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Protein Glycosylation as a Diagnostic and Prognostic Marker of Chronic Inflammatory Gastrointestinal and Liver Diseases

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Glycans are sequences of carbohydrates that are added to proteins or lipids to modulate their structure and function. Glycans modify proteins required for regulation of immune cells, and alterations have been associated with inflammatory conditions. For example, specific glycans regulate T-cell activation, structures, and functions of immunoglobulins; interactions between microbes and immune and epithelial cells; and malignant transformation in the intestine and liver. We review the effects of protein glycosylation in regulation of gastrointestinal and liver functions, and how alterations in glycosylation serve as diagnostic or prognostic factors, or as targets for therapy.

Keywords: Glycan; Glycome; Glycomic; Glycosylation; IBD; Liver Disease.

Protein glycosylation is a post-translational modification that provides proteins with structural diversity required for their interactions with other proteins and cells. The diversity of the glycome (repertoire of glycans structures in an organism) reflects their multiple functions in cells, and glycans act as an interface between the cell surface and the environment.[1] Glycans regulate folding and functions of proteins and lipids.[2] The glycome is affected by genetic and environmental factors, and changes have been associated with development of inflammation. Disruption of genes that encode specific glycosyltransferases causes alterations in the immune system that range from immune suppression to autoreactivity,[2–4] indicating the importance of glycans in regulation of the immune response.

Glycans regulate the cellular and humoral immune responses,[5] including assembly of peptide-loaded major histocompatibility complexes antigens,[6,7] reorganization of T-cell receptor complexes,[8] modulation of immune receptor clustering, endocytosis, receptor signaling,[9] and immunoglobulin functions.[10,11] Some glycan motifs serve as danger-associated molecular patterns or pathogen-associated molecular patterns.[12] The immune system contains different classes of glycan-binding proteins, including C-type lectins, galectins, and siglecs, which are expressed by immune cells and can be secreted. Glycan-binding proteins regulate leukocyte trafficking, pathogen recognition, immune cell activation, and immunosuppression.[13–17] Changes in protein glycosylation are associated with the pathogenesis of diseases, including cancer, infections, and autoimmune diseases.[2,4,18] These include gastrointestinal and liver disease, such as inflammatory bowel diseases (IBDs),[19] liver fibrosis[20] and cirrhosis,[21] nonalcoholic fatty liver disease (NAFLD),[22] gastric cancer,[23] colorectal cancer,[24,25] and hepatocellular carcinoma.[26]

There are many challenges to diagnosis of IBDs and liver diseases, and to predicting responses to treatment or outcomes of patients. Despite recent advances in IBD therapy, a high proportion of patients are refractory to treatment, and half of patients with ulcerative colitis (UC) do not achieve sustained remission.[27] For example, despite the fact that corticosteroids are effective in controlling acute flares of IBD, 20%–30% of patients have only a partial response and 16% do not respond to corticosteroids.[28] It is a challenge to select the best treatment for patients due to the lack of reliable biomarkers of response or adverse events. Biomarkers are also urgently needed to differentiate patients with liver steatosis from those with nonalcoholic steatohepatitis (NASH).

Changes in protein glycosylation might be used as diagnostic and prognostic markers, as well as targets of therapy for chronic inflammatory gastrointestinal diseases. We review how alterations in protein glycosylation affect development of chronic inflammatory gastrointestinal and liver diseases and the application of glycomic analysis to

Abbreviations used in this paper: **AFP, α-fetoprotein; CRC, colorectal cancer; FUT2, fucosyltransferase 2; FUT8, fucosyltransferase 8; GlcNAc, *N*-acetylglucosamine; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IBD, inflammatory bowel disease; Ig, immunoglobulin; IL, interleukin; LC, liquid chromatography; MGAT, mannosyl (α-1,3-)-glycoprotein β-1,2-N-acetylglucosaminyltransferase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Th, T helper; UC, ulcerative colitis.**

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their diagnosis and prediction of patients' response to therapy and outcome.

Glycosylation in T Cells

Glycans regulate T-cell development, activation, signaling, differentiation, and proliferation, as well as thymocyte selection.[8,27,29–31] Alterations in these processes can lead to autoimmune diseases and cancer.[5,32] It is not clear whether alterations in cell glycosylation profiles occur as an early event (inducing) or late event (accelerating) in intestinal inflammation. Intestinal T cells from patients with IBD have glycome profiles that differ from those individuals without IBD, characterized by decreased levels of branched N-glycans[19,30] and increased levels of core fucosylation (the addition of a fucose residue to the core structure of N-linked glycans with an α1,6 linkage).[33] These changes increase T-cell–mediated intestinal immune response.

T cells from inflamed colonic mucosa of patients with active UC have reduced expression of the α-1,6-mannosylglycoprotein 6-β-N-acetylglucosaminyltransferase gene (*MGAT5*) or overexpression of the fucosyltransferase 8 gene (*FUT8*),[30,33] compared with cells from patients without UC. T cells from patients with IBD have a glycome profile that has been associated with a decreased threshold for activation and increased signaling and inflammatory response[30,33] (Figure 1). However, it is not clear whether changes in the glycoprofile of T cells are a cause or a consequence of intestinal inflammation. The cytokines interleukin (IL) 2 and IL7 alter expression of Golgi branching enzymes that control N-glycosylation in T cells.[11] An inflammatory environment would consequently influence N-glycosylation in T cells to affect their function.

Studies of patients with immune-mediated diseases, such as multiple sclerosis, have found that variants in genes, including *IL17RA*, *IL2RA*, *MGAT1,* and *CTLA-4*, along with environmental factors, such as sunlight or vitamin D3 and metabolism, dysregulate Golgi N-glycosylation to promote autoimmunity.[34] This finding indicates that N-glycosylation contributes, as an early event, to pathogenesis of inflammatory diseases. During colitis induction with dextran sodium sulfate (DSS), mice with the disruption of MGAT5 gene develop more severe colitis, with earlier onset, than mice without disruption of this gene, with increased responses of T-helper (Th)1 and Th17 cells.[30] Alternatively, FUT8 knockout mice develop a less-severe colitis than mice that express wild-type FUT8, producing lower levels of Th1 and Th2 cytokines.[33] Mice that express human fucosyltransferase 2 (FUT2) from a transgene have defects in T-cell development and develop spontaneous colitis.[35] Alterations in N-glycosylation therefore dysregulate the T-cell response and contribute to development of colitis.

