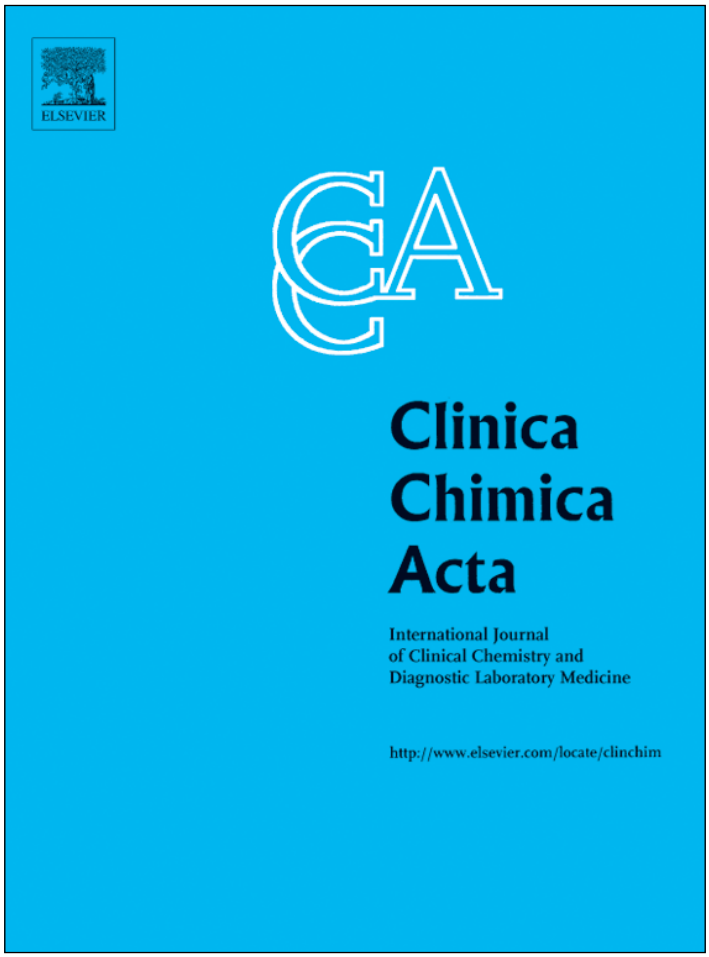

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Review

Glycoprotein biomarkers and analysis in chronic obstructive pulmonary disease and lung cancer with special focus on serum immunoglobulin G

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) and lung cancer are two major diseases of the lung with high rate of mortality, mostly among tobacco smokers. The glycosylation patterns of various plasma proteins show significant changes in COPD and subsequent hypoxia, inflammation and lung cancer, providing promising opportunities for screening aberrant glycan structures contribute to early detection of both diseases. Glycoproteins associated with COPD and lung cancer consist of highly sialylated *N*-glycans, which play an important role in inflammation whereby hypoxia leads to accumulation of sialyl Lewis A and X glycans. Although COPD is an inflammatory disease, it is an independent risk factor for lung cancer. Marked decrease in galactosylation of plasma immunoglobulin G (IgG) together with increased presence of sialic acids and more complex highly branched *N*-glycan structures are characteristic for COPD and lung cancer. Numerous glycan biomarkers have been discovered, and analysis of glycovariants associated with COPD and lung cancer has been carried out. In this paper we review fundamental glycosylation changes in COPD and lung cancer glycoproteins, focusing on IgG to provide an opportunity to distinguish between the two diseases at the glycoprotein level with diagnostic value.

1. Introduction

Chronic obstructive pulmonary disease (COPD) and lung cancer (LC) represent a growing global health problem [1]. Both diseases are primarily associated with cigarette smoking exposure. The prevalence of COPD in lung cancer cases is 42.4% in smokers compared to 21.8% in nonsmokers [2]. However, different environmental factors, such as harmful gases play also important roles in the formation of the diseases [3,4]. COPD is a progressive deterioration of pulmonary functions, which can be characterized by airway obstruction and abnormal and chronic inflammatory responses of the lung to various environmental effects. [5]. Progression of the disease is associated with the enhanced chronic inflammation in the airways and the lung caused by various factors such as inflammatory cytokine release, protease anti-protease imbalance and autoantibody expression [3,6,7]. Abnormal protein glycosylation has been widely documented in various inflammatory diseases, which are accompanied by increased fucosylation and decreased galactosylation and sialylation [8,9].

