GlycA, a novel composite pro-inflammatory glycoprotein biomarker, and its relationship with cardiometabolic disorders

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GLYCA, A NOVEL COMPOSITE PRO-INFLAMMATORY GLYCOPROTEIN BIOMARKER, AND ITS RELATIONSHIP WITH CARDIOMETABOLIC DISORDERS

E.G. Gruppen

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General introduction and aim of the thesis

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ACUTE AND CHRONIC INFLAMMATION

Inflammatory responses are an essential part of the innate (non-specific) immune system [1]. During the acute phase response both local and systemic effects are involved. One of these effects includes changes in the plasma concentrations of a large number of proteins, known as acute phase proteins. During the acute phase response, plasma concentrations of most acute phase proteins rise (positive acute phase proteins), while some decrease (negative acute phase proteins). An acute phase protein has been defined as one whose plasma concentration increases or decreases by at least 25% in response to an inflammatory reaction [2]. These changes are mainly due to changes in their production in the liver. Besides hepatocytes, activated macrophages and neutrophils in peripheral tissues are also able to synthesize and secrete acute phase proteins [3, 4].

Cytokines that are produced during inflammatory processes - with macrophages and monocytes at inflammatory sites as the most important sources [4]- are considered to act as messengers between the local site of injury and the hepatocytes synthesizing the acute phase proteins. Most cytokines have several sources, targets, and functions. Moreover, numerous cytokines can change the production of other cytokines and cytokine receptors [4]. Inteleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are two well-known inflammation-associated cytokines [5]. In addition, IL-6 is the chief stimulator of the production of most acute phase proteins [5-7]. However, the dynamic contribution of various cytokines is difficult to appreciate due to their short half-life [4].

It has been recognized that acute-phase proteins not only appear in acute disease processes but also in chronic conditions. Low-grade systemic inflammation is strongly related to life style factors such as smoking, obesity and dietary habits [8, 9]. Furthermore, a systemic chronic low-grade inflammatory state is a feature of many chronic conditions such a cardiovascular disease (CVD) [10], type 2 diabetes mellitus (T2DM) [11], and the metabolic syndrome (MetS) [12]. In such conditions, the increase in concentrations of acute phase reactants is much more modest compared to the response observed during acute episodes of inflammation. Low–grade inflammation is defined as two- to fourfold elevations in circulating concentrations of pro-inflammatory and anti-inflammatory cytokines, natural occurring cytokine antagonists and acute phase proteins [13]. **Table 1** shows the main differences between acute and chronic inflammation.

Table 1. Features of acute and chronic inflammation.

	Acute inflammation	Chronic inflammation
Onset	Abrupt	Gradual
Duration	Few days	Up to months or years
Major cells involved	Mainly neutrophils	Mainly macrophages and lymphocytes
Local and systemic signs	Prominent	Less prominent
Increase in acute phase proteins	Up to 1000 fold	Two- to fourfold elevations often within the reference range

PROTEIN GLYCOSYLATION

About 70% of all proteins are glycosylated [14]. Of note, many acute phase proteins are heavily glycosylated. Glycosylation is the most abundant post-translational protein modification. It is a highly complex enzymatic process whereby a glycan moiety (oligosaccharide moiety) is added to a protein [15]. Notably, the enzymatic process of protein glycosylation has to be discerned from protein glycation, an irreversible non-enzymatic process [16]. Many changes in glycosylation of proteins have been reported, but most are described in chronic inflammatory conditions. Less information on glycosylation processes is available in acute inflammatory situations [17].

Glycosylation has an impact on protein half-life and function. It increases half-life by increasing stability of proteins through masking sites for cleavage by proteases [18, 19]. Glycan chains are synthesized by glycosyltransferases, whereas specific glycan linkages are hydrolyzed by glycosidases [15]. The sequence of the glycans is determined by specificity of glycosyltransferases, glycosidases and the various nucleotide sugar donors that are available [15]. Moreover, inflammation-induced glycan modifications can alter the structure of the protein, and consequently redirect it to different cell membrane receptors, thereby affecting cellular functioning [20].

Glycosylation can also result in different types of glycans that are attached to proteins. Oligosaccharide structures, i.e. a carbohydrate containing three to ten monosaccharides, are bound to proteins through nitrogen atoms of asparagine or oxygen atoms of serine or threonine side chains, forming N- and O-linked glycoproteins, respectively [21]. Most circulating proteins are N-linked glycoproteins [22] and only these glycoproteins will be discussed here. N-linked oligosaccharides start with N-acetylglucosamine (GlcNAc) and are linked to asparagine residues of proteins [23]. The N-linked acute-phase glycoproteins have carbohydrate structures with 2-4 branches (diantennary, triantennary and tetra-antennary).

MARKERS OF LOW-GRADE SYSTEMIC INFLAMMATION

GLYCA

Until recently, large scale analysis of glycans was cumbersome [24]. However, nowadays, high-throughput proton nuclear magnetic resonance (NMR) spectroscopy is able to quantify inflammatory glycoproteins based on their glycan structure. With NMR technology it is possible to measure subsets of glycoproteins based on their shared glycan moieties. NMR measures the resonance frequency of molecules when placed in a strong magnetic field [25]. This results in a spectrum with peaks that are signals corresponding to specific molecules.

GlycA is such a novel proton NMR spectrometry-based biomarker. GlycA can be measured on a recently developed clinical NMR instrument, the Vantera® Clinical Analyzer, which is able to quantify analytes from NMR spectra collected on serum or plasma [26]. The GlycA signal originates from methyl groups of N-acetylglucosamine (GlcNAc) containing carbohydrate side chains of several abundant glycoproteins [26]. In addition, only the GlcNAc moieties in β(1 → 2) or β(1 → 6) linkage with a preceding mannose give rise to the GlycA NMR signal[26]. Hence, GlycA measures the glycan content of proteins, not protein concentrations as such. Neither the individual concentrations of the proteins involved, nor the differences in glycan structures can be determined by NMR spectroscopy. However, it is possible to make an overall estimate. Otvos et al. did this by multiplying the plasma concentration of each abundant glycoprotein by the number of N-glycosylation sites [26]. The following glycoproteins appear to have glycan chains make major contributions to the measured GlycA signal: α_1-acid glycoprotein, haptoglobin, α_1-antitrypsin, α_1-antichymotrypsin, and transferrin [26, 27]. However, since transferrin is a negative acute phase protein, only the other four glycosylated proteins are presumed to give rise to the increased GlycA signal observed in an inflammatory response [26]. All of the former glycoproteins are acute phase reactants each with a different time-scale, magnitude and direction of concentration change under inflammatory conditions [4, 28, 29]. As a result, GlycA may give a more stable measure of low-grade systemic inflammation that responds more uniformly to diverse inflammatory stimuli compared to other individual acute phase reactants. Notably, application of this NMR spectroscopy technique is confined to high-concentration molecules [30], and species with concentrations lower than approximately 20 μmol/L are not detectable. In addition, the GlycA signal arises mainly from residues that are distal from the site of attachment to the protein. Some of the carbohydrate side chains are close to the protein backbone and are not mobile enough to produce an NMR signal. Another reason why certain N-acetyl methyl signals do not contribute to the measured GlycA signal is constrained glycan chain mobility [26]. In summary, GlycA is a composite

biomarker that senses the integrated concentrations and glycosylation states of several of the most abundant acute-phase proteins in serum.

C-REACTIVE PROTEIN

Clinical assessment of acute inflammation is often performed through quantification of c-reactive protein (CRP). CRP was reported for the first time as an acute phase protein in 1930 [31]. Hepatic synthesis of CRP is regulated by various cytokines such as interleukin-6, interleukin-1 and tumor necrosis factor-α [32]. In healthy subjects, plasma CRP concentrations of around 2 mg/L can be encountered, while CRP values above 10 mg/L are generally used to indicate clinically relevant inflammation [33]. The plasma half-life of CRP is approximately 19 hours and is constant under all conditions of health and disease [34]. Measurement of CRP is useful for different purposes in clinical settings including monitoring infections and assessing the course of severity of inflammatory diseases. Moreover, CRP is much higher in bacterial than in viral infections; therefore measurement of CRP may be useful to differentiate bacterial from viral infections.

Furthermore, there is continued interest in determining whether CRP can be used for CVD risk prediction. While the traditional laboratory method is able to measure CRP with a detection limit of 3-5 mg/L, it lacks sensitivity to assess inflammation within the low-normal range. Therefore, the clinical utility of standard CRP evaluation for CVD risk detection is limited. Several high-sensitivity assays for CRP (hsCRP) are now commercially available and allow measurement of CRP within the low-normal range [35]. Noteworthy, since CRP is an acute phase reactant and has considerable within-individual variability, some studies suggest to use two measurements, optimally 2 week apart, to provide a more stable estimate for CV risk assessment [33]. Besides being associated with CVD risk, circulating levels of CRP have also been associated with prognosis in patients with several types of solid cancers [36]. In general, patients with cancer have been shown to have higher CRP concentrations than healthy controls.

GLYCA AND C-REACTIVE PROTEIN

hsCRP concentrations were found to be strongly correlated with GlycA (r = 0.54) in a large ethnically diverse population [26]. Of note, since CRP is not heavily glycosylated and the plasma concentration is much lower than of α_1-acid glycoprotein, haptoglobin and α_1-antitrypsin, it contributes negligibly to the GlycA signal [26]. Furthermore, during the acute phase response serum concentrations of hsCRP rise and fall within the first few days [4]. In contrast, the serum concentrations of the proteins that contribute to the measured GlycA signal give peak concentrations days after onset of an inflammatory

response [4, 28, 29]. These two biomarkers also differ in biological stability, with GlycA exhibiting far less day-to-day variation than hsCRP [26], which is likely another reflection of its composite origin.

OUTLINE OF THE THESIS

As alluded to above, low-grade systemic inflammation is associated with several adverse effects on health. Since the concentration of most acute-phase proteins increases in inflammatory states, GlycA is expected to be higher in subjects with inflammatory conditions. In this thesis we aim to investigate the role of GlycA as a marker of inflammation with emphasis on cardiometabolic risk markers, CVD, T2DM, MetS and in addition on life expectancy and mortality.

In **Chapter 2** we review recent progress in high-throughput laboratory methods for assessment of glycomics, i.e. the study of glycan structures, and glycoprotein quantification by methods such as mass spectrometry and NMR spectroscopy. We also discuss the clinical utility of glycoprotein and glycan measurements in the prediction of common low-grade inflammatory disorders including CVD, T2DM and cancer, as well as for monitoring autoimmune disease activity.

Lecithin:cholesterol acyltransferase activity (LCAT) is instrumental in high-density lipoprotein (HDL) maturation and remodeling. LCAT may also modify oxidative and inflammatory processes. LCAT was long considered to have cardioprotective properties, but more recently it became clear that high plasma LCAT mass and activity may predict (subclinical) atherosclerosis. MetS is featured by enhanced oxidative stress and low-grade chronic inflammation and also by higher plasma LCAT activity. In **Chapter 3** we examine the extent to which plasma GlycA is elevated in MetS, and determined its relationship with plasma LCAT activity.

Lipoprotein-associated phospholipase (Lp-PLA_2) is secreted by inflammatory cells in the arterial wall and is considered as a cardiovascular risk marker. Given the role of Lp-PLA_2 in stimulating pro-inflammatory cytokines it is plausible to hypothesize that higher plasma Lp-PLA_2 may coincide with higher GlycA concentrations. In **Chapter 4** we compare GlycA and Lp-PLA_2 mass, between subjects without T2DM or MetS and subjects with T2DM and/or MetS. We also test the association of GlycA with Lp-PLA_2 in each group.

Inflammatory processes play a role in the pathogenesis of atherosclerosis and hypertension. In addition, reducing sodium intake has been used a target for CVD prevention. However, the extent to which dietary sodium intake may confer alterations in the inflammatory status is unclear. In **Chapter 5** we determine the cross-sectional associations of the inflammatory markers GlycA and hsCRP with 24-h sodium excretion

in 3,935 subjects of the Prevention of Renal and Vascular End Stage Disease (PREVEND) cohort who were not using antihypertensive medication, lipid lowering drugs, or glucose-lowering treatment.

It has been recently shown that plasma GlycA is independently associated with incident CVD in a large cohort study of initially healthy women [37]. Further, no data are available with respect to the relationship of GlycA with renal function and albuminuria. It is important to determine these relationships, because both lower estimated glomerular filtration rate (eGFR) and higher degrees of albuminuria confer increased risk of cardiovascular morbidity and mortality [38-40]. In **Chapter 6** we determine i) whether GlycA associates with incident CVD in both men and women, and ii) the extent to which the anticipated association of GlycA with future CVD is modified by compromised renal function, as inferred from the eGFR and albuminuria and iii) the extent to which the anticipated association of GlycA with future CVD is attenuated by hsCRP. In **Chapter 7** we evaluate the analytical performance of the GlycA test, measured on the Vantera® Clinical Analyzer and test its prospective association with T2DM in the PREVEND cohort.

It is evident that systemic low-grade inflammation is related to adverse health effects. In **Chapter 8** we determine effects of GlycA and hsCRP on life expectancy in men and women of the PREVEND cohort. The method that we used in this chapter examines mortality against life expectancy as the time base. In **Chapter 9** we examine GlycA and hsCRP with risk of all-cause, cardiovascular, and cancer mortality in the PREVEND cohort.

Finally, **Chapter 10** provides a summary, general discussion, and future perspectives.

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Inflammatory Glycoproteins in Cardiometabolic Disorders, Autoimmune Diseases and Cancer

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2

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Abstract

The physiological function initially attributed to the oligosaccharide moieties or glycans on inflammatory glycoproteins was to improve protein stability. However, it is now clear that glycans play a prominent role in glycoprotein structure and function and in some cases contribute to disease states. In fact, glycan processing contributes to pathogenicity not only in autoimmune disorders but also in atherosclerotic cardiovascular disease, diabetes and malignancy. While most clinical laboratory tests measure circulating levels of inflammatory proteins, newly developed diagnostic and prognostic tests are harvesting the information that can be gleaned by measuring the amount or structure of the attached glycans, which may be unique to individuals as well as various diseases. As such, these newer glycan-based tests may provide future means for more personalized approaches to patient stratification and improved patient care. Here we will discuss recent progress in high-throughput laboratory methods for glycomics (i.e. the study of glycan structures) and glycoprotein quantification by methods such as mass spectrometry and nuclear magnetic resonance spectroscopy. We will also review the clinical utility of glycoprotein and glycan measurements in the prediction of common low-grade inflammatory disorders including cardiovascular disease, diabetes and cancer, as well as for monitoring autoimmune disease activity.

1. Introduction

Given the imperfections in the armamentarium of conventional biomarkers for diagnosis, prognosis, or risk prediction and disease prevention at the individual patient level, there is an ongoing effort using novel high-precision laboratory techniques to discover new biomarkers that will increase the sensitivity and specificity above current clinical tests [1-4]. Glycoproteins play key roles in inflammatory and pathological processes [5-9]. Thus, it is not surprising that investigation of the clinical utility of assays that measure inflammatory glycoproteins has received much attention [10-13]. Besides the clinical information that can be gleaned by quantifying circulating levels of glycoproteins, it is now clear that measurements based on the glycan structures of circulating proteins represent another avenue for improving diagnosis, prognosis and risk prediction of common inflammatory disorders [4, 7, 13-19]. Here we will briefly review the biochemistry and metabolism of glycoproteins, provide insight into the glycoprotein assays that are currently available for clinical use and describe newer high-throughput technologies that are being employed for identifying new glycan-based biomarkers that will add to the current armamentarium and are expected to improve patient care.

2. Glycoprotein biochemistry and rationale for measuring glycoproteins and glycans

Protein glycosylation is the enzyme-mediated post-translational process responsible for the attachment of glycan chains either to the nitrogen of an asparagine residue (N-linkage) or the oxygen of a serine or threonine residue (O-linkage) [8, 20]. While most O-linked glycoproteins remain intracellular or are secreted and become part of the extracellular matrix, most of the abundant proteins in the circulation are N-linked glycoproteins. N-linked glycosylation is initiated in the endoplasmic reticulum and the oligosaccharide chains are further modified via a set of glycosyltransferases in the Golgi apparatus to form the basic glycan structure. The sequence of sugar residues and the overall structure of the oligosaccharide chain depend on the cell type-specific glycosyltransferases and glycosidases and the availability of the various sugar nucleotide donors [20]. Given the vast number of known glycosyltransferases, glycosidases and monosaccharides, and the diversity of linkages that can occur, the molecular structures of protein-bound glycans are remarkably diverse, even before the glycoproteins have been released into the circulatory system [21].

Plasma levels of the majority of circulating glycoproteins rise (positive acute phase proteins) or fall (negative acute phase proteins) during the acute phase response, the systemic reaction to the presence of infection, tissue damage, cancer and pregnancy

[5, 16, 22, 23]. **Table 1** provides examples of both positive and negative acute phase glycoproteins and illustrates the diverse roles they play during an inflammatory reaction. Inflammatory glycoproteins are predominantly synthesized and secreted by hepatocytes but can be produced by activated macrophages and neutrophils in the periphery [5, 15, 17]. While IL-6 is the predominant stimulator of overall glycoprotein production during acute and chronic inflammation, other cytokines such as IL-1β, TNFα, interferon γ, TGFβ and IL-8, stimulate the production of subsets of glycoproteins. Because inflammation is the basis for many autoimmune and chronic low grade inflammatory diseases such as cardiovascular disease (CVD), type 2 diabetes (T2DM) and cancer, glycoproteins play an integral part in the physiology and pathophysiology of these diseases. As a result, many current clinical tests utilize circulating levels of inflammatory glycoproteins (e.g. haptoglobin and α-fetoprotein) for diagnostic or prognostic purposes.

Besides changes in circulating protein levels, the glycan structures of acute phase glycoproteins are dynamically altered by glycosidases, glycosyltransferases and sialyltransferases in the circulation [14, 15]. Post-translational modifications in glycan structures during inflammation include changes in the number of antennary branches, increased sialylation and fucosylation and decreased galactosylation [14-16].

While the glycans of some proteins remain rich in mannose residues, the carbohydrate structures of many N-linked inflammatory glycoproteins become bi-, tri- and tetra-antennary after inflammation-mediated processing [14-16] (**Figure 1**). These branched glycans are rich in N- acetylglucosamine (GlcNAc), N-acetylgalactosamine, sialic acid and fucose residues in a myriad of different arrangements, contributing to the potential diversity of glycan structures [14-17, 20, 21] (**Figure 1**). Therefore, there are both intracellular and extracellular post- translational processes that contribute to the overall diversity of glycan structures that can occur in any one individual. These are also many factors that can influence glycan complexity including: 1) cell-type specific expression of glycosyltransferases, glycosidases, 2) availability of the various monosaccharides, 3) age, 4) gender, 5) epigenetic background, 5) environment (e.g. health, diet, smoking and alcohol consumption) and 6) disease processes (e.g. autoimmune diseases, cancer as well as low-grade inflammatory diseases such as CVD and T2DM) [21, 24].

Although it was once thought that the only purpose for having carbohydrate side-chains on glycoproteins was to aid in protein stability, it has become increasingly clear that glycans play a much more active role in glycoprotein structure and function. Glycans participate in many key biological processes including ligand binding, transport and clearance, cell adhesion, receptor binding and activation and signal transduction [4, 7-9, 14, 15, 20]. Inflammation-induced glycan modifications affect protein folding by masking sites for protease cleavage, preventing proteolysis and extending the circulating half-life of serum proteins [4, 8, 9, 20, 25]. Moreover, they alter a protein's tertiary or

quarternary structure, redirecting it to different cell membrane receptors and changing its downstream cellular effects [4, 8, 9, 15, 20]. These functional alterations may lead to modulation of the immune response or, if modified aberrantly, can lead to autoimmune disease. For example, glycans are a fundamental part of self- versus non-self- recognition and alterations in immunoglobulin G (IgG) glycosylation have been reported in various immune diseases including rheumatoid arthritis (RA) [8, 20]. Therefore, glycans are often casual in the disease process and monitoring these changes may provide pertinent information regarding disease stages. In effect, both desirable and undesirable changes in glycan structure may be exploited for risk assessment, patient stratification, diagnostic or prognostic purposes [4, 13, 18, 24, 26, 27].

Alpha1-acid glycoprotein (AGP), also known as orosomucoid, provides a good example of how changes in glycan structure can affect glycoprotein function and be exploited for diagnostic or prognostic purposes [15]. Normal circulating concentrations of AGP range from

0.6-1.2 mg/mL, and its plasma level is increased up to 50-fold during an acute inflammatory response, making AGP the second most abundant circulating protein (1-3% of plasma protein) [4, 15]. AGP contains 5 sites for N-linked glycosylation and is therefore very high in carbohydrate content (>40%) [4, 15]. During an acute phase response, the lengths of the oligosaccharide chains on AGP increase and are modified from bi- to tri- and tetra-antennary branches, accompanied by an increase in fucosylation and sialylation [4, 15]. Both the immunomodulatory and the binding properties of AGP are strongly dependent on its carbohydrate composition; therefore, inflammation-mediated alterations in glycan structure have a profound effect on AGP function [15].

Increased fucosylation of AGP has been reported in some diseases, allowing measurement of AGP fucosylation to be useful for diagnostic purposes. For example, fucosylated AGP was significantly higher in patients with liver cirrhosis compared to steatosis of the liver, non-alcoholic steatohepatitis (NASH) and fibrosis due to chronic viral- induced hepatitis, suggesting that this glycan marker may be useful for detecting livercirrhosis [15]. Interestingly, AGP glycan modification appears to occur in some inflammatory diseases, but not others. For example, increased AGP glycan branching has been observed in patients with asthma and RA but not in patients with ulcerative colitis [15]. Moreover, glycan structure modifications on AGP led to reduced collagenase-3 activity and collagen binding, which could exacerbate the disease process in RA patients [15] . This may be true for many other circulating inflammatory glycoproteins (**Table 1**). Given the diversity in the numbers of glycoproteins in biological fluids as well as the unique changes that may occur in some diseases and not others, there is likely a wealth of information yet to be mined from glycoproteins as well as their glycans for clinical use [24].

Table 1. Human inflammatory glycoproteins modified during an acute phase response.

Category	Positive Acute Phase Proteins	Molecular Weight (kDa)	Glycosylation Sites (#)	UniProt number*	Adult Concentrations in Serum**
Binding or Transport proteins	α1-acid glycoprotein (AGP/ orosomucoid)	41-43	5	P02763	0.5-1.2 mg/mL
	Haptoglobin	100	4	P00738	0.3 - 3.0 mg/mL
	Ceruloplasmin	151	6	P00450	0.2-0.6 mg/mL
	Mac-2 (or galectin-3) binding protein	85-97	7	Q08380	1.4-16.1 μg/mL
Antiproteases	α1-antitrypsin	52	3	P01009	0.9-2.0 mg/mL
	α2-macroglobulin	179	8	P01023	1.3-3.0 mg/mL
	α1-antichymotrypsin	68	6	P01001	1.5-3.5 mg/mL
	Kallistatin	58	4	P29622	10 μg/mL
Complement system	C2	83	8	P06681	0.02-0.4 mg/mL
	C3	185	3	P01024	0.9-1.8 mg/mL
	C5	190	4	P01031	0.02-0.4 mg/mL
	C1 esterase inhibitor	105	7 N-, 8 O-linked	P05155	0.21-0.39 mg/mL
Coagulation system	Fibrinogen α,β,γ	340	5 N-, 2 O-linked	P02671, -75, -79	1.5-4.0 mg/mL
	Plasminogen	92	1 N-, 2 O-linked	P00747	plasma 120-200 μg/mL
	Vitronectin	140	3	P04004	plasma 110-140 μg/mL
	α2-antiplasmin	70	4	P08697	70 μg/mL in plasma, 47.6 μg/mL in serum
	Prothrombin	72	3	P00734	detection range 0.031 - 32 μg/mL
	Plasminogen activator inhibitor-1 (PAI-1)	43	3	P05121	plasma 5-40 ng/mL
	Tissue plasminogen activator (tPA)	72	3 N-, 1 O-linked	P00750	1-18 ng/mL

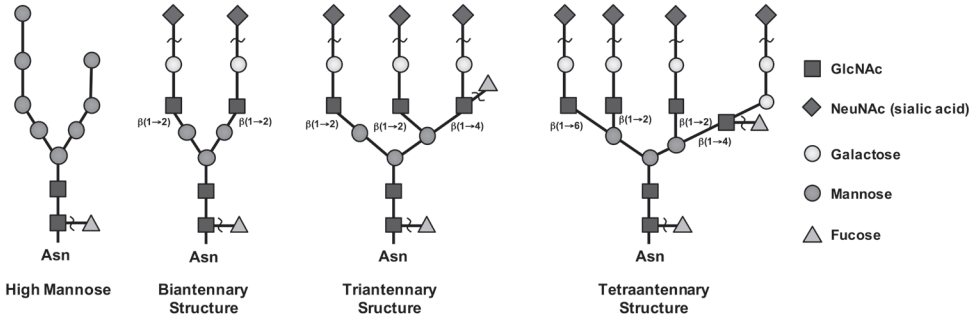
Miscellaneous	Fibronectin	220-440	7 N-, 3 O-linked	P02751	0.3 mg/mL
	Lipoprotein phospholipase A2 (Lp-PLA2)	45	2	P13093	0.5-100 ng/mL
	C-reactive protein (CRP), pentamer	115-120	1†	P02741	hsCRP <1.0 µg/mL; ≥3.0 µg/mL risk for CVD
	Serum amyloid A (SAA)	13.5	0	P0DJ18	0.41 - 300 ng/mL; SAA is not glycosylated.
Category	**Negative Acute Phase Proteins**	**Molecular Weight (kDa)**	**Glycosylation Sites (#)**	**UNIPROT number***	**Adult Concentrations in Serum**
Miscellaneous	Transferrin	76-81	3 N-, 1 O-linked	P02787	2.1-3.6 mg/mL
	Transthyretin	55	1	P02766	0.2-0.4 mg/mL
	α2-HS-glycoprotein (fetuin)	58	2 N-, 4 O-linked	P02765	0.21-0.45 mg/mL
	α-fetoprotein (AFP)	70	1	P02771	<15 ng/mL
	Thyroxine binding protein	54	5	P05543	0.011-0.021 mg/mL
	Coagulation Factor XII	80	2 N-, 7 O-linked	P00748	plasma 0.1-100 ng/mL

*Confirmation of contribution to the acute phase response and the number of sites that are glycosylated was obtained using the UniProtKB/Swiss-Prot database. http://www.uniprot.org/. The UniProt Consortium. UniProt: a hub for protein information Nucleic Acids Res. 43: D204-D212 (2015). For a more comprehensive review of plasma protein glycosylation see reference [4].

**Reference for adult (age 20-60 years) concentrations: C.A. Burtis, E.R. Ashwood, and D.E. Bruns. eds., Tietz Textbook of Clinical Chemistry and Molecular Diagnostics (Fourth edition) Philadelphia, WB Saunders, 2006, Chapter 56 pg. 2251-2302. If no standardized assay is available, a normal detection range was reported from a commercially available ELISA assay.

†Das T. et al., Biochem J. 2003 Jul 15;373(2):345-55.

Figure 1



Examples of N-linked glycans showing mannose-rich as well as bi-, tri-, and tetra-antennary glycan structures. Two N-acetyl glucosamine (GlcNAc) residues occur at the site of protein attachment. Additional GlcNAc residues can be attached via β(1–2), β(1–4) or β(1–6) linkages to mannose residues at the sites of glycan branching. Sialic acid and fucose residues are added or removed during inflammatory processes.

3. Assays of glycoproteins in biological fluids and development of high-throughput assays for glycan measurement

Currently, concentrations of individual inflammatory glycoproteins are determined using immunochemical methods such as enzyme-linked immunosorbent assays (ELISAs), electrochemiluminescence immunoassay (ECLIA), luminex based assays, radioimmunoassays (RIA) and nephelometric assays that quantify the amount of protein present in biological samples (**Table 2**). Such assays are employed to determine protein levels of many of the inflammatory glycoproteins including AGP, haptoglobin, 1-antitrypsin, α2- macroglobulin, α1-microglobulin and β2-microglobulin. While quantifying protein levels remains the mainstay for measurement of inflammatory glycoprotein levels, measuring the glycan portion of inflammatory proteins is becoming increasingly useful for diagnostic purposes. This can be accomplished using lectin-binding ELISAs (**Table 2**) as well as some of the newer high-throughput technologies such as mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) which have recently been introduced to the clinical laboratory.

MS techniques are becoming more common place in clinical laboratories. However, effective analysis of protein-derived circulating glycans is still difficult to accomplish due to the high complexity that is caused by variations in glycan linkage and branching, macro- and micro-heterogeneity. Currently, a combination of methods is often used. Here we describe some of the major MS-based approaches used in glycomics research which may eventually identify new tests for clinical use. Methods for O-linked structures are less well developed compared to methods for N-linked structures and will not be discussed in this review.

Normal phase high performance liquid chromatography (HPLC) is a well-known separation technique that has been used in laboratories for years. In addition, ultra performance liquid chromatography (UPLC) involves HPLC with very high pressure and is one of the newest chromatography technologies in the field of glycomics. UPLC allows high efficiency separations and reduced analysis times [28]. UPLC has the ability to separate glycan isomers. Until recently, UPLC was not widely used in the field of glycan profiling due to the lack of appropriate stationary phases [29, 30]. Hydrophilic interaction liquid chromatography (HILIC) is a separation technique which is related to normal phase HPLC. HILIC columns were originally used for analysis of highly polar analytes and later also for other types of substances including peptides [31] and glycans [32]. A limitation of HILIC- based analyses is the amount of time required per chromatographic run. However, since the introduction of sub-2-µm stationary phases, HPLC or UPLC in combination with HILIC have been used for analyzing glycans [29, 32]. Separation of structural isomers is often achieved which makes HILIC in combination with HPLC or UPLC a valuable tool for structural analysis of oligosaccharides.

Fluorescence detection is a glycan analysis method for quantifying fluorescently labeled glycans. The labeled glycans can be separated by, for example, HILIC and detected by sensitive fluorescence detectors or by MS in some cases. The use of a fluorescence detector enables quantification of even minor glycans. Tagging the glycans with a fluorescent label such as 2-aminobanzamide (2-AB) allows the glycan to be detected even at femtomole levels [33]. Besides 2-AB other fluorescent tags are commercially available. The advantages of 2-AB is that it is compatible with multiple analytical methods including MS which makes it possible to obtain mass and structural information [34].

MS-based detection techniques are promising as enabling methods in the field of glycomics. The glycan can be removed either enzymatically or chemically from the protein. Intact N-linked glycans can be enzymatically split from glycoproteins with an amidase such as peptide-N-glycosidase F [34]. Alternatively, hydrazinolysis can be used for chemical release. MS provides molecular mass and structural information. A wide variety of MS-based techniques are available for glycoconjugate analysis. However, quantification by MS is not always reliable and for some samples there can be overlap from isobaric glycans (discrete isomeric glycan structures that possess the same mass) [33]. MS can be used alone or coupled to separation methods such as HPLC, UPLC, HILIC or capillary electrophoresis to increase the sensitivity [35-37]. Furthermore, matrix assisted laser desorption-ionization-MS and electrospray ionization-MS are often applied. If there are a variety of possible isomers, each one may be discriminated from the other using multistage analyses. However, MS data can be very complex and interpretation requires expertise.

Table 2. Glycoprotein tests for risk assessment, diagnosis or prognosis of various diseases.

Disease	Serum Test	Glycoprotein(s) or Sugar Residue	Assay Type	Clinical Application
Cardiometabolic disorders	hsCRP	High-sensitivity C-reactive protein	Nephelometry	Risk for CVD or all-cause mortality and prognosis for recurrent events in patients with coronary disease or ACS
	Fibrinogen	Fibrinogen	ELISA or activity assay	Detecting blood clotting and bleeding disorders; has been shown to have associations with CVD and all-cause mortality
	Total serum sialic acid	N-acetylneuraminic acid	Colorimetric,enzymatic, chromatographic and fluorescence assays	Risk assessment for CVD or all-cause mortality
	GlycA	N-acetylglucosamine	NMR	Risk assessment for CVD or all-cause mortality
	LecT-Hepa	Lectins that bind to glycans on AGP	Lectin binding	Detecting liver fibrosis in patients with chronic Hepatitis B or C
	Mac-2 BP, Fuc-Hpt	Mac-2 binding protein Fucosylated haptoglobin	ELISA and Lectin-antibody ELISA	Distinguish NASH from fatty liver
Autoimmune diseases	CRP	Conventional C-reactive protein	Nephelometry	Infection, tissue injury, and inflammatory disorders.
	ESR	Fibrinogen and immunoglobulins	Sedimentation rate of red blood cells per hour	Assessment of disease activity in RA
	MBDA	VCAM-1, EGF, VEGF-A, IL-6, TNF-R1, MMP-1, MMP-3, YKL-40, Leptin, Resistin, SAA and CRP	Luminex based assays	Assessment of disease activity in RA
	GlycA	N-acetylglucosamine	NMR	Assessment of disease activity in RA

Cancers	AFP	α-fetoprotein	ECLIA	Diagnosis, staging, detecting recurrence and monitoring of therapy for liver cancer
	PSA, Pro2PSA	Prostate specific antigen	ECLIA	Screening, discriminating prostate cancer from benign disease
	CA125	MUC16 or cancer antigen 125	ECLIA	Monitoring therapy, detecting recurrence of ovarian cancer
	HE4	WFDC2 or human epididymis protein 4	ELISA	Monitoring therapy, detecting recurrence of ovarian cancer
	CA15-3	Sialylated oligosaccharide on MUC1	CMSI	Monitoring therapy for breast cancer
	CA27-29	MUC1 protein levels	CMSI	Monitoring therapy for breast cancer
	CA19-9	Serum Lewis antigen (SLe^a)	RIA	Monitoring therapy for pancreatic and ovarian cancer
	CEA	Cell adhesion glycoproteins	ECLIA	Monitoring therapy, detecting recurrence of multiple cancers
	OVA1	β2-microglobulin, CA125II, apoA-I, prealbumin, transferrin	Immunoassays	Prediction of metastatic ovarian cancer
	ROMA	Combined HE4, CA125II	ELISA and ECLIA	Prediction of metastatic ovarian cancer
	GlycA	N-acetylglucosamine	NMR	Predicting risk of colorectal cancer

More extensive lists of glycoprotein tests and biomarkers can be found in references [12] and [19]. ACS, acute coronary syndrome; AGP, α1-acid glycoprotein; CEA, Carcinoembryonic antigen; CMSI, chemiluminescent microparticle 2-step sandwich immunoassay; CRP, C-reactive protein; CVD, cardiovascular disease; ECLIA, electrochemiluminescence immunoassay; EGF, epidermal growth factor; ELISA, enzyme linked immunosorbent assay; ESR, erythrocyte sedimentation rate; MMP-1, matrix metalloproteinase 1; MMP-3, matrix metalloproteinase 3; NASH, non-alcoholic steatohepatitis; NMR, nuclear magnetic resonance; RA, rheumatoid arthritis; RIA, radioimmunoassay; SAA, serum amyloid A; TNFRI, tumor necrosis factor receptor type I; VCAM-1, vascular cell adhesion molecule 1; VEGF-A, vascular endothelial growth factor A.

Although these techniques have been useful for identifying novel glycan moieties on various glycoproteins, and it has been speculated that these novel assays may eventually be useful for diagnostic purposes, none of the MS-based techniques have been routinely employed in the clinical laboratory to date. Proton (^{1}H) NMR, another high-throughput technological platform that is able to quantify inflammatory glycoproteins based on their glycans, was recently introduced to the clinical laboratory setting [38-43]. Although it is not possible to identify and quantify individual proteins via NMR, it is possible to measure subsets of glycoproteins based on their shared glycan moieties [38, 39, 42]. Protons on the sugar residues in the oligosaccharide chains emit different signals depending on their structural environment. For example, the N-acetyl methylgroup protons emit different NMR signals if they are part of GlcNAc as opposed to N- acetylneuraminic acid (sialic acid), allowing for identification of the various sugar residues based on the chemical shift of their protons, i.e. the position of the signal peak in the NMR spectrum [38, 39]. The complex glycan structures of several acute phase proteins including AGP and transferrin have been determined and catalogued using NMR, allowing for easy identification of the NMR signals for a number of the sugar residues found on inflammatory glycoproteins [38, 39].

Recently, an NMR-based assay called GlycA was developed that quantifies circulating inflammatory glycoproteins based on a subset of mobile GlcNAc residues [42, 44]. In fact, it is only the GlcNAc moieties in (12) or (16) linkage with a preceding mannose that give rise to the GlycA NMR signal at 2.00 0.01 ppm in the *NMR LipoProfile*® test spectra of serum or plasma [38, 42, 45]. It is also possible to quantify the methyl signals from GlcNAc residues at other positions in the bi-, tri-, and tetra-antennary glycans as well as from sialic acid [38, 42, 44, 45]. Therefore, it is possible that there are other NMR signals besides GlycA that, when quantified, may provide useful information for the clinician.

The serum GlycA NMR signal is comprised primarily of contributions from the GlcNAc residues on AGP, haptoglobin, 1-antitrypsin, 1-antichymotrypsin and transferrin [42]. Because plasma concentrations of C-reactive protein (CRP) and cytokines are much lower in comparison and they are not heavily glycosylated, they contribute negligibly to the measured GlycA signal [42]. Reduced glycan mobility is another reason why not all proteins with GlcNAc residues produce observable NMR signals, which is the case for fibrinogen and IgG [42]. Haptoglobin, AGP, 1-antitrypsin and 1-antichymotrypsin are positive acute phase proteins that increase in concentration and glycan complexity in inflammatory states [7, 14, 17], enabling GlycA to be a biomarker of systemic inflammation that is associated with inflammatory markers such as high-sensitivity CRP (hsCRP), fibrinogen, IL-6, serum amyloid A (SAA) and lipoprotein-associated phospholipase A_2 (Lp-PLA_2) [42, 46-51] as well as increased neutrophil activity [52]. It has also been reported that GlycA is related to increased mortality risk [1, 52, 53][Gruppen

et. al. unpublished results]. Therefore, despite similarities in disease associations, GlycA, CRP, fibrinogen and other inflammatory markers likely capture different aspects of the inflammatory response [52]. Moreover, it has been reported that hsCRP, but not GlycA, levels were decreased after statin administration [53]. Therefore, it is clear that GlycA and other inflammatory biomarkers may at least be in part independent, and perhaps even additive, in the clinical information they impart. Furthermore, as a composite biomarker that measures both the increased protein levels and enhanced glycosylation states of the most abundant circulating acute phase proteins, GlycA may be a better reflection of a systemic acute phase response than any single glycoprotein component [42]. For example, assays for measuring individual acute phase proteins, such as hsCRP, often exhibit high intra-individual variability [54-57]. One approach to overcome this issue is to measure multiple inflammatory markers at once. For instance, one can compute a low-grade inflammation score, based on the Z-scores of a number of individual inflammation markers, such as hsCRP, TNF-α, IL-6, IL-8, SAA, soluble intercellular adhesion molecule 1 (sICAM-1), ceruloplasmin and haptoglobin [58]. While useful for research purposes, this computation is not convenient for physician use. GlycA, on the other hand, is already a composite biomarker that simultaneously measures multiple markers, giving it the advantage of having low within-subject biological variation [42].

4. Potential clinical utility for inflammatory glycoprotein assays

4.1 Glycoprotein assays and cardiometabolic disorders

Besides serving as biomarkers of acute or chronic inflammation or infection, elevations of glycoproteins such as hsCRP and fibrinogen are of clinical interest as markers of CVD (**Table 2**). Driving much of this interest is the established role of inflammation in all stages of the atherosclerotic disease process from lesion initiation to progression as well as plaque destabilization [59, 60]. Epidemiologic studies have confirmed the link between systemic inflammation and adverse clinical outcomes by demonstrating consistent, independent associations of hsCRP and fibrinogen with both incident CVD and all-cause mortality [61, 62]. Among the many inflammatory proteins that could serve as clinical indicators of the risk associated with inflammation, hsCRP has been favored due to its stability in fresh and frozen specimens, wide dynamic range, and availability of relatively inexpensive, standardized, and precise high-sensitivity immunoassays [59, 60, 63, 64].

Glycan moieties themselves, such as sialic acid (N-acetylneuraminic acid), the terminal monosaccharide of glycoconjugates, have also been shown to correlate with CVD [65]. Several types of assays have been deployed for the quantification of total serum sialic acid including colorimetric, enzymatic, chromatographic and fluorescence

based assays [65]. Although sialic acid can be found on glycolipids, the majority of serum sialic acid can be found on the glycan chains of AGP, haptoglobin, 1-antitrypsin, 1-antichymotrypsin, ceruloplasmin, fibrinogen and transferrin [65]. Sialic acid was shown to be positively associated with TNFα and IL-6 [65] and multiple studies have shown positive associations of total serum sialic acid with CVD, stroke and mortality [65-68]. A recent study reported that sialic acid was an independent risk marker for CVD during 40 years follow-up among Swedish individuals [69]. Taken together, sialic acid is a marker of systemic inflammation that can be used for risk assessment in subjects with CVD, heart failure and T2DM [65-67, 69, 70].

GlycA, the NMR signal derived from multiple inflammatory glycoproteins, was demonstrated to predict future CVD and T2DM (**Table 2**) [71-73]. GlycA was shown to be related to the leptin/adiponectin ratio, suggesting that adipose tissue-associated low-grade inflammation could be involved in the regulation of inflammatory glycoproteins [49]. Similar to hsCRP, GlycA was found to be higher in subjects with metabolic syndrome and was positively correlated with body mass index (BMI) and insulin resistance determined by homeostasis model assessment of insulin resistance (HOMA-IR) [48-50]. In the Women's Health Study (WHS), GlycA was associated with CVD events, independent of traditional risk factors [71]. In the Prevention of Renal and Vascular End-stage Disease (PREVEND) study, GlycA was associated with incident CVD, defined as the combined end-point of CV morbidity and mortality, independent of clinical and lipid measures as well as renal function [72]. Baseline concentrations of GlycA in the Justification for the Use of Statins in Prevention: an Interventional Trial Evaluating Rosuvastatin (JUPITER) trial were significantly associated with incident CVD events, even when adjusting for established risk factors and a family history of premature coronary heart disease [73]. Remarkably, this association was only slightly attenuated by hsCRP, suggesting that the two biomarkers are reflecting somewhat different pathobiological processes [73]. In addition, GlycA was shown to be associated with future major adverse coronary events and mortality in two different cohorts of patients undergoing coronary angiography [1, 52, 74]. Of note, the association of GlycA with incident T2DM remained statistically significant both in the WHS and PREVEND even after adjusting for traditional diabetes risk factors and hsCRP [43, 75, 76]. Thus evidence is accumulating that GlycA may be a useful biomarker for the assessment of CVD and T2DM risk.

A lectin-based assay, called LecT-Hepa, that exploits the changes in the glycan structure of AGP has been developed to detect liver fibrosis in patients with chronic viral hepatitis and NASH (**Table 2**) [77]. LecT-Hepa is a multi-lectin antibody immunoassay that binds glycosylated AGP [77]. First AGP is immunoprecipitated using a high-throughput, automated protein purification system (ED-01), then a fully automated immunoassay analyzer (HISCL-2000i) is employed to acquire the two glycoprotein binding parameters

(AOL/DSA and MAL/DSA) that are produced by the binding of glycosylated AGP to three lectins isolated from Aspergillus oryzae (AOL), Maackia amurensis (MAL) and Datura stramonium (DSA) [77]. A formula is then used to calculate a score that was reported to correlate with fibrosis stage as determined by liver biopsy [77]. This assay gave comparable, if not better performance than the FIB-4 index, for the diagnosis of significant fibrosis in chronic hepatitis C patients [78] and comparable performance to FibroScan in hepatitis B infected patients [79]. This assay, however, is not yet available in the clinical laboratory.

Recently it was shown that quantification of two inflammatory glycoproteins quantified by ELISA, fucosylated haptoglobin and Mac-2 binding protein (also known as galectin-3 binding protein), may be useful for the diagnosis of NASH and liver fibrosis (**Table 2**) [80]. The authors hypothesized that the fucosylation-based sorting machinery is disrupted in ballooning hepatocytes and that hyperfucosylated glycoproteins are secreted from the liver into serum in the diseased liver. Based on this hypothesis they developed a lectin-based ELISA to quantify fucosylated haptoglobin and showed that this assay was useful for the prediction of ballooning hepatocytes in NASH [80]. They also showed that Mac-2 binding protein, quantified by traditional ELISA, was a good biomarker for liver fibrosis. Moreover, the combination of the two glycoproteins was able to distinguish NASH from simple hepatic steatosis [80].However, additional clinical validation studies are needed to fully understand the clinical usefulness of this combined biomarker test.