Researchers have studied mice without uridine diphosphate–*N*-acetylglucosamine (GlcNAc): βGal β-1,3-N-acetylglucosaminyltransferase 6 (core 3 β1,3-N-acetylglucosaminyltransferase), an enzyme involved in synthesis of core 3–derived O-glycans, and mice without core 1 synthase, glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferase 1 (also called T-synthase). Core 3 β1,3-N-acetylglucosaminyltransferase–deficient mice have colon-specific reductions in mucin 2, increased permeability of the intestinal barrier, and are highly susceptible to dextran sodium sulfate–induced colitis, developing severe forms of the disease.[36] Mice with intestinal epithelial cell-specific deficiency of core 1-derived *O*-glycans developed spontaneous colitis. Their colonic mucosa becomes infiltrated by tumor necrosis factor–producing myeloid cells, in the absence of lymphocytes.[37] The promoter region of the *MGAT3* gene has CpG methylation in $CD3^+$ T cells from colon tissues of patients with IBD compared with healthy individuals.[38] Genetic and epigenetic facts can therefore dysregulate the glycosylation pathway, resulting in altered glycan structures that control T-cell functions and might contribute to IBD pathogenesis.

Glycocalyx

Glycans cover, essentially, all cell surfaces, forming a dense and complex coat of sugar-chains termed *glycocalyx*. This glycocalyx mediates interactions between intestinal cells and the microbiota. Little is known about the contributions of glycans to the composition and function of the intestinal microbiota, and how alterations affect intestinal homeostasis and the mucosa immune response. Exposure of intestinal microbes to glycans, either from diet or intestinal cells, affects their composition and function. Intestinal microbes use glycans from dietary plants, animal-derived cartilage and tissue, and human cells as sources of nutrients and energy.[39] Variations in the glycocalyx or fluctuations in the abundance of dietary glycans might lead to dysbiosis and contribute to development of IBD, colon cancer,[40] NAFLD,[41] alcoholic liver disease,[42] or cirrhosis.[43] Research is needed to clarify the relationship among cell glycosylation, dysbiosis, intestinal inflammation, and associated diseases.

Glycans from cells or the diet can place selective pressures on specific species of bacteria, which can be classified as generalist or specialists.[39] Individuals lacking a functional copy of *FUT2* are known as nonsecretors and have alterations in their intestinal microbiome and increased susceptibility to infection and inflammatory disease, such as Crohn's disease.[44] The *FUT2* genotype of nursing mothers associated with decreased risk for diarrheal diseases in their infants, and milk rich in α1,2-linked fucosyl moieties was proposed to promote the growth of beneficial microbiota species like *Bifidobacteria longum* subsp *Infantis.*[45] Single nucleotide polymorphisms in *FUT2* were associated with increased risk of primary sclerosing cholangitis, which often develops in patients with IBD, alterations to the microbiome, and susceptibility to infectious agents.[46] Loss of core-1– or core-3–derived O-glycans is associated with susceptibility to colitis due to alterations in mucin glycosylation, which increase intestinal epithelial permeability, leading to bacteria accessibility and growth. Mice with loss of core 1–derived O-glycans from intestinal epithelial cells have alterations to the composition of their gut microbiota, with an inverse shift in the abundance of the phyla Bacteroidetes and Firmicutes.[47]

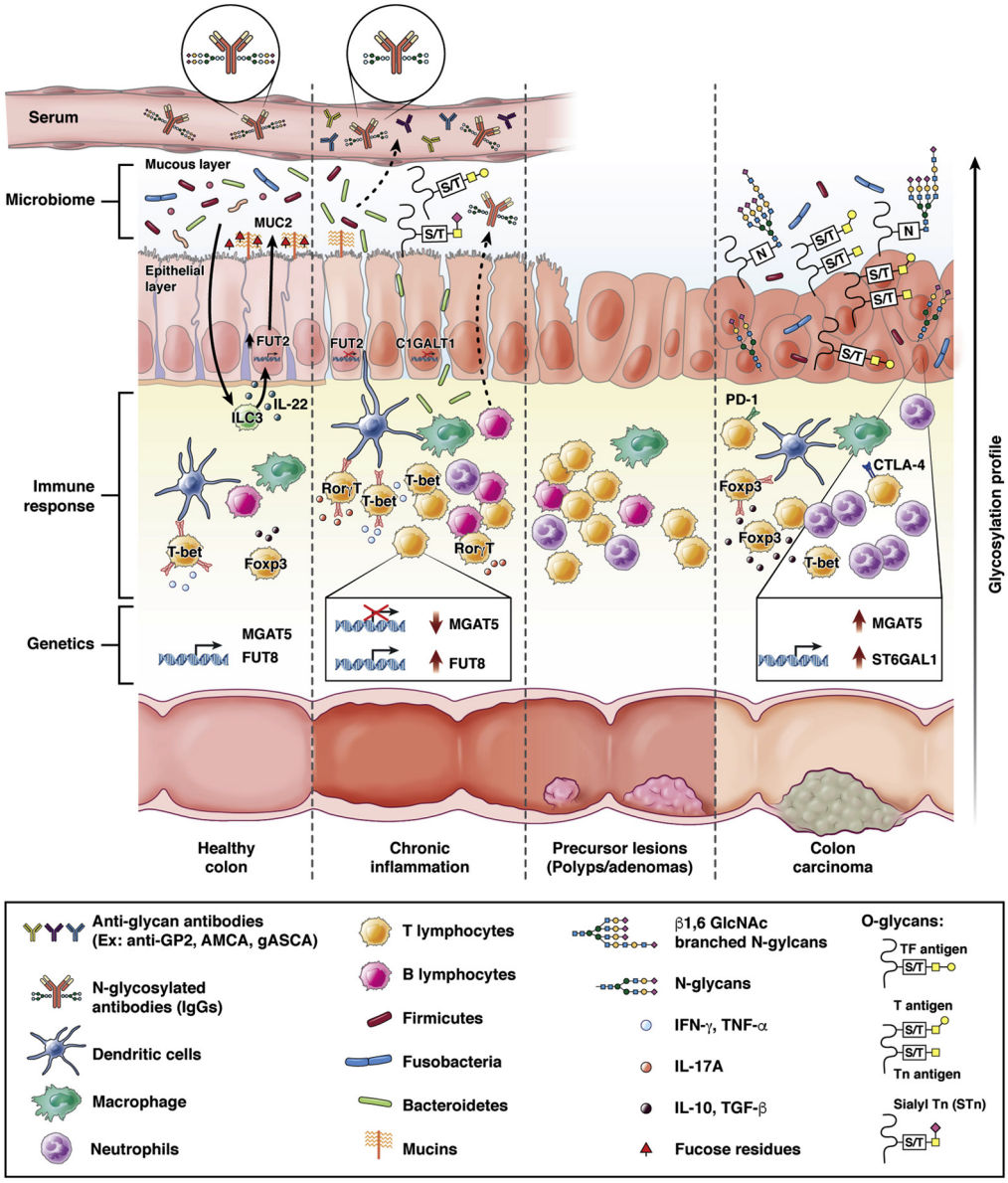


Figure 1. Protein glycosylation in development of IBD. Genetic and environmental factors affect protein glycosylation and its effects on development of inflammation. Decreases in core 3–derived *O*-glycans, increases in core fucosylation, and reductions in branched complex *N*-glycan structures have been associated with development of IBD, affecting interactions between intestinal cells and the microbiota and increasing activation of T cells. Alterations in glycosylation profiles and their associated alterations in the intestinal microbiome can promote inflammation in the intestinal mucosa. Alterations in IgG glycomes in serum samples have been associated with changes in IgG effector functions that lead to intestinal inflammation; studies are needed to determine how these processes contribute to carcinogenesis. Changes in protein glycosylation might be detected in serum samples and used as diagnostic or prognostic factors.