Patients with COPD have a high risk of developing hypoxia and lung cancer because almost all cancerous tissues show inflammation, thus the chronic inflammation in COPD might turn to lung cancer by sharing many common pathways for activation [6,10–13]. Lung cancer is one of the common malignancies with various histological variants that arise from different cell types, such as bronchial epithelium, bronchioles, alveoli, or bronchial mucous glands and the leading cause of cancer-related deaths worldwide [14,15]. This is a heterogeneous disease caused by mutations in oncogenes and comprises various subtypes with pathological and clinical relevance [16]. The most frequent histological subtypes can be classified as non-small cell lung carcinoma (NSCLC), which represents 80–85% of all lung cancers. Squamous cell lung

Abbreviations: ADC, Adenocarcinoma; CE-LIF, Capillary Electrophoresis–Laser Induced Fluorescence; CE-MS, Capillary Electrophoresis–Mass Spectrometry; COPD, Chronic Obstructive Pulmonary Disease; FTICR, Fourier Transform Ion Cyclotron Resonance; Fuc, Fucose; GlcNAc, *N*-acetyl glucosamine; Hex, Hexose; HexNAc, *N*-acetylhexosamine; Neu5Ac, *N*-acetyl neuraminic acid; Neu5Gc, *N*-glycolyl neuraminic acid; HILIC, Hydrophilic Interaction Liquid Chromatography; IgG, Immunoglobulin G; LC, Lung Cancer; LCC, Large Cell Carcinoma; LC-MS, Liquid Chromatography Mass Spectrometry; MRM, Multiple Reaction Monitoring; NET, Neuroendocrine Tumor; NSCLC, Non-Small Cell Lung Carcinoma; PNGase F, Peptide *N*-glycosidase F; PTM, Post Translational Modification; RP-HPLC, Reversed Phase High Performance Liquid Chromatography; SCLC, Squamous Cell Lung Carcinoma; UHPLC, Ultra High Performance Liquid Chromatography

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carcinoma (SCLC) accounts for approximately 20–30% of NSCLC cases, while adenocarcinomas (ADC) comprise about 40–50% of NSCLC cases [5,11]. However, neuroendocrine tumors (NET) that arise in neuroendocrine cells represent 25% of primary lung cancers [17]. These subtypes have become important as determinant of therapy in this disease [18]. Similarly to inflammatory conditions, which are typical for COPD low galactosylation and sialylation of *N*-glycans can be observed in lung cancer as well [19,20]. Several fucosylated tetra-antennary structures with varying degrees of sialylation and the level of outer-arm fucosylation are also increased in inflammatory and malignant lung diseases [21]. Furthermore, fucosylated tri-sialylated tri-antennary glycans show different ratios of sialylation linkages, such as abundance levels of isomers with two and three α2,3-linked sialic acids and an increased abundance of an isomer with two α2,6-linked sialic acids [22].

2. COPD, hypoxia and inflammation

COPD is a progressive disease of the lung during which the risk of alveolar hypoxia and consequent hypoxemia increases [23]. Hypoxia develops due to insufficient blood and concomitant oxygen supply of organs. This condition allows formation of a microenvironment that essentially contributes to the development of inflammation and malignant processes. Recently, considerable efforts have been made to discover the molecular mechanism of hypoxia-induced inflammation in the lung. It has been found that hypoxia is a potent proinflammatory stimulus in systemic organs and may cause damaging inflammatory effects in the lung [24,25]. The extremely low concentration of oxygen induces inflammatory response, which results in an increase in the number of immune cells and the activation of downstream signaling pathways. This process together with regulation of oxygen-dependent gene expression leads to induction of proinflammatory networks including cytokines and chemokines [26]. Hypoxia activates a unique network of genes in the lung, which does not exist in other organs [27]. The members of the CREB (cyclic AMP response element-binding protein) transcription family are selectively activated by phosphorylation under hypoxic conditions. The mechanism of activation is unclear but CREB phosphorylation may supposedly occur via the AKT kinase (Protein kinase B) and not the classic G-protein–mediated signal transduction. AKT kinase is activated in response to a number of cellular signals and CREB phosphorylation may occur directly through the kinase. However, protein kinase C (PKC) and calcium–calmodulin complexes may also take part in the activation process [28,29]. Phosphorylated CREB activates gene transcription in the nucleus that triggers inflammation.