4.2 Glycoprotein assays and autoimmune diseases

RA is an autoimmune disease that manifests itself as severe inflammation in multiple joints, leading to erosions of the cartilage and bone and sometimes causing joint deformity. Joint pain, swelling, and redness are common symptoms of RA. Tight control of disease activity, including monitoring of acute phase proteins is standard of care in RA management [81, 82]. The markers most commonly used to assess RA disease activity are CRP and erythrocyte sedimentation rate (ESR) (**Table 2**) [81, 82]. Both tests have been incorporated into the Disease Activity Score based on 28 joints (DAS28), the core set of measures proposed in the American College of Rheumatology and the American College of Rheumatology/European League Against Rheumatism RA remission criteria [83-85]. However, both CRP and ESR have limitations. For example, ESR is altered by non-inflammatory conditions such as chronic kidney disease, pregnancy, anemia, abnormal red blood cell shape or size, and serum protein concentrations [86]. Because some of these confounding influences are unrelated to RA disease activity, the current treat-to-target recommendations include cautions about the use of ESR for monitoring RA activity [82].

HsCRP exhibits high variability over time, potentially making it less reliable for assessment of RA disease activity at any one time point [55-57]. Moreover, CRP and ESR values are in the normal range in up to half of patients with active disease and they are often discordant with each other. Thus, alternative markers of inflammation whose measurements aren't affected by these factors would be useful for assessing RA disease activity.

Recently a multi-biomarker disease activity (MBDA) blood test was developed to assess disease activity in adult patients with RA (**Table 2**) [87-90]. The test measures 12 inflammatory biomarkers (VCAM-1, EGF, VEGF-A, IL-6, TNF-R1, MMP-1, MMP-3, YKL-40, leptin, resistin, SAA and CRP), including a number of cytokines and acute phase glycoproteins that play key roles in the underlying pathophysiology of RA [87-90]. The MBDA test is based on an algorithm that uses the concentrations of the 12 biomarkers to generate a score that represents the level of RA disease activity on a scale of 1 (lowest activity) to 100 (greatest). Analytical validation studies have proven the MBDA test to be precise and reproducible [87-90]. The MBDA test was developed to correlate with the 28- joint Disease Activity Score (DAS28) and has been clinically validated by correlations with DAS28 and other disease activity measures in independent RA cohorts, with thresholds established for low, moderate and high disease activity [87-90]. Other studies show that the MBDA test tracks responses to treatment with biologic and non-biologic disease-modifying antirheumatic drugs (DMARDs) and may potentially be an indicator of progressive joint damage in patients with RA [87-90]. The MBDA test, however, has not been validated for diagnosing RA.

GlycA may be useful for assessing disease activity and monitoring anti-inflammatory treatment in patients with autoimmune diseases like RA and SLE (**Table 2**). GlycA was shown to be higher in RA and systemic lupus erythematosus (SLE) [47, 91, 92]. In a cross- sectional study that included 166 RA patients and 90 control subjects, GlycA concentrations were higher in RA patients compared to control subjects [47]. Moreover, increased GlycA concentrations were robustly associated with increasing degree of RA disease activity [47]. GlycA was associated with the 28-joint count Disease Activity Score with erythrocyte sedimentation rate (DAS28-ESR) and its components: tender and swollen joint counts, patient-reported global health score, ESR and hsCRP [47]. Additionally, GlycA was significantly correlated with Larsen score, a radiographic scoring of joint disease, whereas hsCRP and ESR were not [47]. GlycA concentrations were not different between rheumatoid factor (RF) positive and negative RA patients, which was expected given that glycosylated immunoglobulins do not contribute to the GlycA NMR signal [42, 47]. Additionally, GlycA was associated with coronary artery atherosclerosis in patients with RA [47]. GlycA levels were also higher in patients with SLE than matched control subjects [91]. In the same study, GlycA levels were positively associated with ESR, hsCRP, E-selectin, sICAM-1 and triglycerides, but not with creatinine, SLE Disease

Activity Index (SLEDAI), SLE Collaborating Clinics (SLICC/ACR) Damage Index, or coronary calcium scores [91]. In a separate SLE cohort, mean GlycA levels were somewhat higher in female patients with high disease activity vs. patients with low or no disease activity and non-afflicted women [42, 92]. In a longitudinal analysis of SLE activity, GlycA increased significantly along with increases in SLEDAI [92]. Taken together, GlycA may have utility for assessing disease activity in patients with autoimmune diseases such as RA and SLE. Given its ability to predict CVD events and its association with coronary artery atherosclerosis, GlycA may also be useful for assessing CVD risk in patients with autoimmune diseases, for whom traditional CVD risk factors such as low density lipoprotein cholesterol (LDL-C) and total cholesterol lack strong CVD associations [93-98].

4.3 Glycoprotein assays and cancer

Cancer is the second most common cause of death in developed countries, with breast and prostate cancer being the most prevalent in the United States [99]. While early detection has helped reduce cancer-related deaths, many cancers are not discovered until they are at a more advanced stage, when prognosis is often not favourable. Most of the clinically used cancer biomarkers are effective when applied to patients with later stage cancers but are often ineffective at detecting early stage cancers. As is the case in other therapeutic areas, single biomarkers have not been identified that have sufficient sensitivity and specificity to be completely reliable. Therefore, there is an urgent need for novel biomarkers with better performance for cancer diagnosis and prognosis. As such, aberrant protein glycosylation is a well-known hallmark of cancer and represents a promising source of new biomarkers that can be used as standalone tests or in composite panels.

Unlike other disease areas, there are several FDA-cleared tests used currently in medical practice that measure glycoproteins as biomarkers of cancer (**Table 2**). The α-Fetoprotein (AFP) test is used for diagnosis, staging, detecting recurrence and monitoring therapy for hepatocarcinoma (HCC) [12]. Serum levels, however, do not allow for discrimination between HCC and benign liver disease [100]. An additional biomarker was developed that is based on a highly fucosylated form of AFP that appears in serum at the stage of liver cirrhosis, just before the onset of HCC [100]. The AFP-1.3 fraction, as it is called, detects both the circulating protein and the increased fucosylation that occurs in patients with liver cancer and has been cleared by the FDA as a marker for early detection of HCC [100]. Additional liver-secreted proteins with promise for early detection of HCC and disease progression are fucosylated GP73, kininogen and haptoglobin [100, 101]. Prostate-specific antigen (PSA) is a test that is used for early detection of prostate cancer. However, the PSA test suffers from the inability to discriminate between prostate cancer and benign prostate hyperplasia [12]. Recent studies showed that altered fucosylation

and sialylation of PSA may be exploited to develop a more specific biomarker that is able to distinguish aggressive from nonaggressive forms of prostate cancer as well as benign hyperplasia [12, 100, 102, 103]. Cancer antigen 125 (also known as CA125, mucin 16 or MUC16) and human epididymis protein 4 (HE4 or WFDC2) are glycoprotein tests that are used for detecting ovarian cancer [12]. CA15-3 and CA27-29 are tests that measure the amount of sialylated glycan or protein levels of mucin 1 (MUC1). These tests are commonly used for breast cancer therapy [12]. Carcinoembryonic antigen (CEA), a test that measures glycoproteins that are involved in cell adhesion, is used for monitoring therapy and detecting recurrence of colon, gastric, pancreatic, lung or breast cancer [12].

None of the single glycoprotein tests is considered optimal; therefore, better biomarker tests are needed for early diagnosis, prognosis and personalized medicine in the cancer field [18]. Multivariate algorithms have been developed that increase specificity and/or sensitivity for cancer detection over single biomarker tests (**Table 2**). The OVA1® test combines the results of β-2 microglobulin, CA125II, apolipoprotein A-I, prealbumin and transferrin into one score of 0-10 [104]. The Risk of Malignancy Algorithm or ROMA™ test combines the results of HE4 enzyme immunoassay (EIA) and CA125 II [105]. Both of these tests measure multiple circulating glycoproteins and have been cleared by the FDA for prediction of malignant ovarian cancer. Additionally, an OVA2® next generation multivariate index assay is currently being evaluated by the FDA for clearance for the same indication. CA19-9 is a cancer associated marker that measures the amount of sialyl Lewis antigen (SLe^a) tetrasaccharide on all circulating inflammatory glycoproteins and has been used to monitor response to therapy in patients with an established diagnosis of pancreatic, colorectal, gastric or biliary cancer [18, 100].

There are many biomarkers with the potential for improving assay performance when included in a multivariable algorithm. For example, galactosylated, fucosylated and poly N- acetyllactosamine glycoforms of α1-antitrypsin have the potential to distinguish between non-small cell lung carcinoma and benign pulmonary disease [12]. Fucosylated α1- antitrypsin also has the potential to distinguish adenocarcinoma from benign pulmonary disease [12] . Fucosylated haptoglobin combined with CEA may be useful as a prognostic biomarker in colorectal cancer [106] and fucosylated haptoglobin alone may be useful for prostate cancer as it correlated with Gleason scores and biochemical recurrence after radical prostatectomy [107]. In addition, GlycA, the marker of circulating GlcNAc residues, was found to be associated with incident colorectal cancer and colorectal cancer mortality but was not associated with breast cancer or mortality from any other cancer in the WHS [108].

The fact that altered glycosylated forms of inflammatory glycoproteins have been associated with acute and chronic inflammatory diseases as well as cancer provokes intriguing questions about the potential links between inflammation and cancer. It

has been hypothesized that chronic inflammation plays a role as a causal factor for the development of some cancers. For example, persistent infection with *Helicobacter pylori* causes chronic atrophic gastritis which may lead to dysplasia and gastric carcinoma [100]. Moreover, there is a well-known connection between colorectal cancer and inflammation; however, it is not yet known whether chronic inflammation exacerbates the progression to colorectal cancer or if colorectal cancer stimulates the secretion of cytokines that then stimulate a chronic inflammatory response [109]. The advent of high-throughput techniques for analyzing glycan structures as well as measuring levels of inflammatory glycoproteins based on both their protein and glycan content should elicit much research to address these questions in the near future.

4.4 Congenital disorders of glycosylation

Further evidence for the importance of glycans in protein function and the potential use of glycan isoforms to increase specificity for disease diagnosis, stems from the study of monogenic disorders in the glycosylation pathway, the Congenital Disorders of Glycosylation (CDG) [19]. Over 100 human genetic disorders have been associated with aberrant glycan metabolism [110]. Because these defective genes affect proteins in a variety of functionally diverse metabolic pathways, the clinical presentation can vary, making differentiation between CDG subtypes quite challenging. Currently, diagnostic tests for CDG are limited to electrophoresis or MS-based tests that characterize the various glycoforms of transferrin [19, 111]. CDG-I mutations are diagnosed by the presence of transferrin with unoccupied glycosylation sites, whereas CDG-II defects are characterized by the presence of transferrin with immature, truncated glycans [19]. N-glycan profiling holds promise for identifying additional glycoprotein biomarkers to aid in the diagnosis of the many CDG that are known to exist [19, 37]. Nevertheless, interpretation of glycan alterations is complicated by fact that the immune response can lead to changes in glycan structure besides those caused by the underlying genetic defect. Therefore, global glycan profiling in complex biological samples for the purpose of diagnosing CDG holds promise, but is not yet useful in the clinical laboratory setting.

5. Conclusions and future perspectives

With the implementation of personalized medicine comes the task of discovering and evaluating new biomarkers that have the potential to improve the performance characteristics of current tests for clinical care. Many tests are being developed to date that support the relevance of high throughput assays for biomarkers presumed to be associated with chronic cardiometabolic disorders like CVD, T2DM and NASH, as well as autoimmune disorders and cancer. Among other techniques, NMR spectroscopy

holds promise to identify subjects at risk for a number of low grade inflammation-associated diseases, and may also have value to predict mortality [1, 42, 53, 112, 113]. As outlined in this review, it is increasingly appreciated that knowledge about alterations in the levels of glycoproteins in biological fluids as such, as well as with respect to the extent and specificity of the various glycan structures may improve risk stratification and identify novel pathogenic pathways. On the one hand, abnormalities in the process of glycosylation can be linked to distinct clinical entities, while on the other hand glycomics will open new avenues from a systems biology perspective. It is anticipated that a glycomics approach will also be helpful to forge a link with genomics, lipidomics, proteomics and metabolomics, especially given the fact that the entities measured in the latter 'omics often contribute to the diversity observed in glycomics [21, 114]. Of further relevance, although it has been surmised that glycan levels are to an important extent genetically determined with environmental factors possibly playing a less important role, it is clear that environmental factors such as smoking and alcohol consumption often lead to measurable differences in glycan structure [21, 24, 115]. Among other challenges, results from glycomics analyses by high-throughput techniques combined with a genome-wide association study (GWAS) approach are required to underpin potentially important novel causal pathways in disease development [116].

The complex chemistry of glycans makes detailed analyses of their structures limited to specialist research laboratories with the most complete structural analyses only being possible using a combination of several advanced analytical techniques. From a clinical perspective there is a quest for technologies to analyze complex samples quickly with minimal need for specialist facilities and technical expertise. However, it is clear that we are moving on a trajectory toward a time when the wealth of information that has yet to be mined from glycoproteins and their glycans will contribute to a more personalized approach to patient care.

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A Novel Protein Glycan Biomarker and LCAT activity in Metabolic Syndrome

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3

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Abstract

Background The cholesterol esterifying enzyme, lecithin:cholesterol acyltransferase (LCAT), is instrumental in high density lipoprotein (HDL) remodelling. LCAT may also modify oxidative and inflammatory processes, as supported by an inverse relationship with HDL anti-oxidative functionality and a positive relationship with high-sensitivity C-reactive protein (hsCRP). GlycA is a recently developed proton nuclear magnetic resonance (NMR) spectroscopy-measured biomarker of inflammation whose signal originates from a subset of *N*-acetylglucoasamine residues on the most abundant glycosylated acute-phase proteins. Plasma GlycA correlates positively with hsCRP, and may predict cardiovascular disease even independent of hsCRP. Here we tested the extent to which plasma GlycA is elevated in metabolic syndrome (MetS), and determined its relationship with LCAT activity.

Materials and methods Plasma GlycA, hsCRP, serum amyloid A (SAA), tumor necrosis factor-α (TNF-α) and LCAT activity were measured in 58 subjects with MetS (including 46 subjects with type 2 diabetes mellitus (T2DM)) and in 45 non-diabetic subjects without MetS.

Results Plasma GlycA was higher in MetS coinciding with higher hsCRP and LCAT activity ($P<0.01$ for each). In all subjects combined, GlycA was correlated positively with hsCRP, SAA and LCAT activity ($P<0.001$ for each), but not with TNF-α. Age- and sex-adjusted multivariable linear regression analysis revealed that GlycA was positively associated with LCAT activity ($P=0.029$), independent of the presence of MetS, T2DM, hsCRP and SAA. GlycA was unrelated to diabetes status.

Conclusion A pro-inflammatory glycoprotein biomarker, GlycA, is higher in MetS. Higher plasma levels of this glycoprotein biomarker relate to increased LCAT activity in the setting of MetS.

Introduction

Human plasma contains lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43), the enzyme which is responsible for the esterification of free cholesterol. LCAT is of critical importance for high density lipoproteins (HDL) maturation and remodelling [1-3]. As a result, LCAT plays a key role in the reverse cholesterol transport pathway by maintaining a concentration gradient for free cholesterol over the cell membrane, thereby promoting transport of peripheral cell-derived cholesterol to extra cellular acceptors [4].

In view of this contribution of LCAT to reverse cholesterol transport, it has long been thought that LCAT may protect against the development of atherosclerosis [4]. However, contradictory data on the impact of LCAT on (subclinical) cardiovascular disease (CVD) have been reported. Partial genetic *LCAT* deficiency was found to be associated with increased carotid intima media thickness (IMT), a well-established biomarker of subclinical atherosclerosis, in a Dutch though not in an Italian cohort [5, 6]. Lower plasma LCAT activity and mass levels do not coincide with increased IMT [7, 8]. Furthermore, it has been demonstrated that neither higher plasma LCAT activity, higher LCAT mass, nor increased plasma cholesterol esterification rate predict a lower incidence of (recurrent) CVD [9-12]. In addition, a Mendelian randomization study did not reveal an independent association of a common variation in *LCAT* with CVD risk [13].

Of note, recent studies have suggested that LCAT affects the potential of HDL to modify oxidative and inflammatory processes [14-18]. LCAT partly inhibits low density lipoprotein (LDL) oxidation [14], but also acts as a potent pro-oxidant during very low density lipoprotein (VLDL) oxidation [15, 16]. In view of the purported contribution of LCAT to HDL functionality [19], and the uncertainty with regard to the impact of LCAT on atherosclerosis development [1, 2, 5-12], it is clinically relevant to more precisely delineate the extent to which low grade chronic inflammation relates to plasma LCAT activity. Nonetheless, except for a positive relationship of plasma high-sensitivity C-reactive protein (hsCRP) levels with LCAT activity [18], and - in apparent contrast - a higher hsCRP in subjects with partial genetic *LCAT* deficiency [6], no data are available with respect to the extent to which low-grade inflammatory responses relate to variation in plasma LCAT activity. In this context it is noteworthy that the metabolic syndrome (MetS) is not only featured by enhanced oxidative stress and low grade chronic inflammation, as evidenced by higher plasma 8-isoprostanes, hsCRP, serum amyloid A (SAA) levels and tumor necrosis factor-α (TNF-α) [7, 20-23], but also by higher plasma LCAT activity [7].

It is increasingly appreciated that protein glycosylation, i.e. the enzymatic process whereby a glycan (carbohydrate) moiety is added to a protein, impacts on many biological processes including inflammatory responses [24-27]. As yet the widespread use of methods to assay plasma glycosylation and/or glycoprotein levels is limited. Proton nuclear magnetic resonance (NMR) spectroscopy has the ability to detect circulating

glycoproteins, the most abundant being acute phase proteins [28], by capturing the NMR signal from the *N*-acetyl methyl protons on the carbohydrate side chains. Recently, a high throughput NMR-based assay has been developed to capture and quantify this NMR signal in plasma and serum, designated GlycA [29, 30]. The major contributors to the GlycA NMR signal are the most abundant circulating acute phase proteins. GlycA has been shown to be positively correlated with hsCRP, and may predict incident CVD, even when taking account of hsCRP and conventional risk CVD factors [30, 31]. Against this background it is plausible to hypothesize that plasma GlycA is higher in MetS and associates positively with LCAT activity.

We, therefore, initiated the present study to determine i) whether plasma GlycA is elevated in subjects with MetS and ii) to assess the relationship of GlycA with plasma LCAT activity.

Patients and Methods

Participants

Reporting of the study conforms to STROBE and the broader EQUATOR guidelines [32]. The study was performed in a university hospital setting. Participants (aged > 18 years) were Caucasian, and were recruited by advertisement in local newspapers. The medical ethics committee of the University Medical Center Groningen, The Netherlands approved the study. All participants provided written informed consent.

Subjects with and without MetS, defined according to the revised NCEP-ATP III criteria [33], participated. Three or more of the following criteria were required for categorization of subjects with MetS: waist circumference > 102 cm for men and > 88 cm for women; hypertension (blood pressure ≥ 130/85 mmHg or use of anti-hypertensive drugs); fasting plasma triglycerides ≥ 1.70 mmol/L; HDL cholesterol < 1.0 mmol/L for men and < 1.3 mmol/L for women; fasting glucose ≥ 5.6 mmol/L. Subjects with type 2 diabetes mellitus (T2DM), previously diagnosed by primary care physicians using guidelines from the Dutch College of General Practitioners (fasting plasma glucose ≥ 7.0 mmol/L and/or non-fasting plasma glucose ≥11.1 mmol/L[34]) were allowed to participate. However, subjects without MetS were not allowed to be previously diagnosed with T2DM. Diabetic subjects who were treated with metformin and/or sulfonylurea were eligible, but subjects using insulin were excluded. Subjects using lipid lowering drugs were also excluded. The use of anti-hypertensive medication was allowed. Further exclusion criteria were clinically manifest CVD, renal insufficiency (estimated glomerular filtration rate < 60 mL/min/1,.73m^2 and/or proteinuria), thyroid disorders, liver disease, current smoking and

pregnancy. Subjects with a hsCRP level > 10 mg/L were additionally excluded to reduce possible bias due to concurrent infection [35].

Physical examination did not reveal pulmonary or cardiac abnormalities. All subjects were studied after an overnight fast. Body mass index (BMI) was calculated as weight divided by height squared (in kg/m^2). Waist circumference was measured between the 10^{th} rib and the iliac crest.

Laboratory analyses

Venous blood samples were collected into EDTA-containing tubes (1.5 mg/mL) for the measurement of plasma lipids and apolipoproteins. Plasma was prepared by centrifugation at 1400 g for 15 min at 4^0C. Blood glucose was measured directly after blood collection. Samples for other assays were stored at -80 ^{0}C until analysis.

NMR spectra were collected from EDTA plasma samples using the NMR Profiler as detailed previously [36]. NMR signal amplitudes originating from the *N*-acetyl methyl group protons of the *N*-acetylglucosamine moieties located on the bi-, tri-, and tetra-antennary branches of plasma proteins, predominantly α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin, were used to calculate the concentrations of GlycA (μmol/L of *N*-acetyl methyl groups). The GlycA NMR signal is centered at 2.00±0.01 ppm in the NMR spectra of plasma, and only *N*-acetylglucosamine with specific glycosidic linkage, namely, β (1>2) or β (1>6) with a preceding mannose residue, contribute to the GlycA signal [29, 30].

The SAA protein concentration was assayed by a monoclonal antibody-based sandwich SAA1 enzyme-linked immunosorbent assay (ELISA) [21, 37]. Human apo-SAA was purified from the HDL3 fraction of acute phase response serum, linked to helix pomatia haemocyanin, and subsequently injected into Balb/c mice to produce monoclonal antihuman-SAA antibodies. The antibodies used in the sandwich ELISA are the capture antibody Reu.86.5, which reacts to all acute phase SAA subtypes, and the coupled to Horseradish peroxidase detection antibody Reu.86.1, which reacts to the major SAA1 subtype. The assay is standardized against the international standard for SAA protein (WHO code 92/680). The lower limit of detection of the assay is 1.6 μg/L.

hsCRP was assayed by nephelometry with a lower limit of 0.175 mg/L (BNII N; Dade Behring, Marburg, Germany). Tumor necrosis factor-α (TNF-α) was measured using Luminex xMAP technology (Lincoplex panel B cat. no. HADK1-61K-B; Linco Research Inc., St Charles, MO, USA).

Plasma LCAT activity was determined using excess exogenous substrate containing [^{3}H]-cholesterol as described previously [12, 38]. In brief, plasma samples were incubated with labelled substrate for 6 h at 37 ^{0}C. Corrections were made for the amount of free

cholesterol in the plasma samples. The reaction was stopped by addition of cold ethanol to the incubation medium. Free and esterified cholesterol were separated using disposable silica columns. [^{3}H]-cholesteryl esters were eluted with hexane. The formation of labelled cholesteryl esters was linear during the 6 h incubation period. Plasma LCAT activity also varies linearly with the amount of plasma used in the incubations, and is strongly correlated with LCAT concentration [39]. Plasma LCAT activity was measured in duplicate. LCAT activity was related to the activity measured in human pool plasma, and was expressed in arbitrary units (AU), corresponding to the percentage of the activity in human pooled plasma (100 AU is equivalent to 48.9 nmol cholesterol esterified per ml plasma per hour).

The intra-assay and inter-assay coefficients of variation of the GlycA, SAA, TNF-α and LCAT assay are all < 5 % and < 7.0 %, respectively.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11875540 and 11876023, respectively; Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04713214; Roche Diagnostics GmbH, Mannheim, Germany). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. LDL cholesterol was calculated by the Friedewald formula if plasma triglycerides were < 4.5 mmol/L. Apoliprotein A-I (ApoA-I) and apoB were assayed by immunoturbidimetry (Roche/Cobas Integra Tina-quant catalog no. 03032566 and 033032574, respectively, Roche Diagnostics).

Glucose was measured with an APEC glucose analyzer (APEC Inc., Danvers, MA, USA).

Statistical analysis

SPSS 20 (version 20.0, SPSS Inc. Chicago, IL, USA) was used for data analysis. Results are expressed as mean ± SD or as median (interquartile range). Because of skewed distribution, logarithmically transformed values of triglycerides, hsCRP and SAA were used for statistical analysis. Differences between subjects with and without MetS were determined by unpaired *t*-tests and Chi-square tests where appropriate. Univariate relationships were determined using Pearson correlation coefficients. Multivariable regression analyses were carried out to disclose those variables which were independently associated with plasma GlycA. To determine whether relationships of GlycA and hsCRP with plasma LCAT activity were different in subjects with MetS *vs*. subjects without MetS interactions terms were calculated as the product terms between the variables of interest. To this end the distribution of plasma LCAT activity was centered to the mean value by subtracting the individual value from the group mean. Interaction terms were considered to be statistically significant at two-sided

P-values<0.10, as recommended by Selvin [40]. Otherwise, the level of significance was set at two-sided *P*-values<0.05.

Results

Fifty eight subjects with MetS and 45 subjects with neither MetS nor T2DM were included in the study (**Table 1**). Forty six of the MetS subjects had T2DM. Of these, 11 used metformin and 9 subjects used sulfonylurea alone, whereas both drugs were used by 15 subjects. Other glucose lowering drugs were not used. Anti-hypertensive medication (mostly angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers and diuretics, alone or in combination) were used by 23 subjects with MetS and by none of the subjects without MetS (*P*<0.001). Two women with MetS and one woman without MetS used estrogens.

Table 1. Clinical characteristics, GlycA, high-sensitive C-reactive protein (hsCRP), serum amyloid A (SAA), plasma glucose, (apo)lipoproteins and lecithin:cholesterol acyltransferase (LCAT) activity in 58 subjects with and 45 in subjects without metabolic syndrome (MetS).

	MetS (n=58)	**No MetS (n=45)**	***P*-value**
Age (years)	58 ± 9	53 ± 9	0.006
Gender (men/women)	31/27	19/26	0.26
Diabetes (yes/no)	46/12	0/45	<0.001
Systolic blood pressure (mm Hg)	147 ± 18	128 ± 20	<0.001
Diastolic blood pressure (mm Hg)	89 ± 9	80 ± 11	<0.001
BMI (kg/m^2)	30.1 ± 4.6	24.9 ± 3.6	<0.001
Waist circumference (cm)	104 ± 13	84 ± 12	<0.001
Glucose (mmol/L)	8.7 ± 2.6	5.7 ± 0.7	<0.001
GlycA (µmol/L)	393 ± 50	364 ± 55	0.006
hsCRP (mg/L)	1.92 (1.25-4.22)	1.36 (0.54-2.61)	0.008
SAA (mg/L)	1.71 (1.11-2.44)	1.15 (0.77-2.38)	0.19
TNF-α (ng/L)	3.91 ± 2.03	3.23 ± 1.27	0.040
Total cholesterol (mmol/L)	5.51 ± 0.96	5.77 ± 0.97	0.19
LDL cholesterol (mmol/L)	3.33 ± 0.84	3.52 ± 0.81	0.26
Non-HDL cholesterol (mmol/L)	4.30 ± 0.95	4.17 ± 1.04	0.53
HDL cholesterol mmol/L)	1.21 ± 0.36	1.59 ± 0.39	<0.001

Triglycerides (mmol/L)	1.18 (0.85-1.66)	1.95 (1.70-2.53)	<0.001
ApoB (g/L)	0.98 ± 0.22	0.94 ± 0.24	0.43
ApoA-I (g/L)	1.32 ± 0.25	1.46 ± 0.21	0.004
Total cholesterol/HDL cholesterol	4.88 ± 1.58	3.87 ± 1.28	0.001
ApoB/apoA-I	0.78 ± 0.30	0.67 ± 0.24	0.046
LCAT activity (AU)	117.3 ± 15.4	105.9 ± 14.4	<0.001

Data in mean ± SD or in median (interquartile range). Apo: apolipoproteins; BMI: body mass index; HDL: high density lipoproteins; LDL: low density lipoproteins; non-HDL: non-high density lipoproteins. TNF-α: tumor necrosis factor-α. LDL cholesterol was calculated in 55 subjects with and in 43 subjects without MetS.

Subjects with MetS were older and had higher blood pressure, BMI, waist circumference and plasma glucose, but sex distribution was not different compared to subjects without MetS (**Table 2)**. Plasma GlycA, hsCRP and TNF-α concentrations were higher in MetS subjects, whereas there was no significant difference in SAA (**Table 2**). Plasma triglycerides were higher, coinciding with lower HDL cholesterol and apoA-I in MetS subjects. Total cholesterol, LDL cholesterol, non-HDL cholesterol and apoB levels were not different between subjects with and without MetS. The total cholesterol/HDL cholesterol and the apoB/apoA-I ratio were higher in MetS subjects. Plasma LCAT activity was increased in MetS subjects (**Table 1**). When excluding T2DM subjects from the analysis, plasma GlycA (404 ± 54 μmol/L) and LCAT activity (114.9 ± 10.6 AU) were still higher in subjects with MetS (n=12) vs. subjects without MetS (n=45; 364 ± 55 μmol/L, *P*=0.027 and 105.9 ± 13.4 AU, *P*=0.035, respectively). Plasma hsCRP (*P*=0.52) and SAA (*P*=0.68) were not different in non-diabetic subjects with MetS vs. subjects without MetS (data not shown).

In all subjects combined, plasma GlycA was correlated positively with hsCRP and SAA, whereas there was also positive correlation of SAA with hsCRP (**Table 2**; **Figure 1**). Similar relationships of GlycA with hsCRP and SAA were found in subjects with and without MetS separately (**Figure 1**), as well as in women (r=0.685, *P*<0.001 and r=0.369, *P*=0.008, respctively) and in men (r=0.515, *P*<0.001 and r=0.407, *P*=0.002, respectively). GlycA was unrelated to TNF-α (**Table 2**). GlycA was correlated positively with systolic blood pressure, BMI, waist circumference as well as with plasma triglycerides, and inversely with HDL cholesterol (**Table 2**). hsCRP was also correlated positively with systolic blood pressure, BMI, waist circumference and plasma triglycerides and inversely with HDL cholesterol. SAA was only correlated positively with diastolic blood pressure and BMI. TNF-α was correlated positively with plasma glucose and triglycerides, and inversely with HDL cholesterol and apoA-I. Of note, GlycA was correlated positively with LCAT

activity in the combined subjects (**Table 2**, **Fig. 2**; *P*=0.51 for the difference in subjects with and without MetS). hsCRP was also correlated positively with LCAT activity. This relationship was marginally different between subjects with and without MetS (*P*=0.067 for the difference in subjects with and without MetS. SAA and TNF-α were unrelated to LCAT activity.

Table 2. Univariate relationships of GlycA, high-sensitivity C-reactive protein (hsCRP), serum amyloid A (SAA) and tumor necrosis factor-α (TNF-α) with clinical variables, plasma glucose, (apo) lipoproteins and lecithin:cholesterol acyltransferase (LCAT) activity in 103 subjects (58 with and 45 without metabolic syndrome; 46 with type 2 diabetes mellitus).

	GlycA	**hsCRP**	**SAA**	**TNF-α**
Age	0.190	0.045	0.017	0.181
Systolic blood pressure	0.205*	0.199*	0.172	0.128
Diastolic blood pressure	0.158	0.158	0.202*	0.065
BMI	0.313***	0.500***	0.252**	0.091
Waist circumference	0.355***	0.415***	0.117	0.093
GlycA		0.597***	0.375***	0.058
hsCRP			0.531***	0.158
SAA				0.165
Glucose	0.187	0.151	0.08	0.262**
Total cholesterol	0.039	-0.027	-0.075	-0.118
LDL cholesterol	0.110	-0.036	-0.070	-0.097
Non-HDL cholesterol	0.146	0.084	-0.100	0.022
HDL cholesterol	-0.254**	-0.260**	0.064	-0.324***
Triglycerides	0.246*	0.275**	-0.010	0.254**
ApoB	0.173	0.110	-0.038	0.038
ApoA-I	-0.201	-0.178	0.008	-0.241*
LCAT activity	0.313***	0.215*	0.013	0.010

Pearson correlation coefficients are shown. hsCRP, SAA and triglycerides are logarithmically transformed. Low density lipoprotein (LDL) cholesterol was calculated in 55 subjects with and in 43 subjects without metabolic syndrome. Apo: apolipoproteins; HDL: high density lipoproteins. *$P<0.05$; **$P\leq0.01$; ***$P\leq0.001$.

Multivariable linear regression analyses were performed to determine the independent association of plasma LCAT activity with GlycA (**Table 3**). In age-, sex and diabetes status-adjusted analysis, plasma GlycA was associated positively with the presence of MetS (Model A) and alternatively with plasma LCAT activity (Model B). When the presence of

MetS, T2DM and plasma LCAT activity were included together in the analysis, plasma GlycA remained associated with LCAT activity, whereas the association with MetS was no longer significant (Model C). When hsCRP and SAA were additionally included in the analysis, plasma GlycA was still related to plasma LCAT activity, but neither to the presence of MetS nor to SAA (Model D). In this analysis, there was a strong positive association of GlycA with hsCRP. Furthermore, there was no positive, independent association of GlycA with the presence of T2DM (Model A, C and D). In additional analyses in which we also took account of the use of anti-hypertensive medication, the use of metformin and of sulfonylurea, similar associations of plasma GlycA with the presence of MetS (cf. Model A; β=0.360, *P*=0.026) or plasma LCAT activity (cf. Model B; β=0.250, *P*=0.019) were observed (data not shown). In these analyses, plasma GlycA was also independently associated with LCAT activity when taking account of the presence of MetS and T2DM (cf. Model C; β=0.215, *P*=0.045), as well as additionally of hsCRP and SAA (cf. Model D; β=0.187, *P*=0.037). Finally, in subsidiary analysis excluding diabetic subjects, GlycA remained associated with LCAT activity taking account of age and sex (cf. Model B; β=0.277, *P*=0.036; data not shown).

Table 3. Multiple linear regression analysis demonstrating relationships of GlycA with plasma lecithin:cholesterol acyltransferase (LCAT) activity, high-sensitive C-reactive protein (hsCRP) and serum amyloid A (SAA) in 103 subjects (58 with and 45 without metabolic syndrome (MetS); 46 with type 2 diabetes mellitus (T2DM)).

	Model A		**Model B**		**Model C**		**Model D**	
	β	***P*-value**	**β**	***P*-value**	**β**	***P*-value**	**β**	***P*-value**
Age	0.125	0.22	0.140	0.15	0.111	0.26	0.133	0.10
Sex (men/women)	0.039	0.69	0.034	0.72	0.034	0.72	0.050	0.52
MetS (yes/no)	0.342	0.034			0.274	0.085	0.221	0.088
T2DM (yes/no)	-0.143	0.37			-0.165	0.29	-0.272	0.034
LCAT activity			0.289	0.003	0.247	0.016	0.185	0.029
Ln hsCRP							0.509	<0.001
Ln SAA							0.112	0.22

hsCRP and SAA levels are logarithmically transformed

Model A: includes age, presence of MetS and presence of T2DM

Model B: includes age, sex and LCAT activity

Model C: includes age, sex, presence of MetS, presence of T2DM and LCAT activity

Model D: includes age, sex, presence of MetS, presence of T2DM, LCAT activity, hsCRP and SAA

Figure 1. Relationships between GlycA, high-sensitivity C-reactive protein (hsCRP) and serum amyloid A in 103 subjects (58 with and 45 without metabolic syndrome (MetS)). Regressions line are from the combined subjects.

A GlycA and hsCRP. Pearson correlation coefficients: all subjects r=0.597, *P* <0.001; subjects with MetS: r=0.584, *P*<0.001; subjects without MetS: r=0.552, *P*<0.001.

B GlycA and SAA. Pearson correlation coefficients: all subjects r=0.375, *P* <0.001; subjects with MetS: r=0.374, *P*=0.004; subjects without MetS: r=0.349, *P*=0.019.

C SAA and hsCRP. Pearson correlation coefficients: all subjects r=0.531, *P* <0.001; subjects with MetS: r=0.387, *P*=0.003; subjects without MetS: r=0.608, *P*<0.001.

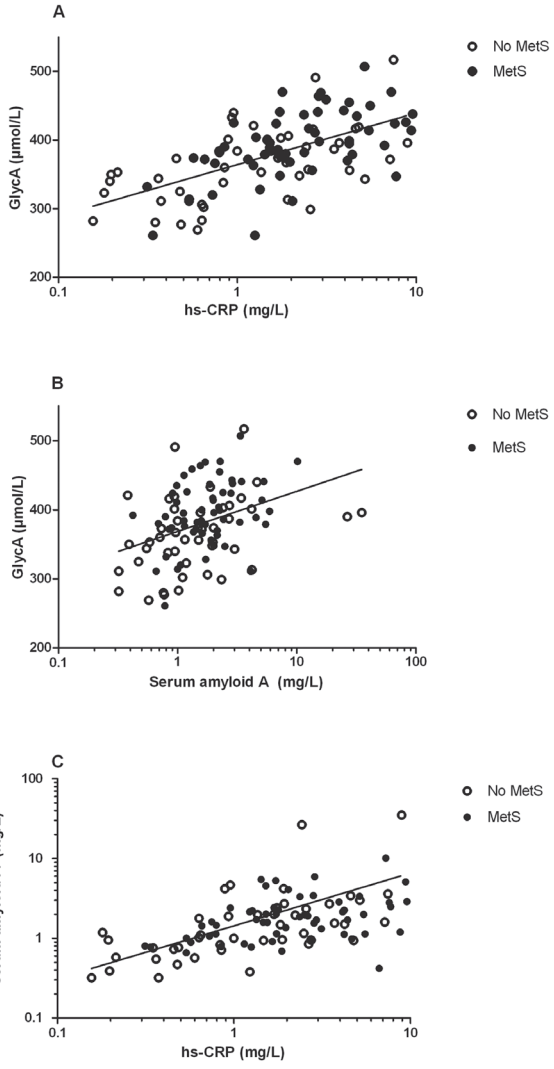
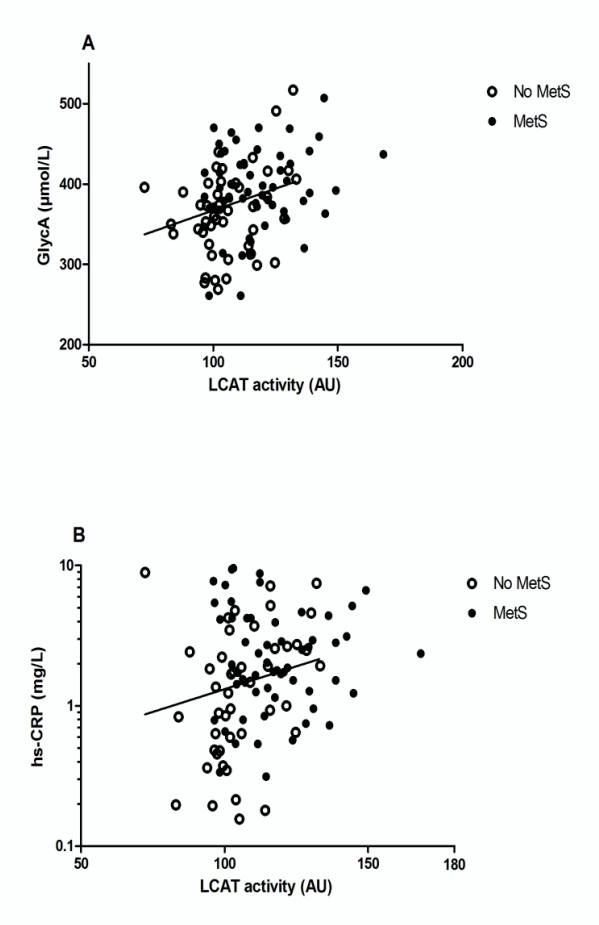


Figure 2. Relationships between GlycA, high-sensitive C-reactive protein (hsCRP) and lecithin: cholesterol acyltransferase (LCAT) activity in 103 subjects (58 with and 45 without metabolic synd. rome (MetS)). Regression lines are from the combined subjects.

A GlycA and LCAT activity. Pearson correlation coefficients: all subjects r=0.313, *P* =0.001; subjects with MetS: r=0.198, *P*=0.14; subjects without MetS: r=0.301, *P*=0.044.

B hsCRP and LCAT activity. Pearson correlation coefficients: all subjects r=0.215, *P*=0.029 ; subjects with MetS: r=-0.008, *P*=0.95; subjects without MetS: r=0.298, *P*=0.047.



Discussion

In the present study, a novel NMR spectroscopy-based glycoprotein biomarker, designated GlycA, was found to be elevated in MetS, but was not positively associated with the presence of T2DM. Like hsCRP, GlycA was correlated positively with (central) obesity, plasma triglycerides and systemic blood pressure, and inversely with HDL cholesterol.

Furthermore, both hsCRP and GlycA were related to higher plasma LCAT activity in univariate analysis. Of note, multivariable linear regression analysis revealed that the association of GlycA with LCAT activity remained present taking account of MetS, diabetes status, hsCRP and SAA. The current observations are in line with the contention that this glycoprotein biomarker signal may reflect a pro-inflammatory state. Our findings would also agree with the hypothesis that high plasma LCAT activity could modify inflammatory processes.

Metabolic profiling provides a promising tool to improve our understanding of abnormalities in multiple pathways that are involved in the pathogenesis of cardiometabolic disorders [41-43]. Glycosylation represents a highly common posttranslational modification of proteins in human biology [25-27]. Most circulating proteins are *N*-linked glycoproteins [25], and during an acute-phase response their glycan moieties are altered consequent to changes in the dynamics between glycosyltransferases and hydrolases [26, 27]. The GlycA signal is predominantly comprised of α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin with specific glycan structures being preferentially detected [29]. Since glycan structures of these acute-phase proteins are modified under chronic inflammatory conditions [25], it is plausible that the integrated plasma concentration of this glycoprotein biomarker reflects a pro-inflammatory state beyond contributions of the individual captured proteins.

Well-documented enhanced low-grade chronic inflammation and increased oxidative stress [19-22, 44, 45] coinciding with increased plasma LCAT activity [7] in MetS formed the basis for our rationale to test whether plasma GlycA is elevated in MetS, and to disclose the extent to which GlycA associates with LCAT activity. The current study demonstrates a strong correlation of GlycA with hsCRP in both women and men, as well as in subjects with and without MetS. Our report also extends recent findings with respect to positive relationships of GlycA with adiposity and plasma triglycerides and an inverse correlation with HDL cholesterol [30]. Additionally, we showed for the first time a positive correlation of GlycA with SAA. Despite expected positive relationships of TNF-α with plasma glucose and triglycerides [46], and its proposed role in affecting protein glycosylation [47], GlycA was unrelated to this pro-inflammatory biomarker.

Remarkably, GlycA was also not significantly correlated with plasma glucose and was not associated positively with diabetes status taking account of the presence of MetS.

Importantly, LCAT acts in a concentration-dependent manner as a potent pro-oxidant during VLDL oxidation, while inhibiting LDL oxidation at the expense of HDL's anti-oxidative properties [15, 16, 48]. In turn, oxidatively modified HDL may have diminished ability to inhibit inflammatory processes [15, 16]. In agreement, elevated plasma LCAT, measured as its enzymatic activity to generate cholesteryl esters from free cholesterol, may result in impaired intrinsic properties of HDL to protect against LDL oxidation *in vitro* [17], and coincide with higher plasma hsCRP levels [18]. In keeping with such a dual role of LCAT in lipoprotein oxidation, markedly enhanced oxidative stress has been demonstrated in *LCAT* deficient mice, while a reduction in oxidative stress is present in mice with combined *LCAT* and *apoE* deficiency [49]. Of relevance, the relationship of GlycA with the presence of MetS was lost in multivariable linear regression analysis, in which we included plasma LCAT activity. This raises the possibility that higher plasma LCAT activity may contribute to MetS-associated GlycA elevations. Since SAA impairs HDL's anti-oxidative functionality by a process which involves displacement of apoA-I from HDL particles [45, 50], we also assessed whether plasma SAA was independently associated with GlycA. In analysis which included plasma LCAT activity and hsCRP, GlycA was not independently associated with SAA, suggesting that the relationship of GlycA with plasma LCAT and hsCRP was stronger than that with SAA. Taken together, it is plausible that the complex roles of LCAT in HDL functionality [1, 3, 14-16, 19] could in part explain why high plasma LCAT is unlikely to protect against atherosclerotic CVD [5-13]. We surmise that GlycA elevations could be the consequence of abnormalities in HDL functionality and at the same time reflect a pro-inflammatory state which as such could contribute to impaired HDL functionality [51, 52].

