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*Review*

# Antibody glycosylation as a potential biomarker for chronic inflammatory autoimmune diseases

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**Abstract:** Glycosylation of immunoglobulins (Ig) is known to influence their effector functions in physiological and pathological conditions. Changes in the glycosylation pattern of immunoglobulin G and autoantibodies in various inflammatory autoimmune diseases have been studied for many years. However, despite extensive research, many questions are still elusive regarding the formation of such differentially glycosylated antibodies and alterations of glycosylation patterns in other immunoglobulin classes for example. Nevertheless, knowledge has been deepened greatly, especially in the field of rheumatoid arthritis. Changes of Ig glycosylation patterns have been shown to appear before onset of the disease and moreover can subject to treatment. In this review, we discuss the potential of detecting Ig glycosylation changes as biomarkers for disease activity or monitoring of patients with chronic inflammatory autoimmune diseases such as antiphospholipid syndrome, rheumatoid arthritis, systemic lupus erythematosus, ANCA-associated vasculitis and Henoch-Schönlein purpura.

**Keywords:** Antibody; Glycosylation; Autoimmunity; Fucosylation; Sialylation; Galactosylation; SLE; RA; APS; ANCA-associated vasculitis; HPS; Cryoglobulins

## 1. Introduction

One of the hallmarks of chronic inflammatory autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), antiphospholipid syndrome (APS) and ANCA-associated vasculitis, is the presence of autoreactive immunoglobulin G (IgG) antibodies

recognizing self-antigens. The arising of disease-driving autoreactive IgG in SLE is thought to be fostered by an impaired clearance of dying cells resulting in a break of self-tolerance and the initiation of an autoimmune response [1]. The glycosylation of the Fc part of IgG is playing an important role in modulating its activity [2]. Additionally, the glycan of the Fc part of IgG can function either as a danger associated molecular pattern (DAMP) or a self-associated molecular pattern (SAMP) leading to an intensification or suppression of the autoreactive immune response, respectively [3]. Thus, analysis of the sugar composition of the glycan tree attached to the highly conserved asparagine 297 ($Asn^{297}$) on the Fc fragment is of major interest (N-glycosylation). Notably, the glycosylation pattern of IgG is not stable. Associations of changed glycosylation patterns and pathological as well as physiological conditions, such as inflammation or aging, have been reported [4-6]. Besides IgG, other immunoglobulin classes show changed glycosylation associated with pathological conditions. For example, altered O-glycosylation of immunoglobulin A1 (IgA1) is reported in Henoch-Schönlein purpura (HSP) with renal involvement and IgA nephropathy [7,8]. Immunoglobulin M (IgM) displays many glycosylation sites that might be potentially altered in autoimmune diseases. However, this needs further investigation.

Determining the exact composition of the glycan tree in the course of autoimmune diseases holds a high diagnostic potential. Here, we describe that profiling Ig glycosylation may serve as a biomarker for chronic inflammatory autoimmune diseases such as SLE, RA, APS, ANCA-associated vasculitis or HSP.

## 2. IgG Fc glycosylation

Among the different classes of antibodies, IgG is the most abundant protein in human serum accounting for about 10–20% of plasma proteins. IgG can be divided further into four subclasses in the order of decreasing abundance: IgG1, IgG2, IgG3 and IgG4 [9]. Each Fc fragment harbors a highly conserved N-glycosylation site at $Asn^{297}$ [10]. Additionally, the variable region of approximately 10–15% of all antibodies is glycosylated [11]. The glycosylation process starts in the endoplasmatic reticulum, but the complex processing of the glycan tree resulting in the addition of various sugar molecules such as fucose, galactose and sialic acid, mainly occurs in the Golgi apparatus [12]. The IgG Fab glycans reportedly affect antigen binding characteristics [13,14] and enable binding of regulatory lectins to IgG [15]. The binding affinity of IgG to Fcγ receptors (FcγR) is not only dependent on the IgG subclass [16], but also modulated by variable glycosylation of the Fc fragment [17]. Besides FcγRs, IgG also binds to the neonatal Fc receptor (FcRn) expressed by placental and intestinal epithelial cells [18]. This is thought to protect IgG from degradation, thereby increasing its half-life and helping to maintain high serum concentrations. However, binding of IgG to FcRn is strictly pH-dependent and the Fc glycans are not involved [19].