On the other hand, commensal bacteria, such as Bacteroides spp can induce epithelial fucosylation,[48] indicating the symbiosis between intestinal cells and microbes.[49]

Variations in glycocalix composition along the gastrointestinal tract[39,50,51] might support survival and growth of subpopulations of bacteria, which degrade different types of glycans, with potential pathologic implications. Changes in intestinal fungi have also been associated with intestinal inflammation and IBD.[52,53] It will be interesting to explore their relationships with glycans in the intestine.

Glycosylation Alterations in Inflammation-Associated Gastrointestinal Cancer

Cancer cells undergo changes in glycosylation patterns[18] that contribute to development and progression of colorectal cancer (CRC). Changes such as the carbohydrate antigen CA 19-9 (sialyl Lewis[a]) or those expressed on carcinoembryonic antigen are used as biomarkers in diagnosis of CRC and in monitoring its progression.[25] The most widely detected glycosylation alterations in colorectal tumors include increased levels of β1,6-branching N-glycans; (poly-)N-acetyllactosamine extensions of N-glycans; and sialylation. Alterations in O-glycans associated with CRC include reductions in core 3 and 4 O-glycans and higher levels of core 1 glycans, (sialyl) T-antigen and (sialyl) Tn-antigen. Changes in glycosylation profile of colon cancer cells have been described and alter their activities to promote tumorigenesis and metastases. Tumor-associated glycans are recognized by glycan-binding proteins (galectins, siglecs, and selectins) on immune cells, modulating the anti-tumor immune response.[24,54]

Patients with IBD are at increased risk for CRC, which accounts for 10%–15% of deaths in patients with IBD.[55]

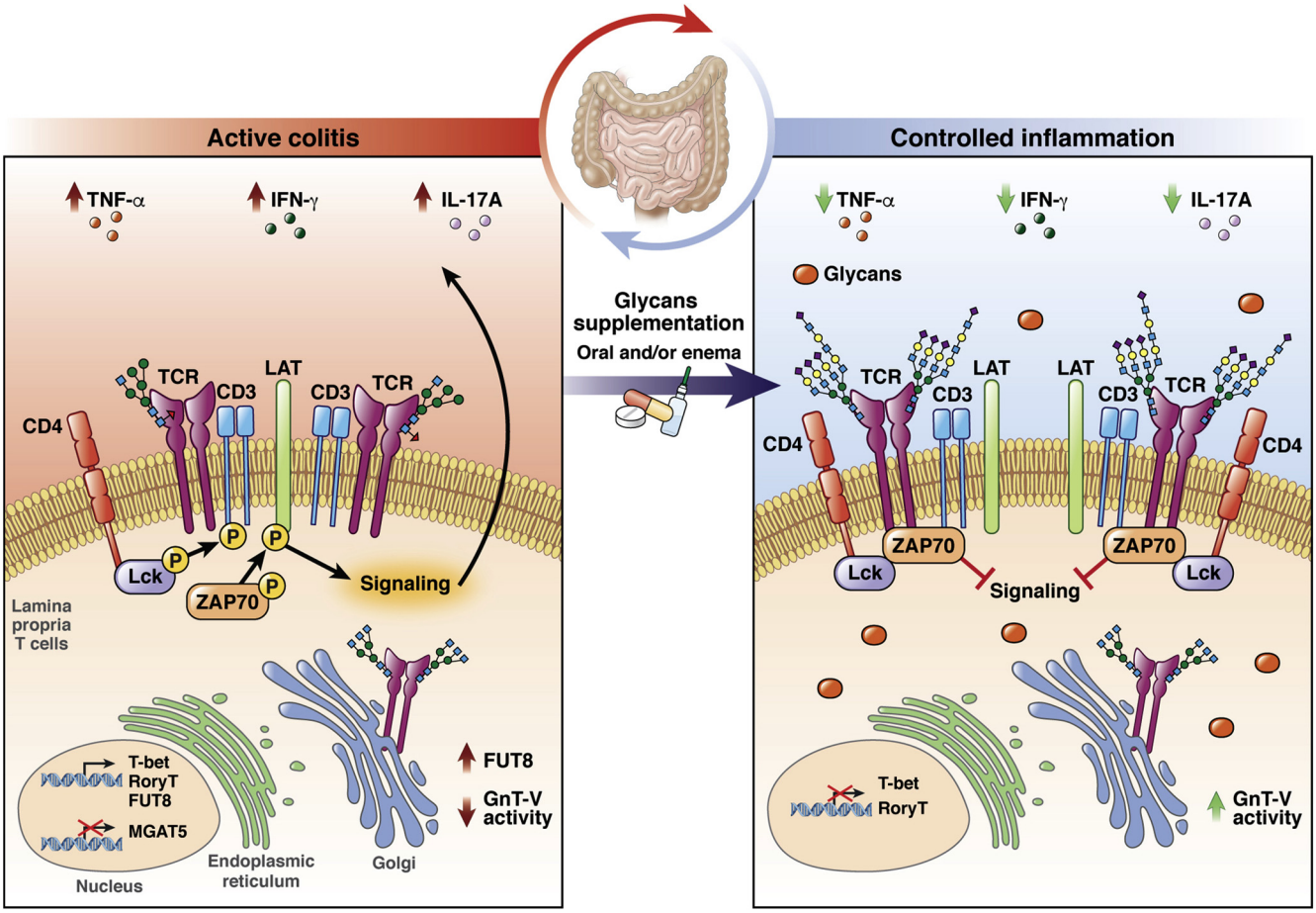


Figure 2. Immune-modulatory properties of glycans in IBD. The complex branched N-glycans have immunosuppressive functions in the intestinal mucosa. Administration of glycans to mice and ex vivo human intestinal T cells, either orally and/or by enemas, alter the T cell–mediated immune response. Increases in β1,6-branching N-glycosylation in intestinal T cells increases their threshold for activation and inhibits their production of inflammatory cytokines, such as tumor necrosis factor, interferon-gamma, and IL17A. This branching N-glycosylation also inhibits T-cell receptor signaling, leading to control of intestinal inflammation and delayed disease progression. Glycans are therefore promising immune-modulatory agents that might be developed for treatment of IBD.

