Endoglycosidase S Enables a Highly Simplified Clinical Chemistry Procedure for Direct Assessment of Serum IgG Undergalactosylation in Chronic Inflammatory Disease

Authors
Dieter Vanderschaeghe, Leander Meuris, Tom Raes, Hendrik Grootaert, Annelies Van Hecke, Xavier Verhelst, Frederique Van de Velde, Bruno Lapauw, Hans Van Vlierberghe, and Nico Callewaert

Correspondence
Nico.Callewaert@ugent.vib.be

In Brief
IgG galactosylation is a biomarker for assessment of chronic inflammatory diseases that has until now not reached the clinic because of complex sample preparation requirements, including IgG purification. We developed a new assay for the measurement of IgG galactosylation which can be performed entirely in liquid phase, without the need for IgG purification, finally paving the way for clinical implementation of this promising biomarker.

Highlights
- Selective release of IgG Fc glycans in crude serum by endoglycosidase S.
- CE-LIF-based measurements of IgG undergalactosylation levels (UGS).
- UGS as a biomarker: Stratification of non-alcoholic steatohepatitis patients and controls.
Endoglycosidase S Enables a Highly Simplified Clinical Chemistry Procedure for Direct Assessment of Serum IgG Undergalactosylation in Chronic Inflammatory Disease

Dieter Vanderschaeghe‡§‡‡, Leander Meuris‡§‡‡, Tom Raes‡§, Hendrik Grootaert‡§, Annelies Van Hecke‡§, Xavier Verhelst¶, Frederique Van de Velde¶, Bruno Lapauw∥∥, Hans Van Vlierberghe¶, and Nico Callewaert‡§**

Over the past 30 years, it has been firmly established that a wide spectrum of (autoimmune) diseases such as rheumatoid arthritis, Crohn's and lupus, but also other pathologies like alcoholic and non-alcoholic steatohepatitis (ASH and NASH) are driven by chronic inflammation and are hallmarked by a reduced level of serum IgG galactosylation. IgG (under)galactosylation is a promising biomarker to assess disease severity, and monitor and adjust therapy. However, this biomarker has not been implemented in routine clinical chemistry because of a complex analytical procedure that necessitates IgG purification, which is difficult to perform and validate at high throughput. We addressed this issue by using endo-β-N-acetylglucosaminidase from Streptococcus pyogenes (endoS) to specifically release Fc N-glycans in whole serum. The entire assay can be completed in a few hours and only entails adding endoS and labeling the glycans with APTS. Glycans are then readily analyzed through capillary electrophoresis. We demonstrate in two independent patient cohorts that IgG undergalactosylation levels obtained with this assay correlate very well with scores calculated from PNGaseF-released glycans of purified antibodies. Our new assay allows to directly and specifically measure the degree of IgG galactosylation in serum through a fast and completely liquid phase protocol, without the requirement for antibody purification. This should help advancing this biomarker toward clinical implementation. Molecular & Cellular Proteomics 17: 2508–2517, 2018. DOI: 10.1074/mcp.TIR118.000740.

IgG glycosylation has been studied for more than 30 years, revealing how glycan composition is altered in patients suffering from chronic inflammatory diseases. Early studies showed that β-1,4-galactosylation on IgG glycans is lowered in individuals with rheumatoid arthritis (RA)1 or primary osteoarthritis (1, 2). Since then, other (chronic) inflammatory diseases have been shown to be associated with IgG undergalactosylation, including several cancers (3, 4), inflammatory bowel disease (5, 6), systemic lupus erythematosus (7) and liver disease (8–10). Remarkably, in RA and hepatitis B virus (HBV) patients, the glycosylation patterns normalize after treatment (11–13). IgG galactosylation can also differentiate between benign non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), a rapidly emerging disease (10). Many drugs for treatment of chronic inflammatory diseases are very expensive (e.g. anti-TNF biologicals) and come with significant side effects. A biomarker for low grade chronic systemic inflammation and objective treatment monitoring would therefore contribute to improved clinical practice.

Despite numerous publications presenting IgG undergalactosylation as a promising biomarker, current clinical tests for inflammation are largely limited to measuring C-reactive protein (CRP) in serum or to historical tests such as the erythrocyte sedimentation rate (ESR). CRP levels fluctuate daily and it is therefore not a suitable marker for measuring chronic disease activity. ESR is a cheap test that is routinely conducted at high throughput but requires fresh blood and is confounded by several interfering factors (14).

Analyzing IgG glycosylation has so far not been feasible in routine clinical chemistry because of the complex and time-consuming procedure that is required. Purifying the antibodies from patients’ sera is a prerequisite and is then usually
followed by a denaturation step and deglycosylation with PNGaseF to assess the sample N-glycosylation profile by MS, ultra-performance liquid chromatography (UPLC) or capillary electrophoresis (CE) (15–17). The mandatory purification step is difficult to implement in routine testing and validation of antibody purity (which can affect the outcome of the test) at high throughput is cumbersome. Attempts to facilitate the purification process have recently resulted in an automated high-throughput IgG purification and N-glycan sample preparation platform (18). Although promising, sample turnaround for 96 samples takes at least 30 h and requires an expensive robotic liquid handling and UPLC system, limiting its potential for clinical implementation.