Several methodological aspects and limitations of our study need to be considered. First, although a considerable number of participating MetS subjects had previously diagnosed T2DM, GlycA remained associated with plasma LCAT activity after exclusion of diabetic subjects. Second, it should be appreciated that the exogenous substrate assay method that we used to assay plasma LCAT activity reflects the amount of active LCAT [38] and closely agrees with its mass concentration [39]. Plasma LCAT activity determined by this method differs considerably from assays that measure the rate of cholesterol esterification in whole plasma [53]. Third, the NMR spectroscopy-based glycoprotein biomarker that we applied in the present study is highly reproducible and has considerable lower day-to-day variability compared to hsCRP [29]. Given the design of this assay to specifically capture *N*-acetylglucosamine moieties on a number of acute-phase proteins, its degree of agreement with other glycan biomarkers should be addressed in future studies. Fourth, inherent to the observational design

of our study, we could not address the nature of the relationships and the possibility of reverse causation. Thus, our study should not be interpreted to indicate that the association of GlycA with plasma LCAT activity implies a direct pathogenic role of LCAT in the metabolism of circulating glycosylated acute-phase proteins. It cannot be excluded that low-grade chronic inflammation, as reflected by higher hsCRP and GlycA concentrations, would influence LCAT synthesis or metabolism. Although LCAT protein is highly glycosylated [54], this possibility seems unlikely since plasma LCAT activity may decrease during an acute-phase response in humans [55].

In conclusion, this study shows that the glycoprotein biomarker, GlycA, is elevated in MetS, and may represent a quantitative marker of a pro-inflammatory state. It is conceivable that increased plasma LCAT activity by adversely affecting HDL functionality could be involved in the pathogenesis of enhanced low-grade inflammation.

Acknowledgments

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Conflict of interest statement

This study was investigator driven. M.A. Connelly and J.D. Otvos are employees of LabCorp. The other authors state no conflict of interest.

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Higher circulating GlycA, a pro-inflammatory glycoprotein biomarker, relates to lipoprotein-associated phospholipase A^2 mass in non-diabetic subjects but not in diabetic or metabolic syndrome subjects

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Abstract

Background Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a cardiovascular risk marker which is in part complexed to low density lipoproteins (LDL), where it exerts pro-inflammatory properties. GlycA is a pro-inflammatory proton nuclear magnetic resonance (NMR) spectroscopy biomarker whose signal originates from a subset of *N*-acetylglucoasamine residues on the most abundant glycosylated acute-phase proteins.

Objective We compared plasma GlycA and Lp-PLA_2 mass between subjects without Type 2 diabetes mellitus (T2DM) or the metabolic syndrome (MetS) and subjects with T2DM and/or MetS. We also tested the relationship of GlycA with Lp-PLA_2 in each group.

Methods Plasma GlycA, Lp-PLA_2mass, high sensitivity C-reactivity protein (hsCRP) and lipids were measured in 40 subjects with neither T2DM nor MetS (group 1) and in 58 subjects with T2DM and/or MetS (group 2).

Results GlycA and hsCRP were higher ($P<0.01$ for each), whereas Lp-PLA_2 was lower in group 2 vs. group 1 ($P<0.001$). GlycA was positively related to hsCRP in each group ($P<0.001$). In contrast, GlycA was correlated positively with Lp-PLA_2 in group 1 (r=0.384, $P=0.015$), but not in group 2 (r=0.045; $P=0.74$; interaction term for difference: $P=0.059$). While Lp-PLA_2 was correlated positively with non-HDL cholesterol and LDL cholesterol in each group ($P\leq0.02$), its inverse relationship with high density lipoprotein cholesterol in group 1 (r=-0.381, $P=0.013$), was absent in group 2 (r=-0.101, $P=0.42$).

Conclusions A pro-inflammatory glycoprotein biomarker, GlycA, is higher in subjects with either T2DM, MetS or both. The normally present positive relationship of GlycA with Lp-PLA_2 is blunted in subjects with T2DM and/or MetS.

1. Introduction

It is well recognized that inflammatory processes are intricately involved in the development of atherosclerosis [1, 2]. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also known as platelet-activating factor (PAF) acetylhydrolase, is secreted by inflammatory cells in the arterial wall [2]. Lp-PLA_2 is able to hydrolyse low density lipoprotein (LDL)-derived oxidized phospholipids, which results in the generation of oxidized free fatty acids, fatty acid hydroperoxides and lysophospholipids [2]. It is likely that Lp-PLA_2 has predominant pro-inflammatory effects, and plays a pathogenetic role in coronary artery plaque vulnerability [2-4]. Plasma Lp-PLA_2 relates positively to carotid artery intima media thickness, an established marker of subclinical atherosclerosis [5]. Importantly, two meta-analyses have demonstrated that plasma Lp-PLA_2 mass and activity predict incident cardiovascular disease (CVD), even independent of established risk factors [6, 7]. Lp-PLA_2 is to a major extent complexed to LDL, as supported by a decrease in plasma Lp-PLA_2 in response to pharmacological and non-pharamacological manoeuvres that lower LDL cholesterol [8, 9], but is to a variable extent also associated with high density lipoproteins (HDL) [2] .

Protein glycosylation, i.e. the enzymatic process whereby a glycan (polysaccharide) moiety is added to a protein, is affected by a number of biological processes including inflammation [10, 11]. Many circulating proteins are *N*-linked glycoproteins [12]. During an acute-phase response their glycan moieties are altered consequent to changes in the dynamics between glycosyltransferases and hydrolases [10]. Proton nuclear magnetic resonance (NMR) spectroscopy has the ability to detect circulating glycoproteins by capturing the NMR signal from the *N*-acetyl methyl protons on the carbohydrate side chains [12, 13]. A high throughput NMR-based assay has been developed to quantify this NMR signal in plasma samples. This so-called GlycA signal is predominantly comprised of several major acute phase proteins, i.e. α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin, with specific glycan structures being preferentially detected [12] . Robust positive correlations of GlycA with high-sensitivity C-reactive protein (hsCRP) have been demonstrated recently, indicating that GlycA can be regarded as a pro-inflammatory glycoprotein biomarker [12-14]. Interestingly, GlycA may confer increased CVD risk, even when hsCRP and established risk factors are taken into account [13]. GlycA may also predict the development of type 2 diabetes mellitus (T2DM) [15].

Given the role of Lp-PLA_2 in stimulating pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin- 6 (IL-6), and the importance of these cytokines for protein glycosylation [16-19], it is plausible to hypothesize that higher plasma Lp-PLA_2 may coincide with higher GlycA levels. In view of the proposed contribution of GlycA to diabetes prediction [15], and the association of higher GlycA levels with the metabolic

syndrome (MetS) [14], it is also relevant to test whether the possible relationship of plasma Lp-PLA_2 with GlycA varies according to diabetes and MetS status.

Against this background we initiated the present study to determine i) the extent to which GlycA is related to plasma Lp-PLA_2, and ii) whether such a relationship is different in subjects with T2DM and/or MetS compared to non-diabetic subjects without MetS.

2. Materials and Methods

2.1 Participants

The study was performed in a university hospital setting. Participants (aged > 18 years) were of Caucasian descent. Participants were recruited by advertisement in local newspapers, and provided written informed consent. The medical ethics committee of the University Medical Center Groningen, The Netherlands, approved the study. Subjects with and without T2DM, and with and without metabolic syndrome (MetS) participated. T2DM had been previously diagnosed by primary care physicians using guidelines from the Dutch College of General Practitioners (fasting plasma glucose ≥ 7.0 mmol/L and/or non-fasting plasma glucose ≥11.1 mmol/L [20]. MetS was defined according to the revised NCEP-ATP III criteria [21]. Three or more of the following criteria were required for categorization of subjects with MetS: waist circumference > 102 cm for men and > 88 cm for women; hypertension (blood pressure ≥ 130/85 mmHg or use of anti-hypertensive drugs); fasting plasma triglycerides ≥ 1.70 mmol/L; HDL cholesterol < 1.0 mmol/L for men and < 1.3 mmol/L for women; fasting glucose ≥ 5.6 mmol/L. Based on these criteria, the participants were divided in 2 groups: the first group consisted of subjects without T2DM and without MetS (group 1), whereas the second group consisted of subjects with either T2DM, MetS or both (group 2). Diabetic subjects who were treated with metformin and/or sulfonylurea were eligible. Subjects using insulin and subjects using lipid lowering drugs were excluded. The use of anti-hypertensive medication was allowed. Further exclusion criteria were clinically manifest CVD, renal insufficiency (estimated glomerular filtration rate < 60 mL/min/1.73m^2 and/or proteinuria), thyroid disorders, liver disease, current smoking and pregnancy.

Physical examination did not reveal cardiac or pulmonary abnormalities. All participants were studied after an overnight fast. Body mass index (BMI) was calculated as weight divided by height squared (in kg/m^2). Waist circumference was measured at the midpoint between the 10^{th} rib and the iliac crest.

2.2 Laboratory analyses

Venous blood samples were collected into EDTA-containing tubes (1.5 mg/mL) for the measurement of plasma lipids and apolipoproteins. Plasma was prepared by centrifugation at 1400 g for 15 min at 4^0C. Blood glucose was measured directly after blood collection. Samples for other assays were stored at -80 ^{0}C until analysis.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11875540 and 11876023, respectively; Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04713214; Roche Diagnostics GmbH, Mannheim, Germany). Non-HDL cholesterol was calculated as the difference between total and HDL cholesterol. LDL cholesterol was calculated by the Friedewald formula if plasma triglycerides were < 4.5 mmol/L. Apolipoprotein A-I (ApoA-I) and apoB were assayed using commercially available assays (immunoturbidimetry; Roche/Cobas Integra Tina-quant catalog no. 03032566 and 033032574, respectively, Roche Diagnostics).

hsCRP was assayed by nephelometry with a lower limit of 0.175 mg/L (BNII N; Dade Behring, Marburg, Germany). Glucose was measured with an APEC glucose analyzer (APEC Inc., Danvers, MA, USA). HbA1c was measured by high-performance liquid chromatography (Bio-Rad, Veenendaal, the Netherlands; normal range: 27-43 mmol/mol).

Plasma Lp-PLA_2 mass was measured using a commercially available turbidimetric immunoassay (PLAC Test, diaDexus cat no 10-0112;diaDexus Inc, San Francisco, CA, USA) on the Modular P device. The manufacturer's instructions for thawing samples were strictly adhered to. In samples with a measured concentration exceeding 360 g/L, the presence of interfering heterofilic antibodies was excluded.

NMR spectra were collected from EDTA plasma samples using the NMR Profiler as described [12]. NMR signal amplitudes originating from the *N*-acetyl methyl group protons of the *N*-acetylglucosamine moieties located on the bi-, tri and tetra-antennary branches of the mentioned plasma proteins were used to calculate the concentrations of GlycA (μmol/L of *N*-acetyl methyl groups). The GlycA NMR signal is centered at 2.00±0.01 ppm in the NMR spectra of plasma, and only *N*-acetylglucosamine with specific glycosidic linkage, namely, β (1>2) or β (1>6) with a preceding mannose residue, contribute to the GlycA signal [12, 13].

The intra-assay and inter-assay coefficients of variation of hsCRP are 3 and 4.5 %, of Lp-PLA_2 4 and 5 % and of GlycA 2 and 3 %, respectively.

2.3 Statistical analysis

SPSS (version 22, SPSS Inc. Chicago, IL, USA) was used for statistical analysis. Data are expressed as mean ± SD or as median (interquartile range). Because of skewed distribution, logarithmically transformed values of hsCRP and triglycerides were used for statistical analysis. Between group differences in clinical and laboratory variables were determined by unpaired T-tests. Dichotomized variables were compared by Chi-square tests. Univariate relationships were determined using Pearson correlation coefficients. Multivariable linear regression analyses were carried out to disclose the independent relationship of GlycA with Lp-PLA_2 mass. In order to evaluate between group differences in the relationship of GlycA with Lp-PLA_2 and of Lp-PLA_2 with the total cholesterol/HDL cholesterol ratio interaction terms between group assignment and Lp-PLA_2 and the total cholesterol/HDL cholesterol were calculated. To this end a distribution of centered to the mean was made for Lp-PLA_2 and for the total cholesterol/HDL cholesterol ratio by subtracting the group mean value from the individual values. The level of significance was set at two-sided *P*-values<0.05. Interaction terms were considered to be statistically significant at two-sided *P*-values<0.10 [22].

3. Results

Forty subjects with neither T2DM nor MetS (group 1) and 58 subjects with either T2DM, MetS or both (group 2) participated. Forty seven subjects from group 2 had T2DM; these participants were also categorized as having MetS. The other 11 subjects from group 2 had MetS only (**Table 1**). In group 2, 11 subjects used metformin and 10 subjects used sulfonylurea alone, whereas both drugs were used by another 15 subjects. Other glucose lowering drugs were not used. Anti-hypertensive drugs (mostly angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers and diuretics, alone or in combination) were used by 23 subjects from group 2. None of the subjects from group 1 used glucose lowering drugs or anti-hypertensive medication. Estrogens were used by 1 woman in group 1 and by 2 women in group 2.

Group 2 subjects were older, had higher blood pressure, were more (centrally) obese and had higher levels of glycemia compared to group 1 subjects (**Table 1**). Sex distribution was not different between the groups. GlycA and hsCRP levels were higher, whereas Lp-PLA_2 was lower in group 2 compared to group 1 (**Table 1**). Total cholesterol, non-HDL cholesterol, LDL cholesterol and apoB levels were not significantly different between the groups. HDL cholesterol and apoA-I levels were lower coinciding with higher triglycerides in group 2 compared to group 1. The total cholesterol/HDL cholesterol ratio was higher in group 2 (**Table 1**). In all subjects combined, GlycA (390±56 vs. 382±52 µmol/L, *P*=0.43), Lp-PLA_2 (317±82 vs. 301±54 µg/L, *P*=0.27) and hsCRP (1.87

(0.84-3.13) vs. 1.75 (0.92-4.22) mg/L, *P*=0.66) were not significantly different between men and women.

Table 1. Clinical and laboratory characteristics in 40 subjects without Type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS) (group 1) and in 58 subjects with either T2DM, MetS or both (group 2).

	Group 1 (n=40)	**Group 2 (n=58)**	***P*-value**
T2DM and MetS	0	47	<0.001
MetS only	0	11	<0.001
Age (years)	54 ± 9	59 ± 9	0.009
Gender (men/women)	24/16	27/31	0.19
Systolic blood pressure (mm Hg)	128 ± 20	146 ± 19	<0.001
Diastolic blood pressure (mm Hg)	80 ± 12	89 ± 9	<0.001
BMI (kg/m^2)	25.1 ± 3.7	30.0 ± 4.5	<0.001
Waist circumference (cm)	84 ± 12	104 ± 13	<0.001
Glucose (mmol/L)	5.6 ± 0.5	8.7 ± 2.5	<0.001
HbA1c (mmol/mol)	34 ± 3	48 ± 9	<0.001
Insulin (mU/L)	5.3 (3.9-7.3)	11.7 (8.5-17.1)	<0.001
GlycA (µmol/L)	366 ± 56	399 ± 48	0.002
hsCRP (mg/L)	1.35 (0.61-2.61)	2.03 (1.31-4.22)	0.004
Lp-PLA_2 (µg/L)	338 ± 63	288 ± 67	<0.001
Total cholesterol (mmol/L)	5.74 ± 0.93	5.55 ± 0.97	0.34
Non-HDL cholesterol (mmol/L)	4.12 ± 0.96	4.33 ± 0.97	0.30
LDL cholesterol (mmol/L)	3.54 ±0.81	3.37 ± 0.88	0.36
HDL cholesterol (mmol/L)	1.62 ± 0.39	1.22 ± 0.36	<0.001
Total cholesterol/HDL cholesterol ratio (mmol/L)	3.77 ± 1.13	4.87 ± 1.55	<0.001
Triglycerides (mmol/L)	1.17 (0.85-1.63)	1.94 (1.69-2.49)	<0.001
ApoB (g/L)	0.93 0± .22	0.98 0.22	0.24
Apo A-I (g/L)	1.47 ± 0.21	1.32 ± 0.25	0.004

Data in mean ± SD or in median (interquartile range). Low density lipoprotein (LDL) cholesterol was calculated in 39 subjects from group 1 and in 55 subjects from group 2. Apo: apolipoproten; BMI: body mass index; HbA1c: glycated hemoglobin; HDL: high density lipoproteins; $HOMA_{ir}$: homeostasis model assessment-estimated insulin resistance.

In group 1 as well as in group 2, GlycA was strongly and positively correlated with hsCRP (**Table 2**, **Fig. 1A**), whereas Lp-PLA_2 was not related to hsCRP in either group (**Table 2**). Of note, GlycA was also correlated positively with Lp-PLA_2 in group1 (r=0.384, *P*=0.015), but no relationship of GlycA with Lp-PLA_2 was observed in group 2 (r=0.045, *P*=0.74; **Table 2**, **Fig. 1B**). This relationship was different between the 2 groups (interaction term: *P*=0.059). In group 1, the relationship of GlycA with Lp-PLA_2 remained significant after adjustment for age and sex (β=0.404, *P*=0.041). In group 2, there was no independent relationship GlycA with Lp-PLA_2 after adjustment for age and sex (β=0.049, *P*=0.72), as well as after additional adjustment for the use of glucose lowering drugs and anti-hypertensive medication (β=-0.015, *P*=0.92).

Table 2. Univariate relationships of GlycA and lipoprotein-associated phospholipase A_2 (Lp-PLA_2) with glycemia, insulin resistance and lipid variables in 40 subjects without Type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS) (group 1) and in 58 subjects with either T2DM, MetS or both (group 2).

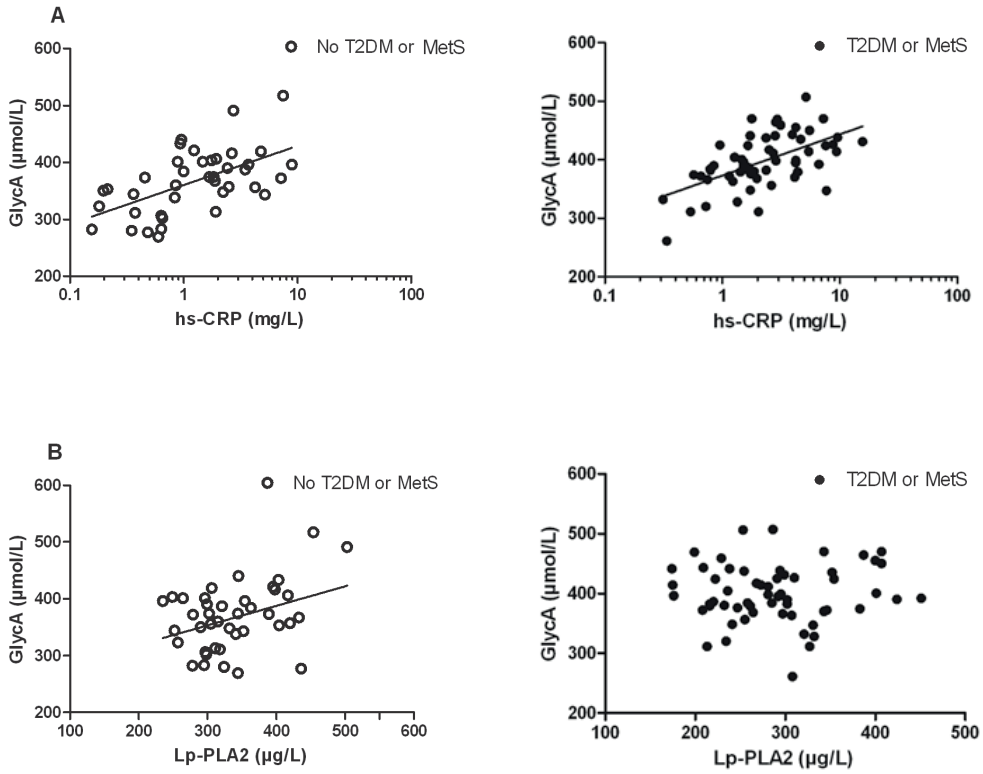
	Group 1 (n=40)		**Group 2 (n=58)**	
	GlycA	**Lp-PLA_2**	**GlycA**	**Lp-PLA_2**
hsCRP	0.570[c]	0.153	0.575[d]	0.165
Lp-PLA2	0.384[b]	-	0.045	-
Glucose	-0.068	0.168	-0.119	-0.273[a]
HbA1c	0.029	0.063	-0.034	-0.115
Total cholesterol	-0.074	0.275	0.230	0.105
Non-HDL cholesterol	0.088	0.421[c]	0.270[a]	0.332[b]
LDL cholesterol	0.030	0.425[c]	0.242	0.349[c]
HDL cholesterol	-0.393[b]	-0.381[b]	-0.108	-0.101
Total cholesterol/HDL cholesterol ratio	0.311	0.449[c]	0.140	0.344[c]
Triglycerides	0.288	0.216	0.185	0.101
Apo B	0.133	0.525[d]	0.268[a]	0.182
Apo A-I	-0.335[a]	0.344[a]	-0.088	0.032

Pearson correlation coefficients are shown. high-sensitivity C-reactive protein (hsCRP) and triglycerides are logarithmically transformed. Low density lipoprotein (LDL) cholesterol was calculated in 39 subjects from group 1 and in 55 subjects from group 2. BMI: body mass index; hsCRP: high-sensitivity C-reactive protein; HbA1c: glycated hemoglobin; HDL: high density lipoproteins. [a]$P<0.05$; [b]$P\leq0.02$; [c]$P\leq0.01$: [d]$P\leq0.001$.

GlycA and Lp-PLA_2 were not significantly correlated with fasting glucose and HbA1c, but there was an inverse relationship of Lp-PLA_2 with glucose in group 2 (**Table 2**).

GlycA was unrelated to (apo)lipoprotein variables in each group, except for an inverse relationship with HDL cholesterol and apoA-I in group 1, and a positive relationship with non-HDL cholesterol and apoB in group 2. Lp-PLA_2 was correlated positively with non-HDL cholesterol, LDL cholesterol and with the total cholesterol/HDL cholesterol ratio in each group. However, there was only an inverse relationship of Lp-PLA_2 with HDL cholesterol and apoA-I in group 1 (**Table 2**). Moreover, the relationship of Lp-PLA_2 with total cholesterol/HDL cholesterol ratio differed between the groups in such a way that for a given total cholesterol/HDL cholesterol ratio Lp-PLA_2 was lower in group 2 (interaction term: P=0.01).

Figure 1. Univariate relationships of GlycA with high-sensitivity C-reactive protein (hsCRP) (A) and of GlycA with lipoprotein-associated phospholipase A_2 (Lp-PLA_2) (B) in 40 subjects without Type 2 diabetes mellitus (T2DM) and metabolic syndrome (MetS), and in 58 subjects with T2DM or MetS.



4. Discussion

The present study shows to our knowledge for the first time that a novel NMR spectroscopy-based glycoprotein biomarker, designated GlycA, is correlated positively with plasma Lp-PLA_2 mass in subjects without T2DM or MetS. In contrast, no such relationship was observed in subjects with T2DM and/or MetS. Furthermore, GlycA was higher, whereas plasma Lp-PLA_2 was lower in subjects with T2DM or MetS. Our findings are, therefore, consistent with the hypothesis that Lp-PLA_2 mass, measured in total plasma, may relate to increased plasma levels of glycosylated acute phase proteins under normal circumstances. This relationship is likely to be disturbed in subjects with increased cardiometabolic risk as indicated by the presence T2DM or MetS. Furthermore, despite the expected strong positive correlation of GlycA with hsCRP, Lp-PLA_2 was unrelated to hsCRP in each group. We interpret this result to be consistent with the possibility that Lp-PLA_2 could specifically contribute to alterations in processes that are involved in glycoprotein metabolism.

Higher GlycA levels in T2DM and/or MetS complement recent findings in other populations [13-15, 23]. Lower plasma Lp-PLA_2 mass levels in association with T2DM have been documented before [5, 6, 24], although higher Lp-PLA_2 mass was reported in MetS as well [25]. The presently observed lower plasma Lp-PLA_2 in T2DM and/or MetS seems to be in part attributable to the relatively mild lipoprotein abnormalities in the participating subjects, as indicated by the lack of higher non-HDL cholesterol and apoB levels. Interestingly, it has also been proposed that Lp-PLA_2 becomes inactivated during LDL oxidation [26]. Whether this phenomenon could explain the inverse relationship of plasma Lp-PLA_2 with the actual fasting glucose level, as observed in the current report, remains unclear. The lack of correlation of plasma Lp-PLA_2 with hsCRP, as a global measure of low-grade systemic inflammation, seems paradoxical in view of its pro-inflammatory properties. Nonetheless, this finding is in line with previous reports comprising much larger patient groups [6, 27].

A potentially important observation of our study is that plasma GlycA was related positively to Lp-PLA_2 in non-diabetic subjects without MetS. Given the absence of a relationship of Lp-PLA_2 with hsCRP, this finding would raise the possibility that Lp-PLA_2 could influence biological pathways involved in protein glycosylation, possibly even apart from effects on inflammatory processes *per se*. It is probable that Lp-PLA_2 has a direct role the generation of pro-inflammatory cytokines. Of note, lysophosphatidylcholine, one of the main products of Lp-PLA_2 hydrolytic activity, is able to stimulate the release of IL-1β and IL-6 from peripheral blood mononuclear cells in a dose-dependent manner [16]. In turn, both IL-1β and IL-6 promote glycosylation of α1-acid glycoprotein, one of the acute phase proteins captured in the GlycA assay [17]. In addition, these interleukins may enhance the expression of glycosyltransferases as evidenced from *in vitro* studies

in several cell lines [18, 19]. Among other possible yet to more precisely delineated pathways, it is, therefore, plausible that higher plasma Lp-PLA_2 may contribute to higher GlycA levels. Obviously, further experimental evidence is needed to more precisely delineate the effect of Lp-PLA_2-mediated interleukin stimulation on this glycoprotein biomarker.

Of note, the physicochemical association of Lp-PLA_2 with HDL is enhanced, whereas Lp-PLA_2 complexed to apolipoproteinB-containing lipoproteins is diminished in T2DM and dyslipidemia [28]. In the current study, there were no inverse correlations of Lp-PLA_2 with HDL cholesterol and apoA-I in subjects with T2DM and/or MetS contrasting the positive relationships in control subjects. Furthermore, Lp-PLA_2 was lower in subjects with T2DM and/or MetS for a given total cholesterol/HDL cholesterol ratio. These findings concur with such a shift in Lp-PLA_2 from apolipoprotein B-containing lipoproteins towards HDL in the context of diabetes and MetS. Importantly, it has been recently shown that higher HDL-associated Lp-PLA_2 predicts decreased risk of cardiac death among subjects with stable coronary heart disease, contrasting the pro-atherogenic effects of Lp-PLA_2 complexed to LDL [29]. Thus, a shift in the distribution of Lp-PLA_2 from apolipoprotein B-containing lipoproteins towards HDL is likely to blunt the pro-inflammatory activity of Lp-PLA_2. This phenomenon may conceivably explain at least in part why GlycA was unrelated total plasma Lp-PLA_2 in subjects with T2DM and/or MetS.

Finally, it can be envisaged that metabolic profiling will help to improve our understanding of thus far incompletely delineated pathways that may be involved in the pathogenesis of cardiometabolic disorders [30, 31]. Although the NMR signal captured in the GlycA assay should be regarded as an integrated plasma glycoprotein biomarker, it is of interest that α1-acid glycoprotein makes part of an NMR-based risk score that predicts all cause and CVD mortality [32].

Several other methodological aspects and limitations of our study need to be considered. First, we carried out a cross-sectional study. Therefore, we cannot address cause-effect relationships or exclude the possibility of reverse causation. Second, we excluded subjects using lipid lowering drugs to circumvent confounding due to LDL cholesterol lowering on plasma Lp-PLA_2 levels [8]. As a probable consequence, subjects with only modest lipoprotein changes were preferentially included. This selection limits extrapolation of our findings to subjects with more severe dyslipidemia. Third, we determined plasma Lp-PLA_2 mass but not activity. Lp-PLA_2 mass levels are less variable between studies than Lp-PLA_2 activity [6]. Of note, higher Lp-PLA_2 activity rather than higher mass may predict CVD in T2DM subjects [33], whereas only Lp-PLA_2 activity was associated with incident CVD in a predominantly obese and glucose intolerant population [34]. In line, it was documented previously that plasma Lp-PLA_2 mass is unrelated to carotid artery intima media thickness in non-diabetic subjects but not

T2DM subjects [5]. Thus, although agreement between Lp-PLA_2 mass and activity assays is generally considered to be sufficient [6], it cannot be ruled out that documentation of Lp-PLA_2 activity would have yielded some additional information. Finally, we did not have data on exercise and diet composition, therefore we cannot completely exclude confounding due to lifestyle factors.

In conclusion, this study shows that GlycA, a novel pro-inflammatory glycoprotein biomarker, relates to higher plasma Lp-PLA_2 mass levels. This relationship appears to be blunted in subjects with T2DM and/or MetS. Since there was no relationship between Lp-PLA_2 and hsCRP our findings would highlight that measurement of pro-inflammatory glycoproteins may provide extra information compared to global assessment of low-grade systemic information.

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GlycA, a novel pro-inflammatory glycoprotein biomarker, and high sensitivity C-reactive protein are inversely associated with sodium intake after controlling for adiposity: PREVEND study

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Abstract

Background The extent to which dietary sodium intake may confer alterations in inflammatory status is unclear. GlycA is a novel pro-inflammatory proton nuclear magnetic resonance spectroscopy biomarker which associates with the development of cardiovascular disease and diabetes.

Objective We determined associations of the inflammatory markers GlycA and high sensitivity C-reactive protein (hsCRP) with 24-h sodium excretion.

Design A cross-sectional population-based study was performed among 3,935 subjects, not using anti-hypertensive medication, lipid lowering drugs or glucose lowering treatment. Urinary sodium excretion was calculated as the mean of two 24-h urine excretions. Linear regression models were used with 24-h sodium excretion as an independent variable and GlycA or Log_e hsCRP as dependent variables.

Results Mean sodium excretion was 143.0±53.4 mmol/24-h. GlycA was 343.6±58.7 µmol/L and hsCRP (geometric mean, 95 % CI) was 1.20 (1.16, 1.25) mg/L, respectively. In age- and sex- adjusted analyses, GlycA and Log_e hsCRP were not significantly associated with 24-h sodium excretion (B: 1.23 (95% CI: -0.67, 3.13), P= 0.21 and 0.03 (95% CI: -0.004, 0.07), P= 0.08, respectively per 1 SD increase). After additional adjustment for body mass index (BMI), both GlycA (B: -2.76 (95% CI: -4.65, -0.86), P= 0.004) and Log_e hsCRP (B: -0.07 (95% CI: -0.11, -0.04), P <0.001) were inversely associated with 24-h sodium excretion. These associations were similar if adjustment was performed for waist circumference instead of BMI, or if additional adjustment was performed for relevant clinical and laboratory variables, and were particularly present in men.

Conclusion The pro-inflammatory biomarkers, GlycA and hsCRP, are inversely related to higher 24-h sodium excretion when taking into account variation in adiposity. These inverse relationships remain present after taking account of other covariates.

Introduction

A high sodium intake has been linked to several major health issues. A lower sodium intake decreases blood pressure (BP) in both hypertensive and normotensive subjects [1, 2]. Therefore, reducing sodium intake has been proposed as a target for cardiovascular disease (CVD) prevention [3, 4]. In addition, high dietary salt intake may cause BP-independent organ damage, such as left ventricular hypertrophy and microalbuminuria [4-6]. The World Health Organization recommends for all adults to consume no more than 86 mmol of sodium per day irrespective of the presence of hypertension [7]. Nonetheless, evidence is inconclusive for linking reduced sodium intake to lower cardiovascular risk [1, 8].

Besides beneficial effects of reduced sodium intake on BP, low dietary sodium could also unfavorably influence pathways that are conceivably involved in cardiometabolic risk. A low sodium intake of less than 50 mmol per day may even be associated with an increased risk for cardiovascular death [9]. A Cochrane review has shown that low sodium intake significantly increases plasma renin, aldosterone and catecholamines, as well as plasma total cholesterol and triglycerides [10].

Inflammatory processes play a role in the pathogenesis of atherosclerosis and hypertension [11, 12]. However, details of the mechanism and the precise inflammatory mediators are not completely understood. High sensitivity C-reactive protein (hsCRP) has been most widely studied as a marker of low-grade systemic inflammation. Indeed, several studies reported an independent association of hsCRP with the risk of CVD and hypertension [13-15]. GlycA is a novel nuclear magnetic resonance (NMR) signal derived from mobile N-acetyl methyl groups, specifically the N-acetylglucosamine and N-acetylgalactosamine moieties, on the carbohydrate side chains of glycosylated proteins [16]. The main contributors to the GlycA signal are α1-acid glycoprotein, α1-antitrypsin, haptoglobin, α1-antichymotrypsin and transferrin [16]. GlycA is regarded as a marker of low-grade systemic inflammation [16-18]. Evidence is accumulating that GlycA may predict CVD as well as incident type 2 diabetes (T2DM) [17, 19-21].

Of interest, dietary salt restriction may relate to alterations in the levels of inflammatory markers. An intervention study demonstrated that reduction of sodium intake was accompanied by an increase in hsCRP [22]. On the other hand, a cross-sectional study showed that higher levels of 24-h sodium excretion were associated with increased levels of serum hsCRP [23]. Remarkably, this association was lost upon adjustment for body mass index (BMI). This finding would raise the possibility that adiposity is an intermediate factor between sodium balance and low-grade systemic inflammation.

Therefore, the aim of the current study was to investigate the association of 24-h sodium excretion with two inflammatory markers, GlycA and hsCRP, within a large

population-based cohort of men and women. Second, we aimed to assess the role of adiposity in the association between sodium intake and inflammatory markers.

Subjects and methods

Study design and population

The Prevention of Renal and Vascular End-Stage Disease (PREVEND) study is a prospective investigation of albuminuria, renal and CVD in a large cohort drawn from the general population. In summary, in 1997 through 1998, all inhabitants of the city of Groningen, The Netherlands, were asked to send in a morning urine sample and to fill out a short questionnaire. Pregnant women and subjects with type 1 diabetes mellitus were excluded. Urinary albumin concentration was assessed in 40,856 responders. Subjects with a urinary albumin concentration of ≥10 mg/L (n=7,768) were invited to participate, of whom 6,000 agreed. Furthermore, 3,394 randomly selected subjects with a urinary albumin concentration <10 mg/L were invited and 2,592 agreed to participate. These 8,592 individuals constitute the PREVEND cohort. For the current study data was used from the second screening round (2001-2003). From the 6,894 subjects participating in the second screening round GlycA and hsCRP were measured in 5,526 subjects. We excluded subjects with missing values for sodium excretion (n=121), subjects who used lipid lowering drugs (n=563), anti-hypertensive medication (n=858) or glucose lowering treatment (n=49). Thus, 3,935 subjects were included in the analysis (**Supplemental Figure 1**). The PREVEND study has been approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands, and was conducted in accordance with the guidelines of the Declaration of Helsinki. All participants provided written informed consent.

Data collection

The procedures at each examination in the PREVEND study have been described in detail previously [24, 25]. In summary, before the outpatient clinic visit, all participants completed a questionnaire regarding demographics, cardiovascular and renal disease history, smoking habits, alcohol consumption and medication use. Information on medication use was combined with information from a pharmacy-dispensing registry, which has complete information on drug of >95% of subjects in the PREVEND study. BMI was calculated as weight (kg) divided by height squared (meter). Waist circumference (WC) was measured on bare skin at the natural indentation between the 10th rib and iliac crest. Smoking status was categorized as never, former and current. Alcohol intake was categorized as almost never, 1-4 drinks per month or 2-7 drinks per week, and ≥1

drink per day. During each examination and during each visit, BP was measured on the right arm, every minute for 10 and 8 min, by an automatic Dinamap XL Model 9300 series device. (Johnson- Johnson Medical, Tampa, FL, USA). Hypertension was defined as a systolic blood pressure (SBP)>140 mmHg or a diastolic blood pressure (DPB)>90 mmHg, or the use of blood pressure-lowering drugs. T2DM was defined as a fasting serum glucose level>7.0 mmol/L, a non-fasting plasma glucose level>11.1 mmol/L, self-report of a physician diagnosis or the use of glucose lowering drugs, retrieved from a central pharmacy registry. Estimated glomerular filtration rate (eGFR) was calculated using the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation [26].

Subjects collected two 24-h urine samples for two consecutive days after having received oral and written instructions. Urinary sodium is given as the mean of the two 24-h urine excretions.

Laboratory measurements

Plasma samples were sent frozen to LipoScience/LabCorp (Raleigh, NC) for testing on the Vantera® Clinical Analyzer. *NMR LipoProfile*® Test spectra were collected and GlycA values were quantified as previously described [27]. Briefly, the GlycA NMR signal originates from the N-acetyl methyl group protons of the N-acetylglucosamine moieties located on the bi-, tri-, and tetra-antennary branches of plasma glycoproteins, predominantly α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1- antichymotrypsin, and transferrin. The amplitude of the combined signals was used to calculate the GlycA concentration (μmol/L of N-acetylmethyl groups) [27]. The coefficients of variation (CVs) for the GlycA assay ranged from 1.3-2.3% (19). hsCRP was measured by nephelometry with a threshold of 0.18 mg/L (BNII, Dade Behring). Plasma glucose was measured as described [6]. Serum total cholesterol was assayed on an automatic analyzer type MEGA (Merck, Darmstadt, Germany) using the CHOD-PAP-method. Measurement of serum creatinine was performed by an enzymatic method on a RocheModular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C concentrations were measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). Urinary albumin concentration was measured by nephelometry with a threshold of 2.3 mg/L, and intra- and inter-assay CVs of 2.2% and 2.6%, respectively, (Dade Behring Diagnostic, Marburg, Germany). Urinary sodium was determined in urine with an MEGA clinical chemistry analyzer (Merck, Darmstadt, Germany).

Statistical analysis

SPSS (version 22.0, SPSS Inc. Armonk, NY: IBM Corp) and STATA version 13.1 (StataCorp, College Station, TX: StataCorp LP) were used for data analysis. A two-sided P-value <0.05 was considered to be statistically significant, except for interaction terms for which the level of significance was set at P <0.10 [28]. Results are presented as mean ± SD, median (interquartile range) and percentages. Skewed data were normalized by natural logarithmic (Log_e) transformation before analyses, which was the case for urinary albumin excretion (UAE) and hsCRP. Univariable relationships between different clinical variables and 24-h sodium excretion were assessed using linear regression. Univariable and multivariable linear regression analysis were carried out to analyze the association of GlycA and hsCRP with 24-h sodium excretion. Since the associations were linear, based on the results of the residual plots, regression coefficients (unstandardized betas) per 1 SD difference in urinary sodium excretion were calculated. Interactions were tested between 24-h sodium excretion and age, sex, menopause and BMI. Mediation analysis was carried out to assess whether BMI (or WC) are potential mediators between urinary sodium excretion and inflammatory markers. Mediation analysis was performed in line with the procedures outlined by Baron and Kenny [29]. Sobel test was used to test statistical significance of mediating effect [30, 31].

Results

Clinical and laboratory characteristics of the total 3,935 subjects that were included in the analysis are shown in **Table 1.** Urinary sodium excretion averaged 143.0±53.4 mmol/24-h in the whole cohort, and amounted to 161.4±57.1 mmol/24-h in men and to 127.5±44.6 mmol/24-h in women. Mean GlycA was 343.6±58.7 µmol/L in all subjects combined and 336.8±57.3 µmol/L in men and 349.4±59.3 µmol/L in women. The geometric mean (95% CI) of hsCRP was 1.20 (1.16, 1.25) mg/L in the total cohort and 1.13 (1.07, 1.19) mg/L for men and 1.27 (1.21, 1.33) mg/L for women. In crude analyses, both GlycA and Log_e hsCRP were positively related to age, sex, BMI, smoking status, hypertension, BP, T2DM, total cholesterol and UAE. GlycA and Log_e hsCRP were inversely related to alcohol intake and eGFR (**Table 1**). The associations of GlycA and Log_e hsCRP with T2DM were no longer significant after adjustment for age, sex and BMI or WC (**Table 1**).

The relationships between different clinical variables and 24-h sodium excretion are presented in **Table 2**. 24-h sodium excretion was positively correlated with BMI, WC, former smoking, hypertension, BP, T2DM, total cholesterol, eGFR and UAE. 24-h sodium excretion was inversely related to age and female sex.

In univariable analyses, GlycA and Log_e hsCRP were not significantly related to 24-h sodium excretion (**Table 3**). There were also no significant associations after adjustment

for age and sex. Of note, these associations became significant after adjustment for BMI alone (B: -5.72 (95% CI: -7.53, -3.91), P <0.001 for GlycA, and B: -0.11 (95% CI: -0.14, -0.08), P <0.001 for hsCRP), and when adjustment for BMI was added to existing multivariable adjustment for age and sex (**Table 3**). These relationships remained significant after further adjustment for relevant clinical and laboratory covariates (**Table 3**). Similar relationships were found if adjustment for BMI was replaced with adjustment for WC (**Supplemental Table 1**). As derived from the age-, sex-, and BMI-adjusted analyses, an increase in 24-h urinary sodium excretion of 53.4 mmol/24-h above the mean (corresponding to a 1 SD increase) was associated with a 2.8 µmol/L (95% CI: -4.65, -0.86), P= 0.004) decrease in GlycA from 343.6 µmol/L to 340.8 µmol/L and a 0.07 mg/L (95% CI: -0.11, -0.04), P <0.001) decrease in hsCRP from a geometric mean value of 1.20 mg/L to 1.12 mg/L. **Figure 1** illustrates the age-, sex- and BMI-adjusted associations of GlycA and Log_e hsCRP with 24-h sodium excretion. **Supplemental Figure 2** shows the age-, sex- and WC-adjusted associations of GlycA and Log_e hsCRP with 24-h sodium excretion.

Table 1. Baseline characteristics and correlates of GlycA and high sensitivity C-reactive protein (n=3,935).

	Total cohort	GlycA			hsCRP		
		Crude	Adjusted for age, sex and BMI	Adjusted for age, sex and waist circumference	Crude	Adjusted for age, sex and BMI	Adjusted for age, sex and waist circumference
Sex (%)							
Male	1797 (45.7)	Ref	Ref	Ref	Ref	Ref	Ref
Female	2138 (54.3)	0.11***	0.13***,1	0.24***	0.05***	0.07***,1	0.22***
Age, years	50.4 ± 11.0	0.17***	0.08***,2	0.10***	0.19***	0.002***,2	0.10***
BMI, kg/m^2	25.9 ± 4.01	0.27***	0.26***,3	0.07*	0.36***	0.34***,3	0.17***
Waist circumference, cm	89.5 ± 11.8	0.24***	0.24***	-	0.33***	0.23***	-
Smoking status (%)							
Never	1240 (31.5)	Ref	Ref	Ref	Ref	Ref	Ref
Former	1552 (39.4)	0.06**	0.02	0.02	0.07***	0.03	0.02
Current	1102 (28.0)	0.21***	0.24***	0.22***	0.13***	0.16***	0.14***
Alcohol intake (%)							
Almost never	854 (21.7)	Ref	Ref	Ref	Ref	Ref	Ref
1-4 drinks per month	679 (17.7)	-0.08***	-0.06***	-0.07***	-0.06**	-0.03	-0.04*
2-7 drinks per week	1321 (33.6)	-0.14***	-0.07***	-0.08***	-0.11***	-0.03	-0.04*
≥1 drinks per day	1047 (26.6)	-0.11***	-0.06*	-0.07***	-0.09***	-0.04*	-0.06**
Hypertension, n (%)							
No	3407 (86.6)	Ref	Ref	Ref	Ref	Ref	Ref
Yes	528 (13.4)	0.13***	0.06***	0.06**	0.14***	0.08*	0.04*

CVD, n (%)							
No	3870 (98.3)	Ref	Ref	Ref	Ref	Ref	Ref
Yes	65 (1.7)	0.01	0.003	0.006	0.04*	0.03	0.03
Systolic blood pressure, mm Hg	121.7 ± 16.2	0.19***	0.12***	0.11***	0.20***	0.08***	0.07***
Diastolic blood pressure, mm Hg	71.7 ± 8.6	0.14***	0.10***	0.09***	0.16***	0.07***	0.06***
T2DM, n (%)							
No	3870 (98.3)	Ref	Ref	Ref	Ref	Ref	Ref
Yes	65 (1.7)	0.05***	0.02	0.02	0.06***	0.02	0.02
Total cholesterol, mmol/L	5.4 ± 1.06	0.20***	0.13***	0.13***	0.15***	0.05***	0.05**
eGFR*crea-cysC*, ml/min/1.73m^2	95.4 ± 14.7	-0.18***	-0.08***	-0.08***	-0.21***	-0.10***	-0.10***
UAE, mg/24-h[1]	8.85 (8.58, 9.03)	0.13***	0.10***	0.09***	0.13***	0.12***	0.12***
hsCRP, mg/L[1]	1.20 (1.16, 1.25)	0.66***	0.63***	0.62***	-	-	-
GlycA, µmol/L	343.6 ± 58.7	-	-	-	0.66***	0.59***	0.59***

Data are numbers (percentages), means ± SD and 1geometric means (95 % confidence interval). Data were obtained by linear regression analysis. Pearson correlation coefficients are given. hsCRP and UAE are logarithmically transformed for correlation analysis. 1Adjusted for age and BMI, 2Adjusted for sex and BMI, 3Adjusted for sex and age. *P<0.05; **P<0.01; ***P≤0.001.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; eGFRcrea-cysC, estimated glomerular filtration rate based on creatinine-cystatin C equation; hsCRP, high sensitivity C-reactive protein; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion.

