Clinical assay for direct assessment of IgG galactosylation in serum using endoglycosidase S.

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Abbreviations

APTS: 8-Aminopyrene-1,3,6-trisulfonic acid

CE: Capillary electrophoresis

CE-LIF: Capillary electrophoresis – laser-induced fluorescence

CI: confidence interval

CRP: C-reactive protein

CV: column volume

EndoS: endo-β-N-acetyl-glucosaminidase S from *Streptococcus pyogenes* = Endoglycosidase S

ESR: erythrocyte sedimentation rate

GlcNAc: N-acetylglucosamine

GNT: GlycoNASHTest

HBV: Hepatitis B virus

IgG: immunoglobulin G

K18F: caspase cleaved cytokeratin-18 fragments

MS: mass spectrometry

NAFLD: non-alcoholic fatty liver disease

NAS: NAFLD activity score

NASH: non-alcoholic steatohepatitis

PNGaseF: Peptide N-glycosidase from *Elizabethkingia meningoseptica* (previously *Flavobacterium meningosepticum)*

RA: rheumatoid arthritis

UGS: UnderGalactosylation Score

UPLC: ultra-performance liquid chromatography

Abstract

A wide spectrum of (autoimmune) diseases such as rheumatoid arthritis (RA), Crohn's disease, systemic lupus erythematosus, but also other pathologies such as alcoholic and non-alcoholic steatohepatitis (ASH and NASH) are driven by chronic inflammation and hallmarked by a reduced level of serum Immunoglobulin G (IgG) galactosylation. IgG undergalactosylation is a promising biomarker to assess the severity of disease, monitor therapy efficacy and adjust therapy accordingly. The main hurdle for clinical implementation is the necessity for purifying the antibodies in order to specifically determine IgG Fc glycan galactosylation. We addressed this issue by using endo-β-N-acetylglucosaminidase from *Streptococcus pyogenes* (endoS) and optimized the reaction conditions in which the enzyme specifically releases the IgG Fc N-glycans in serum. The sample preparation takes two hours and only entails the addition of endoS to serum and subsequent labeling with the fluorophore 8-aminopyrene-1,3,6-trisulphonic acid (APTS). Samples are then readily analyzed on high-throughput DNA sequencers, which are able to analyze up to 96 samples in one hour. We demonstrate in two separate patient cohorts that the degree of IgG undergalactosylation from our assay correlates very well with undergalactosylation scores calculated from N-glycan profiles derived from antibodies that have been purified from serum and deglycosylated with peptide N-glycosidase from *Flavobacterium meningosepticum* (PNGaseF). The presented new assay thus allows to directly and specifically measure the degree of IgG galactosylation in serum without the requirement of isolating the antibodies, and should help in advancing this biomarker towards clinical implementation.

Introduction

Immunoglobulin G (IgG) glycosylation has been studied for more than 30 years, revealing how the glycan composition is altered in patients suffering from chronic inflammatory diseases. Early studies presented that the IgG glycosylation pattern is affected in individuals with rheumatoid arthritis (RA) or primary osteoarthritis[1], showing lowered β-1,4-galactosylation as compared to the N-glycans of healthy controls[1,2]. Since then, other (chronic) inflammatory diseases have been shown to be associated with IgG undergalactosylation (UG), 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) chronic liver disease 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 in developed countries[10]. Many drugs for treatment of chronic inflammatory diseases are extremely expensive (e.g. anti-TNF biologicals) and come with significant side effects. A biomarker for low grade chronic systemic inflammation and objective treatment efficacy monitoring would therefore contribute to improved clinical practice.

Despite the numerous publications presenting IgG undergalactosylation as a promising biomarker in these patients, current clinical tests are still restricted to either measuring C-reactive protein (CRP) concentration in serum or to historical tests such as the erythrocyte sedimentation rate (ESR). CRP levels fluctuate on a daily basis. Therefore, it is not a suitable marker for measuring chronic disease activity. ESR is a cheap test that is routinely conducted at high throughput, but is confounded by several interfering factors[14].

Analyzing IgG glycosylation has so far not been feasible in a clinical context due to the complexity and time-consuming procedure that is required. Purifying the antibodies from patients' sera is a prerequisite and is then usually followed a denaturation step and Peptide N-glycosidase F (PNGaseF) deglycosylation to assess the sample N-glycosylation profile by MS, UPLC or CE. The mandatory purification step is difficult to implement in routine testing and insufficient purity of the antibodies affects the outcome of the test. Attempts to facilitate the purification process have recently resulted in the development of an automated high-throughput IgG purification and N-glycan sample prep platform[15]. Although promising, this method requires an expensive robotic and UPLC system, and sample preparation and analysis for 96 samples takes at least 30 hours, making its clinical implementation difficult.