In 2001, Collin and Olsén (19) described an endo-β-N-acetylgalcosaminidase secreted by the human pathogen Streptococcus pyogenes, designated endoS. EndoS likely suppresses host IgG effector functions, as it can deglycosylate native IgG. It hydrolyzes the N-glycan chitobiose core, leaving a single GlcNAc residue on the IgG heavy chain (20). The enzyme reportedly requires a natively folded IgG for its activity (21) and only cleaves Fc-linked glycans while leaving N-glycans on Fab and other proteins intact (22). Although it is generally accepted that endoS is specific for IgG Fc activity, activity testing in a complex mixture such as serum. Most Fc N-glycans are cleaved by endoS, although glycans with a bisecting GlcNAc have been shown to be resistant (24). The extent to which endoS-released glycans of patients’ sera resemble the total N-glycan profile of the corresponding purified antibodies remains unexplored.

Here we describe a simple and fast, liquid phase, endoS-based assay to specifically measure IgG galactosylation without the need for antibody purification, which overcomes the main bottleneck for widespread clinical use of this biomarker.

EXPERIMENTAL PROCEDURES

Materials—EndoS (IgGZERO™, Genovis, Lund, Sweden) was reconstituted in ultrapure water at 20 U/µl. PNGaseF from Flavobacterium meningosepticum and sialidase from Arthrobacter ureafaciens were recombiantly produced as described previously (25). IgG, IgA, and IgM from human serum (purity > 95%, Sigma-Aldrich, Saint-Louis, MO) were reconstituted in PBS at 10 mg/ml (hereafter named “commercial IgG, IgA, and IgM”).

Patients and Serum Sampling—Serum samples from two independent cohorts (n = 96 and HdpB steroid cohort (26), n = 92) in which patients were enrolled for bariatric surgery, were obtained from an outpatient clinic (Ghent University Hospital). Informed consent was given by all patients and the protocols were approved by the Hospital’s Ethics Committee. The study complies with the requirements of the Declaration of Helsinki. Blood was collected through classic venupuncture directly in serum preparation tubes (BD vacutainer SST tubes with silica clot activator and polymer gel). Serum was prepared according to the manufacturer’s instructions, aliquoted into standard eppendorfs and immediately stored in an internal biobank at −20 °C or −80 °C until the time of analysis. Patient data can be found in the supplemental spreadsheet.

IgG Purification and Serum IgG Depletion—IgG from 30 µl aliquots of sera was purified using a Protein G Spin Plate for IgG Screening (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. Samples were eluted in 420 µl of elution buffer and concentrated/buffer exchanged to 25 µl of PBS with 100 Kda cut-off Amicon® Ultra Centrifugal Filter Devices (Merck, Darmstadt, Germany). A recovery of 83%, determined in a control experiment with pooled human IgG (commercial IgG), was used to set this 25 µl final buffer volume of the purified IgG. Purity and concentration of 12 IgG samples (6 randomly chosen from each cohort) were assessed by SDS-PAGE and A280 measurements to validate this purification procedure (data not shown).

IgG-depleted sera were prepared with the ProteoPrep™ Immunoaffinity Albumin and IgG Depletion Kit (Sigma-Aldrich) according to the manufacturer’s instructions. Depleted sera were concentrated/ buffer-exchanged to PBS with 10 kDa cut-off Amicon® Centrifugal Filter devices to obtain original serum protein concentrations.

Release of N-glycans—N-glycans were released with endoS or PNGaseF. For endoS catalystyzed release, 2.5 µl of serum or purified IgG was incubated with 25 U of endoS for one hour at 37 °C in a total volume of 10 µl in 150 mmol/L NaCl and 50 mmol/L Tris-HCl pH 8.5. For PNGaseF catalystyzed N-glycan release, 3 µl of serum or purified IgG was denatured by incubation at 95 °C for 5 min after adding 2 µl 50 mmol/L Tris-HCl pH 8.5 containing 3.5% SDS. Samples were cooled and incubated for 1 h at 37 °C with 5 µl of deglycosylation mix, containing 11 mU PNGaseF in 50 mmol/L Tris-HCl pH 8.5 and 2% NP40.

IgG Specificity of endoS—To determine the protein specificity of endoS, serum and IgG-depleted serum were incubated with 25 U of endoS for 1 h at 37 °C in buffers with a pH ranging from 5.0 to 9.0: 50 mmol/L NH4Ac (pH 5.0), MES (pH 6.0), sodium phosphate (pH 7.4) or Tris-Cl (pH 8.0, 8.5, and 9.0), each containing 115 mmol/L NaCl.