3. Link between COPD and lung cancer

Increasing number of studies confirmed that there is a link between COPD and lung cancer [30,31]. 40–70% of lung cancer patients had been diagnosed with COPD before development of most subtypes of lung cancer, including ADC, large cell carcinoma (LCC) and particularly small cell lung carcinoma (SCC) and SCLC [2,32]. However, COPD not necessarily leads to lung cancer. A comprehensive study conducted in the United States between 1971 and 1992 indicated that only 6% of mild and moderate/severe COPD cases led unambiguously to lung cancer [33]. According to current research, sufficient amount of data is not available whether each of the subtypes of lung cancer would be linked to COPD as well as smoking and COPD together necessarily leading to the development of a specific or uncommon histological subtype of NSCLC [33–35]. Nevertheless, SCLC has been found to be most closely related to smoking and COPD since COPD increases the risk for SCLC histological subtypes by more than fourfold compared to NSCLC [35]. NSCLC still shows strong correlation with COPD whereby SCC, LCC and ADC are less associated with the disease. Neuroendocrine lung tumors are also closely related to COPD, however, the mutual role of the immune system and the neuroendocrine system in the lower respiratory tracts of COPD patients is scarcely investigated [36]. The neuroendocrine immune control at the site of inflammation occurs via release of neurotransmitters, and various neuropeptides by nerve fibres [37]. These substances have pro-inflammatory activity, but the abnormalities in the neuropeptide pathways in COPD is hardly understood. These observations raise the possibility of common underlying pathological mechanism(s) in the various histological subtypes of lung cancer and COPD, which is corroborated by the fact that COPD is a chronic inflammation and can be a major factor contributing to the progression of lung cancer [30,32,38,39].

Chronic inflammation associated with COPD likely plays a role in the pathogenesis of SCLC and acute exacerbation can be the critical factor in the tumorigenesis of lung cancer. Smoking generates highly reactive molecules through inflammatory and epithelial cells as well as leukocytes within the lung leading to formation of superoxide radicals and hydrogen peroxide. These reactive oxygen species have a key role in sustaining inflammation in COPD through redox-dependent activation of inflammatory transcription factors [40,41]. Inflammation may lead to airway epithelial injury and high cell turnover rates resulting in accumulation of DNA errors and thus amplification of the carcinogenic effects of cigarette smoking [42]. Lung epithelium of heavy smokers finally transforms into a squamous metaplasia phenotype that is associated with the severity of airway obstruction [43].

Further, similar abnormalities have been observed in the glycan structure of various proteins including the members of the immunoglobulin G (IgG) family along with the progress of both diseases. Therefore, accurate and sophisticated molecular diagnostic tools, such as sensitive detection of changes in the glycan structure of glycoprotein biomarkers with Liquid Chromatography Mass Spectrometry (LC-MS), Capillary Electrophoresis Mass Spectrometry (CE-MS) and Capillary Electrophoresis Laser Induced Fluorescence detection (CE-LIF) could help in the early diagnosis. Particularly, differentiating biomarkers could be of great benefit because COPD and lung cancer supposedly have related pathological background, but require different treatments.

4. Glycoproteins in diseases

Co- and post-translational modifications (PTMs), are enzymatically controlled processing events, during which various modifying groups are covalently bound to one or more amino acid residues of the protein released from the ribosome [44]. The most common ones encompass glycosylation, phosphorylation, acetylation, methylation, and sulfation [45]. Most of the human proteins are glycosylated and the glycans play an important role in cellular differentiation and proliferation, apoptosis, oncogenic transformation and metastasis [46–48]. Glycoproteins generally consist of numerous glycosylated variants (glycoforms) of a single polypeptide [49]. Although all proteins undergo the same glycosylation processes most glycoproteins develop characteristic glycosylation patterns and thus heterogeneous populations of glycans at each glycosylation site [49]. Two major types of glycans have been identified. *N*-linked glycans have various carbohydrate moieties attached to the nitrogen atom in the asparagine side chain within a consensus amino acid sequence of Asn-X-Ser/Thr, where X is not proline. *O*-linked glycans contain carbohydrate molecules attached to the oxygen atom of amino acid residues including serine and threonine. *C*-linked glycosylation of proteins have also been described [50]. C-mannosylation differs fundamentally from *N*- and *O*-glycosylation, however, the biochemical details of this modification are still unclear [51]. *O*-GlcNAcylation (*O*-GlcNAc) is also a PTM and involves the attachment of single *O*-linked *N*-acetylglucosamine residue to Ser and Thr residues of cytoplasmic, nuclear and mitochondrial proteins [52,53]. Cycling of *O*-GlcNAc is controlled by two enzymes. OGT (*O*-GlcNAc transferase) transfers O-GlcNAc to the protein, whereby O-GlcNAcase (OGA) catalyses the hydrolysis of this sugar modification. *O*-GlcNAcylation regulates various cellular processes including transcription, translation and