In 2001, Collin and Olsén[16] described an endoglycosidase which is secreted by the human pathogen *Streptococcus pyogenes*, designated endoS. The natural function of endoS is to suppress IgG effector functions by specifically hydrolyzing IgG Fc glycans. Of note, it hydrolyzes the N-glycan chitobiose core, i.e. N-acetylglucosamine (GlcNAc)-GlcNAc, leaving a single GlcNAc residue on the IgG heavy chain[17]. EndoS reportedly requires a natively folded IgG[18] for its activity and only cleaves the Fc-linked glycans while leaving the N-glycans of the Fab domain and other proteins intact[19]. Although it is generally accepted that endoS is specific for IgG Fc fragments, activity towards HIV gp120 has also been reported[20]. Moreover, the specificity of endoS has never been tested 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[21]. 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 assay in which we use endoS to specifically measure IgG galactosylation without the need for purifying the antibody, which is the main bottleneck for widespread clinical use of this biomarker.

Materials & Methods

Materials

Lyophilized recombinant endoS (branded as IgGZERO™) was purchased from Genovis and reconstituted in ultrapure water at 20 U/µl. PNGaseF from *Elizabethkingia meningoseptica* (previously *Flavobacterium meningosepticum*) and sialidase from *Arthrobacter ureafaciens* were recombinantly produced as described by Vanderschaeghe *et al.*[22]. Purified, lyophilized IgG, IgA and IgM, isolated from human serum (purity > 95%) were obtained from Sigma-Aldrich and reconstituted in PBS at 10 mg/ml (hereafter named 'commercial IgG, IgA and IgM').

Patients

Serum samples from two independent cohorts[10,23] (n = 92, n = 96, total n = 188) in which patients were enlisted for bariatric surgery, were obtained from an outpatient clinic (Ghent University Hospital) and stored at -80°C until sample preparation. Informed consent was given by all patients and the protocol was approved by the Ghent University Hospital's Ethics Committee.

IgG purification and serum IgG depletion

IgG from 30 µl aliquots of (patient) sera was purified using a Protein G Spin Plate for IgG Screening (Thermo Scientific) according to the manufacturer's instructions. The

purified samples were eluted in a final volume of 420 µl and concentrated in combination with buffer exchange to PBS with Amicon® Ultra 0.5 ml Centrifugal Filter Devices with a 100 kDa cut-off (Merck Millipore).

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

EndoS or PNGaseF catalysed release of N-glycans

N-glycans were released with either endoS or PNGaseF. For the endoS catalysed release of glycans, 2.5 µl of serum or purified IgG was incubated with 25 U of endoS for one hour at 37°C in a total reaction volume of 10 µl in 150 mM NaCl and 50 mM Tris-HCl pH 8.5.

For PNGaseF catalysed N-glycan release from serum or purified IgG, 3 µl of each sample was first denatured by the addition of 2 µl of denaturing buffer (50 mM Tris-HCl pH 8.5 containing 3.5% SDS), followed by incubation at 95°C for 5 minutes. The samples were then cooled on ice for 5 minutes and incubated for 1 hour at 37°C with 5 µl of a reaction mixture containing 11 mU PNGaseF in 50 mM Tris-HCl pH 8.5 and 2% NP40.

IgG specificity of endoS

To determine the protein specificity of endoS (i.e. does endoS only hydrolyze IgG Fc N-glycans?), serum and IgG-depleted serum were incubated with 25 U of endoS for 1 hour at 37°C in buffers with a pH ranging from pH 5.0 to pH 9.0: 50 mM NH_4Ac (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 150 mM 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 filtering the supernatant through a 0.22 µM filter. The filtered sample was then applied three times over a PBS-equilibrated 0.5 ml peptide M agarose (invivogen) in a 10 ml gravity flow column. The flow through after the third round was collected as IgA-depleted plasma. Then, the column was washed with 20 CV's of PBS and the IgA was eluted with 2.5 ml of 200 mM glycine pH 2.5. The IgA fraction was neutralized with 2.5 ml of 1 M Tris-Cl buffer pH 8.5.

The IgA was then buffer exchanged to PBS and the sample volume reduced to 500 µl with an Amicon 30 kDa centrifugal filter device, the same was done for the IgA-depleted fraction, but with a 3 kDa centrifugal device.

To determine whether endoS also hydrolyses IgA or IgM glycans, 2.5 µl of 10 mg/ml solutions of commercial IgA, IgM or IgG and 2.5 µl samples of IgA purified from patient plasma and IgA-depleted plasma were digested with 25 U of endoS for 1 hour at 37°C in a total reaction volume of 10 µl in 150 mM NaCl and 50 mM NH_4Ac pH 5.0 or in 150 mM NaCl and 50 mM Tris-HCl pH 8.5.

