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Publication Date

2022

DOI

10.3389/fcvm.2022.928566

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Glycosylation of HDL-Associated Proteins and Its Implications in Cardiovascular Disease Diagnosis, Metabolism and Function

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Specialty section:

This article was submitted to
Lipids in Cardiovascular Disease,
a section of the journal
Frontiers in Cardiovascular Medicine

Received: 25 April 2022

Accepted: 09 May 2022

Published: 27 May 2022

Citation:

Romo EZ and Zivkovic AM (2022)
Glycosylation of HDL-Associated
Proteins and Its Implications in
Cardiovascular Disease Diagnosis,
Metabolism and Function.
Front. Cardiovasc. Med. 9:928566.
doi: 10.3389/fcvm.2022.928566

High-density lipoprotein (HDL) particles, long known for their critical role in the prevention of cardiovascular disease (CVD), were recently identified to carry a wide array of glycosylated proteins, and the importance of this glycosylation in the structure, function and metabolism of HDL are starting to emerge. Early studies have demonstrated differential glycosylation of HDL-associated proteins in various pathological states, which may be key to understanding their etiological role in these diseases and may be important for diagnostic development. Given the vast array and specificity of glycosylation pathways, the study of HDL-associated glycosylation has the potential to uncover novel mechanisms and biomarkers of CVD. To date, no large studies examining the relationships between HDL glycosylation profiles and cardiovascular outcomes have been performed. However, small pilot studies provide promising preliminary evidence that such a relationship may exist. In this review article we discuss the current state of the evidence on the glycosylation of HDL-associated proteins, the potential for HDL glycosylation profiling in CVD diagnostics, how glycosylation affects HDL function, and the potential for modifying the glycosylation of HDL-associated proteins to confer therapeutic value.

Keywords: glycosylation, high-density lipoprotein (HDL), O-glycosylation, N-glycosylation, ApoA-I, APOC3, APOE

INTRODUCTION

It has been established across multiple cohorts that high density lipoproteins (HDL) are atheroprotective (1–4). HDL are complex, heterogeneous nanoparticles, with various subclasses, comprised of numerous functional proteins and lipids (5), and have more recently been shown to be highly glycosylated (6) and structurally and compositionally variable in various physiological and pathological states (7, 8). Owing to this high heterogeneity, HDL particles have diverse biological functions including immunomodulatory, anti-inflammatory, antioxidant, antithrombotic, and anti-proteolytic functions among others, which are dependent on their composition (9–13). Protein and lipid composition, as well as particle structure and size, are important known factors driving differences in HDL functional capacity. The role of glycosylation in the differential functionality of HDL particles has only recently started to emerge.

Protein glycosylation is generally an enzymatically driven post-translational modification of newly biosynthesized proteins that occurs in the endoplasmic reticulum and Golgi apparatus

where sugars are attached to proteins by N- or O-linkages, forming glycans (14). N-glycans are attached to a nitrogen atom on the asparagine moiety of the protein whereas O-glycans are bound to the oxygen atom of either threonine or serine (15). Glycans contribute to various biological capacities including protein folding, receptor binding, enzyme activity, and physical properties by lending charge to the protein, and are vastly particular to the type, extent, and specific site of glycosylation (15–19). Protein glycosylation functions as a biological language and is important for biological particle self- and non- self-recognition, molecule transport, and endocytosis (20). In the last 8 years since it was first demonstrated that HDL are highly glycosylated, and specifically sialylated particles (6) (**Figure 1**), there has been a steady increase in the evidence pointing to an important connection between the glycosylation of HDL-associated proteins, and the overall functionality of HDL particles. In this review paper we will discuss the current state of the evidence on the glycosylation of HDL-associated proteins, specifically, where we stand in terms of development of cardiovascular disease (CVD) diagnostics using HDL-glycosylation profiling, how glycosylation of HDL proteins affects HDL function, and the potential for modifying the glycosylation of HDL-associated proteins to confer therapeutic value.

HDL GLYCOSYLATION PROFILING FOR DIAGNOSTIC PURPOSES

One of the problems with HDL particle analysis for diagnostic purposes has been the extreme complexity of these particles and the lack of resolution of older measurement tools. For example, although high HDL-cholesterol (HDL-C) concentrations have been found to be protective against CVD, several large recent studies demonstrated that the relationship between HDL-C concentration and adverse health outcomes tends to follow a U-shaped curve, with both low HDL-C and very high HDL-C being associated with increased cardiovascular (CV) events (21–23). Clearly, it is not simply the measurement of the total amount of cholesterol carried within HDL that is diagnostic, but rather some other aspect of HDL that is critical, whether it be compositional, structural, or functional.