IgA from patient samples was depleted/purified by diluting 500 µl plasma into 2 ml of PBS, followed by centrifugation at 16,000 × g and supernatant filtering (0.22 µm). The sample was then applied three times over 0.5 ml of PBS-equilibrated peptide M agarose (InvivoGen, San Diego, CA). The flow through after the third pass was collected as IgA-depleted plasma. The column was washed with 20 CV’s of PBS and IgA eluted with 2.5 ml of 200 mmol/L glycine pH 2.5 and neutralized with 2.5 ml of 1 M Tris-Cl pH 8.5.

IgA and IgA-depleted plasma were buffer exchanged to 500 µl PBS with 3 and 30 kDa Amicon® centrifugal filter devices respectively.

To determine whether endoS hydrolyzes IgA or IgM glycans, 2.5 µl samples of commercial IgA, IgM, IgG, patient IgA and IgA-depleted plasma were digested with 25 U of endoS for 1 h at 37 °C in a total volume of 10 µl in 50 mmol/L NH4Ac pH 5.0 or 50 mmol/L Tris-HCl pH 8.5, each with 150 mmol/L NaCl.

Labeling and CE Analysis—Released glycans were fluorescently labeled with APTS and analyzed by CE. Sample preparation, exoglycosidase digests (optional) and electrophoreses were per-
formed as previously described (27). Briefly, 5 μl of labeling solution was added to an equal volume of crude digest or dry samples, followed by overnight incubation at 37 °C. Excess label was removed by size-exclusion chromatography. Labeled N-glycans were then optionally desialylated, followed by CE on an ABI3130 DNA sequencer.

Glycan symbolic representations follow the Consortium for Functional Glycomics guidelines (28). Glycan structures, nomenclature and symbols are summarized in supplemental Fig. S1.

**Experimental Design and Statistical Rationale**—The relevant peaks in the glycan profiles were quantified with GeneMapper® v3.7 (Applied Biosystems, Foster City, CA). The level of galactosylation (undergalactosylation score, UGS) from PNGaseF profiles was calculated as the ratio of NGA2F (G0F) glycan over the total peak height (supplemental Fig. S2: PNGase UGS). The UGS from endoS profiles was calculated as the sum of peak heights of non-galactosylated glycans normalized to total peak height, considering the number of antennae for each glycan (supplemental Fig. S2: endoS UGS).

Statistical analyses were performed with R v3.4. The compound patient cohort (n_total = 188) was used in all analyses. Correlations between parameters were addressed with Pearson’s r. Outliers from linear regression were defined as having studentized residuals higher than 3 or lower than −3. The glycan dataset can be found in the supplemental spreadsheet.

**RESULTS**

**Assay and N-glycan Profile Characterization**—To specifically analyze IgG Fc glycan galactosylation in serum using endoS, we developed a new method (Fig. 1), based on procedures for profiling serum N-glycosylation that we previously optimized (25). In contrast to PNGaseF, which is commonly used to remove N-glycans from denatured proteins (Fig. 1, top), endoS activity requires native (dimeric) IgG Fc fragments (Fig. 1, bottom). Only N-glycans from IgG Fc are expected to be released by endoS whereas glycans from the Fab fragment and other serum glycoproteins are not expected to be released, allowing the omission of IgG purification and denaturation steps.

To identify the glycan structures that are released by endoS, we analyzed endoS-released N-glycans from serum of a healthy individual and from IgG that was purified from the same serum sample (Fig. 2A and 2B). These CE profiles revealed the same six major glycan peaks and a few small peaks in both electropherograms. Some other very small peaks were only observed in the electropherogram from serum. These peaks are background peaks (they were also
present in negative control experiments, in which the sample prep was performed without endoS, supplemental Fig. S3). Each of these small peaks constitutes less than 1% of the total signal, and together they are less than 2–3% of the signal in a typical profile (Fig. 2A). Purified IgG, again from the same serum sample, was also digested with PNGaseF for full N-glycan profiling (Fig. 2C). Comparing digests on serum and IgG showed that endoS not only cleaves the same N-glycans in both samples, but we also observed similar relative peak heights (Fig. 2A and 2B). These first results indicated the feasibility of using endoS in serum for specific analysis of IgG glycosylation.

Because endoS hydrolyzes N-glycans in their chitobiose core, all the structures are truncated at the reducing end as compared with PNGaseF-released N-glycans, which hydrolyzes the amide bond between the N-glycan and the asparagine side chain.

The six major endoS-released N-glycan structures were confirmed with exoglycosidase digests and subsequent CE analysis of the resulting fragments (supplemental Fig. S4). The N-glycans were all biantennary complex type N-glycans that are typically present on IgG Fc. We found no structures with bisecting GlcNAc, although about 10% of IgG Fc glycans are modified with such a structure (29). This is consistent with earlier reports that endoS does not hydrolyze glycans with a bisecting GlcNAc (24).

