skeletal muscle in extremely fit athletes.

Ectopic fat in the heart

Obesity and T2DM are both independently associated with fat accumulation in the heart (324), rendering ectopic fat in the heart as a strong predictor of CVD (325, 326), particularly in subjects with T2DM (327). Similar to the liver, excess circulating FFA can also lead to increased triglyceride deposition in the heart. Cardiac tissue mainly utilizes FFA for metabolism, but when delivered in excess of basal myocardial fatty acid oxidation rates can also lead to the accumulation of lipotoxic products (328). In addition to ectopic cardiac myocyte lipid storage, excess FFA can be stored in epiWAT, pericardial fat (between the visceral and parietal pericardia), or PVAT (329). PVAT in particular has a major impact on vascular homeostasis. As a source of several vasoactive mediators, PVAT influences vascular contractility. Healthy PVAT is thought to be a largely anti-inflammatory tissue (330), with characteristics akin to BAT in the areas surrounding the thoracic aorta in particular (331). However, in the setting of obesity, dysfunctional PVAT releases predominantly vasoconstrictive and proinflammatory mediators that negatively influence vascular homeostasis (332–334). Similarly, epiWAT is a source of bioactive molecules that

negatively impact cardiac rhythm and perpetuate an atherogenic environment in obesity (335). Patients with T2DM express higher levels of the LDL and very low-density lipoprotein (VLDL) receptors in epiWAT than non-diabetic control subjects (336), suggesting that altered lipid metabolism in epiWAT could be associated with T2DM.

Ectopic fat in the pancreas

Mounting evidence suggests that excessive fat in the pancreas is associated with an increased risk of metabolic disorders, with reports that nearly 2/3 of the obese population has excessive pancreatic fat (337). Recent studies have connected ectopic pancreatic fat with β-cell dysfunction and T2DM (338–340), which in turn is associated with an increased risk of CVD. Therefore, lipotoxic lipid intermediates may also play a role in increasing the risk of CVD by elevating levels of pancreatic fat, thus leading to T2DM (341). In contrast to skeletal muscle, ectopic pancreatic fat is characterized mostly by adipocyte infiltration rather than intracellular lipid accumulation (342). The accumulation of fat in the pancreas also has been reported to accelerate acute pancreatitis due to increased levels of lipolysis and inflammation (343, 344).

Brown and Beige Adipose Tissue, Inflammation, and the Metabolic Syndrome

Compared with healthy lean controls, obese subjects display reduced BAT content, identified as tissue that actively takes up 2-[^{18}F]fluoro-2-deoxyglucose (FDG) (345). This reduction in active BAT mass appears to be more prevalent in visceral obesity (346, 347). Concurrently, individuals with detectable BAT activity display lower blood glucose, triglyceride and FFA levels, lower glycated hemoglobin (Hb1Ac) levels, and higher HDL cholesterol levels than people with no detectable BAT (348, 349). As discussed in other sections, BAT acts as an important "sink" for excess blood glucose and FFA disposal. Thus, loss of BAT function in association with obesity could contribute to the development of insulin resistance and hyperlipidemia. It has been shown that while cold exposure can activate BAT to a certain degree in obese subjects and those with T2DM, the levels of BAT activation achieved are substantially lower than in healthy lean subjects (350, 351). While BAT is largely resistant to the development of mild obesity-induced local inflammation, BAT inflammation becomes quite pronounced with stronger obesogenic insults (352). Such inflammation can directly upset the thermogenic potential of BAT by impairing its ability to take up glucose (described in more detail in later sections) (353, 354). Whether individuals who inherently possess less active BAT are more prone to obesity and facets of the metabolic syndrome or whether these pathological conditions themselves reduce BAT activity requires further investigation. Regardless, it is still widely believed that strategies that augment BAT or beige activity could represent viable therapeutics to combat metabolic syndrome (355, 356). Efforts to enhance BAT activation in humans consist of intermittent regular cold exposure, introduction of β_3-adrenergic receptor agonists, and exercise (29, 357). However, robust reductions in body weight in humans have not yet been shown to be clinically significant when BAT is activated (358), necessitating further mechanistic studies to elucidate whether BAT activation is a viable target for metabolic improvement in humans.

Whether BAT undergoes similar immune cell changes as WAT under obesogenic conditions is still not clear. In one study, BAT isolated from mice made obese by 13 weeks of high fat diet feeding displayed lower mRNA expression of inflammatory genes, lower immunostaining for macrophage markers F4/80 and CD68, and lower macrophage content by FACS analysis (331). However, subsequent studies have shown that BAT becomes inflamed in obese mice, with increased mRNA expression levels of inflammatory markers *Tnf* and *Emr1* (the gene that encodes the macrophage marker F4/80) (359–361). Such BAT inflammation reportedly lowers the thermogenic potential of this tissue (359), presumably due to increased local insulin resistance (360, 362), which could reduce the glucose and fatty acid oxidizing capacity of BAT.

Similar to BAT, beige adipocyte quantity and functionality appear to be sensitive to local inflammation. A study in which IkB kinase (IKK, an enzyme that is required for NFκB activation and subsequent inflammatory cytokine transcription) was inactivated in mice, not only blunted adipose tissue inflammation and body weight gain, but enhanced WAT browning (363). Similarly, inhibiting a major intracellular mediator of toll-like receptor 4 (TLR4) signaling, interferon regulatory factor 3 (IRF3), blunted WAT inflammation and augmented WAT browning (364). Moreover, it has been shown that the immune cell infiltration of subcutaneous WAT that accompanies obesity directly interferes with the differentiation and/or recruitment of beige adipocytes (365). Thus, accumulating evidence suggests that obesity-associated inflammation hinders the thermogenic and insulin sensitizing effects of both BAT and beige adipocytes.

OBESITY AND INSULIN RESISTANCE

Abundant evidence indicates that adiposity and adipose tissue inflammation are associated with insulin resistance, which refers to a reduced response to binding of insulin to its receptor in peripheral tissues such as adipose tissue and skeletal muscle. This differs from glucose effectiveness, which is uptake of glucose by peripheral tissues in an insulin-independent manner. Insulin inhibits hepatic glucose output and stimulates lipogenesis in the liver, both of which are reduced in the presence of insulin resistance. Such desensitization of insulin signaling pathways also inhibits glucose uptake in peripheral tissues and stimulates lipolysis in adipose tissue. To compensate for reduced insulin sensitivity, insulin secretion is increased in order to maintain euglycemia. If the pancreatic beta cells are unable to secrete sufficient insulin to compensate for the reduced insulin sensitivity (termed beta cell dysfunction), hyperglycemia will ensue, leading to glucose intolerance and eventually T2DM (366). While the precise mechanisms that lead to beta cell dysfunction are not completely understood, ectopic fat accumulation may contribute, as discussed earlier. Nonetheless, ample evidence suggests that excess adiposity and adipose tissue inflammation contribute to insulin resistance [reviewed in (64, 367)]. Many

studies have demonstrated that excess adiposity is correlated with insulin resistance in humans. Cross-sectional studies in men of European, Asian Indian, and American descent have shown that total, visceral, and subcutaneous adiposity, BMI, and waist circumference are all negatively associated with insulin sensitivity (368, 369). As noted earlier, adiposity, especially visceral adiposity, is characterized by adipose tissue inflammation.

Several hypotheses have been put forth to account for the relationship between adipose tissue inflammation and insulin resistance. These include production of pro-inflammatory cytokines by adipocytes and adipose tissue macrophages (discussed previously in the section on WAT Inflammation), excess FFA, decreased adiponectin, increased resistin and retinol binding protein, ceramide accumulation, and ectopic fat accumulation in liver and skeletal muscle (367).

Free Fatty Acids

It was initially hypothesized that excess adiposity promoted insulin resistance due to the accelerated release of FFA by obese adipocytes, which inhibit insulin signaling in liver and muscle due to excessive lipotoxicity and/or ectopic fat storage in these tissues (64), and also contribute directly to beta cell dysfunction (366). It has been shown that adipose tissue mass correlates with circulating FFA in obese humans, with a tendency for individuals with visceral adiposity to have higher FFA turnover (370–372). It has also been reported that individuals with T2DM tend to have elevated FFA levels over non-diabetic controls (373), an effect found to correlate more strongly with insulin sensitivity rather than obesity (374). Consistent with this, one study reported that FFA levels were lower in MHO subjects than those with MUHO (375). In addition to dysregulated energy metabolism, disruption of the endocrine function of obese adipose tissue has now been shown to contribute to insulin resistance, described in more detail below.

Adipokines

Adipocytes in obesity simultaneously secrete lower levels of adiponectin and elevated levels of cytokines and chemokines, such as TNFα, IL-6, MCP-1, and SAA. Not only is there evidence that such inflammatory cytokines contribute directly to insulin resistance in hepatocytes and myocytes (366), they also directly inhibit adiponectin production from adipocytes (376).

There is evidence that hypoadiponectinemia plays a role in obesity-associated T2DM (377–380). Subjects with T2DM exhibit reduced circulating adiponectin levels (379, 380); similarly, MHO subjects have higher circulating adiponectin than those with MUHO (206). Obese mice that are deficient in leptin ($Lep^{Ob/Ob}$ mice) that are engineered to overexpress adiponectin are protected from obesity-associated insulin resistance, despite having elevated adiposity (97). This may be explained by the nature of adipose tissue expansion in these transgenic mice, which had smaller, less inflamed adipocytes and less liver fat content. Similarly, administration of recombinant adiponectin improved glucose tolerance and insulin sensitivity in obese high fat diet-fed or $Lepr^{db/db}$ mice (377).

As discussed in earlier sections, FGF21 is a hormone produced by the liver as well as adipocytes that exerts insulin-sensitizing effects. However, recent evidence has paradoxically suggested an association between serum FGF21 levels and obesity-associated metabolic syndrome (145, 381). FGF21 levels have been reported to be 2-fold higher in MUHO when compared to MHO (148). Moreover, subjects with T2DM were reported to have significantly higher plasma levels of FGF21 than insulin-sensitive controls, with FGF21 levels positively correlated with BMI, HOMA-IR, and Matsuda index, suggesting a strong correlation with insulin resistance (157). Plasma FGF21 levels also correlated strongly with visceral, epicardial, hepatic, and skeletal muscle ectopic fat levels, measured using 64-slice multidetector CT scanning (157). Given that FGF21 has been shown to improve insulin sensitivity and promote negative energy balance (382, 383), some have suggested that obesity and associated metabolic syndrome represent an "FGF21-resistant" state (146). This conclusion was reached based on some observations that circulating FGF21 levels are increased in obesity, with lower FGF21 receptor expression levels on target tissues such as adipose tissue (146, 384). However, this notion has been challenged by evidence that obese subjects are equally responsive to pharmacological administration of FGF21 (384, 385). Thus, it has now been proposed that obesity-associated FGF21 is increased as a compensatory mechanism to preserve insulin sensitivity (386). As such, a clear role for adipocyte-derived FGF21 in obesity and associated metabolic syndrome is still lacking.

Adipose Tissue Plasticity

Evidence suggests that ineffective adipose expansion promotes local inflammation and an insulin resistant phenotype (387). However, sufficient adipogenesis and hyperplasia (i.e., the ability to distribute fat among newly differentiated adipocytes without the need for significant hypertrophy) mitigates such inflammation and subsequent insulin resistance (388). Thus, strategies to increase the recruitment of adipocyte progenitor cells to expand adipose tissue by increasing adipose cell numbers could be protective against the metabolic consequences of obesity.

A key structural and functional component of adipose tissue is made up of extracellular matrix (ECM) molecules, including collagen and proteoglycans such as versican and biglycan, among others (389). Adipose tissue makes large quantities of ECM during active remodeling, as would occur during WAT expansion in obesity (390–392). Obese animal models and humans with obesity and/or T2DM exhibit large increases in visceral WAT ECM content, which can contribute to the local inflammatory milieu (393, 394). To date, most studies of WAT ECM function have centered around collagen, which can form a scaffold that constrains adipocyte expansion due to mechanical stress (391, 392, 395). Targeting ECM components to release adipocytes from such constraints due to excessive ECM production could potentially alleviate the ectopic accumulation of fat that drives the metabolic syndrome.

Visceral Adipose Tissue vs. Hepatic Lipid Accumulation?

While the majority of adipose tissue in humans is localized subcutaneously (396), the volume of visceral adipose tissue is

believed to be a strong predictor of insulin resistance (397), independent from subcutaneous fat quantity (397, 398). The association between insulin resistance and visceral adipose mass is particularly striking in certain ethnic populations, with T2DM rates of 46.6% in Filipino, 14.7% rates in African American, and 9.8% rates in Caucasian populations (398), suggesting a strong genetic component. While visceral adiposity is positively associated with insulin resistance, there is evidence to suggest that it may not be a causal factor. Other conditions associated with visceral adiposity, such as hepatic fat content, may instead drive insulin resistance (292, 399). Some clinical studies have dissociated the glucose metabolic effects of visceral adiposity from hepatic lipid accumulation. In one such study, significant differences in insulin sensitivity in the liver, skeletal muscle, and adipose tissue were reported in obese human subjects who differed in hepatic lipid content, with no such differences observed in obese subjects who differed in visceral adiposity (291). Similarly, in a study in which obese subjects were matched for liver fat content, no differences in indices of glucose metabolism were noted (293). Insulin-sensitive MHO individuals tend to have lower visceral and intrahepatic fat accumulation than their MUHO counterparts (203, 400, 401), providing further evidence that these fat depots contribute to insulin resistance. Collectively, while visceral adiposity and hepatic fat content are both strongly associated with whole-body and tissue-specific insulin resistance, hepatic lipid accumulation may play a more direct role in negatively modulating glucose homeostasis.

Subcutaneous Adipose Tissue

Many studies have suggested that fat distribution is strongly associated with insulin resistance, with visceral adiposity being the strongest predictor of insulin resistance (198, 402, 403). While the detrimental effects of visceral and hepatic lipid accumulation on glucose metabolism are clear, it is also becoming increasingly appreciated that lower body subcutaneous adiposity may be metabolically protective (404–406). Large-volume liposuction of subcutaneous WAT has shown little to no metabolic benefit in human trials (407). Gluteofemoral adipose mass is positively associated with insulin sensitivity in humans, coupled with a slower rate of lipolysis and subsequent FFA release, lower levels of inflammatory cells and cytokines, and elevated adipokines such as leptin and adiponectin (404). Evidence from animal models has suggested that transplantation of subcutaneous WAT into the visceral cavity of recipient mice promotes less body weight and adiposity gain than transplantation with visceral WAT, resulting in greater insulin sensitivity in the liver and endogenous WAT (408). Taken together, a growing body of evidence suggests that adipose tissue and ectopic lipid distribution contribute to whole-body glucose homeostasis.

Brown and Beige Adipose Tissue

With the purported potential to improve glucose homeostasis, interest in BAT and beige adipose tissue as therapeutic targets has increased in recent years. Studies in rodents in which BAT is transplanted into diseased mouse models have shown that transplanted BAT improves insulin sensitivity, glucose metabolism, and obesity (409–411), likely mediated by batokine effects. While the predominant energy source that contributes to brown adipocyte heat production derives from fatty acids (412) (∼90%), with only ∼10% of energy derived from glucose, BAT is still regarded as having a strong impact on glucose homeostasis. As a highly metabolically active organ, BAT contributes to glucose clearance by taking up relatively large amounts of glucose from the circulation, thus reducing insulin secretion by pancreatic β-cells (413). Indeed, individuals that possess detectable BAT have lower fasting glucose concentrations than those without active BAT (414). Glucose disposal through activated BAT occurs by both insulin-dependent and insulin-independent mechanisms (415). For example, the cold exposure-mediated influx of glucose into active BAT has been suggested to be an insulin-independent process (416–418). However, as the insulin receptor is highly expressed in BAT tissue, it is considered to be one of the most sensitive insulin target tissues and thus an important organ for glucose disposal (413). BAT activation further enhances insulin signaling in BAT itself by augmenting insulin-independent glucose uptake associated with thermogenesis and glucose uptake due to insulin signaling. Thus, strategies that activate BAT and beige adipose tissue have the capacity to improve insulin resistance by clearing excess glucose (419–421).

LINKS BETWEEN OBESITY, INSULIN RESISTANCE, AND CVD

Obesity as a Risk Factor for CVD

Several pathologic conditions, including hypercholesterolemia and systemic inflammation, are hypothesized to drive atherosclerotic CVD. With a primary function of sequestering lipotoxic lipids and the known potential for chronic inflammation, obese adipose tissue has emerged as a potential player in the regulation of these atherogenic factors. Obesity has been officially classified as an independent risk factor for CVD by the American Heart Association since 1995, meaning that obesity treatment is likely to lower the incidence of CVD (422). As alluded to in previous sections, people with MHO are at a lower risk of experiencing cardiovascular events than people with MUHO (423), yet those without obesity are at a considerably lower risk for future events. Thus, even a moderate level of weight loss, if sustainable, could potentially lower the risk of adverse CVD events (120). However, some studies have shown that individuals with established CVD and heart failure with moderate degrees of obesity present a more favorable prognosis than those who are normal or underweight, a situation that has been termed the "obesity paradox" (424, 425). Possible reasons include confounding factors such as smoking and the presence of co-morbidities that are associated with lower body weights, or the use of BMI rather than measures of visceral obesity for most studies on the obesity paradox. Despite the obesity paradox in those with established CVD, the following sections will provide information regarding potential links between obesity T2DM and CVD. The

various features of adipose tissue depots, including ectopic fat, and how they contribute to T2DM and CVD are summarized in **Figure 2**. Notably, there are many similarities between adipose depot characteristics that contribute to both T2DM and CVD.

Adipose Depot-Specific Links With CVD

Visceral and Subcutaneous White Adipose Tissue

The accumulation of visceral fat in obesity is associated with the metabolic syndrome, its associated CVD risk factors, and an increased risk for clinical CVD (426). This distribution of WAT has been shown to have the greatest effect on CVD risk and mortality among patients with normal body weight (427). The risk of CVD in the metabolic syndrome has been considered to result from the presence of multiple CVD risk factors such as dyslipidemia (hypertriglyceridemia, an excess of small, dense LDL particles and reduced HDL-cholesterol levels), hypertension, dysglycemia, and a thrombogenic profile that have been reviewed elsewhere (428–430). However, there are several additional potential mechanisms by which visceral WAT might contribute directly to CVD that involve FFA, insulin resistance, and inflammation. Visceral WAT has higher lipolytic activity than subcutaneous WAT due to its having fewer insulin receptors, and thus is a significant source of FFA. Visceral-derived FFA can directly impact the liver via the portal vein, facilitating FFA uptake by the liver and subsequent hepatic insulin resistance. Similarly, excess FFA from visceral fat might directly impair lipid metabolism and lead to dyslipidemia, which increases CVD risk. In obese diabetic subjects, plasma FFA levels have been shown to be elevated compared to BMI-matched non-diabetic subjects (373), supporting the notion that insulin resistance further elevates circulating FFA levels. Moreover, the incidence of T2DM is nearly doubled in patients with the highest levels of FFA (90th percentile) when compared with subjects with the lowest FFA levels (10th percentile) (431). In one study, obese T2DM subjects who had undergone overnight fasting during pharmacological inhibition of lipolysis exhibited improved insulin sensitivity and glucose tolerance (432), providing further evidence for an inhibitory effect of FFA on insulin sensitivity.

The adipokine profile of visceral WAT also contributes substantially to its association with CVD risk. Obese visceral WAT primarily secretes inflammatory cytokines such as resistin, TNFα, IL-6, IL-1β, MCP-1, and SAA, with reduced levels of adiponectin (433). Plasma adiponectin levels are decreased in patients with CVD (434). Adiponectin is believed to contribute to CVD protection by several mechanisms, including the reduction of lipid levels, repressing expression of inflammatory mediators such as VCAM, ICAM, E-selectin, TNFα, and IL-6, and by acting directly on the heart to improve ischemic injury by activating AMPK and subsequently increasing energy supply to the heart (435–438). Adiponectin also stimulates endothelial nitric oxide synthase (eNOS), which maintains healthy vascular tone (439, 440). Thereby, adiponectin would play a protective role in the development of CVD. Conversely, leptin levels are positively associated with acute myocardial infarction, stroke, coronary heart disease, chronic heart failure, and left cardiac hypertrophy (441–445), although the reasons for this remain largely unknown. Leptin receptors are expressed in the heart, indicative of an important impact of direct leptin signaling (446). Resistin is positively associated with systemic inflammatory markers (447), upregulates endothelial expression levels of VCAM-1 and endothelin-1 (448) and promotes the proliferation of smooth muscle cells (449). Resistin also associates positively with coronary artery calcification levels, and negatively with HDL cholesterol (450). Thus, adipose-derived resistin levels could be used to predict the severity of coronary atherosclerosis (450). Similarly, cytokines and chemokines such as those secreted from obese visceral WAT can induce expression of endothelial adhesion molecules (451), recruit macrophages (452), increase thrombosis (453), and reduce vasoreactivity (454), and are positively associated with cardiovascular events (249, 455). While visceral WAT-derived cytokines are associated with these CVD-inducing processes, it is important to note that the direct contribution from visceral WAT is not currently known, as these are also secreted from other tissues.

As discussed in previous sections, in addition to cytokines and exclusive adipokines, WAT is also a source of FGF21. While the liver is considered to be the major source, adipocytes have also been shown to produce FGF21 to varying degrees in response to various stimuli. In addition to its associations with obesity and T2DM, FGF21 levels have also been associated with increased risk for CVD (456–460). Subjects with CVD that also had diabetes exhibited even higher levels of FGF21 (459), suggesting an important role in diabetes-accelerated atherosclerosis. In particular, FGF21 levels have been shown to positively correlate with hypertension and triglyceride levels, and to negatively correlate with HDL-cholesterol levels (461). One study by Lee et al. suggested that plasma FGF21 levels are associated pericardial fat accumulation (462), which suggests that ectopic fat could be a source of FGF21 in metabolic disease. Further studies are needed to discern whether adipocyte- or hepatic-derived FGF21 contribute to these effects. In stark contrast to these effects of physiological FGF21, pharmacological administration of FGF21 in humans and non-human primates reduces blood glucose, insulin, triglycerides, and LDL cholesterol, and increases HDL cholesterol (142, 463, 464). Thus, there is a disconnect between the physiological and pharmacological effects of FGF21 that requires further study.

Epicardial, Perivascular, and Brown Adipose Tissues

It is becoming increasingly clear that adipose tissue expansion contributes directly to obesity-associated cardiovascular disease risk (465). Obesity is accompanied by not only excess visceral adiposity, but also by excess epicardial and perivascular WAT (466). Due to their proximity to the heart, coronary arteries, and other major arterial blood vessels that are prone to atherosclerosis, it is not surprising that epiWAT and PVAT are important regulators of cardiac and vascular. The respective sizes of these adipose depots are associated with risk factors for the metabolic syndrome, including elevated visceral fat content, blood glucose, hypertension, systemic inflammation, insulin resistance, circulating LDL levels, mean arterial pressure, and atherosclerosis (19, 467–471), as well

Type 2 Diabetes Mellitus			Cardiovascular Disease	
Intra-abdonimal WAT:	**Treatments**		**Intra-abdonimal (IA) fat:**	**Treatments**
↑↑ TNFα, IL-6, MCP-1, SAA	M, G, S, AI, P		↑↑ TNFα, IL-6, MCP-1, SAA	M, G, S, AI, P
↑↑ Inflammatory macrophages	M, G, S, AI		↑↑ Inflammatory macrophages	M, G, S, AI
↓ Adiponectin, omentin	L, G, S, T, AI		↓ Adiponectin, omentin	L, G, S
↑ Leptin, resistin	S		↑ Leptin, resistin	S
↑↑ FFA	G, AI		↑↑ FFA	L, B, G
↑ TH1 and cytotoxic T cells				
↓ iNKT cells				
↑ ECM	L, M, G			
↑ Insulin resistance	L, B, M, G, S, T, AI, P			
Subcutaneous fat:			**Subcutaneous fat:**	
↑ TNFα, IL-6, MCP-1, SAA	M, S, AI, P		↑ TNFα, IL-6, MCP-1, SAA	M, S, AI, P
↑ Inflammatory macrophages	M, S, AI, P		↑ Inflammatory macrophages	M, S, AI, P
↓↓ Adiponectin	L, B, M, S, T, AI		↓↓ Adiponectin	L, B, S, AI
↑ ECM	L, M, AI		↑ ECM	AI
↓ Adipose plasticity			↓ Adipose plasticity	
Hepatic fat:			**Hepatic fat:**	
↑↑ TNFα, IL-6, MCP-1, SAA	L, B, G, S, AI		↑↑ TNFα, IL-6, MCP-1, SAA	L, B, G, S, AI
↑↑ Inflammatory Kupffer cells	G		↑↑ Inflammatory Kupffer cells	G, AI
↑ Insulin resistance	L, B, G		↑ CRP	L, G, S, AI
Heart and arterial fat:			**Heart and arterial fat:**	
↑ Cytokines and chemokines	L, B, M, AI		↑ Cytokines and chemokines	L, B, M, G, S, AI
↑ DAG, ceramides	L, B		↑ Factors promoting arrhythmia	
			↓ Vasoactive mediators	L, S, AI
			↓ Contractility	L, B, G, S
Pancreatic fat:			**Pancreatic fat:**	
↑ Inflammation	G		???	
Skeletal muscle fat:			**Skeletal muscle fat:**	
↑ DAG, ceramides	L, B		???	
Brown adipose tissue (BAT):			**Brown adipose tissue (BAT):**	
↓ Mass, metabolic function	M, G, S, AI		↓ Mass, metabolic function	M, G, S, AI
↑ TNFα, macrophages	S, AI		↑ TNFα, macrophages	S, AI
Dysbiotic gut:			**Dysbiotic gut:**	
↑ Systemic inflammation	L, G		↑ Systemic inflammation	L, G
↓ SCFA	L, B, M, T, P		↑ Hyperlipidemia	L, B

FIGURE 2 | Adipose depots and ectopic fat sites and their features that contribute to type 2 diabetes mellitus (T2DM) or cardiovascular disease (CVD). Features of intra-abdominal white adipose tissue (WAT), subcutaneous fat, hepatic fat, heart and arterial fat (inclusive of epicardial, pericardial, and perivascular fat), pancreatic fat, skeletal muscle fat, brown adipose tissue, and a dysbiotic gut that contribute to either T2DM or CVD. Arrows indicate changes in comparison with subjects without T2DM or CVD. The T2DM treatment strategies that have been reported to improve each adipose depot feature are listed under "treatments." Treatments: weight loss due to lifestyle changes (L); weight loss due to bariatric surgery (B); metformin (M); GLP-1 receptor agonists (G); SGLT-2 inhibitors (S); thiazolidinediones (TZDs, T); anti-inflammatory approaches (AI); microbiome modulation with pre- or pro-biotics (P).

as adverse cardiovascular events (472–475). The mechanisms behind these associations include increased secretion of pro-inflammatory cytokines, vasoactive factors, and vascular growth factors (476–478); increased release of lipotoxic FFA (479, 480); increased macrophage content (481); increased oxidative stress (482); and decreased secretion of adiponectin (483), which are triggered by obesity. In a prospective cohort of patients with aortic stenosis, a positive association between epiWAT volume and left ventricular mass was found (484), suggesting that in addition to changes in adipokine secretion, epiWAT could negatively influence cardiac function by placing a restrictive burden on the heart. Mechanisms by which PVAT influences CVD are more nuanced and complex. As an adipose depot that features some characteristics of both WAT and BAT, and with different functions depending on the anatomical location (i.e., thoracic vs. abdominal aortic PVAT), PVAT can play either a cardioprotective or a pathological role (24). As obesity progresses, PVAT can become dysfunctional in that it more resembles WAT, and contributes to a pro-inflammatory and lipotoxic microenvironment that promotes atherosclerosis (485). Similarly to healthy PVAT, BAT provides atheroprotection by serving as a protective "buffer" for the vasculature against lipotoxic FFA (486). However, BAT can become dysfunctional as obesity progresses, undergoing a phenotypic "whitening" switch

that promotes atherosclerosis (487). Thus, while PVAT and BAT play atheroprotective roles in healthy individuals, obesity promotes dysfunction of these depots, blunting this protective effect against CVD.

STRATEGIES FOR REDUCING T2DM AND/OR CVD RISK THAT IMPACT ADIPOSE TISSUE

Strategies for weight loss are multi-faceted, including combinations of diet and lifestyle modifications, pharmaceutical therapy, and various forms of bariatric surgery (488). While there is some debate over this, it is generally believed that small degrees of weight loss in MUHO obese populations can have a dramatic impact on cardiometabolic health (489, 490); thus, strategies that improve obesity are likely to also decrease risk factors for CVD. Similarly, CVD treatment strategies are centered around a combination of pharmaceutical use and lifestyle modifications, which also impact adipose tissue. In this section, we will describe the effects that various CVD treatment strategies have on adipose tissue metabolism and inflammation. How these treatment strategies impact the contributions of particular adipose depot features to T2DM and CVD are listed in **Figure 2**.

Weight Loss

Lifestyle Modifications: Dietary Changes

As most patients with T2DM and/or CVD are overweight or obese, weight loss often is the first strategy to reduce the severity of T2DM and/or CVD. Traditional methods prescribed for weight loss include restricting food intake and increasing energy expenditure. Despite a large number of fad diets that dictate particular proportions of dietary fat, protein, and carbohydrates to facilitate weight loss [summarized in (488, 489)], the simple fact remains that for weight loss to occur, energy balance *must* be negative. Thus, energy intake must be less than energy expended, which includes resting energy expenditure, physical activity, and the thermic effect of food.

It has been previously reported that for every kilogram of body weight lost due to dietary restrictions, visceral adiposity is reduced by around 2–3% (491). Subsequently, additional studies have shown that modest weight loss due to dietary changes in people with overweight or obesity is due to roughly equivalent fat lost from subcutaneous and visceral depots, while the addition of exercise leads to more weight loss from subcutaneous fat as well as loss of ectopic skeletal muscle fat (492–495). The loss of visceral fat is associated with reduced CVD risk factors, including reduced systemic inflammation, total cholesterol, LDL cholesterol, and triglycerides (493, 496), as well as reduced fasting glucose and insulin levels (496, 497). A *post-hoc* analysis from the Look AHEAD study showed that weight loss of ∼10% in overweight or obese subjects with T2DM yielded a 21% lower risk of the primary outcome (including CVD-related death, non-fatal acute myocardial infarction, non-fatal stroke, or hospital admission for angina) (498). As the subjects recruited for the Look AHEAD trial had T2DM, this and other *post-hoc* analyses suggest that weight loss in T2DM subjects also lowers the risk of CVD events (499, 500).