Labelling, sialidase digest and CE analysis

Glycans that were released using any of the methods described above were fluorescently labelled with APTS and analyzed by capillary electrophoresis. APTS-derivatization, removal of excess label, exoglycosidase digests (optional) and capillary electrophoresis were performed as previously described by Laroy *et al.*[24]. Briefly, 5 µl of labelling solution was added to an equal volume of crude digest or dry samples. The

standard labelling reaction was incubated overnight at 37°C and the excess label was removed by size-exclusion chromatography. The labelled N-glycans were then optionally desialylated by the addition of 0.3 mU (IU) sialidase in 2.5 µl of 20 mM NH_4Ac pH 5.0. The sialidase solution was added to an equal volume of clean labelled glycan sample and incubated for 7 hours at 37°C. At this point, samples (native or sialidase treated) were either analyzed by capillary electrophoresis on an ABI3130 DNA sequencer or further processed for exoglycosidase digests, as previously described[24]. When required, samples were diluted in ultrapure water before CE analysis to keep the signal within the dynamic range of the detector.

Glycan symbolic representations are formatted according to the Consortium for Functional Glycomics guidelines (http://www.functionalglycomics.org/). All glycan structures, nomenclature and symbolic representation are summarized in Supplementary Figure 1.

Calculations and statistics

The height of all relevant peaks from the CE profiles was quantified with GeneMapper® v3.7 (Applied Biosystems). The level of agalacto glycans or undergalactosylation score from PNGaseF profiles was calculated as the level of the NGA2F (G0F) glycan normalized to the total peak height (Supplementary figure 2: PNGase UGS). The undergalactosylation score from endoS profiles was calculated as the sum of the peak heights of non-galactosylated glycan peaks normalized to the total peak height, taking into account the number of antennae for each glycan (Supplementary figure 2: endoS UGS).

All statistical analyses were performed with R v3.4. The total patient cohort (n = 188) was used to verify the correlation between endoS and PNGaseF results on IgG

galactosylation. Correlations were addressed with Pearson's r. Outliers from linear regression in the scatter plots were defined as having studentized residuals higher than 3 or lower than -3. The glycan dataset can be found in the Supplementary spreadsheet.

Results

EndoS serum assay and N-glycan profile characterization

To specifically analyse the IgG N-glycosylation in serum using endoS, we developed a new method based on the existing procedure for profiling complete serum N-glycosylation that we previously optimized[22] (Figure 1). In contrast to PNGaseF, which is commonly used to remove all N-glycans from denatured serum proteins (Figure 1, top), endoS activity requires native (dimeric) IgG Fc fragments. EndoS' activity on native IgG allows to omit the purification and denaturation steps (Figure 1, bottom). Only N-glycans from IgG Fc are expected to be released by endoS while leaving Fab fragment glycosylation and the other serum glycoproteins and unaffected.

To identify the glycan structures that are released by endoS, we analysed endoS-released N-glycans from serum of a healthy individual and from IgG that was purified from the same serum sample. The N-glycans were fluorescently labelled with APTS and analysed by capillary electrophoresis (Figure 2A and 2B). CE profiles of endoS samples revealed six major glycan peaks. Purified IgG, again from the same serum sample, was also digested with PNGaseF for full N-glycan profiling (Figure 2C).

Comparing digests on serum and IgG purified from the same serum sample showed that endoS not only cleaved the same N-glycans in both samples, but we also observed similar relative peak heights (Figure 2A and 2B). These results indicate the

feasibility of using endoS in serum for specific analysis of IgG glycosylation, obviating the need for IgG purification. Since endoS hydrolyses N-glycans in their chitobiose core, all the structures are truncated at the reducing end as compared to PNGaseF-released N-glycans, which hydrolyses the amide bond between the N-glycan and the asparagine side chain amine.

All endoS-released N-glycan structures were confirmed with exoglycosidase digests and subsequent CE analysis of the resulting fragments (Supplementary Figure 3). 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[25]. This is consistent with earlier reports that endoS does not hydrolyse glycans with a bisecting GlcNAc[21].

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 (Supplementary Figure 4). This revealed that Fc glycans with a bisecting GlcNAc (peaks indicated in red in Figure 2C) indeed remain intact after endoS treatment. Bisialylated N-glycans (peaks indicated in red in Figure 2C) are not released by endoS, but are originating almost exclusively from the Fab fragments (Supplementary Figure 4), which are no substrate for endoS. The lack of bisialylated N-glycans in the endoS profiles is consistent with reports that less than 0.5% of Fc glycans are bisialylated[25]. Taken together, these observations support the commonly accepted view that endoS is specific for Fc, and that glycans with a bisecting GlcNAc are resistant.

IgG specificity of endoS in serum

To assess whether endoS only cleaves N-glycans from IgG in serum, we performed endoS digests on IgG-depleted serum. We could previously demonstrate that up to 99% of the IgG is removed by this procedure, without affecting other serum proteins[26]. Treating plasma or freshly prepared serum samples with endoS in different pH conditions (pH 5.0 – 9.0), revealed the release of an increasing amount of bi- and monosialylated N-glycans from serum proteins other than IgG with decreasing buffer pH (Figure 3A). This was not the case when using plasma or serum that had been freeze-thawed several times as the starting material (data not shown).