To identify endoS resistant IgG glycans, an endoS digest was performed on commercial whole IgG, followed by separation of the Fab and Fc fragments and analysis of the remaining glycans on both (supplemental Fig. S5). This revealed that Fc glycans with a bisecting GlcNAc (peaks indicated in red in Fig. 2C) indeed remain intact after endoS hydrolysis. Bisialylated N-glycans (peaks indicated in green in Fig. 2C) are not released by endoS, but are originating almost exclusively from the Fab fragments (supplemental Fig. S5), which are no substrate for endoS, as far as our methods can detect. The lack of bisialylated N-glycans in the endoS profiles is consistent with reports that less than 0.5% of Fc glycans are bisialylated (29). Taken together, these observations support the view that, when using purified IgG as the substrate, endoS is specific for the Fc portion. Glycans with a bisecting GlcNAc, which consti-
tute about 10% of Fc glycans, are resistant to endoS hydrolysis.

IgG Specificity of endoS In Serum—To assess whether endoS cleaves only IgG N-glycans in serum, we performed endoS digests on IgG-depleted serum samples. We previously demonstrated that up to 99% of IgG is removed by this procedure, without affecting other serum proteins (30). Treating fresh serum (or plasma) with endoS in different pH conditions (pH 5.0–9.0), revealed the release of an increasing amount of sialylated N-glycans from proteins other than IgG with decreasing pH (Fig. 3A). Exoglycosidase digests showed these peaks to represent mono- and bisialylated, fully galactosylated biantennary complex type glycans. These glycans were not observed when using plasma or serum that had been freeze-thawed several times as the starting material (data not shown). Adding a physiological amount of commercial IgG (10 μg/μl) back to IgG-depleted serum shows that non-IgG N-glycans are prominently present in the profiles at low pH, but not at high pH (supplemental Fig. S6).

Although endoS has been reported to be specific for IgG Fc, these experiments illustrate that this is only true at high pH. We speculated that the enzyme may recognize structurally similar protein substrates at suboptimal pH values. IgA and IgM are the two other immunoglobulins that are present in significant amounts in serum at about 2.5 g/l (IgA) and 1.5 g/l (IgM) (31). Moreover, the observed glycan types are typically abundantly present in IgA glycan profiles (32), but only at low levels in IgG profiles. Therefore, we assessed whether endoS can release IgA or IgM glycans at pH 5.0 and pH 8.5. We first confirmed the pH independence of IgG pro-
files and obtained identical profiles at pH 5.0 or 8.5 (Fig. 3B). EndoS digests at pH 5.0 of IgA purified from a fresh patient plasma sample (Fig. 3C, left) produced the same two sialylated non-IgG glycans as in IgG-depleted serum at pH 5.0. These glycans were absent in the profile of purified IgA digested at pH 8.5 (Fig. 3C, right). EndoS digests of commercial IgA gave the same result (data not shown). Similarly, endoS also produced minute amounts of the monosialylated N-glycan from IgM at pH 5.0 but not at pH 8.5 (supplemental Fig. S7).

To further investigate the contribution of IgA glycans, we compared the glycan profiles of fresh whole and IgA-depleted patient samples (Fig. 3D and 3E) at pH 5.0 and pH 8.5. Although completely removing IgA from the samples proved to be impossible even after several rounds of peptide M chromatography, a significant reduction in the peak height of the contaminating peaks in the pH 5.0 profile can be observed after IgA depletion. This indicates that these peaks are at least partially coming from IgA. Taken together, these experiments indicate that the presence of other immunoglobulins does not interfere with the assay when working at a pH of 8.5 and that endoS is specific for IgG Fc glycans under these conditions and up to the limits of detection of the analytical techniques used.

Further, we tested whether changing the incubation time, endoS concentration or incubation temperature influences the relative peak heights of the peaks used in the UGS calculation (and thus the calculated UGS) and found that the profile is dependent on these parameters only to a very small extent. Under the chosen assay conditions (pH 8.5, 37 °C, 25 units of endoS and 60 min of incubation time for 2.5 µl of serum), the UGS outcome is robust (supplemental Fig. S8).

As a final point, we also confirmed that, under the optimized assay conditions, the presence or absence of serum components does not influence the undergalactosylation score in a cohort of patients and healthy controls with a varying degree of IgG undergalactosylation. To do this, we compared endoS derived profiles from serum versus purified IgG for the total patient cohort. An almost perfect correlation (Fig. 3F; Pearson’s $r = 0.986, p < 0.001$) and a regression line with slope 0.982 and intercept at 0.019 demonstrate that the calculated UGS is the same for serum and purified IgG, underscore the reliability of the assay for use in serum under the selected conditions.