IBD-associated CRC results from a different sequence of genetic alterations than those observed in sporadic cancers, with early p53 mutations and frequent chromosome instability and microsatellite instability. These genetic changes, together with immune dysregulation, alterations to the microbiome, and breaches in the epithelial barrier, contribute to carcinogenesis.[56–58] Curiously, some of the alterations in the cellular glycosylation are similar in IBD and in CRC,[59] such as the increased expression of the oncofetal Thomsen Friedenreich carbohydrate antigen (galactose β1,3*N*-acetylgalactoseamine) in *O*-glycans from mucins extracted from CRC and UC samples,[60] and the increased expression of sialyl Tn that was associated with the risk for cancer development in IBD mucosa.[61,62] Accordingly, mice models lacking core 1 and core 3–derived mucin type *O*-glycans develop spontaneous colitis-associated cancer.[32,63]

Overexpression of β1,6 branching N-glycosylation has been associated with malignancy,[64] cell invasiveness,[18,65–67] and reduced survival times of patients with cancer[68] (Figure 1). Aberrant expression of branched glycans can affect regulation of immune response, although little is known about its effects in patients with IBD-associated CRC. Increasing levels of branching N-glycosylation in T cells through metabolic supplementation with GlcNAc increased their anti-inflammatory properties[30] (Figure 2). Administration of GlcNAc to pediatric patients with refractory IBD increased the intestinal mucus lining and clinical remission.[69] The dual effects of branched glycans as structures that promote carcinogenesis and modify the immune response indicate their possible involvement in development of inflammation-associated cancer (SS Pinho et al, unpublished data, September 2019). Further studies are needed on the relationship between glycosylation alterations and carcinogenesis in patients with IBD, including dysregulation of the immune response and intestinal microbiome.

Glycosylation Alterations in Liver Diseases

Core hyperfucosylation has been observed in serum and liver tissues from patients with cirrhosis[70] and NASH,[71] it

Population	Number of patients	HCC size	Technique	Diagnostic glycan	Diagnostic performance	Reference
HCC in patients with HBV infection	227	TNM classification: T1: n=6 T2: n=28 T3: n=59 T4: n=5	DSA-FACE	GlycoHCCTest ↑ NA3Fb ↓ NA2FB 	AUC, 0.81 Significant correlation with TNM stage	Liu et al [82]
HCC in patients with HCV infection	73	Stage I/II: n=18 Stage III/IV: 33 (AJCC staging)	MALDI-TOF MS	Increased abundance  Decreased abundance 	AUC, 0.960	Goldman et al [155]
HCC	20	Not reported	DSA-FACE	Hemopexin glycan markers ↑ NA3Fbc ↑ NA4Fb  ↓ NA2 	AUC, 0.920	Debruyne et al [86]
HCC	27	Early-stage HCC	Lectin antibody array	Increased fucosylation in complement C3, ceruloplasmin, histidine-rich glycoprotein, CD14 and hepatocyte growth factor	AUC, 0.811	Liu et al [156]
HCC in patients with chronic HBV infection	145	Stage I, 67 Stage II, III, or IV, 69 (AJCC staging)	DSA-FACE	↑ NA3Fb ↓ NG1A2F 	AUC, 0.873	Fang et al [157]
HCC	27	TNM 1/2	Tandem lectin affinity chromato-graphy	↑ Fucosylation & sialylation of serum PON1	AUC, 0.902	Sun et al [158]
HCC	110	Not reported	Lectin ELISA	↑ Fucosylation serum PON1	AUC, 0.803	Zhang et al [74]
HCC (chronic HBV infection, HCV infection, or alcohol use)	50 (18/42/40)	TNM Stage I, 7 Stage II, 24 Stage III, 11 Stage IV, 8	MALDI-QIT-TOF	Increased bifucosylation of haptoglobin	AUC, 0.821–0.843	Zhu et al [79, 159]

Figure 3. Glycome markers of HCC. AJCC, American Joint Committee on Cancer staging system; ELISA, enzyme-linked immunosorbent assay; MALDI-QIT-TOF, matrix-assisted laser desorption/ionization, quadrupole ion trap time of flight; NA2, galactosylated non-fucosylated biantennary glycan; NA3Fb, α1-3-fucosylated triantennary glycan; NA4FB, branching-α1-3-fucosylated tetra-antennary glycan; NG1A2F, agalacto core,1-6,fucosylated biantennary glycan; TNM, tumor node metastases.

seems to be preferentially related to hepatocellular carcinoma (HCC).[72–76] There is evidence that increased fucosylation occurs directly in the tumor.[77] Genomic analysis of HCC showed overexpression of the *FUT8* gene, responsible for core fucosylation, indicating that these glycan alterations promote hepatocarcinogenis, making them biomarkers and potential therapeutic targets.[78] There is evidence that altered expression of fucosyltransferase 1 and β-1,3-galactosyltransferase 5 contribute to development of HCC.[79] High expression of these enzymes in HCC tissues was associated with shorter survival times of patients.[80] B3GALT5 mediates synthesis of a precursor of the SLe^a antigen, which might contribute to HCC development and metastasis.[81]

These findings have been confirmed in clinical studies (Figure 3). Analyses of serum samples from patients with hepatitis B virus–related HCC using the GlycoHCCTest found an increase in α1-3-fucosylated triantennary glycan (involves the SLe^x epitope) and a decrease in bisecting core α-1,6-fucosylated biantennary glycan, compared with serum from patients without HCC (Figure 3).[82] GlycoHCCTest quantifies α1-3-fucosylated triantennary glycan and bisecting core α1-6-fucosylated biantennary glycan by capillary electrophoresis. This test identified patients with HCC with 81% diagnostic accuracy, equal to that of the assay for α-fetoprotein (AFP), and levels of the measured glycans correlated with tumor stage.

Glycoblotting is a platform that integrates mass spectrometry for rapid and efficient glycome profiling of tissues or whole serum.[83] Serum samples from patients with HCC have increased multibranched glycans compared to individuals without HCC. Comparisons of serum samples from patients with cirrhosis with vs without HCC using liquid chromatography (LC) and electrospray ionization tandem

mass spectrometric analysis identified a specific glycome profile in patients with HCC characterized by end products of the GnT-V pathway.[84]

An automated machine for glycoblotting, called the SweetBlot, allows for quantitative profiling of up to 50 glycoforms in human serum. The Sweetblot technology was used to analyze serum samples from 114 patients with HCC and 105 individuals without.[85] The ratio of a tri-sialylated triantennary complex type glycan with fucose residue on a bigalactosylated biantennary fucosylated glycan identified patients with HCC with an area under the curve of 0.810. These glycans showed similarities to those associated with HCC by the GlycoHCCTest.[82] Alterations in glcosylation of specific liver-secreted serum proteins, such as hemopexin,[86] paraoxonase 1 heteroplamon,[87] and vitronectin[88] have also been associated with HCC.