Lifestyle Modifications: Including Exercise

It is well established that aerobic exercise increases fuel mobilization from adipose tissue by increasing lipolysis and subsequent FFA mobilization, which ultimately decreases adiposity and adipocyte size (501–504). Such enhanced fuel mobilization is thought to be highest for visceral WAT (505). Several studies have shown that a high level of fitness (defined by a high activity level with maximal oxygen uptake) negatively associates with visceral adiposity (506–508), even in subjects with obesity and/or T2DM, suggesting that aerobic exercise contributes to a favorable adipose distribution profile that reduces the risk of metabolic syndrome. Hepatic fat is also mobilized and decreased following intense aerobic exercise (509). Studies in mice suggest that not only visceral fat mass is lost with regular exercise, but subcutaneous and brown fat mass are also diminished (510). As expected with fat loss, exercise is coincident with reduced plasma and adipose tissue leptin levels (511–516). The effects of exercise-induced fat loss on adiponectin levels are less clear, with some studies showing no changes in circulating adiponectin levels (517–519), some showing increased plasma adiponectin (520–522), and others showing increased subcutaneous WAT expression of adiponectin mRNA (523–525). A meta-analysis showed that pediatric subjects with obesity exhibit reduced resistin levels following aerobic exercise (526). Little is known about the impact of exercise on FGF21 in obese humans, but one study suggested that aerobic exercise training in obese women reduced circulating FGF21 levels (527). By contrast, studies in rodents have shown that circulating FGF21 levels are not altered by exercise in obese animals (528). Collectively, such exercise-induced changes to WAT distribution and adipokine secretion likely facilitate the observed improvements in insulin sensitivity and CVD risk factors observed with exercise.

While many studies have reported that exercise training increases subcutaneous WAT browning in rodent models of obesity (529–532), there is limited data to support this in humans. Many studies have shown that there is no effect of aerobic exercise training to recruit beige adipocytes in humans (533). However, one study compared subcutaneous WAT from lean, sedentary young men with age- and weight-matched endurance-trained men and reported no differences in beige markers such as *UCP1*, *PGC1A*, or *CIDEA* (534). Another study found evidence of subcutaneous WAT browning (i.e., increased *UCP1* and *CPT1B* expression) in overweight sedentary individuals that had undertaken a 12-week bicycle training program (535). There is some debate about what role brown or beige adipose tissue would play in exercise, if it indeed occurs. It is known that BAT and beige activity is increased when thermogenesis is required, and exercise is a highly thermogenic activity that raises core body temperature, so it is not immediately clear why exercise would increase BAT and/or beige activity. Exercise is known to activate the sympathetic nervous system, which also activates BAT to quickly release stored energy, so it is

possible that BAT activation is secondary to exercise-induced sympathetic activation (536). Nevertheless, further studies are needed to determine what role if any BAT and/or beige adipocytes play in mediating the metabolically beneficial effects of exercise.

Loss of adipose tissue mediated by dietary changes, exercise, liposuction, or bariatric surgery (discussed in the section on Bariatric Surgery) is accompanied by decreased markers of adipose tissue and systemic inflammation (537, 538). Weight loss achieved through calorie restriction and/or exercise resulted in decreased systemic IL-6, CRP, TNFα, MCP-1, soluble intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (273, 539–542). Fat loss by liposuction yielded similar changes in systemic inflammatory markers in one study (543), but did not improve plasma cytokine levels in another (407). The removal of visceral fat from Zucker diabetic fatty rats resulted in dramatic reductions in systemic cytokines (544); this suggests that removing visceral fat, rather than the subcutaneous fat that is routinely removed during liposuction, is more advantageous in terms of resolving inflammation. Many studies also have shown that weight loss following bariatric surgery leads to reductions in systemic inflammatory markers (545), with notable reductions in adipose tissue inflammatory cytokine and macrophage expression (546–548). However, some similar studies do not show improvements in adipose tissue inflammation following various weight loss modalities, such as bariatric surgery or very low-calorie diets (549–551). It has been suggested that pronounced weight loss over time can lead to improvements in adipose tissue inflammation that were not observed in the same subjects following acute moderate weight loss (552). This implies that adipose tissue inflammation during the initial stages of weight loss could be required for the pronounced adipose tissue remodeling required for fat loss (284, 553).

Medications Indicated for the Treatment of T2DM That Lead to Weight Loss

Metformin

Metformin is the most commonly prescribed medication to treat T2DM, particularly in subjects with obesity (554). Metformin has been proposed to lower blood glucose levels through suppression of gluconeogenesis in the liver, activation of AMP-activated protein kinase (AMPK), inhibition of the mitochondrial respiratory chain (complex 1), and by unknown mechanisms in the gut (555, 556). Thus, the precise mechanisms by which metformin lower blood glucose are complex and still evolving. While some diabetes medications have adverse effects on body weight, patients taking metformin often lose a small amount of weight [reviewed in (557)]. Studies in T2DM suggest that metformin may reduce body fat stores and promote a more metabolically healthy fat distribution (558–560). The effect of metformin on adiposity may be partially due to reported nausea and anorexic effects of the drug (561–563). Metformin has been shown to decrease visceral WAT mass, potentially by promoting fatty acid oxidation and/or adaptive thermogenesis (564). With much recent attention focused on BAT as a potential target for obesity treatment, it has recently been shown that BAT is an important effector organ in the glucose-lowering effects of metformin (565). Some studies have reported increases in omentin following metformin therapy, which could be due to visceral fat loss (566). Metformin also reduces hepatic steatosis through inhibition of ApoA5 and steroyl-CoA desaturase-1 (SCD1) which combine to limit *de novo* lipid synthesis, which is partially mediated by its actions on AMPK and liver X receptor (LXR) activity (567, 568). It also has been suggested that metformin reduces ECM remodeling that is dysregulated in obesity (see previous section on adipose tissue plasticity), and reduces lipogenesis (564).

In addition to the increasingly recognized anti-obesity effects of metformin, its ability to improve CVD risk is also becoming apparent (569). A recent meta-analysis suggested that metformin could contribute to a 16% decrease in all-cause mortality, but may also contribute to a 48% increased risk of stroke (570). The mechanism may include improvements in the lipid profile, such as mild reductions in plasma VLDL cholesterol and triglycerides with slight elevations in HDL cholesterol (571). In addition, metformin has been shown to have anti-inflammatory properties, reported to reduce circulating CRP and MCP-1, reduce NFκB activity, and to reduce advanced glycation end products (AGE) (572–576).

GLP-1 receptor agonists

Glucagon-like peptide-1 (GLP-1) is a peptide hormone that is continuously secreted at low levels during fasting by intestinal L cells. Consumption of a meal enhances GLP-1 secretion, which functions to reduce plasma glucose levels by stimulating insulin secretion from pancreatic beta cells. In addition, GLP-1 receptors are abundant in brain areas that control food intake regulation, such as the hypothalamus, where GLP-1 functions to reduce the drive to eat (577, 578). Thus, several GLP-1 receptor agonists have been developed to mimic the glucose-lowering and anorexic effects of GLP-1 to treat obesity and T2DM.

Liraglutide, a GLP-1 receptor agonist, has shown efficacy in not only glucose control, but also in promoting weight loss and reduced waist circumference based on results from the Liraglutide Effect and Action in Diabetes (LEAD) study (579–581). Liraglutide has also been shown to reduce total adiposity, and specifically visceral fat mass (582, 583). While initially described as being devoid of GLP-1 receptors (584), it has now been confirmed that adipocytes express the GLP-1 receptor (585, 586). Adipose tissue may therefore be an additional target for GLP-1 receptor agonists to promote adipose remodeling by unknown mechanisms. In addition to its effects on body weight and glucose metabolism, GLP-1 receptor agonists may also provide protection against CVD (587). The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial showed that liraglutide lowered the risk of myocardial infarction and non-fatal stroke among patients with T2DM that had high CVD risk (587). GLP-1 receptor agonist treatment has been shown to protect against atherosclerosis in animal models and in humans, potentially by lowering plasma lipids and by reducing circulating CRP and soluble ICAM-1 levels (588–590). Liraglutide, when administered in combination with metformin as indicated for the treatment

of T2DM, has been shown to reduce epicardial WAT volume with simultaneous increased omentin expression (591). Thus, liraglutide may provide cardioprotection through reduced levels of ectopic fat, lipids, and inflammation.

SGLT-2 inhibitors

Inhibitors of the sodium-glucose cotransporter 2 (SGLT-2) have been shown to reduce blood glucose levels in subjects with T2DM by enhancing urinary glucose excretion (592). The SGLT-2 inhibitor empagliflozin, alone and in combination with the GLP-1 receptor agonist liraglutide, has been shown to reduce CVD risk (593), as well as cardiovascular death to a greater extent than statins alone (488). Empagliflozin also is associated with decreased hypertension, reduced arterial stiffness, and decreased vascular resistance (594, 595). In both rodents and humans with non-alcoholic fatty liver disease, SGLT-2 inhibitors have been shown to reduce ectopic liver fat by blunting *de novo* hepatic lipogenesis (596–599), with reduced alanine transaminase (ALT) and aspartate transaminase (AST) levels (600), two markers of hepatic metabolic stress. Furthermore, empagliflozin is associated with weight loss in humans when administered in combination with other therapeutics, such as metformin, thiazolidinediones, and sulfonylureas (601–603). In rodents, SGLT-2 inhibitors have been shown to suppress high fat diet-induced weight gain and to markedly reduce obesity-induced inflammation in WAT, potentially by increasing fat oxidation and the recruitment of beige adipose tissue (604, 605). Thus, in addition to correcting hyperglycemia, SGLT-2 inhibitors can also impact adipose tissue physiology; whether this is through direct or indirect mechanisms remains to be elucidated.

Bariatric surgery

Bariatric surgical techniques, including Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy, are widely acknowledged to be the most effective treatment strategies for obesity, achieving relatively low levels of obesity remission (606). Within the first year of surgery, some patients experience the loss of around half of their adipose tissue mass (607), often with roughly equivalent losses from subcutaneous and visceral WAT (608, 609). As weight loss progresses, studies have shown that later weight loss is largely from visceral depots (610–612), an effect that correlates with the degree of diabetes remission (609). It has also been reported that ectopic skeletal muscle and pancreatic fat are reduced following bariatric surgery (610, 613, 614), which could contribute to improved glucose metabolism. Studies in humans have reported that subcutaneous adipocytes become smaller following bariatric surgery, resembling adipocytes from lean individuals, but that total adipocyte number remains unchanged (615, 616). Little is known regarding the size and number of visceral adipocytes, which are extremely difficult to sample from humans. As expected with reduced adipocyte size, leptin levels have been shown to decrease following bariatric surgery, while adiponectin has been shown to increase in some studies (617, 618), but not in others (549, 550). Whether changes in adipokine secretion are important for the sustained metabolic improvements following bariatric surgery or whether they simply reflect the adipose remodeling remain to be elucidated. However, it is worth noting that one study has shown that adiponectin levels are elevated only 2 weeks following bariatric surgery, before significant weight loss has occurred, suggesting that adipokine responses may be independent from weight loss (619).

Following bariatric surgery, obesity-associated systemic inflammation persists for as much as 1 month, as indicated by IL-6 and CRP levels (549, 550, 620). Some of this inflammation has been attributed to the surgery itself (545). However, by 6 to 12 months post-surgery, circulating IL-6, CRP, and MCP-1 are typically reduced below pre-surgery levels (548, 549, 620–627), an effect that may be due to fat loss. Importantly, it is not yet clear what effect weight loss due to bariatric surgery has specifically on adipose tissue inflammation. Some studies have reported reduced levels of adipose tissue inflammation following 15–17% weight loss mediated by bariatric surgery (547, 548, 628, 629), while others have shown no changes in adipose tissue inflammation following 7–37% weight loss (549, 550, 630). With insulin sensitivity being substantially improved in all of these studies, these latter studies present a potential disconnect between adipose tissue inflammation and insulin sensitivity that requires further study. However, it must be noted that the adipose tissue sampled in these studies was from subcutaneous depots, due to ease of sampling. Given that visceral WAT is more prone to inflammatory changes, it is possible that visceral WAT inflammation is more impacted by bariatric surgery than subcutaneous WAT.

Bariatric surgery has been shown to upregulate FGF21 in humans, an effect that appears to be specific to RYGB-induced weight loss, as this effect is not observed following weight loss due to caloric restriction or sleeve gastrectomy (631–635). Importantly, it is not known if such FGF21 derives from the liver or adipose tissue. One study has shown that increased FGF21 is associated with improved HOMA-IR in RYGB subjects, an effect that remains when adjusted for adiposity (634), introducing the possibility that elevated FGF21 levels serve to impact glucose homeostasis. Given that FGF21 has been shown to be elevated in obesity, and in particular in subjects with insulin resistance (634), the notion that FGF21 levels would become even further elevated following RYGB surgery, a procedure which rapidly improves insulin sensitivity, represents a paradox. It has been proposed that obesity-associated increased FGF21 levels reflect a "spill-over" from cells that are experiencing metabolic stress (636); however, this hypothesis does not explain the further increased FGF21 levels that accompany RYGB.

Various forms of bariatric surgery have been shown to evoke long-term benefits including sustained and considerable weight loss as well as rapid and sustained remission of T2DM and reduced risk of CVD-related mortality (489). A recent meta-analysis has estimated that on average, patients exhibit a 48% reduction in macrovascular events with a 79% reduction in mortality more than 5 years following bariatric surgery (637). Similarly, long-term follow-up (>17 years) post-surgery in the Swedish Obesity Study showed a 32% reduction in macrovascular complications in T2DM subjects, with 29% fewer myocardial infarctions and a 29% decrease in stroke incidence (638, 639). Bariatric surgery also is associated with improved hypertension, but not a reduced risk of incident hypertension

(640). Interestingly, the CRP reduction observed following bariatric surgery was most pronounced in subjects that regained the most insulin sensitivity (624), suggesting an important link between improved glucose metabolism and CVD.

Thiazolidinediones (TZDs)

TZDs are synthetic peroxisome proliferator-activated receptor gamma (PPARγ) activators that have been used to treat T2DM for decades (641–643). The ability of TZDs to improve insulin resistance is clear; however, TZDs also promote adipogenesis and subsequent weight gain (644–647), and are thus not popular choices among patients who don't want to gain weight, even if it is "metabolically healthy" weight gain. The mechanism for such improvements in insulin sensitivity in the face of weight gain appears to be through the induction of adiponectin by TZDs (648), which has known insulin-sensitizing properties as described above.

Activation of PPARγ by TZDs not only enhances adipogenesis, it also alleviates inflammatory cytokine secretion associated with obesity (649) and reduces ectopic fat deposition in tissues such as the liver and skeletal muscle (650). There appears to be a reciprocal relationship between inflammatory cytokines and adiponectin. For example, *in vitro* experiments in cultured adipocytes revealed that treatment with adiponectin reduces cytokine secretion (651, 652), while treatment with cytokines drastically reduces adiponectin expression and secretion (648, 653, 654). Due to greater adipose lipid storage potential, TZDs should therefore reduce plasma triglyceride levels, which appears to be the case for pioglitazone but not rosiglitazone (655–657). This may in part account for the beneficial cardiovascular effects of pioglitazone in a clinical trial (658).

Anti-Inflammatory Approaches

Characteristic features of MUHO and the metabolic syndrome include adipose tissue and systemic inflammation, which may play a role in the pathogenesis of atherosclerotic CVD. Therefore, an approach that inhibits inflammation would seem logical.

The CANTOS trial, in which CVD events were reduced using an IL-1β antagonist, canakinumab (659), was the first successful proof of concept study using an anti-inflammatory approach for the prevention of recurrent CVD events. A more recent study showed that colchicine, an old drug that has powerful anti-inflammatory properties, reduced recurrent ischemic events when administered after a myocardial infarction (660). Statins, which inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) to reduce LDL cholesterol levels, also have anti-inflammatory properties (661–664). Whether this anti-inflammatory effect of statins plays a role in the well-documented effect of statins in inhibiting clinical CVD events and CVD mortality (665, 666) is unknown. Even less is known about the effect of statins on inhibiting inflammation in adipose tissue, although statins have been shown to reduce epicardial fat accumulation (667). A clue to the potential role of statins in adipose tissue inflammation is provided by the recent demonstration that myeloid-specific deletion of HMG-CoA reductase improved glucose tolerance in obesity induced by a high fat diet, as a result of decreased macrophage recruitment into adipose tissue (668). These changes occurred independently of weight loss and provide impetus for further studies on the effect of statins on adipose tissue inflammation. Regardless, the effect of statins on adipose tissue inflammation is an area that warrants further investigation.

Modulating the Gut Microbiota

The trillions of bacteria that reside within our digestive tract, termed gut microbiota, play an important symbiotic role in shaping our metabolic health. The specific bacterial populations that inhabit our gut can have substantial metabolic impact in relation to obesity, as it is becoming increasingly recognized that that the gut microbiota may contribute to the pathology of obesity (669–671). Dysbiosis, or microbial imbalance in the body, has been associated with obesity in both humans and mice, and can be reversed with weight loss (672–675). Germ-free mice that do not possess gut microbiota are protected from diet-induced obesity and insulin resistance (676, 677), and the obesity phenotype can be conferred by transplantation of cecal contents from obese mice into lean germ-free mice (678), suggesting that the "obese microbiome" is sufficient to cause obesity. It is known that gut bacteria can influence distinct host organ systems indirectly and specifically through the release of particular microbial metabolites such as bile acids, short-chain fatty acids (SCFA), and others. Adipose tissue is a notable target of these microbial metabolites (679). As such, treatments that target the microbiome and modulate microbial metabolism could improve metabolic health.

There is growing evidence that gut dysbiosis can contribute directly to atherosclerotic CVD (669–671, 680). Gut microbial imbalance could modulate atherosclerosis by several mechanisms, including but not limited to: (1) promotion of metabolic endotoxemia due to decreased intestinal barrier integrity, leading to systemic inflammation; (2) altered cholesterol metabolism through the modification of bile acid metabolism, or (3) microbial production of specific beneficial or harmful metabolites with local and/or systemic activity, such as short-chain fatty acids (SCFA). These processes are described below.

Metabolic Endotoxemia

Gut dysbiosis has been associated with elevated intestinal permeability, or a "leaky gut" (681–683). Increased intestinal permeability allows inflammatory bacterial components to enter the systemic circulation to trigger an inflammatory response in diverse tissues such as the liver and adipose tissue. Obese mice and humans have been shown to exhibit gut dysbiosis (670), with increased proportions of endotoxin-producing gut bacteria and elevated circulating levels of lipopolysacharide that correlate with metabolic disease state such as obesity or T2DM (684, 685). Such metabolic endotoxemia is reduced following antibiotic treatment (681) or RYGB surgery-induced weight loss (627). Thus, a compromised intestinal barrier may contribute to systemic inflammation that is characteristic of obesity and CVD (686).

Bile Acid Metabolism

Gut dysbiosis contributes to dysregulated bile acid metabolism (687), leading to hyperlipidemia and hyperglycemia (688, 689). Bile acids produced by the liver facilitate the absorption of dietary fat in the small intestine, and are known to regulate lipid and glucose metabolism through the FXR (690, 691). FXR activation by bile acids initiates a negative feedback pathway, such that bile acid synthesis is inhibited when FXR is activated. Intestinal microbiota are capable of producing secondary bile acids from ∼5 to 10% of hepatic-derived bile acids that affect host physiology by serving as FXR agonists, thus resulting in a smaller bile acid pool due to the inhibition of primary bile acid synthesis (692, 693). Subjects with obesity and/or T2DM have been shown to have fewer plasma secondary bile acids in comparison to healthy control subjects (694), an effect that may reflect the altered gut microbial composition observed in obesity. Adipocytes express the major G-protein-coupled bile acid receptor, TGR5, and FXR (695–697), and obesity is accompanied by reduced FXR expression in WAT (697); thus, bile acid signaling to adipose tissue could play a role in modulating adipose tissue inflammation and/or lipid metabolism (698). Secondary bile acids have been shown to exert an anti-inflammatory phenotype in macrophages and hepatocytes (699–701). Bariatric surgery increases plasma bile acid concentrations before any significant weight loss has been achieved (702–704). Metabolic benefits from bariatric surgery, including weight loss and improved glucose metabolism, were absent in mice lacking the TGR5 receptor (705), suggesting an important role for bile acids in the metabolic improvements associated with bariatric surgery. Indeed, adipocyte TGR5 is required for adipogenesis and a metabolically healthy adipokine profile, including secretion of adiponectin and repression of inflammatory cytokines (706, 707). Similarly, deficiency of FXR promotes adipocyte dysfunction, exemplified by impaired adipogenesis, defective insulin signaling, and reduced lipid storage capacity (697). Collectively, these previous studies suggest that intact bile acid signaling is required for adipocyte homeostasis. Thus, equilibrium between dietary-intestinal- and microbiome-intestinal-derived bile acids is important for metabolic health associated with lipid metabolism. The gut microbiota composition and metabolism are therefore important contributors to metabolic health.

SCFA Metabolism

SCFA, including predominantly acetate, propionate, and butyrate, are produced in the gut to varying degrees, depending on the fermentable carbohydrate-based substrates available (i.e., dietary fiber quantity and type) and the particular bacterial populations that are present (i.e., microbiota composition). SCFA serve as signaling molecules to remote organ systems, with impacts on autonomic regulation of systemic blood pressure, systemic inflammation, and other cellular functions. Dysbiotic gut bacteria that is observed in metabolic pathologies such as obesity and T2DM has been characterized by taxonomic shifts that produce fewer SCFA, with notably less butrate produced in the gut (708–710). Evidence from pre-clinical models suggests that SCFA administration could improve metabolic disease states such as obesity, T2DM, and atherosclerosis (711–714). Adipocytes express high levels of key receptors for SCFA, including GPR43 (715). Genetic deletion of GPR43 from adipocytes results in spontaneous obesity, while overexpression of adipocyte GPR43 protects mice from obesity (713). As such, adipose homeostasis can be directly modulated by the gut microbial composition and subsequent SCFA profile. Health benefits of giving SCFA to obese rodents include weight loss (712), improved glucose metabolism, reduced inflammation (716–719), and reduced LDL-cholesterol (720, 721), among others.

Gut Inflammation/Adipose Tissue Cross Talk

The gut microbiota are now considered to be a distinct organ system with endocrine properties that can directly and profoundly modulate the host immune system (722, 723). Under healthy conditions, the commensal ("normal") gut microbiota play a prominent role in host homeostatic immunity, an essential function to limit the pathogenic potential of gut microbes, via innate and adaptive mechanisms (724, 725). When gut bacteria become dysbiotic, resulting immune deficiencies may contribute to the pathogenesis of obesity, T2DM, and CVD (726). The precise mechanisms by which gut microbiota modulate host immunity [reviewed in (709)] are beyond the scope of this review. However, some mechanisms by which microbial-derived metabolites can modulate adipose tissue function will be described herein. SCFA such as butyrate have been shown to dampen subcutaneous and visceral WAT inflammation by inhibiting NFκB activation (727, 728). Similarly, secondary bile acids negatively correlate with inflammatory pathways in WAT, suggesting an anti-inflammatory effect (729). Bacterial endotoxin, circulating levels of which increase during metabolic diseases that exhibit metabolic endotoxemia, readily promotes adipose tissue inflammation by activating toll-like receptor 4 (TLR4), which is highly expressed in adipocytes as well as macrophages (730). Thus, various metabolites produced by the gut microbiota are known to modulate adipose tissue inflammation directly through the circulation.

Probiotics, Prebiotics, and Synbiotics

Probiotics, prebiotics, and the combined synbiotics could provide an avenue for increased endogenous production of secondary bile acids and/or particular SCFA by modulating the composition of the gut microbiota. Probiotics are commercial preparations of live bacteria designed to be ingested, with the intention of colonizing the gut with the ingested bacteria, or at a minimum to confer a health benefit to the host. Prebiotics, on the other hand, are non-digestible dietary substrates designed to promote an abundance of gut-healthy bacteria, with inferred benefit to the host (731). Synbiotics are preparations that combine particular pre- and pro-biotics, as it is becoming clear that defined fiber substrates increase probiotic colonization efficiency. Pre- and pro-biotics and synbiotics are relatively inexpensive alternatives to conventional CVD medications, with fewer side effects (732). Mechanisms by which pre- and pro-biotic-mediated changes in the gut microbiota may improve adipose function are still emerging, but may include the promotion of an anti-inflammatory milieu (including reducing intestinal

permeability to decrease circulating endotoxins), enhancing fat oxidation, recruitment of beige adipocytes, increased energy expenditure, and improved lipoprotein profile, which collectively could improve insulin sensitivity and reduce ectopic fat to combat T2DM and CVD (733–739). While it is generally accepted that particular pre- and pro-biotics reduce diet-induced weight and adiposity gain in animal models (736, 740–743), human intervention studies to date showing efficacy of probiotic treatment are still emerging (744, 745), warranting further study (746).

CONCLUDING REMARKS

Obesity results in many changes to adipose tissue, including adipocyte hypertrophy and hyperplasia, infiltration of inflammatory cells, changes in the ECM, and altered adipokine secretion patterns. A critical determinant of whether obesity is likely to lead to metabolic complications such as insulin resistance, the metabolic syndrome, T2DM and CVD is the site where adiposity increases, particularly intra-abdominal, epicardial and perivascular depots, as well as other ectopic sites such as liver, skeletal muscle and pancreas. Ectopic fat accumulation at these sites demonstrate different metabolic, adipokine, and inflammatory profiles from excess white adipose tissue that accumulates subcutaneously, which is predominantly in a lower body distribution and contributes to a less unhealthy form of obesity. Several mechanisms by which these metabolic and inflammatory changes to different adipose tissue depots could influence the metabolic syndrome and its downstream consequences are potential targets for intervention. Various strategies for the treatment of T2DM and/or CVD, including lifestyle- and surgically-mediated weight loss as well as pharmacological or naturopathic methods, also have notable impacts on adipose tissue, which are important to consider.

AUTHOR CONTRIBUTIONS

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REVIEW
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Receptor for Advanced Glycation End Products (RAGE) and Mechanisms and Therapeutic Opportunities in Diabetes and Cardiovascular Disease: Insights From Human Subjects and Animal Models

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Obesity and diabetes are leading causes of cardiovascular morbidity and mortality. Although extensive strides have been made in the treatments for non-diabetic atherosclerosis and its complications, for patients with diabetes, these therapies provide less benefit for protection from cardiovascular disease (CVD). These considerations spur the concept that diabetes-specific, disease-modifying therapies are essential to identify, especially as the epidemics of obesity and diabetes continue to expand. Hence, as hyperglycemia is a defining feature of diabetes, it is logical to probe the impact of the specific consequences of hyperglycemia on the vessel wall, immune cell perturbation, and endothelial dysfunction—all harbingers to the development of CVD. In this context, high levels of blood glucose stimulate the formation of the irreversible advanced glycation end products, the products of non-enzymatic glycation and oxidation of proteins and lipids. AGEs accumulate in diabetic circulation and tissues and the interaction of AGEs with their chief cellular receptor, receptor for AGE or RAGE, contributes to vascular and immune cell perturbation. The cytoplasmic domain of RAGE lacks endogenous kinase activity; the discovery that this intracellular domain of RAGE binds to the formin, DIAPH1, and that DIAPH1 is essential for RAGE ligand-mediated signal transduction, identifies the specific cellular means by which RAGE functions and highlights a new target for therapeutic interruption of RAGE signaling. In human subjects, prominent signals for RAGE activity include the presence and levels of two forms of soluble RAGE, sRAGE, and endogenous secretory (es) RAGE. Further, genetic studies have revealed single nucleotide polymorphisms (SNPs) of the *AGER* gene (*AGER* is the gene encoding RAGE) and *DIAPH1*, which display associations with CVD. This Review presents current

knowledge regarding the roles for RAGE and DIAPH1 in the causes and consequences of diabetes, from obesity to CVD. Studies both from human subjects and animal models are presented to highlight the breadth of evidence linking RAGE and DIAPH1 to the cardiovascular consequences of these metabolic disorders.

Keywords: diabetes, obesity, cardiovascular disease, peripheral arterial disease, RAGE, DIAPH1

INTRODUCTION

We reported the discovery of the receptor for advanced glycation end products (RAGE; gene name is *AGER*) in 1992 on account of this molecule's ability to bind the products of non-enzymatic glycation and oxidation of proteins/lipids, the advanced glycation end products, or AGEs (1). AGEs are not solely biomarkers of a hyperglycemic and pro-inflammatory/pro-oxidative state; rather, they also play mediating roles in the pathogenesis of diabetic complications, in large part through their interactions with RAGE. Various AGEs are also generated in highly heated and processed foods (2). Hence, AGE interaction with RAGE ensues both from endogenously-formed AGE adducts, as well as from dietary AGE sources. RAGE is expressed on multiple types of cells, such as vascular cells, immune cells, neurons, cardiomyocytes, adipocytes, glomerular epithelial cells or podocytes, lung epithelial cells, and a wide range of transformed cells, both in animal models and human subjects (3–5).

The pivotal discovery in the biology of RAGE was the finding that RAGE bound a diverse series of ligands beyond AGEs, such as members of the S100/calgranulin family, high-mobility group box 1 (HMGB1), lysophosphatidic acid (LPA) and oligomeric forms of amyloid beta peptide (Aβ) and islet amyloid polypeptide (IAPP) (6–10). These ligands bind to the extracellular domains of RAGE in a heterogeneous manner; although the extracellular V-type immunoglobulin (Ig) domain binds to many of the ligand families, the binding sites on the V-domain are multiple and spatially distinct. Further, ligands may also bind at the extracellular C1 and C2-type Ig domains, thereby further diversifying the complexity of the RAGE-ligand interactions (11–14).

Soluble forms of the receptor have also been described (15). Identified as "sRAGEs," these forms of RAGE have been found in plasma, and in other fluid compartments, such as synovial fluid, cerebrospinal fluid, and bronchoalveolar lavage fluid, as examples (15–18). There are two major forms of sRAGE that result from distinct mechanisms. Most of the circulating sRAGE results from cell surface-cleavage of the full-length receptor by species such as matrix metalloproteinases (MMPs) and a disintegrin and metalloprotease domain-containing protein 10 (ADAM10) (19). G-protein coupled receptor (GPCR) activity has also been linked to the production of sRAGE (20). The other form of sRAGE, known as endogenous secretory or esRAGE, represents a less prevalent form of the sRAGE in plasma and is a product of a splice variant of *AGER* (21).

In the absence of endogenous kinase activity, the means by which the RAGE cytoplasmic domain signals and impacts transcriptional programs and cellular functions remained elusive until the discovery that this RAGE intracellular domain binds the formin, Diaphanous1 (DIAPH1), and that this interaction is essential for RAGE signaling in multiple cell types (22). The cytoplasmic domain of RAGE, particularly through its amino acids R366/Q367, binds to the formin homology 1 (FH1) domain of DIAPH1; mutation of these amino acids to alanine residues or knock-down of *Diaph1* results in loss of this binding and loss of RAGE ligand (but not non-RAGE ligand)-mediated signaling in smooth muscle cells (SMCs) and transformed cells, respectively (22, 23). Others, using super-resolution stochastic optical reconstruction microscopy (STORM) and single-particle tracking (SPT), independently confirmed the interaction of the cytoplasmic domain of RAGE with DIAPH1 (24).