We verified the relevance of the non-IgG derived glycans by adding a physiological amount of commercial IgG (i.e. a final concentration of 10 µg/µL) to the depleted serum (Figure 3B). The relative abundance of non-IgG N-glycans in the profiles was prominent at pH 5.0 to 6.0, but as good as absent from pH 8.0 and higher. The identities of the two peaks were confirmed by exoglycosidase digests. They are bisialylated and monosialylated, fully galactosylated biantennary glycans.

Although endoS has been reported to be specific for IgG Fc, these experiments illustrate that under some conditions this is not the case. It is possible that the enzyme recognizes structurally similar substrates at suboptimal pH values. IgA and IgM are the two other immunoglobulins that are present in significant amounts in human serum at about 2,5 µg/µl for IgA and 1,5 µg/µl for IgM[27]. Moreover, the observed glycan types are typically present at high abundance in PNGaseF derived IgA glycan profiles, but only at low levels in IgG glycan profiles. Therefore, we assessed whether endoS is able to release IgA or IgM glycans at pH 5.0 and pH 8.5. We first confirmed that endoS profiles of (commercial) IgG are independent of the pH. Figure 3C indeed shows that identical profiles are obtained for IgG at pH 5.0 or 8.5. EndoS digests at pH 5.0 of IgA

purified from a fresh patient plasma sample (Figure 3D, left) produced the same two sialylated non-IgG glycans as we found in IgG-depleted serum at pH 5.0. These glycans were absent in the pH 8.5 profile of the purified IgA (Figure 3D, right). EndoS digests of commercial IgA gave the same result (data not shown). IgM at pH 5.0 but not at pH 8.5 also produced minute amounts of the monosialylated N-glycan (data not shown).

To further investigate the contribution of IgA glycans, we compared the glycan profiles of fresh whole and IgA-depleted patient samples (Figure 3E and 3F) 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 due to the presence of 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.

As a final point, we also confirmed that, under the 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 undergalactosylation. To do this, we performed an endoS digest on all the serum samples and compared this to purified IgG digested with endoS, from the same samples. An extremely high correlation (Figure 3G; Pearson's $r = 0.986$, $p < 0.001$) and a regression line with slope 0.982 and intercept at 0.019 indicate that the presence of serum glycoproteins does not influence the UGS outcome, underscoring the robustness of the assay in the selected conditions.

EndoS enables direct serum IgG galactosylation assessment

Classically, IgG Fc galactosylation levels have always been determined through PNGaseF and, depending on the analysis method, neuraminidase digested, purified whole IgG. There seems to be no general 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, either normalized to the total signal or to the monogalactosylated glycan peak NG1A2F (G1F, sometimes G1)[28,29]. Another approach is to take the contribution of all individual antennae on all glycans in the profile into account[30]. In most cases, Fab fragment glycans also contribute to the calculation to some extent. On the other hand, under the conditions of our assay, endoS specifically cleaves N-glycans from IgG Fc fragments, while leaving Fab glycans intact. As a result, the measured undergalactosylation score (UGS) might not be equal to that obtained from a PNGaseF digest. However, 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% of which are glycosylated. In other words, Fab glycosylation is not expected to be a major confounder and UGS calculated from endoS profiles should correlate well with scores calculated from PNGaseF derived profiles. For the comparisons presented here, we decided to use the G0F level normalized to the total glycan signal for PNGaseF derived profiles, as this seems to be the most prevalent way of calculating galactosylation levels.

To assess the performance of our assay, we analysed the IgG glycosylation in a cohort of 188 patients and healthy controls with varying levels of galactosylation. We calculated the UGS from profiles obtained by treating serum with endoS (and neuraminidase) or treating IgG purified from serum with PNGaseF and sialidase. We found a very good correlation between the two methods (Figure 4A; Pearson's r =

0.945, $p < 0.001$). When working with sialidase digested endoS glycans, the correlation is similar (Figure 4B; 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 in the figure (these are the same samples in both experiments). Out of these three outliers, two can be attributed to samples in which the IgG purification was suboptimal: the PNGaseF profiles from these samples contained relatively higher levels of triantennary glycan as compared to the rest of the cohort from which they were drawn. Triantennary glycans are not present on IgG and these glycans are thus considered to be an impurity from contaminating serum proteins. For these samples, it is thus likely that the galactosylation level determined from the 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 by our assay, without the need for IgG purification.

Discussion

In current clinical practice inflammation is predominantly determined by measuring C-reactive protein values in serum. The short half-life (18 hours) of CRP cause fluctuations on a daily basis. Therefore, CRP is only suitable for assessing acute inflammation. For ESR, which is routinely performed at very high frequency, 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.