Several dozen IgG Fc glycoforms have been discovered so far in the serum of healthy controls. The most common are fucosylated species without galactose (IgG-G0), with one or two galactose residues (IgG-G1 or IgG-G2, respectively), or with two galactose and a single sialic acid residue (IgG-G2S1) [20]. It is generally accepted that the IgG Fc fragment is critical for complement activation and FcγR-mediated effector functions, e.g., the release of reactive oxygen species as well as chemokines and cytokines involved in establishing and maintaining tissue inflammation [21]. Importantly, the glycosylation patterns of IgG can alter during acute inflammation and aging resulting in an increase of IgG-G0 glycoforms [4,6]. Conversely, these G0 glycoforms decrease during pregnancy correlating with a reduction of inflammatory flares in pregnant women with

arthritis [22]. In the context of autoimmunity, IgG glycoforms lacking branched fucose residues display a 10- to 50-fold enhanced affinity for activating mouse FcγRIV and human FcγRIIIA, respectively [23]. Engaging these receptors, results in an enhanced cytotoxic or phagocytic activity [23].

## 3. IgG glycosylation in patients with APS

APS is an autoimmune disease that manifests clinically as recurrent venous or arterial thrombosis [24]. It is characterized by persistently elevated levels of autoreactive IgG recognizing the plasma protein β2-Glycoprotein 1 (β2GP1).

The affinity of autoantibodies present in patients with chronic inflammatory diseases is directly associated with their pathogenicity [25]. The affinity depends on distinct oligosaccharides attached to the N-glycosylation site of the autoantibody [26]. These oligosaccharides determine auto-antibody function and therefore their pathogenicity as they are able to modulate FcγR binding. We have recently shown that the pathogenicity of human anti-β2GP1 IgG depends on the glycosylation of the Fc fragment [27]. Healthy children displayed high serum levels of anti-β2GP1 antibodies, which were significantly higher than in the sera of patients with APS. This suggests a more efficient clearance of anti-β2GP1 immune complexes in healthy children without the induction of an accompanying inflammatory reaction or coagulatory event. Strikingly, anti-β2GP1 immune complexes showed significantly higher sialylation in healthy children compared to those of patients with APS. Therefore, the pathogenicity of autoreactive IgG in APS is likely to be related to a lower sialylation status, which reportedly aggravates inflammation. Thus, higher sialylation of autoreactive IgG might protect asymptomatic children from developing symptomatic APS. This is further supported by the finding that anti-β2GP1 IgG is also higher sialylated in the sera of asymptomatic seropositive adults compared to IgG from patients with APS. Sialylation was examined for total serum anti-β2GP1 IgG. Differences in subclasses between healthy children, patients with APS, and NHD were assessed independently showing only a significant difference in subclasses for IgG1 and IgG3 compared to NHD and patients with APS. However, these higher levels may be a reflection of the total IgG1 and IgG3 levels in children which decrease with age [28,29]. Anti-β2GP1 IgG2 was significantly enriched in patients with APS which is in accordance with already published data [30-33]. Therefore, anti-β2GP1 IgG2 may not only be a marker of the disease but also involved in its pathogenicity. However, the differences in subclass levels of IgG are unlikely to contribute to the protection of healthy children as sialylation levels of total IgG are significantly different between the sample groups [27].

However, what triggers changes in the sialylation status and how quick they appear, is still elusive. Differences in IgG glycosylation between patients and healthy controls are unlikely to result from differential glycosylation of IgG subclasses, however, cannot be fully excluded. Therefore, further investigation regarding the glycosylation pattern of different IgG subclasses is needed. After clarifying these questions, routine analysis of the IgG sialylation status may present a valuable diagnostic tool to predict the conversion from asymptomatic to symptomatic adults.

## 4. IgG glycosylation in patients with RA

Abnormal N-glycosylation patterns of IgG in the context of a pathological condition have already been reported in patients with RA over 30 years ago by Parekh et al. [34]. It is now generally accepted that autoantibodies characteristic for RA, such as anti-citrullinated peptide antibodies (ACPA) or rheumatoid factor (RF), display a pro-inflammatory N-glycosylation pattern exhibiting

low levels of galactose and sialic acid [35]. Importantly, these changes in the glycosylation pattern of autoantibodies are already detectable before the clinical onset of the disease assigning valuable diagnostic properties to it [36]. Mechanistically, the conversion to a pro-inflammatory glycosylation pattern, reflected by an increase of G0 glycoforms, leads to a higher accessibility of terminal N-acetyl glucosamine residues (GlcNAc) to mannose binding protein (MBP). This in turn can activate the complement cascade leading to chronic inflammation [37].