For more sophisticated measurements of HDL structure, composition, and function, it is imperative to first isolate the HDL particles and purify them from other potentially contaminating components. Because HDL particles are so small (7–12 nm in diameter) as to overlap with many plasma proteins in terms of their size (e.g., ferritin), and because they are close in density to other lipoprotein particles and even extracellular vesicles, they are difficult to isolate and purify. According to multiple proteomic studies HDL could carry as few as 12 key proteins or up to an excess of 200 proteins (24, 25) depending on how they are isolated (24, 26, 27). Various methods, and combinations of these methods, have been used to isolate HDL including ultracentrifugation, size exclusion chromatography, immunoaffinity precipitation, and asymmetrical flow field flow fractionation. More recently, methods combining these different

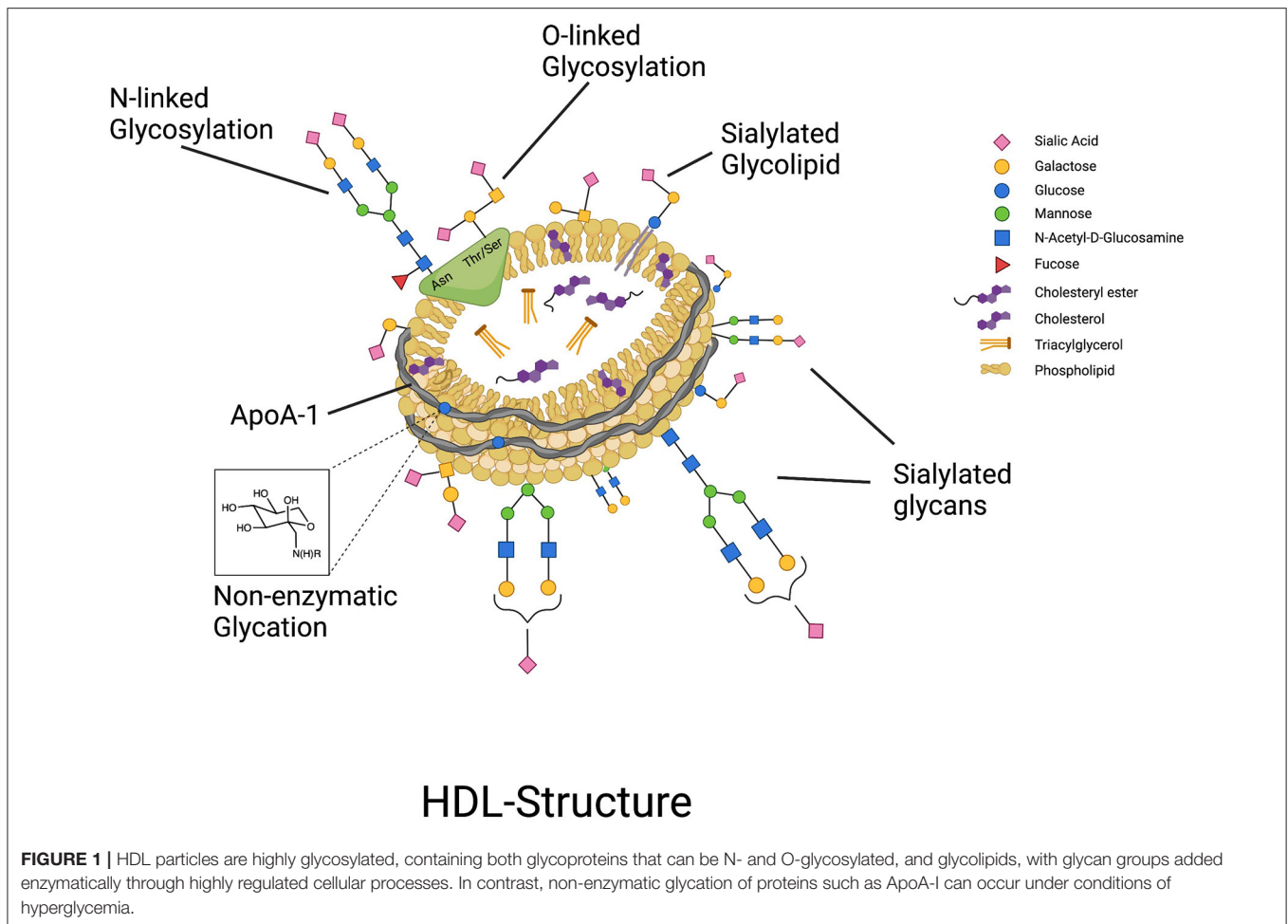
approaches have been used to improve the overall yield and purity of HDL particles while preserving their structural and functional integrity (28–30), including an optimized, validated method using sequential flotation density ultracentrifugation followed by size exclusion chromatography which yields highly purified HDL fractions (5).