IgG Fc glycosylation changes are uniquely suitable for monitoring chronic inflammation because IgG has a half-life of 24 days in humans. 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. Many publications over the last 30 years reported a decrease in IgG galactosylation in the vast majority of chronic necro-inflammatory diseases. So far, all available assays require purification of IgG followed by complex analytics, making the biomarker unsuitable for routine clinical chemistry use.

Here we used endoglycosidase S from a pathogenic bacterium to cleave the N-glycans of IgG Fc. We show that this enzyme can be used in a highly complex sample such as serum. In contrast to the generally accepted notion that endoS is specific for IgG Fc, we found that the enzyme's specificity in serum depends on the pH of the reaction buffer. It seems that in the pH range of 5.0-8.0, endoS is not entirely IgG specific, but can also release some IgA N-glycans and minute amounts of some IgM N-glycans. The specificity of endoS for IgG Fc drops with increasingly lower pH values of the buffer. However, under the conditions of our assay (at a pH of 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 the sample preparation. Moreover, since 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 conditions of our assay, UGS assessed by endoS should be more reliable than UGS from PNGaseF profiles of purified IgG.

We confirmed that the outcome of the endoS-based assay is not dependent on the presence of other glycoproteins by comparing endoS profiles of serum and purified

IgG in a cohort of patients and healthy controls and found an almost identical outcome for both methods.

When employing CE as an analysis method, the glycans are often treated with sialidase to obtain a profile that is easier to interpret and quantify. The endoS derived glycan profile is quite simple, rendering the use of sialidase for proper peak identification and quantification unnecessary. We also showed that neuraminidase treatment of endoS-released glycans does not change the outcome of our assay and therefore propose to omit it in the final protocol. This results in an even faster and cheaper workflow.

Our new endoS-based assay thus represents an important breakthrough towards the clinical implementation of the proposed biomarker. It encompasses a fast and simple, Fc-glycan specific sample preparation and subsequent analysis on high-throughput DNA sequencers. The DNA sequencer-based analysis method can also be readily adapted to cheap CE-based microfluidics platforms and clinical CE analysers, as we have shown before[22], paving the road for an easy clinical implementation.

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Competing interests statement

D.V., T.R. and N.C. disclose competing interests due to filing of a patent application.