Recently, Gińdzieńska-Sieśkiewicz et al. reported that changes in the glycosylation pattern of IgG in RA are subject to change under methotrexate treatment [38]. IgG galactosylation and sialylation levels were significantly lower in patients suffering from RA before treatment compared to healthy controls and significantly increased after therapy. Furthermore, differences in the N-glycosylation pattern were strongly associated with disease activity based on disease activity scoring. Moreover, reactivity of the fungus-derived fucose-specific Aleuria Alantia Lectin (AAL) was significantly higher in patients with RA before treatment compared to healthy controls and decreased slightly after treatment. Therefore, detection of changes in IgG galactosylation and sialylation might be used as a therapeutic marker reflecting the effectiveness of the treatment.

Additionally, we reported recently that the Fc sialylation status of random IgG and disease-specific IgG autoantibodies determines the bone architecture of patients with RA [39]. Patients exhibiting lower sialylation or galactosylation levels of total IgG and disease-specific autoantibodies had significantly decreased bone volume compared to patients with higher levels of sialylation or galactosylation. Desialylated immune complexes might enhance osteoclastogenesis *in vitro* and *in vivo* by binding to FcγRII and FcγRIII on pre-osteoclasts.

Assessing the glycosylation of IgG, especially terminal galactose, in individuals susceptible to developing RA might be a promising predictive approach, since changes in the glycosylation pattern of autoantibodies are already detectable before onset of the disease. Early detection of these changes might lead to earlier diagnosis of the disease and consequently early treatment. This might prevent excessive reduction of the bone volume.

## 5. IgG glycosylation in patients with SLE

It has been found already 25 years ago that serum IgG derived from patients with SLE displays abnormal galactosylation [40]. Importantly, low levels of IgG galactose in patients were also present in their family members being unaffected by any autoimmune disease. Additionally, an increased reactivity of IgG immune complexes with AAL, representing higher fucosylation at the N-glycan core, has not only been observed in patients with RA, but also in patients suffering from SLE [41]. In this study, the increased exposure of fucose residues positively correlated with disease severity. The authors suggested that the increased accessibility of glycans towards lectins may alternatively target the immune complexes to lectin receptors of effector cells and thereby modify their clearance and biological effects. Using ultra-performance liquid chromatography it was demonstrated that patients with SLE harbor a changed IgG glycome showing lower levels of galactosylation and sialylation [42]. These changes might be associated with an altered immunosuppressive potential of the immune complexes since galactosylation [43] and sialylation [44] reportedly regulate the pro- and anti-inflammatory actions of IgG, respectively.

Furthermore, we have recently shown that the sialylation pattern of anti-histone IgG is impacting the clearance of opsonized Secondary NEcrotic Cells (SNEC) by phagocytes [45]. Pathogenic anti-histone IgG was mainly present in the non-sialylated fraction and only non-sialylated

anti-SNEC IgG favored the uptake of SNEC by polymorphonuclear cells (PMN). Unexpectedly, this uptake was not accompanied by a pro-inflammatory cytokine response in whole blood cultures. Strikingly, sialylated IgG significantly reduced the production of the pro-inflammatory cytokines IL-8 and IL-6, but induced an increase of TNF-α and IL-1β. We suggest that higher sialylation of anti-histone IgG derived from patients with SLE shifts the response to SNEC towards a milder inflammation. However, a contribution of the different subclasses of IgG cannot be excluded, since this study has been conducted on total anti-histone IgG.

Autoantibodies from patients with SLE are predominantly of the subclass IgG1 [46], which is also the major IgG subclass in healthy adults [9]. However, Bijl et al. showed that IgG subclass distribution varies with renal or extra-renal disease manifestation and relapses in patients with SLE [47]. Anti-dsDNA and anti-nucleohistone autoantibodies were predominantly of the subclass IgG1, but at the moment of relapse, autoantibodies of the IgG2 and IgG3 subclass were more frequently present in patients with renal disease compared to those with extra-renal manifestations.