Table 2. Univariable linear regression analysis between various clinical and laboratory variables and urinary sodium excretion (mmol/24-h) (n=3,935).

	Pearson's correlation coefficient
Sex	
Male	Ref
Female	-0.32***
Age, years	-0.08***
BMI, kg/m^2	0.23***
Waist circumference, cm	0.30***
Smoking status	
Never	Ref
Former	0.06**
Current	-0.03
Alcohol intake	
Almost never	Ref
1-4 drinks per month	-0.008
2-7 drinks per week	0.06**
≥1 drinks per day	0.05*
Hypertension	
No	Ref
Yes	0.07***
Systolic blood pressure, mm Hg	0.14***
Diastolic blood pressure, mm Hg	0.14***
T2DM	
No	Ref
Yes	0.03*
Total cholesterol, mmol/L	0.03*
eGFR*crea-cysC*, ml/min/1.73m^2	0.12***
Log_e UAE, mg/24-h	0.18***

Pearson correlation coefficients are given. *P<0.05; **P<0.01; **P≤0.001.

Abbreviations: BMI, body mass index; eGFRcrea-cysC, estimated glomerular filtration rate based on creatinine-cystatin C equation; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion.

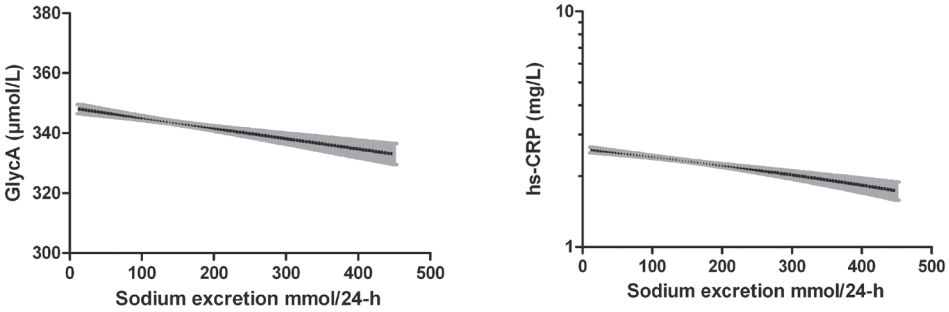
Table 3. Univariable and multivariable associations of GlycA and high sensitivity C-reactive protein (hsCRP; Log_e transformed) with sodium excretion (per 1 SD increase)[1] in the total cohort (n=3,935) and in men (n=1,797) and women (n=2,138) separately.

	GlycA (dependent variable)		hsCRP (dependent variable)	
	B per 1 SD increase in sodium excretion (95% CI)	**P-value**	**B per 1 SD increase in sodium excretion (95% CI)**	**P-value**
Total cohort				
Crude	-1.81 (-3.65, 0.02)	0.05	-0.009 (-0.05, 0.03)	0.62
Model 1	1.23 (-0.67, 3.13)	0.21	0.03 (-0.004, 0.07)	0.08
Model 2	-2.76 (-4.65, -0.86)	0.004	-0.07 (-0.11, -0.04)	<0.001
Model 3	-2.30 (-4.16, -0.44)	0.02	-0.07 (-0.10, -0.03)	<0.001
Model 4	-2.74 (-4.65, -0.84)	0.005	-0.08 (-0.11, -0.04)	<0.001
Model 5	-2.86 (-4.76, -0.96)	0.003	-0.08 (-0.12, -0.04)	<0.001
Model 6	-2.86 (-4.76, -0.96)	0.003	-0.08 (-0.11, -0.04)	<0.001
Men				
Crude	-1.95 (-4.60, 0.70)	0.15	-0.02 (-0.07, 0.04)	0.56
Model 1	-1.11 (-3.73, 1.50)	0.40	0.007 (-0.04, 0.06)	0.78
Model 2	-3.74 (-6.45, -1.02)	0.007	-0.09 (-0.14, -0.04)	<0.001
Model 3	-3.19 (-5.84, -0.55)	0.02	-0.09 (-0.14, -0.04)	0.001
Model 4	-3.54 (-6.21, -0.87)	0.01	-0.09 (-0.14, -0.04)	0.001
Model 5	-3.68 (-6.33, -1.02)	0.007	-0.09 (-0.14, -0.04)	<0.001
Model 6	-3.72 (-6.37, -1.06)	0.006	-0.09 (-0.14, 0.04)	<0.001
Women				
Crude	2.47 (-0.04, 4.99)	0.05	0.04 (-0.01, 0.09)	0.15
Model 1	3.66 (1.17, 6.15)	0.004	0.06 (0.008, 0.11)	0.02
Model 2	-0.62 (-3.03, 1.79)	0.61	-0.04 (-0.09, 0.004)	0.08
Model 3	-0.24 (-2.60, 2.13)	0.84	-0.04 (-0.09, 0.006)	0.09
Model 4	-0.28 (-2.67, 2.12)	0.82	-0.04 (-0.08, 0.01)	0.14
Model 5	-0.21 (-2.60, 2.18)	0.86	-0.04 (-0.08, 0.01)	0.15
Model 6	-0.19 (-2.58, 2.20)	0.88	-0.03 (-0.08, 0.01)	0.16

[1] SD change in urinary sodium excretion corresponds to 54.3 mmol/24-h (57.1 mmol/24-h for men and to 44.6 mmol/24-h for women). Data were obtained by linear regression analysis. Unstandardized betas (B) are given with corresponding 95% confidence intervals (95% CI). There was significant interaction of sex with 24-h sodium excretion impacting on both GlycA and hsCRP (P for interaction: 0.001 and 0.06).

Model 1: Crude + age and sex (was only adjusted for age in the sex-stratified analysis).
Model 2: Model 1 + BMI
Model 3: Model 2 + smoking status (never, former, current) and alcohol consumption (almost never, 1-4 drinks per month, 2-7 drinks per week, ≥ drinks per day)
Model 4: Model 3 + SBP, eGFR*crea-cysC* and UAE
Model 5: Model 4 + total cholesterol
Model 6: Model 5 + CVD and T2DM
Abbreviations: BMI, body mass index; CVD, cardiovascular disease; eGFR*crea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; hsCRP, high sensitivity C-reactive protein; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion.
Significant interaction of sex with 24-h sodium excretion impacting on both GlycA and hsCRP (in multivariable adjusted analyses: P for interaction: 0.001 and 0.06).

Figure 1. Association between sodium excretion (mmol/24-h) and GlycA and hsCRP adjusted for age, sex and BMI in 3,935 subjects. Data were fit by linear regression analysis; the grey areas indicate the 95% confidence intervals.

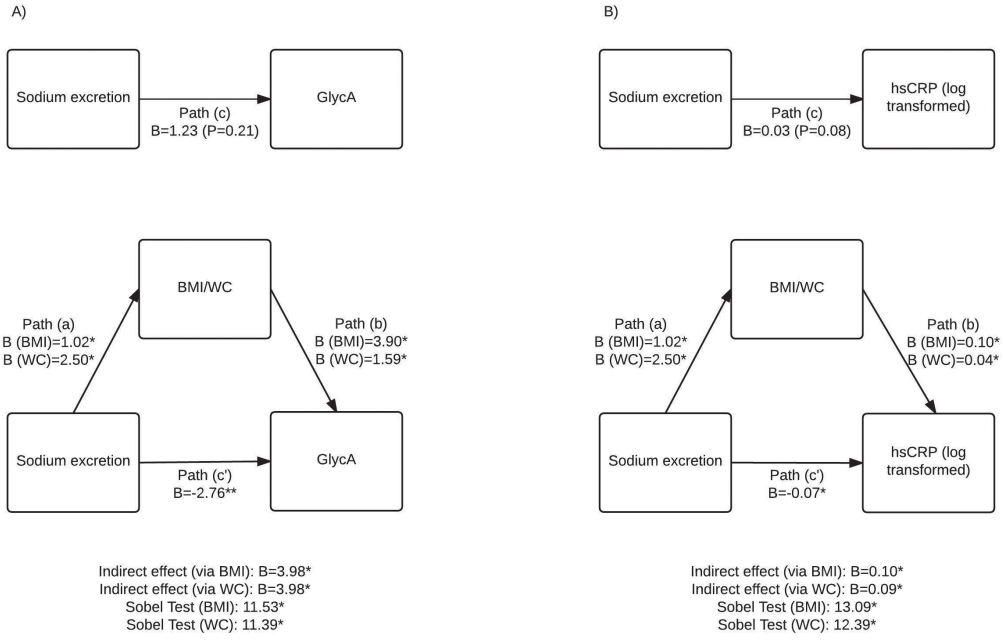


There were no statistically significant interactions by either age, BMI or menopause (interactions: P>0.10 for all, data not shown). However, there was a significant interaction between sex and 24-h sodium excretion impacting on both GlycA and hsCRP (in multivariable adjusted analyses: P for interaction: 0.001 and 0.06). We, therefore, also performed sex-stratified analyses. Baseline characteristics for men and women separately are presented in **Supplemental Table 2**. **Table 3** shows the sex-stratified linear regression analyses. Similar to the non-stratified analyses, in crude stratified analyses, there was neither a significant association of GlycA with 24-h sodium excretion in men (B: -1.95 (95% CI: -4.60, 0.70), P= 0.15) nor in women (B: 2.74 (95% CI: -0.04, 4.99), P= 0.05, **Table 3**). However, after adjustment for age and BMI, the association between 24-h sodium excretion and GlycA became significant in men (B:-3.74 (95% CI: -6.45, -1.02), P= 0.007), but not in women (B: -0.62 (95 % CI: -3.03, 1.79), P= 0.61; model 1, **Table 3**). Results for Log_e hsCRP were comparable with the association being significant in men after adjustment for age and BMI (B: -0.09 (95% CI: -0.14, -0.04), P <0.001; model 1, **Table 3**). Furthermore, adjustment for smoking status, alcohol consumption, SBP, eGFR,

UAE, cholesterol, CVD, and T2DM did not materially alter the results in both the analyses with GlycA and Log_e hsCRP as outcome (**Table 3**, model 5).

Mediation analysis suggested that BMI could represent a possible contributor involved in both the association between GlycA and Log_e hsCRP with 24-h sodium excretion (**Figure 2**). The analysis of the mediating role of WC in the association between GlycA and Log_e hsCRP with 24-h sodium excretion showed similar results.

Figure 2. Mediation from body mass index (BMI) and waist circumference (WC) in the association between sodium excretion (per 1 SD increase) and GlycA (A), and high sensitivity C-reactive protein (hsCRP; Log transformed) (B), controlling for age and sex.



Sobel test was used to test statistical significance of mediating effect. The indirect effect (through BMI and WC) is calculated as a*b. *P<0.001, **P=0.004

Discussion

In a large cohort of men and women, lower levels of GlycA and hsCRP were associated with higher levels of 24-h sodium excretion if measures of adiposity were taken into account. BMI is an appropriate measure for overweight and obesity, but cannot be used to distinguish between lean body mass, adipose tissue or body fat distribution [32]. Of note, adjustment for WC gave essentially similar results, which suggests that it is indeed (visceral) fat accumulation tissue that modifies the association.

The results of our study are in line with a recent intervention study of Nakandakare et al. who studied the effects of a week on a control diet (160 mmol sodium per day) followed by 3 weeks on low sodium intake (60 mmol sodium per day) [22]. Non-pharmacologically treated hypertensive adult patients were included in that study, while subjects with severe hypertriglyceridemia, obesity, diabetes, alcohol abuse and the use of any drug that could interfere with lipid metabolism were excluded. hsCRP, IL-6 and TNF-α concentrations were all elevated on the low dietary sodium intake. In contrast, a cross-sectional study found that higher hsCRP levels were positively associated with 24-h sodium excretion [23]. The difference in results between the previous observational study and our current study may at least in part be explained by differences in the populations studied. In the earlier study, there were slightly more men and the mean age of the investigated population was lower compared to our population. Even more importantly, in the previous observational study, no information was available on the use of medication such as statins [23], which are known to reduce systemic inflammation [33].

The mean 24-h sodium excretion was 143 mmol in our population, which is higher than the current daily recommendation of 86 mmol sodium as currently proposed the World Health Organization [7], and of 103 mmol sodium as proposed by the Health Council of the Netherlands [34]. However, when the data for the present study were collected the daily recommendation in the Netherlands was 155 mmol of sodium [35]. The age-, sex- and BMI-adjusted calculated changes in GlycA and hsCRP in relation to differences in urinary sodium excretion as determined by linear modelling were rather small and should be placed in the context of the average 24-h sodium excretion in the population studied. For reasons that are not clear at present the associations of GlycA and hsCRP with 24-hour sodium excretion were only statistically significant in men.

As expected, urinary sodium excretion was lower in women and in older subjects, and was positively related to BP [6, 10, 36]. Urinary sodium excretion was also positively related to BMI and WC [6]. These anticipated relationships with adiposity indices require adjustment for these variables. It is, however, noteworthy that the associations of GlycA and hsCRP were uncovered after adjustment for either BMI or WC. In comparison, the Nottingham survey showed a positive relationship of hsCRP with urinary sodium

excretion in a crude analysis that lost significance after adjustment for BMI [23]. The reasons responsible for this adiposity-mediated shift in the association of inflammation markers with sodium intake deserve further study. Mediation analysis in our study suggested that BMI or alternatively WC could influence the association between GlycA and hsCRP and 24-h sodium excretion. However, it should be emphasized that this analysis does not provide evidence of causation. It can be envisaged that unmeasured dietary factors that may coincide with an increase in sodium intake could also predispose to adiposity but our findings should be regarded as hypothesis generating.

Why may a lower sodium intake be associated with a higher burden of circulating inflammation markers? Among other possibilities, a lower sodium intake activity activates the renin-angiotensin-aldosterone system [2, 10]. Both a low sodium diet and infusion of angiotensin I result in lower plasma levels of adiponectin, an adipokine with well delineated anti-inflammatory properties [37]. Importantly, the angiotensin II type I receptor is expressed in adipose tissue, and blockade of this receptor attenuates in part the decrease in circulating adiponectin consequent to obesity and aging [38]. In addition, it may be relevant that lipoprotein-associated phospholipase A_2 (Lp-PLA_2), a pro-inflammatory enzyme which is predominantly complexed to apolipoprotein B-containing lipoproteins, decreases in response to a short term dietary sodium challenge [39]. In this regard, it is noteworthy that the novel pro-inflammatory glycoprotein biomarker, GlycA, has been recently found to be positively related to Lp-PLA_2 in subjects without T2DM or the metabolic syndrome [40]. In view of the effect of Lp-PLA_2 to increase interleukins which are able to enhance protein glycosylation [41-44], it is conceivable that Lp-PLA_2 could be involved in the association of sodium intake with plasma GlycA levels.

Several limitations of our study warrant consideration. First, the cross-sectional nature of our study limits the ability to conclude a causal relationship between 24-h sodium excretion and inflammation. Also it is unknown whether changes in urinary sodium excretion relate to changes in circulating inflammation markers overtime. Second, subjects of the PREVEND cohort is predominantly comprised of white people. Our results can therefore not be easily extrapolated to other ethnicities. This may be important since some data suggests that black and Asian people are more sensitive to sodium reduction with respect to BP changes than white people [10]. Third, subjects with a urinary albumin concentration of ≥10 were overrepresented in the PREVEND study. However, adjustment for UAE did not alter the results, which makes it unlikely that this overrepresentation influenced the observed findings. Fourth, no information about other dietary habits which could coincide with higher sodium intake were available.

Strengths of our study include the relative large cohort of predominantly healthy men and women and extensive information about possible confounding factors, such

as the presence of CVD and the use of medications that could alter the degree of low-grade chronic inflammation. In addition, two 24-h urine collections were obtained from each participant. Repeated 24-h sodium excretion is considered as the reference standard for sodium intake estimation [45]. Since 90 to 95% of the sodium ingested is excreted in the urine, 24-h sodium excretion is a good approach to estimate sodium intake [45]. Although 24-h sodium overcomes limitations of recall bias, which is a major problem when estimating dietary sodium intake from dietary questionnaires, limitation of 24-h urine collection is the high frequency of incomplete sample collection.

In conclusion, in this large population-based cohort of men and women, GlycA and hsCRP were not significantly related to 24-h sodium excretion in crude analyses. Notably, in age- and sex-adjusted analyses taking account of BMI or alternatively of WC, lower GlycA and hsCRP levels were both associated with higher 24-h sodium excretion, and these relationships remained present after taking account of other potential covariates.

Acknowledgments

The GlycA measurements were performed by LipoScience/LabCorp (Raleigh, North Carolina, USA) at no cost.

Conflict of interest statement

MAC and JDO are employees of LabCorp. EGG, PV, SJLB and RPFD have no conflict of interest to declare.

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Supplemental Table 1. Univariable and multivariable associations of GlycA and Log_e hsCRP with sodium excretion per 1 SD increase adjusted for waist circumference instead of body mass index[1].

	GlycA (dependent variable)		**hsCRP (dependent variable)**	
	B per 1 SD increase in sodium excretion (95% CI)	**P-value**	**B per 1 SD increase in sodium excretion (95% CI)**	**P-value**
Crude	-1.81 (-3.65, 0.02)	0.05	-0.009 (-0.05, 0.03)	0.62
Model 1	1.23 (-0.67, 3.13)	0.21	0.03 (-0.004, 0.07)	0.08
Model 2	-2.74 (-4.62, -0.86)	0.004	-0.07 (-0.10, -0.03)	<0.001
Model 3	-2.17 (-4.02, -0.32)	0.02	-0.06 (-0.10, -0.03)	0.001
Model 4	-2.59 (-4.49, -0.69)	0.007	-0.07 (-0.11, -0.03)	<0.001
Model 5	-2.72 (-4.61, -0.83)	0.005	-0.07 (-0.11, -0.04)	<0.001
Model 6	-2.73(-4.62, -0.84)	0.005	-0.07 (-0.11, -0.03)	<0.001

[1] SD change in urinary sodium excretion corresponds to 54.3 mmol/24-h. Unstandardized betas (B) are given with corresponding 95% confidence intervals (95% CI).

Model 1: Crude + age and sex

Model 2: Model 1 + waist circumference

Model 3: Model 2 + smoking status (never, former, current) and alcohol consumption (almost never, 1-4 drinks per month, 2-7 drinks per week, ≥ drinks per day)

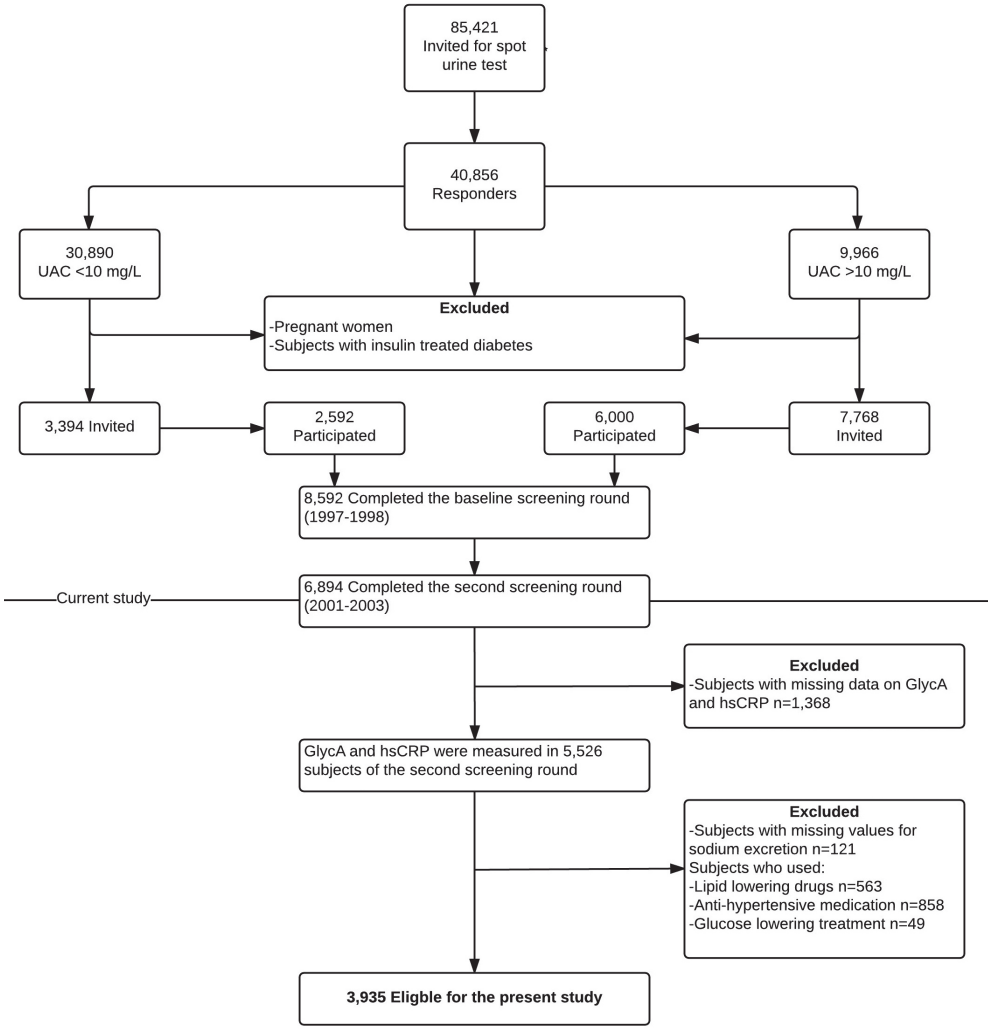
Model 4: Model 3 + SBP, eGFR*crea-cysC*, UAE

Model 5: Model 5 + total cholesterol

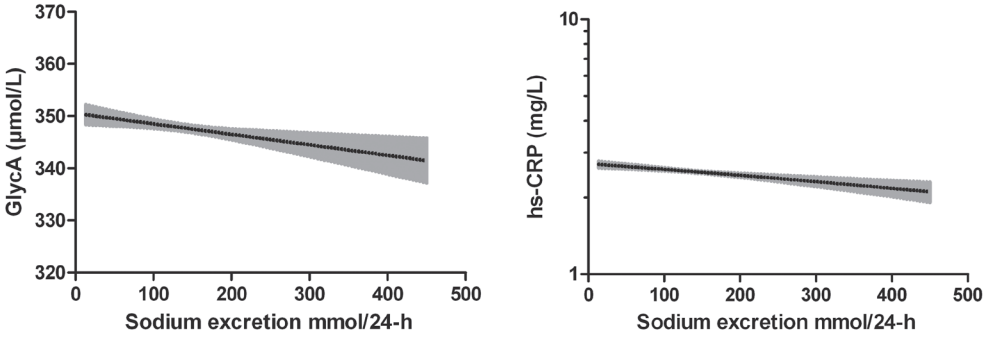
Model 6: Model 5 + CVD and T2DM

Abbreviations: CVD, cardiovascular disease; eGFR*crea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; hsCRP, high sensitivity C-reactive protein; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion.

Supplemental Figure 1. Flowchart of PREVEND participants included or excluded for the purpose of this study.



Supplemental Figure 2. Association between sodium excretion (mmol/24-h) and GlycA and hsCRP adjusted for age, sex and waist circumference. Data were fit by linear regression analyses; the grey areas indicate the 95% confidence intervals.



GlycA, a Pro-Inflammatory Glycoprotein Biomarker, and Incident Cardiovascular Disease: Relationship with C-reactive protein and Renal Function

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Abstract

Objective GlycA is a novel nuclear magnetic resonance spectroscopy-measured biomarker of systemic inflammation. We determined whether GlycA is associated with incident cardiovascular disease (CVD) in men and women, examined whether this association with CVD is modified by renal function, and compared this association with high sensitivity C-reactive protein (hsCRP).

Research design and methods A prospective cohort study was performed among 4,759 subjects (PREVEND study) without a history of CVD and cancer. Incident CVD was defined as the combined endpoint of cardiovascular morbidity and mortality. Cox regression analyses were used to examine associations of baseline GlycA and hsCRP with CVD.

Results 298 first CVD events occurred during a median follow-up of 8.5 years. After adjustment for clinical and lipid measures the hazard ratio (HR) for CVD risk in the highest GlycA quartile was 1.58 (95% CI, 1.05-2.37, P for trend=0.004). This association was similar after further adjustment for renal function (estimated glomerular filtration rate and urinary albumin excretion). After additional adjustment for hsCRP, GlycA was still associated with incident CVD (HR: 1.16 per SD change (95% CI, 1.01-1.33), P=0.04). Similar results were obtained for hsCRP (HR per SD change after adjustment for GlycA: 1.17 (95% CI 1.17 (95% CI, 1.01-3.60), P= 0.04). CVD risk was highest in subjects with simultaneously higher GlycA and hsCRP (fully adjusted HR: 1.79 (95% CI, 1.31-2.46), P<0.001).

Conclusion GlycA is associated with CVD risk in men and women, independent of renal function. The association of GlycA with incident CVD is as strong as that of hsCRP.

Introduction

It is increasingly recognized that protein glycosylation, i.e. the enzymatic process whereby a glycan (polysaccharide) moiety is added to a protein, affects many physiological processes including the innate immune system, thereby modulating inflammatory responses [1-3]. Circulating glycosylated acute phase proteins are elevated in various inflammatory and autoimmune disorders [4]. It is well known that atherosclerotic cardiovascular disease (CVD) is featured by enhanced low grade inflammation [5, 6]. To date, numerous studies have shown that higher circulating levels of acute phase proteins, in particular high sensitivity C-reactive protein (hsCRP), predict future development of cardiovascular disease (CVD) [4, 5, 7, 8], although the possibility that CRP may play a causal role in derangements of inflammatory processes involved in the pathogenesis of atherosclerosis has been questioned [9, 10].

GlycA is a recently developed nuclear mass resonance (NMR) spectroscopy-derived biomarker of systemic inflammation [11, 12]. This NMR signal arises from the *N*-acetyl methyl groups of the *N*-acetylglucosamine residues located on specific glycan branches of circulating plasma proteins, mainly α1-acid glycoprotein (oromucosoid), haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin. It has been established that GlycA is strongly correlated with hsCRP, which supports the contention that GlycA is a marker of low-grade systemic inflammation [11, 13].

Interestingly, it has been recently shown that plasma GlycA is independently associated with incident CVD in a large cohort study of initially healthy women [13]. In this report, the association of GlycA with incident CVD was similar to that of hsCRP. Of note, the associations of GlycA and hsCRP with incident CVD were attenuated after mutual adjustment for these inflammatory markers, which raises the possibility that GlycA and hsCRP are biomarkers that reflect in part common processes involved in atherosclerosis development. At present it is unknown whether the association of GlycA with future development of clinically manifest CVD also holds true for men. Of further importance, no data are available with respect to the relationship of GlycA with renal function and albuminuria. It is important to determine these relationships, because both lower estimated glomerular filtration rate (eGFR) and higher degrees of albuminuria confer increased risk of cardiovascular morbidity and mortality [14-16]. Moreover, low grade chronic inflammation as inferred from higher hsCRP predicts a decline in renal function, and associates with albuminuria [14, 17, 18].

We therefore initiated the present study to determine i) whether GlycA associates with increased CVD risk in both men and women, ii) the extent to which the anticipated association of GlycA with future CVD is modified by renal function abnormalities, as inferred from eGFR and albuminuria and iii) the extent to which the anticipated association of GlycA with future CVD is attenuated by hsCRP, representing a widely

used marker of low-grade chronic inflammation. To this end a prospective evaluation was performed among participants of the Prevention of Renal and Vascular ENd-Stage Disease (PREVEND) study, a prospective investigation of albuminuria, renal and cardiovascular disease in a large, predominantly Caucasian population.

Materials and Methods

Study design and population

Details of the PREVEND study are described elsewhere [19, 20]. In summary, in 1997 through 1998, all inhabitants of the city of Groningen, The Netherlands, between the ages of 28 and 75 years (85,421 subjects) were asked to send in a morning urine sample and to fill out a short questionnaire. Pregnant women and subjects with type 1 diabetes mellitus were excluded. The urinary albumin concentration was assessed in 40,856 responders. Subjects with a urinary albumin concentration ≥10 mg/L (n=7,768) were invited to participate, of whom 6,000 were enrolled. In addition, a randomly selected group with a urinary albumin concentration of <10 mg/L (n=3,394) was invited to participate in the cohort, of whom were 2,592 were enrolled. These 8,592 individuals constitute the PREVEND cohort. The second screening took place from 2001 through 2003 (n=6,894), which was the starting point of the present evaluation. The PREVEND study has been approved by the Medical Ethics Committee of the University Medical Center Groningen, and is performed in accordance with Declaration of Helsinki guidelines. All participants provided written informed consent. GlycA and hsCRP were measured in 5,526 subjects in whom previously unthawed samples were available. For the present study subjects with a history of CVD (n=349) and cancer (n=418) at baseline were excluded, leaving 4,759 subjects for the analysis.

Follow-up and outcome

Follow-up time was defined as the period between assessment at the second screening round (baseline) and first CVD event, loss to follow-up, or the end of follow up time (01-01-2011), whichever came first. If a person had moved to an unknown destination, the date on which the person was dropped from the municipal registry was used as the census date.

Data on mortality were obtained from the municipal register, and the cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics. Information for cardiovascular morbidity was obtained from PRISMANT, the Dutch national registry of hospital discharge diagnoses. All data were coded according to the International

Classification of Diseases, the Ninth Revision (ICD-9) was used for data until 01-01-2009, after this date, data were coded according to the Tenth Revision (ICD-10). CVD was defined as the combined endpoint of incident cardiovascular morbidity and mortality which includes the following events: acute myocardial infarction, acute and subacute ischaemic heart disease, occlusion or stenosis of the precerebral or cerebral arteries or the following procedures: coronary artery bypass grafting, percutaneous transluminal coronary angioplasty or other vascular interventions (i.e. percutaneous transluminal angioplasty or bypass grafting of the aorta and peripheral vessels). Cardiac events were defined as fatal/nonfatal myocardial infarction, ischemic heart disease, coronary artery bypass grafting and percutaneous transluminal coronary angioplasty.

Baseline measurements and definitions

Body mass index (BMI) was calculated as weight (kg) divided by height squared (meter). Smoking status was categorized as never, former and current. Alcohol intake was categorized as almost never, 1-4 drinks per month or 2-7 drinks per week, and ≥1 drink per day. Blood pressure was measured with an automatic Dinamap XL Model 9300 series device (Johnson-Johnson Medical, Tampa, FL, USA). Hypertension was defined as a systolic blood pressure (SBP) >140 mmHg or a diastolic blood pressure (DPB) >90 mmHg, or the use of blood pressure-lowering drugs. Type 2 diabetes mellitus (T2DM) was defined as a fasting serum glucose level >7.0 mmol/L, a non-fasting plasma glucose level >11.1 mmol/L, self-report of a physician diagnosis or the use of glucose lowering drugs, retrieved from a central pharmacy registry. eGFR was calculated using the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation from 2012 [21].

Laboratory measurements

Fasting blood samples were provided and stored at -80 °C. NMR spectra were collected from EDTA plasma samples using the Vantera® Clinical Analyzer [22]. The GlycA NMR signal is derived from the *N*-acetyl methyl protons of *N*-aceylated carbohydrate side chains of serum glycoproteins (predominantly, α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin) [11]. The GlycA NMR signal is centered at 2.00 ± 0.01 ppm in the NMR spectra of plasma, and only *N*-acetylglucosamine with specific glycosidic linkage, namely, β (1>2) or β (1>6) with a preceding mannose residue, contribute to the GlycA signal [11].

hsCRP was measured by nephelometry with a threshold of 0.18 mg/L (BNII, Dade Behring). Plasma glucose was measured as described [6]. Serum total cholesterol was assayed on an automatic analyser type MEGA (Merck, Darmstadt, Germany) using the

CHOD-PAP-method. Triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) were measured on a Beckman Coulter AU Analyzer. Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Measurement of serum creatinine was performed by an enzymatic method on a RocheModular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C concentrations were measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). Urinary albumin concentration was measured by nephelometry with a threshold of 2.3 mg/l, and intra- and inter-assay coefficients of variation of 2.2% and 2.6%, respectively, (Dade Behring Diagnostic, Marburg, Germany).

Statistical analysis

Statistical analyses were performed using statistical software SPSS version 22.0 (SPSS Inc, Chicago, IL) and STATA version 13.1 (StataCorp, College Station, TX: StataCorp LP). Normally distributed data were expressed as mean ± SD and skewed data as median [interquartile range]. Subject characteristics and laboratory variables were calculated across sex-stratified quartiles of GlycA. P-values across quartiles of GlycA were determined by linear regression for continuous variables or chi-square test for categorical variables. Skewed data were normalized by logarithmic transformation before analyses, which was the case for triglycerides, UAE and hsCRP. Univariable linear regression analyses are presented as standardized beta with corresponding P-value.

Kaplan-Meier curves with log-rank tests were used to estimate survival curves of GlycA and hsCRP levels, either separately or joint, on CVD outcome. Cox proportional hazards regression analyses were used to determine the risk for incident CVD events according to quartiles of GlycA and hsCRP, as well as per 1 SD increase of GlycA and log hsCRP. The assumption of proportional hazards for baseline predictors was investigated by inspecting the Schoenfeld residuals. Multivariable analyses were conducted using Cox regression models including the covariates age, sex, BMI, alcohol intake, smoking status, prevalent T2DM, use of lipid lowering drugs, use of anti-hypertensive medication, SBP, total cholesterol, HDL cholesterol, triglycerides, eGFR, and UAE. Tests of trend across quartiles were conducted by assigning the median value for each quartile as its value and treating this as a continuous variable. Additionally, we also evaluated the association between GlycA and hsCRP and cardiac events only, reasoning the numbers of cerebrovascular and peripheral vascular endpoints were too low to allow for a meaningful subanalysis. Possible effect modification was explored by including the interaction terms between GlycA or hsCRP and age or sex in the multivariable adjusted models.

The additional value of GlycA and hsCRP for CVD risk prediction was assessed by discrimination using Harrell's C-statistic. The theoretical maximum of 1.0 indicates

perfect prediction and a value of 0.5 indicates that patients are correctly classified in 50% of subjects (no discrimination) [23].

The joint associations of GlycA and hsCRP on outcome were evaluated by dichotomizing the distribution of GlycA and hsCRP, according to cut points of the highest quartile of GlycA (>387 μmol/L) and the highest quartile of hsCRP (>2.95 mg/L) in both sexes combined, to test if both biomarkers in the highest range had a different association with first CVD event vs. both these biomarkers in the lowest range or one of these biomarkers in the lowest range.

Given the enrichment of subjects with elevated urinary albumin excretion in the PREVEND population, we also performed a secondary analysis in which we accounted for the sampling design of the study, with respect to enrichment of subjects with a urinary albumin concentration >10 mg/L, by specifying stratum-specific baseline hazard functions.

Interaction terms were considered to be statistically significant at two-sided P-values<0.10 [24]. Otherwise, the levels of significance was set at two-sided P-values <0.05.

Results

The baseline characteristics of the 4,759 subjects according to sex-stratified quartiles of GlycA are presented in **Table 1**. The mean age of the study population was 52.7 ± 11.8 years. Subjects in the highest quartile of GlycA were older and smoked more frequently compared to subjects in the lowest GlycA quartile. Hypertension and T2DM were more prevalent among subjects in the highest quartile. Levels of hsCRP, total cholesterol, non-HDL cholesterol, triglycerides and UAE were higher, whereas HDL cholesterol and eGFR were lower in subjects in the highest quartile vs. subjects in the lowest GlycA quartile.

The relationship between GlycA and hsCRP is shown in **Fig 1**. Both GlycA and hsCRP were higher in women than in men (**Table 2**). Univariable linear regression analyses showed that age, BMI, smoking, hsCRP, glucose, total cholesterol, non-HDL cholesterol, triglycerides and UEA were positively associated with GlycA, whereas alcohol intake, HDL cholesterol and eGFR were inversely associated with GlycA levels (**Table 2**). GlycA was higher in subjects using anti-hypertensive drugs, lipid lowering drugs and glucose lowering medication. Similar associations were observed for hsCRP.

Table 1. Baseline characteristics according to sex-stratified quartiles of GlycA concentrations in 4,759 participants of the PREVEND study

	Quartiles of GlycA, µmol/L				
	1 **♂≤301** **♀≤311**	**2** **♂302-335** **♀312-350**	**3** **♂336-377** **♀351-392**	**4** **♂≥378** **♀≥393**	**P-value**
Participants, n	1186	1182	1205	1186	
Age, years	48.6±10.6	52.2±11.8	54.5±11.9	55.5±11.8	<0.001
Female, n (%)	641 (54.0)	642 (54.3)	657 (54.5)	641 (54.0)	0.99
BMI, kg/m^2	24.6±3.4	26.1±3.7	27.2±4.1	28.3±4.9	<0.001
Smoking, n (%)					<0.001
Never	480 (40.4)	376 (31.8)	316 (26.2)	287 (24.2)	
Former	490 (41.3)	519 (43.9)	514 (42.7)	446 (37.6)	
Current	201 (16.9)	273 (20.1)	360 (29.9)	441 (37.2)	
Alcohol intake, n (%)					<0.001
Almost never	222 (18.7)	243 (20.6)	321 (26.6)	363 (30.6)	
1-4 drinks per month	190 (16.0)	214 (18.1)	202 (16.8)	191 (16.1)	
2-7 drinks per week	454 (38.3)	391 (33.1)	363 (30.1)	323 (27.2)	
≥1 drinks per day	310 (26.1)	321 (27.2)	309 (25.6)	299 (25.2)	
Hypertension, n (%)	177 (14.9)	296 (25.0)	436 (36.2)	507 (42.7)	<0.001
Lipid lowering drug use, n (%)	40 (3.4)	58 (4.9)	103 (8.5)	128 (10.8)	<0.001
SBP, mmHg	118.4±15.3	123.8±18.2	127.8±19.7	130.6±18.9	<0.001
DBP, mmHg	70.2±8.6	72.4±8.8	74.0±9.0	74.7±8.7	<0.001
T2DM, n (%)	21 (1.8)	38 (3.2)	74 (61)	106 (8.9)	<0.001
Blood pressure-lowering drug use, n (%)	102 (8.6)	170 (14.4)	277 (23.0)	333 (28.1)	<0.001
Use of glucose-lowering drugs, n (%)	6 (0.5)	19 (1.6)	40 (3.3)	61 (5.3)	<0.001
hsCRP, mg/L	0.52 [0.26-0.95]	0.98 [0.56-1.70]	1.75 [0.93-3.19]	3.61 [1.86-7.10]	<0.001
Glucose, mmol/L	4.7±0.9	4.9±0.9	5.1±1.1	5.3±1.3	<0.001
Total cholesterol, mmol/L	5.2±1.0	5.4±1.0	5.6±1.0	5.7±1.1	<0.001
Non-HDL cholesterol, mmol/L	3.8±1.0	4.1±1.0	4.3±1.0	4.5±1.1	<0.001

HDL cholesterol, mmol/L	1.4±0.3	1.3±0.3	1.2±0.3	1.3±0.3	<0.001
Triglycerides, mmol/L	0.84 [0.64-1.70]	1.03 [0.78-1.97]	1.22 [0.88-1.70]	1.39 [1.02-1.86]	<0.001
eGFR*crea-cysC*, ml/min per 1.73 m^2	98.2±13.9	93.6±16.4	90.7±16.3	88.0±18.1	<0.001
eGFR*crea-cysC,* ml/min per 1.73 m^2, categorical					<0.001
≥90 ml/min per 1.73 m^2	845 (71.2)	689 (58.3)	631 (52.4)	541 (45.6)	
≥60 ml/min per 1.73 m^2	276 (23.3)	383 (32.4)	450 (37.3)	471 (39.7)	
<60 ml/min per 1.73 m^2	7 (0.6)	24 (2.0)	43 (3.6)	75 (6.3)	
UAE, mg/ 24h	7.1 [5.7-10.3]	7.6 [5.8-11.4]	8.4 [6.1-13.3]	8.9 [6.1-17.9]	<0.001
UAE, mg/24H, categorical					<0.001
≥30	40 (3.4)	80 (6.8)	109 (9.0)	186 (15.7)	
<30	1141 (96.2)	1096 (92.7)	1090 (90.5)	996 (84.0)	

Data are expressed as mean ± SD, median [IQR] or in number (n) and %. P-values are calculated by linear regression analysis or χ^2 analysis. Data with respect to smoking and alcohol consumption are missing in 56 (1.2%) and 43 (0.9%) of the subjects, respectively. Abbreviations: *BMI,* body mass index*;* eGFR*crea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; *DBP,* diastolic blood pressure; *SBP,* systolic blood pressure; *T2DM*, type 2 diabetes mellitus; *HDL-cholesterol,* high density lipoprotein cholesterol; *hsCRP;* high sensitive- C-reactive protein; *UAE,* urinary albumin excretion; *PREVEND,* Prevention of REnal and Vascular ENd-stage Disease.

Figure 1. Scatter plot showing the correlation between GlycA and high sensitivity C-reactive protein (hsCRP) (univariate correlation coefficient: 0.67)

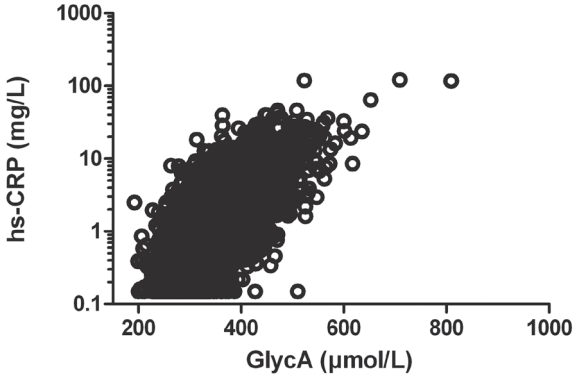


Table 2. Univariate associations of clinical parameters with GlycA and hsCRP

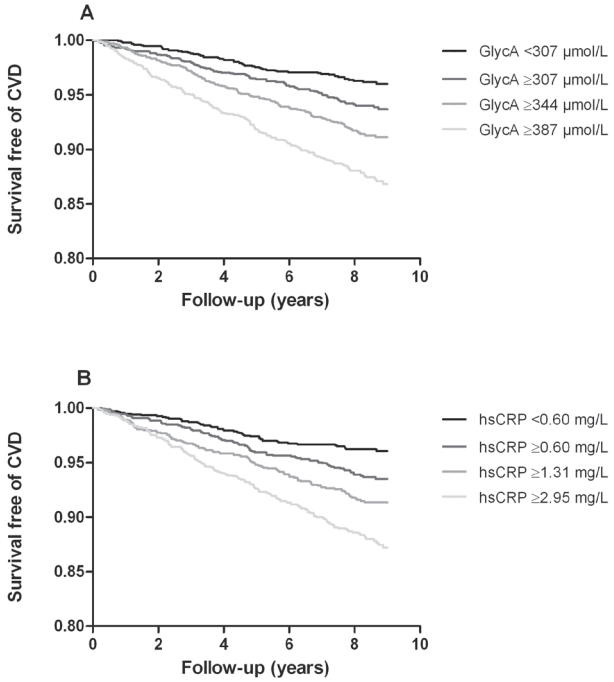
	GlycA		hsCRP	
Clinical parameter	**Std B**	**P-value**	**Std B**	**P-value**
Age	0.22	<0.001	0.25	<0.001
Sex				
Males	*Reference*		*Reference*	
Females	0.10	<0.001	0.04	<0.001
BMI, kg/m^2	0.31	<0.001	0.39	<0.001
Smoking				
Never	*Reference*		*Reference*	
Former	0.06	0.001	0.08	0.001
Current	0.19	<0.001	0.12	<0.001
Alcohol intake				
Almost never	*Reference*		*Reference*	
1-4 drinks per month	-0.09	<0.001	-0.07	<0.001
2-7 drinks per week	-0.16	<0.001	-0.12	<0.001
≥1 drinks per day	-0.13	<0.001	-0.09	<0.001
SBP, mmHG	0.22	<0.001	0.23	<0.001
DBP, mmHg	0.16	<0.001	0.18	<0.001
Hypertension	0.23	<0.001	0.23	<0.001
Type 2 diabetes mellitus	0.13	<0.001	0.12	<0.001
Use of blood pressure lowering drugs				
No	*Reference*		*Reference*	
Yes	0.20	<0.001	0.20	<0.001
Use of lipid lowering drugs				
No	*Reference*		*Reference*	
Yes	0.12	<0.001	0.06	<0.001
Use of glucose lowering drugs				
No	*Reference*		*Reference*	
Yes	0.12	<0.001	0.09	<0.001
hsCRP, mg/L	0.67	<0.001	-	-
GlycA	-		0.67	<0.001

Glucose, mmol/L	0.17	<0.001	0.17	<0.001
Total cholesterol, mmol/L	0.17	<0.001	0.13	<0.001
Non-HDL cholesterol, mmol/L	0.21	<0.001	0.17	<0.001
HDL cholesterol, mmol/L	-0.13	<0.001	-0.17	<0.001
Triglycerides, mmol/L	0.26	<0.001	0.21	<0.001
eGFRcrea-cysC (ml/min per 1.73 m^2)	-0.23	<0.001	-0.25	<0.001
UAE, mg/24h	0.18	<0.001	0.17	<0.001

Data are presented as standardized B coefficient (std B) with corresponding P-value. Abbreviations: *BMI,* body mass index*; eGFRcrea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; *DBP,* diastolic blood pressure; *SBP,* systolic blood pressure; *HDL-cholesterol,* high density lipoprotein cholesterol; *hsCRP*; high sensitive- C-reactive protein; *UAE,* urinary albumin excretion.