Once HDL particles are isolated, the analysis of their glycosylation status can be performed. Pioneering work in lipoprotein glycobiology establishing analytical methods for profiling the glycome of HDL particles revealed for the first time that HDL has both N- and O-linked glycosylation and is distinctly highly sialylated (6). Glycosylation analysis is a complex chemical approach traditionally using mass spectrometry combined with various extraction methods such as enzymatic digestion, chemical cleavage, and liquid chromatography (31–35). HDL glycosylation can be profiled in several ways: (1) the glycans can be enzymatically or chemically cleaved, followed by mass spectrometry (6), (2) site-specific glycoprofiling can be performed by tandem mass spectrometry analysis of protease-digested samples (6, 36, 37), and (3) hydrophilic interaction liquid chromatography profiling can be performed, which uses a combination of the three main types of liquid chromatography for separation and profiling of glycan-containing peaks (38, 39).

To date, no large studies examining the relationships between HDL glycosylation profiles and CVD outcomes have been performed. However, small pilot studies provide promising preliminary evidence that such a relationship may exist. For example, in a small pilot study performed by our group, differences in HDL glycan composition were able to differentiate between individuals at equal risk for CVD based on clinical parameters (i.e., total cholesterol, low-density lipoprotein-cholesterol (LDL-C), HDL-C, etc.) who were found to have arterial occlusion vs. not (37). The role of HDL glycosylation in CV health is starting to be recognized as a promising new research field (40). Larger cohort studies investigating the relationships between HDL glycoprofiles and CV outcomes across factors including age, sex, and ethnicity are needed, and have the potential to add greatly to our ability to detect individuals at risk for CVD earlier when disease prevention measures are the most likely to be effective.

HOW GLYCOSYLATION OF HDL PROTEINS AFFECTS HDL FUNCTION

Most of the known HDL-associated proteins are glycosylated, and only a few are found to be non-glycosylated. In **Table 1** we provide information on the N- and/or O-glycosylation status, sites of attachment, and number of unique glycans attached for several HDL proteins for which this information has been confirmed by extensive MS/MS analysis. Whereas, here are many putative sites for O-glycosylation (i.e., any Ser or Thr residue) on any given protein, whether O-glycans are actually attached must be confirmed by advanced MS analysis. Thus, although several HDL-associated proteins, such as ApoC-I have Ser or Thr residues that could in theory be O-glycosylated, in **Table 1** we report only those that have been demonstrated to



beO-glycosylated by MS measurement of isolated HDL fractions derived from a starting volume of 500 μ L of plasma. It is possible that some proteins (e.g., PLTP) are present at such low abundance in isolated HDL that their glycoforms fall below the limits of detection. Thus, to further investigate the glycosylation status of these low-abundance HDL proteins future experiments involving enrichment for these proteins will be required. Other proteins, such as ApoA-I, have been reported to be glycosylated in the literature, however, we do not include it in **Table 1** because based on detailed MS analysis the O-glycosylation could not be confirmed. In the following section we review what is currently known about the impact of glycosylation of several key HDL-associated on overall HDL metabolism and function, for which there is currently information. As the field evolves this list will doubtless grow and a more comprehensive picture of the extent and diversity of glycans attached to HDL-associated proteins will emerge.

Apolipoprotein A-I

ApoA-I, the major structural, defining HDL apoprotein accounting for around 70% of total HDL protein mass, plays a key role in lipid and cholesterol metabolism and is highly associated with cardioprotection (42). Despite ApoA-I being

reported to possibly be glycosylated (43–45) extensive mass spectrometry-based (MS)-based profiling demonstrated that there is no evidence of ApoA-I glycosylation (25). ApoA-I does not have the consensus sequence for N-glycosylation (AsnXxxSer/Thr/Cys, where Xxx can be any amino acid except proline), and whereas O-glycosylation is possible on any serine or threonine residue, detailed analysis of isolated HDL has not yielded any confirmed O-glycosylated peptides on ApoA-I. It is possible that ApoA-I O-glycosylation can occur in certain conditions or disease states, however MS-based analysis has never confirmed the existence of this to date. ApoA-I can, however, be non-enzymatically or chemically glycosylated (44), which has been found to be deleterious for its function.

Apolipoprotein A-II

ApoA-II is the second most abundant HDL apoprotein, representing as much as 20% of total protein mass (42), and has been shown to have important implications for CV health though results were historically inconsistent and controversial. For instance one early study showed that low serum ApoA-II was a marker of atheroprotection in patients with non-insulin-dependent-diabetes mellitus (46) but conversely another study showed that elevated levels of ApoA-II were proatherogenic

TABLE 1 | Glycosylation status of HDL-associated proteins with confirmed glycosylation sites.