Taken together, routinely analyzing the exposure of fucose and sialylation on serum immune complexes may present a valuable biomarker for monitoring disease activity in SLE. However, further studies addressing the role of IgG autoantibody subclasses are needed, especially in patients with renal manifestations of SLE compared to those with extra-renal implications at the moment of a relapse.

## 6. IgG glycosylation in other autoimmune diseases

Some forms of systemic vasculitis, an inflammatory disease characterized by inflammation and necrosis of blood vessels, are strongly correlated with the presence of anti-neutrophil cytoplasmic antibodies (ANCA) [48]. These autoantibodies specifically target contents of the azurophilic granules of neutrophils, such as myeloperoxidase or serine proteinase-3, and are involved in the pathogenesis of ANCA-associated vasculitis. Neutrophils are activated by binding of ANCA IgG to FcγRIIa and FcγRIIIb [49,50]. This suggests that the glycosylation status of IgG could influence the inflammatory process driving the disease. In 2002, Holland et al. reported that patients with ANCA-associated vasculitis display a deficiency in IgG galactosylation showing agalactosylated IgG (IgG-G0) as the major glycoform in patients [51]. The IgG subclass involved in these ANCA-containing immune complexes was not examined in this study. In a different study the total subclass distribution of IgG in patients with active vasculitis has been found to be similar to healthy controls [52]. However, the subclass distribution of ANCA showed enrichment for IgG3 and depletion of IgG2. In clinical remission, ANCA IgG2 levels were increased and ANCA IgG3 levels were decreased. Therefore, testing the serum of patients with ANCA-associated vasculitis not only for the glycosylation status, but also for the subclass distribution of ANCA may be a useful biomarker for monitoring the disease status.

## 7. Glycosylation of other immunoglobulin classes

Contrary to IgG, IgM displays five N-linked glycosylation sites on its heavy μ chain (at $Asn^{171}$, $Asn^{332}$, $Asn^{395}$, $Asn^{402}$ and $Asn^{563}$) resulting in a total of 51 N-glycosylation sites on pentameric IgM and 60 N-glycosylation sites on hexameric IgM. $Asn^{171}$, $Asn^{332}$, and $Asn^{395}$ are occupied by fucosylated, mono-sialylated complex glycans, whereas $Asn^{402}$ and $Asn^{563}$ are comprised entirely of oligomannosidic structures [53,54]. 20% of total serum IgM has been shown to bind mannose-binding lectin (MBL), however, only when it is not bound to antigen [53]. Thus,

antigen-bound IgM pentamers do not activate the complement cascade via the lectin pathway. Instead, binding of MBL may facilitate removal of IgM aggregates by opsonization.

There is not much known regarding changes of the glycosylation profile of IgM antibodies under pathological conditions. Recently, changes in IgG and IgM antibody glycosylation during ablation-induced immune response to cancer have been investigated. The study aimed to establish antibody glycosylation as a potential biomarker for diagnosis, prognosis and disease treatment. Antibody glycosylation varied with cancer type and the authors suggest that glycosylation patterns are indicative of an immune system that is unable to prevent different types of cancer [55]. In another study, sialylated N-linked glycans have been shown to induce the internalization of natural anti-lymphocyte IgM by T cells causing inhibition of T cell responses [56]. The authors conclude that sialylated N-glycans play a key role in inducing IgM-mediated immune suppression. However, if altered glycosylation of IgM has an impact in inflammatory autoimmune diseases still needs to be elucidated.

Human serum IgA is extensively glycosylated via both, N-linkages (IgA1, IgA2) and O-linkages (IgA1) [57]. IgA1 contains two conserved N-glycosylation sites ($Asn^{263}$ and $Asn^{459}$) on its Fc part of which over 90% are sialylated contrary to IgG of which only <10% shows enrichment in sialic acid. IgA1 additionally contains nine potential O-glycosylation sites in its proline-rich hinge region between the Fab and Fc regions [58]. The occupancy of these sites in normal serum IgA1 is not known, however, it is thought to be tissue-specific [59,60].