EndoS Enables Direct Serum IgG Galactosylation Assessment—Classically, IgG Fc galactosylation levels have been determined through PNGaseF mediated deglycosylation of purified whole IgG. There seems to be no consensus on the choice of glycans for calculating the level of galactosylation. Often, the peak height or area of NGA2F (also known as G0F, sometimes just G0) is used, most often normalized to the total signal or to the monogalactosylated glycan peak NG1A2F (G1F, sometimes G1) (33, 34). Another approach is to take the contribution of the galactosylation status of individual antennae on all glycans in the profile into account (35). In most cases, Fab fragment glycans also contribute to the calculation to some extent as PNGase is not specific for the Fc-linked N-glycan. On the other hand, under the conditions of our assay, endoS specifically cleaves N-glycans from IgG Fc fragments and we cannot detect any release of Fab glycans. As a result, the measured undergalactosylation score (UGS) may not be exactly equal to that obtained from a PNGaseF digest. Given that only 15–25% of Fab fragments are glycosylated, they contribute much less to the total IgG glycan content than do Fc fragments (100% glycosylated). In other words, Fab glycosylation is not expected to be a major confounder. However, because the UGS calculated from endoS profiles is expected to be insensitive to Fab glycosylation, it should be an even better approach for determining UGS. For the comparisons presented here, we decided to use the G0F level normalized to the total glycan signal for PNGaseF derived profiles, as this appears to be the most commonly used way of calculating galactosylation levels (supplemental Fig. S2).

To assess the performance of our assay on patient serum samples, we analyzed IgG glycosylation in a total cohort of 188 patients and healthy controls with varying levels of galactosylation. We calculated the UGS from profiles obtained by either treating whole serum with endoS (and sialidase) or treating IgG purified from serum with PNGaseF and sialidase. We found a very good correlation between the two methods irrespective of the use of sialidase (Fig. 4A; no sialidase, Pearson’s $r = 0.945, p < 0.001$ and Fig. 4B; sialidase, Pearson’s $r = 0.951, p < 0.001$).

Data points that we identified as outliers in a residuals analysis (studentized residuals $> 3$) are indicated in red and represent the same samples in both experiments. Two outliers can be attributed to suboptimal IgG purification: the PNGaseF profiles from these samples contained relatively higher levels of triantennary glycan as compared with the rest of the cohort from which they were drawn (data not shown). Triantennary glycans are not present on IgG (36) and these glycans are thus considered to be an impurity from contaminating serum proteins. For these samples, it is likely that the UGS derived from PNGaseF profiles is incorrect. For the last outlier we found no straightforward explanation.

Taken together, these strong correlations show that the galactosylation state of IgG in serum can be reliably measured with endoS, without the need for IgG purification.

To demonstrate the use of the assay for disease stage assessment of patients with chronic inflammatory disease, we analyzed the subset of patients from the NASH cohort ($n = 63$), for whom histological liver data were available, which is needed for disease staging of chronic liver disease. We calculated the mean and 95% confidence intervals for the undergalactosylation scores in each group, both for PNGaseF-derived UGS from purified IgG (Fig. 5A) and for endoS-derived UGS (no sialidase) from serum (Fig. 5B). In patients with
histological features that indicate progression toward NASH, but no full-fledged NASH yet (“borderline NASH”), a trend toward higher values is already noticeable. As demonstrated by ROC analysis (Fig. 5C), endoS UGS (AUC = 0.819) can equally well distinguish NASH patients from non-NASH controls as PNGaseF derived UGS (AUC = 0.798).
In current clinical practice, inflammation is predominantly determined by measuring CRP values in serum. However, the short half-life (18 h) of CRP causes fluctuations daily. Therefore, CRP is only suitable to assess acute inflammation. For ESR, which is frequently used in routine clinical chemistry, the most restricting limitation is that it requires fresh blood. Moreover, it has several analytical interferences that may confound the results (14). Hence, there is an unmet need for a marker that measures cumulative exposure to inflammation.

Changes in IgG Fc glycosylation are uniquely suitable for monitoring chronic inflammation because IgG has a half-life of 24 days. The inflammatory processes that lead to IgG glycosylation changes need to persist for at least one to two t_{1/2} of the IgG pool to become noticeable. Since the first report in the context of RA in 1985 (1), many publications reported a decrease in IgG galactosylation in most chronic necro-inflammatory diseases (2–9) and recently very large studies have been reported on this glycosylation change (37, 38). In these same studies, increases in IgG Fc glycan core fucosylation have also been associated with increased chronic inflammation in the context of specific diseases (38).

So far, all assays require IgG purification followed by complex analytics, making the biomarker rather unsuitable for routine clinical chemistry.

Here we used endoglycosidase S from a pathogenic bacterium to cleave the N-glycans of IgG Fc. In this context, we should note that, because of the specific action of endoS, information on Fc glycan core fucosylation is lost, as this part of the glycan is left on the protein backbone.