In vivo, DIAPH1 has been linked to numerous *in vivo* settings in which RAGE ligands and RAGE have been implicated, such as neointimal expansion after vessel injury, hypoxia-mediated damage, myocardial ischemia, diabetes-associated nephropathy, cancer, responses to infection (such as *Listeria monocytogenes*), and immune/inflammatory responses (25–33). Key downstream effectors of DIAPH1 relevant to cellular perturbation include activation of pathways such as the following: RhoGTPases, such as CDC42, RAC1, and RHOA; glycogen synthase kinase3β (GSK3β) and AKT; Rho-associated, coiled-coil-containing protein kinase (ROCK), Serum Response Factors (SRF); and SRF-dependent genes, such as *Egr1*, *Tagln,* or *c-fos* (25–33).

In the sections to follow, recent findings linking RAGE to both the pathogenesis and complications of diabetes, particularly in the setting of cardiometabolic dysfunction and disease, will be discussed. Recent developments in the generation of a novel class of RAGE/DIAPH1 antagonists will be presented, as well as opportunities for biomarking cardiometabolic disease through the lens of the RAGE signaling pathway in human subjects.

CVD, DIABETES, AND RAGE/DIAPH1

In both types 1 and 2 diabetes (T1D, T2D), CVD remains a leading cause of morbidity and mortality (34–36). When diabetes is combined with MI or stroke, the mortality rate for affected patients is nearly doubled, leading to an estimated reduction in life expectancy of ∼12 years (37). Beyond management of lipids and blood pressure and modulation of life style, major gaps in the therapeutic armamentarium in diabetes and CVD still exist, underscoring the critical need for disease-modifying therapies for these disorders. To follow is a review of common manifestations of CVD and the links to the RAGE/DIAPH1 pathway.

Atherosclerosis

Numerous studies have illustrated that RAGE is expressed in both non-diabetic and diabetic atherosclerotic lesions in human subjects, but that the expression is higher in diabetes and co-localizes with markers of lesional oxidative and inflammatory stress (38, 39). An ever-growing series of published work associates RAGE with atherosclerosis, both in human subjects and in animal models.

Studies in Human Subjects

Levels of sRAGEs have been extensively studied in human subjects to test associations of the RAGE pathway to diabetes and CVD. In a study of T1D subjects and healthy control subjects studied at baseline (age 8–18 years) and after 5 years of follow-up, levels of sRAGE and esRAGE declined with aging, in a manner independent of sex, diabetes, or pubertal stage. In the diabetic subject group, the levels of sRAGE and esRAGE were positively associated with carotid intima-media thickness (IMT) and baseline sRAGE was negatively associated with levels of C-reactive protein (CRP) at the follow-up testing (40). The authors concluded that high levels of baseline sRAGE might protect from inflammation 5 years later, but no protection from abnormalities of arterial stiffness or wall thickness was noted (40).

Recent studies have probed if levels of sRAGE in patients with metabolic dysfunction but without diagnosed diabetes provided surrogate markers for incipient atherosclerosis. Levels of esRAGE were examined in non-diabetic subjects with metabolic disease, in whom 1-h glucose tolerance testing (GTT) revealed a high serum post-glucose load level of $\geq$155 mg/dl. In these individuals, lower levels of esRAGE and higher levels of RAGE ligand S100A12 were observed vs. control subjects, in whom 1-h post-glucose load level was <155 mg/dl, in parallel with increased pulse wave velocity (PWV) and carotid IMT (41). These data suggested heterogeneity of metabolic dysfunction among subjects within normal limits of glucose tolerance, which might be linked to the RAGE pathway. In a separate study, subjects without a previous history of diabetes were stratified into three groups: controls, pre-diabetes, and new-onset T2D. The prediabetic subjects displayed lower levels of esRAGE and higher levels of S100A12 compared to controls; in the subjects with lower esRAGE, peripheral blood mononuclear cells (PBMCs) demonstrated lower levels of the esRAGE splice variant, suggesting that the lower systemic levels of esRAGE could be accounted for, at least in part, by lower transcription of this splice variant (42). Statistical analyses revealed that age, glycosylated hemoglobin and esRAGE were the major determinants of IMT and levels of S100A12 and blood pressure (systolic) were the main determinants of PWV (42).

In a 3-year longitudinal study of 1,002 subjects with CVD, 933 underwent testing for sRAGE levels, which were then segregated by quartiles. After 3 years follow-up, 16% of the subjects demonstrated a new CVD event (MI, stroke, and CVD death). The patients with the highest quartile of sRAGE displayed the highest incidence of recurrent CVD events, even after correction for confounders for CVD (43).

Collectively, these recent studies add to a large body of reports on the relationship between sRAGEs and diabetes and CVD and suggest the following insights: (1) High levels of sRAGEs may be protective, at least in early stages of disease or, perhaps, in periods of active exacerbation of acute CVD events; and (2) even after the discernment of early metabolic vulnerability subsets in subjects without diagnosed diabetes, the levels of sRAGEs may align with markers of CVD risk. These considerations underscore that long-term prospective studies in subjects without and with varying degrees of metabolic dysfunction are required to fully test if the levels of sRAGEs, including both sRAGE and esRAGE, correlate with CVD predilection, first events and recurrent events.

Studies in Animal Models

Early *in vivo* studies in animal models of diabetes and atherosclerosis were performed in mice devoid of *Apoe* and rendered T1D-like with streptozotocin; these mice developed accelerated atherosclerosis in the hyperglycemic state (44). Daily treatment with recombinant sRAGE (by intraperitoneal injection) resulted in a reduction in the development of accelerated atherosclerosis in diabetic mice devoid of *Apoe*, without effects on levels of glucose or lipids. In a distinct study, treatment of diabetic mice devoid of *Apoe* with established atherosclerosis with sRAGE resulted in halting the progress of diabetic atherosclerosis (45). In mice devoid of *Apoe* or *Ldlr*, and in transgenic mice expressing cytoplasmic domain-deleted RAGE [in endothelial cells (ECs)] or in mice with global genetic deletion of *Ager*, significant attenuation in atherosclerosis, irrespective of the diabetic state, but particularly in animals with hyperglycemia, was observed (46–48). Studies using transgenic mice in which the cytoplasmic domain of RAGE was deleted in ECs revealed prominent roles for EC RAGE in endothelial function and signal transduction. RAGE ligands specifically upregulated inflammatory markers, such as Vascular Cell Adhesion Molecule 1 (VCAM1) in ECs from wild-type aorta, but not in ECs lacking the RAGE cytoplasmic domain (46). Bu and colleagues analyzed the transcriptome of the aortas of mice devoid of *Apoe* with or without simultaneous deletion of *Ager* in the T1D state. A significant RAGE-dependent modulation of the ROCK1 branch of the TGF-β signaling pathway in SMCs was uncovered in these analyses, suggesting that SMC RAGE contributed importantly to diabetic atherosclerosis in mice through ROCK1 signaling (47).

Key roles for myeloid *Ager* in diabetic atherosclerosis were also uncovered through bone marrow transplantation studies (49). In macrophages, RAGE ligand-RAGE interaction significantly attenuated cholesterol efflux to APOA1 and HDL and downregulated the cholesterol transporters *Abca1* and *Abcg1*, at least in part through PPAR-γ-dependent regulation of these transporters in both murine bone marrow derived macrophages (BMDMs) and human THP1 cells (50). Beyond genes regulating cholesterol efflux, significant attenuation of vascular inflammation was observed upon deletion of *Ager* in diabetic atherosclerotic mice.

Vascular Calcification

Diabetes is associated with significant acceleration of vascular calcification, due at least in part to the pathogenic effects of hyperglycemia and oxidative stress (51–54).

Studies in Human Subjects

The ligand-RAGE axis has been explored in vascular calcification in human subjects. In 199 patients on hemodialysis in whom vascular calcium scores were obtained (49.2% of the subjects had diabetes), circulating levels of sRAGE were negatively associated with calcium score independent of the level of S100A12 and inflammatory markers (55). In non-diabetic subjects undergoing hemodialysis, levels of esRAGE were significantly lower than those of control subjects and correlated negatively with the degree of aortic calcification (56). In SMCs isolated from the saphenous veins of patients undergoing coronary artery bypass grafting (CABG), exposure to high levels of glucose resulted in NADPH oxidase- and Protein Kinase C-dependent translocation of HMGB1 to the nucleus, which increased calcification through an NF-κB-dependent regulation of bone morphogenetic protein 2 (BMP2) (57). Consistent with roles for RAGE ligands in these processes, exposure of vascular SMCs to AGEs increased calcification, at least in part through activation of p38 mitogen-activated protein kinase (MAPK) (58, 59). Collectively, these considerations suggest that RAGE signaling contributes to vascular calcification in diabetic and non-diabetic settings, presumably on account of the generation of RAGE ligands such as AGEs and other pro-inflammatory/pro-oxidative ligands in conditions such as advanced renal disease.

Studies in Animal Models

Studies in mouse models using various means to facilitate calcification underscored roles for RAGE in these processes *in vivo*. Mice devoid of *Apoe* or mice devoid of *Apoe* and *Ager* were subjected to either chronic kidney disease (CKD) or sham surgery and subsets of these animals were fed a high phosphate diet. After 12 weeks of CKD, RAGE ligands AGEs, and S100/calgranulins were increased in the serum of the *Apoe* null mice with a significant increase in *Ager* mRNA in the CKD vessels vs. controls. Vascular calcification was increased in the CKD *Apoe* null mice, in parallel with increased expression of *Runx*, which was lower in mice devoid of *Ager* (60). *In vitro,* stimulation of SMCs with RAGE ligand S100A12 stimulated mineralization and osteoblast transformation, which was inhibited by *Ager* deletion in these cells (60). In other studies, direct mediating roles for S100/calgranulins in the pathogenesis of vascular calcification were illustrated by studies in which transgenic mice overexpressing RAGE ligand S100A12 subjected to CKD demonstrated increased vascular calcification through NADPH oxidase-dependent mechanisms (61).

In valvular calcification (mitral valve and aortic valve) associated with CKD, an upregulation of FGF23 in the heart and vascular tissues was observed selectively in S100 transgenic mice with CKD but not in CKD wild-type or CKD *Ager* null S100 transgenic mice, thereby implicating S100/RAGE in upregulation of FGF23 and pro-inflammatory factors contributing to vascular calcification (62).

In summary, the accumulation of RAGE ligands in both diabetes and non-diabetes states of CKD exacerbates vascular calcification, at least in part through RAGE. The apparently potent mediating influence of the S100/calgranulins, demonstrated directly through the study of S100-transgenic mice, underscores the multi-ligand contributions of the RAGE pathway to CVD and calcification.

Peripheral Arterial Disease

Peripheral arterial disease (PAD) is increased in patients with T2D and contributes to amputations and substantial morbidity and mortality in affected subjects (63, 64). Unlike cardio- and cerebrovascular disease, PAD is not fully explained by traditional risk factors, perhaps on account of the fact that endothelial, neuropathic and immune/infection-related perturbations also contribute importantly to this disorder and the frequent accompaniment of impaired wound healing (65).

Studies in Human Subjects

Accumulating evidence links the ligand-RAGE pathway to the pathogenesis of PAD (66). RAGE ligands S100A12 and carboxymethyllysine (CML)-AGE are elevated in the circulation of subjects with PAD vs. control subjects (67) and, interestingly, in the infrainguinal vein tissue used for vascular grafting in this disorder, the proportion of the tissue stained for AGE, CML, RAGE, and S100A12 was similar in patients with and without diabetes (67), suggesting that non-glucose-related factors also led to the recruitment of the RAGE pathway in PAD. Examination of levels of sRAGE in patients with CAD and/or PAD revealed that the lowest overall levels were observed in patients with both disorders (68) vs. either disorder alone, suggesting that the factors mediating disease in both areas differed, at least in part.

In a population-based cohort study, levels of S100, RAGE ligands, and esRAGE (collectively referred to as RAGE score) were examined in 106 subjects with PAD with and without amputation. The authors reported that higher levels of plasma S100A12 and the overall RAGE score were associated with shorter amputation-free survival in T2D patients, suggesting that the RAGE pathway contributed to the severity of PAD (65).

Studies in Animal Models

These concepts have been tested in animal models of PAD, using unilateral hind limb ischemia as a means to introduce ischemic injury to the peripheral vascular system. In those studies, key endpoints include angiogenesis responses and detection of blood flow by Laser Doppler Imaging techniques. In a mouse model of hind limb ischemia, using multimodal imaging with molecularly targeted nanoparticles, a significant increase in RAGE expression accompanied hind limb ischemia vs. the sham limb (69). Others showed that RAGE imaging (using an anti-RAGE antibody fragment) was enhanced in diabetic vs. non-diabetic hind limb ischemia (70).

Global deletion of *Ager* and administration of anti-RAGE antibodies have been shown to improve angiogenesis and blood flow recovery in diabetic mice; in mice globally devoid of *Ager*, the beneficial effects were observed in hind limb ischemia induced in both diabetic and non-diabetic animals (71–73).

The model of hind limb ischemia underscored interesting distinctions vis-à-vis RAGE signaling in atherosclerosis vs. the peripheral vascular system. Whereas, it was shown that immune cell content (macrophages and T cells) was significantly reduced in the atherosclerotic lesions of diabetic *Apoe* null mice

devoid of *Ager* vs. diabetic *Apoe* null mice expressing *Ager* (47), macrophage content in the peak period of immune cell infiltration into the ischemic hind limb was significantly higher in *Ager* null vs. wild-type animals, in both the diabetic and non-diabetic states (71). In parallel, the ischemic hind limb muscle tissue of *Ager* null mice displayed significantly higher mRNA transcripts for *Ccl2* and *Egr1*, genes involved in inflammatory cell recruitment and pro-inflammatory mechanisms (71).

Despite the apparent differences in RAGE impact on immune cell content in atherosclerosis vs. hind limb ischemia, in both settings, deletion of *Ager* (and administration of anti-RAGE antibodies in T1D mice subjected to hind limb ischemia) resulted in reduced vascular disease. These findings underscore the complexity and plasticity of RAGE signaling in immune cells in distinct vascular depots and in *in vivo* conditions and suggest that niche-specific cues regulate RAGE ligands and RAGE responses in distinct forms of vascular injury.

Atrial Fibrillation

Cardiac rhythm abnormalities such as atrial fibrillation (AF) accompany disorders of the cardiovasculature. It has been suggested that the incidence of AF is higher in diabetic patients, especially those with longer disease duration or poor glycemic control and that RAGE ligand AGEs, through their ability to increase stiffness, oxidative stress, and fibrosis contribute to this phenomenon (74). Markers of the ligand-RAGE pathway were investigated in human subjects with AF. When comparing 38 patients with AF vs. 59 in normal sinus rhythm, it was shown that levels of fluorescent AGEs and sRAGE were higher in the AF vs. control subjects, especially in non-diabetic patients (75). The markers of AGEs and sRAGE correlated with left atrial dimensions in that study (75). In a distinct study, levels of plasma sRAGE and esRAGE were found to be higher in Caucasian patients with persistent AF vs. paroxysmal AF (76). In another study examining the effects of therapeutic intervention, higher plasma levels of sRAGE were independently associated with reduced rate of recurrence of AF after catheter ablation in diabetic patients (77).

In contrast, in the Atherosclerosis Risk in Communities (ARIC) study, 1068 participants were studied who had baseline sRAGE values determined at the time of study entry. Multiple measures of inflammation were also obtained in these subjects. Compared to the highest quartile of sRAGE, the lowest quartile of sRAGE was associated with the higher baseline levels of inflammatory markers (hsCRP, white blood cell count and fibrinogen). However, when viewed prospectively (6-year change in inflammatory markers), there was no association with sRAGE. Moreover, no significant associations of sRAGE levels were noted with the risk for AF (78).

Collectively, these studies suggest that at least in certain populations and certain conditions, levels of sRAGE may be biomarkers for AF. More work is needed to definitively test these concepts.

Thrombotic Disorders

There is published evidence suggesting links between diabetic complications and thrombosis and platelet pathobiology. For example, examination of mean platelet volume (MPV) revealed that this measure was higher in human T2D subjects with uncontrolled hyperglycemia and a statistically significant association between MPV and albuminuria was also documented (79). High levels of glucose were linked to increased NETosis (neutrophil extracellular traps) and it is suggested that NETosis is associated with T2D (80). In a meta-analysis of the adverse outcomes occurring after Percutaneous Coronary Intervention (PCI) involving >139,000 subjects, it was identified that short-term stent thrombosis, but not long-term stent thrombosis, was significantly higher in subjects with diabetes vs. the non-diabetic control subjects (81). Others studied platelets and their characteristics in diabetes and reported that in T2D, significantly higher platelet activation and markers of hypercoagulation were observed, with increased platelet expression of GP11b/IIIa receptors (82). However, the MEGA study (Multiple Environmental and Genetic Assessment) failed to identify relationships between self-reported diabetes, fasting levels of blood glucose, and venous thrombosis (83).

Emerging evidence links RAGE and at its ligands, AGEs, S100/calgranulins, and HMGB1, to thrombosis and thrombotic disorders. For example, increased expression of HMGB1 has been observed in a number of thrombosis-related diseases such as CAD, stroke, PAD, disseminated intravascular coagulation (DIC), and venous thrombosis (84). The biology of HMGB1 is complex in that, in addition to binding to RAGE, HMGB1 is also a ligand for some of the toll-like receptors (TLRs) (85). Recent studies have probed potential mechanistic links between RAGE and thrombotic disorders.

Studies in Human Subjects

In the prothrombotic disorder known as anti-phospholipid syndrome (APS), it has been established that one of the main targets of the anti-phospholipid antibody is β_2 glycoprotein 1 (or anti-β_2-GP1). When platelets and monocytes obtained from healthy human subject donors were incubated with anti-β_2-GP1, upregulation of RAGE was noted as well as altered cellular location of HMGB1 (86). In serum studies, levels of sRAGE and HMGB1 were significantly higher in patients with APS vs. controls and there was a direct correlation between the levels of HMGB1 and disease duration (86).

Other studies illustrated that HMGB1 binds to platelets and that platelet activation resulted in upregulation of RAGE expression and that HMGB1 was highly expressed in platelet-rich human coronary artery thrombi (87). In ECs obtained from human saphenous veins, incubation with AGEs increased neutrophil adhesion and generation of reactive oxygen species (ROS) and treatment with simvastatin reduced these prothrombotic stimuli and reduced RAGE expression (88). ECs from diabetic patients treated with simvastatin resulted in reduced expression of RAGE, neutrophil adhesion and ROS (88). However, other studies tested if the levels of platelet HMGB1 were associated with outcomes in symptomatic CAD. The authors reported that there were no differences in platelet expression of HMGB1 when comparing patients with stable CAD, unstable CAD, non-ST segment elevation myocardial infarction (NSTEMI), or ST segment elevation myocardial

infarction (STEMI) (89). Further, there were no correlations between left ventricular ejection fraction (LVEF) amongst the subjects that also suffered MI.

HMGB1 derived from platelets also affected monocyte behavior; it was shown that HMGB1 trigged monocyte migration via RAGE and suppressed monocyte apoptosis through a TLR4-dependent activation of the MAPK pathway in these cells (90). Hence, platelet HMGB1-RAGE interactions might impact on distinct cell types, which collectively contribute to the increased risk and severity of CVD.

Studies in Animal Models

In animal models, neutrophil-derived S100A8/A9, through liver production of IL6, promoted production of thrombopoietin, which resulted in reticulated thrombocytosis, was found to be increased in diabetic animals, but reduced by lowering of blood glucose using dapagliflozin or by blocking the binding of S100A8/A9 to RAGE using paquinimod, in mice (91). These authors correlated their findings in human diabetic subjects in whom reticulated thrombocytosis correlated both with the levels of glycosylated hemoglobin and S100A8/A9 (91).

Other studies in mice illustrated that disulfide HMGB1 facilitated monocyte recruitment and, through RAGE, stimulated the formation of the prothrombotic NETs; this process then exposed additional HMGB1 on their extracellular DNA strands to propagate the prothrombotic effects of HMGB1 and NETs (92).

Collectively, these studies link RAGE and its ligands to platelet perturbation and to upregulation of prothrombotic mechanisms, which are both associated with and independent of diabetes, but linked to the complications of diabetes in the cardiovascular system.

Myocardial Infarction

MI is a critical complication of diabetes, which occurs to accelerated rates and degrees in patients with diabetes. Studies in animal models have forged insights into roles for the ligand-RAGE axis in the pathogenesis of diabetic CVD and MI. Emerging insights from human subjects now link this axis to MI as well.

Studies in Human Subjects

Studies in human subjects with MI and related CVD disorders have been probed for the ligand-RAGE axis. In subjects from Japan with T2D, baseline clinical, and biochemical data were examined and prospectively evaluated for the association between those parameters and CVD events over a mean follow-up period of 5.6 years with 25 new CVD events reported during that time. In a tertile analysis, the risk for CVD events rose with increasing levels of sRAGE; a multivariate Cox proportional hazards regression analysis showed that even after correction for typical coronary risk factors, serum sRAGE levels remained independently associated with CVD (93). In a registry of patients enrolled during 2009–2011 with acute MI, the mean values of fluorescent AGEs and CRP did not differ between diabetic and non-diabetic subjects with MI; however, a direct association between AGE levels and CRP was observed only in diabetic, but not non-diabetic patients (94). In patients who had received statins before their MI, however, this relationship disappeared (94), suggesting that statin therapies might mitigate the impact of proinflammatory stimuli. In a distinct study in patients with acute coronary syndrome (ACS), plasma levels of sRAGE were significantly lower in subjects with ACS vs. stable angina pectoris. These authors showed, however, that in the subjects with ACS, the levels of sRAGE did not correlate with the number of affected vessels (95). These considerations suggest that sRAGE might be a biomarker of plaque destabilization but not necessarily the extent of plaque burden in human CAD.

Others examined a group of subjects with T2D undergoing sirolimus-stent PCI. The primary endpoint for the study was the MACCE, or major adverse cardio-cerebral events, which included the following: cardiac death, non-fatal MI or non-fatal stroke during a 2-year period of follow-up. The secondary endpoint of the study was the need for clinically-driven repeat revascularization during the 2-year period. The authors monitored levels of circulating glycated albumin and esRAGE and found that both glycated albumin and esRAGE predicted long-term clinical outcomes; specifically, elevated serum glycated albumin and reduced esRAGE were associated with poor clinical outcomes in this patient group (96). In a distinct study in patients with T2D undergoing drug-eluting stent implantation, the relationship between plasma levels of sRAGE and in-stent restenosis were probed and measured at the time of the stent implantation. The authors reported that plasma levels of sRAGE were significantly higher in the T2D patients with in-stent restenosis vs. control T2D subjects; interestingly, the levels of glycosylated hemoglobin, CRP, and IGF-1 (insulin-like growth factor 1) did not differ between the groups (97). When the authors performed multivariate regression analysis, they found that plasma levels of sRAGE and mean stent diameter <2.0 mm significantly predicted in-stent restenosis (97).

A critical complication of acute MI is the development of cardiogenic shock. As it was shown that higher levels of monocyte RAGE and lower levels of plasma sRAGE were linked to higher mortality in cardiogenic shock, the effects on levels of MMP9 and Tissue Inhibitors of Metalloproteinases (TIMPs) were tested, as MMPs have been shown to contribute to the production of sRAGE through the cleavage of its extracellular domains. MMP9 activity was found to be increased in acute MI survivors but reduced in subjects with acute MI who developed cardiogenic shock (98). Further, MMP9 activity was found to correlate inversely with RAGE expression on monocytes. Collectively, the above considerations suggest that sRAGE might serve as a biomarker in acute MI with respect to prognosis and that maintenance of effective MMP9 activity may serve to stabilize sRAGE production in MI complicated by cardiogenic shock.

With respect to RAGE ligand HMGB1, plasma levels of this factor were shown to be related to infarct size and to residual left ventricular function after MI (99).

Studies in Animal Models

Studies in the isolated perfused heart and in *in vivo* MI triggered by occlusion/reperfusion of the left anterior coronary artery in rats and/or mice have shown that blockade of RAGE,

using either sRAGE or in genetically modified mice, that is *Ager* null mice or transgenic mice expressing cytoplasmic domain-deleted RAGE (in macrophages or ECs) is protective against ischemia/reperfusion (I/R) injury in diabetic and non-diabetic animals (100–102). In those studies, reduced infarct size, reduced myocardial necrosis, and increased cardiac function and ATP recovery accompanied blockade of the RAGE axis. In cultured cardiomyocytes, induction of hypoxia/reoxygenation (H/R) stimulated RAGE-dependent activation of JNK MAP kinase and dephosphorylation of GSK-3β, which was prevented in cells devoid of *Ager* or upon treatment with sRAGE in wild-type cardiomyocytes (103).

Others employed a rat model of MI and cultured cardiomyocytes to study the S100/calgranulin-RAGE interactions and showed that S100B via RAGE may contribute to cardiomyocyte apoptosis via activation of ERK1/2 MAPK and p53 signaling (104). In a distinct study in T1D mice, coronary artery ligation was performed in wild-type and S100B-deleted mice. Diabetes and MI induction each alone induced expression of S100B and RAGE in the heart; but in the post-MI myocardium, only in diabetic mice, the expression of S100B was attenuated. In the diabetic S100B-deleted mice post-MI, increased dilation of the left ventricle was noted compared to diabetic wild-type mice, in parallel with increased impairment of cardiac function, expression of GLUT4 and systemic levels of AGE (105). Collectively, those studies suggested that S100B expression may beneficially modulate cardiac metabolism post-MI in diabetes. Distinct studies also implicated ligand HMGB1 in experimental cardiac MI, at least in part through RAGE, in both diabetic and non-diabetic animals (106). Hence, identification of the timing of the actions of the specific RAGE ligands in the chronic vs. acute setting appears to be essential in order to discern the optimal conditions for RAGE antagonism in MI.

Finally, studies have begun to examine the potential roles of the formin, DIAPH1, the cytoplasmic domain binding partner of RAGE, in myocardial I/R injury. After induction of I/R in wild-type mice, DIAPH1 expression was upregulated; in cultured H9C2 and AC16 cardiomyocytes, H/R also upregulated expression of DIAPH1 (32). Consistent with mediating roles for DIAPH1 in myocardial injury, global deletion of *Diaph1* reduced infarct size and preserved cardiac function after experimental MI when compared to the *Diaph1*-expressing control animals. In H9C2 cells, silencing of *Diaph1* in H/R reduced expression of the sodium-calcium exchanger and increased sarcoplasmic calcium ATPase activity (32).

Fat Depots, RAGE, Obesity, and CVD

Recent work has highlighted roles for distinct fat depots, such as brown adipose tissue, subcutaneous adipose tissue (SAT), visceral (omental/epididymal), perivascular adipose tissue (PVAT), and epicardial adipose tissue (EAT) in cardiometabolic fate in human subjects and animal models. Intriguingly, RAGE contributes to metabolic perturbation via its expression in multiple fat depots.

Studies in Human Subjects

In human omental adipose tissue, obesity was associated with increased accumulation of CML-AGE RAGE ligand and RAGE expression compared to lean individuals; interestingly, it was reported that CML-AGE levels were reduced in the circulation of obese subjects, which was proposed to be due to tissue trapping of CML-AGE in the adipose tissue, at least in part on account of higher RAGE expression (107). These decreased levels of circulating CML-AGE were also shown to correlate with insulin resistance (107).

Increasingly, epicardial adipose tissue or EAT has been linked to CVD (108, 109). In 33 human subjects undergoing open-heart surgery, EAT was retrieved for analyses. As RAGE expression rose, increased EAT thickness, reduced expression of GLUT4, adiponectin and glyoxalase1 (GLO1), and elevated levels of HMGB1, TLR4, and MYD88 were observed (110), suggesting that the ligand-RAGE axis may be associated with EAT adiposity and metabolic dysfunction. In a distinct study, SAT and EAT were obtained for RNA-sequencing from 5 T2D patients with CAD and 3 subjects without CAD with or without T2D undergoing cardiac surgery. 592 genes were differentially expressed in diabetic vs. non-diabetic EAT; there were no changes in the transcriptome between diabetic and non-diabetic SAT (110). The diabetic EAT-associated genes were largely linked to inflammation (IL1B and IL6); KEGG pathway analysis placed these differentially-expressed genes in the TNF, NF-κB, and AGE-RAGE pathways (110).

Imaging modalities have also been employed to track epicardial fat volume (EFV), paracardial fat volume (PFV) and perivascular fat (PVAT) in 66 consecutive patients (33 with diabetes) and multivessel CAD included for study. In diabetes, higher EFV was observed; at the transcript level, patients with diabetes displayed significantly higher RAGE expression in EAT (111).

Studies in Animal Models

Similar observations have been made in animal models; even prior to the onset of high fat diet-induced obesity and the development of insulin resistance in wild-type mice, RAGE ligands are upregulated in the metabolic organs (112). On account of these observations, the potential roles of RAGE in diet-induced obesity were studied by feeding mice a high-fat diet (60% kcal from fat).

In mice globally devoid of *Ager*, mice fed a 60% high-fat diet were significantly protected from the gain in body mass that accompanied the feeding of this diet in wild-type mice (112). In parallel, the *Ager* null mice were protected from insulin resistance that accompanied obesity in the wild-type animals; this was determined both through insulin tolerance tests (intraperitoneal injections of glucose) and through the hyperinsulinemic euglycemic clamp (112). Indirect calorimetry studies revealed that food intake did not differ between the two genotypes of mice fed the high-fat diet, but energy expenditure was significantly higher in the mice devoid of *Ager* vs. the control animals. Pharmacological blockade of RAGE, using sRAGE, in wild-type mice, significantly suppressed weight gain when compared to vehicle upon introduction of sRAGE either immediately at the time of the high-fat diet feeding or 3 weeks after the initiation of the high-fat diet (112). These results led

to the direct testing of roles for RAGE in regulation of energy expenditure, focusing on the adipocyte.

Ager floxed mice were bred into the *Adiponectin (Adipoq)* Cre recombinase background, which resulted in deletion of *Ager* in both brown and white adipose tissue adipocytes. These mice were significantly protected from high-fat diet-induced obesity and from cold-induced loss of body temperature when compared to *Ager* floxed control mice, in which RAGE was expressed in the adipocytes (113). The underlying mechanisms were traced to RAGE ligand-RAGE-dependent suppression of lipolysis and thermogenic programs (such as expression of *Ucp1*) in these settings, through reduced phosphorylation of p38 MAP kinase and hormone sensitive lipase (HSL) (113). On account of the fact that the *Adipoq* Cre recombinase mice could not discern mechanistic roles for RAGE in white vs. brown adipose tissue, adipose tissue transplantation of either brown or subcutaneous white adipose tissue from these mice, or their controls, were introduced into wild-type mice C57BL/6J recipients. Transplantation of adipocyte *Ager*-deficient brown or subcutaneous white adipose tissue protected the recipient mice from obesity induced by high-fat feeding via upregulation of thermogenic programs. Interestingly, in both cases, the native brown or white adipose tissue of the recipients of the *Ager*-deleted adipocytes (white or brown) displayed increased expression of UCP1 protein by immunostaining, suggesting that the transplanted tissue conferred its beneficial effects, at least in part through paracrine mechanisms that directly and beneficially affected the native brown and subcutaneous white fat depots (113).