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Figures and Legends

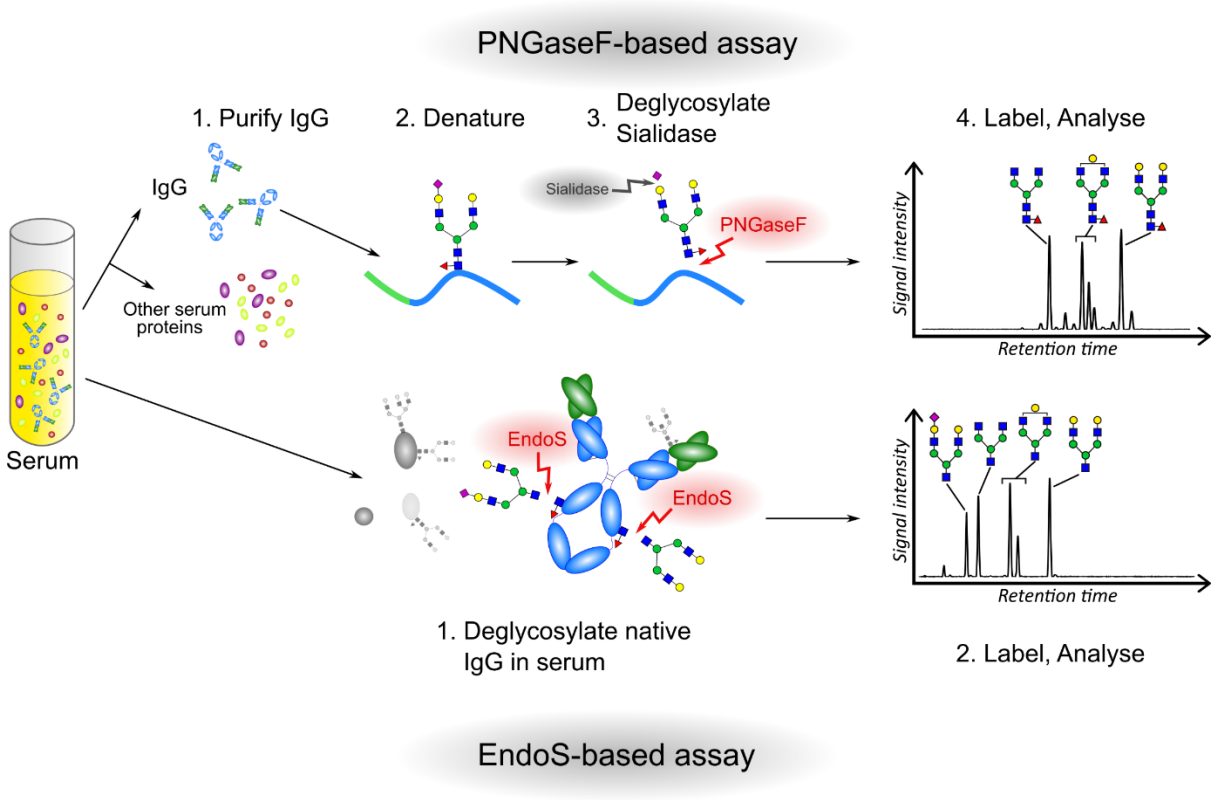

Figure 1: Overview of the assay. In traditional PNGaseF-based assays to assess IgG Fc glycan galactosylation levels, IgG has to be purified from serum, denatured and then deglycosylated (and optionally desialylated) before the glycans can be analysed. We here propose a new assay, based on the use of endoS, in which the purification and denaturation steps can be omitted, as well as the use of sialidase. Due to endoS' specificity for IgG Fc glycans on folded IgG, the resulting profile can be used to determine IgG Fc galactosylation levels.

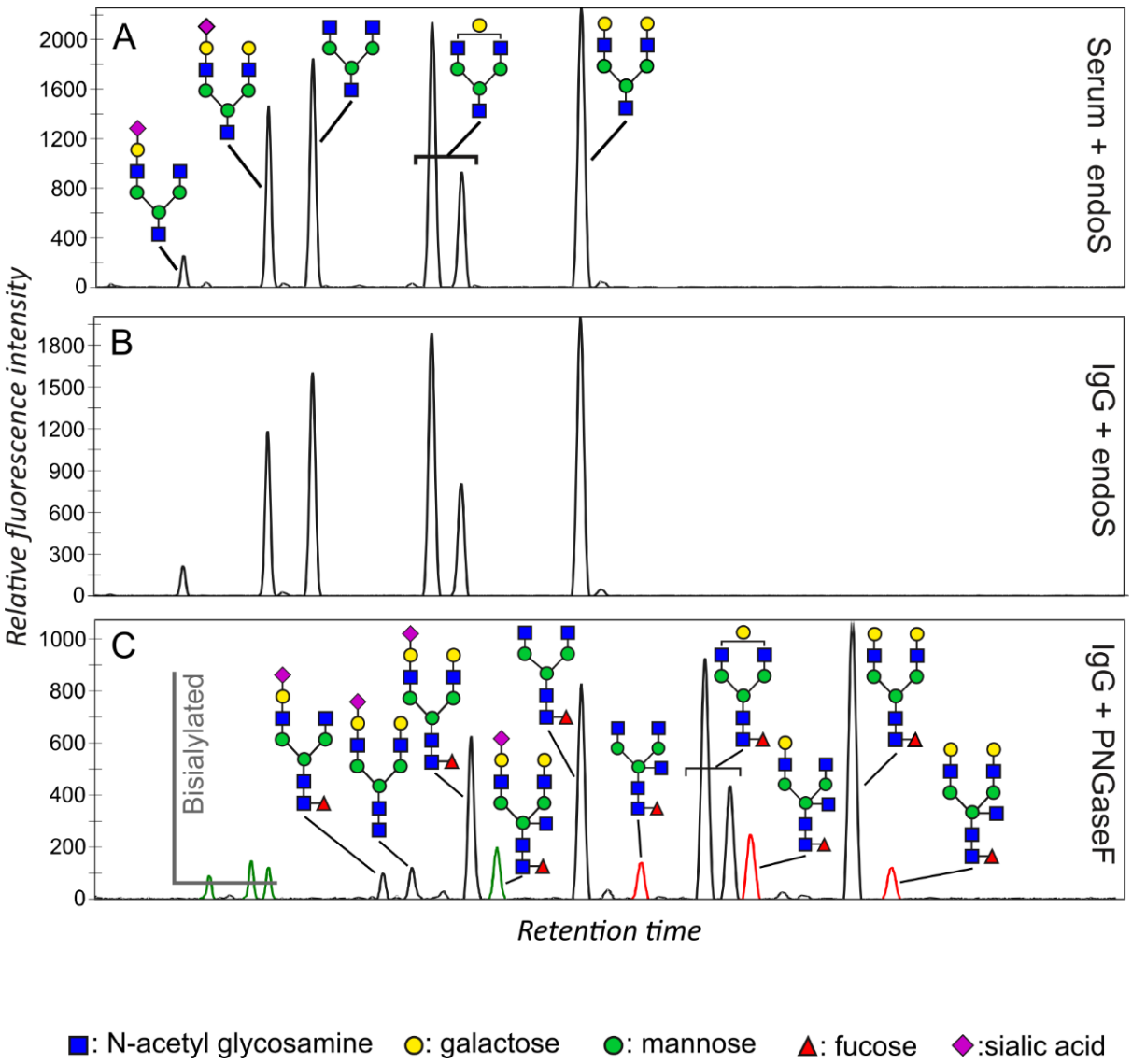

Figure 2: CE-LIF profiles of endoS and PNGaseF digested N-glycans. A) EndoS-released N-glycans from serum of a healthy individual. **B)** EndoS-released N-glycans from purified whole IgG of the same individual. **C)** PNGaseF-released glycans from purified whole IgG of the same individual. Peaks in red represent the bisecting GlcNAc-containing structures on the Fc fragment that are resistant to endoS hydrolysis. Bisialylated glycans and the bisected monosialylated N-glycan (indicated in green) are almost exclusively coming from Fab and were thus not hydrolysed by endoS (see Supplementary Figure 4 for additional data on the origin of the different glycan peaks).