Protein	N-glycans	O-glycans	Sites of attachment
Alpha-1-antichymotrypsin (AACT)	8	0	Asn106, Asn127, Asn271
Alpha-1-antitrypsin (A1AT)	18	0	Asn70, Asn107, Asn271
Alpha-1B-glycoprotein (A1BG)	1	0	Asn179
Alpha-2-HS-glycoprotein (FETUA or A2HSG)	17	2	Asn156, Asn176, Thr346
Apolipoprotein A-II (APOA2)	0	4	Ser35, Ser88, Thr95
Apolipoprotein C-III (APOC3)	0	21	Thr94
Apolipoprotein D (APOD)	28	0	Asn65, Asn98
Apolipoprotein E (APOE)	0	40	Ser215, Thr307/Ser308*, Ser76/Thr83*, Ser129/Thr130*, Thr194, Ser197, Ser263, Thr289/Ser290* Ser296 (25, 41)
Apolipoprotein F (APOF)	0	3	Ser269, Thr273/Thr27*
Apolipoprotein M (APOM)	9	0	Asn135
Clusterin (CLUS or APOJ)	10	0	Asn86, Asn291, Asn374
Complement C1s subcomponent (C1S)	2	0	Asn174
Complement C3 (C3)	4	0	Asn85
Hemopexin (HPX)	6	0	Asn187, Asn453, Asn240/Asn246*
Heparin cofactor 2 (HCF2)	2	0	Asn49
Kininogen-1 (KNG1)	4	0	Asn169, Asn205
Lecithin-cholesterol acyltransferase (LCAT)	1	0	Asn108
Serum amyloid A-4 (SAA4)	7	0	Asn94
Serum paraoxonase/arylesterase 1 (PON1)	8	0	Asn253, Asn324

Proteins included in this table include only those with glycosylation sites confirmed to actually express glycans at those sites by mass spectrometry analysis of isolated HDL fractions from a starting volume of 500 μ L of plasma, as described in (25). HDL-associated proteins that have been reported to be glycosylated previously, and/or have putative sites but that either could not be confirmed by mass spectrometry or are present at low abundance such that they fall under the limit of detection, are not reported in this table.

*For these sites, the site of attachment could not be disambiguated thus both possible attachment sites are reported.

(47). However, more recently a large prospective study ($n = 912$) showed that ApoA-II was indeed inversely associated with future risk for coronary artery disease (CAD) and was exerting antiatherogenic properties (48). ApoA-II binds to phospholipid transfer protein (PLTP) on HDL (49), suggesting that it plays an important role in the remodeling of HDL particles. ApoA-II contributes to structural properties of HDL (50) and its presence on HDL enhances ATP-binding cassette transporter-1 (ABCA-1)-mediated efflux, suggesting that ApoA-II can contribute to structural changes in ApoA-I, and improve functionality of the HDL particle (51). Like ApoA-I, ApoA-II does not contain the consensus sequence for N-linked glycosylation, however it has been shown to be O-glycosylated (25, 52). The glycosylation of ApoA-II contributes to its association affinities since sialylated ApoA-II preferentially associates with smaller HDL whereas non-sialylated ApoA-II associates with all sizes of HDL (53). In a recent study in patients who were equally at risk for CAD based on traditional biomarkers and who were then diagnosed as either having CAD or not using diagnostic coronary arteriography, ApoA-II was significantly lower in CAD patients compared to patients without CAD (7). In children given a lipid rich dietary supplementation there was no difference in ApoA-II glycosylation between groups, but the analysis did confirm that ApoA-II indeed had multiple glycoforms (25). It is currently unknown what the role of glycosylation in ApoA-II function is, and whether the extent of sialylation drives the binding of ApoA-II to smaller HDL particles or whether higher sialylation

is reflective of a particular pathway of metabolism that is linked with the production of small particles.

Apolipoprotein C-III

ApoC-III is a critical metabolic protein whose glycosylation status has long been known to be an important determinant of its function. ApoC-III is a small (8 kDa) O-glycosylated apoprotein whose glycans can be capped with 0, 1, or 2 sialic acids and thus is often denoted as, ApoC-III₀, ApoC-III₁, and ApoC-III₂ accordingly. Because of the negative charge conferred by the sialic acids the ApoC-III glycoforms have differential migration on gel (18), which enabled the study of its glycosylation much earlier than more advanced MS-based tools became available. ApoC-III is synthesized in the liver and intestine and found on very-low-density-lipoproteins (VLDL), chylomicrons, LDL and HDL and is a multifunctional protein whose primary functions are to hinder apolipoprotein E (ApoE) mediated hepatic uptake of lipoproteins, and to inhibit lipoprotein lipase, a key enzyme that catalyzes the hydrolysis of triacylglycerols from lipoproteins to free fatty acids and monoacylglycerol fragments (54). ApoC-III has gained considerable attention due to its relationship with CV health and the strong correlation with ApoC-III overexpression and CVD due to its involvement in hypertriglyceridemia (55, 56).