Collectively, these results identified a natural function for RAGE in energy conservation mechanisms and suggested that the cardiometabolic effects of the RAGE signaling pathway are active even before the development of T2D.

Increasingly, studies in human subjects demonstrate genetic associations of *AGER* and *DIAPH1* SNPs to disease. In the sections to follow, we detail the findings on *AGER* and *DIAPH1* SNPs in cardiometabolic disease.

RAGE AND DIAPH1 AND SNPS—DEEPENING THE CONNECTIONS TO HUMAN SUBJECTS

AGER SNPs

Multiple SNPs of the *AGER* gene have been described; among the most common include the following: rs2070600, rs1800624, rs1800625, rs184003, and a 63 bp deletion (114, 115). The rs2070600 represents the nucleotide change 244G>A and at the amino acid level, Gly82Ser (114). This *AGER* SNP was of particular interest on account of the fact that the Gly82Ser is within the V-type Ig domain, that is, the extracellular domain encompassing much of the ligand binding capacity (116). Structurally, the Gly82Ser SNP promotes N-linked glycosylation of Asn81, which is important for RAGE ligand binding (117). Indeed, *in vitro,* cultured cells bearing the RAGE 82S allele displayed enhanced binding affinities for RAGE ligands in the S100/calgranulin family, and upon RAGE ligand stimulation, exaggerated expression of cytokines and MMPs was observed in G82S- vs. G82G-transfected cells, suggestive of an amplified inflammatory response (116). With respect to human subjects and inflammatory disease, a case-control study revealed that there was an increased prevalence of the 82S allele in patients with rheumatoid arthritis (RA) compared with control subjects (116).

Other SNPs, specifically, rs1800624 (-388T>A), rs1800625 (-442T>C), and rs1051993 (-1435G>T); and a 63 base pair deletion (-421_-359) reflect promoter variants and rs184003 (822+49G>T) affects intron 7–8 (114).

Review of the literature suggests that the links of *AGER* SNPs to cardiovascular disease (CVD) may be dependent on ethnicity (114). For example, extensive studies in the Chinese Han population suggested that the rs2070600 SNP was significantly associated with increased risk of all-cause mortality and acute myocardial infarction (MI) (118). Ma and colleagues performed a meta-analysis of 16 eligible studies reporting on rs2070600 SNP and CVD; they reported an association between this SNP and coronary artery disease (CAD) and ischemic stroke (IS) in the Chinese population, but not in non-Chinese populations (119). Until studies examining larger groups of subjects are examined to fully test this *AGER* SNP and potential relationships to CVD, studies of these specific populations in China may, therefore, shed light on mechanisms of RAGE-dependent predilections to CVD.

DIAPH1 SNPs

Compared to *AGER*, at least to date, less is reported with respect to *DIAPH1* SNPs and human disease. However, a report linked a *DIAPH1* SNP to a blood-related disorder. The R1213* variant of *DIAPH1* was associated with sensorineural hearing loss and a disorder of platelets, called macrothrombocytopenia (MTP), in which cytoskeletal abnormalities in megakaryocytes (platelet precursors) and platelets were described (120). This mutation, which affects the autoregulatory domain of DIAPH1, results in constitutive activation of DIAPH1 (120).

Recently, *DIAPH1* SNPs were linked to stroke. One of the *DIAPH1* SNPs, Rs7703688T>C was significantly associated with increased risk of ischemic stroke, $p = 4.139 \times 10^{12}$, which was further validated in an additional group (121). Further, in a small artery occlusion (SAO) subtype of stroke, *DIAPH1* expression in affected tissue displayed trends to increased levels in subjects with the rs25019 genotype, $p_{trend} = 0.048$ (121). These findings add to the connections between DIAPH1 and human subjects in the context of CVD.

Collectively, these data, from animal models and human subjects suggest roles for RAGE/DIAPH1 in cardiometabolic disease. Hence, efforts to target RAGE pharmacologically might provide substantial benefit in obesity, metabolic dysfunction, diabetes, and diabetic complications.

TARGETING RAGE THROUGH BLOCKADE OF RAGE-DIAPH1 INTERACTION

As indicated earlier in this review, the extracellular domains of RAGE (V, C1, and C2-type Ig domains) bind the diverse classes of ligands in distinct sites; although the V-type Ig domain is the chief

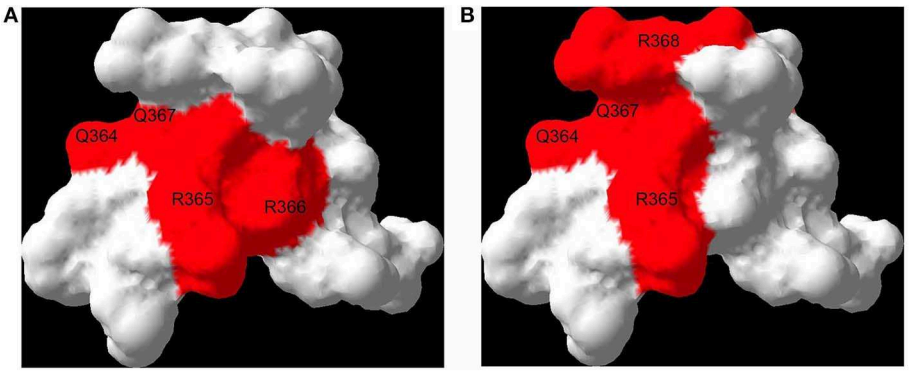

FIGURE 1 | RAGE binds DIAPH1: effect of a small molecule antagonist. Both DIAPH1 and a small molecule RAGE antagonist bind to the proximal sites on the RAGE cytoplasmic domain, suggesting a mechanism of RAGE inhibition. Interaction surfaces of DIAPH1-RAGE [see (23)] **(A)** and small molecule RAGE antagonist-RAGE [see (124)] **(B)** are mapped onto a solution structure of RAGE cytoplasmic domain (PDB code 2lmb). Affected residues are labeled. The residues are numbered based on the full length RAGE.

site for ligand interaction, the C1- and C2-type Ig domains are reported to bind ligands as well (11–14). Hence, it was perhaps not surprising that the small molecule known as Azeliragon failed to show benefit in a Phase III clinical trial in Alzheimer Disease (AD) for subjects with mild cognitive impairment (MCI) when compared with placebo-treated patients (122). In AD, beyond RAGE ligand Aß, it is known that multiple classes of pro-inflammatory and pro-oxidative RAGE ligands are enriched in the AD brain (123, 124).

In this context, the demonstration of the binding of the RAGE cytoplasmic domain to DIAPH1 in a discrete manner, with a binding pocket <200 Å, paved the way for the development of small molecule antagonists for this interaction as a means to block RAGE signaling (23). Accordingly, a 59,000 small molecule library was screened to identify inhibitors of the interaction of the RAGE cytoplasmic domain with DIAPH1; 11 such molecules were reported on the basis of their ability to bind to the RAGE cytoplasmic domain and block DIAPH1 binding (**Figure 1**); the ability to block RAGE ligand-mediated signaling stimulated by multiple different classes of ligands; the ability to block RAGE ligand, but not non-RAGE ligand-mediated cellular migration in SMCs; the ability to reduce I/R injury in the isolated perfused heart model; and the ability to block the proinflammatory actions of RAGE ligands (CML-AGE) injected into wild-type mice (125). Further development and refinement of the key scaffolds identified from that work is underway at this time. If successful, such efforts may result in the development of a novel class of RAGE antagonists.

PERSPECTIVES AND CHALLENGES

Despite multiple advances for therapeutic interventions in CVD, gaps in therapies remain for subjects with diabetes, in whom traditional treatments do not afford the same degree of protection as they do in non-diabetic subjects. In this context, the identification of diabetes-specific disease-modifying pathways is essential to fill the chasm in therapeutic opportunities for patients with diabetes. Further, the identification of the optimal timing for intervention with disease-modifying agents is essential in order to maximize potential benefits, even if preventive therapies are, ultimately, deemed more realistic, as they are for hyperlipidemia and hypertensive disorders, for example.

Hence, in diabetes, it is logical that targeting the consequences of the defining feature of the disorder, that is, hyperglycemia, may yield benefit. The acute and long-term effects of high glucose, manifested in part by the formation of AGEs, is one such target. As AGEs form and accumulate both endogenously and through dietary ingestion, and as AGEs represent a highly heterogeneous class of structures, the direct targeting of AGEs may be challenging. For this reason, antagonizing the cellular effects of AGEs may be more comprehensive and feasible. Complicating this notion, however, is the finding that the chief receptor for AGEs, RAGE, is a multi-ligand receptor whose ligands display significant promiscuity in their binding modes to the extracellular domains of RAGE. Thus, inhibiting the interaction of the RAGE cytoplasmic domain with DIAPH1 may reflect a superior approach by curating the effects of diverse ligands signaling through RAGE/DIAPH1. Indeed, significant advances in the development of protein-protein interaction inhibitors (PPI) in other settings bolster promise for this proposed approach (126, 127).

Of note, studies in human subjects underscore that the measurement of the levels of sRAGE and/or esRAGE might provide a biomarker to track the activity of the RAGE pathway (**Figure 2**). In chronic disease without exacerbation, the levels of sRAGEs appear, in general, to be lower than those in control subjects. However, it is plausible that in exacerbations of chronic disease, such as acute cardiac events, levels of sRAGEs might rise, perhaps in response to increased MMP and ADAM activities, although these relationships remain to be definitively discerned. It is clear, however, that studies testing both sRAGE and esRAGE, in a serial manner, prior to and at the time of and after acute cardiac events will be required to fully discern the reasons for the apparent variability of these markers.

Beyond immediate benefits for antagonizing RAGE/DIAPH1 in cardiometabolic disease in the periphery, it is plausible

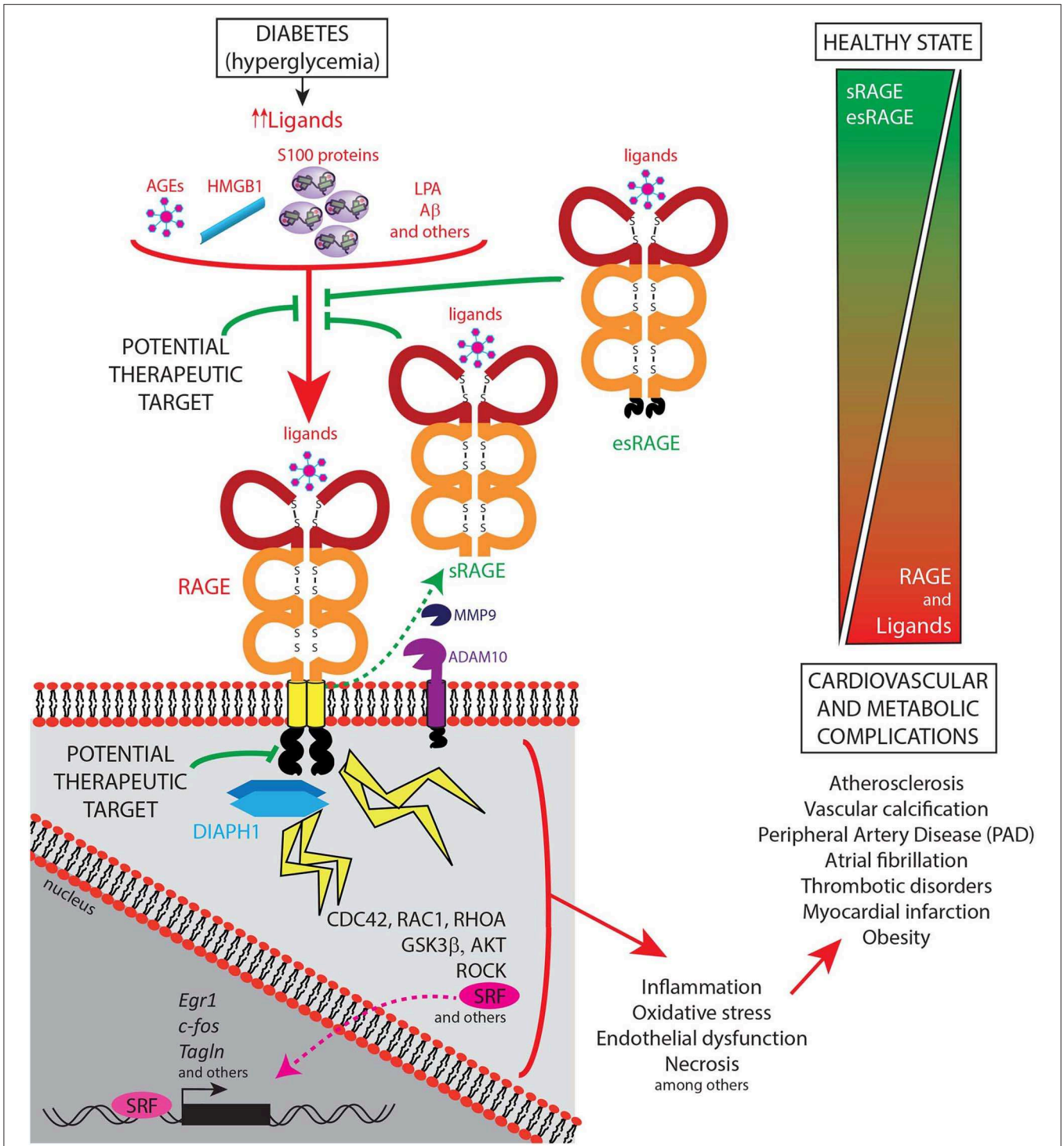

FIGURE 2 | Schematic representation of the ligand-RAGE-DIAPH1 axis and its role in diabetic cardiometabolic complications. The receptor for advanced glycation end products (RAGE; gene name is *AGER*) mainly acts through its known ligands, such as AGEs, HMGB1, S100 family of proteins, LPA, and Aβ, which bind the RAGE extracellular domains. The cytoplasmic domain of RAGE interacts with its cytoplasmic effector protein, Diaphanous1 (DIAPH1), thereby activating multiple downstream regulators and stress responses, as illustrated in the figure. Cellular stressors such as inflammation, oxidative stress, endothelial dysfunction, and necrosis amongst others, are well-known induce cardiovascular and metabolic complications such as atherosclerosis, vascular calcification, peripheral artery disease, atrial fibrillation, thrombotic disorders, myocardial infarction, and obesity. In contrast, soluble forms of RAGE, including sRAGE, that results from cell surface-cleavage of the full-length receptor by Matrix Metallopeptidase-9 (MMP9) and A Disintegrin And Metalloproteinase Domain-Containing Protein-10 (ADAM10), and esRAGE, a product of a splice variant of *AGER*, have been demonstrated to show a protective role in cardiometabolic complications, at least in part, by preventing the RAGE ligands from binding to the cell surface receptor, and, therefore, reducing the RAGE-DIAPH1 signaling activation. Hence, currently identified potential therapeutic targets include blocking the binding of the ligands to the receptor and by interruption of the RAGE-DIAPH1 interaction.

that targeting the diverse signaling platform through DIAPH1 and stimulated by RAGE ligands may be broadly beneficial, including in the central nervous system. Beyond AGEs and proinflammatory ligands, RAGE is also a receptor for oligomeric forms of Aß, which is strongly implicated in the pathogenesis of AD (128). The relatively recent designation of VCID, or "vascular contributions to cognitive impairment and dementia" culls the collective impact of aging, age- and lifestyle-related disorders (such as hyperlipidemia, hypertension, elevated fasting blood glucose, and obesity) and AD into a schema that suggests that these disorders may complicate and exacerbate each other, with a final common manifestation of "dementia" (129–131). Potentially biomarked by "white matter hyperintensities" or WMHs by imaging techniques, VCID may represent the product of the sum total of vascular/cognitive risks in individuals. It was recently shown that DIAPH1 is upregulated in the brains of AD vs. age-matched control human subjects; that its expression co-localizes with that of RAGE; and that the AD-specific upregulation of DIAPH1 was localized to microglia (132), the endogenous/resident yolk sac-derived immune/inflammatory cells of the brain. In this context, the demonstration of roles for RAGE ligands/RAGE in pathological aging, vascular and cognitive disturbances may suggest broader benefits for RAGE/DIAPH1 antagonism in aging and cardiometabolic disease.

In summary, the identification of fundamental roles for RAGE in energy conservation mechanisms, which go awry in nutrient excess, thereby contributing to the development of obesity in high-fat feeding in mice; and in the propagation of chronic tissue-damaging pro-inflammatory mechanisms, lay the framework for the potential benefits for RAGE antagonism both in the causes and consequences of diabetes and its complications, particularly in CVD. Despite the finding that the highest levels of RAGE expression are in the lung, a plethora of evidence suggests that RAGE plays pathogenic roles in this organ, as RAGE is implicated in such disorders as allergic airway inflammation and asthma, pulmonary fibrosis, lung cancer, chronic obstructive pulmonary disease, acute lunginjury, pneumonia, cystic fibrosis, and bronchopulmonary dysplasia (133). We speculate that therapeutic interruption of RAGE, post-development and in the setting of the mature lung, and with partial antagonism to be achieved by pharmacological means, is very likely to be safely tolerated in the lung and in the overall organism. Further, the observations that deletion of *Ager* is protective in polymicrobial sepsis, massive liver injury and in most forms of infection (134, 135), at least in animal models, suggest that targeting RAGE may, on balance, exert salutary benefits in chronic diseases such as diabetes. These concepts remain to be tested definitively in human subjects with diabetes and cardiometabolic disease and the results of these investigations are eagerly awaited.

AUTHOR CONTRIBUTIONS

AMS wrote the first draft of the manuscript and completed all of the editing. LE-G, RL-D, GY, LR, SR, PG, AS, and RR provided critical comments on the manuscript and edited the manuscript.

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Conflict of Interest: AMS, RR, and AS hold submitted and pending patents on intellectual property (small molecule antagonists of RAGE/DIAPH1), which are discussed in this manuscript.

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Glycemia and Atherosclerotic Cardiovascular Disease: Exploring the Gap Between Risk Marker and Risk Factor

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There is consistent, unequivocal and reproducible epidemiological evidence derived from diverse populations that various indices of glycemia (fasting plasma glucose, post-prandial or post oral glucose challenge plasma glucose, HbA1c) are associated with an increased risk of atherosclerotic cardiovascular disease (ASCVD), even in the prediabetic state. Furthermore, there is abundant experimental evidence demonstrating that hyperglycemia *per se* accelerates and aggravates the atherosclerotic process, providing biological plausibility to the concept that hyperglycemia is causally related or a true risk factor for ASCVD. Two studies in particular, DCCT and UKPDS, that enrolled a younger cohort of patients with type 1 diabetes or an older cohort with newly diagnosed type 2 diabetes, respectively, showed trends toward a reduction in ASCVD. The reductions in ASCVD reached statistical significance only after prolonged follow up, and when differences in HbA1c were no longer maintained (referred to by some as a "legacy effect"). More recent studies in those with established type 2 diabetes, in which glycemic control was improved by a variety of strategies, failed to demonstrate reductions in ASCVD. The gap in evidence supporting hyperglycemia as a true causative risk factor for ASCVD or simply a risk marker for some other confounding causative factor is discussed in this review. We conclude that hyperglycemia does appear to be at least partially causative of ASCVD (i.e., an ASCVD risk factor). We discuss how this evidence can be incorporated into an overall therapeutic strategy to prevent ASCVD in those with prediabetes and established diabetes.

Keywords: cardiovascular risk, diabetes mellitus, glycemia, atherosclerosis, macrovascular complications

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INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of morbidity and mortality in people living with diabetes. Advances in the prevention and management of ASCVD have reduced mortality (1). Epidemiological studies have consistently supported a strong association between various indices of glycemia (fasting plasma glucose, post-prandial or post oral glucose challenge plasma glucose, HbA1c) and risk of ASCVD. However, there is still much debate as to whether hyperglycemia should be regarded as a risk marker (i.e., associated but not causative) or a true risk factor (i.e., a causative factor for ASCVD).

Both risk factors and risk markers are associated with increased risk, however risk factors are defined by the ability to reduce the risk by correction of the variable. Although intuitively, in view of the tight association between glycemia and ASCVD and biological plausibility based on experimental evidence, one might assume that improved glycemic control would reduce the risk of ASCVD, most studies have failed to demonstrate a robust reduction in ASCVD with improved glycemic control. Long-term follow-up of glycemic control studies have provided new insights that shed additional light on the relationship between glycemia and ASCVD. This brief review does not provide a comprehensive review of all available evidence but instead highlights key evidence to illustrate important relationships. First, we will briefly review cross-sectional population data and experimental evidence of pathophysiological links, the latter providing biological plausibility for hyperglycemia as a causative factor for ASCVD. Next, we will discuss the results of the major, prospective, randomized, controlled, clinical trials of the treatment of hyperglycemia by various modalities that have for the most part failed to demonstrate a robust reduction in ASCVD as the primary study outcome. Based on this evidence we will address the gap that exists between hyperglycemia as a risk marker vs. risk factor for ASCVD and will speculate on the factors that may explain this discrepancy.

GLYCEMIA AS A CONSISTENT AND POWERFUL RISK MARKER FOR ASCVD—RESULTS FROM CROSS-SECTIONAL POPULATION STUDIES

Numerous studies in those with diabetes have demonstrated unequivocally that hyperglycemia is a potent risk marker for ASCVD (2, 3). Studies have also demonstrated that hyperglycemia in the non-diabetic range is associated with ASCVD (4). In the Glucose Tolerance in Acute Myocardial Infarction study, glucose intolerance was the single most powerful predictor of cardiovascular complications and death (5). Similar results were reported in the Asian Pacific study, showing that higher fasting plasma glucose levels correlate with increased risk for stroke and ischemic heart disease (IHD) (6). In addition to the fasting or peak glucose levels affecting ASCVD risk, a higher variability of glucose levels was shown to correlate with carotid intima media thickness (7) and with oxidative stress (8). Similarly, post-prandial glucose has also been associated with a higher CVD risk (9–11).

In the Multiple Risk Factor Intervention trial, regardless of any other ASCVD risk factors, patients with diabetes had an odds ratio of 2–4 for ASCVD mortality, in comparison to patients without diabetes (12). This was further validated in the European Prospective Investigation of Cancer and Nutrition (EPIC Norfolk) (13) and in the Atherosclerosis Risk in Communities (ARIC) analysis (14). In summary, this non-exhaustive list of studies that have linked hyperglycemia, even in the non- or pre-diabetic range, to increased risk for ASCVD, provide convincing evidence that hyperglycemia is a powerful risk marker for ASCVD.

POTENTIAL PATHOLOGICAL MECHANISMS OF HYPERGLYCEMIA—BUILDING A CASE FOR BIOLOGICAL PLAUSIBILITY

Numerous studies have examined potential pathophysiological mechanisms whereby hyperglycemia may be directly or indirectly implicated in accelerating the atherosclerotic process. A number of reviews in this issue of Frontiers in Cardiovascular Medicine and others have discussed various aspects of this topic in detail (15). Here we will briefly highlight the most prominent mechanisms that have been described.

Prolonged hyperglycemia is observed both in type 1 and type 2 diabetes. Endothelial cells have limited ability to regulate glucose influx, leaving them more vulnerable to high intracellular glucose concentrations, excessive reactive oxygen species (ROS) production and oxidative stress. Excessive ROS production is believed to play a key role in the cellular dysfunction observed in the person with diabetes, linked to the many processes that promote atherosclerosis, cardio-toxicity and insulin resistance (16). ROS promotes the generation of Methylglyoxal, the major contributor to generation of a heterogenous group of chemical moieties known as advanced glycation end products (AGEs). AGEs contribute to endothelial dysfunction, vasoconstriction and pro-inflammatory and pro-atherogenic changes, that underlie atherosclerotic plaque rupture and thrombus formation (17). Increase glucose flux divert glucose from glycolysis to alternative pathways such as polyol and hexosamine pathways, exacerbating glycation and protein kinase C activation (18, 19). ROS have been shown to activate PKC isoforms, again contributing to endothelial dysfunction (20).

High intra-cellular Ca2+ levels, induced by ROS activate NFAT (nuclear factor of activated T cells). NFAT activation has been linked to accelerated atherosclerosis, cardiac toxicity and inflammation (21). The pro-inflammatory milieu promotes atherosclerosis and plaque rupture (22), however it also disrupts pancreatic beta cell function, aggravating hyperglycemia (23), which in turn generates oxidative stress that provokes an inflammatory response (24–26), forming a vicious cycle. Finally, hyperglycemia has also been shown to promote activity of 12- and 15-lipoxygenase, enzymes that react with fatty acids and generate lipid metabolites that are pro-atherogenic (19).

While prolonged hyperglycemia is observed in both type 1 and 2 diabetes, type 2 diabetes caries marked insulin resistance and hyperinsulinemia, with further deleterious effects. Insulin resistance is associated with an increase in free fatty acid (FFA), further contributing to ROS production (27). Additionally, insulin resistance decreases the myocardium ability to utilize glucose, due to a decrease in glucose transporter 4 (GLUT4) expression (28). Together, it makes the myocardium dependent on fatty acid as a source of energy, generating more cardiac toxicity (25).

The above-mentioned intracellular pathways interfere with key endothelial functions, including loss of nitric oxide vasodilatory effect, increased expression of inflammatory cytokines and adhesion molecules (29, 30). An increase in endothelial cell permeability allows the infiltration of monocytes into the intimal layer, influx of lipoproteins and the ultimate generation of foam cells, the basis of the fatty streak and eventually the atherogenic plaque. People with diabetes consistently have an atherogenic plaque characterized by a higher lipid concentration and more inflammatory cells (31), making the plaque more prone to rupture and thrombus formation.

In addition to vascular injury, patients with diabetes also present a pro-coagulable state, with a decrease in the fibrinolytic pathway and heightened platelet activity (32). Hyperinsulinemia and hyperglycemia have been linked to higher levels of plasminogen activator inhibitor-1 (PAI-1), factor VII, XII and fibrinogen and lower levels of tissue plasminogen activator (tPA), favoring a pro-coagulant state (33). In addition, chronic hyperglycemia causes an increase in glycoprotein IIb-IIIa and P-selectin expression on the platelet surface, and an increase in the sensitivity of PY212, an activating platelet receptor, all promoting platelet activation and thrombus formation (34).

Furthermore, neurologic injury that may result from hyperglycemia *per se*, could potentially impair the sensation of myocardial ischemic symptoms in those with diabetes, leading to late diagnosis and treatment, which further contributes to the ASCVD morbidity and mortality. In summary, numerous pathophysiological mechanisms have been described that provide biological plausibility to the notion that hyperglycemia can directly promote atherosclerosis and worsen the outcome of cardiovascular events.

THE DCCT AND UKPDS TRIALS FAILED TO DEMONSTRATE SIGNIFICANT REDUCTIONS IN ASCVD BUT DID DEMONSTRATE A "LEGACY" EFFECT AFTER PROLONGED FOLLOW UP (TABLE 1)

The Diabetes Control and Complications Trial (DCCT), which enrolled relatively young adults with type 1 diabetes (T1D), demonstrated a non-significant trend toward reduced CV events with improved glycemic control (average HbA1c in the intensively insulin-treated group was 7% and that in the usual care group was 9%). Of note, the relatively young age of the DCCT study participants was reflected in a low overall incidence of ASCVD (0.5 in the treated patients vs. 0.8 event per 100 patient-years in the control group), thus making the study

TABLE 1 | Effects on CV outcomes of landmark randomized controlled trials with the goal of intensifying glycemic control.

	ADVANCE (35)	ACCORD (36, 37)	VADT (38)	UKPDS (39)	DCCT (40, 41)
Number of patients	11,140	10,251	1,791	5,102	1,441
Mean Age (years)	66	62.2	60.4	53.3	26.9
nitial BMI	28	32	31.3	27.5	23.5
HbA_{1c} Achieved (%) Intensive vs. standard	6.5 vs.7.3	6.4 vs.7.5	6.9 vs. 8.4	7 vs. 7.7	7 vs. 9
Mean FU (years)	5	3.5	5.6	10	6.5
CV outcome	MACE: - Non-fatal MI - Non-fatal stroke - CV death	MACE: - Non-fatal MI - Non-fatal stroke - CV death	Composite CV events: - MI - Stroke - CV death - CHF - Inoperable CAD - Surgical intervention for CVD - Amputation for ischemic gangrene	Myocardial Infarction	MACE: - Non-fatal MI - Non-fatal stroke - CV death
Duration and severity of diabetes	Vascular disease or risk factor 8 years of diabetes	CVD or 2 risk factors m 10 years of diabetes	Poorly controlled 11.5 years of diabetes	New onset type 2 diabetes	5.9 years of type 1 diabetes
Risk reduction for CV outcome	*HR* = 0.94 (0.84–1.06) *P* = 0.32	HR = 0.9 (1.04–0.78) P = 0.16	*HR* = 0.87 (0.73–1.04) *P* = 0.14	*RR* = 0.84 (0.71–1) *P* = 0.053	NS
Glucose lowering drugs	Gliclazide, metformin, thiazolidinediones, acarbose, or insulin	Metformin, sulfonylureas, meglitinides, thiazolidinediones, α-glucosidase inhibitors, insulin, and exenatide	Glimepiride, metformin, rosiglitazon, and insulin	Chlorpropamide, glibenclamide, glipizide, metformin, and insulin	Insulin pump or injections

CV, cardiovascular; RR, Relative Risk; HR, hazard ratio; FU, follow up; CHF, congestive heart failure; CI, Confidence Interval; MI, myocardial infarction; BL, baseline; NS, Non significant; MACE, Major adverse cardiovascular events; CAD, Coronary artery disease.

underpowered to demonstrate a reduction in ASCVD (40). With prolonged follow up of an additional 9 years following unblinding of the main study results (EDIC study), a significant reduction in ASCVD events was demonstrated in the original intensive insulin treatment group (41, 42), despite the fact that the initial difference in glycemic control was not maintained during follow up. Similarly, the initial analysis of the UK Prospective Diabetes Study (UKPDS) trial of newly diagnosed individuals with T2D failed to demonstrate that more aggressive vs. standard glycemic control using a variety of glucose lowering therapeutic modalities significantly reduced ASCVD ($p = 0.052$), while a clear reduction in microvascular complications was observed (39). The average HbA1c in the intensively treated UKPDS cohort was 7% and that in the usual care cohort was 7.9%, with HbA1c rising with time in parallel in both cohorts. Metformin treatment in obese patients in the UKPDS study was shown to reduce the incidence of myocardial infarction (MI) by 39% ($p = 0.01$). However, the metformin-treated cohort was relatively small and the results would need to be replicated in a larger study population to confidently confirm that metformin is antiatherogenic (43). Following completion and unblinding of the UKPDS study population, a long-term follow-up study (UKPDSFU) continued to follow some of the study population with no specific intervention in terms of glycemic control. With a follow-up of 10 years, a significant reduction of 15% in the incidence of MI and 17% reduction in mortality from diabetic complications was demonstrated in the original intensively treated group, despite the fact that the original study difference in glycemic control became non-significant 1 year after termination of the initial study (44). These results of the UKPDSFU and the EDIC study pointed to a legacy effect of early glycemic control that became evident 10 years after the initial study ended, despite no ongoing difference in treatment between the groups during this follow up time period (42). A study with a follow-up of 18 years showed a similar reduction in ASCVD related mortality both in patients with type 1 and type 2 diabetes, age adjusted HR of 5.2 and 4.9, respectively (45). There has been much speculation regarding the mechanism of the legacy effect. Hyperglycemia for prolonged periods has been shown to generate an inflammatory and fibrotic gene signature in endothelial and smooth muscle cells, with evidence of specific epigenetic changes. Interested readers are referred to a number of excellent reviews on the topic (46–48). Of note, as discussed in the next section, a similar "legacy effect" has not been replicated in other clinical trials.