After a median follow-up period of 8.5 (7.9-9.0) years 298 CVD events occurred (6.3 %); 210 (70.5%) of these events were cardiac. Kaplan Meier curves for GlycA and hsCRP are shown in **Fig 2**. The Schoenfeld residuals did not suggest deviations from proportionality, supporting the assumption of proportional hazards. Associations of GlycA and hsCRP with CVD risk are shown in **Table 3**.

Figure 2. Kaplan-Meier curves showing incident cardiovascular events according to GlycA, P≤0.001 by log-rank test (A) and high sensitivity C-reactive protein (hsCRP), P≤0.001 by log-rank test (B)



In crude analysis, there was a robust dose response effect of GlycA on incident CVD (P for trend<0.001). Results remained essentially similar after additional adjustment for age and sex (model 1). Further adjustment for BMI, alcohol intake, smoking status (model 2), as well as T2DM, SBP, lipid lowering drug, anti-hypertensive medications (model 3) and lipid levels (model 4) did not result in any substantial change. Additional adjustment for eGFR and UAE (model 5) did not alter the results. Although the association of GlycA with incident CVD when evaluated as P for trend was no longer significant after adjustment for hsCRP, GlycA remained independently associated with CVD when evaluated per SD change (model 6). Results for CRP were comparable to those for GlycA. As with GlycA the association of hsCRP with incident CVD remained present taking account of eGFR and UAE (model 5). Also, when evaluated per SD change the association of hsCRP with incident CVD was still significant after adjustment for GlycA (model 6). There were no statistically significant interactions between GlycA or hsCRP and age or sex on CVD outcome [interactions: P>0.10 for all, data not shown].

When the analyses were restricted to the cardiac domain (210 events) GlycA was associated with incident cardiac events in analyses adjusted for clinical covariates, lipids, eGFR and UAE (**S1 Table**), both when evaluated as P for trend (HR 1.72 (95% CI, 1.02-2.89), P=0.009) and as per SD change (HR 1.23 (95 % CI, 1.07-1.40), P=0.003). In analysis in which we additionally adjusted for hsCRP (cf. model 6, **Table 3**), GlycA was still associated with incident cardiac events (HR 1.19 (95 % CI, 1.01-1.42), P=0.04), whereas the fully adjusted association of hsCRP with cardiac events was not significant (HR 1.05 (95 % CI, 0.87-1.26), P=0.62).

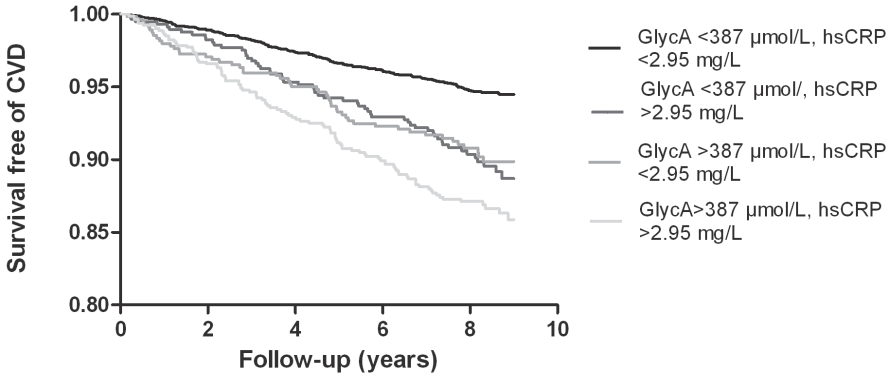
The Harrell's C-index for the Cox regression model with age and sex was 0.77 (95% CI: 0.75-0.80), indicating that 77% of the patients were correctly classified. Addition of GlycA or hsCRP to this basic model resulted in a significant improvement in the C-statistic (0.016 (95% CI, 0.006-0.02), P=0.001 and 0.012 (95% CI, 0.003-0.022), P=0.01, respectively). However, on top of a Cox regression model which included age, sex, BMI, smoking status and alcohol use, addition of neither GlycA, nor hsCRP resulted in a significant change in the C-statistic (0.005 (95% CI: -0.001-0.012), P=0.10 and 0.003 (95% CI, -0.003-0.009), P=0.34).

We next performed a joint analysis based on dichotomized subgroups with higher and lower GlycA or hsCRP in both sexes combined. Kaplan-Meier curves for the 4 prespecified groups of high and low GlycA or hsCRP are shown in **Fig 3**. In crude as well as in age-and sex-adjusted analysis higher GlycA (>387 μmol/L) and higher hsCRP (>2.95 mg/L) both alone and combined were associated with increased CVD risk compared with the reference group of subjects with lower GlycA and lower hsCRP (**Table 4**). After multivariable adjustment, simultaneous occurrence of higher hsCRP and higher GlycA

(GlycA > 387 µmol/L and hsCRP >2.95 mg/L) was still associated increased CVD risk, whereas the association of either higher GlycA or higher hsCRP with incident CVD had lost significance (**Table 4**).

In secondary analysis, in which we accounted for the design of the PREVEND study, the association between GlycA and hsCRP and CVD events was still significant after adjustment for clinical covariates, lipids and eGFR (**S2 Table).** Furthermore, after adjustment for hsCRP, the association between GlycA and CVD events remained significant when analyzed per 1 SD change (HR 1.16 (95% CI, 1.01-1.34), P=0.04); however, the P for trend was no longer statistically significant (P for trend= 0.10). Likewise, the fully adjusted association of hsCRP with CVD events was borderline significant when expressed per SD change (HR 1.16 (95% CI: 1.00-1.35), P=0.05).

Fig 3. Kaplan-Meier curves of incident cardiovascular events according to joint levels of higher GlycA and hsCRP, *P*≤0.001 by log-rank test



Higher levels of GlycA and hsCRP were defined as >387 µmol/L for GlycA and >2.95 mg/L for hsCR in both sexes combined. *hsCRP*, high sensitivity C-reactive protein.

Table 3. Association between GlycA and hsCRP levels and cardiovascular event in 4,759 participants (298 events) of the PREVEND study

	Quartile 1	Quartile 2	P-value	Quartile 3
GlycA				
Participants (n)	1163	1213		1193
Range, µmol/L	<307	≥307-343		≥344-386
No. of cases (%)	35 (3.0)	58 (4.8)		85 (7.1)
Person years	9342	9652		9393
Crude	(reference)	1.60 [1.05-2.44]	0.03	2.41 [1.63-3.58]
Multivariable model 1	(reference)	1.27 [0.83-1.94]	0.26	1.91 [1.28-2.84]
Multivariable model 2	(reference)	1.16 [0.76-1.78]	0.50	1.60 [1.06-2.40]
Multivariable model 3	(reference)	1.09 [0.71-1.68]	0.68	1.43 [0.95-2.15]
Multivariable model 4	(reference)	1.02 [0.66-1.57]	0.94	1.29 [0.85-1.95]
Multivariable model 5	(reference)	1.04 [0.66-1.64]	0.88	1.41 [0.91-2.19]
Multivariable model 6	(reference)	0.94 [0.59-1.50]	0.80	1.19 [0.76-1.87]
hsCRP				
Participants (n)	1187	1190		1191
Range, mg/L	<0.60	≥0.60-1.30		≥1.31-2.94
No. of cases (%)	37 (3.1)	54 (4.5)		83 (7.0)
Person years	9507	9485		9333
Crude	(reference)	1.46 [0.96-2.22]	0.08	2.28 [1.55-3.36]
Multivariable model 1	(reference)	1.05 [0.69-1.60]	0.82	1.43 [0.96-2.11]
Multivariable model 2	(reference)	0.96 [0.63-1.48]	0.87	1.13 [0.75-1.71]
Multivariable model 3	(reference)	0.91 [0.59-1.40]	0.67	1.06 [0.70-1.60]
Multivariable model 4	(reference)	0.84 [0.55-1.29]	0.43	0.95 [0.63-1.44]
Multivariable model 5	(reference)	0.93 [0.59-1.47]	0.75	1.11 [0.71-1.72]
Multivariable model 6	(reference)	0.87 [0.55-1.37]	0.87	0.97 [0.62-1.51]

Hazard ratios were derived from Cox proportional hazards regression models.
Multivariable model 1: crude model + age, sex
Multivariable model 2: model 1 + BMI, alcohol intake, smoking status
Multivariable model 3: model 2 + type 2 diabetes mellitus, systolic blood pressure, lipid lowering drugs and anti-hypertensive medications
Multivariable model 4: model 3 + total cholesterol, HDL cholesterol, triglycerides.
Multivariable model 5: model 4 + eGFRcrea-cystatin C, UAE
Multivariable model 6: model 5 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).
Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses. *Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable. ** 1 SD is 60.4 µmol/L for GlycA and 1.1 mg/L for hsCRP (hsCRP was natural log transformed). Abbreviations: *BMI,* body mass index*; eGFRcrea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; *HDL-cholesterol,* high density lipoprotein cholesterol; *hsCRP,* high-sensitivity C-reactive protein; *UAE,* urinary albumin excretion.

P-value	Quartile 4	P-value	P for trend*	Per 1 SD**	P-value
	1190				
	≥387				
	120 (10.1)				
	8988				
<0.001	3.56 [2.44-5.19]	<0.001	<0.001	1.53 [1.39-1.67]	<0.001
0.001	2.75 [1.88-4.04]	<0.001	<0.001	1.41 [1.29-1.54]	<0.001
0.02	2.09 [1.40-3.13]	<0.001	<0.001	1.34 [1.21-1.48]	<0.001
0.09	1.84 [1.22-2.76]	0.003	<0.001	1.28 [1.16-1.42]	<0.001
0.23	1.58 [1.05-2.37]	0.03	0.004	1.24 [1.12-1.38]	<0.001
0.12	1.75 [1.13-2.70]	0.01	0.001	1.27 [1.14-1.42]	<0.001
0.45	1.31 [0.81-2.12]	0.27	0.18	1.16 [1.01-1.33]	0.04
	1191				
	≥2.95				
	124 (10.4)				
	9050				
<0.001	3.52 [2.44-5.08]	<0.001	<0.001	1.65 [1.48-1.84]	<0.001
0.08	2.22 [1.53-3.23]	<0.001	<0.001	1.47 [1.31-1.66]	<0.001
0.55	1.69 [1.13-2.51]	0.01	<0.001	1.36 [1.19-1.54]	<0.001
0.79	1.53 [1.02-2.28]	0.04	0.001	1.33 [1.16-1.51]	<0.001
0.82	1.34 [0.89-2.01]	0.16	0.003	1.28 [1.12-1.47]	<0.001
0.66	1.55 [1.01-2.40]	0.05	0.001	1.34 [1.16-1.54]	<0.001
0.88	1.19 [0.75-1.90]	0.46	0.10	1.20 [1.01-1.43]	0.04

Table 4. Associations of joint GlycA and hsCRP levels and cardiovascular events in 4,759 participants (298 events) of the PREVEND study

	Low GlycA and low hsCRP	Low GlycA and high hsCRP	P-value	High GlycA and low hsCRP	P-value	High GlycA and high hsCRP	P-value
Participants (n)	3079	503		479		688	
No. of cases (%)	135 (4.4)	45 (8.9)		39 (8.1)		79 (11.5)	
Crude	(reference)	2.09 [1.49-2.93]	<0.001	1.93 [1.35-2.75]	<0.001	2.81 [2.13-3.71]	<0.001
Model 1	(reference)	1.64 [1.17-2.30]	0.004	1.68 [1.18-2.40]	0.004	2.41 [1.82-3.20]	<0.001
Model 2	(reference)	1.38 [0.96-1.98]	0.08	1.27 [0.87-1.85]	0.22	1.79 [1.31-2.46]	<0.001

Higher levels were defined as greater than the upper quartile, GlycA >387 µmol/L and hsCRP >2.95 mg/L.

Multivariable model 1: Crude + age, sex.

Multivariable model 2: Model 1 + BMI, alcohol intake, smoking status, diabetes mellitus type 2, lipid lowering drugs, anti-hypertensive medications and systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, eGFRcrea-cysC and UAE.

Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

Abbreviations: *BMI,* body mass index*; HDL-cholesterol,* high density lipoprotein cholesterol; eGFR*crea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; *hsCRP,* high-sensitivity C-reactive protein; *UAE,* urinary albumin excretion.

Discussion

This prospective study among 4,759 men and women demonstrates that a recently developed high throughput NMR spectroscopy-based glycoprotein biomarker, designated GlycA, is associated with incident CVD. This association was not significantly different in men and women, and was independent of clinical risk factors and plasma lipids. Moreover, this association was not appreciably attenuated after further adjustment for eGFR and albuminuria. Of note, the extent to which GlycA predicted CVD risk was comparable to that for hsCRP. When these pro-inflammatory biomarkers were included together in the analyses, their associations with incident CVD were attenuated. Furthermore, CVD risk was highest in subjects with simultaneously higher GlycA and hsCRP, which suggests that these biomarkers may have additive potential in predicting CVD risk. Taken together, the present findings underscore the proposed role of altered protein glycan dynamics in inflammatory processes that may conceivably play a role in the development of atherosclerotic CVD.

hsCRP is a single biomarker of low-grade systemic inflammation. In contrast, the GlycA assay represents an NMR signal derived from residues within the carbohydrate side-chains of several of the most important acute-phase proteins [11, 12, 25]. It should be noted that the GlycA assay is not designed for determination of the relative contributions of the individually captured glycosylated protein moieties to the NMR signal. Interesting though, one of the acute phase proteins included in the NMR signal is α1-acid glycoprotein, which has been shown to be an independent predictor of cardiovascular morbidity and mortality in other large cohorts [26, 27]. A potential advantage of the GlycA measurement is that its level is less variable than that of hsCRP [11]. Glycan structures of acute-phase proteins are modified under chronic inflammatory conditions [1, 11]. The robust positive correlation of GlycA with hsCRP, as reinforced here, supports the contention that this glycoprotein biomarker reflects a pro-inflammatory state [11, 12]. The positive relationships between GlycA and CVD risk factors, such as obesity, smoking, hypertension as well as total cholesterol, non-HDL cholesterol and triglycerides, and its inverse correlation with HDL cholesterol are in full agreement with recent reports [11, 13, 28]. Moreover, GlycA was higher in T2DM subjects, although it appears that the extent to which GlycA varies according to glucose tolerance status is modest [28]. Very similar relationships of these variables were found for hsCRP. The proposed pathogenic role of low-grade systemic inflammation in renal function loss, and the association of hsCRP with albuminuria [17, 18, 29-32] led us to determine the relationships of GlycA with eGFR and albuminuria. Of further relevance, acute-phase proteins, such as α1-acid glycoprotein and α1-antitrypsin which are captured by the GlycA assay, are present in human urine [33, 34]. α1-Acid glycoprotein is correlated with albuminuria, and its urinary excretion is increased in diabetic nephropathy [34]. In hemodialysis patients, circulating levels of α1-acid glycoprotein are inversely associated with serum albumin, which in turn may predicts mortality [35]. A potentially relevant novel finding of our study is that GlycA, like hsCRP, was inversely associated with eGFR

and positively with albuminuria. Given the relevance of even mild degrees of chronic kidney disease and albuminuria for the development of atherosclerotic CVD [14, 15] and the presently observed relationship of GlycA with renal function abnormalities, it is remarkable that the association of GlycA with incident CVD was not attenuated after adjustment for eGFR and albuminuria. This observation raises the possibility that the impact of alterations in glycoprotein metabolism and of renal functional changes on CVD may at least in part be attributable to distinct biological pathways.

It is evident that our central finding that GlycA predicts incident CVD independent of clinical and laboratory covariates agrees with recent results from the Women's Health Study [13]. We were also able to demonstrate that GlycA improved CVD risk classification when added to a simple model including age and sex. However, the extent to which adding GlycA improved CVD risk classification did not reach significance in analysis taking account of established CVD risk factors. Thus, it is anticipated that results from additional case-cohort studies are required to more robustly discern whether GlycA may independently improve prediction of new onset CVD.

Several methodological aspects of our study should be addressed. We consider the comprehensive assessment of laboratory variables, including lipoprotein fractions, eGFR and albuminuria in a large population of men and women a strength of our study. Second, as in previous reports [6, 13, 36, 37], we only included subjects without clinically manifest cardiovascular disease at baseline. Thus the association of GlycA with recurrent CVD remains to be established. Third, the PREVEND study population consists of almost only Caucasians. Therefore, the applicability of the current results to other ethnicities remains uncertain. Fourth, subjects with elevated urinary albumin excretion are overrepresented in the PREVEND cohort. Of note, the association of GlycA with incident CVD remained present after adjustment for eGFR and albuminuria, and a secondary analysis taking account of the design of the PREVEND study showed similar results. Nonetheless, we cannot completely rule out the possibility of residual confounding. Finally, it should be emphasized that the process of glycosylation should be discerned from protein glycation, whereby a sugar molecule is covalently bound to a protein via a non-enzymatic mechanism.

In conclusion, this prospective cohort study involving both men and women demonstrates that GlycA, a new pro-inflammatory glycoprotein biomarker is associated with future CVD events, independent of clinical and laboratory variables including renal function. The association of GlycA with incident CVD is as strong as that of hsCRP. CVD risk was highest in the context of simultaneously higher GlycA and hsCRP, suggesting that these biomarkers may have additive potential in predicting CVD.

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S1 Table. Association between GlycA and hsCRP levels and cardiac events in 4,759 participants (210 events) of the PREVEND study

	Quartile 1	Quartile 2	P-value	Quartile 3
GlycA				
Participants (n)	1163	1213		1193
Range, µmol/L	<307	≥307-343		≥344-386
No. of cases (%)	25 (2.1)	44 (3.6)		59 (4.9)
Person years	9342	9652		9393
Crude	(reference)	1.70 [1.04-2.78]	0.03	2.35 [1.47-3.75]
Multivariable model 1	(reference)	1.39 [0.85-2.27]	0.19	1.95 [1.22-3.13]
Multivariable model 2	(reference)	1.27 [0.77-2.10]	0.35	1.64 [1.01-2.67]
Multivariable model 3	(reference)	1.21 [0.73-2.00]	0.47	1.47 [0.90-2.41]
Multivariable model 4	(reference)	1.10 [0.67-1.83]	0.70	1.29 [0.79-2.11]
Multivariable model 5	(reference)	1.07 [0.62-1.85]	0.80	1.43 [0.85-2.40]
Multivariable model 6	(reference)	1.03 [0.60-1.79]	0.91	1.33 [0.78-2.29]
hsCRP				
Participants (n)	1187	1190		1191
Range, mg/L	<0.60	≥0.60-1.30		≥1.31-2.94
No. Of 'cases' (%)	30 (2.5)	42 (3.5)		59 (5.0)
Person years	9507	9485		9333
Crude	(reference)	1.40 [0.88-2.24]	0.16	2.00 [1.29-3.11]
Multivariable model 1	(reference)	1.03 [0.64-1.64]	0.92	1.29 [0.83-2.02]
Multivariable model 2	(reference)	0.94 [0.58-1.52]	0.80	1.01 [0.63-1.61]
Multivariable model 3	(reference)	0.88 [0.54-1.42]	0.60	0.95 [0.59-1.51]
Multivariable model 4	(reference)	0.79 [0.49-1.28]	0.33	0.81 [0.51-1.30]
Multivariable model 5	(reference)	0.91 [0.54-1.53]	0.71	0.97 [0.59-1.61]
Multivariable model 6	(reference)	0.85 [050-1.43]	0.53	0.85 [0.51-1.42]

Multivariable model 1: crude model + age, sex
Multivariable model 2: model 1 + BMI, alcohol intake, smoking status
Multivariable model 3: model 2 + diabetes, lipid lowering drugs, anti-hypertensive medications and systolic blood pressure.
Multivariable model 4: model 3 + total colesterol, HDL colesterol, triglycerides
Multivariable model 5: model 4 + eGFRcrea-cystatin C, UAE
Multivariable model 6: model 5 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).
Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

P-value	Quartile 4	P-value	P for trend*	Per SD**	P-value
	1190				
	≥387				
	82 (6.9)				
	8988				
<0.001	3.41 [2.18-5.34]	<0.001	<0.001	1.47 [1.32-1.65]	<0.001
0.006	2.80 [1.78-4.42]	<0.001	<0.001	1.38 [1.24-1.54]	<0.001
0.05	2.16 [1.34-3.49]	0.002	<0.001	1.30 [1.15-1.47]	<0.001
0.12	1.90 [1.17-3.09]	0.009	0.002	1.25 [1.10-1.41]	0.001
0.31	1.55 [0.95-2.52]	0.08	0.04	1.19 [1.04-1.36]	0.01
0.18	1.72 [1.02-2.89]	0.04	0.009	1.23 [1.07-1.40]	0.003
0.30	1.54 [0.87-2.73]	0.14	0.11	1.19 [1.01-1.42]	0.04
	1191				
	≥2.95				
	79 (6.6)				
	9050				
0.002	2.77 [1.82-4.21]	<0.001	<0.001	1.51 [1.33-1.73]	<0.001
0.26	1.82 [1.18-2.80]	0.006	<0.001	1.36 [1.18-1.57]	<0.001
0.97	1.35 [0.85-2.13]	0.20	0.04	1.23 [1.05-1.44]	0.01
0.81	1.22 [0.77-1.94]	0.40	0.08	1.20 [1.02-1.41]	0.02
0.39	1.01 [0.63-1.61]	0.97	0.28	1.13 [0.96-1.34]	0.13
0.92	1.22 [0.74-2.03]	0.43	0.13	1.20 [1.01-1.42]	0.04
0.53	0.93 [0.54-1.61]	0.80	0.97	1.05 [0.86-1.30]	0.62

*Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable. ** 1 SD is 60.4 µmol/L for GlycA and 1.1 mg/L for hsCRP (hsCRP was natural log transformed). Abbreviations: *BMI,* body mass index*; eGFRcrea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; *HDL-cholesterol,* high density lipoprotein cholesterol; *hsCRP,* high–sensitivity C-reactive protein; *UAE,* urinary albumin excretion.

S2 Table. Association between GlycA and hsCRP levels and cardiovascular events in 4,759 participants (298 events) of the PREVEND study, accounting for the sampling design of the study (presence or absence of a urinary albumin concentration >10 mg/L) by specifying stratum-specific baseline hazard functions

	Quartile 1	Quartile 2	P-value	Quartile 3
GlycA				
Participants (n)	1163	1213		1193
Range, µmol/L	<307	≥307-343		≥344-386
No. of cases (%)	35 (3.0)	58 (4.8)		85 (7.1)
Person years	9342	9652		9393
Crude	(reference)	1.60 [1.05-2.43]	0.03	2.39 [1.61-3.54]
Multivariable model 1	(reference)	1.27 [0.83-1.93]	0.28	1.87 [1.25-2.78]
Multivariable model 2	(reference)	1.04 [0.66-1.64]	0.87	1.43 [0.93-2.22]
Multivariable model 3	(reference)	0.95 [0.60-1.51]	0.82	1.21 [0.77-1.91]
hsCRP				
Participants (n)	1187	1190		1191
Range, mg/L	<0.60	≥0.60-1.30		≥1.31-2.94
No. of cases (%)	37 (3.1)	54 (4.5)		83 (7.0)
Person years	9507	9485		9333
Crude	(reference)	1.45 [0.95-2.20]	0.08	2.29 [1.55-3.37]
Multivariable model 1	(reference)	1.04 [0.68-1.58]	0.87	1.41 [0.95-2.10]
Multivariable model 2	(reference)	0.90 [0.57-1.41]	0.64	1.07 [0.69-1.65]
Multivariable model 3	(reference)	0.84 [0.53-1.32]	0.44	0.93 [0.60-1.45]

Multivariable model 1: crude + age and sex.

Multivariable model 2: model 1 + BMI, alcohol intake, smoking status, diabetes, lipid lowering drugs, anti-hypertensive medications, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, eGFRcrea-cystatin C.

Multivariable model 3: model 2 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).

Triglycerides and hsCRP were log transformed when used as a continuous variable in the analyses.

*Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable. ** 1 SD is 60.4 µmol/L for GlycA and 1.1 mg/L for hsCRP (hsCRP was natural log transformed).

Abbreviations: *BMI,* body mass index*; eGFRcrea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; *HDL-cholesterol,* high density lipoprotein cholesterol; *hsCRP,* high–sensitivity C-reactive protein; *UAE,* urinary albumin excretion.

P-value	Quartile 4	P-value	P for trend*	Per SD**	P-value
	1190				
	≥387				
	120 (10.1)				
	8988				
<0.001	3.49 [2.39-5.08]	<0.001	<0.001	1.51 [1.38-1.66]	<0.001
0.002	2.66 [1.81-3.91]	<0.001	<0.001	1.40 [1.28-1.53]	<0.001
0.11	1.75 [1.14-2.71]	0.01	0.001	1.27 [1.14-1.42]	<0.001
0.40	1.33 [0.82-2.15]	0.24	0.10	1.16 [1.01-1.34]	0.04
	1191				
	≥2.95				
	124 (10.4)				
	9050				
<0.001	3.47 [2.41-5.01]	<0.001	<0.001	1.64 [1.47-1.83]	<0.001
0.09	2.16 [1.49-3.15]	<0.001	<0.001	1.46 [1.29-1.64]	<0.001
0.76	1.51 [0.98-2.31]	0.06	0.001	1.33 [1.16-1.53]	<0.001
0.76	1.15 [0.72-1.83]	0.55	0.11	1.19 [1.00-1.42]	0.05

GlycA, a Marker of Acute Phase Glycoproteins, and the Risk of Incident Type 2 Diabetes Mellitus: PREVEND study

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Abstract

Background GlycA is a recently developed glycoprotein biomarker of systemic inflammation that may be predictive of incident type 2 diabetes mellitus (T2DM).

Methods Analytical performance of the GlycA test, measured on the Vantera® Clinical Analyzer, was evaluated. To test its prospective association with T2DM, GlycA was measured in 4,524 individuals from the PREVEND study and a survival analysis was performed with a mean follow-up period of 7.3 years.

Results Imprecision for the GlycA test ranged from 1.3-2.3% and linearity was established between 150-1588µmol/L. During the follow-up period, 220 new T2DM cases were ascertained. In analyses adjusted for relevant covariates, GlycA was associated with incident T2DM; hazard ratio (HR) for the highest vs. lowest quartile 1.77 [95% Confidence Interval (CI): 1.10-2.86, P=0.01], whereas the association of high sensitivity C-reactive protein (hsCRP) with T2DM was not significant. GlycA remained associated with incident T2DM after additional adjustment for hsCRP; HR 1.71 [1.00-2.92, P=0.04]. A multivariable adjusted analysis of dichotomized subgroups showed that the hazard for incident T2DM was highest in the subgroup with high GlycA and low hsCRP (P=0.03).

Conclusions The performance characteristics of the GlycA test reveal that it is suitable for clinical applications, including assessment of the risk of future T2DM.

1. Introduction

GlycA is a recently developed nuclear magnetic resonance spectroscopy (NMR)-measured biomarker of systemic inflammation [1-7]. The GlycA NMR signal arises from the N-acetyl glucosamine (GlcNAc) residues on the carbohydrate side-chains of acute phase proteins, mainly α1-acid glycoprotein (orosomucoid), α1-antitrypsin, α1-antichymotrypsin, haptoglobin and transferrin [1, 6]. Hence, GlycA is a composite biomarker that integrates both the protein levels and glycosylation states of the most abundant acute phase proteins present in the circulation [1]. GlycA has been demonstrated to be associated with common markers of inflammation such as high sensitivity C-reactive protein (hsCRP), fibrinogen, interleukin-6 and serum amyloid A [1-7]. Recently it was reported that GlycA is related to the leptin/adiponectin ratio, suggesting that adipose tissue-associated low-grade inflammation could be involved in the regulation of acute phase proteins [5]. Similar to hsCRP, GlycA was found to be higher in subjects with metabolic syndrome and was positively correlated with body mass index (BMI) and insulin resistance as assessed by homeostasis model assessment (HOMA-IR) [4, 5, 7].

GlycA is elevated in several chronic inflammatory diseases and associated with disease incidence [8-11]. GlycA was elevated in patients with various autoimmune diseases and was associated with disease activity and coronary atherosclerosis in rheumatoid arthritis patients [3, 6, 12, 13]. In the Women's Health Study (WHS) [8], the Prevention of Renal and Vascular End-stage Disease (PREVEND) study [9] and the JUPITER trial [10], GlycA was associated with incident cardiovascular disease (CVD) events, independent of traditional risk factors. GlycA was also found to predict incident type 2 diabetes mellitus (T2DM) in a large population of healthy women [11]. These data raise the possibility that GlycA provides added value for the evaluation of CVD and diabetes risk.

Recently a clinical NMR instrument, the Vantera® Clinical Analyzer, has been developed that addresses the limiting factors of research instruments and allows lipoprotein measurements to be performed in the clinical laboratory [14]. The Vantera Clinical Analyzer, a Food and Drug Administration (FDA)-cleared in vitro diagnostic device, is able to quantify additional analytes from the *NMR LipoProfile*® test spectra, including GlycA and metabolites such as branched chain amino acids [14, 15]. Although the GlycA assay has been previously described [1], the analytical performance of the GlycA assay on the Vantera Clinical Analyzer, the instrument that will be reporting GlycA results as a clinical diagnostic test, has not been reported to date.

The aims of the present study were two-fold: first, to document the analytical performance of the GlycA test on the Vantera Clinical Analyzer and second, to determine the extent to which GlycA predicts incident T2DM in a large population of men and

women. To this end, a prospective analysis was carried out among predominantly white PREVEND participants.

2. Materials and methods

2.1 Study design

The PREVEND study was approved by the local medical ethics committee, University Medical Center Groningen, The Netherlands; each participant provided written informed consent. Details of the study design and recruitment have been described elsewhere [16].

2.2 Study participants

Briefly, the PREVEND study is a Dutch cohort drawn from the general population of the city of Groningen. After exclusion of subjects with insulin-treated diabetes and pregnant women, all subjects with a urinary albumin concentration ≥10 mg/L were invited to participate (n=7,768), of whom 6,000 accepted. In addition, a random sample of 2,592 individuals with a urinary albumin concentration <10 mg/L was included. These 8,592 subjects (aged 28-75 years) completed the baseline survey (1997-1998). The second screening, which was the starting point of the current study, took place between 2001 and 2003 (n=6,894). GlycA and hsCRP were measured in 5,526 subjects from the second screening in whom samples of sufficient quality and quantity were available. For the current study subjects with missing data on diabetes at baseline (n=40), subjects with prevalent T2DM at baseline (n=358) and those with missing data on follow-up (n=604) were excluded, leaving 4,524 subjects for the present analysis .

2.2.1 Follow-up and outcome

Follow-up time was defined as the period between the second screening round (baseline) and the date of ascertainment of T2DM. Follow-up time was censored at 8.5 years. In case a person moved to an unknown destination, census date was date of removal from the municipal registry. Incident cases of diabetes was ascertained if one or more of the following criteria were met: 1) fasting plasma glucose ≥7.0 mmol/L (126 mg/dL); 2) random sample plasma glucose ≥11.1 mmol/L (200 mg/dL); 3) self-report of a physician diagnosis of T2DM and 4) initiation of glucose-lowering medication use, retrieved from a central pharmacy registry [17, 18].

2.2.2 Laboratory analysis in PREVEND

Venous blood was obtained at each screening round after an overnight fast. Plasma samples were prepared by centrifugation at 4°C. EDTA plasma samples were stored at -80°C until thawed for testing. Plasma samples from the second screening were sent frozen to LipoScience, Laboratory Corporation of America Holdings for testing on the Vantera Clinical Analyzer. NMR spectra were collected and GlycA values were quantified as described above [1]. Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured on a Beckman Coulter® AU680 Analyzer. hsCRP and glucose were determined using standard laboratory protocols [19].

2.2.3 Statistical analyses for the PREVEND study

All statistical analyses were carried out using SPSS version 22.0 (SSPS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD), median (interquartile range) and percentages. For all analyses, two-sided P values <0.05 were considered statistically significant, except for interaction terms for which the level of significance was set at P <0.10. Baseline characteristics were calculated across sex-stratified quartiles of GlycA. P-values across quartiles of GlycA were determined by linear regression for continuous variables or chi-square test for categorical variables.

Cox proportional hazards regression analysis was performed to examine the associations across quartiles calculated in the whole study population of GlycA and hsCRP with the risk of developing T2DM. In addition, hazards were calculated per 1 SD increment of GlycA and hsCRP. Hazard ratios (HR) were expressed with 95% confidence intervals (CI). TG and hsCRP were log transformed when used as a continuous variable in the analysis. In order to test if each of the biomarkers in the highest range had a different association with incident T2DM vs. both biomarkers or one of these biomarkers in the lowest range the joint associations of GlycA and hsCRP with outcome were evaluated by dichotomizing the distribution of GlycA and hsCRP according to cut points at the highest quartile of GlycA (>384 µmol/L) and the highest quartile of hsCRP (>2.83 mg/L).

Given the enrichment of subjects with microalbuminuria in the PREVEND population, we also performed a secondary analysis in which we accounted for the sampling design of the study, with respect to enrichment of subjects with a urinary albumin concentration >10 mg/L, by specifying stratum-specific baseline hazard functions.

2.3 Analytical Validation Studies for the GlycA test

For the analytical validation studies, serum samples were collected from healthy volunteers in the United States of America (USA). These studies were cleared by an Institutional Review Board. For both the analytical validation and PREVEND studies, all

donors signed consent forms and the studies were conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.3.1 Specimen collection and preparation

Control serum pools and specimens were purchased and prepared as previously described [14, 15]. All studies were performed in *NMR LipoProfile*® (LipoScience) serum separator tubes (#456293/455232; Greiner Bio-One®), also known as LipoTubes® (LipoScience), unless otherwise indicated.

2.3.2 Acquisition of NMR spectra and data processing

NMR spectra were acquired at the Clinical Laboratory Improvement Amendments (CLIA) approved laboratory at Laboratory Corporation of America® Holdings (Raleigh, NC) as previously described using the Vantera Clinical Analyzer, a 400 MHz NMR spectrometer [14, 15]. Typically, two levels of serum controls were included at the beginning and end of each specimen run. Data acquisition on the Vantera was accomplished in a similar fashion to the NMR Profiler platform with the exception that water was suppressed using the WET solvent suppression technique [14, 20, 21]. NMR spectral data were acquired as 3 blocks of 4 scans for a total acquisition time of 48 seconds and a sweep width of 4496.4 Hz and 9024 data points.

The signal peak appearing at 2.00 ppm, named GlycA, which lies outside of the spectral region used to calculate the lipoprotein information, was quantified as previously described in detail [1]. GlycA is not a homogeneous signal from a single molecular species, but rather a composite signal arising from the superposition of slightly offset N-acetyl methyl group resonances from a subset of mobile GlcNAc residues on the glycan branches of abundant glycoproteins [6]. It is only the GlcNAc residues in $\beta(1\rightarrow2)$ or $\beta(1\rightarrow6)$ linkage with a preceding mannose residue that give rise to N-acetyl methyl resonances at the 2.00 ±0.01 ppm GlycA position [1]. The units for the GlycA signal represent the number of GlcNAc residues in µmol/L. These particular residues can be found on many glycosylated proteins however, the glycoproteins that circulate at >10 µM and are highly glycosylated make the largest contributions to the composite GlycA signal (e.g. α1-acid glycoprotein (orosomucoid), α1-antitrypsin, α1-antichymotrypsin, haptoglobin and transferrin) [1, 6].

2.3.3 Assay performance testing

Sensitivity, imprecision and linearity were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines as previously described [14, 22-24]. For linearity testing, the high GlycA serum pool was obtained by the addition of α1-acid glycoprotein, the most abundant protein that contributes to the GlycA signal [1].

2.3.4 Method and tube comparisons

Method comparison studies consistent with CLSI guidelines were performed to ensure that the performance of the GlycA test on the Vantera Clinical Analyzer was similar to the GlycA test run on the NMR Profiler [1]. Samples (n=631) were tested in singlicate on the reference (Profiler) and comparator (Vantera Clinical Analyzer) NMR systems over a period of 5 days. The correlation between results generated on the two NMR platforms was evaluated using Deming regression analysis. Blood from 46 donors was drawn into three different tubes: LipoTube (serum), BD Vacutainer® serum tube (red top, no gel barrier) and K_2EDTA plasma tube. GlycA was measured for a total of 50 specimens. Results for conventional serum and EDTA plasma tubes were compared to results for the LipoTube by linear regression.

2.3.5 Reference interval and interfering substance studies

To determine the reference range for the GlycA assay, samples from healthy adult men and non-pregnant women between the ages of 18 and 84 (n=450) were collected in BD Vacutainer serum tubes (red top, no gel barrier). A description of this study population has been reported [14]. The GlycA reference range was estimated using non-parametric analyses with reference limits at the 2.5th and 97.5th percentiles according to the nonparametric method described in CLSI guidelines [25]. The reference intervals for men and women were compared by assessing their median values by Mann-Whitney test. A total of 7 endogenous and 23 exogenous substances were tested in vitro for possible assay interference consistent with CLSI guidelines [26], as previously described [14, 15].

2.3.6 Stability testing

2.3.6.1 Refrigerated stability- Serum samples obtained from three separate studies of 10 donors each (n=30) were used to assess stability of GlycA. Samples were stored at 4°C and aliquots were tested daily for 12 days. Daily mean results for all donors were evaluated with acceptable differences falling within ±10% of the day 0 (draw day) mean.

2.3.6.2 Room temperature stability- Serum samples obtained from 12 donors were used to assess the stability of GlycA at room temperature over time. Samples were allowed to sit at room temperature for 24 hours. Aliquots of the serum samples were tested at the following time points: 0, 1, 2, 4, 6, 8 and 24 hours.

2.3.6.3 Freeze-thaw stability- A set of 3 pooled serum samples (low, mid and high analyte concentrations) was used to assess the stability of GlycA after 3 freeze-thaw cycles. One aliquot from each of the 3 samples within the set was tested fresh and 3 aliquots from each were frozen at -80°C. After 24 hours, all aliquots were thawed unassisted at room temperature for 1 hour. Once thawed, 1 aliquot

from each of the 3 samples was tested while the remaining sets of aliquots were returned to the freezer. This process continued until data for 3 freeze-thaw cycles were collected.

2.3.7 Statistical analysis

Analytical validation data was calculated using either Excel Analyse-it® or GraphPad Prism version 6.0 software.

3. Results

3.1 Analytical performance of the GlycA assay

The analytical performance of the GlycA assay, as measured on the Vantera Clinical Analyzer, was assessed for the ability to accurately quantify the NMR signal at 2.00 ppm in spectra acquired from serum samples. Because the GlycA signal arises from circulating glycosylated proteins and it is not possible to remove all proteins from a serum specimen, limits of blank (LOB) and detection (LOD) could not be determined by traditional means. However, testing of five serum pools, with GlycA ranging from 57 to 166 µmol/L, gave CVs <7.6% and a functional sensitivity or limit of quantitation (LOQ) of 18 µmol/L. Because the calculated LOQ was lower than the lowest concentration measured, 57 µmol/L should be considered the LOQ for GlycA. Serum pool samples with three varying concentrations of GlycA (low, intermediate and high) were tested for intra-assay (within-run) and inter-assay (within-lab) precision. The CV for the GlycA assay ranged from 1.3-1.8% for within-run and 1.9-2.3% for within-lab imprecision (**Table 1**). In order to test the ability of the assay to measure the GlycA NMR signal, the measured values (n=10 serum pools) were plotted against the actual or expected concentrations. Linearity of GlycA was demonstrated throughout the reportable range of 150 to 1588 µmol/L with a correlation coefficient (R^2) of 0.998 (**Fig. 1A**).

The linear regression for the GlycA data (n=631), with the Profiler results as the reference method, produced a slope (95% CI) of 1.00 (0.99 to 1.01), intercept of 4.60 (-1.15 to 10.35), and correlation coefficient (R^2) of 0.983 (data not shown). Deming regression produced a slope of 1.01 (1.00 to 1.02) and an intercept of 0.09 (-5.12 to 5.30) (**Fig. 1B**). Differences between the GlycA values and the Deming regression line (residuals) were plotted against the estimated concentration of GlycA. The points on the residuals plot were randomly dispersed around the horizontal axis, suggesting that the results of the two assays were linearly related with no significant bias and no apparent outliers (**Fig. 1C**).

Serum specimens from a population of healthy individuals (n = 450) were used to determine the reference interval for the GlycA test. **Table 2** shows the distribution of

GlycA values in this population. The mean GlycA value was 386 ± 60 µmol/L, the median was 379 µmol/L and the reference interval (2.5-97.5th percentile) was 288-518 µmol/L (**Table 2**). In women, the mean GlycA value was 395 ± 60 µmol/L, the median was 388 µmol/L, and the reference interval was 299-522 µmol/L. In men, the mean GlycA value was 370 ± 57 µmol/L, the median was 366 µmol/L and the reference interval was 273-487 µmol/L. There was a statistically significant difference between the means and medians for men and women (both P-values <0.0001).

Table 1. Within-run and within-laboratory imprecision for GlycA measured on the Vantera Clinical Analyzer.

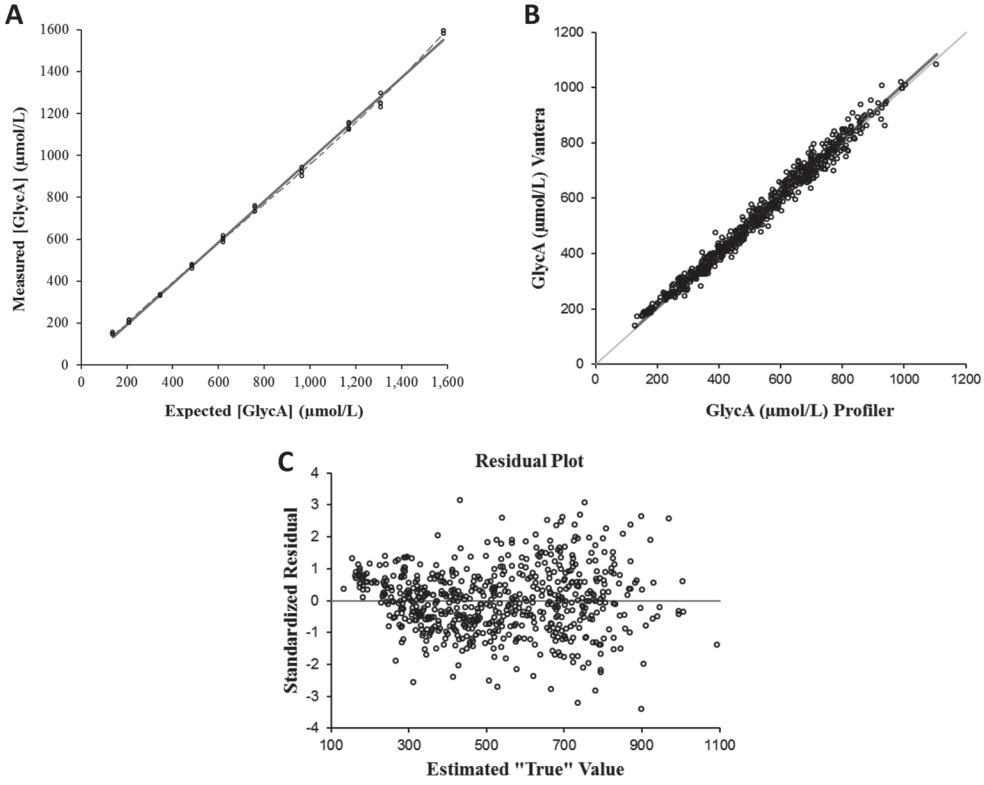
	GlycA (µmol/L)		
	Low	**Intermediate**	**High**
Within-run[a]			
Mean	344.1	460.9	649.9
SD	6.3	7.7	8.7
CV	1.8%	1.7%	1.3%
Within-laboratory[b]			
Mean	342.6	463.7	667.0
SD	7.9	10.3	12.5
CV	2.3%	2.2%	1.9%

[a] Based on 1 run of 20 tests.[a] Based on CLSI guidelines tested using 3 serum pools, 2 runs per day in duplicate, for 20 days (total n = 80). CV: coefficient of variation.

Table 2. Distribution of GlycA observed in a reference population.