Though the association between elevated ApoC-III concentration and CVD has been established for some time, the focus has been primarily on the role of ApoC-III in VLDL metabolism, however, recently a relationship between

ApoC-III and HDL has emerged. For example, CVD patients have increased HDL ApoC-III content (57, 58). Changes in sialylation in the more common glycoforms of ApoC-III have been observed in multiple conditions including uremia, obesity, kidney disease, cancers and diabetes (8, 59–62). The enzyme from the GalNAc-transferase family Golgi-localized polypeptide N-acetyl-D-galactosamine-transferase 2 isozyme (GALNT2) initiates the first step in the O-glycosylation of ApoC-III, as well as several other lipoprotein-associated targets including ApoE, PLTP, and angiopoietin-like 3 (ANGPTL3) (63). Loss of function of GALNT2 was found to be associated with extremely low HDL concentrations (64), highlighting the importance of O-glycosylation of critical apoproteins and related proteins involved in lipoprotein remodeling in HDL metabolism. Elevated circulating levels of triglycerides (TG) are a risk factor for CVD (65) which is positively correlated with circulating ApoC-III concentrations (66, 67). High-throughput mass spectrometric immunoassay found that increased plasma TG levels were associated with higher ratio of ApoC-III₁ over ApoC-III₂ (68). Importantly, it is already well-known that the sialylation state of ApoC-III associated with LDL particles is responsible for its binding affinity to cell surface receptors, with ApoC-III₂ being preferentially cleared by heparan sulfate proteoglycans and conversely ApoC-III₁ being more effectively cleared by the LDL receptor and other receptors in the LDL receptor family (69). It is currently unknown whether and how the sialylation state of ApoC-III associated with HDL particles influences the binding of those HDL to cell surface receptors.

The glycosylation of ApoC-III is more complex than was previously thought. In addition to the known glycosite at position Alanine-74 (Ala)-74 (70) and the three possible non-sialylated and sialylated glycans attached at this site (6), our group identified a total of 20 glycoforms most of which were fucosylated and nearly half were sialylated (15). Interestingly, 13 unique glycoforms of ApoC-III were significantly enriched in HDL particles compared to serum, with the HDL-associated glycoforms being more highly sialylated (15). These findings suggest that either ApoC-III glycosylation state modifies its affinity for a specific lipoprotein class, or that the metabolism of ApoC-III and its exchange between the circulating lipoproteins is reflected in its glycosylation. Research is needed to better understand the mechanisms driving these intriguing findings about the links between ApoC-III glycosylation and its association with HDL vs. the ApoB containing lipoproteins, and the unique role of ApoC-III in HDL particle metabolism.

In a recent study comparing the site-specific glycosylation of ApoC-III in patients across the spectrum from healthy, to those with metabolic syndrome to diabetic patients with chronic kidney disease on hemodialysis, ApoC-III was differentially glycosylated in patients with metabolic syndrome and diabetic hemodialysis compared to controls (37). Patients with chronic kidney disease who were on hemodialysis and patients with metabolic syndrome had HDL that were significantly more enriched in ApoC-III especially in di-sialylated ApoC-III (ApoC-III₂) compared to the control group (37). Importantly, HDL ApoC-III glycosylation was able to distinguish between HDL that suppressed vs. increased IL-6 secretion by monocytes stimulated

with lipopolysaccharide (LPS), when clinical biomarkers such as total cholesterol, LDL cholesterol, C-reactive protein (CRP), glucose and blood pressure were not discriminatory in this immunomodulatory ability (37). These intriguing preliminary findings suggest that ApoC-III glycosylation may play an important role in directing the immunomodulatory capacity of HDL particles.