IMPROVEMENTS IN GLYCEMIA IN THREE LANDMARK CLINICAL TRIALS THAT DEMONSTRATED NO SIGNIFICANT BENEFICIAL EFFECT IN PREVENTING ASCVD (TABLE 1)

The above-mentioned studies prompted studies that examined the effect of even more stringent glycemic control to near-normal levels and whether the benefit of glycemic control was limited to newly diagnosed patients with diabetes. Three major studies compared stringent glycemic control in patients with T2D: the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study (36), the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) study (35) and the Veterans Affairs Diabetes Trial (VADT) study (38). All three studies achieved a significant and sustained difference in glycemia between intensive and standard treatment arms: in the ACCORD trial: HbA1c 6.4 vs. 7.5%, the ADVANCE: HbA1c 6.4 vs. 7.0%, the VADT: HbA1c 6.9 vs. 8.5%. All three studies failed to demonstrate a significant benefit on ASCVD risk, with minor trends toward reduction (6–13%) in CV events, that did not reach statistical significance (35, 38, 49, 50). Moreover, the ACCORD study was terminated after 3.5 years due to a 22% increase in overall mortality, demonstrating a possible harmful effect of intensive glycemic control (49). As mentioned above, in contrast to the "legacy effect" demonstrated in the DCCT and UKPDS studies, prolonged follow-up of these studies failed to demonstrate a similar legacy effect. In the VADT study the trend toward reduced risk for ASCVD disappeared as the difference in glycemic control became insignificant (51). A 6-year post trial follow-up of the ADVANCE trial, showed no difference in the risk of death from any cause or major macrovascular events, between the intensive- and standard glucose control groups (52). In the ACCORD trail, a 17 months follow-up after the early termination, also failed to demonstrate a significant difference in major cardio-vascular events between the groups (53). A longer follow up of 9 years did show an increase in cardiovascular-related death, but no change in all-cause death and non-fatal cardiovascular events (54).

EXPLAINING THE GAP BETWEEN GLYCEMIA AS A RISK MARKER VS. RISK FACTOR

Several explanations for the absence of robust cardiovascular benefit of improved glycemic control in the ACCORD, ADVANCE and VADT trials have been suggested, however none has been validated in a study designed to test these hypotheses. Therefore, we can only speculate on the mechanisms of the gap between risk marker and risk factor.

The first, obvious potential speculation is that hyperglycemia is not directly atherogenic in humans (despite *in vitro* and animal experimental findings) but instead is associated with a confounding factor that is the causative factor accelerating atherosclerosis. There are many potential candidate factors that could be operative in persons affected by diabetes. To name a few; hyperlipidemia, hypertension, chronic inflammation, prothrombotic state, microalbuminuria or overt nephropathy, and endothelial dysfunction. The obesity, insulin resistance and hyperinsulinemia of Type 2 diabetes may also be implicated.

Secondly, the long standing hyperglycemia in the ACCORD, ADVANCE and VADT trials might have caused diffuse and irreversible atherosclerosis and cardiovascular injury, which were beyond the beneficial effect of later improvements in glycemic control. This is again in contrast to the study population of the UKPDS and DCCT that recruited younger patients in the DCCT and newly diagnosed patients in the UKPDS. Evidence

supporting this hypothesis was found where patients with a higher level of coronary atherosclerosis measured by coronary-artery calcium score showed a lesser reduction in ASCVD events (55). The proatherosclerotic changes that may occur as a direct result of hyperglycemia may not be reversible by glucose lowering after a prolonged duration of hyperglycemia. Along these lines, subgroup analysis of the studies mentioned above demonstrated that intensive treatment benefited predominantly the younger and newly diagnosed population, similar to the cohort of the UKPDS and DCCT trials (41, 43).

Another hypothesis is that cardiovascular events triggered by severe hypoglycemia may offset any potential cardiovascular benefits that may occur with intensive glycemic control. Hypoglycemia was hypothesized but not proven to play a role in the excess mortality observed in the ACCORD trial. This was mainly due to the low target HbA1C (<6) which was suspected as a cause for excess hypoglycemia events, and the association between hypoglycemia and triggering of cardiac events (56). However, in a later analysis of the ACCORD data, aimed to better identify the population(s) at higher risk of mortality, only three significant interactions were found between baseline characteristics and effects of intensive vs. standard glycemia treatment on mortality: self-reported history of neuropathy, higher HbA1c and aspirin use (36). Furthermore, the risk of death appeared to be greater with the intensive compared to the standard strategy only when the average HbA1c was >7% (57). Obesity and polypharmacy with potential drug interactions were also suggested as possible explanations of the increased mortality.

It is feasible that cardiovascular benefits may only be seen when improvements from very poor to improved glycemic control are achieved but not when improving those who have milder degrees of hyperglycemia. Although the DCCT and UKPDS trials did not demonstrate significant reductions in ASCVD outcomes during the trials, they did demonstrate reductions after prolonged follow up.

Another possible cause for the lack of benefit was the aggressive treatment of other risk factors with statins, aspirin and ACE inhibitors in the more recently conducted ACCORD, ADVANCE, and VADT trials. Reduced mortality and morbidity to below what is expected in patients with T2D might have diminished the added effect of aggressive glycemic control. This was further evident in the STENO-2 trial. Intensified multifactorial intervention with tight glycemic control and the use of ACE inhibitors, aspirin, and lipid-lowering agents has been shown to reduce ASCVD events by 59%, CV related mortality by 57%, and all-cause mortality by 46% (58). Of note, the UKPDS and DCCT studies were performed before the widespread use of statins and ACE inhibitors, again suggesting that the beneficial effect of tight glycemic control is less evident when other risk factors such as hypertension and hypercholesterolemia are treated.

CONCLUSIONS

There is a gap between hyperglycemia as a consistent, reproducible risk marker demonstrated in numerous epidemiological studies and the somewhat underwhelming evidence of reduction in ASCVD in glucose lowering intervention trials. When considering all available evidence, hyperglycemia does appear to be at least partially causative (i.e., an ASCVD risk factor). Reductions in ASCVD have been demonstrated in patients with more recently diagnosed T2D or in younger patients with T1D. This is in keeping with the abundant experimental evidence that has demonstrated biological pathways in which hyperglycemia *per se* can accelerate the atherosclerotic process. The benefit is relatively small, however, and takes many years to manifest, in contrast to the more rapid and robust cardiovascular benefits of other therapies such as LDL lowering, antihypertensive therapy, inhibition of the renin angiotensin system and two of the newer classes of glucose lowering therapies, the SGLT2 inhibitors and GLP-1 receptor agonists. Given the major benefits of glucose lowering in reducing diabetic microvascular complications and the added small benefit of glucose lowering in reducing macrovascular events, optimization of glycemic control remains the cornerstone of diabetes therapy, especially in the context of microvascular complications. One should take heed, however, of the risks of aggressive control of hyperglycemia that have been well-demonstrated in numerous clinical trials, particularly that of severe hypoglycemia. As lowering glucose levels appears not to be the most effective measure to reduce ASCVD complications and aggressive control has not resulted in a clear benefit in those with long standing diabetes and milder levels of hyperglycemia and even might result in excess mortality, practicing physicians must carefully balance benefits vs. risks, as has been aptly highlighted in all national diabetes guidelines. Finally, as macrovascular complications start to develop well before clinically evident diabetes is diagnosed, non-glucose and modifiable ASCVD risk factors such as hypercholesterolemia, hypertension and smoking cessation should be aggressively implemented in the pre-diabetic phase.

AUTHOR CONTRIBUTIONS

AN wrote the manuscript. PS and CX critically reviewed the manuscript. GL wrote and reviewed the manuscript.

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REVIEW
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Postprandial Glucose Spikes, an Important Contributor to Cardiovascular Disease in Diabetes?

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Clinical trials investigating whether glucose lowering treatment reduces the risk of CVD in diabetes have thus far yielded mixed results. However, this doesn't rule out the possibility of hyperglycemia playing a major causal role in promoting CVD or elevating CVD risk. In fact, lowering glucose appears to promote some beneficial long-term effects, and continuous glucose monitoring devices have revealed that postprandial spikes of hyperglycemia occur frequently, and may be an important determinant of CVD risk. It is proposed that these short, intermittent bursts of hyperglycemia may have detrimental effects on several organ systems including the vasculature and the hematopoietic system collectively contributing to the state of elevated CVD risk in diabetes. In this review, we summarize the potential mechanisms through which hyperglycemic spikes may increase atherosclerosis and how new and emerging interventions may combat this.

Keywords: diabete, hyperglyacemia, inflammation, RAGE (receptor for advanced glycation end products), hematopoeis

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INTRODUCTION

Although the pathophysiological mechanisms through which individuals develop type 1 and type 2 diabetes are different, both conditions are characterized by elevated blood glucose levels and share a similar elevated risk of cardiovascular mortality (1). Despite the fact that benefits from intensive glucose-lowering treatment were obvious in reducing microvascular complications in initial trials such as the Diabetes Control and Complications Trial (DCCT) (type 1 diabetes), the Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) trials (type 2 diabetes) showed no obvious benefit from glucose lowering treatment in the short term (2). Even though new glucose-lowering treatments, such as glucagon-like peptide 1 (GLP1) agonists and sodium glucose receptor 2 uptake inhibitors (SGLT2i) reduce major adverse cardiovascular events (MACE), it is already evident that most of the therapeutic benefit is achieved independently of their reduction in HbA1c (3). Therefore, the extent to which a reduction of hyperglycemia in diabetes directly reduces cardiovascular disease (CVD) remains controversial. Although others have found a modest reduction of coronary heart disease by glucose-lowering treatment in large meta-analyses (4, 5), the effects are somewhat disappointing with no reduction of all-cause mortality being identified. The reasons for this are not completely understood, but it is thought that benefits of glucose-lowering

treatments may be partly counterweighed by an increased occurrence of severe hypoglycemic episodes associated with intensive insulin therapy. In reality, controlling traditional CVD risk factors including blood pressure and plasma lipids still remains the most successful strategy to reduce CVD mortality in diabetes (6). However, statins are less effective in people with diabetes, even if cholesterol levels are lowered equally (7), suggesting there is still a large unmet medical need for optimized cardiovascular risk-management in diabetes. The phenomenon that diabetes is characterized by high glucose levels and an increased risk of CVD, but glucose lowering treatment fails to effectively reduce this risk, is often referred to as the glucose paradox (8).

The fact that glucose lowering treatment in people with diabetes has not convincingly reduced the high risk of CVD does not rule out the possibility that high glucose levels are causally involved in the development of CVD. Studies investigating the long-term benefits of glucose lowering treatment have revealed a modest benefit of glucose-lowering treatment in both type 1 (9) and type 2 diabetes (10), and suggest that prolonged reductions are required to achieve this benefit. Epidemiological studies have also suggested that postprandial spikes of high glucose levels may be a more robust determinant of CVD risk than average glucose levels (11–15). These episodes of high glucose levels increase oxidative stress, which in turn has several detrimental downstream effects, activating immune cells, and keeping the vasculature in a persistent state of elevated risk of cardiovascular events (**Figure 1**). To further support this hypothesis, postprandial blood glucose (PBG) levels are more predictive for CVD than HbA1c levels. Even in people without diabetes, PBG levels independently predict CVD in the non-diabetic glucose range. If these individuals were grouped into the lowest (69–107 mg dL^{-1}) vs. the highest (150–194 mg dL^{-1}) PBG, there was a 27% increased risk of CVD in those that had poorer PBG control. Interestingly, these studies suggest that this association is a continuum for PBG, while for fasting plasma glucose levels there seemed to be a threshold effect at 100 mg/dL (5.6 mmol/L) (16). Along with the amplitude of the glucose spike, the duration outside of the "normal" range is also likely to be important. Recently it was shown that people with pre-diabetes spent equal amounts of time (∼50% of the day) as individuals with type 2 diabetes outside of the optimal glucose range (17). Therefore, we suggest that people with diabetes (both type 1 and type 2), glucose-lowering treatment strategies directed at increasing the time in the desired glucose range may be more effective in reducing CVD in diabetes.

In this review, we will summarize the findings that lead us to this hypothesis, discussing human studies as well as mouse models of transient hyperglycemia (i.e., akin to exploring the glucose effects of PBG spikes). We propose that expansion of myeloid cells plays a central role in this process, and we will discuss potential mechanisms, which are initiated when glucose reaches toxic concentrations that may contribute to our understanding of the glucose paradox. These include but are by no means limited to; epigenetic memory, protein modifications [advanced glycation endproducts (AGEs)], and the pattern recognition receptor for AGEs (RAGE).

IMPORTANCE OF CONTINUITY TO GLYCEMIC CONTROL: POSTPRANDIAL GLUCOSE CONCENTRATIONS MAY CAUSE THE MOST HARM IN DIABETES

Elevation of fasting and PBG levels in diabetes are determined by distinct mechanisms of defective insulin secretion and/or signaling (18), and may occur independently of each other. Generally, upon food consumption, the incretins, glucose-dependent insulinotropic peptide (GIP), and glucagon-like peptide (GLP-1) are released by intestinal L cells to suppress glucagon release and stimulate insulin secretion. It has been appreciated for decades that the incretins are responsible for 50–70% of the insulin release following an oral glucose challenge, while this dramatically drops to ∼20% in people with type 2 diabetes (19). Thus, controlling PBG is more challenging in these people. Controlling carbohydrate intake, food composition, and promoting physical activity to lower PBG in people with diabetes is important, along with interventional care (20, 21).

The association between fasting plasma glucose and risk of CVD is not linear (6), suggesting that there is a certain threshold above which glucose is less tolerated and becomes toxic. Previous studies have shown that glucose levels after an oral glucose tolerance test (OGTT) are more strongly associated with carotid intima media thickness (cIMT), a marker of atherosclerosis, rather than fasting plasma glucose or HbA1c (22). In fact, post OGTT glucose levels were also more strongly associated with cardiovascular mortality than HbA1c (23). Moreover, pharmacological interventions with glyburide or repaglinide, that reduce postprandial hyperglycemia, promote regression of cIMT, suggesting an improvement in atherosclerotic CVD burden (24). Therefore, diabetic individuals predisposed to experiencing hyperglycemic spikes may be at increased risk of developing CVD.

Unfortunately, research to establish the causal effect of hyperglycemia on CVD, independent of other risk factors associated with diabetes, has been hampered by the lack of appropriate animal models (25). Most experimental models of diabetes are complicated by concomitant changes in the lipid spectrum (generally elevated VLDL/LDL and triglycerides), which makes interpretations about the specific effects of high glucose *per se* on cardiovascular disease particularly difficult to conclude. For example, the streptozotocin (STZ)-induced model of type 1 diabetes diabetes consistently accelerates atherosclerosis and vascular inflammation in the $Apoe^{-/-}$ mouse. Importantly however, diabetes in this model is characterized by concomitant increases in lipid levels, when a fed chow (26) or a high fat/low cholesterol diet (27), potentially confounding any effects of high

Abbreviations: AGEs, Advanced glycation endproducts; CVD, Cardiovascular disease; CMPs, Common myeloid progenitors; cIMT, Carotid intima media thickness; HbA1c, Glycated hemoglobin; GLO1, Glyoxalase 1; HDACi, Histone-deacetylase inhibitors; RAGE, Receptor for advanced glycation endproducts; ROS, Reactive oxygen species; SGLT2i, Sodium glucose cotransporter 2 inhibitor; STZ, Streptozotocin.

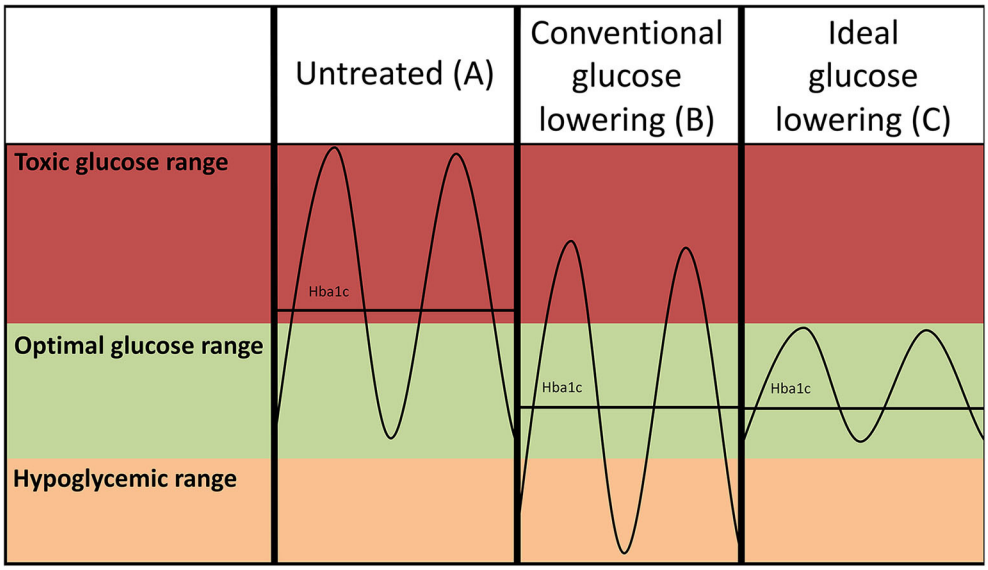

FIGURE 1 | Hypothetical glucose curves of an untreated patient with diabetes **(A)** and after conventional intensive glucose lowering (insulin-based) treatment regimens **(B)**. Lowering of average glucose values may come at the expense of increased risk of hypoglycemia as the amplitude of the glucose excursions is not suppressed. Ideal glucose lowering strategies **(C)** selectively decrease postprandial glucose spikes, but do not increase risk of hypoglycemia. Please note that such a strategy reduces the amplitude of the glucose excursions, but may not necessarily further reduce HbA1c as the average glucose levels are not further reduced.

glucose alone. While these studies are important in describing the role of hyperglycemia, the mechanisms contributing to accelerated atherogenesis by hyperglycemia, independent of cholesterol, remain unclear.

However, there are some models that allow the investigation of the effects of hyperglycemia on atherogenesis without the confounding effects of hypercholesterolemia. In a model of viral-induced type 1 diabetes in $Ldlr^{-/-}$ mice fed a cholesterol-free diet, Renard et al. (28) reported that hyperglycemia, independent of changes in plasma cholesterol, increased atherosclerotic lesions. This finding was also observed in another model where $Ldlr^{+/-}$ mice were employed. Rendering these mice diabetic with STZ and feeding a cholesterol/cholic acid-containing diet produced similar cholesterol levels between the diabetic and non-diabetic mice and still resulted in accelerated atherosclerotic lesion formation (29). Conversely, while the induction of diabetes in *Ldlr/Apoa-I* double knockout mice fed a cholesterol-enriched diet also had no effect on lipid levels, these mice did not develop larger atherosclerotic lesions (30). However, it must be noted that the deletion of *Apoa-I* (i.e., no HDL), could have altered the lesions in the non-diabetic group.

We also designed a model to isolate the effects of hyperglycemia from lipids, but in a clinically relevant model of atherosclerotic lesion regression. This was done by establishing lesions in $Ldlr^{-/-}$ mice by feeding them a modified Western Type Diet (WTD; 0.15% cholesterol as opposed to 0.2%) for 16 weeks and then switching the mice to a chow diet to lower plasma cholesterol and induce lesion regression. A group of mice were made diabetic with STZ in this period and importantly, plasma cholesterol levels were reduced to similar levels compare to control mice. In this study we also treated a group of diabetic mice with a sodium glucose cotransporter 2 inhibitor (SGLT2i), isolating the effects of glucose. We made the discovery that hyperglycemia impaired atherosclerotic lesion regression by promoting enhanced monocyte production from the bone marrow, causing their persistent entry into the atherosclerotic plaque (31) (**Figure 2**). This suggests that inflammatory changes in at least type 1 diabetes models are largely mediated by hyperglycemia, and not merely by changes in insulin or lipid levels.

In addition to the above-mentioned studies reporting into long-term effects of high glucose on atherosclerosis, several *in vivo* models to assess the acute effects of high glucose on the vasculature have also been developed. Firstly, increasing glucose levels up to 6 h in mice during a hyperglycemic clamp induced the sustained expression of inflammatory genes in the vasculature even after 6 days of normoglycemia (32). Interestingly, these effects were recapitulated by merely administering a series of glucose boluses in non-diabetic mice (33) suggesting that the dynamics of the glucose excursion following glucose administration may an important predictor of cardio-metabolic health. In fact, we recently showed that the repeated administration of such hyperglycemic spikes robustly increases atherosclerosis and myeloid cell expansion in otherwise normoglycemic $Apoe^{-/-}$ mice, while their lipid profile and HbA1c remained unaltered (34).

In conclusion, experimental studies have supplied evidence that glucose contributes to increased atherosclerosis in diabetes independently of changes in lipids or insulin. However, the relative contribution of glucose to atherogenesis seems to differ greatly between mouse models and the use of atherosclerosis-prone diets. Profound hyperlipidemia in some models may

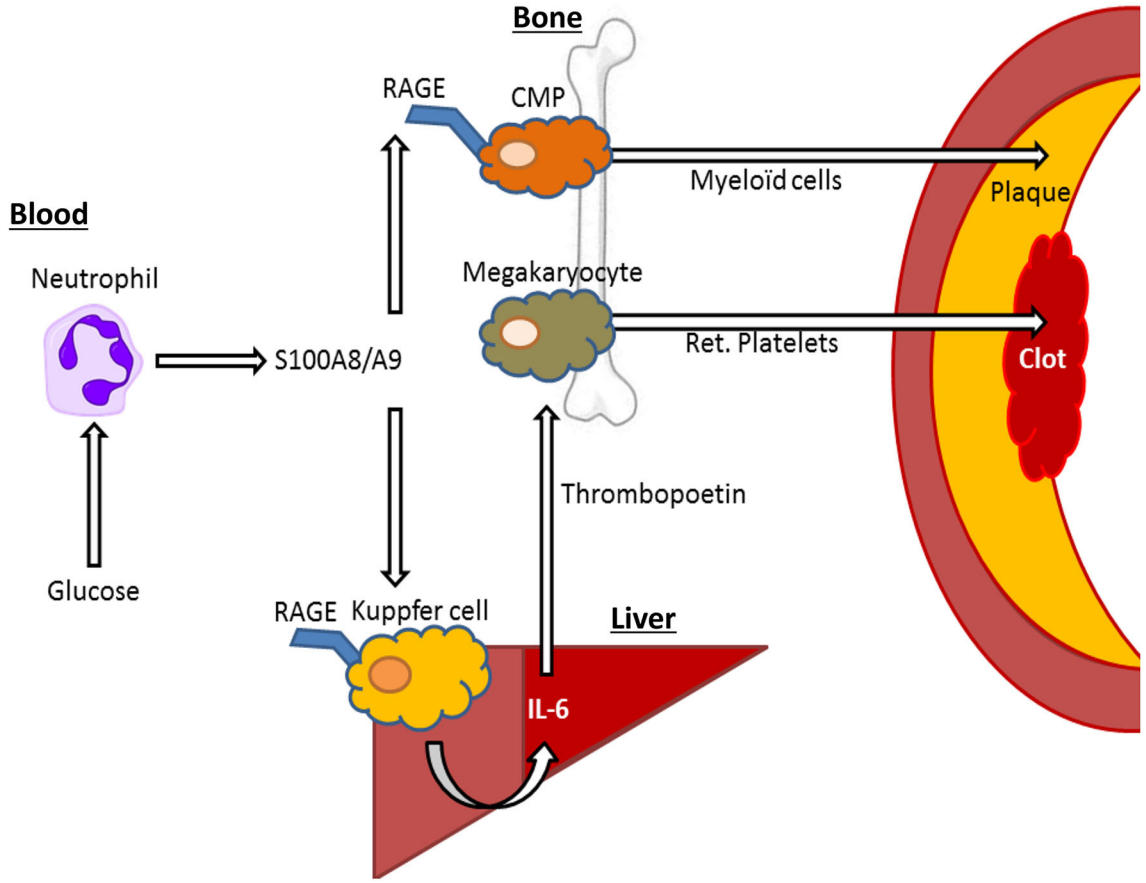

FIGURE 2 | S100A8/A9—RAGE interactions on Kupffer cells and common myeloid progenitor (CMP) cells may drive elevated cardiovascular risk by hyperglycemia. In response to hyperglycemia, neutrophils release S100A8/A9, interacting with RAGE in Kupffer cells in the liver, leading to IL-6 mediated thrombopoietin release by hepatocytes that in turn leads to increased reticulated (ret.) platelet production, that contribute to atherothrombosis. Furthermore, S100A8/A9 interacts with RAGE on CMPs, leading to increased differentiation into neutrophils and monocytes (myeloid cells), which migrate to the plaque promoting plaque growth and inhibiting plaque regression.

"mask" any contributions by high glucose, and this phenomenon remains poorly understood. As lipid metabolism differs greatly between mice and humans (35), the extent to which these findings translate to humans is unknown. Future studies to address this issue are clearly warranted.

WHICH TISSUES ARE SENSITIVE TO HIGH GLUCOSE WITH RELEVANCE TO CVD?

Atherosclerosis is a systemic vascular disease, in which a complicated interplay between the pre-existing cells of the vasculature and recruited inflammatory cells occurs, forming an advanced atherosclerotic lesion. Experimental studies have identified several cell types including endothelial cells and immune cells that are especially sensitive to high glucose levels.

The endothelium appears particularly sensitive to changes in glucose, likely due to its constant exposure to blood glucose fluctuations. Despite expressing the insulin receptor, endothelial cells are unable to restrict the amount of glucose they uptake, as the glucose transporter 1 (GLUT-1) is not down-regulated in response to high glucose (36). Furthermore, more recent findings indicate that increases in circulating myeloid cells, derived from bone marrow progenitors, are directly influenced by changes in blood glucose and are not merely due to secondary responses to vascular injury. For example, in response to high blood glucose levels, neutrophils secrete the damage associated molecular pattern (DAMP) molecules such as S100A8/A9 which interact with RAGE on myeloid progenitor cells in the bone marrow to promote monocytosis and impair lesion regression (31) (**Figure 2**). Exposing neutrophils to high levels of glucose results in their activation and release of S100A8/A9 via increased glycolytic flux and generation of reactive oxygen species (34). Diabetes, through neutrophil-released S100A8/A9, also causes liver inflammation (37). This occurs through interaction with RAGE on Kupffer cells, in turn promoting the production of IL-6, which stimulates thrombopoietin (TPO) production in the liver. We found that the increased production of TPO promoted platelet production, which appeared to be responsible for reticulated thrombocytosis, commonly seen in people with

diabetes (37) (**Figure 2**). These immature platelets are often more reactive and inflammatory that their mature counterparts and can accelerate atherosclerosis, as we revealed in diabetes and through genetic models of enhanced production (37–39). Thus, we suggest that neutrophils are an innate sensor of hyperglycemia to promote glucose-induced inflammation.

TRANSIENT HIGH GLUCOSE EXPOSURE MAY CONTRIBUTE TO SUSTAINED RISK OF CVD

In an attempt to explain the lack of efficacy and/or delayed response in glucose lowering treatment in reducing CVD risk in diabetes, several authors have postulated that high glucose induces a metabolic memory (or causes a so-called legacy effect). According to this hypothesis, a high glucose induced phenotype is "remembered" by the vasculature, which in an individual with diabetes develops into a persistently increased risk of CVD, even when blood glucose is lowered (**Figure 1B**). Support for a metabolic memory to high glucose arose from the findings from the long-term follow-up of the DCCT. Despite the fact that a difference in HbA1c was no longer detectable between the intensive glucose lowering and control group after the initial trial period, the individuals from the intensive glucose-lowering treatment group did display a reduced risk of CVD at long-term follow-up relative to the control group (9). Furthermore, STZ treated *Apoe*$^{-/-}$ mice in which their diabetes regressed and thus were only transiently hyperglycemic, displayed equal plaque development compared to STZ injected *Apoe*$^{-/-}$ mice that remained diabetic (40). Although the mechanisms that contribute to metabolic memory are still far from clear, the endothelium seems particularly susceptible to metabolic memory. For example, transient exposure of cultured endothelial cells to high glucose was sufficient to induce a persistent increase in p65 expression, a major subunit of the NF-κB complex, (32). In fact, when non-diabetic mice were subjected to a hyperglycemic clamp, the increased p65 expression in endothelial cells isolated from the aorta persisted for up to 1 week. This memory appears to be associated with the accumulation of reactive oxygen species (ROS), as ROS production continued for at least 1 week after normalization of glucose and was paralleled by an increase in inflammatory gene expression, which could be reduced with a ROS inhibiting agent (41). Whether glucose imparts significant epigenetic modification in hematopoietic cells (stem or mature) that directly influence atherosclerosis is yet to be fully understood, but could also be an important mechanism in which transient high glucose levels contribute to CVD.

Recently, we explored PBG spikes using a model termed "transient intermittent hyperglycemia" in mice, by injecting glucose four times in a day spaced 2 h apart (34). We discovered that exposure to these PBG spikes 1 day per week accelerated atherogenesis, which was driven by enhanced myelopoiesis. The transient rises in blood glucose activated circulating neutrophils through glycolysis and oxidative stress, which support previous hypotheses.

AGEs

AGEs are irreversible, long-term sugar modifications of proteins. Therefore, it is likely that once formed in the vasculature during hyperglycemia, they lead to a long-term disruption of cellular function even after blood glucose has been normalized. The accumulation of AGEs, first described as consequence of heating it (42), was considered mostly a slow and passive process *in vivo* (43), especially for AGEs such as pentosidine or glucosepane which have been linked to crosslinking of vascular collagen, leading to vascular remodeling, arterial stiffness and which, in turn, may increase cardiovascular risk (44). Indeed, in line with this concept, Monnier et al. (45) showed lower AGE levels in skin biopsies of DCCT subjects that received intensive glucose-lowering treatment.