We showed that, under optimized conditions, this enzyme is sufficiently specific that it can be used in a highly complex sample such as serum. We found that the enzyme’s specificity depends on the pH of the reaction buffer. In the pH range of 5.0–8.0, endoS is not entirely IgG specific, as it also releases some IgA N-glycans and minute amounts of IgM N-glycans. The specificity of endoS for IgG Fc drops with increasingly lower pH values of the buffer. However, under optimal conditions (at pH 8.5 or higher) we only detected glycans that were derived from IgG Fc fragments.

Whole IgG UGS measurements by PNGaseF strongly rely on the purity of the IgG, which inherently is a potential problem during sample preparation. Moreover, because Fab and Fc glycans are distinct but overlapping, contribution of Fab N-glycans can never be completely excluded as a confounding factor, even when using purified IgG. Because of its specificity for IgG Fc under the assay conditions, UGS assessed by endoS should be more reliable than UGS from PNGaseF profiles of purified IgG.

By comparing endoS profiles of serum and purified IgG in a cohort of patients and healthy controls, we confirmed that the outcome of the endoS-based assay is not dependent on the presence of other glycoproteins: we found an almost identical outcome for both methods.

How does the fact that Fc glycans carrying a bisecting GlcNAc are resistant to endoS hydrolysis influence the outcome of our assay? Because these glycans are not part of the UGS calculations from endoS profiles, but they are in PNGaseF-based UGS calculations, one might expect that they change the correlation between PNGaseF-based and endoS-based UGS calculations. However, IgG undergalactosylation of glycans with and without bisecting GlcNAc is strongly correlated so removing these glycans from the UGS determination does not change the validity of the assay method (supplemental Fig. S9).

Our new endoS-based assay thus represents an important advance toward the clinical implementation of the well-studied IgG undergalactosylation as a correlate of chronic inflammation. It encompasses a fast and simple, Fc-glycan specific sample preparation and subsequent analysis on high-throughput CE analyzers, e.g., on capillary DNA sequencers. The analytical step can also be readily adapted to cheap CE-based microfluidics platforms as we have shown before (25) and indeed, as we are currently working on for similar assays, on clinical CE analyzers (39), paving the road for implementation in clinical chemistry routine. We envisage that this new assay will find use in diagnosis of the presence of chronic inflammation (e.g., as it accompanies the senescence processes associated with old age) and in monitoring of responses to anti-inflammatory drug treatments.

* This work was supported by the FP7 Ideas: European Research Council (grant number ERC-2013-CoG-616966).
** To whom correspondence should be addressed: Unit for Medical Biotechnology, VIB-UGhent University Center for Medical Biotechnology, Technologielpark 927, B-9052 Gent-Zwijnaarde. Tel.: +32-9-331-36-30; Fax: +32-9-331-36-09; E-mail: Nico.Callewaert@ugent.vib.be.

This article contains supplemental material.
* Authors contributed equally.
** Authors contributions: D.V., L.M., T.R., H.G., and N.C. designed research; D.V., L.M., T.R., H.G., and A.V.H. performed research; D.V., L.M., T.R., H.G., and N.C. analyzed data; D.V., L.M., T.R., H.G., and N.C. wrote the paper; X.V., F.V.d.V., B.L., and H.V.V. contributed new research; D.V., L.M., T.R., H.G., and A.V.H. performed research; D.V., L.M., T.R., H.G., and A.V.H. contributed new reagents/analytic tools.

REFERENCES
1. Parekh, R. B., Dwek, R. A., Sutton, B. J., Fernandes, D. L., Leung, A., Stanworth, D., Rademacher, T. W., Mizuochi, T., Taniguchi, T., Matsuta, K., Takeuchi, F., Nagano, Y., Miyamoto, T., and Kobata, A. (1985) Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. Nature 316, 452–457
2. Axford, J. S., Mackenzie, L., Lydyard, P. M., Hay, F. C., Isenberg, D. A., and Rott, I. M. (1987) Reduced B-cell galactosyltransferase activity in rheumatoid arthritis. Lancet 2, 1486–1488
3. Saldova, R., Wormold, M. R., Dwek, R. A., and Rudd, P. M. (2008) Glycosylation changes on serum glycoproteins in ovarian cancer may contribute to disease pathogenesis. Dis. Markers 25, 219–232
4. Ren, S., Zhang, Z., Xu, C., Guo, L., Lu, R., Sun, Y., Guo, J., Qin, R., Qin, W., and Gu, J. (2016) Distribution of IgG galactosylation as a promising
Fast and Direct Assessment of IgG Galactosylation In Serum

5. Go, M. F., Schroenholzer, R. E., and Tomana, M. (1994) Deficient galactosylation of serum IgG in inflammatory bowel disease: correlation with disease activity. J. Clin. Gastroenterol. 18, 86–87