Remarkably, the best glcosylation marker of HCC is probably the oldest: AFP:L3, the fucosylated L3 fraction to total AFP ratio. This α-6 core fucosylation is detectable using *Lens culinaris* agglutinin lectin and the *L culinaris* agglutinin-reactive fraction of AFP (AFP-L3). This test identifies patients with HCC more accurately than measurement of AFP.[89] So changes in glycosylation associated with HCC include increased activity of α1-6 fucosyltransferase in serum, leading to formation of AFP-L3; increased GnT-III, resulting in a bisecting GlcNAc (used to detect early-stage HCC); and up-regulation of GnT-V, leading to increased branching and formation of tetra-antennary glycans (involved in metastasis).[90] Most glycomic biomarkers detect large liver tumors with high levels of sensitivity, but have not been adequately tested for detection of small tumors.

Glycome analyses might also be used to predict patient outcomes or response to therapy.[91] The GlycoCirrhoTest, which measures the abundance of bisecting GlcNAc residues and triantenarry glycans on serum proteins, can identify patients with cirrhosis and determine their risk for HCC. This test identified patients at risk for HCC with a hazard ratio of 12.1 (95% confidence interval, 2.8–51.6) and patients with a low risk of HCC with a negative predictive value of 97%.[92] In patients with compensated cirrhosis, higher levels of Wisteria floribunda agglutinin-reactive colony-stimulating factor 1 receptor were associated with rapid development of HCC.[93] These tools might be used in HCC screening. Pretreatment glycome profiles have been associated with outcomes of treatment in patients with HCC.[94] Glycome alterations are therefore consistent and will be useful in diagnosis of HCC and determining patient prognoses.

Clinical Effects

The glycosylation profile of plasma immunoglobulin (Ig) G has been associated with clinical features of IBD.[95] In human serum, there are multiple glycoforms of IgG, resulting from the diverse glycan structures at the IgG crystallizable fragment (Fc) region. The Fc region of antibodies interacts with Fc receptors (such as FcγRs) on immune cells to activate signaling pathways and responses to antigens. The type of glycan attached to the Fc regions of IgGs determines which type of receptor they bind: Fcs that bind to activating FcγRs promote inflammatory responses and Fcs that bind to inhibitory FcγRs activate anti-inflammatory responses.

Patients with rheumatoid arthritis have loss of a terminal galactose (agalactosylation) on total IgG that appears in the circulation before disease onset.[96–99] In support of this observation, serum levels of agalactosyl IgGs are increased in patients with Crohn's disease and correlate with levels of C-reactive protein, a marker of inflammation.[100] The agalactosyl fraction of the fucosylated IgG oligosaccharides (G0F/G2F) was increased in serum samples from patients with Crohn's disease or UC vs controls and associated with disease severity.[101] In fact, the glycosylation profiles of IgG differ between patients with UC and Crohn's disease, with differences in fucosylation, galactosylation, and bisection, in association with disease severity.[95,102] Therefore, there is heterogeneity in glycosylation patterns of IgG glycome among different groups of patients with IBD, with different levels of severity and response to treatment. The serum glycome might therefore be used in determining prognoses of patients with IBD. Studies are needed to determine whether changes in IgG glycome are a proxy of bowel damage and level of immune dysregulation, and whether they can be detected before disease development to identify patients at risk or in diagnosis.

Serum antibodies against glycans can be detected and used as markers of autoimmune and inflammatory disorders. For example, anti-glycoprotein 2, anti-mannobioside carbohydrate IgG, and anti-*Saccharomyces cerevisiae* (recognizing an oligomannosidic epitope)[103] are markers used in early diagnosis of IBD (to distinguish UC from Crohn's disease) to determine disease activity and to predict disease course (development of aggressive disease)[104,105] (Figure 1). Interestingly, a higher proportion of non-affected relatives of patients with Crohn's disease test positive for anti-glycan antibodies than individuals with no relatives with IBD.[106] Most interestingly, anti-glycan antibodies have been detected years before a diagnosis of Crohn's disease and are associated with complicated disease before its onset.[107] The serum glycome is therefore a valuable source of diagnostic and prognostic markers of inflammatory disorders, including IBDs.

Changes in glycosylation patterns in the mucosa might also be prognostic factors for patients with IBD.[108] Patterns of branched N-glycans in inflammatory infiltrate of the intestinal mucosa of patients with UC[19] have been associated with response to therapy. Levels of branched N-glycans in colon biopsies collected when patients received a diagnosis of UC correlated with response to standard therapy, identifying patients who did not respond to conventional therapy with 75% specificity. Combined analysis of this glycobiomarker and level of C-reactive protein increased in the accuracy of identification of patients who did not respond to standard therapy.[108] Variations in IgG glycosylation are also associated with resistance of intravenous Ig treatment.[109]

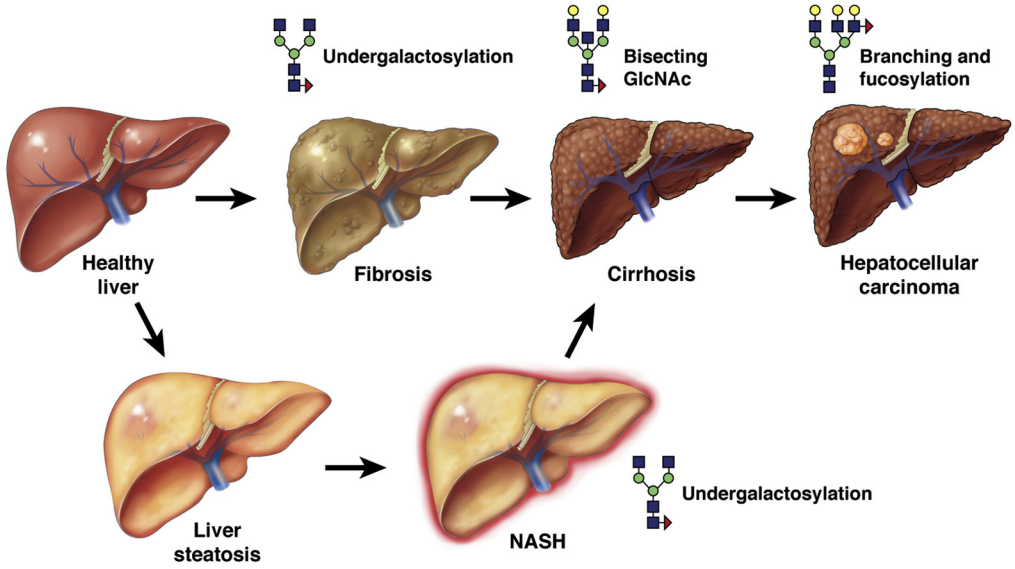


Figure 4. Overview of serum glycomic changes in chronic liver disease. The development of liver fibrosis is associated with increased under-galactosylation of *N*-glycans. The establishment of cirrhosis is characterized by the increased appearance of bisecting GlcNAc residues. The development of HCC is characterized by increased branching of glycans and increase branch fucosylation. In patients with NAFLD, the appearance of NASH is characterized by the increased presence of under-galactosylated glycans.