Furthermore, rapid AGE formation from highly reactive intermediates of glycolysis is now considered the major mechanism of AGE formation *in vivo* (46). Methylglyoxal (MGO) has been identified as the major precursor for AGEs (47). MGO is a byproduct of glycolysis, and in conditions of intracellular hyperglycemia leads to a rapid increase of AGEs (36). MGO is increased in diabetes, closely tracks with PBG levels (48) and is associated with CVD in type 1 (49) and type 2 diabetes (50). Prevention of MGO and AGE accumulation by overexpressing glyoxalase 1 (GLO1) (51), the major enzyme to detoxify MGO, prevented endothelial dysfunction (52) and ROS formation (51) in STZ treated rats. MGO has not only been linked to the modification of cytosolic proteins, but also has been shown to directly modify and damage DNA (53). In fact, a link between accumulation of MGO and development of a epigenetic memory has been made by El-Osta et al. Overexpression of GLO1 prevented the persistent increase of p65 expression in endothelial cells following transient hyperglycemia, while GLO1 knockdown mice, displayed increased p65 expression in their endothelial cells even when normoglycemic (32). This is perhaps not surprising, given that MGO accumulation is closely linked to formation of ROS (52). The lowering of AGEs has been proposed as a treatment for diabetic CVD, as AGE inhibiting compounds, such as alagebrium, and pyridoxamine have been shown to reduce atherosclerosis in diabetic *Apoe*$^{-/-}$ mice (26). However, it is important to note that AGE levels within the atherosclerotic lesions of *Ldlr*$^{-/-}$ mice failed to correlate with lesion size (54), thus it is still debated where AGEs impart their atherogenic effects.

While elevated AGEs (specifically the AGE moieties N$^{\varepsilon}$-(carboxyethyl) lysine (CML) and pentosidine) are observed in people (55) and animals with type 1 diabetes (51), plasma AGE [CML, pentosidine and N$^{\varepsilon}$-(carboxyethyl) lysine (CEL)] levels are not increased in individuals with type 2 diabetes (56). This discrepancy may be explained by the fact that formation of AGEs is a complex and heterogeneous process, and AGEs may also be formed as a result of glucose-independent processes such as lipid-oxidation and other types of ROS (57). Furthermore, AGE levels (of CML and 5-hydro-5-methylimidazolone (MG-H1) are strongly increased in unstable plaques, but these plaque AGE levels were not associated with plasma glucose levels, and were not higher in individuals with type 2 diabetes (58). Together,

these findings indicate that AGE levels are not determined by hyperglycemia alone. In fact, plaque CML and MG-H1 levels may be produced in response to plaque inflammation and hypoxia, rather than hyperglycemia (58). Nonetheless, plasma levels of CML and CEL were strongly associated with incident CVD in both individuals with type 1 diabetes (59), and type 2 diabetes (60), although in type 2 diabetes this association became much stronger for CML after adjustment for BMI. This finding is likely explained by the fact that obese adipose tissue traps certain circulating AGEs (CML in particular) in a RAGE-dependent manner, reducing their plasma levels (61). Interestingly, although crosslinking AGE pentosidine was associated with CVD in type 1 diabetes, this association was not present in type 2 diabetes (60, 62). Taken together, these results also underline that type 1 and type 2 diabetes are metabolically distinct diseases, and therefore yield different patterns of AGEs.

Few clinical studies have been performed to investigate whether AGE-lowering compounds are suitable for use in humans. Unfortunately, aminoguanidine, the best known AGE-inhibitor induced glomerulonephritis in a small subset of subjects at higher dosages (63), and its use in clinical studies has therefore been discontinued. Other well-known AGE inhibiting compounds, such as alagebrium, have been used in small clinical trials with mixed results (64). It remains to be tested if these or any other AGE-lowering compounds will reduce CVD endpoints in individuals with either type 1 or type 2 diabetes (64), but based on these initial studies, efforts should be made to develop new AGE-lowering compounds with stronger AGE inhibition, but fewer non-glycation directed properties, yielding a more favorable toxicity profile.

RAGE

A number of studies have shown that RAGE plays a pivotal role in the development of atherosclerotic lesions in diabetes, as deletion of RAGE almost completely reduces the extent of atherosclerosis in diabetic $Apoe^{-/-}$ mice (65). Since RAGE deletion also reduces atherosclerosis in non-diabetic mice, its protective effects on atherosclerosis cannot be fully derived from prevention of high glucose dependent effects. We showed that glucose induced monocytosis, a major determinant of plaque growth, was almost completely dependent on hyperglycemia-dependent RAGE signaling in the bone marrow (31). However, RAGE did not mediate increased monocytosis in a model of obesity and only mild hyperglycemia (66), suggesting a threshold above which glucose levels induce RAGE signaling. RAGE may thereby mediate a sustained inflammatory response induced by hyperglycemic spikes by releasing increased amounts of monocytes in the circulation. Further complexity for the involvement of RAGE in glucose mediated vascular damage was discerned as hematopoietic or tissue (non-hematopoietic) deletion of RAGE equally inhibited atherosclerosis in diabetic $Apoe^{-/-}$ mice (67). Perhaps the link is that RAGE is associated with sustained NF-κB activation (68), and as such, a major link between hyperglycemia, ROS, and inflammation.

There is also compelling evidence that RAGE is associated with diabetes and CVD in humans. RAGE expression is increased in plaques of the carotid artery of individuals with type 2 diabetes (69), which can be lowered with statin treatment (70). Although the exact consequence of increased RAGE expression in human atheroma is not clear, it does not seem to influence or predict plaque vulnerability directly, as the expression of RAGE does not differ between stable or ruptured plaque segments (58). However, RAGE has been shown to co-localize with MMP9, a major marker for plaque rupture (70).

In addition to its role as the receptor for AGEs, RAGE is also a well-recognized pattern recognition receptor that can interact with various inflammatory molecules, such as S100A8/A9, S100B, and HMGB1. It is difficult to discern which ligand for RAGE plays the most important role in the development of CVD, as S100A8/A9, S100B, and HMGB1 as well as the AGEs have all been shown to increase due to high glucose (71), and are associated with CVD (58, 62, 72). S100A8/A9 appear to be the important biological ligands of RAGE, at least in driving monocyte and neutrophil production from the bone marrow. Transplantation of $S100a9^{-/-}$ bone marrow into WT mice that were subsequently made diabetic with STZ were protected from hyperglycemia-induced leukocytosis (31). Additionally, depletion of neutrophils (the main source of S100A8/A9) in diabetic mice normalized S100A8/A9 levels and normalized circulating blood monocyte levels but had no effect on the expression of RAGE on the bone marrow common myeloid progenitors CMPs (31). This suggests that at least in respect to monocyte production, other RAGE ligands (i.e., AGEs and HMGB1) are insufficient in stimulating RAGE signaling in the CMPs to produce monocytes. Further, when we isolated neutrophils from mice with diabetes or diabetes + SGLT2i we observed that lowering blood glucose normalized the expression of *S100a8* and *S100a9*, without impacting *Hmgb1* expression, providing more evidence for the importance of S100A8/A9 compared to HMGB1 (31). In line with our hypothesis that glucose and PBG spikes drive atherogenesis in mice, these same pathways were initiated in mice exposed to transient intermittent hyperglycemia (34).

APPROACHES TO REDUCE POSTPRANDIAL HYPERGLYCEMIA AND REDUCE CVD

Traditionally, intensive insulin-based regimens have been hampered by concomitant increase of hypoglycemia (**Figures 1A,B**), and intensification of glucose-control with insulin use has even been linked to an increase of all-cause mortality in vulnerable individuals with type 2 diabetes (73). Higher frequencies of hypoglycemia are associated with a higher risk of CVD (74). The mechanism through which hypoglycemia increases risk of CVD is not completely understood, but profound sympathetic activation in response to low glucose, leading to strong hemodynamic changes, is presumed to play a large role (75). New approaches to lower glucose have been developed to reduce postprandial hyperglycemia with a lower risk of hypoglycemia events. The underlying concept would be

to lower glucose excursions (**Figure 1C**) rather than lowering average glucose which comes at the expense of increased risk of hypoglycemia (**Figure 1B**). A novel approach to refine insulin use is the development of a bionic pancreas (76). This allows for continuous glucose monitoring such that insulin as well as glucagon release are automated, yielding much more precise glucose control. Furthermore, as mentioned below SGLT2i and GLP1 agonists, which effectively lower hyperglycemia, also appear to avoid hypoglycemic events. Since their striking cardiovascular effects have mainly been addressed in cardiovascular safety studies, at short follow-up times, it is actually possible their true benefits will be even greater at longer duration of use, as benefits of glucose-lowering treatments take a long time to cause clinically meaningful results.

Sodium Glucose Co-transporter 2 Inhibitors

Arguably one of the most important drug developments over the past decade to treat people with diabetes is the SGLT2i's. SGLT2i's empagliflozin, dapagliflozin, and canagliflozin, have been linked to striking reductions in heart failure and chronic kidney disease in their land-mark cardiovascular safety studies (EMPA-REG, DECLARE, CANVAS). Their effect on HbA1c however, is overall modest, and their effect on MACE is most likely mediated by glucose-independent, mainly hemodynamic, mechanisms (77). Although SGLT2i do not carry an inherent risk of hypoglycemia, their use has been linked to increased risk of a (normoglycemic) ketoacidosis, warranting careful use of these compounds in type 1 diabetes. Nonetheless, continuous glucose monitoring studies have revealed interesting effects of these compounds on glucose variability.

The effect of SGLT2i on hyperglycemic excursions seems to be largely influenced by the context in which they are given. For instance, empagliflozin monotherapy was found not to reduce postprandial glucose excursions or 24-h glucose variability (78), while when added to insulin therapy empagliflozin significantly lowered glucose excursions in type 2 and even in type 1 diabetes. Sotagliflozin, an inhibitor of both the SGLT1 and SGLT2 receptor, has also been shown to substantially decrease the number of hyperglycemic episodes in individuals with type 1 diabetes (79). These beneficial effects on glucose variability are likely achieved in large part due to decreased insulin dosages leading to less pronounced glucose peaks, and less offshoot effects by hypoglycemia, since SGLT2i mainly increase renal glucose excretion.

GLP-1 Agonists

GLP-1 agonists (e.g., liraglutide, semaglutide, exenatide, etc) lower HbA1c by 1–2% points compared to usual care plus placebo and reduce cardiovascular disease (80, 81). The profile of their beneficial cardiovascular effect differs considerably from SGLT2i's, with less pronounced reductions in heart failure and more overall reduction in CVD. This likely reflects the distinct mechanism of action of GLP-1 agonists compared to SGLT2i's. Given the pleiotropic effects of GLP-1 agonists, the precise mechanisms of action leading to cardiovascular risk reduction is unclear. Interestingly, the FLAT-SUGAR Trial has shown that exenatide on top of long-acting insulin reduced glucose excursions more than a four daily insulin regimen (82). Thus, reductions in glucose excursions may be, at least in part, an important cardio-protective mechanism offered by GLP-1 agonists and warrants further investigation.

Designer Cytokines

Over the past few decades, an appreciation of the interaction between cytokines and metabolic regulation has evolved. While most cytokines relay important signals around the body during times of inflammation, some cytokines also serve important basal roles. Once such cytokine is IL-6, known to promote inflammation under many conditions, but also to be increased during bouts of exercise with positive effects on metabolism via GLP-1 (83). A recent study from the Febbraio group took the novel approach of generating a cytokine-like molecule that combined IL-6 and the leukemia inhibitor factor receptor (LIFR) binding domain of ciliary neurotrophic factor (CNTF) (84). This unique designer cytokine, termed IC7Fc, was able to retain the positive metabolic, without the inflammatory effects of IL-6, while also preserving CNTF's effects on satiety. This was achieved by the molecule preferentially docking to the gp130 cytokine signaling receptor with either the LIFR or the IL-6R. When administered to mouse models of obesity and diabetes, IC7Fc was able to prevent weight gain and in turn improve glucose tolerance and hyperglycemia, along with protecting the liver from steatosis (84). Positive effects were also seen in the musculoskeletal system. However, no cardiovascular outcomes were reported which is critical in the development and approval of therapies against metabolic diseases. Although, one would hypothesize that, given the strikingly positive effects on metabolism, IC7Fc may also reduce CVD. Another interesting observation that leads us to hypothesize that IC7Fc could protect against CVD was the positive effects on the bone. Extending this observation, we ponder if IC7Fc treatment would impact the hematopoietic stem cell microenvironment to retain stem cells and prevent unwanted extramedullary hematopoiesis which has been shown to directly influence atherosclerosis (85, 86). This new class of therapy in the area of cytokines, opens up other avenues where cytokines may be utilized to have positive effects on metabolism. These could include NLRP1/IL-18 axis and IL-33, both of which can have multiple roles in inflammation, but potent anti-obesity effects (87). Whether targeting any of these pathways could limit glycemic variation and ultimately reduce CV events is an exciting prospect and remains to be explored.

CONCLUSION

Evidence suggests some reduction in CVD risk by long-term glucose lowering treatments in people with diabetes. This effect however, seems limited to certain specific subpopulations, and is minor in type 2 diabetes with a complicated risk spectrum at best, with some studies even reporting harmful effects due mostly to hypoglycemia (74). Nonetheless, epidemiological research now suggests that postprandial high glucose "spikes" as opposed to high average glucose levels are a more important determinant in CVD development in diabetes. Therefore, we argue that new

glucose lowering strategies should be more directed against the reduction of postprandial spikes, than of HbA1c and/or average glucose, as such a strategy should avoid concomitant episodes of hypoglycemia. The extent to which HbA1c captures transient hyperglycemic episodes seems to be highly dependent on the type of diabetes, and degree of overall glycemic control.

Based on the transient hyperglycemia experiments in mice, we speculate that hyperglycemic spikes may be sufficient to sustain an individual's increased risk of CVD. Importantly, pre-clinical studies have also established that hyperglycemia plays a causal role in the development of CVD in diabetes and even impairs the resolution of lesions in the setting of cholesterol lowering. This can be reversed by the administration of a SGLT2i's. If this translates into clinical outcomes with SGLT2i's and other new interventions, without the risk of hypoglycemia, this would suggest that such compounds would be attractive combat CVD in diabetes. Whether the SGLT2i can sufficiently reduce hyperglycemic spikes and prevent its consequences in humans remains to be determined. In humans, their use has been showed to significantly reduce hyperglycemia (88), and therefore, clinical trials evaluating their effect on cardiovascular risk are eagerly awaited. The limited risk-reducing effects from insulin treatment have been attributable to the low compliance issues caused by subsequent weight gain and episodes of hypoglycemia. Hopefully, the development of the bionic pancreas will improve benefits of intensive insulin use. Whether the bionic pancreas will have any place in the management of advanced type 2 diabetes remains to be determined.

Compounds erasing epigenetic marks, inhibiting formation of AGEs and signaling of the RAGE receptor may provide promising therapeutic targets for treating the consequences of hyperglycemic spikes in diabetes. The molecular mechanisms on which these compounds operate are closely linked and may form a positive feedback loop through formation of ROS. Now, large clinical trials are needed to evaluate whether these new compounds actually reduce the risk of cardiovascular disease in humans with both type 1 and type 2 diabetes, bearing in mind that the results may differ in the two very different diseases. Furthermore, additional mechanisms, beyond the score of the current review, such as impaired collateral vessel formation, may play a role in the detrimental effects of hyperglycemic spikes.

CLINICAL PERSPECTIVE

Based on experimental studies, several strategies have been identified to reduce the risk of CVD in people with diabetes. These include reducing hyperglycemic spikes, epigenetic marks, accumulation of AGEs, and inhibiting RAGE ligands. However, we are still awaiting the evaluation of compounds intervening with these pathways in large-scale clinical trials.

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All authors contribute to writing and editing the manuscript.

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REVIEW
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Clinical Approach to Assessment and Amelioration of Atherosclerotic Vascular Disease in Diabetes

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Atherosclerotic cardiovascular disease is increased on average 2–3-fold in people with diabetes as compared to their non-diabetic counterparts and is the major cause of the increased morbidity and mortality in this disease. There is however heterogeneity in cardiovascular risk between individuals based on demographic, cardiometabolic and clinical risk factors in the setting of hyperglycemia, insulin resistance and obesity that needs to be taken into consideration in planning preventive interventions. Randomized clinical trials of agents or procedures used for amelioration of augmented CVD risk in diabetes have been pivotal in providing evidenced-based treatments. Improvement in hyperglycemia in both type 1 and type 2 diabetes is considered to be central in the prevention of microvascular and macrovascular complications although selected antihyperglycemic agents have demonstrated beneficial as well as possible deleterious off-target effects. Lowering low density lipoprotein cholesterol, treating hypertension and stopping smoking each play important roles in preventing cardiovascular disease in diabetes as they do in the general population and low dose aspirin is overall beneficial in high risk individuals. Hypertriglyceridemia may represent another important marker for augmented cardiovascular risk in diabetes and newer agents targeting dyslipidemia appear promising. The fall in cardiovascular events over the past two decades offers hope that modern intervention strategies as well as novel approaches such as those targeting inflammation may contribute to a continued reduction of cardiovascular disease in people with diabetes.

Keywords: diabetes, cardiovascular disease, risk assessment, prevention, clinical trials

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INTRODUCTION

It has been recognized for decades that people with diabetes have an increased risk for atherosclerotic vascular disease (ASCVD). The Framingham Study was one of the early studies that reported that cardiovascular disease (CVD) events in those with diabetes was increased 3-fold in men and 4-fold in women (1). Coronary heart disease (CHD) rates were double in men and 3 times higher in women with diabetes than their non-diabetic counterparts, with similar excess rates for stroke except that that these sex differences were reversed. Event rates for peripheral vascular disease and heart failure (HF) were increased even more, especially in women (8–10-fold). It has become clear that ASCVD is the leading cause of morbidity and mortality in diabetes and its health and economic burden has grown with the epidemics of obesity and diabetes. Furthermore, it has

become clear that while augmented atherosclerosis is the major factor underlying the high rates of CVD in diabetes, structural and functional abnormalities of cardiac muscle and its autonomic innervation have a major influence on morbidity and mortality, particularly in older people (2). As a consequence, understanding the nature of CVD and developing strategies for its prevention and treatment in people with diabetes has become a priority.

HETEROGENEITY IN THE RISK FOR ASCVD IN DIABETES

In 2001, the National Cholesterol Education Panel in its Adult Treatment Panel III guidelines recommended that adults with diabetes and without CVD be considered a CHD risk equivalent, assigning a 10 year ASCVD risk of at least 20% (3). However, it subsequently became evident that while this may be true in older people with long-standing diabetes (4) there is significant heterogeneity of risk for ASCVD in people with diabetes (5, 6). Among key determinants of risk are demographic factors such as age, sex, race/ethnicity, and socioeconomic status, duration and type of diabetes, and the number and severity of major risk factors including hyperglycemia itself, as well as risk enhancers, some of which are specific to diabetes and others that are not (**Table 1**). It is also likely that genetic factors play an important role. How these factors interact to accelerate atherosclerosis in diabetes is incompletely understood.

TABLE 1 | Known associations between demographic, clinical and cardiometabolic risk factors and increased atherosclerotic cardiovascular disease (ASCVD) risk in diabetes.

Factor	Direction of association with ASCVD risk
Demographic	
Age	Increased
Sex	Women have a greater increase in relative risk; men have a greater increase in absolute risk
Race/Ethnicity	South Asians have greater risk
Socioeconomic	Increased in lower socioeconomic groups
Duration of diabetes	Increased
Major risk factors	
LDL-C	Increased with no apparent threshold for risk
Hypertension	Increased from a systolic blood pressure of 120 mm Hg
Smoking	Increased
HDL-C	Decreased in population studies, but HDL function may be a better risk factor
Hyperglycemia	Increases risk but studies are confounded by off-target effects of anti-hyperglycemic agents; findings clearest in type 1 diabetes
Insulin resistance	Increased
Dyslipidemia	Hypertriglyceridemia associated with increased risk
Risk enhancers	Increased (See **Table 2** for list)

LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.

Pathophysiologic Issues

The central, clinically relevant pathophysiologic abnormalities in diabetes are hyperglycemia, insulin deficiency and insulin resistance and the accompanying alterations in metabolic fluxes. While hyperglycemia defines diabetes, varying only in severity, insulin resistance coupled with defective insulin secretion is typically found in type 2 diabetes whereas type 1 diabetes is caused by severe insulin deficiency. Obesity which is linked to the development of type 2 diabetes, is a major determinant of insulin resistance. Obesity is also increasingly being recognized as a feature of type 1 diabetes as intensive insulinization is often associated with weight gain. It is the interplay of hyperglycemia and insulin resistance and the accompanying metabolic alterations complicated by obesity that is thought to drive oxidative stress, subclinical inflammation, and a procoagulant state, which leads to the functional and structural tissue changes that characterize cardiovascular damage in type 1 and type 2 diabetes (7).

Demographic Factors

As for the general population, the absolute risk for ASCVD in diabetes increases with age in both type 1 and type 2 diabetes (8, 9) although the relative risk is highest in young adults and then falls with age. Women appear to lose their relative protection from CHD and stroke and have a greater relative risk compared to men but this falls as they age such that the prevalence of ASCVD becomes similar in elderly men and women with diabetes (10). Most minority groups have lower rates of ASCVD compared to Caucasians except for South Asians, a point that has been emphasized in a recent American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines report (11), and socioeconomic status is associated with higher mortality in type 2 diabetes (12). While the basis for these differences in effects of demographic factors on ASCVD risk in diabetes is poorly understood, their clinical relevance is significant.

Onset and Duration of Diabetes

ASCVD risk is related to duration of diabetes independent of aging (13) although it is confounded by age. Onset of diabetes is usually obvious in type 1 diabetes, particularly when this develops in children and adolescents in whom ASCVD is rare before age 30 years (14) but the onset of type 2 diabetes is more insidious and diabetes may be present for years before clinical diagnosis. Added to this the incidence of type 2 diabetes has been increasing in obese children and adolescents and it is likely that their ASCVD risk will be substantial in young and mid-adulthood (15) although definitive evidence is not yet available. Also uncertain is how much ASCVD risk is increased in newly diagnosed diabetes in the elderly. Underlying these considerations is our lack of understanding of the impact of the pre-clinical phase of diabetes on atherosclerosis. Depending upon how it is defined, up to 1 in 3 individuals have prediabetes and many of these individuals will develop type 2 diabetes (16). Hence the origin of accelerating atherogenesis likely begins early in the course of development of type 2 diabetes and there is evidence that people with prediabetes already have modestly increased ASCVD risk (17) thus offering

an opportunity for intervention in this early, identifiable phase of type 2 diabetes.

Risk Factors and Risk Enhancers

Major Risk Factors

The major ASCVD risk factors, hypercholesterolemia, cigarette smoking and hypertension are strongly related to development of ASCVD in diabetes as in the general population, although compared to non-diabetic subjects matched for these three risk factors, the incidence of CHD mortality remains 2-fold increased in diabetes, indicating the importance of other determinants of risk (18). Low density lipoprotein cholesterol (LDL-C) levels are similar in diabetes to those without diabetes, but the frequency of hypertension is ∼2-fold increased in diabetes. Clinical management of these major risk factors together with treatment of hyperglycemia constitutes the basis for primary and secondary prevention of ASCVD in diabetes.

Hyperglycemia and Insulin Resistance

Much of the underlying substrate for ASCVD risk is likely to be related to hyperglycemia, insulin resistance and obesity and the accompanying pro-inflammatory and procoagulant states. The degree of hyperglycemia is related to CVD risk in populations without known diabetes (19) although the associations are attenuated after adjustment for other risk factors and this is true for obesity as well. More compelling in those with established diabetes is the evidence that improving hyperglycemia reduces ASCVD in clinical trials of anti-hyperglycemic agents as discussed below, although this is confounded by off-target effects of the antidiabetic medications. There is also evidence that insulin resistance is associated with ASCVD (20). However, this evidence is based mostly on epidemiologic assessments of insulin resistance which incorporate glucose values and are imperfect surrogate measures of insulin resistance—particularly in diabetes, and have generally not led to clinically useful risk assessment or intervention strategies with the possible exception of dyslipidemia.

Dyslipidemia—an Atherogenic Tetrad

Insulin resistance in type 2 diabetes is thought to be a key determinant of hypertriglyceridemia and reduced high density lipoprotein cholesterol (HDL-C), both common abnormalities in type 2 but not type 1 diabetes and they have been associated with ASCVD risk in type 2 diabetes (21). The HDL-C level is inversely and strongly related to ASCVD, and is included together with age, sex, total cholesterol, blood pressure (BP), and presence or absence of smoking in the risk factor algorithms used to quantify ASCVD risk in diabetes (22–24). Lack of success in clinical trials to raise HDL-C pharmacologically has led to the notion that the basis for the strong inverse association between HDL-C and ASCVD may be related to HDL dysfunctionality (25), which is not sufficiently captured by the HDL-C value in high risk states where HDL may be dysfunctional. In support of this concept, very high HDL-C was shown paradoxically to be a direct risk factor for ASCVD in type 1 diabetes (26) in whom HDL-C levels tend to be elevated (27). Triglyceride levels are a less powerful risk factor for ASCVD in diabetes, and triglyceride-lowering with pharmacologic agents has not been shown to be associated with a reduction of ASCVD events. More likely hypertriglyceridemia is a marker for other metabolic abnormalities such as dysfunctional HDL, atherogenic small dense LDL and remnant lipoprotein particles, making up an atherogenic tetrad (28, 29). Methods that efficiently quantify lipoprotein subfractions have demonstrated that selected subfractions are strongly correlated with insulin resistance and are currently in clinical use although it remains for them to be shown to be independent predictors of ASCVD in diabetes.

TABLE 2 | Risk enhancers for atherosclerotic cardiovascular disease (ASCVD) (14).

Specific to Diabetes	General
RISK ENHANCERS	
Long duration (≥10 years for type 2 diabetes mellitus or ≥20 years for type 1 diabetes mellitus	Family history of premature ASCVD
Albuminuria ≥30 mcg of albumin/mg creatinine	LDL-C levels >160 mg/dl
eGFR <60 mL/min/1.73 m^2	Metabolic syndrome
Retinopathy	Chronic kidney disease
Neuropathy	History of preeclampsia or premature menopause in women
Ankle brachial index <0.9	Chronic inflammatory disorders
	High-risk ethnicity such as South Asian ancestry
	Triglyceride levels persistently >175 mg/dl
	If measured, elevations in apolipoprotein B (may be useful if hypertriglyceridemia >200 mg/dl persists
	High sensitivity C reactive protein >2 mg/L
	Lipoprotein(a) levels with elevations above 125 nmol/L (50 mg/dl) (especially useful in those with a family history of ASCVD)
	Reduced ankle brachial index

eGFR, estimated glomerular filtration rate.

Risk Enhancers

The concept of risk enhancers was recently incorporated into risk assessment (24) to include factors that are not typically included in risk factor algorithms yet are sufficiently associated with ASCVD event rates to warrant consideration in risk assessment (**Table 2**). Of relevance to individuals with diabetes these include hypertriglyceridemia, elevated apolipoprotein B as a marker of increased numbers of atherogenic particles, and chronic kidney disease, which is common in diabetes due to development of diabetic nephropathy, manifesting as albuminuria or as reduced glomerular filtration rate. Subclinical tests of peripheral vascular and coronary artery disease such as the ankle brachial index obtained by Doppler ultrasound and the coronary calcium score measured by computerized tomography are strongly related to future occurrence of ASCVD in diabetes

although their clinical utility is unclear. Lastly the presence of any form of diabetic microangiopathy whether it be retinopathy, peripheral neuropathy, or nephropathy are all associated with increased risk of ASCVD possibly because of common pathways for vascular damage (30–32) and should be considered in risk assessment.

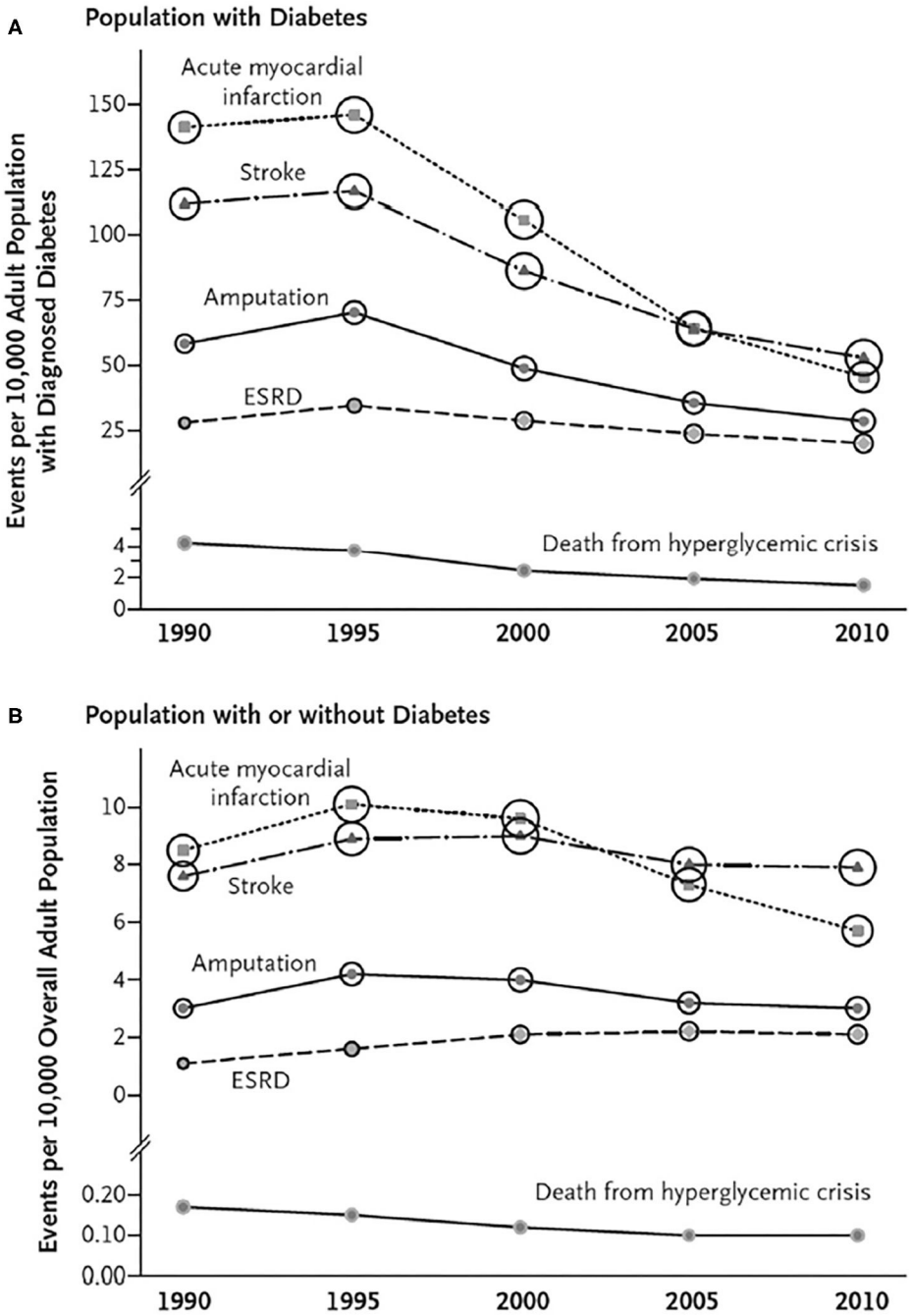

FIGURE 1 | Trends in Age-Standardized Rates of Diabetes-Related Complications among U.S. Adults with and without Diagnosed Diabetes, 1990–2010. For rates of myocardial infarction, stroke, and leg amputation, numerators are from the National Hospital Discharge Survey; for rates of end-stage renal disease (ESRD), numerators are from the U.S. Renal Data System, and for rates of death from hyperglycemic crisis, numerators are from the National Vital Statistics System. Denominators are from the National Health Interview Survey **(A)** and the U.S. Census Bureau **(B)**. Circle size is proportional to the absolute number of cases (e.g., the number of cases of acute myocardial infarction ranges from 140,122 in 1990 to 135,743 in 2010, and the number of cases of death from hyperglycemic crisis ranges from 2890 in 1990 to 2361 in 2010). **(A)** Shows trends for persons with diabetes, and Panel B shows trends for persons with or without diabetes [From (33); with permission].