	GlycA (µmol/L)		
Percentile	**All (n=450)**	**Men (n=158)**	**Women (n=292)**
0	245	245	269
2.5	288	273	299
25	346	340	350
50	379	366	388
75	420	394	434
97.5	518	487	522
100	605	605	599

Figure 1. A. Results of linearity testing for the Vantera GlycA assay, solid blue line = linear fit, dotted grey line = polynomial fit; B. Comparison of Vantera and NMR Profiler methods for quantification of GlycA, solid blue line = Deming fit, grey line = identity; C. Residual plot for the Deming regression.



Seven endogenous substances normally found circulating in blood and 22 exogenous substances, previously used to test for interference with quantification of LDL-P [14] and HDL-P [15], were evaluated for potential interference with the GlycA test. Only hemoglobin, at levels which may occur in grossly hemolyzed specimens (≥20 mg/dL), reduced GlycA concentrations by approximately 20%.

Several types of specimen collection tubes were compared to LipoTubes for their suitability in the GlycA assay. Linear regression analyses were performed and the resulting lines had slopes of 0.94 (R^2=0.99) for plain red-top serum tubes (no gel barrier) and 0.98 (R^2=0.98) for EDTA plasma tubes. Plain red-top serum showed no significant bias based on either 95% confidence intervals around the correlation slope and intercept or estimation of bias from Bland-Altman residual plots. Measurements from EDTA plasma specimens, however, were on average 3-5% lower than from serum specimens.

The stability of GlycA as measured on the Vantera Clinical Analyzer was evaluated in 30 serum samples stored for up to 12 days at 4°C. Measurements were deemed acceptable if they were within 10% of the day 0 mean GlycA. Results demonstrated GlycA was stable out to day 12 at 4°C with changes ≤3.7% and no trend toward higher or lower values (data not shown). Additional stability studies revealed that GlycA values were stable up to 24 hours at room temperature, when specimens were frozen up to 24 months and after being frozen and thawed up to 3 times. When preparing serum specimens, centrifugation could be delayed up to 24 hours, when refrigerated after clotting, without experiencing a significant change in GlycA values.

3.2 Associations of GlycA with incident T2DM in PREVEND

Of the 6,894 PREVEND participants that completed the second round of screening, 4,524 subjects were included in this study. Subjects were excluded if they were missing data for GlycA, hsCRP or information regarding prevalent T2DM, at baseline or follow-up. Subjects were also excluded if they had T2DM at baseline. After a median (interquartile range [IQR]) follow-up period of 7.3 (5.9-7.6) years, 220 incident cases of T2DM were ascertained. Baseline clinical and laboratory characteristics of the cohort are shown in **Table 3**. Participants with higher levels of GlycA were more likely to be older and tended to have a higher BMI, blood pressure, glucose and hsCRP levels. They also had higher TC and TG levels and lower HDL-C, and were more likely to be on lipid lowering medications. Additionally, they were more likely to be current smokers and less likely to consume alcohol.

Cox proportional hazards regression was used to evaluate the association of GlycA and hsCRP with incident T2DM (**Table 4**). GlycA predicted incident T2DM in models adjusted for age, sex, and additionally for BMI, alcohol intake, smoking status, lipid lowering drugs, anti-hypertensive medication, systolic blood pressure (SBP), TC, HDL-C and TG (models 1-4). The association of GlycA with incident T2DM remained present after additional adjustment for baseline glucose (model 5). When hsCRP was added to the model, the relationship of GlycA with T2DM was still significant (model 6). Comparable results were obtained when GlycA was examined per 1 SD change, although statistical significance was lost after adjustment for hsCRP (data not shown; model 6, HR 1.14 [95% CI: 0.95-1.36, P = 0.16]). hsCRP was also associated with T2DM in models adjusted for age, sex and additionally for BMI, alcohol intake, smoking status, lipid lowering drugs, anti-hypertensive medication and SBP (**Table 4**, models 1-3), but significance was lost after further adjustment for lipids, baseline glucose and GlycA (**Table 4**, models 4-6). When examined per 1 SD change, hsCRP was also not significantly associated with incident T2DM in analysis in which we accounted for GlycA (data not shown; model 6, HR 1.03 [95 % CI: 0.87-1.23, P = 0.71]).

Furthermore, in secondary analyses in which the design of the PREVEND study with preferential inclusion of subjects with elevated urinary albumin excretion was taken into account, GlycA was still associated with incident T2DM after adjustment for hsCRP (**Supplementary Table 1**, model 6; HR 1.80 [95% CI: 1.05-3.07, P = 0.03]), whereas hsCRP did not predict T2DM after adjustment for GlycA (**Supplementary Table 1**, model 6; HR 1.22 [95% CI: 0.68-2.20, P = 0.86]).

Table 3. Baseline characteristics according to sex-stratified quartiles of GlycA in 4,524 participants of the PREVEND study.

	Quartiles of GlycA, µmol/L				
	1 **men<301** **women<312**	**2** **men≥301** **women≥312**	**3** **men ≥334** **women ≥349**	**4** **men ≥376** **women ≥391**	**P-value**
Participants, n	1134	1117	1146	1127	
GlycA, µmol/L	284 [267-296]	324 [315-332]	362 [351-381]	418 [398-448]	<0.001
Female, n (%)	599 (52.8)	578 (51.7)	597 (52.1)	588 (52.2)	0.81
Age, years	48.7±10.5	52.0±11.6	54.2±11.8	55.4±11.7	<0.001
BMI, kg/m^2	24.5±3.4	26.0±3.7	27.0±3.9	28.0±4.7	<0.001
Smoking, n (%)					<0.001
Never	456 (40.7)	357 (32.4)	281 (24.8)	263 (23.5)	
Former	475 (42.4)	500 (45.4)	501 (44.2)	445 (39.8)	
Current	189 (16.9)	245 (22.2)	351 (31.0)	409 (36.6)	
Alcohol intake, n (%)					<0.001
Almost never	202 (18.0)	223 (20.2)	296 (26.0)	323 (28.9)	
1-4 drinks per month	192 (17.1)	195 (17.6)	196 (17.2)	188 (16.8)	
2-7 drinks per week	425 (37.8)	370 (33.5)	337 (29.6)	318 (28.5)	
≥1 drinks per day	305 (27.1)	318 (28.8)	309 (27.2)	287 (25.7)	
Lipid lowering drug use, n (%)	44 (3.9)	74 (6.6)	117 (10.2)	154 (13.7)	<0.001
SBP, mm Hg	118.6±15.3	123.2±17.2	127.0±19.5	129.7±18.9	<0.001
DBP, mm Hg	70.3±8.7	72.2±8.6	73.8±9.0	74.4±8.8	<0.001
Blood pressure-lowering drug use, n (%)	105 (9.3)	176 (15.8)	260 (22.7)	316 (28.0)	<0.001
hsCRP, mg/L	0.50 [0.25-0.93]	0.93 [0.53-1.55]	1.66 [0.93-3.11]	3.43 [1.80-6.85]	<0.001
Glucose, mmol/L	4.7±0.6	4.8±0.6	4.9±0.6	5.0±0.7	<0.001

TC, mmol/L	5.2±1.0	5.4±1.0	5.5±1.0	5.7±1.08	<0.001
HDL-C, mmol/L	1.3±0.3	1.3±0.3	1.2±0.3	1.2±0.3	<0.001
TG, mmol/L	0.85 [0.64-1.18]	1.02 [0.77-1.40]	1.20 [0.88-1.64]	1.35 [0.98-1.83]	<0.001

Data are expressed as mean ± SD, median [IQR] or proportion n (%). P values are calculated by linear regression or χ^2 analysis. Diabetes was defined as fasting plasma glucose level >7.0 mmol/L or non-fasting plasma glucose level >11.1 mmol/L or use of antidiabetic medication. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HDL-C high density lipoprotein cholesterol; hsCRP, high sensitivity C-reactive protein; PREVEND, Prevention of REnal and Vascular ENd-stage Disease; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

Subsequently, we performed a joint analysis based on dichotomized subgroups with high and low GlycA or hsCRP (**Table 5**, **Figure 3**). In crude analysis, the hazard of incident T2DM was highest in the subgroup with high GlycA and high hsCRP and lowest in the subgroup with low GlycA and low hsCRP. After adjustment for age and sex, the hazard for incident T2DM remained the same in the subgroups with high GlycA and high hsCRP and high GlycA and low hsCRP, and was only slightly attenuated in the subgroup with high hsCRP and low GlycA compared to the subgroup with low GlycA and low hsCRP. Notably, after adjustment for age, sex, BMI, alcohol intake, smoking status, lipid lowering drugs, antihypertensive medications, SBP, lipids and baseline glucose, the hazard of incident T2DM was only significantly increased in the subgroup with high GlycA and low hsCRP.

Table 4. Association of GlycA and hsCRP with incident type 2 diabetes mellitus in the PREVEND study (n=4,524; 220 events).

	Quartile 1	Quartile 2	P value	Quartile 3	P value	Quartile 4	P value	P for trend*
GlycA	**<306**	**306-340**		**341-383**		**≥384**		
Participants (n)	1104	1147		1133		1140		
No. of 'cases' (%)	26 (2.4)	40 (3.5)		63 (5.6)		91 (8.0)		
Person years	7505	7554		7257		6998		
Crude	1	1.53 [0.93-2.51]	0.09	2.52 [1.60-3.99]	<0.001	3.81 [2.46-5.89]	<0.001	<0.001
Model 1	1	1.40 [0.86-2.30]	0.18	2.24 [1.41-3.55]	0.001	3.39 [2.18-5.28]	<0.001	<0.001
Model 2	1	1.22 [0.74-2.00]	0.44	1.75 [1.09-2.79]	0.02	2.14 [1.34-3.41]	0.001	<0.001
Model 3	1	1.17 [0.71-1.92]	0.55	1.52 [0.95-2.44]	0.08	1.82 [1.13-2.91]	0.01	0.004
Model 4	1	1.08 [0.66-1.77]	0.77	1.33 [0.82-2.13]	0.25	1.55 [0.97-2.48]	0.07	0.005
Model 5	1	1.23 [0.73-2.06]	0.44	1.64 [1.01-2.66]	0.05	1.77 [1.10-2.86]	0.02	0.01
Model 6	1	1.22 [0.72-2.05]	0.46	1.61 [0.98-2.66]	0.06	1.71 [1.00-2.92]	0.05	0.04
hsCRP	**<0.58**	**0.58-1.25**		**1.26-2.82**		**≥2.83**		
Participants (n)	1132	1130		1134		1128		
No. of 'cases' (%)	20 (1.8)	41 (3.6)		74 (6.5)		85 (7.5)		
Person years	7703	7485		7148		6977		
Crude	1	2.11 [1.24-3.60]	0.006	4.03 [2.46-6.60]	<0.001	4.75 [2.92-7.74]	<0.001	<0.001
Model 1	1	1.85 [1.08-3.16]	0.03	3.27 [1.98-5.39]	<0.001	3.93 [2.40-6.45]	<0.001	<0.001
Model 2	1	1.43 [0.83-2.45]	0.20	2.12 [1.27-3.53]	0.004	2.10 [1.25-3.54]	0.005	0.02
Model 3	1	1.37 [0.80-2.36]	0.25	1.94 [1.16-3.24]	0.01	1.88 [1.11-3.17]	0.02	0.06

Model 4	1	1.18 [0.68-2.03]	0.56	1.64 [0.98-2.75]	0.06	1.56 [0.92-2.64]	0.10	0.10
Model 5	1	1.14 [0.65-1.99]	0.65	1.58 [0.93-2.68]	0.09	1.47 [0.86-2.51]	0.16	0.26
Model 6	1	1.08 [0.62-1.89]	0.79	1.45 [0.84-2.48]	0.18	1.20 [0.67-2.17]	0.54	0.91

Hazard ratios were derived from Cox proportional hazards regression models. TG and hsCRP were log transformed when used as a continuous variable in the analysis. *Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.

Model 1: crude model + age, sex

Model 2: model 1 + BMI, alcohol intake, smoking status

Model 3: model 2 + lipid lowering drugs, anti-hypertensive medication and SBP

Model 4: model 3 + TC, HDL-C, TG

Model 5: model 4 + baseline glucose

Model 6: model 5 + hsCRP (for GlycA analysis) and GlycA (for hsCRP analysis)

Abbreviations: BMI, body mass index; HDL-C, high density lipoprotein cholesterol; hsCRP, high–sensitivity C-reactive protein; PREVEND, Prevention of REnal and Vascular ENd-stage Disease; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

Table 5. Joint analysis of GlycA and hsCRP categories on incident type 2 diabetes mellitus.

	Low GlycA Low hsCRP	Low GlycA High hsCRP	P value	High GlycA Low hsCRP	P value	High GlycA High hsCRP	P value
Participants (n)	2938	468		458		660	
No. Of 'cases' (%)	101 (3.4)	29 (6.2)		34 (7.4)		56 (8.5)	
Crude	1	1.89 [1.25-2.86]	0.002	2.32 [1.57-3.42]	<0.001	2.74 [1.98-3.80]	<0.001
Model 1	1	1.68 [1.11-2.54]	0.02	2.16 [1.46-3.19]	<0.001	2.54 [1.82-3.53]	<0.001
Model 2	1	1.19 [0.77-1.83]	0.44	1.57 [1.04-2.36]	0.03	1.24 [0.86-1.78]	0.25

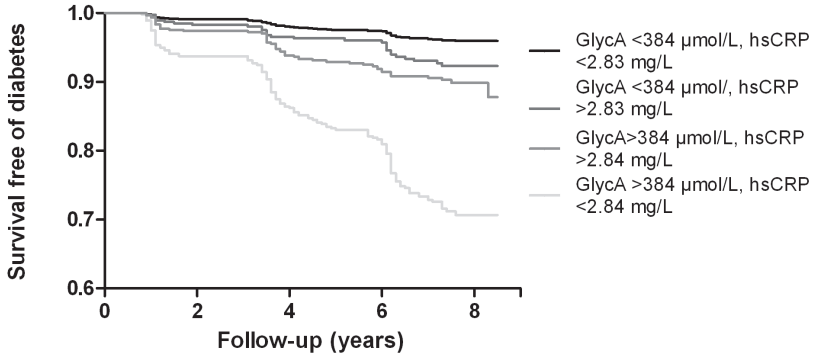
Higher levels were defined as greater than the upper quartile for GlycA >384 µmol/L and hsCRP >2.83 mg/L.

Model 1: crude model + age, sex

Model 2: model 1 + BMI, alcohol intake, smoking status, lipid lowering drugs, anti-hypertensive medication, SBP, baseline glucose and TC, HDL-C, TG.

Abbreviations: BMI, body mass index; HDL-C, high density lipoprotein cholesterol; hsCRP, high–sensitivity C-reactive protein; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

Figure 3. Kaplan-Meier curves of diabetes according to joint levels of GlycA and hsCRP. High levels of GlycA were defined as higher than top quartile, i.e. >384 µmol/L for GlycA and >2.84 mg/L for hsCRP.



4. Discussion

Our prospective study is the first to show that GlycA, a marker of systemic inflammation, is associated with incident T2DM in a general population of both men and women. In PREVEND participants GlycA was an independent predictor of T2DM even after adjusting for traditional diabetes risk factors and hsCRP. Recently Akinkuolie et al. reported that GlycA was associated with risk of incident T2DM in a population of initially healthy women enrolled in the WHS [11]. In the WHS study, the associations of GlycA and hsCRP with incident T2DM were attenuated but remained statistically significant after adjusting for diabetes risk factors. hsCRP remained significantly associated with incident T2DM, while the association with GlycA was no longer significant after further mutual adjustment

[11]. In our study, after adjusting for diabetes risk factors as well as mutual adjustment, GlycA remained significantly associated with incident T2DM whereas the association of hsCRP was no longer significant. Moreover, in PREVEND the associations of GlycA with future T2DM were similar for men and women, while the hsCRP associations appeared to be somewhat stronger in women than in men. The association of hsCRP with incident T2DM in men was attenuated after adjustment for medications (model 2; HR 1.13 [95% CI: 0.94-1.35. P = 0.19) but remained significant in women even after further adjustment for medications and lipids (model 3; HR 1.28 [95% CI: 1.04-1.59. P = 0.02) and was only attenuated after further adjustment for baseline glucose (model 4; HR 1.21 [95% CI: 0.97-1.51. P = 0.10). The fact that the association of hsCRP, but not GlycA, with incident T2DM is stronger in women than in men may explain, at least in part, the differences in results between these two studies.

GlycA may provide complementary and possibly superior information to hsCRP for the prediction of future T2DM. GlycA is unique in that it is an NMR signal derived from residues within the carbohydrate side-chains of multiple acute phase reactants [1]. Not only are the synthesis and secretion of these proteins increased during inflammation, but their glycan structures are dynamically altered [27-30]. For example, the carbohydrate side-chains of α1-acid glycoprotein, one of the acute phase glycoproteins captured in the GlycA signal, become highly branched and contain additional GlcNAc residues in the context of chronic inflammation and cancer [27-31]. It is plausible that GlycA represents a composite biomarker with the potential to have disease associations that differ, or are even stronger, than individual inflammatory markers [2]. In this regard, it is also relevant that GlycA is less variable within subjects than hsCRP [1]; in our study, GlycA remained significantly associated with incident T2DM even after adjusting for diabetes risk factors and hsCRP.

Low grade inflammation is known to trigger the development of insulin resistance and loss of β-cell function, both of which are proposed to be implicated in the pathogenesis of T2DM (for review: [32-35]). Accumulating evidence suggests that carbohydrate metabolism and the immune system are intricately linked. Nutritional excess leads to enhanced systemic inflammation via multiple mechanisms including obesity and ensuing adipose tissue inflammation and alterations in the intestinal microbiome [32]. In turn, tissue and circulating inflammatory mediators may be causally implicated in the development of insulin resistance and β-cell dysfunction, factors that are key for the progression to T2DM [32-35]. Previous studies revealed that there are increases in individual components of the innate immune system, including cytokines, complement factors and acute phase proteins, in subjects with T2DM [36]. In addition, several of these inflammatory markers have been shown to be associated with incident T2DM [37-41]. Therefore, it makes biological sense that GlycA, a unique multi-marker of systemic inflammation, would be independently related to diabetes risk.

It may be possible to combine GlycA with other NMR-measured metabolic and lipoprotein biomarkers of diabetes risk in order to increase the specificity of the prediction for progression to T2DM. For example, specific alterations in the lipoprotein particle distribution occur with insulin resistance and metabolic syndrome [42-44]. In addition, branched chain amino acids such as valine are associated with incident T2DM [45-47]. Because GlycA, valine and lipoprotein particle information can be quantified simultaneously from the same NMR spectra of serum or plasma, it would be straightforward to combine these pieces of information in a single algorithm that would predict a patient's risk of progressing to T2DM. Studies investigating the utility of GlycA in this regard are ongoing.

The successful development of a high-throughput method to measure GlycA on a fully automated platform allows NMR technology dissemination into the routine clinical laboratory setting and creates the opportunity for NMR-based testing across a broader range of clinical applications [14, 15]. The performance characteristics of the GlycA test on the presently used device underscores that it is suitable for clinical applications. Furthermore, high-throughput measurements of GlycA allow both physician use as well as efficient exploration of additional disease associations in large clinical studies like PREVEND.

We acknowledge several strengths and weaknesses in our study. Our study included a large number of participants. Notably, the PREVEND study was designed to study the impact albuminuria on renal and cardiovascular outcome. Thus, subjects were preferentially recruited on the basis of their urinary albumin concentrations; approximately two thirds having urinary albumin concentrations ≥10 mg/L and one third having <10 mg/L at the start of this cohort study. For this reason we performed a secondary analysis taking account of the design of the PREVEND study. This analysis revealed no differences in the results or conclusions that were drawn for the entire population. This finding is also relevant because albuminuria itself may confer increased risk of diabetes development [48]. In addition, it should be appreciated that most PREVEND participants were of Caucasian descent, possibly limiting extrapolation of our findings to other populations. Nonetheless, we were able to extend the results obtained in an initially healthy large population of US women [11].

In conclusion, GlycA, an assay that is available for testing on the Vantera® Clinical Analyzer, may be useful as a diagnostic indicator for risk of T2DM, alone or in combination with other NMR-measured analytes, particularly in a population with abnormal urinary albumin.

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Author's conflict of interest disclosure

MAC, JWD, SPM, IS and JDO are employees of LipoScience, Laboratory Corporation of America® Holdings, a company that is marketing the Vantera® Clinical Analyzer and the GlycA assay for clinical diagnostic use.

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Supplementary Table 1. Association of GlycA and hsCRP with incident type 2 diabetes mellitus in the PREVEND study (n=4,524; 220 events) accounting for the sampling design (presence or absence of a urinary albumin concentration >10 mg/L) by specifying stratum-specific baseline hazard functions.

	Quartile 1	Quartile 2	P value	Quartile 3	P value	Quartile 4	P value	P for trend*
GlycA	<306	≥306		≥341		≥384		
Participants (n)	1104	1147		1133		1140		
No. of 'cases' (%)	26 (2.4)	40 (35)		63 (5.6)		91 (8.0)		
Person years	7504.80	7553.52		7257.32		6998.09		
Crude	(reference)	1.53 [0.93-2.51]	0.09	2.51 [1.59-3.97]	<0.001	3.74 [2.42-5.78]	<0.001	<0.001
Model 1	(reference)	1.40 [0.85-2.30]	0.19	2.21 [1.39-3.51]	0.001	3.29 [2.11-5.13]	<0.001	<0.001
Model 2	(reference)	1.22 [0.74-2.01]	0.43	1.74 [1.09-2.78]	0.02	2.11 [1.32-3.37]	0.002	<0.001
Model 3	(reference)	1.18 [0.72-1.94]	0.52	1.53 [0.95-2.45]	0.08	1.82 [1.14-2.91]	0.01	0.005
Model 4	(reference)	1.09 [0.66-1.79]	0.74	1.33 [0.83-2.14]	0.23	1.56 [0.97-2.49]	0.06	0.006
Model 5	(reference)	1.30 [0.77-2.19]	0.33	1.73 [1.06-2.83]	0.03	1.85 [1.14-2.99]	0.01	0.008
Model 6	(reference)	1.29 [0.76-2.18]	0.35	1.71 [1.03-2.83]	0.04	1.80 [1.05-3.07]	0.03	0.03
hsCRP	<0.58	≥0.58		≥1.26		≥2.83		
Participants (n)	1132	1130		1134		1128		
No. of 'cases' (%)	20 (1.8)	41 (3.6)		74 (6.5)		85 (7.5)		
Person years	7703.12	7485.37		7147.97		6977.27		
Crude	(reference)	2.08 [1.22-3.55]	0.007	4.02 [2.45-6.59]	<0.001	4.68 [2.88-7.62]	<0.001	<0.001
Model 1	(reference)	1.83 [1.07-3.13]	0.03	3.25 [1.97-5.36]	<0.001	3.85 [2.34-6.31]	<0.001	<0.001
Model 2	(reference)	1.42 [0.83-2.44]	0.21	2.12[1.28-3.53]	0.004	2.10 [1.25-3.52]	0.005	0.02
Model 3	(reference)	1.37 [0.80-2.35]	0.26	1.95 [1.17-3.25]	0.01	1.88 [1.12-3.18]	0.02	0.06

Model 4	(reference)	1.18 [0.69-2.03]	0.55	1.64 [0.98-2.75]	0.06	1.57 [0.93-2.64]	0.09	0.10
Model 5	(reference)	1.16 [0.66-2.03]	0.61	1.58 [0.93-2.69]	0.09	1.50 [0.87-2.57]	0.14	0.23
Model 6	(reference)	1.09 [0.62-1.92]	0.76	1.45 [0.84-2.49]	0.18	1.22 [0.68-2.20]	0.51	0.86

Hazard ratios were derived from Cox proportional hazards regression models. TG and hsCRP were log transformed when used as a continuous variable in the analysis. *Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.

Model 1: crude model + age, sex

Model 2: model 1 + BMI, alcohol intake, smoking status

Model 3: model 2 + lipid lowering drugs, anti-hypertensive medications and SBP

Model 4: model 3 +TC, HDL-C, TG

Model 5: model 4 + baseline glucose levels

Model 6: model 5 + hsCRP (for GlycA analysis) and GlycA (for hsCRP analysis)

Abbreviations: BMI, body mass index; HDL-C, high density lipoprotein cholesterol; hsCRP, high–sensitivity C-reactive protein; PREVEND, Prevention of REnal and Vascular ENd-stage Disease; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

Higher Plasma GlycA, a Novel Pro-inflammatory Glycoprotein Biomarker, is Associated with Reduced Life Expectancy: the PREVEND study.

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Clin Chim Acta. 2019;488:7-12

Abstract

Objective Elevated circulating levels of pro-inflammatory biomarkers are associated with adverse health effects, but the extent to which enhanced low-grade inflammation influences remaining life expectancy (LE) is uncertain. GlycA is a novel pro-inflammatory marker. We determined effects of GlycA and high sensitivity C-reactive protein (hsCRP) on LE.

Methods GlycA and hsCRP were determined in 5,526 subjects. LE was compared in the upper quartile of both GlycA and hsCRP vs. the respective lower three quartiles combined, adjusted for LE of individuals in the Dutch general population of the same birth cohort and sex.

Results Median follow up was 8.5 years [interquartile range 7.9-9.0], during which 348 (6.3%) subjects had deceased. LE at the end of follow up was lower in the highest vs. the lower three quartiles of GlycA ($P<0.001$) and hsCRP ($P<0.001$). Both men as well as women in the highest GlycA quartile had reduced LE vs. the lowest three quartiles combined ($P<0.001$ and $P=0.02$). For hsCRP, this was only observed in men ($P<0.001$) but not in women ($P=0.67$).

Conclusions This population-based cohort study demonstrates that higher plasma levels of GlycA were associated with reduced LE in men and women. With regard to hsCRP this only applied to men.

1. Introduction

Glycosylation is one the most common post-translational modifications of proteins and is influenced by many biological processes, including inflammation [1]. GlycA is a novel nuclear magnetic resonance (NMR) spectroscopy measured marker of inflammation, which identifies N-acetyl glycan groups on enzymatically glycosylated acute phase proteins (primarily α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin) [2, 3]. GlycA concentrations are robustly correlated with those of well described inflammatory biomarkers such as high sensitivity C-reactive protein (hsCRP) [2, 4].

Several studies have shown that GlycA is positively correlated with cardiometabolic risk factors such as elevated body mass index (BMI) [5], insulin resistance [6] and components of the metabolic syndrome [7]. Furthermore, GlycA is higher in patients with chronic inflammatory conditions [8]. Elevated levels were found to be associated with incident type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) in several large prospective studies [9-12]. Notably, all of these associations were independent of traditional risk factors.

Besides being a marker of chronic inflammatory diseases, GlycA is also associated with all-cause and cause specific mortality, independently of established risk factors [13-15]. The study of Lawler et al. showed that cardiovascular disease (CVD), colorectal and lung cancer mortality were all significantly associated with elevated levels of GlycA among initially healthy subjects [15]. Another study showed that higher GlycA levels were associated with increased risk of incident colorectal cancer and colorectal cancer mortality, but not with breast cancer or mortality from other cancers [14].

It is evident that elevated levels of GlycA are related to adverse health effects. Notably however, it is uncertain how this influences remaining life expectancy, an increasingly used approach to determine the overall effect of a certain condition on survival [16-18]. We initiated the present study to determine differences in life expectancy in men and women of the Prevention of Renal and Vascular End Stage Disease (PREVEND) cohort with higher vs. lower levels of GlycA and hsCRP. Our method interrogates mortality against life expectancy as the time base. As the individual life expectancies at the time of sampling were different, we used left-censoring as well as right-censoring in the calculations of subjects at risk for Kaplan-Meier curves and log-rank statistics.

2. Material and methods

2.1 Study design and population

This study was part of the PREVEND study, a large-scale, observational, general population cohort study bases in the Netherlands. The study began in 1997. Details of the study design and recruitment have been described in previous reports [19, 20]. In

brief, 40,856 individuals (47.8%) completed a questionnaire on demographics, history of cardiovascular and metabolic outcomes, medication use, and pregnancy before their first visit and collected an early morning urine sample in a vial to measure urinary albumin concentration. Those who were unable or unwilling to participate, individuals using insulin, and pregnant women were excluded. The baseline PREVEND participants were recruited from a total of 6,000 individuals with a urinary albumin concentration of 10 mg/L or greater and a random control sample of individuals with a urinary albumin concentration of <10 mg/L (n = 2592). In total, 8,592 individuals constitute the PREVEND cohort and completed an extensive examination between 1997 and 1998. The second screening took place from 2001 through 2003 (n=6,894), which was the starting point of the present evaluation. We excluded 1,368 individuals with missing data on GlycA and/or hsCRP, leaving 5,526 subjects available for the analyses.

The PREVEND study was approved by the local medical ethics committee of the University Medical Center Groningen in accord with the Declaration of Helsinki.

2.2 Measurements and laboratory analysis

BMI was calculated as weight (kg) divided by height squared (m^2). Smoking status was defined as self-reported never smoker, former smoker, or current smoker (<6, 6–20, or >20 cigarettes/day) and alcohol intake as no/rarely, 1 to 4 drinks/month, 2 to 7 drinks/week, 1 to 3 drinks/day, and 3 or more drinks/day. Blood pressure was measured with an automatic Dinamap XL Model 9300 series device (Johnson-Johnson Medical, Tampa, FL, USA). Hypertension was defined as systolic blood pressure of ≥ 140 mm Hg, a diastolic blood pressure of ≥ 90 mm Hg, or both or the use of antihypertensive agents. Type 2 diabetes mellitus (T2DM) was defined as a fasting serum glucose level ≥ 7.0 mmol/L, a non-fasting plasma glucose level ≥ 11.1 mmol/L, self-report of a physician diagnosis or the use of glucose lowering drugs, retrieved from a central pharmacy registry. Estimated glomerular filtration rate (eGFR) was calculated using the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation from 2012 [21].

Fasting blood samples were provided and stored at -80 °C. NMR spectra were collected from EDTA plasma samples using the Vantera® Clinical Analyzer. The GlycA NMR signal is derived from the N-acetyl methyl protons of N-acetylated carbohydrate side chains of serum glycoproteins (predominantly, α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin) [2] . The GlycA NMR signal is centered at 2.00 ± 0.01 ppm in the NMR spectra of plasma, and only N-acetylglucosamine with specific glycosidic linkage, namely, β (1>2) or β (1>6) with a preceding mannose residue, contribute to the GlycA signal [2].

hsCRP was measured by nephelometry with a threshold of 0.18 mg/L (BNII, Dade Behring). Plasma glucose was measured as described [6]. Serum total cholesterol was

assayed on an automatic analyser type MEGA (Merck, Darmstadt, Germany) using the CHOD-PAP-method. Triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) were measured on a Beckman Coulter AU Analyzer.

The intra-assay and inter-assay coefficients of variation for hsCRP and GlycA are 3% and 4.5%, and 2% and 3%, respectively.

2.3 Statistical analysis

Baseline characteristics are presented according to sex-specific GlycA and hsCRP quartiles. Continuous data are presented as mean with SD or as median with interquartile range (IQR) in case of skewed distribution. Categorical data are presented as numbers with percentages. Pearson correlation coefficients were calculated between GlycA and logarithmically transformed hsCRP. Life expectancy was calculated as follows. The median residual life time (EXP) was derived from gender specific mortality reports provided by the Dutch Central Office of Statistics (CBS) (http://www.CBS.nl). The starting point was the date of blood collection of the individuals at the second screening round. For any person this date was substituted by the median life expectancy of individuals in the general population with the same age, gender and year of birth. This standardization of survival time enables us to compare mortality at the same life expectancy, thereby omitting influence of other risk factors due to age. Because individual life expectancies at start of follow-up were different, we had to use left-censoring (apart from right censoring) in the calculation of number of persons at risk for Kaplan-Meier curves and log-rank statistics. The time base used in the graphs is negative life expectancy (-EXP). Differences were tested using the log-rank test with left and right censoring. Results were calculated for the whole group and for men and women separately. Life expectancy was compared in the upper quartiles of GlycA and hsCRP vs. the lower three quartiles combined. This cut-off was chosen prior to the analyses to be able to evaluate the effect of higher GlycA and hsCRP. Two-sided P-values <0.05 were considered statistically significant. Analyses were performed using SPSS statistics for Windows, Version 23.0 (Armonk, NY: IBM Corp) and Microsoft Excel 2010 for Windows.

3. Results

Baseline characteristics of the 5,526 subjects according to sex specific quartiles of GlycA are presented in **Table 1**. The mean age of the subjects at start of the study was 53.6±12.1 years. 324 (5.9%) of the subjects had T2DM and 349 (6.3%) had a history of CVD. Subjects in the highest quartile of GlycA were older. Levels of total cholesterol, triglycerides and UAE were higher, whereas HDL cholesterol and eGFR were lower in subjects in the highest quartile vs. subjects in the lowest quartile. Hypertension, T2DM

and a history of CVD were more prevalent among subjects in the highest quartile. Baseline characteristics according to sex specific quartiles of hsCRP were comparable to those for GlycA (**Table 2**).

Table 1. Characteristics of the 5,526 subjects of the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study according to sex-stratified quartiles of GlycA

	Q1	Q2	Q3	Q4	*P* for trend
Number of subjects	1387	1386	1373	1380	
GlycA, µmol/L					
Men	<305	≥305	≥339	≥382	
Women	<314	≥314	≥353	≥394	
Age	49.1±10.8	53.2±12.0	55.4±12.2	56.8±12.1	<0.001
Sex, n (%)					0.97
Men	655 (47.2)	668 (48.2)	653 (47.6)	657 (47.6)	
Women	732 (52.8)	718 (51.8)	720 (52.4)	723 (52.4)	
BMI, kg/m^2	24.7±3.4	26.2±3.7	27.3±4.2	28.3±4.9	<0.001
Smoking status, n (%)					<0.001
Non smoker	541 (39.5)	437 (31.9)	335 (24.7)	311 (22.8)	
Former smoker	588 (42.9)	611 (44.6)	610 (45.0)	539 (39.5)	
Current smoker	242 (17.7)	322 (23.5)	412 (30.4)	513 (37.6)	
Alcohol consumption					0.22
<10 g/d	1010 (73.4)	990 (72.1)	1004 (73.7)	1033 (75.6)	
≥10 g/d	366 (26.6)	383 (27.9)	358 (26.3)	333 (24.4)	
Hypertension, n (%)	229 (16.5)	417 (30.1)	548 (39.9)	652 (47.2)	<0.001
Lipid lowering drug use, n (%)	59 (4.3)	111 (8.0)	172 (12.5)	227 (16.4)	<0.001
History of CVD, n (%)	35 (2.5)	81 (5.8)	98 (7.1)	135 (9.8)	<0.001
History of cancer, n (%)	82 (5.9)	80 (5.8)	87 (6.3)	89 (6.4)	0.86
T2DM, n (%)	24 (1.7)	51 (3.7)	105 (7.6)	144 (10.4)	<0.001
Family history of CVD, n (%)	623 (44.9)	677 (48.8)	701 (51.1)	713 (51.7)	0.001
Blood pressure lowering drug use, n (%)	135 (9.7)	264 (19.0)	372 (27.1)	459 (33.3)	<0.001
Glucose lowering drug use, n (%)	8 (0.6)	28 (2.0)	59 (4.3)	80 (5.8)	<0.001
SBP, mm Hg	119.0±15.7	124.5±18.2	128.5±19.7	131.3±19.6	<0.001
DBP, mm Hg	70.4±8.7	72.6±8.8	74.0±8.9	74.7±8.9	<0.001

Total cholesterol, mmol/L	5.16±1.00	5.40±1.00	5.52±1.05	5.64±1.12	<0.001
HDL cholesterol, mmol/L	1.34±0.32	1.28±0.30	1.23±0.31	1.20±0.30	<0.001
Triglycerides, mmol/L	0.85 [0.64-1.18]	1.05 [0.79-1.44]	1.24 [0.90-1.72]	1.39 [1.03-1.87]	<0.001
eGFR*crea-cysC*, ml/min/1.73m^2	98.6 [88.4-107.7]	94.2 [81.8-104.6]	91.2 [79.0-101.7]	87.5 [75.5-99.7]	<0.001
UAE, mg/24h	7.20 [5.68-10.42]	7.83 [5.80-12.27]	8.53 [6.11-14.36]	9.50 [6.28-20.58]	<0.001

Data are numbers (percentages), means (SD) or medians [interquartile range (IQR)]. P-values were calculated by linear regression analysis or χ2 analysis. Triglycerides and UAE were logarithmically transformed for analysis. Data with regard to smoking and alcohol consumption were missing in 65(1.2%) and 49 (0.9%) of the subjects, respectively. Abbreviations: BMI: Abbreviations: BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR*crea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; HDL, high density lipoproteins; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion.

Table 2. Characteristics of the 5,526 subjects of the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study according to sex-stratified quartiles of hsCRP

	Q1	**Q2**	**Q3**	**Q4**	**P-value**
Number of subjects	1387	1386	1373	1380	
hsCRP, mg/L					
Men	<0.62	≥0.62	≥1.31	≥2.82	
Women	<0.64	≥0.64	≥1.43	≥3.33	
Age	49.1±10.8	53.2±12.0	55.4±12.2	56.8±12.1	<0.001
Sex, n (%)					
men	682	683	682	683	
women	744	745	744	745	
BMI, kg/m^2	24.2±3.1	26.2±3.5	27.5±4.0	28.6±5.0	<0.001
Smoking status, n (%)					<0.001
Non smoker	521 (37.8)	425 (31.2)	355 (26.1)	323 (23.8)	
Former smoker	535 (38.8)	611 (44.8)	625 (45.9)	577 (42.6)	
Current smoker	323 (23.4)	328 (24.0)	382 (28.0)	456 33.6(	
Alcohol consumption					0.23
<10 g/d	1004 (72.5)	998 (73.0)	1004 (73.6)	1031 (75.8)	
≥10 g/d	381 (27.5)	369 (27.0)	360 (26.4)	330 (24.2)	
Hypertension, n (%)	255 (18.2)	391 (28.4)	541 (39.4)	659 (47.9)	<0.001
Lipid lowering drug use, n (%)	93 (6.7)	125 (9.1)	175 (12.7)	176 (12.8)	<0.001
History of CVD, n (%)	48 (3.4)	68 (4.9)	88 (6.4)	145 (10.5)	<0.001

Table 2. Continued

	Q1	Q2	Q3	Q4	P-value
History of cancer, n (%)	86 (6.2)	79 (5.7)	93 (6.8)	80 (5.8)	0.66
T2DM, n (%)	34 (2.4)	51(3.7)	95 (6.9)	144 (10.5)	<0.001
Family history of CVD, n (%)	667 (47.7)	683 (49.5)	680 (49.5)	684 (49.7)	0.68
Blood pressure lowering drug use, n (%)	148 (10.6)	237 (17.2)	373 (27.1)	472 (34.3)	<0.001
Glucose lowering drug use, n (%)	16 (1.1)	30 (2.2)	53 (3.9)	76 (5.5)	<0.001
SBP, mm Hg	119±15	125±18	129±19	131±21	<0.001
DBP, mm Hg	70±9	73±9	74±9	75±9	<0.001
Total cholesterol, mmol/L	5.18±1.00	5.45±1.04	5.60±1.09	5.50±1.05	<0.001
HDL cholesterol, mmol/L	1.34±0.31	1.29±0.32	1.24±0.30	1.19±0.29	<0.001
Triglycerides, mmol/L	0.89 [0.66-1.25]	1.06 [0.78-1.52]	1.24 [0.90-1.74]	1.31 [0.96-1.76]	<0.001
eGFR*crea-cysC*, ml/ min/1.73m^2	99.7 [89.5-108.5]	94.2 [82.6-104.0]	89.8 [67.6-100.4]	88.1 [74.6-100.16]	<0.001
UAE, mg/24h	7.10 [5.66-10.20]	7.90 [5.88-1.62]	8.45 [5.93-14.96]	9.70 [6.37-19.85]	<0.001

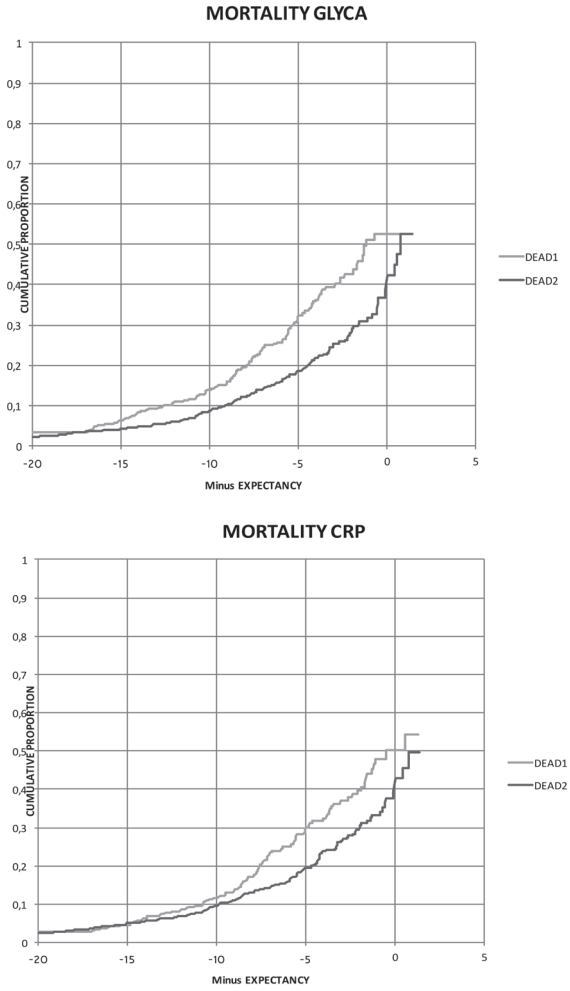
Data are numbers (percentages), means (SD) or medians [interquartile range (IQR)]. P-values were calculated by linear regression analysis or χ2 analysis. Triglycerides and UAE were logarithmically transformed for analysis. Data with regard to smoking and alcohol consumption were missing in 65(1.2%) and 49 (0.9%) of the subjects, respectively. Abbreviations: BMI: Abbreviations: BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR*crea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; HDL, high density lipoproteins; hsCRP, high sensitivity C-reactive protein; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion.

Mean levels of GlycA and median levels of hsCRP of the subjects were 352.3±62.0 µmol/L and 1.36 [0.62-3.08] mg/L, respectively. In the whole group as well as in men and women separately there was a strong positive correlation between GlycA and hsCRP (whole group: r=0.67 P<0.001; men: r=0.65, P<0.001; women r=0.68, P<0.001). Both GlycA and hsCRP were higher in women than in men (357.1±61.4 µmol/L vs.347.0±62.3 µmol/L, P<0.001 and 1.41 [0.63-3.25] mg/L vs. 1.31 [0.62-2.84] mg/L, P=0.04, respectively).

At the end of a median follow-up of 8.5 [IQR, 7.9-9.0] years, 348 subjects had deceased, of which 74 (21.3%) from CVD, 168 (48.3%) from malignancy and 106 (30.5%) from other causes. Life expectancy was compared in the upper quartiles of GlycA and hsCRP vs. the lower three quartiles combined. At start of the study, subjects in the highest quartile of GlycA were older (**Table 1**, P<0.001). The same was true for subjects in the highest quartile of hsCRP compared to the lowest three quartiles combined (**Table 2**, P<0.001). Life expectancy at the end of follow up was lower in the highest quartile vs. the lower three quartiles of GlycA (P<0.001) and hsCRP (P<0.001). Fifty percent of the deceased

subjects in the highest GlycA quartile had died 1.18 years earlier than expected; this was 0.78 years later for the lowest three GlycA quartiles combined. Of the highest hsCRP quartile 50% had deceased 0.48 years earlier than expected; for the lower three hsCRP quartiles combined this was 1.41 years later.

Figure 1. Kaplan-Meier estimates of overall survival. The survival times were adjusted for sex, age and birth cohort. The time base represents the minus life expectancy in years. The lines show the distribution of the life expectancy at death (n=348). The green lines show the life expectancy of patients in the highest quartiles of A. GlycA, B. hsCRP ("dead 1"). The purple lines show the life expectancy of subjects in the lower three quartiles combined of A. GlycA and B. hsCRP ("dead 2"). Life expectancy at the end of follow up was lower in the highest quartile vs. the lower three quartiles of GlycA (P<0.001) and hsCRP (P<0.001).



Analyses in men (249 deceased subjects) and women (99 deceased subjects) separately showed that life expectancy was significantly different in both men and women in the highest quartile vs. those in the lower quartiles of GlycA (P<0.001 and P=0.02, respectively). For hsCRP, men in the highest quartile vs. the lower three quartiles had lower life expectancy (P<0.001). However, no difference in life expectancy was found when we compared women in the highest quartile vs. women in the lowest three quartiles of hsCRP (P=0.67).