Treatment of Inflammatory Bowel Diseases

The optimization and control of N-glycan profiles are important for the efficacy of the antibodies.[110] Glyco-engineered Igs with increased sialylation of Fc had a 100-fold increase in anti-inflammatory activity in a mouse model of arthritis.[111] Intestinal T cells isolated from patients with UC and treated with GlcNAc had increases in branching glycosylation and reduced proliferation, T-cell receptor signaling, and Th1 and Th17 cell-mediated responses, including decreased production of tumor necrosis factor, interferon-gamma, and IL17A.

Oral and enema administration of GlcNAc to mice with severe colitis (due to disruption of *MGAT5*) reduced the severity and progression of colitis due to control in the intestinal immune response[30] (Figure 2). The effects of GlcNAc are being tested in a phase 2 trial of patients with IBD. Nineteen years ago, oral GlcNAc was reported to promote mucus production in intestinal tissues of children with severe treatment-resistant IBD; 8 of 12 children given GlcNAc achieved clinical remission.[69] Given the effects of GlcNAc as an immunomodulatory agent[30] and promoter of epithelial barrier integrity,[69] it is worth exploring in IBD prevention and treatment (Figure 2). Overall, glycans could be developed as low-cost, nontoxic, targeted therapies (alone or in combination with other agents) for IBD.

Diagnostic and Prognostic Markers of Liver Disease

Aberrations in protein folding affect their functions in liver cells.[112] Sinusoidal cells and hepatocytes have surface receptors that are modified by glycans. Changes in glycosylation patterns affect receptor concentration and distribution and have been associated with liver diseases (cirrhosis and HCC). Certain glycoproteins accumulate in the circulation of patients with liver diseases.[113] Liver disease is an attractive field for the study of the serum glycome because most N-glycans found in whole serum are attached to serum proteins produced in the liver[21] and to a lesser extent to Igs,[20,70,114] which are produced by B cells. Alterations in serum glycan structures have been reported in patients with liver diseases[20,22,90,92,114,115,116] (Figure 4). Some glycomic-based markers have potential for clinical use (Figures 3, 5, and 6).

Fibrosis and Cirrhosis

Fibrosis is associated with increased levels of under-galactosylated IgGs.[20,70,117] Profiles of protein N-glycans were generated from patient serum samples using 8-aminopyrene-1,3,6-trisulfonic acid-labeled (N-glycan) profiling on a high-throughput DNA sequencer (DNA sequencer-assisted fluorophore-assisted capillary electrophoresis).[118,119] Serum samples from patients with chronic hepatitis C virus (HCV)[20] or hepatitis B virus infection[120,121] had gradual increases in proportions of under-galactosylated core fucosylated glycans and a decrease in the proportion of triantennary N-glycans was observed. Other groups reported an increased core fucosylation and the presence of bisecting GlcNAc residues using matrix-assisted laser desorption/ionization, quadrupole ion trap time of flight analysis.[122] Glyco-alterations, including fucosylation and desialylation, on specific serum proteins are also present during fibrosis development like α1-acid glycoprotein.[123] The Fast-Lec Hepa, an automated immune assay that measures fibrosis-related alterations in glycosylation of serum

Population	Number of patients	Technique	Diagnostic glycan	Diagnostic performance (fibrosis level metavir)		Reference
				F2 or higher	F4 (cirrhosis)	
Chronic HCV infection	400	DSA-FACE	GlycoFibroTest ↑ NGA2FB ↓ NA3 	F2–F4 AUC, 0.71		Vanderschaeghe et al [20]
Chronic HBV infection	173 Ishak scores 0–2, 87 Ishak scores 3–6, 86	DSA-FACE	GlycoFibroTest ↑ NGA2FB ↓ NA3 		AUC, 0.710	Gui et al [121]
Chronic HBV infection	128	DSA-FACE	↑ NGA2FB ↓ NA3 	AUC, 0.736	AUC, 0.754	Qu et al [120]
Chronic HBV infection	46 Ishak 1, 10 Ishak 2, 9 Ishak 3–4, 10 Ishak 5, 8 Ishak 6, 9	MALFI-TOF	Fibro-glyco index increase of 2 core-fucosylated glycans and 1 penta-antennary glycan decrease of NA2	AUC, 0.912	AUC, 0.911	Kam et al [122]
Chronic HCV infection Cirrhosis in patients without HCV infection HCC	Ishak 1–2, 24 Ishak 3–5, 19 Ishak 6, 57 34 87	Lectin fluorophore-linked immunosorbent assay	↑ Anti-Alpha-Gal epitope on IgG based on ↑ serum glycans NGA2F, NA2F, NA2FB	AUC, 0.900	AUC, 0.930	Mehta et al [70]
Chronic HBV and HCV infection	125 F0–1, 33 F2, 32 F3, 31 F4, 29	Multi-lectin assay (12 lectins)	Cirrhosis: increased α1-3 fucosylation of AGP	AUC, 0.760	AUC, 0.900	Kuno et al [135]
Chronic HCV infection	175	Lectin antibody immunoassay	LectHepa Increased α1-3 fucosylation of AGP	AUC, 0.730	AUC, 0.950	Kuno et al [124]
Chronic HCV infection	183	Lectin antibody immunoassay	LectHepa Increased α1-3 fucosylation of AGP	AUC, 0.802	AUC, 0.929	Ito et al [126]
Chronic HCV infection	209 F0–F1, 82 F2, 52 F3, 40 F4, 35	Automated chemiluminescence enzyme immunoessay (HISCL)	FastLec-Hepa Increased α1-3 fucosylation of AGP	Fibrosis ≥ F2 AUC, 0.797	AUC, 0.910	Kuno et al [125]
Chronic HCV (n=160) or HBV infection (n=21) Alcohol abuse (n=12)	160 F0–F1, 82 F2, 52 F3, 40 F4, 35	Automated chemiluminescence enzyme immunoessay (HISCL)	FastLec-Hepa Increased α1-3 fucosylation of AGP	AUC, 0.812	AUC, 0.795	Toshima et al [153]
Fibrotest	Meta-analysis (HCV, HBV, alcohol, NASH)			AUC, 0.840	AUC, 0.840	Poynard et al [136]
FIB-4 HCV infection (n=592)	F0, 73 F1, 470 F2, 185 F3, 85 F4, 61		Age, platelets, level of alanine or aspartate aminotransferase	≥ F3: AUC, 0.85	AUC, 0.91	Vallet-Pichard et al [137]
Chronic liver disease	F0–F3, 52 F4, 48	DSA-FACE	GlycoCirrhoTest ↑ NA2FB ↓ NA3 		AUC, 0.87	Callewaert et al [21]