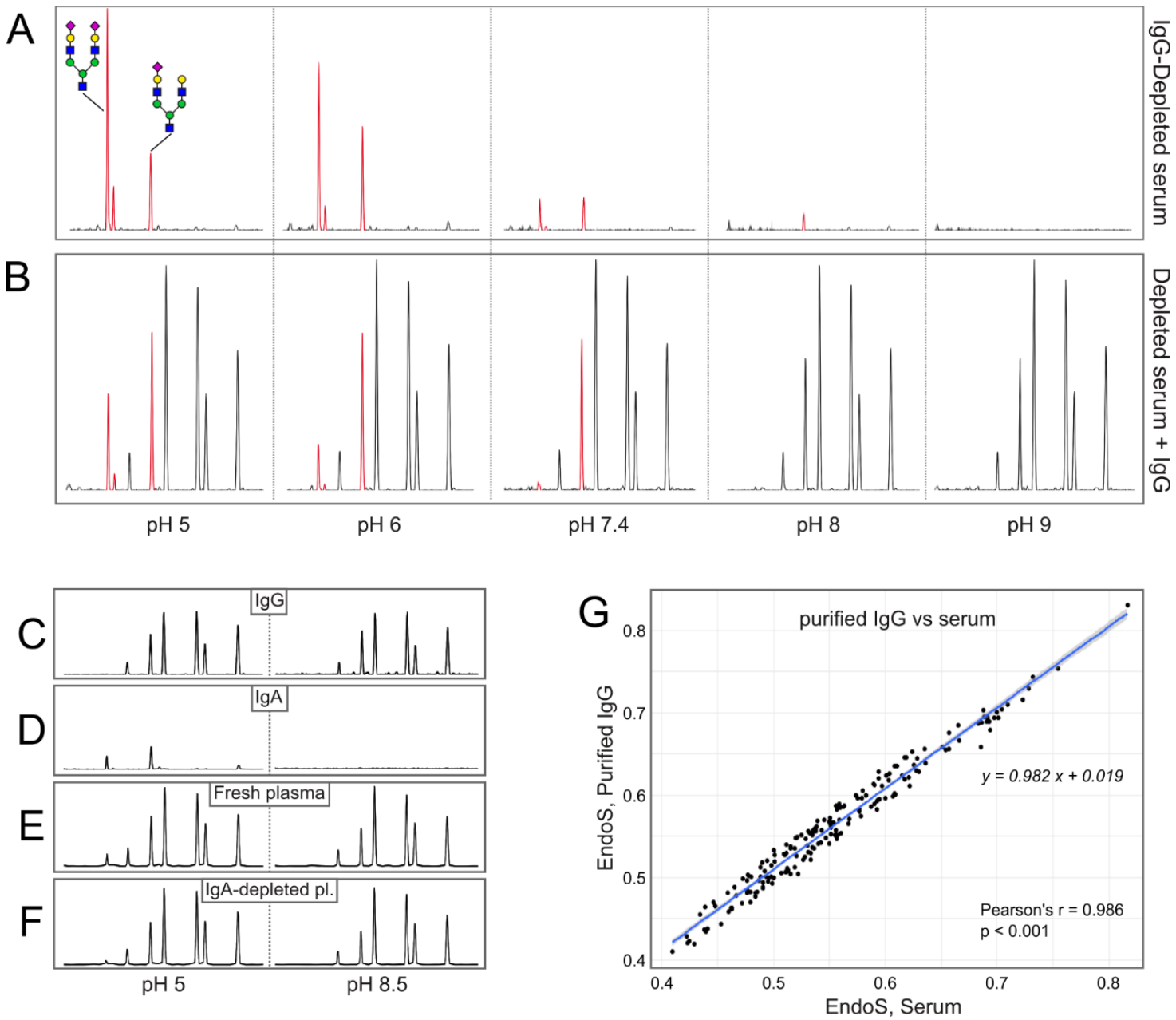
A
B
IgG-Depleted serum
Depleted serum + IgG
pH 5
pH 6
pH 7.4
pH 8
pH 9
C
D
E
F
IgG
IgA
Fresh plasma
IgA-depleted pl.
pH 5
pH 8.5
G
purified IgG vs serum
y = 0.982 x + 0.019
Pearson's r = 0.986
p < 0.001
EndoS, Purified IgG
EndoS, Serum
0.4
0.5
0.6
0.7
0.8