Henoch-Schönlein purpura (HSP) is a systemic vasculitis of childhood that affects males twice as often as females [61]. Nevertheless, it can also affect adults with a more severe disease outcome with gastrointestinal and renal involvement as the major morbidities in adults [62]. The disease is characterized by IgA-dominant immune complexes in smaller venules, capillaries and arterioles and hence was renamed IgA vasculitis. HSP is associated with abnormalities in IgA1 glycosylation, one of the two subclasses of IgA. IgA1 is an unusual serum protein as it contains a series of O-linked glycans in the hinge region distinguishing it from IgGA2 and other immunoglobulins [63]. Allen et al. reported in 1998 that these alterations in IgGA1 glycosylation are solely observed in patients with IgA nephropathy and HSP with renal involvement, while IgA1 O-glycosylation is normal in patients with other forms of renal disease [8]. In a previous study, Saulsbury already reported that the O-linked oligosaccharides of IgA1 from patients with HSP are deficient in sialic acid [7]. IgA1 additionally shows a reduced galactosylation in patients with either HSP nephritis or IgA nephropathy [64]. The exposure of N-acetylgalactosamine (GalNAc) residues at the surface of IgA1 leads to the formation of a novel antigen thus inducing an IgG-mediated immune response. Interestingly, patients have been described who develop HSP due to an IgA paraprotein-producing malignant melanoma [65,66]. Even though these cases are rare, an altered O-glycosylation of IgA1 with decreased sialylation has been described which was accompanied by the presence of IgA ANCA in one case [65].

In HSP the IgA1 subclass seems to play a major role in disease pathogenesis and a strong correlation between alterations in IgA1 O-glycosylation and renal involvement has been reported. Therefore, monitoring IgA1 galactosylation and sialylation levels in children and adults may be a useful biomarker for predicting renal involvement in HSP. However, if detection of IgA1 glycosylation is also a tool to predict disease severity, especially in adults, needs to be further investigated.

## 8. Glycosylation of Cryoglobulins

Cryoglobulins are either monoclonal immunoglobulins or Ig immune complexes, which precipitate if the temperature drops under body temperature and redissolve upon warming [67].

These cryoglobulins are usually detected by visual inspection of precipitates formed in cooled serum compared to serum at 37 °C. Importantly, detection of these cryoglobulins using flow cytometry has been shown to be more sensitive with equal specificity [68]. These Ig immune complexes are often found in sera of patients with autoimmune diseases such as RA or SLE. Both, human and murine IgG3 has the unique property to self-associate via Fc-Fc interactions which is necessary but not sufficient to confer cryoglobulin activity [69]. Employing variants of an IgG3 anti-IgG2a cryoglobulin derived from an autoimmune MRL-*Fas*$^{lpr}$ mouse, Kuroki et al. discovered that diminished cryoglobulin activity was associated with increased galactosylation levels in the CH2 oligosaccharide side chain. The same group also reported that terminal sialylation of the IgG3 cryoglobulin limited its cryoglobulin activity and therefore attenuated cryoglobulin-mediated glomerulonephritis [70]. Therefore, higher galactosylation and sialylation of IgG3 cryoglobulins seems to play a protective role and highlights the general anti-inflammatory role attributed to increased galactosylation and sialylation. Accordingly, reduced contents of sialic acid in cryoglobulins are reported in several cases of human cryogobulinemia [71,72]. Additional studies have to be performed to verify the potential of detection of galactosylation and sialylation of IgG3 cryoglobulins as a biomarker for disease activity in patients with autoimmune diseases. Furthermore, it needs to be investigated if cryoglobulin glycosylation correlates with cryoglobulin activity, disease severity, and disease pathogenesis.