Figure 5. Glycome-based markers of fibrosis and cirrhosis. As a comparator Fibrosis-4 and FibroTest, which are already used in clinical practice, have been included in the table. All patients had histologic grading of liver disease. Symbols used in the structural formulas are: *blue square* indicates β-linked N-acetylglucosamine (GlcNAc); *yellow circle* indicates β-linked galactose, *red triangle* indicates α/β-1,3/6-linked fucose; *green circle* indicates α/β-linked mannose, purple rhomboid: (α-2,3/6 linked) N-acetylneuraminic acid. AGP, α1-acid glycoprotein; AUC, area under the curve; DSA-FACE, DNA sequencer-assisted fluorophore-assisted capillary electrophoresis; FIB-4, Fibrosis-4; HBV, hepatitis B virus; MALFI-TOF, matrix assisted laser desorption/ionisation time-of-flight analyzer; NA2, galactosylated non fucosylated biantennary glycan; NA2FB, bisecting core α1-6-fucosylated biantennary glycan; NA3, triantennary glycan; NGA2F, core fucosylated agalactosylated biantennary glycan; PON, paraoxonase 1 heteroplasmon.

hyperglycosylated galectin 3 binding protein (LGALS3BP, also called MAC-2-BP),[124–126] shows an excellent correlation with fibrosis stage.

Glycosylation changes are markers of not only fibrosis, but of inflammatory processes, such as chronic HCV infection, that lead to fibrosis. Increased levels of MAC-2-BP are attributed to increased hepatic stellate cell activation[127] and decreased during treatment of HCV infection. In serum samples from patients with biliary atresia, levels of MAC-2-BP fluctuate based on the presence of cholangitis of portal

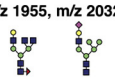
Population	Number of patients	Technique	Diagnostic glycan	Diagnostic performance	Reference
NAFLD and NASH	NAFLD, 9 NASH, 38	DSA-FACE	GlycoNashTest ↑ NGA2F ↓ NA2 	NASH AUC, 0.743	Chen et al [141]
NAFLD and NASH	NAFLD, 199 NASH, 76	DSA-FACE	GlycoNashTest ↑ NGA2F ↓ NA2 	NASH AUC, 0.66–0.75	Blomme et al [22]
Pediatric NAFLD and NASH	NAFLD, 5 NASH, 46	DSA-FACE	GlycoNashTest ↑ NGA2F ↓ NA2 	NASH AUC, 0.72	Blomme et al [154]
NAFLD and NASH	NAFLD, 15 NASH, 42	Sweetblot (integrated glycoblotting and MS)	m/z 1955, m/z 2032  m/z 2584 	NASH AUC, 0.833, 0.863, and 0.866	Yamasaki et al [142]
NAFLD and NASH (no liver biospy)	NAFLD, 19 NASH, 107	Lectin Ab ELISA	Fucosylated haptoglobin	NASH AUC, 0.734	Kamada et al [71]
NAFLD and NASH	Training cohort: NAFLD, 29 NASH, 95 Validation cohort: NAFLD, 169 NASH, 213	Anti-lectin ELISA	Fucosylated haptoglobin and MAC-2-BP	AUC, 0.854	Kamada et al [144]

Figure 6. Glycome-based markers of NAFLD and NASH. AUC, area under the curve; DSA-FACE, DNA sequencer-assisted fluorophore-assisted capillary electrophoresis; ELISA, enzyme-linked immunosorbent assay; MS, mass spectrometry; NA2, galactosylated non fucosylated biantennary glycan; NGA2F, core fucosylated agalactosylated biantennary glycan.

hypertension,[128] in support of the hypothesis that they are markers of inflammation rather than fibrosis.

The GlycoFibroTest measures ratios of N-glycans in serum of patients with chronic HCV infection and results correlate with fibrosis stage determined by histologic analysis. Significant decreases in GlycoFibroTest results are observed by 4 weeks after initiation of treatment with direct-acting antiviral agents for HCV infection and persist in patients with a sustained virologic response to treatment (X Verhelst et al, unpublished data, March 2018). Similar findings were observed in analyses of results from Fast-lec Hepa test in patients with chronic HCV infection.[125] Furthermore, decreases in FIB-4,[129] aspartate aminotransferase-to-platelet ratio index,[129] and FibroTest scores,[130] and in transient liver stiffness measurements[131] (markers of fibrosis) were reported at the end of treatment of patients with chronic HCV infection. We find it surprising that fibrosis can begin to resolve in the short timeframe of the treatment period.

In addition to markers of fibrosis and inflammation, serum glycome signatures have been described in patients with cirrhosis. The GlycoCirrhoTest[21] measures proportions of bisecting GlcNAc containing N-glycans and proportions of triantennary N-glycans. Its results can distinguish patients with compensated cirrhosis from patients with chronic liver disease without cirrhosis with 79% sensitivity and 86% specificity. This bisecting GlcNAc is the end product of GnT-III,[132] an enzyme that is overabundant in hepatocytes in cirrhotic nodules.[132–134] Serum samples from patients with cirrhosis have increased levels of colony-stimulating factor 1 receptor, which can be measured by an antibody-lectin sandwich enzyme-linked immunosorbent assay.[135]

So can glycomic markers more accurately detect liver fibrosis and liver cirrhosis than other biomarkers? A glycomic-based test for fibrosis identifies patients with advanced fibrosis (F3–F4) with a diagnostic accuracy of at least 80%—comparable to that of other serum tests for fibrosis, such as FibroTest[136] and Fibrosis-4[137] test, which are used in clinical practice. Furthermore, combined results from the FibroTest and GlycoCirrhoTest had increased performance compared with the single test for diagnosis of cirrhosis.[21]

Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis

Markers are needed to distinguish NAFLD from NASH,[138] which still requires liver biopsy analysis.[139,140] DNA sequencer-assisted fluorophore-assisted capillary electrophoresis analysis of glycomic signatures of serum samples was able to distinguish patients with NASH from patients with NAFLD. This GlycoNASHTest is based on an increase of a core fucosylated agalactosylated biantennary glycan and a decrease of galactosylated nonfucosylated biantennary glycan.[22,141] Its diagnostic performance is comparable to that of the assays to measure K18F, currently the best available noninvasive assay for detection of NASH. Interestingly, the value of the GlycoNashTest increased significantly according to the level of lobular inflammation but not with fibrosis stage. A highly simplified assay for endo-β-*N*-

acetylglucosaminidase from *Streptococcus pyogenes* (endoS) has been developed.[114] This should help advance this biomarker toward clinical implementation.