4. Discussion

This prospective study comprising 5,526 PREVEND study participants demonstrates that higher levels of plasma GlycA and hsCRP are associated with reduced life expectancy. Furthermore, when the analyses were carried out for men and women separately, men as well as women in the highest GlycA quartile had lower life expectancy compared to the lowest three quartiles combined. However, for hsCRP, the association remained only significant in men.

Despite the fact that GlycA and hsCRP are both markers of low-grade systemic inflammation, there are also some noteworthy differences. First, while GlycA is a composite biomarker that integrates both the increased protein levels and enhanced glycosylation states of the most abundant circulating acute phase proteins, hsCRP is a single biomarker of low-grade systemic inflammation. Second, as a result of the foregoing, GlycA has lower intra-individual variability compared to hsCRP and provides a less variable measure of inflammation [2]. Third, hsCRP is an early acute phase protein while the proteins that give rise to the GlycA signal rise later in the acute phase response [22]. Notably, hsCRP circulates at lower concentrations and contributes negligibly to the measured GlycA signal itself [2].

Although this is the first study that investigated the association between GlycA and life expectancy, previous studies have already revealed an association between GlycA and mortality. GlycA was significantly associated with all-cause, CVD, colorectal and lung cancer mortality in 27,524 initially healthy subjects of the Women's Health study (WHS) [15]. Notably, participants with a history of cancer were not excluded in that report. In addition, the all-cause mortality associations were replicated in an independent cohort of 12,527 subjects of the JUPITER study [15]. The WHS showed an association between GlycA and colorectal cancer (CRC) incidence and mortality. The CRC results were replicated in 6,784 men and women from the MESA study [14]. Numerous reports have shown an association between hsCRP and mortality in initially healthy subjects. An overview article of 13 prospective cohort studies of in total nearly 63,000 men and women has shown that elevated hsCRP levels are predictive of near-term and long-term

mortality [23]. Additionally, several studies also found an association between elevated hsCRP levels and cardiovascular mortality [24-31].

Remarkably, in the current study hsCRP was only associated with reduced life expectancy in men. Another study in elderly men and women examined whether interleukin-6 (IL-6) and hsCRP were associated with relative survival time and age at death (adult lifespan) [32]. Higher levels of both markers predicted reduced survival time and shorter life span among older men. For women, increased IL-6 levels were associated with lower survival time and shorter lifespan but only in those who were not using estrogens at the time of measurement. Interestingly, in that study hsCRP was again only associated with reduced survival time and lifespan in men but not in women [32]**.** Although there is no certain explanation for the discrepant results for men and women in our study, it reinforces the idea that GlycA and hsCRP likely capture different aspects of the inflammatory response. Furthermore, due to the low number of deceased females in the highest hsCRP quartile it seems plausible that we did not have enough power to find an association.

Several other methodological considerations need to be discussed. Strengths of this study include the prospective design and considerable follow-up period. The method that we use to examine life expectancy has been demonstrated to give useful information with respect to other disease conditions such as thyroid cancer, and effects of thyroid function status [16, 17]**.**

We consider it a strength that it allows to compare survival rates with individuals in the general Dutch population with the same sex and year of birth, enabling to reduce the influences of other risk factors due to age [17]. This study also has certain limitations. First, the cohort predominantly consists of Caucasian individuals. Therefore, our results may not be generalizable to other population. Second, due to the limited number of deaths it was not feasible to conduct cause specific life expectancy analyses with sufficient precision.

In conclusion, this large population based cohort study of men and women demonstrates that higher levels of GlycA and hsCRP were associated with reduced life expectancy. In addition, when the analyses were performed for men and women separately, both men and women in the highest quartiles of GlycA had a lower life expectancy. However, for hsCRP, only men in the highest quartile vs. the lower three quartiles combined had significant lower life expectancy.

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Declaration of interest

MAC is an employee of LabCorp. GlycA measurements were performed by LabCorp (Raleigh, North Carolina, USA) at no cost.

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GlycA, a novel pro-inflammatory glycoprotein biomarker is associated with mortality: Results from The PREVEND study and meta-analysis.

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Abstract

Objective Chronic diseases are associated with an inflammatory response. We determined the association of two inflammatory markers, GlycA and high sensitivity C-reactive protein (hsCRP), with overall and cause-specific mortality in a cohort of men and women.

Methods Cox regression analyses were used to examine associations of GlycA and hsCRP with all-cause, cancer and cardiovascular mortality in 5,526 subjects (PREVEND cohort; average follow-up 12.6 years).

Results GlycA was associated with all-cause mortality (n=838), independent of clinical risk factors and hsCRP (hazard ratio 1.43 (95% confidence interval (CI): 1.09-1.87) for top vs. bottom quartiles). For hsCRP, the association with all-cause mortality was non-significant after adjustment for GlycA. GlycA and hsCRP were associated with cancer mortality in men (n=248), but not in women (n=132). Neither GlycA nor hsCRP was independently associated with cardiovascular mortality (n=201). In a meta-analysis of seven population-based studies, including 8,153 deaths, the pooled multivariable-adjusted relative risk of GlycA for all-cause mortality was 1.74 (95% CI: 1.40-2.17) for top vs. bottom quartiles. The association of GlycA with all-cause mortality was somewhat stronger than that of hsCRP. GlycA and hsCRP were not independently associated with cardiovascular mortality. The associations of GlycA and hsCRP with cancer mortality were present in men, but not in women.

Conclusions GlycA is significantly associated with all-cause mortality. GlycA and hsCRP were each not independently associated with cardiovascular mortality. The association of GlycA and hsCRP with cancer mortality appears to be driven by men.

Introduction

Accumulating evidence shows that there may be a link between systemic low-grade inflammation and major adverse health issues. Numerous studies have shown an association between low-grade inflammation and life style factors such as obesity[1], exercise[2], smoking[3] and diet[4]. In addition, enhanced low-grade inflammation may play a role in the etiology of chronic diseases such as cardiovascular disease (CVD) [5], type 2 diabetes (T2D) [6] and cancer [7].

GlycA and high-sensitivity C-reactive protein (hsCRP) are both markers of low-grade systemic inflammation. While GlycA is a composite biomarker that senses the glycosylation states of several of the most abundant acute-phase proteins [8], hsCRP is a single marker of low-grade systemic inflammation. GlycA is determined using nuclear magnetic resonance (NMR) spectroscopy; the signal comes from N-acetyl methyl groups mostly bound to acute phase proteins (mainly: α1-acid glycoprotein (oromucosoid), haptoglobin, α1-antitrypsin, α1-antichymotrypsin and transferrin) [8]. GlycA and hsCRP were found to be rather strongly correlated with each other [8-10], but hsCRP is not highly glycosylated, thus it contributes negligibly to the measured GlycA signal.

GlycA has been found to be associated with incident CVD events as well as with new onset T2D in multiple large studies [9, 11-15]. Interestingly, its association with CVD and with incident T2D remained present after adjustment for hsCRP, suggesting that the association of GlycA with adverse cardiometabolic outcomes is as at least as strong as that with hsCRP. Of further interest, GlycA has been shown to be associated with cancer incidence in the Women's Health Study (WHS) [16] and with cancer- hospitalization and mortality in the Multi-Ethnic Study of Atherosclerosis (MESA) [11].

We have recently shown that higher levels of GlycA were associated with reduced life expectancy[17]. Furthermore, the association of GlycA with all-cause mortality has been evaluated in a number of studies. GlycA was associated with all-cause mortality in high-risk populations of subjects with established CVD or with several cardiovascular risk factors [18-20]. Comparable results on GlycA and all-cause mortality were found in general population-based studies [11, 21]. However, published studies on GlycA and all-cause mortality showed effect sizes ranging from 1.30-2.40. The current study will investigate how the variability in effect size might be explained. Further, limited data is available with regard to GlycA and cause-specific mortality.

Hence, the aims of the current study were i) to examine the associations of GlycA and hsCRP with all-cause, CVD and cancer mortality in the Prevention of Renal and Vascular End-Stage Disease (PREVEND) cohort, a general predominantly Caucasian population of both men and women, ii) to report on a meta-analysis of published evidence on the association of GlycA with all-cause mortality.

Subjects and methods

Study design and population

The Prevention of Renal and Vascular End-Stage Disease (PREVEND) study was designed to investigate the natural course of increased levels of urinary albumin excretion and its relation to renal and CVD in a large cohort drawn from the general population. In short, in the period 1997 to1998, all inhabitants of the city of Groningen (The Netherlands) aged 28 to 75 years were asked to send in a morning urine sample and to fill out a short questionnaire. Pregnant women and subjects with type 1 diabetes mellitus were excluded. Urinary albumin concentration was assessed in 40,856 (47.8%) responders. Subjects with a urinary albumin concentration of ≥10 mg/L (n=7,768) were invited to participate, of whom 6,000 agreed. Furthermore, 3,394 randomly selected subjects with a urinary albumin concentration <10 mg/L were invited and 2,592 agreed to participate. These 8,592 individuals constitute the actual PREVEND cohort.

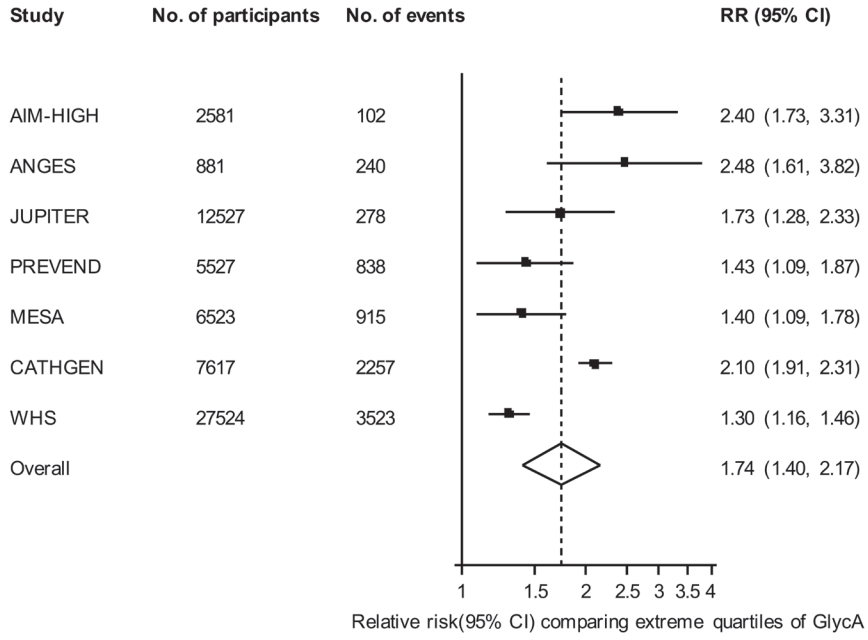
For the current study, data was used from the second screening round (2001-2003) in which 6,894 subjects participated. GlycA and hsCRP were measured in 5,526 subjects of the second screening round (**Supplemental Figure 1**). The PREVEND study has been approved by the medical ethics committee of the University Medical Center Groningen, The Netherlands, and was conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent.

Mortality Data

The cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Dutch Central Bureau of Statistics (CBS). Causes of death were coded according to the 10th revision of the International Classification of Diseases. Survival time for the participants was defined as the period from the date of blood collection of the participant at the second screening round to the date of death from any cause or January first 2017, until which date information about specific causes of death follow-up information was available.

If a person had moved to an unknown destination, the date on which the person was dropped from the municipal registry was used as the census date. Cardiovascular mortality was defined as a cardiovascular event leading to or directly causing death. Qualifying cardiovascular events were ICD-10 codes I10-I99, which include myocardial infarction, stroke, abdominal aortic aneurysm, pulmonary embolism, arrhythmias, myocarditis, cardiomyopathy, cardiac arrest, hearth failure, cerebrovascular diseases and intraoperative and post procedural complications and disorders of circulatory system. Cancer mortality was defined as death due to any type of malignancy (ICD-10 codes C00-C97).

Figure 1. Relative risks for mortality comparing extreme quartiles of GlycA in published studies.



ANGES: Angiography and Genes Study; AIM-HIGH trial: Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides and Impact on Global Health Outcomes; CATHGEN, CATheterization GENetics; CI, confidence intervals (bars); JUPITER, Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin; MESA, Multi-Ethnic Study of Atherosclerosis; PREVEND, Prevention of Renal and Vascular End-stage Disease; RR, relative risk; WHS, Women's health study.

Data collection

The procedures at each examination in the PREVEND study have been described in detail previously [22]. In short, before the outpatient clinic visit, all participants completed a questionnaire regarding demographics, cancer, cardiovascular and renal disease history, smoking habits, alcohol consumption and medication use. Cancer incidence was established by computerized record linkage with the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA: Dutch Pathology Registry) [23]. A history of cancer was defined as any type of malignancy, indicated by the patient in the questionnaire or obtained by PALGA.

Information on medication use (including oral contraceptive use and hormone replacement therapy) was combined with information from a pharmacy-dispensing registry, which has complete information on drug usage of >95% of subjects in the PREVEND study. Body mass index (BMI) was calculated as weight (kg) divided by height

squared (meter). Smoking status was categorized as never, former and current. Alcohol intake was categorized as <10 gram or ≥10 gram per day. T2D was defined as a fasting serum glucose level>7.0 mmol/L, a non-fasting plasma glucose level>11.1 mmol/L, self-report of a physician diagnosis or the use of glucose lowering drugs, retrieved from a central pharmacy registry. Estimated glomerular filtration rate (eGFR) was calculated using the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation [24].

Laboratory measurements

Plasma samples were sent frozen to LipoScience/LabCorp (Morrisville, NC) for testing on the Vantera® Clinical Analyzer. *NMR LipoProfile®* Test spectra were collected and GlycA values were quantified as previously described [8, 14, 25]. In short, the GlycA NMR signal comes from the N-acetyl methyl group protons of the N-acetylglucosamine moieties located on the bi-, tri-, and tetra-antennary branches of plasma glycoproteins, mainly α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1- antichymotrypsin, and transferrin. The coefficients of variation (CVs) for the GlycA assay ranged from 1.3-2.3%. hsCRP was measured by nephelometry with a threshold of 0.18 mg/L (BNII, Dade Behring). Plasma glucose was measured using standard laboratory protocols [26]. Serum total cholesterol was assayed on an automatic analyzer type MEGA (Merck, Darmstadt, Germany) using the CHOD-PAP-method. Measurement of serum creatinine was performed by an enzymatic method on a Roche Modular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C concentrations were measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). Urinary albumin concentration was measured by nephelometry with a threshold of 2.3 mg/L, and intra- and inter-assay CVs of 2.2% and 2.6%, respectively, (Dade Behring Diagnostic, Marburg, Germany).

Statistical analysis

SPSS (version 22.0, SPSS Inc. Armonk, NY: IBM Corp) and STATA version 13.1 (StataCorp, College Station, TX: StataCorp LP) were used for data analysis. Results are presented as mean ± SD, median (interquartile range) and percentages. Skewed data were normalized by natural logarithmic (Log_e) transformation before analyses, which was the case for urinary albumin excretion (UAE) and hsCRP. Baseline characteristics were calculated across sex-stratified quartiles of GlycA. P-values across quartiles of GlycA were determined by linear regression for continuous variables or chi-square test for categorical variables. To study the association of GlycA and hsCRP with mortality, we fitted Cox proportional hazard models to the data. Tests of trend across quartiles were

conducted by assigning the median value for each quartile as its value and treating this as a continuous variable. Results are summarized by hazard ratios (HRs), with 95% confidence intervals. Possible effect modification was explored by including the interaction terms between GlycA or hsCRP and age, sex or smoking in the multivariable adjusted models. Interaction terms were considered to be statistically significant at two-sided P-values<0.10 [27]. Otherwise, the levels of significance was set at two-sided P-values <0.05.

Meta-analysis of published studies

Studies that determined the association between GlycA and all-cause mortality, published in full text before December 17, 2018 (date last searched), were identified through electronic searches not limited to the English language using MEDLINE and EMBASE. Reference lists from included articles were scanned as well. Summary measures were presented as relative risks (RR) with 95% CI intervals and were pooled using a random effects model to minimize the effect of between-study heterogeneity. We assessed heterogeneity using the Cochrane χ^2 statistic and the I^2 statistic.

Results

Table 1 shows the baseline characteristics according to sex-stratified quartiles of GlycA. The mean age of the subjects was 53.6±12.1 years at baseline and 52.4% were women. Mean GlycA was 352±62 µmol/L and median [IQR] hsCRP was 1.36 [0.62-3.08] mg/L. BMI, blood pressure, glucose, total cholesterol, triglycerides, hsCRP and UAE increased, whereas HDL cholesterol and eGFR decreased when GlycA levels were higher. Subjects with elevated GlycA were more likely to have comorbid conditions such as hypertension, CVD history, cancer history and T2D.

Table 1. Baseline characteristics according to sex-stratified quartiles of GlycA concentrations in 5,526 participants of the PREVEND study.

	Quartiles of GlycA, µmol/L				
	1 **♂ ≤304** **♀ ≤313**	**2** **♂ 304-388** **♀ 313-352**	**3** **♂ 339-382** **♀ 353-394**	**4** **♂ >382** **♀ >394**	**P-value**
Participants, n	1387	1386	1373	1380	
Age, years	49.1±10.8	53.2±12.0	55.4±12.2	56.8±12.1	<0.001
Female, n (%)	732 (52.8)	718 (51.8)	720 (52.4)	723 (52.4)	0.99
BMI, kg/m^2	24.7±3.4	26.2±3.7	27.3±4.1	28.3±4.9	<0.001
Smoking, n (%)					<0.001
Never	541 (39.0)	437 (31.5)	335 (24.4)	311 (22.5)	
Former	588 (42.4)	611 (44.0)	610 (44.4)	539 (39.1)	
Current	242 (17.4)	322 (23.2)	412 (30.0)	513 (37.2)	
Alcohol intake, n (%)					<0.001
<10 g/d	1339 (96.5)	1318 (95.1)	1297 (94.5)	1295 (93.8)	
>10 g/d	37 (2.7)	55 (4.0)	65 (4.7)	71 (5.1)	
Hypertension, n (%)	229 (16.5)	417 (30.1)	548 (39.9)	652 (47.2)	<0.001
DBP, mm Hg	70.4±8.7	72.6±8.8	74.0±8.9	74.7±8.9	<0.001
SBP, mm Hg	119.0±15.7	124.5±18.2	128.5±19.7	131.3±19.6	<0.001
History of CVD	35 (2.5)	81 (5.8)	98 (7.1)	135 (9.8)	<0.001
History of cancer	26 (1.9)	27 (1.9)	34 (2.5)	45 (3.3)	0.011
History of T2D, n (%)	34 (2.0)	69 (4.1)	134 (8.0)	182 (11.0)	<0.001
Lipid lowering drug use, n (%)	59 (4.3)	111 (8.0)	172 (12.5)	227 (16.4)	<0.001
Blood pressure-lowering drug use, n (%)	135 (9.7)	264 (19.0)	372 (27.1)	459 (33.3)	<0.001
Use of glucose-lowering drugs, n (%)	8 (0.8)	28 (2.0)	59 (4.3)	80 (5.8)	<0.001
hsCRP, mg/L	0.53 [0.27-0.99]	1.00 [0.57-1.78]	1.78 [0.98-3.25]	3.80 [1.96-7.30]	<0.001
Glucose, mmol/L	4.7±0.9	4.9±0.9	5.1±1.2	5.3±1.5	<0.001
Total cholesterol, mmol/L	5.2±1.0	5.4±1.0	5.5±1.1	5.6±1.1	<0.001
HDL cholesterol, mmol/L	1.3±0.3	1.3±0.3	1.2±0.3	1.2±0.3	<0.001

Triglycerides, mmol/L	0.85 [0.64-1.18]	1.05 [0.79-1.44]	1.24 [0.90-1.72]	1.39 [1.03-1.87]	<0.001
eGFR (ml/min per 1.73 m^2)	90.9±15.6	85.9±17.4	83.4±17.6	80.3±19.7	<0.001
UAE, mg/24h	7.2 [5.7-10.4]	7.8 [5.8-12.3]	8.5 [6.1-14.4]	9.5 [6.3-20.6]	<0.001

Data are expressed as mean ± SD, median [IQR] or proportion n (%). P values are calculated by linear regression or χ^2 analysis. Abbreviations: *CVD,* cardiovascular disease; *BMI,* body mass index*; eGFRcrea-cysC*, estimated glomerular filtration rate based on creatinine-cystatin C equation; *DBP,* diastolic blood pressure; *SBP,* systolic blood pressure; *HDL-cholesterol,* high density lipoprotein cholesterol; *hsCRP*; high sensitive- C-reactive protein; *LDL cholesterol*, low density cholesterol; *UAE,* urinary albumin excretion; *PREVEND,* Prevention of REnal and Vascular ENd-stage Disease.

All-cause mortality

During an average follow-up of 12.6 years, 838 deaths were recorded. Associations of GlycA and hsCRP with all-cause mortality are shown in **Table 2**. GlycA was significantly associated with all-cause mortality in a crude model, as well as after adjustment for age, sex, BMI, alcohol consumption and smoking status. Further adjustment for T2D, blood pressure, use of lipid lowering drugs, anti-hypertensive medication use and lipids did not substantially change the hazard associated with GlycA. Results remained essentially the same after further adjustment for CVD and cancer history (model 4) and renal function (model 5). Of note, the P for trend was still significant after adjustment for hsCRP. Results for hsCRP were comparable to those for GlycA. However, after adjustment for GlycA the P for trend was no longer significant. In addition, there were no statistically significant interactions between GlycA or hsCRP and age, sex or smoking on outcome [interactions: P>0.10 for all].

Cardiovascular mortality

During the follow-up period 201 subjects died due to CVD events (**Table 3**). GlycA was significantly associated with CVD mortality in a crude model as well as after adjustment for age, sex, BMI, alcohol intake and smoking status. The association was attenuated after adjustment for T2D, systolic blood pressure, lipid lowering drugs and anti-hypertensive medication (model 2). In addition, statistical significance was lost after adjustment for lipid levels. This was also true for the analyses with hsCRP as independent variable. There were no significant interactions for each marker with age, sex or smoking with CVD mortality.

Table 2. Association between GlycA and hsCRP levels and all-cause mortality in 5,526 participants (838 deaths) of the PREVEND study.

	Quartile 1	Quartile 2	P- value	Quartile 3	P-value	Quartile 4	P-value	P for trend*
GlycA								
Participants (n)	1362	1395		1363		1406		
Range, µmol/L	<309	≥309		≥346		≥388		
No. of deaths (%)	101 (7.4)	180 (12.9)		228 (16.7)		329 (23.4)		
Crude	Ref.	**1.77 [1..39-2.26]**	**<0.001**	**2.34 [1.86-2.96]**	**<0.001**	**3.37 [2.70-4.22]**	<0.001	**<0.001**
Model 1	Ref.	1.11 [0.87-1.42]	0.42	**1.37 [1.08-1.74]**	**0.01**	**1.78 [1.41-2.25]**	**<0.001**	**<0.001**
Model 2	Ref.	1.04 [0.81-1.33]	0.75	1.23 [0.96-1.56]	0.10	**1.56 [1.23-1.98]**	**<0.001**	**<0.001**
Model 3	Ref.	1.085 [0.84-1.39]	0.55	1.26 [0.99-1.62]	0.06	**1.65 [1.30-2.10]**	**<0.001**	**<0.001**
Model 4	Ref.	1.07 [0.84-1.38]	0.57	1.25 [0.98-1.60]	0.08	**1.58 [1.24-2.02]**	**<0.001**	**<0.001**
Model 5	Ref.	1.08 [0.84-1.39]	0.54	1.24 [0.97-1.58]	0.09	**1.51[1.19-1.93]**	**0.001**	**<0.001**
Model 6	Ref.	1.07 [0.83-1.37]	0.61	1.20 [0.93-1.55]	0.17	**1.43[1.09-1.87]**	**0.009**	**0.002**
hsCRP								
Participants (n)	1373	1388		1384		1381		
Range, mg/L	<0.62	≥0.62		≥1.36		≥3.08		
No. of deaths(%)	107 (7.8)	165 (11.9)		247 (17.8)		319 (23.1)		
Crude	Ref.	**1.53 [1.20-1.95]**	**0.001**	**2.37 [1.89-2.97]**	**<0.001**	**3.16 [2.54-3.93]**	**<0.001**	**<0.001**
Model 1	Ref.	0.93 [0.73-1.19]	0.57	1.24 [0.98-1.57]	0.08	**1.53 [1.22-1.93]**	**<0.001**	**<0.001**
Model 2	Ref.	0.95 [0.74-1.21]	0.66	1.19 [0.94-1.51]	0.14	**1.42 [1.13-1.80]**	**0.003**	**<0.001**
Model 3	Ref.	0.94 [0.73-1.20]	0.60	1.19 [0.93-1.51]	0.17	**1.36 [1.07-1.73]**	**0.011**	**0.01**

Model 4	Ref.	0.95 [0.74-1.22]	0.69	1.22 [0.96-1.55]	0.10	**1.45 [1.14-1.83]**	**0.002**	**<0.001**
Model 5	Ref.	0.93 [0.72-1.19]	0.56	1.16 [0.91-1.47]	0.24	1.27 [1.00-1.62]	0.052	**0.005**
Model 6	Ref.	0.91 [0.71-1.16]	0.44	1.10 [0.86-1.41]	0.44	1.16 [0.88-1.51]	0.29	0.09

Hazard ratios were derived from Cox proportional hazards regression models.
Model 1: crude model + age, sex, BMI, alcohol intake (<10g/d or >10 g/d) and smoking status (never, former current).
Model 2: model 1 + diabetes, systolic blood pressure, lipid lowering drugs and anti-hypertensive medications.
Model 3: model 2 + total cholesterol, HDL cholesterol and triglycerides.
Model 4: Model 3 + history of CVD and history of cancer
Model 5: Model 4 + $eGFR_{creatinine\ cystatin\ C}$ and UAE
Model 6: Model 5 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).
Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses.
*Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.
Abbreviations: *BMI*, body mass index; *HDL-cholesterol*, high density lipoprotein cholesterol; *CVD*, cardiovascular disease; *hsCRP*, high–sensitivity C-reactive protein; *UAE*, urinary albumin excretion; *PREVEND*, Prevention of REnal and Vascular ENd-stage Disease.

Table 3. Association between GlycA and hsCRP levels and cardiovascular mortality in 5,526 participants (201 deaths) of the PREVEND study.

	Quartile 1	Quartile 2	P- value	Quartile 3	P-value	Quartile 4	P-value	P for trend*
GlycA								
Participants (n)	1362	1395		1363		1406		
Range, µmol/L	<309	≥309		≥346		≥388		
No. of deaths (%)	21 (1.5)	47 (3.4)		51 (3.7)		82 (5.8)		
Crude	Ref.	**2.23 [1.33-3.73]**	**0.002**	**2.53 [1.52-4.20]**	**<0.001**	**4.05 [2.51-6.55]**	**<0.001**	**<0.001**
Model 1	Ref.	1.24 [0.74-2.09]	0.41	1.25 [0.74-2.09]	0.41	**1.78 [1.09-2.93]**	**0.02**	**0.008**
Model 2	Ref.	1.08 [0.64-1.81]	0.78	0.96 [0.57-1.62]	0.88	1.32 [0.80-2.19]	0.28	0.15
Model 3	Ref.	1.13 [0.66-1.93]	0.65	0.98 [0.57-1.67]	0.93	1.39 [0.83-2.34]	0.21	0.13
Model 4	Ref.	1.08 [0.63-1.84]	0.79	0.93 [0.54-1.60]	0.80	1.26 [0.75-2.13]	0.39	0.26
Model 5	Ref.	1.06 [0.62-1.81]	0.83	0.90 [0.53-1.55]	0.72	1.14 [0.67-1.93]	0.64	0.56
Model 6	Ref.	1.04 [0.61-1.78]	0.89	0.86 [0.49-1.50]	0.60	1.05 [0.59-1.85]	0.88	0.85
hsCRP								
Participants (n)	1373	1388		1384		1381		
Range, mg/L	<0.62	≥0.62		≥1.36		≥3.08		
No. of deaths (%)	29 (2.1)	33 (2.4)		60 (4.3)		79 (5.7)		
Crude	Ref.	**1.13 [0.69-1.86]**	**0.63**	**2.12 [1.36-3.31]**	**0.001**	**2.89 [1.89-4.42]**	**<0.001**	**<0.001**
Model 1	Ref.	0.60 [0.36-0.99]	0.045	0.93 [0.58-1.47]	0.75	1.16 [0.74-1.81]	0.53	**0.019**
Model 2	Ref.	0.59 [0.36-0.98]	0.041	0.85 [0.54-1.34]	0.48	0.97 [0.62-1.53]	0.91	0.16
Model 3	Ref.	0.59 [0.35-0.98]	0.042	0.88 [0.55-1.40]	0.59	1.01 [0.64-1.61]	0.97	0.12

Model 4	Ref.	0.58 [0.35-0.97]	0.037	0.83 [0.52-1.34]	0.45	0.90 [0.56-1.44]	0.65	0.34
Model 5	Ref.	0.54 [0.32-0.91]	0.02	0.78 [0.48-1.25]	0.30	0.77 [0.47-1.25]	0.28	0.73
Model 6	Ref.	0.54 [0.32-0.91]	0.02	0.78 [0.48-1.26]	0.30	0.76 [0.45-1.30]	0.32	0.73

Hazard ratios were derived from Cox proportional hazards regression models.

Model 1: crude model + age, sex, BMI, alcohol intake (<10g/d or >10 g/d) and smoking status (never, former current).

Model 2: model 1 + diabetes, systolic blood pressure, lipid lowering drugs and anti-hypertensive medications.

Model 3: model 2 + total cholesterol, HDL cholesterol and triglycerides.

Model 4: Model 3 + history of CVD and history of cancer.

Model 5: Model 4 + $eGFR_{creatinine\ cystatin\ C}$ and UAE

Model 6: Model 5 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).

Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

*Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.

Abbreviations: BMI, body mass index; HDL-cholesterol, high density lipoprotein cholesterol; *CVD*, cardiovascular disease; hsCRP, high–sensitivity C-reactive protein; UAE, urinary albumin excretion; *PREVEND,* Prevention of REnal and Vascular ENd-stage Disease.

Cancer mortality

In total, 380 participants died due to malignancies (248 men and 132 women). **Table 4** shows the associations of GlycA and hsCRP with cancer mortality. GlycA was associated with cancer mortality in analyses adjusted for clinical covariates, lipids, and renal function. After additional adjustment for hsCRP, the P for trend remained statically significant. Results for hsCRP were comparable to those for GlycA; however in the final model, when adjusted for GlycA, the P for trend was no longer significant.

There was a significant interaction between hsCRP and sex with cancer mortality (P for interaction 0.002, tested in a model with age and sex). **Supplemental Table 1** shows the sex-stratified analysis of hsCRP with cancer mortality. hsCRP was significantly associated with cancer mortality in men but not in women. The interaction term between GlycA and sex was also significant in a model with age and sex (P for interaction 0.045). In sex-stratified analyses, GlycA was found to be significantly associated with cancer mortality in men but not in women (**Supplemental Table 2**).

Exploratory analyses between GlycA and hsCRP with lung cancer mortality (57 deaths) are presented in **Supplemental Table 3**. GlycA was associated with lung cancer mortality in a crude model as well as after multivariable adjustments for clinical variables and hsCRP. The association between hsCRP and lung cancer mortality was no longer significant after adjustment for age, sex, BMI, alcohol intake and smoking status (model 1). Exploratory analyses between GlycA and hsCRP with death attributable to gastrointestinal cancer (56 deaths) are displayed in **Supplemental Table 4**. After multivariable adjustment, GlycA but not hsCRP was significantly associated to gastrointestinal cancer.

Meta-analysis of published studies

We identified six population-based prospective cohort studies that had reported associations between circulating GlycA and all-cause mortality risk (**Table 5**). Including the current study, the pooled analysis involved seven studies comprising 63,180 participants and 8,153 all-cause mortality events. The pooled random effects multivariable adjusted RR for all-cause mortality when comparing the top versus bottom quartiles of GlycA levels was 1.74 (95% CI: 1.40 to 2.17). Significant heterogeneity was noted (I^2=88%, 95% CI: 78 to 94%; P<0.001) (**Figure 1**). When studies with high CVD risk populations were excluded [18-20], the RR for all-cause mortality comparing extreme quartiles of GlycA was 1.37 (95% CI: 1.24 to 1.52). Heterogeneity was reduced to non-significance (I^2=8%, 95% CI: 0 to 86%; P=0.354). On exclusion of the study which comprised of only women, the RR for all-cause mortality comparing extreme quartiles of GlycA was 1.84 (95% CI: 1.52 to 2.23).

Table 4. Association between GlycA and hsCRP levels and cancer mortality in 5,526 participants (380 events) of the PREVEND study.

	Quartile 1	Quartile 2	P- value	Quartile 3	P-value	Quartile 4	P-value	P for trend*
GlycA								
Participants (n)	1362	1395		1363		1406		
Range, µmol/L	<309	≥309		≥346		≥388		
No. of deaths (%)	46 (3.4)	80 (5.7)		114 (8.4)		140 (10.0)		
Crude	Ref.	**1.73 [1.20-2.48]**	**0.003**	**2.56 [1.82-3.61]**	**<0.001**	**3.13 [2.25-4.37]**	**<0.001**	**<0.001**
Model 1	Ref.	1.14 [0.79-1.65]	0.49	**1.62 [1.14-2.30]**	**0.007**	**1.78 [1.26-2.53]**	**0.001**	**<0.001**
Model 2	Ref.	1.12 [0.78-1.62]	0.55	**1.57 [1.10-2.24]**	**0.013**	**1.72 [1.21-2.44]**	**0.002**	**<0.001**
Model 3	Ref.	1.17 [0.81-1.70]	0.41	**1.62 [1.13-2.32]**	**0.009**	**1.83 [1.28-2.62]**	**0.001**	**<0.001**
Model 4	Ref.	1.18 [0.82-1.72]	0.38	**1.64 [1.14-2.34]**	**0.007**	**1.81 [1.26-2.59]**	**0.001**	**<0.001**
Model 5	Ref.	1.20 [0.83-1.75]	0.33	**1.63 [1.14-2.34]**	**0.008**	**1.80 [1.26-2.59]**	**0.001**	**<0.001**
Model 6	Ref.	1.16 [0.79-1.68]	0.45	**1.49 [1.03-2.17]**	**0.036**	**1.55 [1.03-2.32]**	**0.034**	**0.023**
hsCRP								
Participants (n)	1373	1388		1384		1381		
Range, mg/L	<0.62	≥0.62		≥1.36		≥3.08		
No. of deaths (%)	38 (2.8)	84 (6.1)		119 (8.6)		139 (10.1)		
Crude	Ref.	**2.19 [1.49-3.21]**	**<0.001**	**3.20 [2.22-4.61]**	**<0.001**	**3.85 [2.69-5.51]**	**<0.001**	**<0.001**
Model 1	Ref.	1.46 [0.99-2.15]	0.056	**1.84 [1.26-2.68]**	**0.002**	**2.09 [1.43-3.05]**	**<0.001**	**0.001**
Model 2	Ref.	**1.48 [1.01-2.19]**	**0.047**	**1.84 [1.26-2.69]**	**0.002**	**2.06 [1.41-3.00]**	**<0.001**	**0.001**
Model 3	Ref.	**1.48 [1.00-2.19]**	**0.048**	**1.86 [1.27-2.73]**	**0.001**	**2.05 [1.40-3.01]**	**<0.001**	**0.002**
Model 4	Ref.	1.47 [0.99-2.17]	0.055	**1.83 [1.25-2.68]**	**0.002**	**1.98 [1.35-2.91]**	**0.001**	**0.004**

Model 5	Ref.	**1.48 [1.00-2.19]**	**0.049**	**1.82 [1.24-2.68]**	**0.002**	**1.96 [1.33-2.89]**	**0.001**	**0.006**
Model 6	Ref.	1.43 [0.97-2.12]	0.073	**1.71 [1.15-2.53]**	**0.007**	**1.71 [1.12-2.61]**	**0.014**	0.13

Hazard ratios were derived from Cox proportional hazards regression models.

Model 1: crude model + age, sex, BMI, alcohol intake (<10g/d or >10 g/d) and smoking status (never, former current).

Model 2: model 1 + diabetes, systolic blood pressure, lipid lowering drugs and anti-hypertensive medications.

Model 3: model 2 + total cholesterol, HDL cholesterol and triglycerides.

Model 4: Model 3 + history of CVD and history of cancer.

Model 5: Model 4 + $eGFR_{creatinine\ cystatin\ C}$ and UAE

Model 6: Model 5 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).

Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

*Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.

Abbreviations: *BMI*, body mass index; *HDL-cholesterol*, high density lipoprotein cholesterol; *CVD*, cardiovascular disease; *hsCRP*, high–sensitivity C-reactive protein; *UAE*, urinary albumin excretion; *PREVEND*, Prevention of REnal and Vascular ENd-stage Disease.

Table 5. Characteristics of prospective studies evaluating associations between GlycA and all-cause mortality.

Author, Publication Year	Name of Study	Location of study	Baseline year	Baseline age range	% Male
Current Study	PREVEND	Netherlands	2001-2003	28-75	47.6
Duprez, 2016	MESA	USA	2000-2002	45-84	47.0
Lawler, 2016	WHS	USA	2005-2006	>45	0.0
Lawler, 2016	JUPITER	USA	2003-2006	women≥60 men≥50	64.0
McGarrah, 2017	CATHGEN	USA	2001-2011	>20	62.4
Otvos, 2017	AIM-HIGH trial	USA and Canada	2006-2010	≥45	85.8
Kettunen, 2018	ANGES	Finland	September 2002-March 2004	Not specified	64.0
Total					

Abbreviations: ANGES: Angiography and Genes Study; AIM-HIGH trial: Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides and Impact on Global Health Outcomes; PREVEND, Prevention of Renal and Vascular End-Stage Disease; MESA, Multi-Ethnic Study of Atherosclerosis; WHS, Women's Health Study; JUPITER, Justification for the Use of Statins in Primary Prevention: an Intervention Trial Evaluating Rosuvastatin; CATHGEN, CATheterization GENetics.

Duration of Follow-up	No. of participants	No. of deaths	Variables adjusted for
12.6 years	5527	838	Age, sex, body mass index, alcohol consumption (<10g/d or >10 g/d), smoking status (never, former, current), diabetes, systolic blood pressure, lipid lowering drugs, anti-hypertensive medications, history of CVD, history of cancer, $eGFR_{creatinine\ cystatin\ C}$, and urinary albumin excretion and hsCRP
12.1 years	6523	915	Age, race, sex, and clinic, while the full model adds height, heart rate, systolic blood pressures, diastolic blood pressure, blood pressure lowering medication, BMI, former and current smoking, diabetes, cholesterol lowering medication, total cholesterol, HDL-cholesterol, triglycerides, low eGFR [< 60 mL _ min−1 _ (1.73 m2)−1]
20.5 years	27524	3523	Age, race, smoking (current or former), alcohol use (≥1 drink per day), history of hypertension, family history of myocardial infarction, body-mass index, LDLc, HDLc, glycated hemoglobin and hsCRP.
2.0 years	12527	278	Age, race, smoking (current or former), alcohol use (≥1 drink per day), history of hypertension, family history of coronary heart disease, body-mass index, LDLc, HDLc, glycated hemoglobin and hsCRP.
7.0 years	7617	2257	Age, sex, race, BMI, diabetes, hypertension, smoking, hyperlipidemia, and LDL-P, and additionally for presence of CAD and ejection fraction.
1.0 year	2581 (1 year postbaseline)	102	Age, sex, diabetes history, and treatment assignment
12 years	881	240	Age, sex, albumin, VLDL-diameter, citrate, thrombocyte count, hemoglobin levels, left ventricular hypertrophy and ejection fraction
	63,180	8,153	

Discussion

The present study demonstrates a significant association of GlycA with all-cause mortality, independent of established risk factors and potential confounders. Our pooled finding from the meta-analysis including 63,180 participants and 8,153 deaths reinforces the validity and generalizability of the findings. The observed heterogeneity among these studies was explained by three studies reporting on high risk populations. The positive association of hsCRP with all-cause mortality was attenuated to non-significance after adjustment for GlycA. GlycA and hsCRP were each not independently associated with cardiovascular mortality. In addition, sex-stratified analyses revealed that the positive association of GlycA and hsCRP with cancer mortality was only present in men, but not in women. The association of higher GlycA with increased total cancer mortality was in part attributable to an increased risk of lung and gastrointestinal cancer mortality.

GlycA represents a subset of acute-phase reactants, including α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin, and transferrin [8]. Its composite nature likely has the advantage of giving more stability compared to a single and more variable marker such as hsCRP [8]. Based on the meta-analysis [11, 18-21], individuals with the highest GlycA levels were found to have a 74% greater risk to die from any cause compared to individuals with the lowest GlycA category. Consistent with our findings, α1-acid glycoprotein, one of the major contributors to the GlycA signal, was found to be associated with all-cause mortality in two large cohorts in Estonia and Finland [28]. In line, Ritchie et al. recently showed that of GlycA's constituent glycoproteins, α1-antitrypsin was the strongest predictor for future disease risk and mortality [29]. Our analyses in the PREVEND study furthermore showed that hsCRP was also associated with all-cause mortality. Notably, however, significance was lost after adjustment for GlycA, suggesting that the association of GlycA with all-cause mortality was stronger.

The association of hsCRP with CVD has been firmly established in numerous studies [30, 31]. Although we did find statistically significant trends of GlycA and hsCRP with CVD mortality in unadjusted models, these associations were abolished by controlling for age, sex and lifestyle factors. This may indicate that elevations in GlycA and hsCRP are non-specific responses to environmental stimuli and may not be related directly or indirectly with the pathogenesis of cardiovascular mortality. In line with this, results from Mendelian randomization studies indicate that *CRP* variants do not independently confer increased CVD risk [32, 33]. Moreover, whether CRP should be used to screen asymptomatic persons is still a matter of debate [34, 35]. Notably, in the PREVEND study, we have shown before that GlycA and hsCRP were associated with incident CVD, using a combined endpoint of CVD- morbidity and mortality [9]. Overall, results of the current study, therefore, suggest that these associations are mainly driven by CVD morbidity. In our cohort, 24% of all deaths were attributable to cardiovascular causes, which is

comparable with cardiovascular death rates from the entire Dutch population in 2016 [36]. This supports the idea that the PREVEND study is a representative reflection of the general Dutch population.

The current study showed that GlycA and hsCRP were positively associated with total cancer mortality in men and not in women. This finding is in agreement with the results of an earlier meta-analysis, including six studies comprising a total of 55,721 participants and 3,180 deaths due to cancer, which observed an effect of elevated hsCRP on cancer related mortality only in men [31]. The non-significant association in women may be attributable to lower statistical power or heterogeneity of different types of cancer. In addition, a cross-sectional study of postmenopausal women using hormone replacement therapy (HRT) showed increased CRP levels compared to women not taking HRT [37]. Comparable results were found in young adult women using low dose oral contraceptives [38]. HRT and oral contraceptive use may cause elevated levels of both GlycA and hsCRP in women with relatively healthy lifestyles, which might attenuate the effect on cancer mortality risk. However, in our study, exclusion of 358 women on HRT therapy and oral contraceptives did not alter the results [data not shown]. Further studies are needed to investigate the mechanisms behind the lack of statistical significance between systemic low-grade inflammation and cancer mortality in women. In addition, subgroup analysis results suggested that GlycA might also be predictive for lung cancer mortality. A similar observation was reported by Duprez et al. who found that GlycA was associated with lung cancer mortality in an analysis including 107 deaths [11]. Furthermore, in an exploratory analysis, we showed a significant association between GlycA and gastrointestinal cancer mortality. Interestingly, results of the WHS showed that GlycA was associated with colon cancer mortality in initially healthy women [16].

The strengths of our study include analyses of primary data as well as a meta-analysis of all available published cohorts on GlycA and all-cause mortality so far. Furthermore, the PREVEND study has measurement on comprehensive number of lifestyle and biological markers that enabled adequate adjustment for potential confounders. On the other hand, our study also has some limitations to consider. First, our findings were based on a single baseline measurement of hsCRP and GlycA. However, the study of Ritchie et al. showed stable GlycA elevations for periods of up to a decade [39]. Second, as an observational study, it does not allow for identification of underlying causes. Finally, our meta-analysis was based on study-level data and did not involve individual participant data, which might give more reliable risk estimates compared to study-level data.

In conclusion, in this prospective study involving both men and women, the relative risk for all-cause mortality increased significantly with each increasing quartile of baseline GlycA level. GlycA and hsCRP were not independently associated with

cardiovascular mortality in this study. The association of GlycA and hsCRP with cancer mortality appears to be driven by men.

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Conflict of interest statement

MAC is an employee of LabCorp.

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Supplemental Table 1. Sex-stratified analyses between hsCRP and cancer mortality in 5,526 participants (380 events) of the PREVEND study.