Apolipoprotein E

ApoE may well be one of the most influential proteins in lipoprotein biology, and in metabolic health overall. Genome-wide association studies across multiple geographic regions have irrefutably identified APOE, which directs lipoprotein metabolism both peripherally and in the central nervous system, as the single strongest genetic marker of extreme longevity across multiple, multi-ethnic cohorts (70). APOE genotype is a major risk factor for a number of age-related pathologies including CVD and Alzheimer's disease (71, 72). ApoE exists in three isoforms, ApoE2, ApoE3, and ApoE4, with ApoE4 conferring increased risk for both CVD and Alzheimer's (73–75). Importantly, it is well-known that compared to ApoE3 the ApoE4 isoform has a reduced ability to induce cholesterol efflux (76, 77), and has a higher binding affinity for VLDL than HDL particles, altering its metabolic fate (78). Unlike the intracellular fate of ApoB-100, which is largely degraded upon uptake via the LDL receptor, as much as 80% of ApoE internalized as part of VLDL particles is recycled and re-secreted as part of HDL particles (79). This recycling and re-secretion pathway is not exclusive to hepatocytes, and instead has been demonstrated to occur across a wide variety of cell types (79). Importantly, when internalized as part of TG-rich lipoproteins via receptors in the LDL receptor family, ApoE4 is more likely to be retained in the cell than recycled and re-secreted as part of HDL particles compared to ApoE3, resulting in diminished concentrations of ApoE4 in circulation and reduced cholesterol efflux (76). The endocytic vesicles involved in ApoE recycling were identified to contain sialyltransferase enzymes (80), suggesting that addition of sialic acid residues to ApoE glycan structures may be a critical step in directing ApoE from internalized TG-rich particles to re-secreted HDL particles. In support of this hypothesis, it has been found that HDL-associated ApoE is more highly sialylated than VLDL-associated ApoE (81).

ApoE was found to be glycosylated in 1979 (82), with 6 sialylated glycoforms identified (83). ApoE does not contain the consensus amino acid sequence for N-linked glycosylation, and instead is O-glycosylated with mucin-type glycans at the originally characterized site at Threonine¹⁹⁴ (Thr194), which is not essential for ApoE secretion (84). More recently, additional glycosylation sites have been identified, including one at Thr²¹² (85), and 3 additional sites were identified at Serine²⁹⁰ (Ser²⁹⁰), Thr²⁸⁹ and Ser²⁹⁶ in ApoE secreted by macrophages isolated from peripheral blood mononuclear cells of a single donor with ApoE3/E3 genotype (86). It was recently shown that ApoE in fact has two more glycosites, for a total of 7 mucin-type O-glycosylation sites, with glycans ranging from simple GlcNAc to biantennary structures

containing sialylation and fucosylation (87). Evidence regarding the importance of ApoE glycosylation in lipoprotein function is starting to emerge, building on the established evidence that ApoE structure impacts the metabolism of lipoproteins (41, 76). An aberrantly glycosylated variant of ApoE causes defective binding to the LDL receptor (88). ApoE is highly sialylated when associated with HDL compared to serum, and its sialylation state is involved in mediating ApoE's binding affinity to HDL vs. VLDL (81, 89). ApoE glycosylation was shown to be considerably different in cerebral spinal fluid (CSF) than in serum (90) and its extent of sialylation in CSF affects ApoE binding to amyloid beta, thus influencing the development of plaque formation and Alzheimer's disease (91) and suggesting that glycosylation of ApoE may be tissue-specific (90). Importantly, it was recently demonstrated that site-specific glycoproteins of HDL-associated ApoE are correlated with HDL functional capacity (87), strongly suggesting that ApoE glycosylation is important for HDL function. ApoE isoform-specific glycoproteomics has not yet been performed and will likely be important in distinguishing ApoE genotype-specific effects on disease risk.

Alpha-1 Antitrypsin

A1AT is an acute phase protein mainly synthesized by the liver, which acts as a protease inhibitor, and which has been shown to increase dramatically during inflammation and has also been found to persist post infection (92). Recent work showed that statins can also induce A1AT concentrations, and that association of A1AT with HDL protects the protein and enhances its anti-proteolytic activity in the context of the highly oxidative environment of the acute phase response (93). Post translational modifications of A1AT contribute to changes in conformation that may influence its function (94). Differential glycoforms of A1AT have been reported in patients with various types of lung cancers and are used in lung cancer diagnosis (95). Sialylation variations of A1AT have also been observed in patients with COVID-19 (96).

A1AT is N-glycosylated, and its site-specific glycosylation profiles differ when associated with HDL compared to serum (15). A1AT glycosylation is critical for its secretion by monocytes (97), is differential between serum and hepatocytes (98), and has increased fucosylated biantennary glycans in the serum of hepatocellular carcinoma patients (99). The site-specific glycosylation profiles of A1AT were highly differential between diabetic chronic kidney disease patients on hemodialysis compared to patients with metabolic syndrome and healthy controls: kidney disease patients had a higher proportion of monofucosylated to non-fucosylated glycans, and a lower proportion of di-sialylated glycans on A1AT (37). In the same study, HDL particles that attenuated the amount of Interleukin-6 (IL-6) secreted by LPS-stimulated monocytes had higher amounts of A1AT as well as lower amounts of several disialylated glycans across multiple sites, suggesting A1AT and its specific glycoproteins are involved in mediating HDL immunomodulatory function (37). A disialylated A1AT glycopeptide was also positively correlated with cholesterol efflux capacity in healthy young adults (87), and in young children from Ghana (25).