The Sweetblot technique, an automated protocol for integrated glycoblotting and mass spectrometry, detects a combination of 3 glycans in serum (2 core fucosylated and 1 nonfucosylated triantennary glycans) and can distinguish patients with NASH from those with only steatosis (area under the curve, 0.83).[142] Levels of these glycans correlate with levels of lobular inflammation, ballooning, and steatosis. Serum samples from patients with NASH have increases in fucosylated MAC-2-BP and fucosylated haptoglobin compared to patients with simple steatosis.[71,143,144] The GlycoNashTest and assays that measure fucosylated MAC-2-BP and FucHpt were superior to tests to measure CK-18 in detection of NASH, using liver biopsy analysis as the reference standard.[22,71,143,145]

Liver Transplantation

Liver transplantation is the best option for many patients with end-stage liver disease, and has excellent long-term results.[146] Decreasing donor organ quality increases the risk of complications, including the occurrence of primary nonfunction, requiring urgent repeated transplantation.[147] Changes in glycosylation of proteins in perfusate, the solution used to transport the donor organ to the acceptor, detected shortly before transplantation were associated with the development of primary nonfunction after liver transplantation.[116] These changes might be used as biomarkers of nonfunctioning livers before transplantation and prevent futile liver transplantations. In the first year after liver transplantation, serum protein glycosylation patterns are independently associated with organ survival (X Verhelst et al, unpublished data, September 2019).

Newer Developments in Glycome Analysis

Reliable glycan profiling of complex mixtures requires detection of a wide range of glycans with high levels of sensitivity.[148] Analyses must be transferrable to affordable high-throughput systems that can operate in an automated fashion in the routine clinical laboratory environment. Lectin and antibody–lectin microarrays might be used in profiling. Lectins are carbohydrate-binding proteins that recognize motifs of oligosaccharide structure, such as a monosaccharide, a certain linkage, combination of monosaccharides.[149] Lectin arrays and lectin-based flow cytometry methods are useful for initial assessments of differences in glycomes between samples, or to screen surfaces of living cells. However, there is a need to design sufficiently robust automated immunoassays. An example is the fast-lec Hepa test for liver fibrosis.[125] One major disadvantage is that it is straightforward to identify glycan structural alterations based on lectin binding data. This makes it difficult to associate biochemical changes with pathologies. Also, it is a challenge to produce recombinant forms of many of the most useful lectins, so they require purification from their natural source. This is a disadvantage for manufacturing of diagnostic tests.

Direct glycan structure profiling tools, like mass spectrometry, are used extensively in glycome research because they provide useful information with a detailed structure analysis. However, mass spectrometry is less suitable for routine clinical chemical analyses, due to its higher level of complexity and cost vs available alternatives (LC and capillary electrophoresis).[150,151] Moreover, accurate quantification of glycans by mass spectrometry requires careful operation and often involves chemical glycan derivatization, which is difficult to implement. LC analysis of fluorophore-labeled glycans has been developed and provides a robust method with a wide dynamic range for glycan profiling.[149] Several laboratories use this method for glycan profiling. LC is difficult to parallelize and can be time-consuming if large series of samples need to be analyzed.[152]

This problem is overcome with capillary electrophoresis. High-throughput analysis methods on multicapillary analyzers of various types have been developed to analyze the N-glycome of serum and glycoprotein sample types (perfusate of liver transplants, urine, and others).[118,119] Sample preparation is performed in a single 96-well plate; multicapillary instruments originally designed for DNA analysis can analyze up top 96 samples simultaneously in 30 minutes. This technique is excellent for high-throughput glycomic profiling. As with LC, to enable sensitive detection, the glycans need to be derivatized with a fluorophore through simple chemistry.

For capillary electrophoresis analysis, methods have been developed to enable glycan release from the protein followed by this labeling, without any intermittent purifications. This allows the researcher to start with a serum sample and prepare it for capillary electrophoresis profiling of the N-glycans on the proteins in serum simply by adding reagents, followed by incubation, which can be fully automated. Furthermore, this analysis can be transferred to capillary electrophoresis analyzers, which are already used for serum protein profiling in most modern clinical chemistry laboratories, enabling straightforward implementation in clinical practice. The Glyco Liver Profile (Helena Biosciences, Newcastle, UK) is the first regulated clinical diagnostic test that has become available to routine clinical chemistry laboratories and is based on this technology. The Glyco Liver Profile allows for analysis of several glycan biomarkers used to analyze liver tissues—particularly the GlycoCirrhoTest.[21] Other tests for specific quantification of alterations in IgG glycosylation associated with chronic inflammation are also under development based on this platform.[114]

Future Directions

Glycans regulate protein and cell functions. Changes in the glycome have been associated with many gastrointestinal and hepatic diseases. Alterations in glycan structure cause pathologic events that contribute to development of disease, and these can be used as biomarkers for diagnosis and prediction of disease progression and response to therapy. Supplementation with glycans has been shown to

reduce features of colitis in mice and control T cell–mediated immune response in human IBDs.

Combining data generated by advanced glycomic and glycoproteomic analyses (see the Human Glycome Project, https://human-glycome.org/) with data on the human genome, epigenome, microbiome, and immune profiles will increase our understanding of how specific glycans induce or modify gastrointestinal and liver disease phenotypes. Applying these findings to longitudinal and prospective clinical studies would allow us to generate glycan-based markers for use in diagnosis and prognosis, as well as identify targets for therapy. It will be important to incorporate glycome analyses into clinical studies and the therapeutic decision-making process of gastrointestinal and liver diseases.

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Conflicts of interest
These authors disclose the following: Xavier Verhelst is listed as co-inventor on a patent owned by Ghent University (Belgium) for a glycomics-based biomarker for the prediction of primary nonfunction after liver transplantation. Hans Van Vlierberghe is listed as co-inventor on a patent owned by Ghent University (Belgium) for a glycomics-based biomarker for the prediction of primary non function after liver transplantation. Nico Callewaert is listed as a co-inventor on a patent on GlycoCirrhoTest that is owned by VIB vzw and has been licensed to Helena Biosciences. The remaining authors disclose no conflicts.

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