6. Theodoratou, E., Campbell, H., Ventham, N. T., Kolarich, D., Pucil-Baković, M., Zoldić, V., Fernandes, D., Pemberton, I. K., Rudan, I., Kennedy, N. A., Wuhler, M., Nimmo, E., Annesa, V., McGovern, D. P. B., Satsangi, J., and Lauc, G. (2014) The role of glycosylation in IBD. Nat. Rev. Gastroenterol. Hepatol. 11, 588–600

7. Tomana, M., Schroenholzer, R. E., Reveille, J. D., Arnett, F. C., and Koopman, W. J. (1992) Abnormal galactosylation of serum IgG in patients with systemic lupus erythematosus and members of families with high frequency of autoimmune diseases. Rheumatol. Int. 12, 191–194

8. Collin, M., Van Vlierberghe, H., Van Hecke, A., Laroy, W., Delanghe, J., and Contreras, R. (2004) Noninvasive diagnosis of liver cirrhosis using DNA sequence- based total serum protein glyconomics. Nat. Med. 10, 429–434

9. Blomme, B., Van Steenkiste, C., Grassi, P., Haslam, S. M., Dell, A., Callewaert, N., and Van Vlierberghe, H. (2011) Alterations of serum protein N-glycosylation in two mouse models of chronic liver disease are hepatic and not B cell driven. Am. J. Physiol.-Gastrointest. Liver Physiol. 300, G833–G842

10. Blomme, B., Franque, S., Trepo, E., Libbrecht, L., Vanderschaeghe, D., Callewaert, N., and Van Vlierberghe, H. (2012) N-glycan based biomarker distinguishing non-alcoholic steatohepatitis from steatosis independently of fibrosis. J. Hepatol. 57, 1322–1330

11. Van Beneden, K., Coppetières, K., Laroy, W., De Keyser, F., Hoffman, I. E., Van den Bosch, F., Cruyssen, B. V., Drennan, M., Jacques, P., Rottiers, P., Verbruggen, G., Contreras, R., Callewaert, N., and Elewaat, D. (2009) Reversible changes in serum immunoglobulin galactosylation during the immune response and treatment of inflammatory autoimmune arthritis. Ann. Rheum. Dis. 68, 1360–1365

12. Gildzietówna-Siekielkiewicz, E., Radziejewska, I., Domysławska, I., Klimiuk, T., Blomme, B., Francque, S., Trepo, E., Libbrecht, L., Vanderschaeghe, D., Callewaert, N., and Van Vlierberghe, H. (2011) Alterations of serum protein N-glycosylation in two mouse models of chronic liver disease are hepatic and not B cell driven. Am. J. Physiol.-Gastrointest. Liver Physiol. 300, G833–G842

13. Ho, C.-H., Chien, R.-N., Cheng, P.-N., Liu, J.-H., Liu, C.-K., Su, C.-S., Wu, I.-C., Li, C.-I., Tsai, H.-W., Wu, S.-L., Liu, W.-C., Chen, S.-H., and Chang, T.-T. (2015) Aberrant serum immunoglobulin G glycosylation in chronic hepatitis B is associated with histological liver damage and reversible by antiviral therapy. J. Hepatol. 62, 111–117

14. Siemons, L. M., Fernandez-Merino, C., and Vidal, C. (2008) High level plasma N-glycome profiling using multipleplex labelling and UPLC with fluorescence detection. Analyst 133, 4670–4673

15. O'Flaherty, R., Trbojević-Akmacić, I., Greville, G., Rudd, P., and Lauc, G. (2011) High throughput plasma N-glycome profiling using multiplexed labelling and UPLC with fluorescence detection. Analyst 136, 2358–2363

16. Knežević, A., Bones, J., Krčun, S. K., Gornik, O., Rudd, P. M., and Lauc, G. (2011) High throughput plasma N-glycome profiling using multiplexed labelling and UPLC with fluorescence detection. Analyst 136, 4670–4673

17. Wang, T., Hoi, K. M., Stöckmann, H., Wan, C., Sim, L. C., Shi Jie Tay, N. H. B. K., Poo, C. H., Woen, S., Yang, Y., Zhang, P., and Rudd, P. M. (2018) LC/MS-based intact IgG and released glycan analysis for biotherapeutic glycoproteins. Analyst 143, 573–582

18. Stöckmann, H., Adamczyk, B., Hayes, J., and Rudd, P. M. (2013) Automated, high-throughput IgG-antibody glyco profiling platform. Anal. Chem. 85, 8841–8849

19. Collin, M., and Olsén, A. (2001) EndoS, a novel secreted protein from Streptococcus pyogenes with endoglycosidase activity on human IgG. EMBO J. 20, 3046–3055

20. Trastoy, B., Lomino, J. V., Pierce, B. G., Carter, L. G., Günther, S., Giddens, J. P., Snyder, G. A., Weiss, T. M., Weng, Z., Wang, L.-X., and Sundberg, E. J. (2014) Crystal structure of Streptococcus pyogenes EndoS, an immunomodulatory endoglycosidase specific for human IgG antibodies. Proc. Natl. Acad. Sci. 111, 6714–6719