These findings suggest an important connection between HDL A1AT glycosylation, particularly disialylated A1AT glycans, and HDL functionality.

Alpha-2-HS-Glycoprotein

A2HSG is a hepatically derived protein found in plasma and associated with HDL particles (24). Several studies have shown that A2HSG is critically important for CV health (100–103), playing a particularly important role in preventing vascular calcification, and emerging as an independent risk factor of CVD and all-cause mortality (100). A2HSG is differentially glycosylated in patients with chronic pancreatitis and pancreatic cancer (104). Site-specific analysis of HDL-associated A2HSG revealed that it is highly sialylated and decorated with both N- and O-glycans at multiple sites (6). In patients with chronic kidney disease HDL were enriched with non-sialylated A2HSG, and non-sialylated A2HSG was enriched in HDL particles that enhanced IL-6 secretion by LPS-stimulated monocytes (37). Interestingly, A2HSG concentrations were lower in HDL compared to serum but specific glycoforms were significantly more enriched in HDL than in serum (15). Multiple A2HSG glycopeptides were positively correlated with HDL cholesterol efflux capacity and immunomodulatory capacity in healthy adults (87), and in young children in Ghana supplemented with a lipid-based nutrient supplement (25).

Lecithin-Cholesterol Acyltransferase

LCAT functions as a key enzyme in reverse cholesterol transport and HDL particle maturation by esterifying free cholesterol with a fatty acid from phosphatidylcholine (lecithin), which allows HDL particles to carry a larger cholesterol load as cholesteryl esters (CE) in the core of the particle (105). LCAT is strongly linked with CV health and disease (106). ApoA-I is a potent activator of LCAT (107). Mutations in the LCAT gene lead to altered function of the enzyme resulting in elevated levels of TG and reduced HDL-C, which can lead to atherosclerotic pathology (108). The glycosylation of LCAT has been known since the 1990's, with both N-linked and O-linked glycoforms identified (109, 110), and with important implications for LCAT function (111). The glycosylation of LCAT is critical for its structural stability and function (112). Loss of glycosylation at several sites resulted in loss of function but loss of glycosylation at site 408 increased the activity of the enzyme (113). Desialylation of LCAT by neuraminidase resulted in considerable alteration of LCAT activity, reducing cholesterol esterification and concomitantly reducing the size of HDL (114). Depending on LCAT glycoform LCAT binds preferentially to HDL or ApoB-containing lipoproteins (115). These findings provide strong evidence that LCAT glycosylation is imperative for overall lipoprotein metabolism as well as cholesterol efflux and transport globally, as well as metabolism and efflux capacity of HDL particles in particular.

Cholesterol Ester Transfer Protein

CETP is a critical mediator of lipid transfer between HDL and ApoB-containing lipoproteins, which in the context of high TG concentrations, transfers CE from HDL in exchange for

TG from ApoB-lipoproteins, thereby enriching HDL particles with TG and altering their metabolism (116, 117). Loss of function genetic mutations in CETP and lower concentrations of CETP are associated with lower LDL-C and increased HDL-C, and lower risk of CVD, which has made CETP a major pharmacological target for CVD and atherosclerosis prevention (116, 118). CETP is highly sialylated with four N-linked glycoforms (119). A major form of serum CETP lacking glycosylation at Asparagine341 (Asn341) was shown to have markedly increased functionality compared to other forms (119, 120). Defective sialylation of CETP in heavy alcohol drinkers showed a significant reduction in the function of CETP compared to controls (121). Patients with a congenital disorder of glycosylation of the glycosyltransferase enzyme beta-1,4-galactosyltransferase 1 have defectively glycosylated CETP with reduced functionality, and larger HDL than healthy controls (122). CETP is a minor component of HDL, whose function is to temporarily associate with HDL while bridging between the HDL and ApoB particle between which the exchange of lipids occurs, thus it is often missed as an HDL-associated protein depending on the HDL isolation method and sensitivity of the protein detection method (24). However, its importance in lipid metabolism and strong links with CVD make it an important protein whose content and glycosylation when associated with HDL particles is an area of focus for future studies.