	Quartile 1	Quartile 2	P- value	Quartile 3	P-value	Quartile 4	P-value	P for trend*
Men								
Participants (n)	657	665		652		659		
Range, mgl/L	<0.62	≥0.62		≥1.31		≥2.84		
No. of deaths (%)	18 (2.7)	49 (7.4)		74 (11.3)		107 (16.2)		
Crude	Ref.	**2.74 [1.60-4.70]**	**<0.001**	**4.45 [2.66-7.45]**	**<0.001**	**6.68 [4.06-11.01]**	**<0.001**	**<0.001**
Model 1	Ref.	**1.85 [1.07-3.19]**	**0.027**	**2.50 [1.48-4.25]**	**0.001**	**3.11 [1.86-5.22]**	**<0.001**	**<0.001**
Model 2	Ref.	**1.89 [1.10-3.27]**	**0.022**	**2.55 [1.50-4.33]**	**0.001**	**3.11 [1.85-5.24]**	**<0.001**	**<0.001**
Model 3	Ref.	**1.90 [1.10-3.29]**	**0.021**	**2.57 [1.51-4.37]**	**<0.001**	**3.06 [1.81-5.16]**	**<0.001**	**<0.001**
Model 4	Ref.	**1.90 [1.10-3.29]**	**0.022**	**2.54 [1.49-4.33]**	**0.001**	**3.01 [1.78-5.10]**	**<0.001**	**<0.001**
Model 5	Ref.	**1.93 [1.11-3.34]**	**0.019**	**2.55 [1.50-4.35]**	**0.001**	**2.98 [1.76-5.06]**	**<0.001**	**<0.001**
Model 6	Ref.	**1.86 [1.07-3.22]**	**0.028**	**2.37 [1.38-4.08]**	**0.002**	**2.57 [1.46-4.53]**	**0.001**	**0.019**
Women								
Participants (n)	727	719		722		725		
Range, mg/L	<0.63	≥0.63		≥1.41		≥3.25		
No. of deaths (%)	21 (2.9)	32 (4.5)		41 (5.7)		38 (5.2)		
Crude	Ref.	1.52 [0.88-2.63]	0.14	**1.95 [1.15-3.30]**	**0.013**	**1.84 [1.08-3.13]**	**0.025**	0.08

Model 1	Ref.	1.02 [0.58-1.79]	0.95	1.13 [0.65-1.98]	0.66	1.09 [0.61-1.95]	0.78	0.82
Model 2	Ref.	1.04 [0.59-1.83]	0.89	1.09 [0.62-1.90]	0.76	1.07 [0.59-1.92]	0.83	0.90
Model 3	Ref.	1.09 [0.62-1.93]	0.77	1.15 [0.65-2.05]	0.63	1.12 [0.61-2.05]	0.71	0.85
Model 4	Ref.	1.04 [0.59-1.85]	0.89	1.12 [0.63-2.00]	0.70	1.07 [0.59-1.96]	0.82	0.91
Model 5	Ref.	1.04 [0.59-1.86]	0.86	1.12 [0.62-2.00]	0.71	1.08 [0.58-1.98]	0.82	0.90
Model 6	Ref.	1.01 [0.57-1.81]	0.96	1.06 [0.58-1.92]	0.86	0.95 [0.481.90]	0.88	0.77

Hazard ratios were derived from Cox proportional hazards regression models.

Model 1: crude model + age, sex, BMI, alcohol intake (<10g/d or >10 g/d) and smoking status (never, former current).

Model 2: model 1 + diabetes, systolic blood pressure, lipid lowering drugs and anti-hypertensive medications.

Model 3: model 2 + total cholesterol, HDL cholesterol and triglycerides.

Model 4: Model 3 + history of CVD and history of cancer.

Model 5: Model 4 + $eGFR_{creatinine\ cystatin\ C}$ and UAE.

Model 6: Model 5 + GlycA

Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

*Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.

Abbreviations: *BMI*, body mass index*; HDL-cholesterol*, high density lipoprotein cholesterol; *CVD,* cardiovascular disease; *hsCRP,* high–sensitivity C-reactive protein; *UAE,* urinary albumin excretion; *PREVEND,* Prevention of REnal and Vascular ENd-stage Disease.

Supplemental Table 2. Sex-stratified analyses between GlycA and cancer mortality in 5,526 participants (380 events) of the PREVEND study.

	Quartile 1	Quartile 2	P- value	Quartile 3	P-value	Quartile 4	P-value	P for trend*
Men								
Participants (n)	655	644		667		667		
Range, µmol/L	<305	≥305		≥338		≥381		
No. of deaths (%)	30 (4.6)	47 (7.3)		70 (10.5)		101 (15.1)		
Crude	Ref.	**1.64 [1.04-2.60]**	**0.034**	**2.41 [1.57-3.70]**	**<0.001**	**3.72 [2.48-5.59]**	**<0.001**	**<0.001**
Model 1	Ref.	**1.04 [0.65-1.66]**	**0.19**	**1.34 [0.86-2.06]**	**0.19**	**1.82 [1.19-2.79]**	**0.006**	**<0.001**
Model 2	Ref.	**1.02 [0.64-1.63]**	**0.94**	**1.32 [0.85-2.05]**	**0.21**	**1.78 [1.16-2.74]**	**0.009**	**<0.001**
Model 3	Ref.	**1.06 [0.66-1.70]**	**0.81**	**1.37 [0.87-2.13]**	**0.17**	**1.87 [1.20-2.90]**	**0.006**	**<0.001**
Model 4	Ref.	**1.07 [.67-1.72]**	**0.78**	**1.36 [0.87-2.12]**	**0.18**	**1.84 [1.18-2.87]**	**0.007**	**0.001**
Model 5	Ref.	**1.08 [0.67-1.73]**	**0.76**	**1.35 [0.86-2.11]**	**0.19**	**1.82 [1.16-2.84]**	**0.009**	**0.001**
Model 6	Ref.	0.99 [0.62-1.60]	0.98	1.14 [0.71-1.81]	0.59	1.35 [0.82-2.22]	0.24	0.12
Women								
Participants (n)	712	715		743		723		
Range, µmol/L	<313	≥313		≥352		≥394		
No. of deaths (%)	18 (2.5)	27 (3.8)		45 (6.1)		42 (5.8)		
Crude	Ref.	1.49 [0.82-2.70]	0.19	**2.43 [1.41-4.20]**	**0.001**	**2.35 [1.35-4.07]**	**0.002**	**0.001**
Model 1	Ref.	1.06 [0.58-1.94]	0.86	1.49 [0.84-2.64]	0.17	1.38 [0.76-2.49]	0.29	0.20
Model 2	Ref.	1.05 [0.57-1.92]	0.88	1.39 [0.78-2.47]	0.26	1.29 [0.71-2.34]	0.41	0.33
Model 3	Ref.	1.09 [0.59-2.00]	0.79	1.40 [0.78-2.52]	0.27	1.36 [0.74-2.52]	0.32	0.27
Model 4	Ref.	1.09 [0.59-2.00]	0.79	1.47 [0.81-2.64]	0.20	1.40 [0.76-2.58]	0.28	0.22

Model 5	Ref.	1.10 [0.60-2.02]	0.77	1.47 [0.82-2.65]	0.20	1.41 [0.76-2.60]	0.27	0.22
Model 6	Ref.	1.11 [0.60-2.06]	0.73	1.53 [0.83-2.81]	0.18	1.51 [0.76-3.01]	0.24	0.19

Hazard ratios were derived from Cox proportional hazards regression models.

Model 1: crude model + age, sex, BMI, alcohol intake (<10g/d or >10 g/d) and smoking status (never, former current).

Model 2: model 1 + diabetes, systolic blood pressure, lipid lowering drugs and anti-hypertensive medications.

Model 3: model 2 + total cholesterol, HDL cholesterol and triglycerides.

Model 4: Model 3 + history of CVD and history of cancer.

Model 5: Model 4 + $eGFR_{creatinine\ cystatin\ C}$ and UAE.

Model 6: Model 5 + hsCRP

Triglycerides, UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

*Tests of trend across increasing quartiles were conducted by assigning the median for each quartile as its value and treating this as a continuous variable.

Abbreviations: *BMI*, body mass index; *HDL-cholesterol*, high density lipoprotein cholesterol; *CVD*, cardiovascular disease; *hsCRP*, high–sensitivity C-reactive protein; *UAE*, urinary albumin excretion; *PREVEND*, Prevention of REnal and Vascular ENd-stage Disease.

Supplemental Table 3. Association between GlycA and hsCRP levels and lung cancer mortality in 5,526 participants (57 events) of the PREVEND study.

	Per 1 SD increment	**P- value**
GlycA		
Crude	**1.61 [1.33-1.95]**	**<0.001**
Model 1	**1.34 [1.07-1.67]**	**0.011**
Model 2	**1.33 [1.06-1.67]**	**0.015**
Model 3	**1.34 [1.07-1.67]**	**0.011**
Model 4	**1.38 [1.02-1.88]**	**0.040**
hsCRP	Per 1 SD increment	
Crude	**1.48 [1.19-1.84]**	**<0.001**
Model 1	1.18 [0.92-1.52]	0.19
Model 2	1.17 [0.91-1.50]	0.23
Model 3	1.18 [0.92-1.51]	0.19
Model 4	0.96 [0.70-1.33]	0.81

Hazard Ratios are given per 1 SD increment in GlycA and hsCRP

Model 1: crude model + age, sex, BMI, alcohol intake (<10g/d or >10 g/d) and smoking status (never, former current).

Model 2: model 1 + history of CVD and history of cancer.

Model 3: model 2 + $eGFR_{creatinine\ cystatin\ C}$ and UAE.

Model 4: Model 3 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).

UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

1 SD is 62 µmol/L for GlycA and 5.22 for hsCRP. Abbreviations: *BMI,* body mass index*; CVD*, cardiovascular disease; *hsCRP,* high–sensitivity C-reactive protein; *UAE,* urinary albumin excretion; *PREVEND,* Prevention of REnal and Vascular ENd-stage Disease.

Supplemental Table 4. Association between GlycA and hsCRP levels and gastrointestinal cancer mortality in 5,526 participants (56 events) of the PREVEND study.

	Per 1 SD increment	P- value
GlycA		
Crude	**1.31 [1.04-1.65]**	**0.023**
Model 1	**1.39 [1.09-1.75]**	**0.007**
Model 2	**1.40 [1.10-1.79]**	**0.007**
Model 3	**1.42 [1.11-1.83]**	**0.006**
Model 4	**1.41 [1.01-1.98]**	**0.043**
hsCRP		
Crude	1.22 [0.97-1.55]	0.094
Model 1	1.25 [0.97-1.61]	0.08
Model 2	1.24 [0.97-1.60]	0.09
Model 3	1.25 [0.97-1.61]	0.08
Model 4	1.01 [0.73-1.41]	0.95

Hazard Ratios are given per 1 SD increment in GlycA and hsCRP

The following organs belong to the category of gastrointestinal: bile duct, esophagus, colon + rectum, stomach, pancreas, small intestine and liver.

Model 1: crude model + age, sex, BMI, alcohol intake (<10g/d or >10 g/d) and smoking status (never, former current).

Model 2: model 1 + history of CVD and history of cancer.

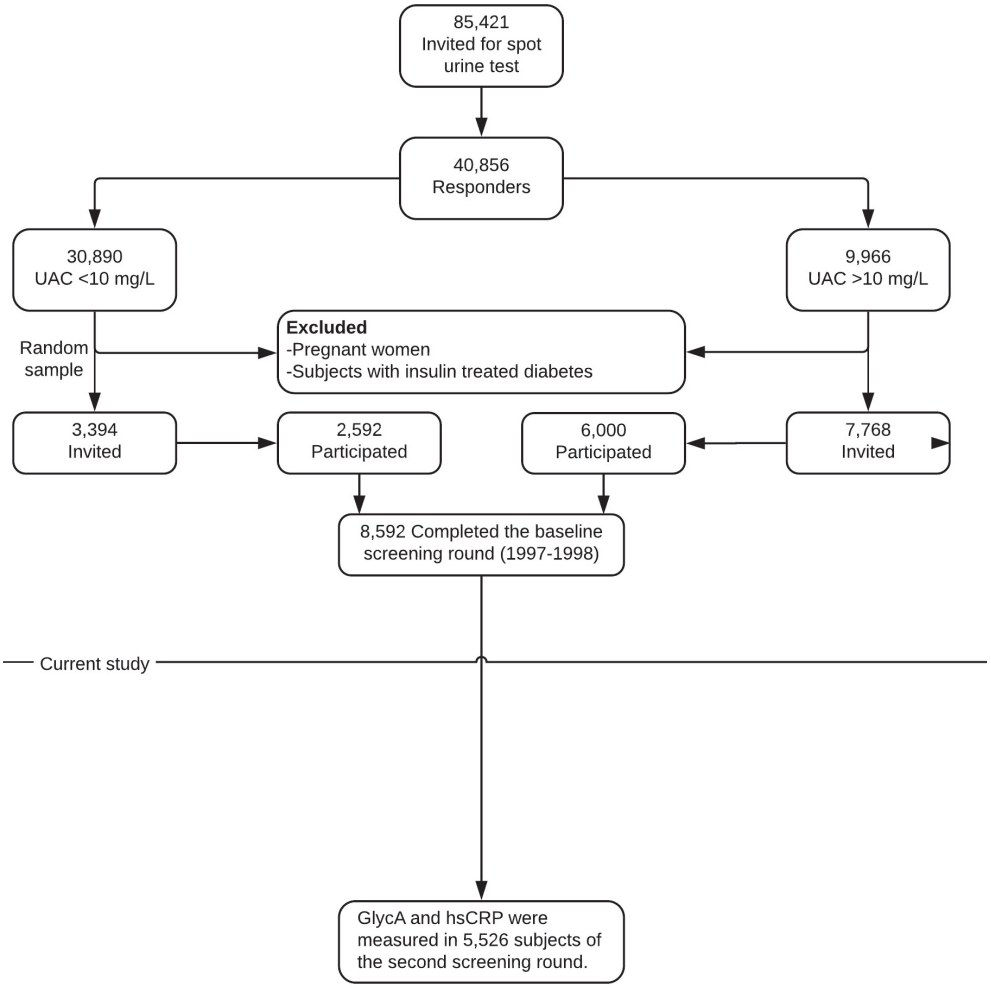
Model 3: model 2 + $eGFR_{creatinine\ cystatin\ C}$ and UAE.

Model 4: Model 3 + hsCRP (for GlycA analyses) + GlycA (for hsCRP analyses).

UAE and hsCRP were log transformed when used as a continuous variable in the analyses.

1 SD is 62 µmol/L for GlycA and 5.22 mg/L for hsCRP. Abbreviations: *BMI*, body mass index*; CVD*, cardiovascular disease; *hsCRP,* high–sensitivity C-reactive protein; *UAE,* urinary albumin excretion; *PREVEND,* Prevention of REnal and Vascular ENd-stage Disease.

Supplemental figure 1



Summary, general discussion, and future perspectives

10

SUMMARY

The first reaction after immunological danger is the innate, non-specific response. In contrast to the adaptive immune system, the innate response is not based on immune memory. Inflammation is an important part of the innate immune response. Acute-phase reactants are commonly measured to assess the severity of inflammation. Moreover, many acute phase reactants are heavily glycosylated. Glycosylation is the most diverse post-translational protein modification that can modulate protein function [1]. In this process, a glycan (oligosaccharide) moiety is added to a protein, thereby producing glycoproteins. Most proteins are N-linked glycoproteins [2]. N-linked proteins start with N-acetylglucosamine (GlcNAc) and are linked to asparagine residues of proteins [3]. Changes in glycosylation have been reported in many inflammatory diseases [4].

Widespread availability of methods to measure glycoprotein levels for clinical use is still limited. Since glycoproteins are a combination of a protein and oligosaccharides, and have diverse glycan structures and compositions, they are complex to analyze. With high-throughput proton nuclear magnetic resonance (NMR) spectroscopy it is possible to quantify inflammatory glycoproteins based on their glycan structure. An NMR spectroscopy method, called GlycA, has been recently developed which detects the concentration of N-acetyl methyl groups, specifically from the N-acetylglucosamine and N acetylgalactosamine moieties, on the carbohydrate side chains of glycosylated proteins. The measured amplitude reflects the plasma protein glycosylation [5]. The units for the GlycA signal are the number of N-acetylglucosamine (GlcNAc) residues expressed in μmol/L. Acute-phase proteins which circulate at >10 μmol/L and which are highly glycosylated contribute to the GlycA signal. These are mainly α1-acid glycoprotein (also known as orosomucoid), haptoglobin, α1-antitrypsin and α1-antichymotrypsin [5]. Therefore, GlycA is considered to be a measure of inflammation [5]. It is noteworthy that there is a strong correlation between GlycA and high sensitivity C-reactive protein (hsCRP), which is widely used to assess the course of inflammatory reactions [5].

The general aim of this thesis was to evaluate the role of GlycA as a marker of inflammation with emphasis on cardiometabolic risk markers, cardiovascular disease (CVD), type 2 diabetes (T2DM), the metabolic syndrome (MetS) and in addition on life expectancy and mortality.

In **Chapter 2**, we provide a review with the aim to provide detailed information about the recent developments in laboratory methods for the study of glycan structures and glycoprotein quantification. Furthermore, the clinical utility of glycoprotein and glycan measurements in low-grade inflammatory diseases such as CVD, T2DM and cancer was reviewed.

In **Chapter 3,** we tested the extent to which plasma GlycA is elevated in MetS and established its relationship with lecithin:cholesterol acyltransferase (LCAT) activity.

LCAT is a plasma enzyme which esterifies free cholesterol and plays a role in the remodeling and the metabolism of high-density lipoprotein cholesterol. GlycA, high sensitivity C-reactive protein (hsCRP), serum amyloid A (SAA), tumor necrosis factor-α (TNF-α) and LCAT were determined in 58 subjects with MetS (of which 46 had T2DM) and in 45 subjects without diabetes or MetS. We observed that plasma GlycA, hsCRP and TNF- α concentrations were higher in MetS subjects, whereas there was no significant difference in SAA. Furthermore, GlycA was not correlated to TNF- α and was unrelated to diabetes status. GlycA was significantly associated with LCAT activity, also after taking account of MetS, diabetes status, hsCRP and SAA. These findings agree with the concept that high GlycA levels reflect a pro-inflammatory state, and provide a link between lipid metabolism and enhanced low grade chronic inflammation.

In **Chapter 4**, we showed that GlycA and hsCRP were higher, while Lp-PLA_2 was lower in subjects with T2DM and/or MetS compared to subjects with neither T2DM nor MetS. Lp-PLA_2, also known as platelet-activating factor acetylhydrolase, is secreted by inflammatory cells in the arterial wall and is considered as a CVD risk marker. GlycA was positively correlated with plasma Lp-PLA_2 mass in subjects without T2DM or MetS. Notably, this association was not observed in subjects with T2DM or MetS. Our findings, therefore, agree with the hypothesis that Lp-PLA_2 may relate to increased plasma levels of glycosylated acute-phase proteins under normal circumstances. However, this relationship is likely to be disturbed in subjects with increased cardiometabolic risk as indicated by T2DM or MetS. In addition, Lp-PLA_2 was not correlated with hsCRP in each group. This observation indicates that Lp-PLA_2 might influence pathways involved in protein glycosylation, possibly distinct from effects on inflammatory processes *per se*.

Subsequent studies were performed using data of the Prevention of Renal and Vascular End-Stage Disease (PREVEND) cohort. The PREVEND study is designed to investigate the natural course of microalbuminuria and its relation with renal and cardiovascular disease in the general population. The cohort consists of male and female inhabitants of the city of Groningen, the Netherlands. The participants were aged 28 to 75 years at baseline. Pregnant women and diabetic subjects using insulin were excluded.

Reduced sodium intake has beneficial effects on blood pressure. However, low dietary sodium could also unfavorably influence pathways that are involved in cardiometabolic risk. In **Chapter 5,** we determined cross-sectional associations of GlycA and hsCRP with 24-h sodium excretion. A total number of 3,935 subjects who were not using anti-hypertensive medication, lipid lowering drugs, or a glucose-lowering treatment were included. Lower concentrations of GlycA and hsCRP were associated with higher amounts of 24-h sodium excretions if measures of adiposity were taken into account.

The association of GlycA and hsCRP with incident CVD was described in **Chapter 6**. Here, we also examined whether this association is modified by renal function. A prospective

cohort study was performed among 4,759 subjects without a history of CVD and cancer. During a median follow-up time of 8.5 years, 298 CVD events occurred. GlycA and hsCRP were both associated with incident CVD, independently of clinical risk factors and plasma lipids. Moreover, the association was not substantially attenuated after adjustment for renal function. CVD risk was highest in subjects with simultaneously higher GlycA and hsCRP levels.

In **Chapter 7,** we prospectively determined the association of GlycA and hsCRP with incident T2DM. GlycA was associated with incident T2DM in both men and women, even after adjustment for diabetes risk factors and hsCRP. In women, the association remained statistically significant, even after further adjustment for medications and lipids. In addition, we also tested the analytical performance of the GlycA assay in this chapter. The performance characteristics of the GlycA test underscored that this assay is suitable for clinical applications.

In **Chapter 8,** we examined how elevated levels of GlycA and hsCRP were influencing remaining life expectancy. We found that men and women in the highest GlycA quartile had lower life expectancy compared to the lowest three quartiles combined. For hsCRP this was only true in men.

In **Chapter 9,** we investigated the association of GlycA with all-cause and-cause specific mortality. GlycA was significantly associated with all-cause mortality. GlycA and hsCRP were each not independently associated with cardiovascular mortality. We found an association of GlycA and hsCRP with cancer mortality, particularly in men. Moreover, the association of higher GlycA with increased total cancer mortality was in part attributable to an increased risk of lung and gastrointestinal cancer mortality. Second, our pooled finding from a meta-analysis which included 63,180 participants and 8,153 deaths, disclosed that individuals in the highest GlycA quartile were having 74% greater risk to die from any cause compared to individuals with the lowest GlycA quartile.

Methodological considerations

Several methodological issues regarding the studies should be acknowledged. First, given the observational design of our studies, we could only determine associations between independent variables and outcome variables. So, we cannot draw conclusions about causality. Second, although we adjusted for multiple possible confounding factors in our studies, residual confounding cannot be ruled out. Finally, our findings were based on a single baseline measurement of hsCRP and GlycA. However, a previous study showed stable GlycA elevations for periods of up to a decade [6].

GENERAL DISCUSSION

GlycA and cardiometabolic disorders

Several cross-sectional studies indicated that GlycA might be a reliable marker of cardiometabolic risk. GlycA is related with adipose tissue associated low-grade chronic inflammation as is indicated by a positive correlation between GlycA and body mass index (BMI), insulin resistance and the leptin to adiponectin ratio [7, 8]. In line with this, we showed that GlycA is associated with the cardiovascular risk marker Lp-PLA_2. Recently, a study in The Multi-Ethnic Study of Atherosclerosis (MESA) cohort showed that suboptimal cardiovascular health was associated with higher GlycA levels [9]. Another report from MESA revealed that GlycA was associated with prevalent carotid artery plaques and other markers of atherosclerosis burden [10, 11]. Furthermore, several cross-sectional studies showed positive associations between GlycA and coronary disease among patients with psoriasis, rheumatoid arthritis, and HIV infection [12-14].

In this thesis, we have shown that GlycA was associated with incident CVD in the PREVEND study. This association was independent of clinical risk factors and plasma lipids. In addition, the association was as strong as that of hsCRP and only slightly attenuated by hsCRP, suggesting that GlycA provides CVD risk information beyond hsCRP. Furthermore, we used the C-statistics to assess the additional value of a GlycA and hsCRP to CVD risk prediction [15, 16]. Notably, when GlycA or hsCRP were added to a model which included age, sex, BMI, smoking status and alcohol intake, neither addition of GlycA nor addition of hsCRP could significantly improve C-statistics. The observation that minor elevations of single markers such as GlycA and hsCRP do not result in additional predictive value over traditional risk factors, can be explained by the fact that CVD is a complex disease with multiple antecedents. In addition, our study provides evidence that GlycA and hsCRP additively associate with incident CVD, suggesting that when patients have both high GlycA and hsCRP, they are at higher risk of future CVD events than when either one or both of the inflammatory markers are low. These findings support the rationale of adding multiple biomarkers that reflect different disease pathways in assessing CVD risk.

Importantly, GlycA has been found to be associated with incident CVD in multiple large prospective studies in both general populations and high-risk populations. GlycA predicted adverse cardiovascular events in subjects in the Women's Health and JUPITER, independent of traditional risk factors [17, 18]. Furthermore, GlycA was also associated with CVD in subjects of the AIM-HIGH trial, CATHGEN and Intermountain Heart Collaborative studies, which are cohorts including high-risk subjects [19-21].

The causal role of hsCRP in CVD has been widely studied. A Mendelian randomisation study with data from 47 studies concluded that hsCRP is unlikely to be a causal factor

in CVD [22]. CRP is more likely a biochemical response to the subtle perturbations in the pathogenic pathways of CVD. However, many clinical trials highlighted that the benefits of lipid lowering are increased when low concentrations of both LDL cholesterol and hsCRP are achieved by statins [23-25]. Recently, the Canakinumab Anti-Inflammatory Thromobosis Outcomes Study showed that reductions in major adverse cardiovascular event rates, independent of lipids, can be achieved by targeting systemic inflammation [26, 27]. Dual goals of inflammation reduction together with cholesterol reduction appear to provide the greatest benefit when it comes to the prevention of atherosclerotic events. Notably, responsiveness to drug treatment is different for GlycA and hsCRP. While hsCRP levels are lowered by statin and niacin therapy, both drugs do not lower GlycA levels [17, 21]. On the other hand, in a small study of 16 patients with psoriasis, GlycA levels were reduced after antitumor necrosis factor therapy [28].Besides the association of GlycA with incident CVD, elevated GlycA levels have been shown to be associated with incident T2DM in a large population of healthy women [29]. We are the first to demonstrate that GlycA is also associated with incident T2DM in a general population of both men and women without known preexisting T2DM.

GlycA as a marker of mortality

Elevated GlycA levels have been shown to be associated with mortality in several studies. GlycA was shown to be associated with mortality in secondary prevention cohorts [20, 21, 30]. Furthermore, some studies demonstrated that CVD and cancer mortality were all significantly associated with elevated levels of GlycA [31, 32]. Results of previous studies were confirmed within this thesis, in which we showed that GlycA was associated with all-cause and cancer mortality. Importantly, the mortality risk was independent of underlying disease status. Notably, the positive association of hsCRP with all-cause mortality was attenuated to non-significance after adjustment for GlycA. Of further interest, the association of GlycA with cancer mortality was only present in men. Similar results were found in a large meta-analysis, which reported an effect of higher hsCRP levels on cancer mortality only in men [33]. This finding might be explained by insufficient statistical power or by heterogeneity of effects of different types of cancer. In addition, we have shown that higher levels of GlycA and hsCRP were associated with reduced life expectancy. Inflammatory markers such as hsCRP tend to increase with age, presumably reflecting the increasing incidence of subclinical pathologies [34]. Importantly, in our life expectancy study, we used a method that is independent of age, suggesting that these findings are not explained by increasing age. However, it is not clear how these findings might improve outcomes.

Differences between GlycA and hsCRP

Nowadays, the most widely used measured acute phase protein is CRP. CRP is commonly determined by a high-sensitivity assay which is able to detect very low levels of CRP, high-sensitivity CRP (hsCRP) [35]. hsCRP has been favored because of the availability of precise, relatively inexpensive and widely distrubuted assays [36, 37]. While hsCRP is a single biomarker of low-grade systemic inflammation, GlycA is a composite biomarker that integrates both the increased protein levels and enhanced glycosylation states of the most abundant circulating acute phase proteins [5]. A disadvantage is that it is currently unknown whether the associations with GlycA can be attributed to the concentrations of these proteins, their glycosylation, or their acetylation profiles. An advantage is that GlycA is measured on a high-throughput device, with an assay time of only 90 seconds [38]. Moreover, there is no need for sample preparation before analysis on the clinical instrument [38]. In terms of costs, odds are still in favor of hsCRP, with around €5,- for an hsCRP measurement and around €30,- for a GlycA measurement, this may change when numbers of assays to be performed increase.

Most studies reported correlation coefficients of hsCRP and GlycA between 0.40 and 0.70 [9, 21, 39, 40]. As mentioned before, the associations of GlycA with incident CVD and T2DM were often only marginally attenuated after adjustment for hsCRP [18, 29]. Hence, it is likely that both markers capture at least in part different aspects of the inflammatory response. Furthermore, the physiological changes that characterize the acute-phase response increase not uniformly in all patients with the same inflammatory conditions [41]. This might in part be explained by differences in the patterns of production of specific cytokines in different pathological states.Therefore, it's likely that a composite biomarker like GlycA may be a more comprehensive biomarker of the inflammatory response since it captures multiple acute phase proteins.

It should also be noted that both markers have different characteristics. hsCRP is an early acute phase protein with a half-life of ~19 hours [42]. The proteins that give rise to the GlycA signal (α1-acid glycoprotein, haptoglobin, α1-antitrypsin, α1-antichymotrypsin) rise later in the acute phase response [41]. The highest values are seen several days after onset of an acute inflammatory response, and fall slowly over weeks [41]. In addition, GlycA showed positive associations with a diverse range of pro- and anti-inflammatory cytokines, suggesting that GlycA is able to reflect changes to many circulating cytokine patterns [43]. Two of the major contributors to the GlycA signal, α1-acid glycoprotein and haptoglobin, are mainly synthesized by hepatocytes but extrahepatic synthesis has also been reported [44, 45]. This indicates that besides the liver extrahepatic sources also contribute to elevated GlycA levels.

Finally, GlycA was shown to have much lower intra-individual variability compared with hsCRP if measurements are repeated over a period of five weeks. The within-

subject CV for GlycA was 4.3% and 29.2% for hsCRP [5]. Since the acute-phase response is a continuum, rather than an on-off phenomenon, it if difficult to have a precise boundary between normal and abnormal hsCRP levels. For practical use it seems useful to consider hsCRP of less than 3 mg/L as normal. hsCRP levels higher than 10 mg/L reflect clinically significant inflammatory states. When used to estimate a person's risk of developing CVD, hsCRP levels of less than 1 mg/L are considered low risk, levels between 1 mg/L and 3 mg/L moderate risk and levels greater than 3 mg/L are regarded as high vascular risk when interpreted in the context of other risk factors. Since, the within-subject standard deviation of hsCRP is 1.2 mg/L, this means that in a subject with an observed hsCRP of 2 mg/L (moderate risk), solely on the basis of day to day variation, a new measurement could easily result in a value of 0.8 mg/L or 3.2 mg/L, with the subject now being categorized in the low (1 mg/l) or high (3 mg/l) range, depending on the value obtained [46]. Therefore, some guidelines recommend two sequential measures when hsCRP is used for cardiovascular risk assessment [22, 47]. To our knowledge, no studies looked at multiple GlycA measurements in the context of CVD risk prediction. However, as mentioned above, the biological variability of GlycA is much lower than that of hsCRP with weekly measurements over a 5-week period [5].

CONCLUSION AND FUTURE PERSPECTIVE

Since inflammation is the basis for many low grade systemic inflammatory diseases such as CVD, T2DM and cancer, glycoproteins likely play a role in the pathophysiology of these diseases. In this thesis, we examined the role of GlycA in the context of MetS, CVD, T2DM, life expectancy and mortality. Overall, the results of this thesis support the contention that this glycoprotein biomarker reflects a pro-inflammatory state, in part alike, but also complementary to hsCRP. Due to its composite nature, GlycA has lower analytical imprecision and lower intra-individual variability, compared to established markers such as CRP. However, since the etiologies of chronic diseases are complex processes, it may be important to not only focus on a single marker, but also to investigate potential interactions and joint effects of risk factors. Noteworthy, GlycA and lipoproteins can be quantified from the same NMR spectra of serum or plasma. Therefore, it is possible to combine GlycA along with standard clinical assessments and other NMR-measured metabolic and lipoprotein biomarkers to increase the specificity of the prediction for progression to CVD or T2DM. This can be of relevance to guide the selection and intensity of interventions intended to prevent progression to CVD or T2DM. For a novel marker to have a clinical role, there must be a widely available and affordable diagnostic test with reproducible assay characteristics appropriate for application in patient-related purposes. Currently, NMR-based equipment is not available in most clinical laboratories in Europe. However, the clinical approach of targeting treatment to those who genuinely profit would be a major step toward personalized medicine.

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Summary in Dutch

Inleiding

In tegenstelling tot het adaptieve immuunsysteem, kent het aangeboren immuunsysteem geen geheugen. Ontsteking is een belangrijk onderdeel van het aangeboren immuunsysteem. Acute fase eiwitten worden vaak in het bloed gemeten om de mate van ontsteking te beoordelen. Veel van deze acute fase eiwitten zijn sterk geglycosyleerd [1]. Bij dit enzymatische proces wordt een oligosaccharide (suikerketen) aan een eiwit toegevoegd, waardoor glycoproteïnen ontstaan. De meeste eiwitten zijn N-gekoppelde glycoproteïnen [2]. N-gekoppelde glycoproteïnen beginnen met N-acetylglucosamine (GlcNAc) op asparagine delen van de eiwitten [3]. Veranderingen in glycosylatie komen veel voor bij ontstekingsziekten [4].

Methoden voor het meten van glycoproteïnen-niveaus voor klinisch gebruik zijn nog steeds beperkt. Omdat glycoproteïnen een combinatie van een eiwit en oligosacchariden zijn, en uiteenlopende glycanstructuren en samenstellingen hebben, zijn ze complex om te analyseren. Met "nuclear magnetic resonance" (NMR) spectroscopie is het mogelijk om inflammatoire glycoproteïnen te kwantificeren op basis van hun oligosaccharide structuur. Recent is een NMR-spectroscopie-bepaling ontwikkeld, genaamd GlycA, die de concentratie van N-acetylmethylgroepen detecteert. Het signaal is voornamelijk afkomstig van de N-acetylglucosamine- en N-acetylgalactosamine delen, in de koolhydraatzijketens van geglycosyleerde eiwitten. De gemeten amplitude weerspiegelt de plasmaproteïne glycosylatie [5]. De eenheden voor het GlycA-signaal zijn het aantal N-acetylglucosamine (GlcNAc)-residuen, uitgedrukt in µmol/L. Acute-fase-eiwitten die circuleren in een concentratie > 10 µmol/L en die sterk geglycosyleerd zijn dragen bij aan het GlycA-signaal. Dit zijn voornamelijk α1-acid glycoproteïne (ook bekend als orosomucoid), haptoglobine, α1-antitrypsine en α1-antichymotrypsine [5]. Daarom wordt GlycA beschouwd als een marker van ontsteking [5]. Er is een sterke correlatie tussen GlycA en high sensitivity C-reactive protein (hsCRP). CRP is een acuut fase eiwit en wordt in de kliniek veel gebruikt om de mate van ontsteking te bepalen. CRP wordt vaak gemeten met een hoog sensitieve test (hsCRP) waarmee laaggradige ontstekingsreacties aangetoond kunnen worden. Dit proefschrift heeft als doel de rol van GlycA, als een marker voor inflammatie, te bestuderen met de nadruk op cardiometabole risicofactoren, hart- en vaatziekten, type 2 diabetes mellitus, het metabool syndroom, levensverwachting en mortaliteit.

Dit proefschrift

Hoofdstuk 2 van dit proefschrift bevat een overzichtsartikel waarin we recente ontwikkelen beschrijven op het gebied van het bestuderen van glycoproteïnen en glycan structuren. Ook wordt de klinische bruikbaarheid van glycoproteïnen bij laaggradige ontstekingsziekten zoals hart- en vaat ziekten, type 2 diabetes mellitus en kanker beschreven.

In **Hoofdstuk 3** hebben we beschreven in hoeverre plasma GlycA verhoogd is bij deelnemers met het metabool syndroom. Verder hebben de associatie tussen GlycA en lecithine:cholesterol acyltransferase (LCAT) activiteit vastgesteld. LCAT is een enzym dat vrij cholesterol verestert en een rol speelt bij het remodelleren en van HDL cholesterol. GlycA, hsCRP, Serum Amyloid A (SAA), Tumornecrosefactor-alfa (TNF-α), en LCAT werden bepaald bij 58 deelnemers met het metabool syndroom (waarvan 46 met type 2 diabetes mellitus) en 45 deelnemers zonder diabetes en zonder het metabool syndroom. TNF-α is een cytokine en speelt een belangrijke rol bij de ontstekingsreactie in het lichaam. SAA is net als CRP een acute fase eiwit. We vonden dat GlycA, hsCRP en TNF-α concentraties hoger waren in deelnemers met het metabool syndroom. We vonden geen verschil in SAA. GlycA was niet gecorreleerd met TNF-α, maar GlycA was significant geassocieerd met LCAT activiteit, ook als we rekening hielden het metabool syndroom, diabetes status, hsCRP en SAA. Deze resultaten zijn in overeenstemming met het idee dat GlycA een inflammatoire marker is. Verder laat deze studie een relatie zien tussen laaggradige ontsteking en het cholesterol metabolisme.

In **Hoofdstuk 4** laten we zien dat GlycA en hsCRP hoger waren bij deelnemers met type 2 diabetes mellitus en/of het metabool syndroom ten opzichte van gezonde deelnemers. Verder hebben we in dit hoofdstuk aangetoond dat lipoproteïne-geassocieerd fosfolipase A2 (Lp-PLA_2) lager was in deelnemers met het metabool syndroom en/of type 2 diabetes mellitus vergeleken met gezonde deelnemers. Lp-PLA_2 wordt geproduceerd en afgegeven door inflammatoire cellen in de vaatwand en wordt beschouwd als een biomarker voor hart- en vaatziekten. GlycA was positief gecorreleerd met plasma Lp-PLA_2 bij deelnemers zonder hetzij type 2 diabetes mellitus of het metabool syndroom. Deze associatie werd niet gevonden bij deelnemers met type 2 diabetes mellitus of het metabool syndroom. Dit komt overeen met de hypothese dat onder normale omstandigheden Lp-PLA_2 gerelateerd is aan geglycosyleerde acute fase eiwitten. Het lijkt erop dat deze associatie verstoord is bij deelnemers met een verhoogd cardiometabool risico (deelnemers met type 2 diabetes mellitus of het metabool syndroom). Lp-PLA_2 was in beide groepen niet gecorreleerd met hsCRP. Dit duidt er op dat Lp-PLA_2 invloed heeft op het glycosyleren van eiwitten, mogelijk deels onafhankelijk van laaggradige inflammatie.

De volgende studies zijn in het PREVEND cohort uitgevoerd. De PREVEND studie is een grote, op de algemene bevolking gebaseerde cohortstudie. De deelnemers waren bij aanvang allemaal 28-75 jaar.

In **Hoofdstuk 5** onderzochten we de associatie tussen GlycA en hsCRP met 24-uurs urine zout excretie. In totaal werden 3935 deelnemers geïncludeerd die geen bloeddrukverlagende medicatie, lipiden verlagende middelen of glucose verlagende middelen gebruikten. Lagere concentraties van GlycA en hsCRP waren geassocieerd met hogere waardes van 24-uurs urine zout excretie als we rekening hielden met variatie in body mass index of buikomvang. We vonden dat deze associatie voornamelijk aanwezig was bij mannen. Ook na correctie voor andere klinische variabelen bleef deze associatie bestaan.

In **Hoofdstuk 6** hebben we de associatie tussen GlycA en hsCRP met het optreden van hart- en vaatziekten onderzocht. In dit hoofdstuk hebben we ook onderzocht of deze associatie beïnvloed wordt door nierfunctie. Hiervoor hebben we een prospectieve studie uitgevoerd onder 4759 deelnemers zonder hart- en vaatziekten of kanker. We vonden dat GlycA en hsCRP beide geassocieerd waren met hart- en vaatziekten, onafhankelijk van bekende risico factoren. De associatie werd niet in belangrijke mate beïnvloed door de nierfunctie. Het risico op hart- en vaatziekten was het grootst bij deelnemers die zowel een hoog GlycA als een hoog hsCRP hadden.

Hoofdstuk 7 bevat een studie naar de associatie tussen GlycA en hsCRP en het ontwikkelen van type 2 diabetes mellitus. GlycA was geassocieerd met type 2 diabetes mellitus incidentie bij mannen en vrouwen, onafhankelijk van diabetes risicofactoren en hsCRP. Bij vrouwen bleef deze associatie statistisch significant, zelfs na correctie voor lipiden en medicatie gebruik. In dit hoofdstuk hebben we ook de analytische prestaties van GlycA getest. De prestatiekenmerken van de GlycA test ondersteunen dat deze test geschikt is voor klinische toepassingen.

In **Hoofdstuk 8** hebben we onderzocht hoe verhoogde waarden van GlycA en hsCRP de resterende levensverwachting beïnvloeden. We vonden dat mannen en vrouwen in het hoogste GlycA kwartiel kortere levensverwachting hadden in vergelijking met de laagste drie kwartielen gecombineerd. Voor hsCRP gold dit alleen in mannen.

In **Hoofdstuk 9** onderzochten we het verband tussen GlycA en hsCRP en de kans op overlijden. In deze studie vonden we dat GlycA geassocieerd was met overlijden door alle oorzaken. GlycA en hsCRP waren beide niet significant geassocieerd met cardiovasculaire mortaliteit. De associatie tussen GlycA en hsCRP met kanker mortaliteit was alleen aanwezig bij mannen. Onze meta-analyse met 63180 deelnemers en 8153 overledenen liet zien dat deelnemers in het hoogste GlycA kwartiel een 74% hoger risico hebben om te overlijden in vergelijking met deelnemers in het laagste GlycA kwartiel.

Conclusie en toekomstperspectieven

Toegenomen inflammatie is een kenmerk van veel ziekten zoals hart- en vaatzieken, type 2 diabetes mellitus en kanker. Daarom is het aannemelijk dat glycoproteïnen een rol spelen bij de pathofysiologie van deze ziekten. In dit proefschrift hebben we de rol van GlycA onderzocht in de context van het metabool syndroom, hart- en vaatziekten, type 2 diabetes mellitus, levensverwachting en mortaliteit. We hebben laten zien dat, GlycA deels vergelijkbaar is met hsCRP maar deels ook complementair is aan hsCRP met betrekking tot deze eindpunten. GlycA heeft een hogere analytische nauwkeurigheid en lagere intra-individuele variabiliteit in vergelijking met bestaande biomarkers zoals hsCRP. Omdat de etiologie van chronische ziekten complex is, kan het belangrijk zijn om niet te focussen op afzonderlijke markers, maar ook om potentiële interacties en gezamenlijke effecten van risicofactoren te onderzoeken. Belangrijk is dat GlycA en lipoproteïnen gekwantificeerd kunnen worden uit hetzelfde NMR spectrum van serum of plasma. Daarom is het mogelijk om GlycA te combineren met standaard klinische bepalingen en/of andere NMR metabolieten, om zo de specificiteit naar de ontwikkeling van hart- en vaatziekten of type 2 diabetes mellitus te vergroten. Dit kan van belang zijn als leidraad bij de selectie en intensiviteit van interventies die bedoeld zijn om de progressie naar hart- en vaatziekten en type 2 diabetes mellitus te voorkomen. Om een nieuwe marker klinische relevant te laten zijn, moet er een algemeen beschikbare en betaalbare diagnostische test zijn met reproduceerbare test karakteristieken die geschikt is voor patiënt gerelateerde doeleinden. Momenteel zijn NMR apparaten niet beschikbaar in de meeste Europese klinische laboratoria. Desalniettemin zou het richten van behandelingen op degene die hier het meeste van kunnen profiteren een grote stap zijn in de richting van gepersonaliseerde geneeskunde.

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Dineke,
augustus 2019

About the Author

ABOUT THE AUTHOR

Eke Gerdina (Dineke) Gruppen was born on October 10th 1991 in Emmen, The Netherlands. In 2008 she graduated from the Hondsrugcollege. The same year she started her study Nutrition and Dietetics at the Hanzehogeschool Groningen. After graduating in Groningen, Dineke continued her education at the VU University in Amsterdam studying Health Sciences. She received her Master's degree in 2014 with the specialization Nutrition and Health. In the same year she started with her PhD-fellowship under supervision of dr. R.P.F. Dullaart and prof. dr. S.J.L. Bakker at the Department of Internal Medicine of the University Medical Center in Groningen.

The work of this thesis led to several oral and poster presentations at national and international congresses. Dineke further developed her skills as a researcher by completing the training for the Graduate School of Medical Sciences GUIDE and the training for Epidemiologist B by the Netherlands Epidemiological Society.
In the final year of her PhD trajectory she started working at the Department of Internal Medicine, Division of Nephrology of the University Medical Center Groningen as research coordinator for an EIT Health project called VALDITHI (the Validation of an Innovative Dietary Intake Tool for Healthcare Implementation).

List of publications

LIST OF PUBLICATIONS

Gruppen EG, Connelly MA, Otvos JD, Bakker SJ & Dullaart RP. A novel protein glycan biomarker and LCAT activity in metabolic syndrome. *European journal of clinical investigation* 2015 45 850-859.

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