21. Collin, M., and Olsén, A. (2001) Effect of SpeB and EndoS from Streptococcus pyogenes on human immunoglobulins. Infect. Immun. 69, 7178–7189

22. Huang, W., Giddens, J., Fan, S.-Q., Toonstra, C., and Wang, L.-X. (2012) Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. J. Am. Chem. Soc. 134, 12308–12318

23. Goodfellow, J. J., Baruah, K., Yamamoto, K., Bonomelli, C., Krishna, B., Harvey, D., Kao, D., Crispin, M., Scanlan, C. N., and Davis, B. G. (2012) An endoglycosidase with alternative glycan specificity broadens glycoprotein remodelling. J. Am. Chem. Soc. 134, 8030–8033

24. Goetze, A. M., Zhang, Z., Liu, L., Jacobsen, F. W., and Flynn, G. C. (2011) Rapid LC-MS screening for IgG Fc modifications and allelic variants in blood. Mol. Immunol. 49, 338–352

25. Vanderschaeghe, D., Szekrényes, A., Wenz, C., Gassmann, M., Naik, B., and Yiu, H., Delanghe, J., Guttmann, A., and Callewaert, N. (2014) High-throughput profiling of the serum N-glycome on capillary electrophoresis-microfluidics systems: toward clinical implementation of GlycoHepatoTest. Anal. Chem. 82, 7408–7415

26. Bondt, A., Rombouts, Y., Selman, M. H. J., Hensbergen, P. J., Reiding, K. R., Hazes, J. M. W., Dolhain, R. J. E. M., and Wuhler, M. (2014) Immunoglobulin G (IgG) Fab glycosylation analysis using a new mass spectrometric high-throughput screening method reveals pregnancy- associated changes. Mol. Cell. Proteomics 13, 3029–3039

27. Vanderschaeghe, D., Laroy, W., Sablon, E., Halton, P., Hecke, A., Delanghe, J., and Callewaert, N. (2009) GlycoFibroTest is a highly performant liver fibrosis biomarker derived from DNA sequence-based serum protein glycomics. Mol. Cell. Proteomics 8, 986–994

28. González-Quintela, A., Alende, R., Gude, F., Campos, J., Rey, J., Meijide, L. M., Fernandez-Merino, C., and Vidal, C. (2008) Serum levels of immunoglobulin G1 Fc N-glycan are related with pregnancy complications. J. Biomed. Biotechnol. 2008, 86837

29. Kao, D., Lux, A., Schaffert, A., Lang, R., Altmann, F., and Nimmerjahn, F. (2017) IgG subclass and vaccination stimulus determine changes in antigen specific antibody glycan diversity in mice. Eur. J. Immunol. 47, 2070–2079

30. Jeong, S. E., Selman, M. H. J., Aedeignka, A. A., Amoaoh, A. S., Van Riet, E., Kruize, Y. C. M., Raynes, J. G., Rodriguez, A., Boakye, D., von Mutius, E., Knulst, A. C., Gennetin, J., Cooper, P. J., Hokke, C. H., Wuhler, M., and Yazdanbakhah, M. (2016) IgG1 Fc N-glycan glycosylation as a biomarker for immune activation. Sci. Rep. 6, 28207

31. Wuhler, M., Stamat J. C., van de Geijn, F. E., Koeleman, C. A. M., Verrips, C. T., Dolhain, R. J. E. M., Hokke, C. H., and Deelder, A. M. (2007) Glycosylation profiling of immunoglobulin G (IgG) subclasses from human serum. Proteomics 7, 4070–4081
37. Šimurina, M, de Haan Vučković, N., Kennedy, F., NAStambuk Falck, J., Trbojević-Akmačić, D., Clerc, I., Razdorov, F., Khon, G., Latiano, A., D'Inca, A., Danese, R., Targan, S., Landers, S., Dubinsky, C., Inflammatory Bowel Disease Biomarkers Consortium, M., McGovern Annese, D. P. B., Wuhrer, V., and Mand Lauc, G. (2018) Glycosylation of immunoglobulin G associates with clinical features of inflammatory bowel diseases. Gastroenterol. 154, 1320–1333

38. Plomp, R., Ruhaak, L. R., Uh, H.-W., Reiding, K. R., Selman, M., Houwing-Duistermaat, J. J., Slagboom, P. E., Beekman, M., and Wuhrer, M. (2017) Subclass-specific IgG glycosylation is associated with markers of inflammation and metabolic health. Sci. Rep. 7, 12325

39. Helena Biosciences Europe. Glyco Liver Profile. http://www.helena-biosciences.com/en/clinical-electrophoresis/capillary/glyco-liver-profile (Accessed February 2018)