Phospholipid Transfer Protein

The primary function of PLTP is to transfer phospholipids from ApoB containing TG-rich lipoproteins to HDL (123, 124). As a key modulator of HDL size, composition, and concentration PLTP has gained considerable attention for its role in the development of CVD (125). PLTP overexpression has been reported to be an independent risk factor for CAD and is associated with type II diabetes and obesity (126). Two forms of PLTP have been described that have high and low phospholipid transfer activity, which may explain the conflicting findings of the association between PLTP and pro- vs. anti-atherogenic effects (127). Higher concentrations of the low-activity PLTP type may be the driver of the pro-atherogenic effects, and PLTP glycosylation may play a critical role in the function and activity of the protein. Human PLTP has 6 N-linked and 2 O-linked glycoforms (123). Multiple earlier studies showed that tunicamycin treatment disrupts the ability of cells to secrete PLTP, suggesting glycosylation is necessary for synthesis and secretion (128, 129). A later study confirmed that inhibition of PLTP N-glycosylation affected its structural stability and markedly reduced its ability to be excreted resulting in the non-glycosylated PLTP being intracellularly degraded (125). Much like CETP, PLTP is a protein that temporarily associates with HDL particles to mediate the exchange of material between HDL and ApoB-containing lipoproteins, thus the ability to detect its presence on HDL depends on the nature of the HDL isolation method. Although PLTP is a minor constituent of HDL particles and thus measuring its glycosylation may be limited without enrichment prior to analysis, its content and glycosylation profile are likely to be important factors in overall HDL metabolism.

POTENTIAL FOR MODIFYING THE GLYCOSYLATION OF HDL-ASSOCIATED PROTEINS TO CONFER THERAPEUTIC VALUE

Given the growing evidence that HDL glycosylation may be critically involved in both metabolism and function, with implications for both CVD diagnosis and treatment, the potential for HDL-based therapeutics targeting HDL glycosylation is compelling. Strategies to reduce CVD risk and prevent or reverse CVD by increasing the concentration of HDL particles have been largely disappointing. Increasing the number of HDL particles through pharmacological means (e.g., CETP inhibitors, niacin), has met with some success, however the ability to further reduce residual CVD risk following LDL-lowering with statins has been difficult to achieve (130, 131). Several additional HDL modifying therapies, including injection with reconstituted HDL particles, ApoA-I, as well as extracorporeal HDL lipid depletion, where HDL particles are removed from plasma, exogenously delipidated, and then reinfused, have similarly met with modest success despite promising results in animal trials (132, 133). Thus, novel therapeutic approaches to increase not just the concentration but also the function of HDL particles remain an important area of research. The potential for dietary and pharmacological strategies to improve HDL function via modulation of HDL glycoprofiles is tantalizing given the growing evidence of the importance of HDL glycosylation in its function. Several recent studies show promising results for the modification of HDL glycosylation through diet. Whereas, the glycosylation of HDL-associated ApoE was not affected by a short-term intervention with Mediterranean vs. fast food diet, the glycosylation of HDL-associated ApoC-III was significantly altered in just 4 days (87). Specifically, disialylated ApoC-III (ApoC-III₂) was increased after the Mediterranean diet whereas nonsialylated ApoC-III (ApoC-III₀) was increased after 4 days of consuming a diet enriched in saturated fat and simple sugars and depleted in fiber (87). These alterations were associated with HDL cholesterol efflux capacity as well as immunomodulatory capacity (ability to suppress cytokine secretion in stimulated monocytes) (87). In young children in Ghana supplemented with a lipid nutrient supplement, HDL glycopeptides that were altered by the supplement were correlated with HDL cholesterol efflux capacity (25). There is also evidence that targeting GALNT2 activity may be a viable strategy to alter the glycosylation of HDL-associated proteins and thus increase HDL concentration and function (64, 134). While this research area is very new, early tantalizing evidence provides support for the idea that the alteration of HDL glycoprofiles via dietary or pharmacological interventions may be a viable strategy for improving the functional capacity of HDL particles and thus improving CV outcomes.

CONCLUSION

While the study of HDL glycosylation is still in a nascent state, emerging evidence suggests that differential glycoprofiles

of HDL-associated proteins may be diagnostic and may reveal new mechanisms in lipoprotein-mediated aspects of CVD. In order to uncover glycan-based disease biomarkers newly developed glycan analytical methods need to be applied to large, comprehensively characterized, and preferably genotyped cohorts with known CV outcomes. Basic cell and molecular biology studies are also needed to better understand how glycosylation affects HDL metabolism and function, so that the potential for modifying the glycosylation of HDL-associated proteins through intervention to confer therapeutic value can be realized. In the last 10 years there has been progress toward developing the fundamental methodologies for both the isolation of HDL from plasma and the analysis of HDL glycosylation especially using MS. This field is now ripe for major discoveries utilizing these tools in the areas of glycan-based HDL CVD biomarkers, novel CVD disease

mechanisms, and ultimately, novel HDL-based therapeutics for cardioprotection.

AUTHOR CONTRIBUTIONS

ER and AZ had significant contribution to the content, design, and preparation of this manuscript. Both authors have reviewed and approved the submission of this document.

FUNDING

The project described was supported by the National Institute of Aging of the NIH (R01AG062240, UH3CA241694) and the USDA National Institute of Food and Agriculture, Hatch project (CA-D-NUT-2242-H). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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