RECENT TRENDS IN THE PREVALENCE OF ASCVD IN DIABETES

Data collected over the past three decades on the incidence of cardiovascular disease in diabetes indicates a significant decline in myocardial infarction (MI), stroke and leg amputation in the US and similar findings have been reported from other high income countries (**Figure 1A**) while this is not observed in the general population (**Figure 1B**) (34). Although this may have been influenced by earlier diagnosis of diabetes over time leading to an overall healthier population with diabetes, it is possible that improvements in management strategies that reduced ASCVD risk may have played a role. The fact that CVD event rates have been demonstrated to be strongly related to whether the LDL-C, BP and the glycosylated hemoglobin (HbA1c) level as a measure of glycemic control were at their respective targets supports this contention (35). In contrast to the fall in the incidence of occlusive atherosclerotic events, the incidence of HF with its attendant high morbidity and mortality has been increasing (36). Most cases are thought to be due to CHD, but in recent years it has become apparent that the combined effects of diabetes, obesity and aging cause cardiomyopathic changes leading to loss of left ventricular compliance and a form of HF (HF with preserved ejection fraction) that does not respond well to conventional therapies for HF with reduced ejection fraction that typically results from MI (37, 38).

These findings provide an incentive to initiate effective, evidence-based interventions in people with diabetes particularly in view of the fact that the mortality rate after a first event in those with diabetes is significantly increased compared to those without diabetes (39). The approaches to prevention of ASCVD in diabetes described below form the basis for similar recommendations from both United States and European society guidelines (22–24, 40).

APPROACH TO PREVENTION OF ASCVD

Weight Reduction

Despite the fact that intensive lifestyle modification achieving prolonged moderate weight loss and increased physical activity in type 2 diabetes has been shown in a controlled clinical trial to produce favorable changes in CVD risk factors, it did not lead to a reduction in major adverse cardiovascular events (MACE) over a 10 year period (41). Greater degrees of weight reduction are achieved by bariatric surgery and in a large controlled but non-randomized study in which the control group received standard diabetes and obesity management, the gastric bypass surgery group had fewer MI events but not stroke (42). In a more recent controlled randomized study, gastric bypass had significant and sizable benefit for HF and renal disease outcomes but not on MI or stroke suggesting that the benefits of weight reduction for diabetic complications are greatest for cardiac and renal dysfunction rather than for atherosclerotic events (43).

Hyperglycemia

Sulfonylureas, Metformin, Insulin

The first definitive study to show that improving glycemic control in type 2 diabetes lowered the risk of complications, tested the effects of intensified treatment with the sulfonylurea insulin secretagogues as primary therapy to which insulin could be added, vs. standard care with diet and addition of sulfonylureas to treatment only to prevent severe hyperglycemia. While intensified treatment lowered the risk of microvascular complications over the 10 period, the effect on MI did not quite reach significance (44). Importantly though the effect of newly introduced metformin, which inhibits hepatic glucose overproduction through an effect on AMP kinase, did show a beneficial effect in a parallel smaller substudy, but not when combined with sulfonylurea drugs (45), raising questions about the use of sulfonylurea agents for prevention of ASCVD. However, long term follow-up of the original intensified treatment group during which the HbA1c levels in the intensive and standard groups were no longer different, found a significant reduction in MI events suggesting the existence of a legacy effect of improved glycemia that has been attributed to metabolic "memory" (46). Similar long-term follow-up findings were obtained after intensive insulinization in young adults with type 1 diabetes. Although there was no benefit on ASCVD during a 6.5 year period of intensive glycemic vs. standard control, after a further 12 years of follow-up when HbA1c values became similar in the two groups, total CVD events were reduced by 42% and MACE by 57% in the intensively treated group (47). These data in type 1 diabetes are the best evidence that improved glycemic control reduces ASCVD risk, because the two study groups received treatment with the same agent, namely insulin. There have been no equivalent studies with insulin only in type 2 diabetes.

Thiazolidinediones

The issue of possible deleterious off-target effects became a further concern after rosiglitazone, the first of the thiazolidinediones, which activate peroxisome proliferator activated ɤ-receptors (PPARɤ), was found to be associated with an increase in MI and CVD death (48). Subsequently pioglitazone, a thiazolidinedione with somewhat more favorable effects on CVD risk factors thought to be related to differences from rosiglitazone in binding to PPARɤ, was shown to have beneficial effects on MACE and especially stroke in type 2 diabetes (49, 50). However, both agents increase risk for HF, at least in part through water retention (51).

DPP4 Inhibitors, GLP-1 Agonists, and SGLT2 Inhibitors

More recently the newer antihyperglycemic agents namely the dipeptidyl peptidase 4 inhibitors (DPP-4i), the glucagon like peptide-1 agonists (GLP-1a) and the sodium/glucose transporter 2 inhibitors (SGLT2i) have all been tested in clinical trials for non-inferiority to standard treatments with pre-existing agents on ASCVD outcomes, as is now required for new antidiabetic agents by the US Food and Drug Association because of concern for deleterious off-target effects. Compared to therapy with older agents, DPP-4i had no effect on ASCVD events other than an increase in HF long-term attributable mainly to the SAVOR-TIMI trial with saxagliptin (52). However, clinical trials with GLP-1a have demonstrated that overall these agents modestly reduce MACE by 8% but not CVD death or HF (53). By

contrast SGLT2i's reduced HF and CVD death by 24% in patients with pre-existing ASCVD, lowered recurrent ASCVD events by 14% events and decreased the worsening of renal disease by 26% (54). They are also fairly effective in lowering BP (55) which likely contributes to their beneficial effects. Furthermore, their benefit for cardiorenal outcomes especially HF resembles the findings noted after gastric bypass and points to possible common mechanisms that tie these two forms of therapy together in prevention of cardiorenal complications. Although there were small differences in HbA1c between the test and standard care groups in these studies, these were not found to account for the beneficial effects of the GLP-1a and SGLT2i and so these benefits are considered to be off-target protective cardiovascular effects though they are not well-understood (56).

Clinical Guidelines for ASCVD Reduction Through Glucose Lowering (Figure 2)

The studies with sulfonylurea, metformin, insulin, and the thiazolidinediones provide support for the recommendation that improvement of glycemic control has long term benefits on ASCVD risk in both type 1 and 2 diabetes although they did not point to a clear target for this treatment. In addition they raised questions about active treatment differences between sulfonylureas and metformin and with rosiglitazone that pointed to possible deleterious off-target effects on ASCVD. Subsequent large observational studies suggest that sulfonylurea agents are associated with a higher incidence of CVD and death than metformin (57) that has relevance given that these two drugs are still the most commonly used antidiabetic medications in type 2 diabetes in part because of their inexpensiveness. The possible deleterious effects of sulfonylurea agents may be due to their inhibition of pre-ischemic conditioning (58); for rosiglitazone the mechanism is unknown. More recently three large clinical trials using various combinations of available antidiabetic medications including insulin but with minimal use of GLP1a and SGLT2i compared intensive vs. standard glycemic treatment aimed at reaching HbA1c values below what has become the usually accepted HbA1c target of 7% as a measure of good glycemic control (59). They showed trends but no significant benefit for CHD events and in one of them there was actually an increase in mortality forcing the trial to be stopped. These trials also drew attention to the risks of hypoglycemia in sulfonylurea and insulin treated patients since its incidence was increased in these studies. Although it was unclear from these studies whether increased hypoglycemia contributed to the lack of benefit, prospective studies have demonstrated that severe hypoglycemia is accompanied by an increased risk of CHD (60). Overall, when these data were included in a meta-analysis with the earlier studies, an average reduction of HbA1c from 7.8–6.9% was associated with a significant reduction of 15% in CHD outcomes (61).

Based on the current evidence, GLP-1a and/or SGLT2i typically with metformin are favored for glycemic management for type 2 diabetes in those with established ASCVD and possibly in those with high risk for ASCVD, with the goal of achieving an HbA1c of <7% if this can be done safely (22, 23, 40).

Dyslipidemia (Figure 2)

LDL-C

People with type 2 diabetes and a small number with type 1 diabetes in the 40–75 year age group were included in most

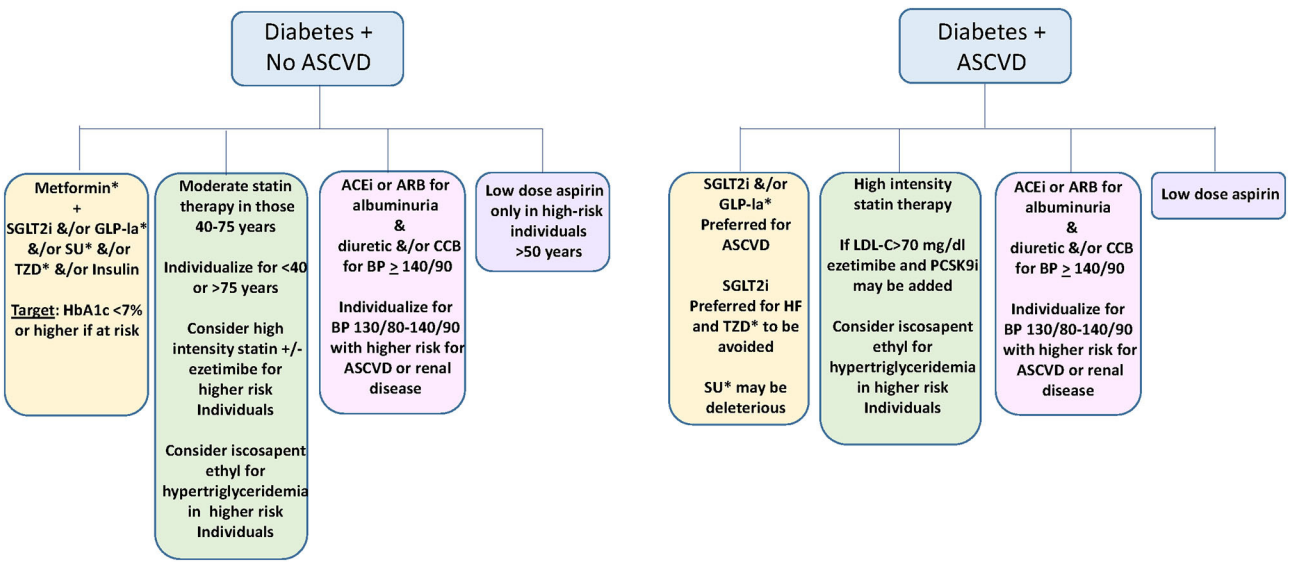

FIGURE 2 | Suggested approach to medical prevention and amelioration of ASCVD in diabetes (22–24, 41). Yellow; Glycemic control. Green: Lipid management. Pink; Blood pressure management. Violet; Use of low dose aspirin. SGLT2i, sodium glucose transporter 2 inhibitor; GLP-1a, Glucagon like peptide 1 agonist; SU, sulfonylurea; TZD, thiazolidinedione; ACEi, Angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker; LDL-C, low density lipoprotein cholesterol. *not used in type 1 diabetes.

of the placebo controlled trials with statins and benefitted in a similar manner to those without diabetes, although because of their higher ASCVD event rates, the absolute reduction in events was always greater in those with diabetes, both in primary and secondary prevention studies (62). There have been 3 primary prevention trials conducted in large cohorts with diabetes and average LDL-C levels, and another that recruited individuals with and without prior ASCVD (63). All used moderate intensity statin therapy which lowers LDL-C ∼30% and overall they demonstrated that ASCVD relative risk was lowered 25% with no apparent difference in benefit between type 1 and 2 diabetes. This led to the recommendation that moderate intensity statin therapy is indicated for adults with diabetes aged 40–75 years. Assessment of ASCVD risk using quantitative risk assessment algorithms is not deemed necessary for this decision although these algorithms may be useful in refining risk assessment in individual patients. Furthermore, since the residual risk for ASCVD events remained in the intermediate risk range after moderate intensity statin-treated individuals, high intensity statin therapy which lowers LDL-C ∼50% and which has been shown to lead to incremental benefit (62) is preferred for primary prevention in those with multiple risk factors as is recommended for patients with established ASCVD (22–24, 40).

Ezetemibe, an intestinal cholesterol absorption inhibitor, may be added to reach this goal if necessary in view of its incremental effectiveness when added to a statin (64). For secondary prevention in very high risk individuals into which category older patients with diabetes fall, an LDL-C target of <70 mg/dl has been proposed which may require the addition of inhibitors of propeptide convertase subtilisin/kexin 9 (PCSK9i). PCSK9i prevent the action of this protein to promote intracellular catabolism of the LDL receptor (22–24, 40) and like ezetimibe, PCSK9i have been shown to further reduce ASCVD events in high-risk statin treated individuals with diabetes in proportion to its additive LDL-C lowering (65, 66). Since there are very little or no data on the benefit of pharmacologic LDL-C lowering in people with diabetes below the age of 40 years or those older than 75 years, this decision is left to medical judgement based on perceived benefit vs. safety (24).

Triglyceride

Since hypertriglyceridemia and reduced HDL-C are common in type 2 diabetes despite dietary recommendations aimed at losing weight through reduction of refined carbohydrate and saturated fat, and likely contribute to ASCVD risk, triglyceride-lowering agents such as fibric acid derivatives and high dose omega 3 fatty acid preparations have been evaluated for their utility in preventing CVD in type 2 diabetes. These agents have been largely unsuccessful in demonstrating benefit for ASCVD in placebo-controlled clinical trials although they have generally not been specifically tested in hypertriglyceridemic subgroups with diabetes. Secondary analyses from the fibrate trials have suggested possible benefit for fibrate therapy in those with triglyceride values >200 mg/dl and HDL-C levels <35 mg/dl (67). In a recent clinical trial, icosapent ethyl (68), a synthetic derivative of the omega 3 fatty acid eicosapentaenoic acid was compared to placebo in a large statin-treated cohort either with CHD or type 2 diabetes without ASCVD and one risk factor, and with triglyceride levels >135 mg/dl. There was a 25% relative risk reduction in ASCVD events including on CVD death unrelated to the amount of triglyceride lowering achieved, suggesting the benefit was related to other effects of the specific omega 3 fatty acid used. This agent is now being recommended for high risk individuals with diabetes on statin treatment with residual hypertriglyceridemia (24). Trials with apo C-III, and angiopoioetin-like-3 antisense oligonucleotides are yielding promising results for treatment of hypertriglyceridemia that may yield benefit for ASCVD risk (69, 70). In addition studies with an antisense oligonucleotide nucleotide to lipoprotein (a) may become important in reducing risk related to this risk enhancer (71).

Hypertension (Figure 2)

Controlled clinical trials have clearly demonstrated that lowering BP to <140/90 reduces the risk of both microvascular and ASCVD complications in cohorts with diabetes (23). Reducing weight and lowering sodium intake lowers blood pressure but is usually insufficient. Pharmacologic treatment should begin with any of either an angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB), a calcium channel blocker, or a diuretic all of which individually have been shown to reduce ASCVD events in clinical trials mostly conducted in cohorts with large diabetes subgroups. For those with albuminuria or CKD, agents that reduce intraglomerular pressure such as an ACEi or ARB are favored because of their specific benefits for progression of renal disease (22–24) but many patients require multidrug therapy. Since the association between BP and ASCVD risk begins at values below 140/90, several clinical trials have tested more intensive treatment aimed at achieving lower BP targets. Overall there may be additional benefit for stroke and microvascular disease outcomes but not clearly for CHD events in those with diabetes, and there was an increased likelihood of drug side-effects, so one recommendation is to treat to a target of 140/90 with individualization for more intensive treatment to 130/80 in individuals with higher risk such as those with established ASCVD and renal disease (22, 23). Others have proposed more uniform treatment to a target of 130/80 in people with diabetes (40).

Aspirin (Figure 2)

Low dose aspirin's antiplatelet effect has been shown to be effective in reducing MACE. The relative risk reduction is about 25%, and stronger for MI than ischemic stroke but the risk of serious hemorrhagic complications particularly in the elderly although small, is a significant safety concern especially in primary prevention where the absolute risk for ASCVD events is considerably less than in those with established CVD. Accordingly while low dose aspirin is recommended to prevent recurrent ASCVD in diabetes, use of aspirin in primary prevention is proposed for those with diabetes in the 50–70 year age range who have at least one additional risk factor for ASCVD including renal disease (23, 24, 40).

Anti-inflammatory Agents

In a placebo-controlled clinical trial in a statin treated cohort with elevated high sensitivity C reactive protein level as a

measure of subclinical inflammation with a past history of MI, canakinumab, a monoclonal antibody to interleukin 1Lβ, reduced MACE by 15% in the mid-range dose although there was a higher incidence of fatal infections (72). Forty percent of the cohort had diabetes and the relative risk reduction in this subgroup was 10% which did not reach significance. Although not ready for clinical use, this study provides clinical evidence for the concept that inhibiting a pathway of inflammation may reduce ASCVD events. A subsequent trial with methotrexate an immunosuppressant and inhibitor of IL-6 binding was not effective, while colchicine, a microtubule inhibitor with anti-inflammatory effects reduced total CVD events although this was only significant for coronary revascularization and stroke (73, 74).

CONCLUSION

During the past 20 years significant advances have been made in understanding the relationship between clinically relevant risk factors in relation to age, sex and type and duration of diabetes and the augmentation of ASCVD in diabetes. These have led to the application of interventions targeting glycemic control, LDL-C lowering, BP lowering and the prothrombotic state that have demonstrated effectiveness in individual clinical trials to lower rates of ASCVD events. Incorporation of these findings into clinical guidelines has likely contributed to the fall in prevalence of MI, stroke and amputation in diabetes. Although there has been only one long-term controlled clinical trial evaluating the combined effects of multiple risk factor interventions on vascular complications in diabetes, it demonstrated a 53% reduction in CVD death and a 59% reduction in total CVD events over a 13.3 year follow-up (75). Widespread application of the guidelines combined with earlier diagnosis of diabetes together with newer developments of novel pharmacologic agents should strengthen and broaden efforts to improve quality of life and longevity in people with diabetes.

AUTHOR CONTRIBUTIONS

RG was solely responsible for preparing and writing the review.

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Plasma Amino Acids and Residual Hypertriglyceridemia in Diabetic Patients Under Statins: Two Independent Cross-Sectional Hospital-Based Cohorts

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Objective: The objective of the study was to investigate the relationship of amino acid metabolism with hypertriglyceridemia in diabetic patients under statins free of prior cardiovascular diseases.

Methods: Two independent cross-sectional hospital based cohorts, i.e., Liaoning Medical University First Affiliated Hospital (LMUFAH, $n = 146$) and the Second Affiliated Hospital of Dalian Medical University (SAHDMU, $n = 294$) were included in the current analysis. Hypertriglyceridemia was defined as triglyceride $\geq$1.7 mmol/L, and well-controlled LDL-C was defined as <2.6 mmol/L. The adjusted ORs (95% CI) of circulating metabolic measures for hypertriglyceridemia were assessed using logistic regression. Pooled results of metabolites with the same direction of association in both cohorts were combined using inverse variance-weighted fixed-effect meta-analysis. Difference of identified metabolites in patients with and without hypertriglyceridemia were also obtained in the context of LDL-C.

Results: Patients, 86 and 106, were with hypertriglyceridemia in LMUFAH and SAHDMU, respectively. We observed that elevated alanine, asparagine, leucine, and valine were consistently associated with increased hypertriglyceridemia in both cohorts. In fixed-effect pooled analysis, the OR (95% CI) per SD increase was 1.71 (1.32–2.20) for alanine, 1.62 (1.20–2.19) for asparagine, 1.64 (1.22–2.20) for leucine, and 1.62 (1.22–2.13) for valine (all P values ranged from 0.0018 to <0.0001); adjusting for C-peptide attenuated effect sizes of Ala, Leu, and Val for hypertriglyceridemia. The difference were robust in groups with well- or bad-controlled LDL-C.

Conclusion: Among 23 amino acids, alanine, asparagine, leucine, and valine were positively associated with increased residual risk of hypertriglyceridemia in diabetic patients with statin treatment.

Keywords: hypertriglyceridemia, amino acids, statins, type 2 diabetes, cardiovascular diseases

INTRODUCTION

Hypertriglyceridemia (HTG) is defined as triglyceride ≥1.7 mmol/L. Recent evidence suggest that HTG, as a risk factor for cardiovascular diseases (CVD), is independent of low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) (1–3). Patients with type 2 diabetes (T2D) are more likely to have dyslipidemia and elevated CVD risk (4, 5). For the prevention of CVD, lipid-lowering agents are generally recommended for people with diabetes (6, 7). As first-line lipid-lowering treatment, statins can markedly lower LDL-C by blocking synthesis of cholesterol in the liver, and reduce CVD risk subsequently (8, 9). By contrast, statins' effect on triglyceride (TG) is moderate, with high dose of statins reducing 20–40% of TG (10, 11). Substantial residual HTG contributes to increased risk of CVD even among diabetics with well statin-controlled LDL-C (8, 12, 13). High dosage of statins can induce a range of side effects related to rhabdomyolysis, cognitive impairment, hepatotoxicity, and so on (14, 15). So instead of intensive use of statins, combined medication may be a better option for patients with poor response to current statin therapy. Additional efforts are needed to identify these subjects and explore potential new targets for their TG lowering.

Liquid chromatography-mass spectrometry (LC-MS) enables high-throughput analysis of metabolites and provides novel insight into metabolic pathway discovery (16). Amino acids are important components for protein synthesis and play significant roles in a number of physiological processes including energy production, inflammation, signaling, insulin resistance, redox, and so on (17–20). In this connection, amino acids were identified as new biomarkers of chronic conditions such as diabetes, CVD, and obesity (16, 21–23). Metabolites have the potential of clinical utility with regard to assessing therapeutic effectiveness and response (24). Given that both TG and amino acids are closely linked to obesity and CVD development in T2D, we would like to know whether plasma-free amino acids can identify diabetic subgroups that can or cannot respond well to the TG-lowering property of statins and perform as potential novel therapeutic targets.

In this study, we aimed to assess (1) if amino acids are associated with residual hypertriglyceridemia in diabetic patients with statin treatment and (2) if the relationships remain in the context of LDL-C. Two independent cross-sectional hospital-based cohorts were included in the current analysis.

MATERIALS AND METHODS

Study Populations

The study involved individuals from two cross-sectional hospital-based cohorts in China. Details of the two cohorts are as follows:

Liaoning Medical University First Affiliated Hospital

The details of this cohort are described elsewhere (25). Briefly, from May 27, 2015 to August 3, 2016, serum metabolites were quantified from 1,032 consecutive diabetic patients. Clinical information was retrospectively extracted from electronic medical records. Among 1,032 patients, 288 patients were excluded due to lack of complete information on TG, LDL-C, and HDL-C. Of the remaining 744 patients, 287 with prior coronary heart disease (CHD) and stroke were excluded. Among the remaining 457 patients, 146 were with statin treatment, and 311 were without statin treatment. Finally, the main analysis included 146 individuals (1) with complete information on TG, LDL-C, and HDL-C, (2) with complete metabolomic profile, (3) without prior CHD and stroke, and (4) with statin treatment (**Figure 1**). The protocol of the study was approved by the Ethics Committee for Clinical Research of Liaoning Medical University First Affiliated Hospital (LMUFAH). Informed consent was waived due to the nature of the retrospective study, which was in accordance with the Helsinki Declaration of 1964 and its later amendments.

The Second Affiliated Hospital of Dalian Medical University

From April 2018 to April 2019, a total of 1,024 consecutive diabetic subjects were admitted into The Second Affiliated Hospital of Dalian Medical University (SAHDMU) and agreed to participate in this research. As in LMUFAH, electronic medical records were collected. Serum metabolites were quantified in 748 of them. Subsequently, six patients were excluded due to lack of complete information on TG, LDL-C, and HDL-C. Of the remaining 741 patients, 110 with prior CHD and stroke were excluded. The remaining 631 patients included 294 with statin treatment and 338 without statin treatment. The final analysis was limited to 294 individuals (1) with complete information on TG, LDL-C, and HDL-C, (2) with complete metabolomic profile, (3) without prior CHD and stroke, and (4) with statin treatment (**Figure 1**). The Ethics Committee for Clinical Research of SAHDMU approved the ethics of the study, and all the participants provided informed written consent.

Data Collection and Definitions

T2D was diagnosed by the 1999 World Health Organization's criteria (26) or treated with antidiabetic drugs. CHD was defined as having a history of angina with abnormal electrocardiogram or on stress test, myocardial infarction, angina coronary artery bypass graft surgery, or angioplasty; stroke was defined as non-fatal subarachnoid hemorrhage, intracerebral hemorrhage, or other unspecified intracranial hemorrhage and ischemic stroke; HTG was defined as triglyceride ≥1.7 mmol/L; treatment goals was <2.6 mmol/L for LDL-C, <1 mmol/L in male or <1.3 mmol/L in female for HDL-C, and <7% for glycated hemoglobin (HbA1c) (6).

Other available information in the current analysis included age, sex, current smoking, current drinking, body mass index (BMI), systolic blood pressure (SBP), fasting C-peptide (only available in SAHDMU), duration of diabetes, diabetic nephropathy (DN), diabetic retinopathy (DR), and use of antidiabetic agents. BMI was calculated as the ratio of weight in kilograms to height squared in meters; DR was evaluated by bilateral retinal photography and was defined as the presence of microaneurysms, retinal hemorrhages, soft exudates, hard exudates, or vitreous hemorrhage; DN was defined as persistent albuminuria, progressive reduction in glomerular filtration rate,

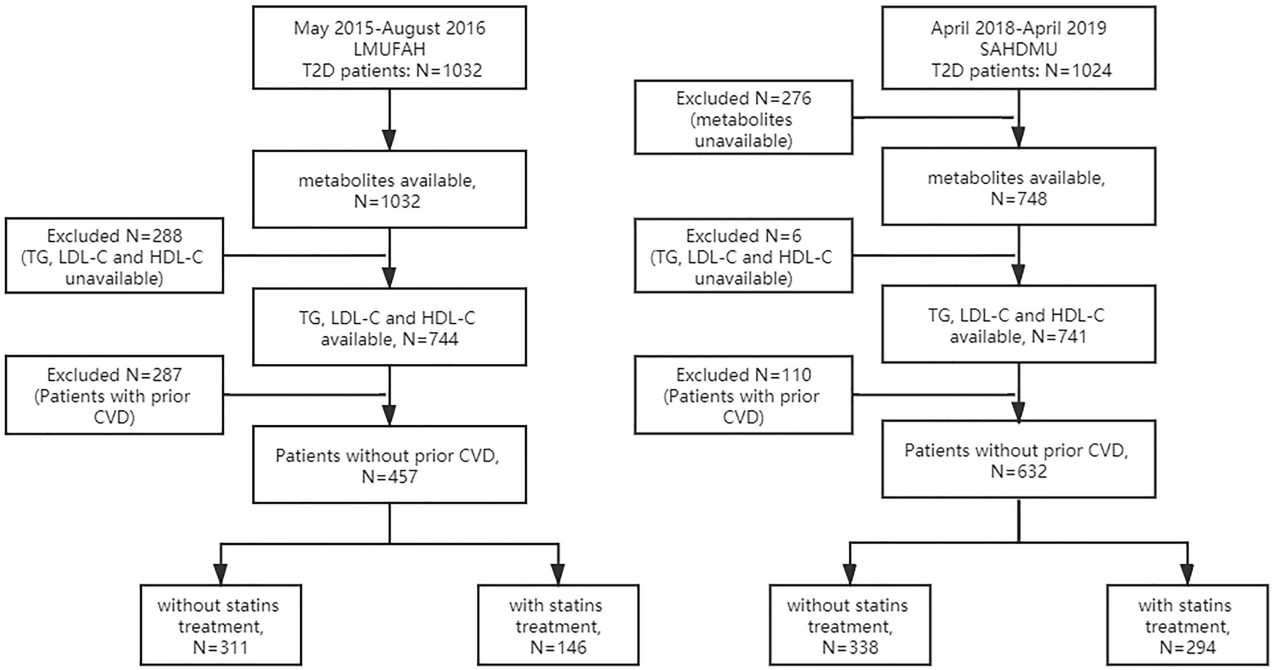

FIGURE 1 | Flow diagram of selection of subjects included in this study. LMUFAH, Liaoning Medical University First Affiliated Hospital; SAHODMU, the Second affiliated hospital of Dalian Medical University; T2D, type 2 diabetes; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CVD, cardiovascular diseases, including coronary heart disease and stroke.

and hypertension judged by clinicians (27); antidiabetic agents included insulin and other oral antidiabetic agents.

Amino Acid Quantification

Details of the metabolomics assessment method were published previously (28). Briefly, 8 h of fasting blood sample was collected at admission. A total of 23 amino acids, i.e., alanine (Ala), asparagine (Asn), leucine (Leu), phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr), valine (Val), arginine (Arg), glycine (Gly), proline (Pro), threonine (Thr), citrulline (Cit), glutamine (Gln), histidine (His), lysine (Lys), methionine (Met), serine (Ser), ornithine (Orn), glutamate (Glu), aspartate (Asp), piperamide (Pip), cysteine (Cys), and homocysteine (Hcy), were detected via LC-MS. AB Sciex 4000 QTrap system (AB Sciex, Framingham, MA, USA) was used to conduct direct injection MS metabolomic analysis. Analyst v1.6.0 software (AB Sciex) was used for data collection. ChemoView 2.0.2 (AB Sciex) was used for data preprocessing. Isotope-labeled internal standard samples were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Standard samples of the amino acids were purchased from Chrom Systems (Grafelfing, Germany).

Statistical Analysis

Characteristics of participants in two cohorts were described and compared according to TG levels (TG <1.7 vs. ≥ 1.7 mmol/L). Continuous data with normal distribution were expressed as the mean $\pm$ standard deviation (SD), while data with skewed distribution were presented as median with interquartile range (IQR). Normality was tested by checking the Q-Q plot. Categorical data were presented as n (%). Differences between subjects with optimal TG and HTG were compared by Student's t-test (or Mann–Whitney U test when appropriate) for continuous variables and Chi-square test (or fisher test if appropriate) for categorical variables. False discovery rate (FDR) was calculated for multiple comparisons of 23 amino acids and $q < 0.05$ was defined as statistically significant.