Figure 3: IgG specificity of EndoS in serum at different pH values. A) At pH values below 8.0, EndoS released bi- and monosialylated biantennary glycans from IgG-depleted serum samples. These glycans were not released from IgG-depleted serum samples at pH values above 8. **B)** When commercial IgG was added back to the depleted serum samples at physiological concentrations (10 µg/µl), it became apparent that the non-IgG glycans contributed considerably to the profile at pH values below 8, but not at higher pH values. **C)** The relative peak heights in the N-glycan profile of commercial IgG (purity > 95%) were independent of the assay buffer. **D)** Profiles of IgA, purified from patient samples. EndoS releases the same bi- and monosialylated biantennary N-glycans as found in IgG-depleted serum (Figure 3A) at pH 5.0. At pH 8.5, endoS does not hydrolyse any IgA glycans. **E)** Profiles of whole plasma from the same patient are pH dependent: at pH 8.5 a profile similar to the commercial IgG profile is observed. At pH 5.0, the profile contains IgG peaks as well as the same peaks as in the IgA profile and the depleted serum (Figure 3A and 3D). **F)** EndoS profiles of the same sample after IgA-depletion results similar profiles at either pH and also similar to the IgG profile. Note that 100 % IgA depletion is extremely difficult to achieve. Consequently, a small peak representing IgA-derived glycans can still be observed in the pH 5.0 profile of IgA-depleted samples. **G)** Serum and purified IgG from 188 patients and healthy controls were digested by endoS. The samples were labelled and analysed on an ABI3130 DNA sequencer. The UGS scores were calculated from the CE profiles. Scatterplots of undergalactosylation scores correlate very well for whole serum and IgG purified from the same serum samples, indicating that the presence of serum (glyco)proteins and other serum components do not change the assay outcome.

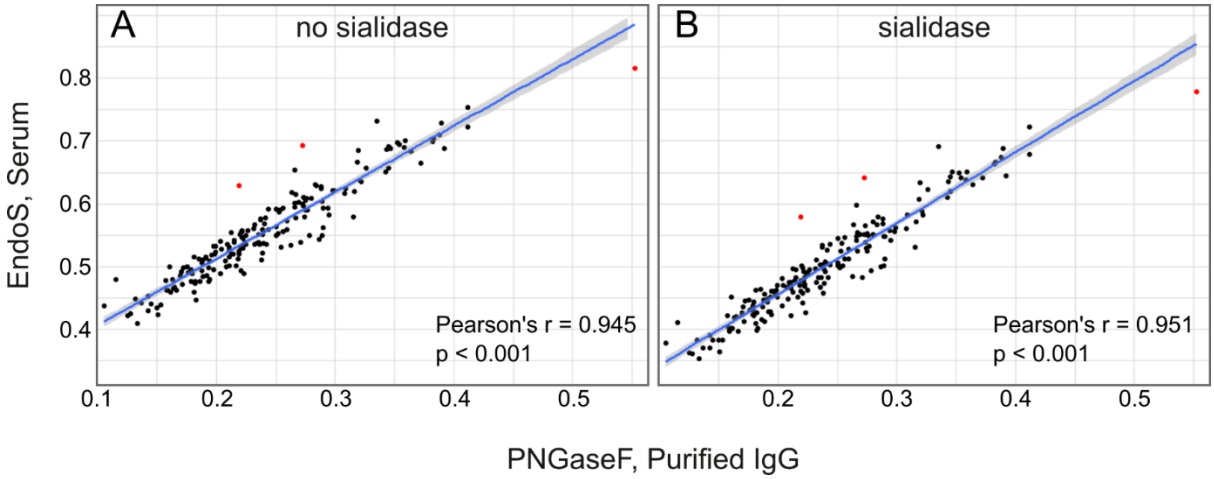

Figure 4: Correlation between galactosylation levels calculated from serum derived profiles versus purified IgG derived profiles. Serum and purified IgG from 188 patients and healthy controls were digested by endoS or PNGaseF. The samples were labelled and analysed on an ABI3130 DNA sequencer. The UGS scores were calculated from the CE profiles. Scatterplots of undergalactosylation scores are shown for endoS profiles of serum versus PNGaseF derived profiles for purified IgG from the same serum samples. **A)** undergalactosylation scores calculated from endoS-derived profiles versus PNGaseF-derived profiles. EndoS-derived glycans were not treated with sialidase. **B)** undergalactosylation scores calculated from endoS-derived profiles versus PNGaseF-derived profiles. EndoS-derived glycans were treated with sialidase prior to analysis.

Pearson's r correlation coefficients are shown in the right lower corner of all panels, along with p-values for each set. The linear regression lines (blue) with their 95% confidence intervals (grey) are also shown for each comparison.

The three datapoints depicted in red were identified as outliers by studentized residuals > 3. Two out of these represent samples in which the IgG purification was suboptimal.