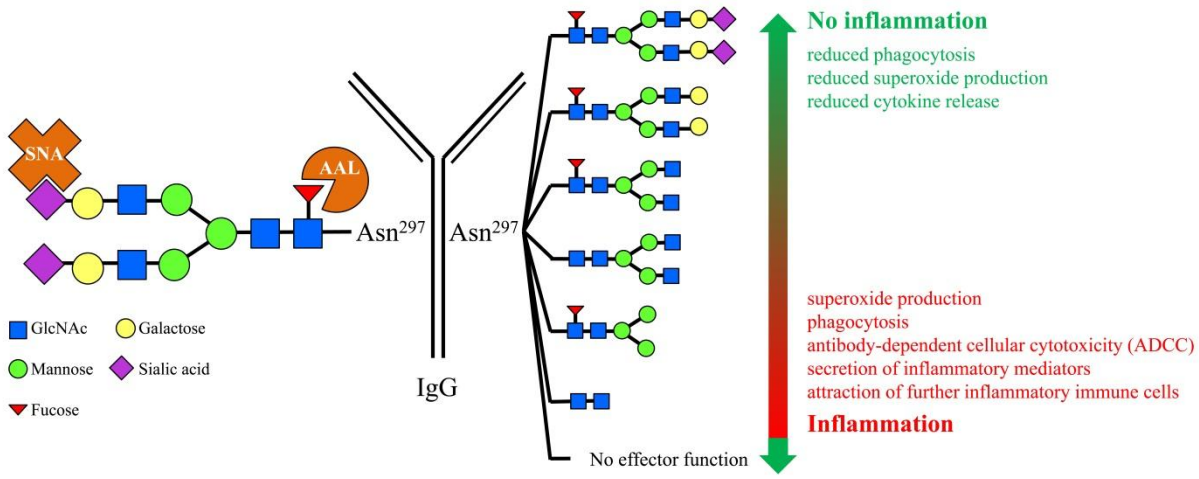


**Figure 1. Impact of Asn$^{297}$-linked glycan tree on FcγR-mediated effector functions of IgG.** The composition of the IgG glycan tree linked to the conserved amino acid asparagine 297 (Asn$^{297}$) influences IgG-mediated cellular inflammatory responses regarding phagocytosis, superoxide production, cytokine release and antibody-dependent cellular cytotoxicity (ADCC). Distinct sugar moieties are recognized by lectins such as Sambucus Nigra Agglutinin (SNA) and Aleuria Alantia Lectin (AAL), specific for sialic acid and fucose, respectively.

## 9. Conclusion

Differential modification of the glycan tree has been found to influence IgG activity and effector functions (Figure 1) and plays a role in several pathological conditions (Table 1). Changes in the IgG glycosylation profile are associated with chronic inflammatory autoimmune diseases and are

reported to be even present before onset of the disease as it is the case in RA. Therefore, exploiting the differential glycosylation pattern of IgG as a biomarker for diagnostic and prognostic purposes may be a useful approach, since glycosylation is easily detectable employing lectin-based enzyme-linked immunosorbent assays (ELISA) specifically binding to distinct sugar residues in serum IgG. However, validation of glycosylation as a true biomarker requires the conduction of additional studies in this field. To date, changes in IgG and IgA1 glycosylation patterns during the course of disease and under treatment are best characterized in RA and HSP. However, if this knowledge also holds true for other chronic inflammatory autoimmune diseases and other immunoglobulin classes, such as IgM, still needs to be investigated.

**Table 1. Alterations of antibody glycosylation associated with different pathological conditions.**

| Disease | Antibody type/specificity | Glycosylation alteration | Reference |
|---|---|---|---|
| Anti-Phospholipid Syndrome | anti-β2GP1 immune complexes | Decreased sialylation | [27] |
| Rheumatoid arthritis | Total serum IgG/IgG1 | Decreased galactosylation | [34,36-38] |
| Rheumatoid arthritis | Total serum IgG | Decreased sialylation | [35,38] |
| Rheumatoid arthritis | Total serum IgG | Increased Fc core fucosylation | [38] |
| Rheumatoid arthritis | ACPA | Decreased galactosylation | [36] |
| Rheumatoid arthritis | ACPA-IgG1 | Increased Fc core fucosylation | [36] |
| Systemic Lupus Erythematosus | Total serum IgG | Decreased galactosylation | [40] |
| Systemic Lupus Erythematosus | IgG immune complexes | Increased Fc core fucosylation | [41] |
| Systemic Lupus Erythematosus | Total serum IgG | Decreased galactosylation | [42] |
| Systemic Lupus Erythematosus | Total serum IgG | Decreased sialylation | [42] |
| Systemic Lupus Erythematosus | Anti-histone IgG | No sialylation | [45] |
| ANCA-associated Vasculitis | Total serum IgG | Decreased galactosylation | [51] |
| Henoch-Schönlein Purpura | IgGA1 | Decreased sialylation (O-linked oligosaccharides) | [7] |
| Henoch-Schönlein Purpura | IgGA1 | Decreased sialylation (O-linked oligosaccharides) | [64] |
| Tumor | Total serum IgG and IgM | Cancer-specific | [55] |
| Cryoglobulin-associated glomerulonephritis | IgG3 anti-IgG2a rheumatoid factor | Decreased sialylation | [70] |

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**Conflict of interest**

The authors declare no conflicts of interest.

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