According to FDR, four amino acids with the same direction of association in both cohorts were selected into further analysis. The ORs (95% CI) of four circulating metabolic measures with HTG were assessed using logistic regression. Before introducing into regression models, all metabolites were scaled to SD concentrations separately for each cohort. A structured adjustment scheme was used to control for confounders: model 1, adjusted for age and sex; model 2, adjusted for variables in model 1 plus BMI, duration of diabetes, and DN; and model 3, adjusted for variables in model 2 plus HDL-C, LDL-C, and HbA1c. To explore whether insulin action mediated part of the effects of amino acid metabolism, we also adjusted for variables in model 3 plus C-peptide in model 4. Pooled results from individual cohorts were combined using inverse variance-weighted fixed-effect meta-analysis.

Pearson or Spearman correlation was used to calculate the coefficients within selected metabolites and clinical biochemical parameters, i.e., four amino acids, TG, HDL-C, LDL-C, HbA1c, and C-peptide.

TABLE 1 | Clinical and biochemical characteristics of participants in two cohorts according to TG levels.

	LMUFAH			SAHODMU		
	TG < 1.7 mmol/L	**TG ≥ 1.7 mmol/L**	***P***	**TG < 1.7 mmol/L**	**TG ≥ 1.7 mmol/L**	***P***
N	60	86		106	188	
TG, mmol/L	1.13 (0.83–1.35)	2.48 (2.03–3.20)	<0.0001	1.11 (0.88–1.42)	2.68 (2.04–3.87)	<0.0001
Age, years	56.0 ± 11.2	55.0 ± 12.2	0.6047	62.2 ± 11.0	57.8 ± 12.7	0.0011
Sex, male	33 (55.0)	48 (55.8)	0.9224	62 (58.5)	89 (47.3)	0.0663
Duration of diabetes, years	5 (0–11)	5 (0–10)	0.8364	11 (4–17)	8 (2–15)	0.0754
Body mass index, kg/m2	24.4 ± 3.4	26.3 ± 3.4	0.0016	26.6 ± 4.5	27.0 ± 3.5	0.3727
Current smoking	25 (41.7)	33 (38.4)	0.6890	25 (23.6)	39 (20.7)	0.5710
Current drinking	20 (33.3)	33 (38.4)	0.5333	10 (9.4)	22 (11.7)	0.5488
Systolic blood pressure, mmHg	140.0 ± 24.2	138.0 ± 22.5	0.6073	152.2 ± 19.8	148.9 ± 21.3	0.1947
HbA1c, %	10.3 ± 2.3	9.8 ± 2.2	0.1774	8.9 ± 2.2	9.2 ± 2.1	0.1747
≥7.0	47 (94.0)	70 (90.9)	0.7389	83 (78.3)	162 (86.2)	0.0822
HDL-C, mmol/L	1.17 ± 0.36	1.09 ± 0.29	0.1886	1.41 ± 0.42	1.08 ± 0.31	<0.0001
<1.00 in male or <1.30 in female	35 (58.3)	55 (64.0)	0.4920	23 (21.7)	115 (61.2)	<0.0001
LDL-C, mmol/L	3.13 ± 0.96	3.56 ± 1.04	0.0118	2.97 ± 0.80	2.68 ± 0.97	0.0062
≥2.60	42 (70.0)	72 (83.7)	0.0486	81 (76.4)	103 (54.8)	0.0002
Fasting C-peptide, ng/ml	-	-	-	1.12 (0.81–1.55)	1.75 (1.26–2.34)	0.0002
Antidiabetic agents	60 (100.0)	83 (96.5)	0.2688	101 (95.3)	176 (93.6)	0.6147
Diabetic nephropathy	23 (38.3)	21 (24.4)	0.0714	51 (48.1)	87 (46.3)	0.7619
Diabetic retinopathy	14 (23.3)	17 (19.8)	0.6042	26 (25.2)	58 (31.2)	0.2868

TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HbA1c, glycated hemoglobin; LMUFAH, Liaoning Medical University First Affiliated Hospital; SAHODMU, the Second affiliated hospital of Dalian Medical University.

Data are mean ± standard deviation, median (IQR), or n (%).

P values were derived from independent-samples Student t test for normally distributed variables, Mann-Whitney U test for skewed distributions, Chi-square test (or fisher test if appropriate) for categorical variables. P < 0.05 was defined as statistically significant.

To explore associations between amino acids and residual HTG in the context of LDL-C, we also repeated Student's *t*-test (or Mann–Whitney *U* test when appropriate) in subjects with LDL-C <2.6 mmol/L and LDL-C ≥2.6 mmol/L separately. Box plot was used to exhibit the distribution and difference visually. The same procedures were conducted in groups with either normal or abnormal HbA1c, and groups with either normal or abnormal HDL-C.

Given that we did not collect a specific dose of statins, to eliminate possible impact of statins on association between amino acids and TG, we also compared metabolic profile in subjects without statin treatment according to TG levels and obtained ORs (95% CI) using logistic regression as well.

All analyses were performed using SAS version 9.4 (SAS institute Inc., Cary, NC, USA) and R version 3.6.2.

RESULTS

Characteristics of the Study Population

The characteristics of the study participants are shown in **Table 1**. Characteristic distribution was different in two cohorts. In LMUFAH, among 146 subjects, 86 of them were with HTG. The 146 patients had a mean age of 55.4 (SD: 11.5) years and median duration of T2D of 5 (IQR: 0–10) years. Compared with patients with normal TG, patients with HTG had higher BMI and LDL-C. In SAHODMU, 106 of 294 subjects were with HTG. Mean age of 294 patients was 59.0 (SD: 12.2) years, and median duration of diabetes was 9 (IQR: 3–16) years. Compared with patients with normal TG, patients with HTG was younger and had lower HDL-C, LDL-C, and higher fasting C-peptide. Difference of other characteristics were not statistically significant in each cohort (**Table 1**).

Differences in Individual Amino Acids According to Triglyceride Levels

We observed four metabolites, i.e., Ala, Asn, Leu, and Val, demonstrating significant associations (all FDRs <0.05) in the same direction with HTG in both cohorts. Other amino acids were similar between patients with and without HTG (**Table 2**).

For the subgroup with LDL-C <2.6 mmol/L, the directions of associations between amino acids and HTG were consist with the directions in the total group. However, in LMUFAH, the only difference in Ala was significant ($P < 0.05$). We speculated a non-significant difference in other three amino acids derived from a small sample size ($N = 32$); in SAHODMU, the difference in Asn, Leu, and Val were significant ($P < 0.05$), while the difference in Ala was not significant (**Figure 2**). For the subgroup with LDL-C ≥2.6 mmol/L, the directions of associations between amino acids and HTG were also consist with the directions in the total group, and all differences were significant except for Asn in SAHODMU (**Figure 3**).

TABLE 2 | Plasma amino acids levels in two cohorts according to TG levels.

	LMUFAH			SAHODMU		
	TG < 1.7 mmol/L	**TG ≥ 1.7 mmol/L**	***q***	**TG < 1.7 mmol/L**	**TG ≥ 1.7 mmol/L**	***q***
Ala, μmol/L	119.70 ± 37.28	136.70 ± 40.31	0.0109	164.90 ± 50.98	185.70 ± 57.04	0.0092
Arg, μmol/L	8.67 (5.24–15.01)	10.56 (5.77–16.65)	0.4471	3.36 (1.88–4.61)	2.80 (1.86–4.56)	0.8663
Asn, μmol/L	72.23 ± 19.55	84.12 ± 24.09	0.0219	69.60 ± 16.67	78.22 ± 25.54	0.0029
Asp, μmol/L	27.59 ± 12.17	29.86 ± 10.99	0.3966	25.79 ± 10.53	28.14 ± 12.30	0.3157
Cit, μmol/L	21.19 ± 6.94	20.91 ± 7.32	0.8926	24.67 (20.48–30.63)	22.17 (17.18–29.75)	0.0157
Cys, μmol/L	1.24 ± 0.58	1.31 ± 0.58	0.5591	1.33 (0.80–1.87)	1.29 (0.85–1.94)	0.8663
Gln, μmol/L	6.31 (4.71–8.71)	7.40 (5.79–9.14)	0.1734	8.50 (5.72–11.06)	7.97 (5.83–11.02)	0.8663
Glu, μmol/L	87.99 (75.81–102.94)	94.78 (83.50–111.10)	0.0843	129.08 (104.02–157.22)	129.58 (107.28–153.97)	0.8663
Gly, μmol/L	184.30 ± 72.09	210.10 ± 80.46	0.1592	165.10 (141.94–185.09)	162.88 (144.52–186.65)	0.3157
Hcy, μmol/L	7.91 (6.45–8.63)	7.72 (6.40–8.16)	0.3291	8.57 (8.02–9.27)	8.48 (8.01–9.17)	0.8663
His, μmol/L	41.28 (32.77–66.60)	47.31 (34.91–77.75)	0.2578	64.27 (42.37–93.25)	67.07 (42.16–99.20)	0.7181
Leu, μmol/L	120.00 ± 39.89	145 ± 48.52	0.0219	111.34 (95.16–126.09)	122.35 (106.21–148.89)	0.0023
Lys, μmol/L	130.30 ± 60.30	131.30 ± 59.29	0.9194	134.32 (99.20–173.80)	137.53 (86.14–191.94)	0.8663
Met, μmol/L	16.47 (13.99–21.43)	16.37 (14.21–21.10)	0.6456	14.80 ± 3.95	14.40 ± 5.14	0.8663
Orn, μmol/L	16.57 (12.44–23.93)	17.49 (12.95–22.57)	0.9194	11.24 (9.11–14.28)	11.53 (8.41–14.98)	0.8751
Phe, μmol/L	42.62 ± 12.05	47.34 ± 11.45	0.0823	40.38 ± 12.05	38.95 ± 11.19	0.7181
Pip, μmol/L	128.37 (98.83–182.87)	124.67 (92.52–153.26)	0.3966	191.60 ± 76.82	194.00 ± 94.09	0.8663
Pro, μmol/L	486.90 ± 178.80	520.70 ± 186.90	0.3966	423.40 ± 153.00	496.90 ± 166.20	0.0023
Ser, μmol/L	51.21 (42.53–63.04)	51.25 (44.69–67.80)	0.4112	45.53 ± 10.59	45.11 ± 12.07	0.8663
Thr, μmol/L	23.26 ± 7.18	25.46 ± 6.82	0.1592	24.54 ± 7.59	23.97 ± 7.46	0.8663
Trp, μmol/L	44.17 ± 12.47	48.39 ± 13.61	0.1592	39.38 ± 11.21	41.78 ± 12.91	0.3157
Tyr, μmol/L	43.86 (34.27–54.65)	46.71 (38.20–56.51)	0.2593	51.84 ± 15.04	52.81 ± 17.29	0.8663
Val, μmol/L	129.70 ± 33.28	147.20 ± 36.53	0.0284	141.60 ± 32.37	157.50 ± 43.92	0.0029

TG, triglyceride; LMUFAH, Liaoning Medical University First Affiliated Hospital; SAHODMU, the Second affiliated hospital of Dalian Medical University; Ala, aanine; Asn, asparagine; Leu, leucine; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; Val, valine; Arg, arginine; Gly, glycine; Pro, proline; Thr, threonine; Cit, citrulline;Gln, glutamine; His, histidine; Lys, lysine; Met, methionine; Ser, serine; Orn, ornithine; Glu, glutamate; Asp, aspartate; Pip, piperamide, Cys, cysteine; Hcy, homocysteine.

Data are mean ± standard deviation, median (IQR), or n (%).

False discovery rate was calculated for multiple comparisons and q < 0.05 was defined as statistically significant.

Besides, in subgroups with different HbA1c or HDL-C, the differences in these four amino acids were robust too, although some of them were not statistically significant (majority with marginal significance) due to their small sample sizes (**Supplementary Figures 1, 2**).

Correlations Within Selected Amino Acids and Clinical Biochemical Parameters

Amino acids were positively associated with each other and TG in both cohorts. There were only slight or no correlations between amino acids and HbA1c, with negative direction in LMUFAH and positive direction in SAHODMU. C-peptide was positively associated with amino acids (correlation coefficients ranged from 0.16 to 0.25) (**Figure 4**).

Associations Between Selected Amino Acids and Hypertriglyceridemia

As shown in **Table 3**, the associations of these four metabolites with HTG risk remained after further adjustments of traditional risk factors, i.e., age, sex, BMI, duration of diabetes, and DN. In fixed-effect pooled analysis, the ORs (95% CI) of per SD increase were 1.49 (1.2–1.85) for Ala, 1.75 (1.35–2.26) for Asn, 1.74 (1.35–2.23) for Leu, and 1.58 (1.24–2.00) for Val (all *P* values ranged from 0.0003 to <0.0001) (model 2). When further adjusted for HDL-C, LDL-C, and HbA1c, the pooled effect sizes were 1.71 (1.32–2.20) for Ala, 1.62 (1.20–2.19) for Asn, 1.64 (1.22–2.20) for Leu, and 1.62 (1.22–2.13) for Val (all *P* value ranged from 0.0018 to <0.0001). Adjusting for C-peptide attenuated associations in model 3, except for Asn (Ala, 7.9%; Leu, 2.7%; Val, 8.3%).

In diabetic patients without statin treatment, the associations between amino acids and HTG were in accordance with findings in the group with statin therapy, although the effect sizes were attenuated (**Supplementary Tables 1, 2**).

DISCUSSION

In this cross-sectional investigation in two independent hospital-based studies of targeted metabolomics and HTG risk, we identified four amino acid metabolites, including Ala, Asn, Leu, and Val, consistently associated with increased risk of HTG despite statin use. Besides, the associations were robust in the context of LDL-C, suggesting that abnormal amino

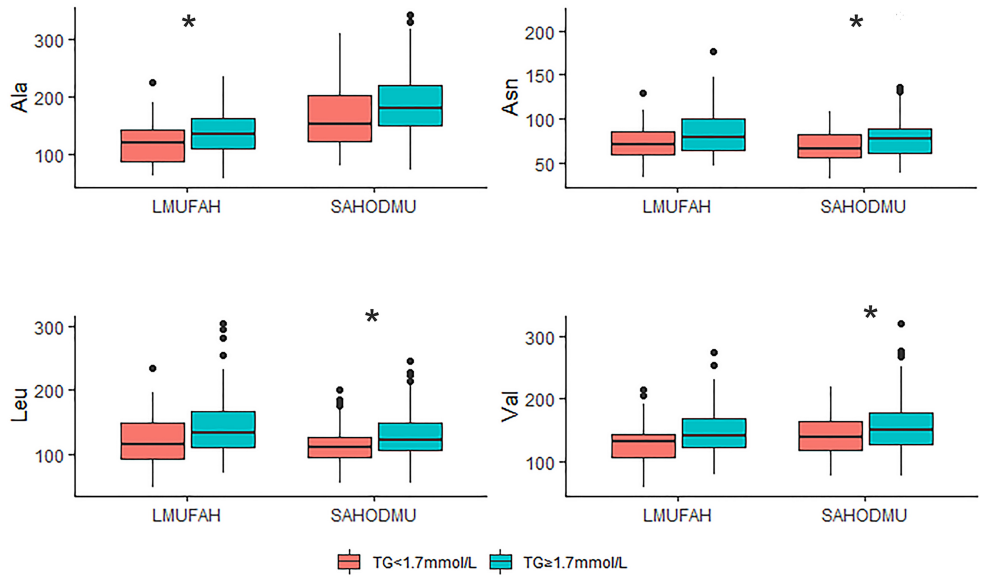

FIGURE 2 | Plasma amino acids levels in patients with LDL-C <2.6 mmol/L according to TG levels. LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; LMUFAH, Liaoning Medical University First Affiliated Hospital; SAHODMU, the Second affiliated hospital of Dalian Medical University; Ala, Alanine; Asn, Asparagine; Leu, Leucine; Val, Valine. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

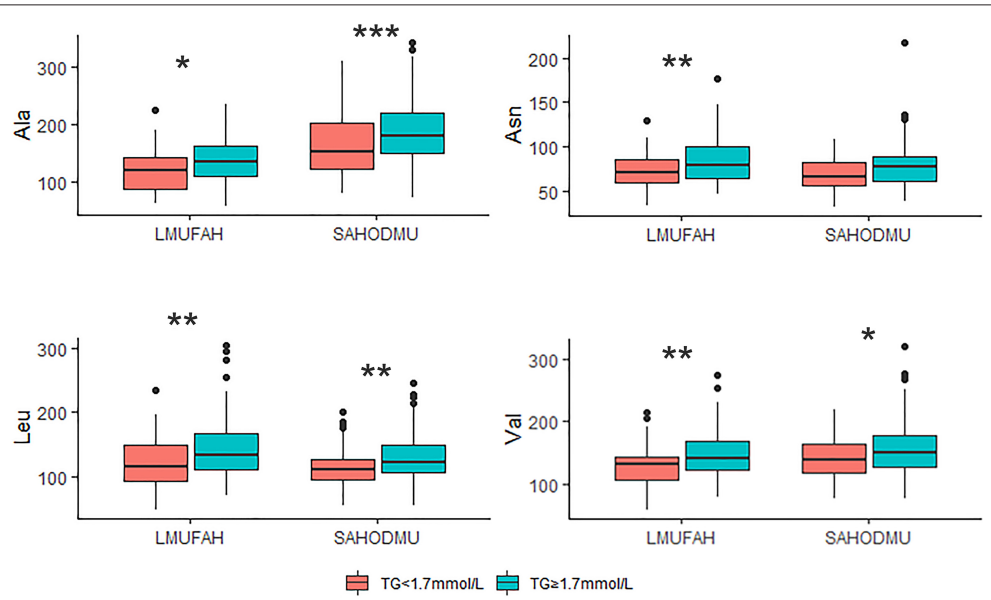

FIGURE 3 | Plasma amino acids levels in patients with LDL-C ≥ 2.6 mmol/L according to TG levels. LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; LMUFAH, Liaoning Medical University First Affiliated Hospital; SAHODMU, the Second affiliated hospital of Dalian Medical University; Ala, Alanine; Asn, Asparagine; Leu, Leucine; Val, Valine. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

acid metabolism contributed to residual HTG ignorant of LDL-C levels.

Although the relationships between amino acids and lipid abnormality were yet completely clarified, their associations have been investigated in many studies. Compared with healthy controls, patients with abnormal BMI or non-alcoholic fatty liver disease have profound perturbation of amino acid metabolism (29, 30). Several cross-sectional and longitudinal studies also showed that amino acid signature significantly predicted future hypertriglyceridemia in children (31, 32). Among these altered amino acids, branched-chain amino acids (BCAAs) was the most robust regarding linkage to lipid abnormality.

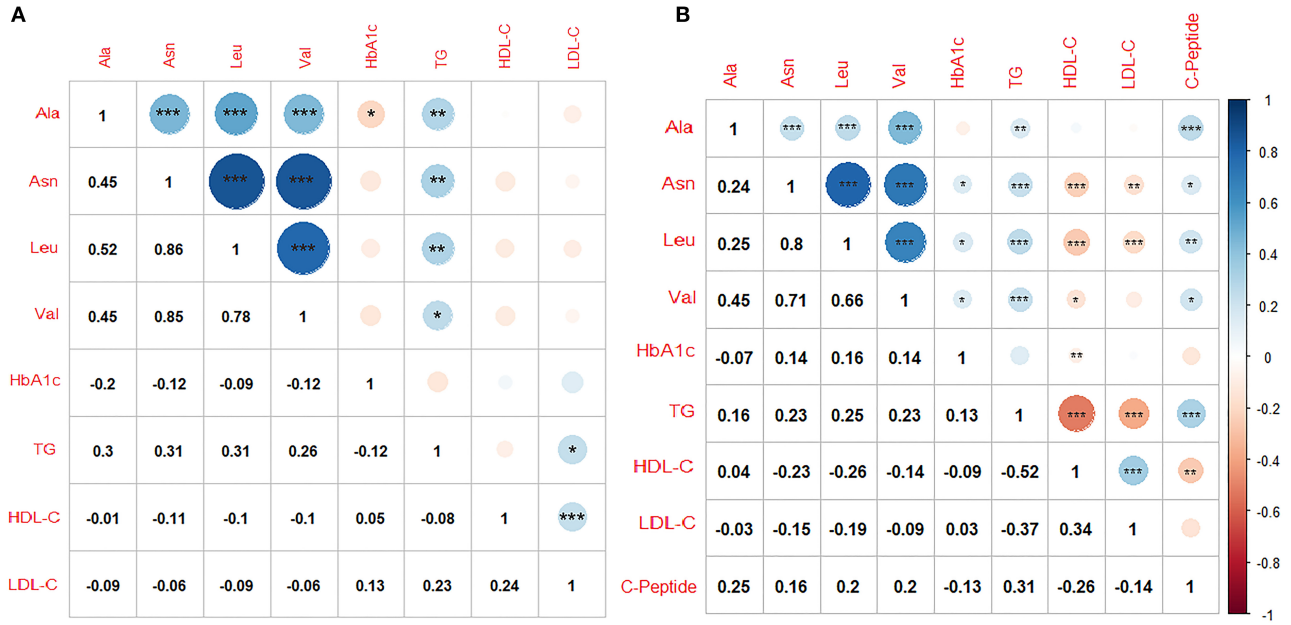

FIGURE 4 | Correlations within metabolites and clinical biochemical parameters. **(A)** Liaoning Medical University First Affiliated Hospital; **(B)** the Second affiliated hospital of Dalian Medical University. Ala, Alanine; Asn, Asparagine; Leu, Leucine; Val, Valine; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HbA1c, glycated hemoglobin; Color blue, positive correlation coefficients; color red, negative correlation coefficients; Pearson or Spearman correlation was used to calculate the correlation coefficients; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Generally, after absorption from the intestines, BCAAs are first transaminated to branched-chain keto acids (BCKAs) by branched-chain amino acid transaminase. Then BCKAs are oxidized by branched-chain α-keto acid dehydrogenase (BCKDH), the rate-limiting enzyme complex. Subsequently, downstream products with further enzymes were involved in metabolism and provide many physiological benefits via mechanisms such as regulating β-cell function and adipose tissue metabolism (33, 34). However, emerging evidence has revealed that impaired adipose BCAA catabolic pathway with suppressed BCKDH activity (i.e., accumulation of circulating BCAAs and BCKAs) can lead to β-cell dysfunction through mechanisms including chronic hyperactivation of mammalian target of rapamycin (mTOR) signaling, oxidative stress, and so on (18, 35). In this connection, insulin promotes storage of TG in adipose tissues and reduces circulating level and ectopic storage. Conversely, insulin resistance and deficiency in T2D accelerates lipolysis in adipocytes and excessive secretion of TG-rich lipoprotein such as very low-density lipoprotein cholesterol (VLDL-C) and LDL-C from the liver (5). Previous studies found that BCAA was positively associated with plasma TG in non-diabetic cohorts (31, 32, 36), which is consistent with our findings in the present diabetic group. Moreover, we also found that C-peptide, a byproduct of proinsulin and a good predictor of insulin resistance (37), only mediated partial effects of BCAA on TG. This finding is also in accordance with earlier prospective research in non-diabetic young to elderly population, where associations between BCAA and HTG remained significant even after controlling for insulin resistance (32, 38). Our study further emphasizes the complex pathological mechanisms of BCAA beyond insulin resistance.

Apart from BCAA, we also identify a positive association between Ala and TG as several previous studies in non-diabetes (30, 39). Ala plays a key role in the glucose–alanine cycle between tissues and the liver. Briefly, in muscle and other tissues, pyruvate accepts an amino group from glutamate through the action of alanine aminotransferase (ALT), forming alanine, and α-ketoglutarate. In fasting, Ala can also derive from muscle protein breaking down. The alanine enters the bloodstream and then the liver, where the ALT reaction takes place in reverse, and generates glucose subsequently (40). Thus, elevated Ala in the current study may be a marker of enhanced muscle glycolysis, muscle protein breaking down, or liver injury. In accordance with this assumption, impaired carbon metabolism, and liver function always accompany rising TG (41, 42).

Findings regarding Asn in our study was opposite to previous research: Takashina et al. classified 83 subjects with normal glucose tolerance as obese or non-obese, and as visceral obesity or non-visceral obesity, and analyzed correlations between 23 plasma amino acids and obesity. They found that obesity or visceral obesity was negatively associated with Asn (43). In a case-control study of Iranian adults, compared with 100 controls, 200 obese patients had lower levels of Asn (29). As we speculated before, the discrepancy may derive from population heterogeneity. Although Asn may perform as a protective factor in these two scenarios, accelerated Asn consumption leads to a decreasing Asn level, while activating asparagine synthetase gene (ASNS) may lead to increasing circulating

TABLE 3 | Associations between amino acids and hypertriglyceridemia in two cohorts.

	LMUFAH		SAHODMU		Pooled	
	OR (95% CI)	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Model 1						
Ala	1.59 (1.11–2.28)	0.0122	1.51 (1.16–1.97)	0.0024	1.54 (1.24–1.90)	<0.0001
Asn	1.98 (1.27–3.08)	0.0024	1.64 (1.19–2.25)	0.0025	1.75 (1.35–2.26)	<0.0001
Leu	1.99 (1.29–3.06)	0.0018	1.79 (1.31–2.46)	0.0003	2.37 (1.62–3.47)	<0.0001
Val	1.76 (1.19–2.59)	0.0047	1.51 (1.14–2.01)	0.0045	1.59 (1.27–2.01)	<0.0001
Model 2						
Ala	1.47 (1.02–2.13)	0.0399	1.50 (1.15–1.97)	0.0028	1.49 (1.20–1.85)	0.0003
Asn	1.77 (1.15–2.71)	0.0091	1.74 (1.25–2.42)	0.0011	1.75 (1.35–2.29)	<0.0001
Leu	1.73 (1.11–2.69)	0.0147	1.74 (1.29–2.35)	0.0003	1.74 (1.35–2.23)	<0.0001
Val	1.55 (1.03–2.32)	0.0344	1.59 (1.19–2.14)	0.0020	1.58 (1.24–2.00)	0.0002
Model 3						
Ala	1.60 (1.04–2.46)	0.0340	1.77 (1.29–2.41)	0.0004	1.71 (1.32–2.20)	<0.0001
Asn	1.91 (1.16–3.15)	0.0114	1.47 (1.01–2.15)	0.0456	1.62 (1.20–2.19)	0.0018
Leu	2.03 (1.18–3.49)	0.0105	1.50 (1.06–2.13)	0.0233	1.64 (1.22–2.20)	0.0010
Val	1.71 (1.05–2.77)	0.0309	1.57 (1.11–2.21)	0.0104	1.62 (1.22–2.13)	0.0008
Model 4						
Ala	-	-	1.63 (1.17–2.27)	0.0040	-	-
Asn	-	-	1.47 (0.99–2.21)	0.0595	-	-
Leu	-	-	1.46 (1.01–2.09)	0.0420	-	-
Val	-	-	1.44 (1.01–2.04)	0.0425	-	-

TG, triglyceride; LMUFAH, Liaoning Medical University First Affiliated Hospital; SAHODMU, the Second affiliated hospital of Dalian Medical University; OR, odds ratio; CI, confidence interval; Ala, Alanine; Asn, Asparagine; Leu, Leucine; Val, Valine.

Logistic regression was used to obtain per standard deviation increased OR.

Model 1, adjusted for age and sex.

Model 2, adjusted for variables in model 1 plus body mass index, duration of diabetes and diabetic nephropathy.

Model 3, adjusted for variables in model 2 plus high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and glycated hemoglobin.

Model 4, adjusted for variables in model 3 plus C-peptide.

Asn (25, 44). More investigations are warranted to clarify the difference.

Genetic and epidemiologic evidence have provided robust evidence for the causal role of HTG for CVD risk (2). In terms of lowering TG, fibrates, niacin, and fish oil have better performance than statins (10, 45). A combination of statins and other lipid-lowering agents was considered in mixed dyslipidemia, which raises some safety concerns. For example, niacin may increase the risk of diabetes (46); fibrates may compound rhabdomyolysis induced by statins (47); fish oil was proved to attenuate cardiovascular diseases and NAFLD in the general person; however, its effect in the diabetic group is controversial (48). More targets, especially in patients with diabetes, are required for TG management. In the present study, the adverse effects of amino acids on TG were not eliminated by statins, so amino acids may provide additional benefits beyond statins.

Our study has significant implications for clinical practice. As stated above, controlling cardiometabolic risk factors plays a central role in CVD prevention of patients with diabetes. TG management was recently recommended, whereas agents including statins reduced only partial HTG risk. Amino acids can be novel targets in diabetic subjects with statin therapy. Our study found associations between amino acids and residual HTG risk, which may have partially been mediate by insulin resistance, suggesting that patients with residual HTG may benefit from the regulation of amino acid metabolism. Agents targeting amino acids can be an option for combinations with statins. Besides, more intensive treatment on insulin resistance may also be recommended in the absence of hypoglycemia. Apart from clinical practice, our study also generated new hypotheses for basic science: First, the mechanism linking amino acids with TG requires more investigations. Second, lipid abnormalities may explain some links between amino acids and increased CVD risk in T2D.

There are several limitations in our study too. First, provided the nature of cross-sectional study design, the causal relationship cannot be established, and prospective cohorts are warranted. Second, we did not collect the dose and frequency of statins. Instead, we repeated the analysis in the subgroups without statin treatment. The associations between amino acid and HTG still existed. So the difference in amino acids metabolism between patients with and without HTG may not be derived from disparity of dose and frequency of statin therapy. Third, a large amount of observations have revealed that amino acid metabolism is often deregulated in diabetic patients. Diabetes may bias our finding. Nevertheless, a previous study in non-diabetic young to elder subjects also found robust relationships between amino acids and TG.

In conclusion, we detected positive associations between four amino acids, i.e., Ala, Asn, Leu, and Val, and residual HTG risk in patients with diabetes and statin treatment. Prospective researches are needed to confirm the findings, and experimental studies are needed to elucidate the underlying mechanism that will shed light on the prevention of HTG, subsequently, CVD in diabetes with statins.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The protocol of the study was approved by the Ethics Committee for Clinical Research of LMUFAH and the Ethics Committee for Clinical Research of SAHDMU. Informed consent of LMUFAH was waived due to the nature of the retrospective study, which was in accordance with the Helsinki Declaration of 1964 and its later amendments. And all the participants in SAHDMU provided informed written consent.

AUTHOR CONTRIBUTIONS

PY, HS, and H-HL designed the study and put forward the idea. H-HL and SW analyzed the data, wrote the first draft, and revised the paper. Y-FC, X-YS, MH, and Z-ZF gave comments on metabolomics and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.605716/full#supplementary-material

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Conflict of Interest: X-YS and MH were employed by company RSKT Biopharma Inc., Dalian, Liaoning, China.

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