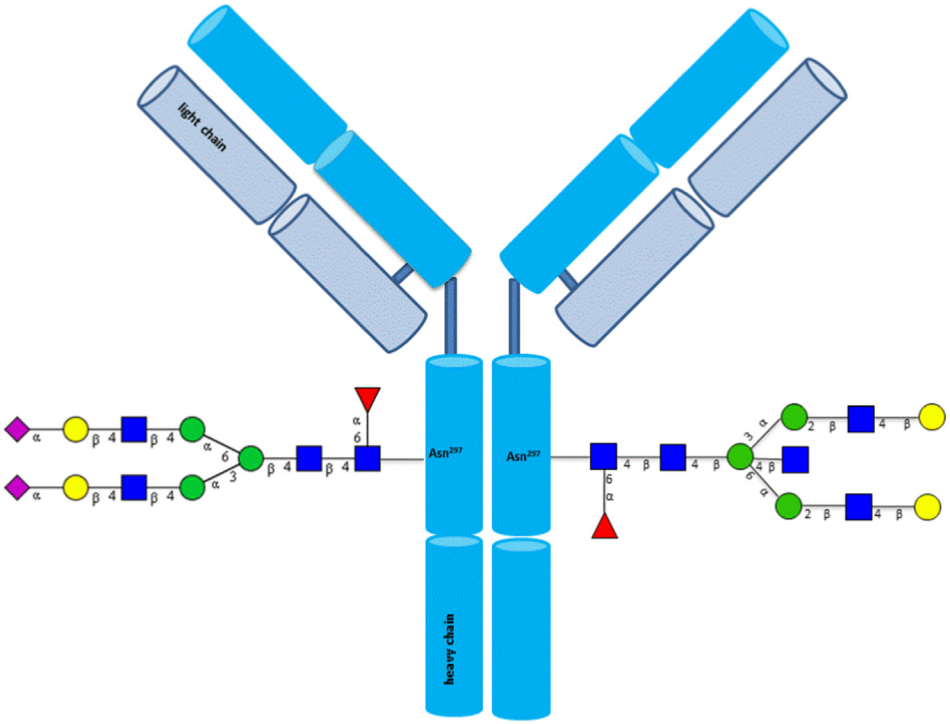

Fig. 1. Glycan structures of human IgG. Core glycan components: ■ N-acetyl glucosamine, ● Mannose. Variable glycan components: ● Galactose, ■ N-acetyl glucosamine, ▲ Fucose, ◆ Sialic acid. Numbers and Greek letters indicate the position of a linkage between individual sugar residues.

signal transduction and metabolism [54]. Cancer and many other diseases are associated with aberrant glycosylation patterns of proteins on both *N*-linked and *O*-linked carbohydrate chains that was first described by Meezan in 1969 and has been confirmed by other scientists [55–59]. Formation of these aberrant structures can be attributed to the changes in the expression level of glycosyltransferases and glycosidases. One of the most common changes in the glycan structure in cancer is an increase in the size and branching of *N*-linked glycans. This increased branching is often attributed to the increased activity of *N*-acetylglucosaminyl transferase V (GlcNAc-TV) that leads to β1,6 GlcNAc branching [60,61]. The increased branching results in formation of additional sites for terminal sialic acid residues, which together with a corresponding upregulation of sialyl transferases, leads to an increase in overall sialylation [62].

Fucosylation occurs by attaching fucose residues to both *N* and *O*-glycans. Its regulation is complicated and involves various fucosyltransferases, GDP-fucose (Guanosine 5′-diphospho-β-L-fucose) synthetic enzymes and GDP fucose transporters. Fucosylation can occur in α1-2, α1-3, α1-4 and α1-6 positions on the oligosaccharides of *N* and *O*-glycans [63]. It has been shown that lack of core fucose on the conserved (ASN297) oligosaccharide site of human IgG1 enhances antibody-dependent cellular cytotoxicity (ADCC), offering an opportunity for therapy with fucosylated antibodies in various diseases [64]. Increased level of core fucosylation has been described in various pathological conditions, such as inflammation and cancer [63]. For instance, stage-dependent changes in IgG fucosylation and an association of several IgG glycoforms with the survival of cancer suggest that IgG glycosylation is related to pathogenesis of cancer and progression of the disease [65].

5. COPD, hypoxia and lung cancer specific glycan biomarkers

Various COPD biomarkers have been discovered, which can be classified as plasma proteins originally from lungs and inflammation-related glycoproteins that are associated with some of the features of the diseases [66,67]. The glycoprotein surfactant protein-D (SP-D) and the Club (Clara) cell protein 16 (CC-16) are synthesized specifically in the lung and can be detected in the blood of COPD and healthy patients [68]. SP-D modulates immune and inflammatory responses in the lung and plays a role in protecting against viral infection [69]. It is a potential inflammatory biomarker because it is synthetized predominantly in the respiratory tract [70]. However, the *N*-glycosylation of SP-D changes in COPD compared to healthy control and the exacerbation of the disease generates further alterations in the glycan structure. Monitoring these alterations may help in better understanding of the inflammation that influences the glycan structure of proteins in the lung in the course of the disease.

CC-16, a 16 kD homodimeric protein secreted by bronchiolar club cells is also a predominant protein in the lung that has shown promise as a biomarker of disease activity [71]. It protects the respiratory tract against oxidative stress and inflammation within the airways [72]. CC-16 expression decreases with lung injury and smoking and is associated with accelerated decline in lung function [71]. Because of its presumed relationship to inflammation CC-16 has been proposed as a biomarker in the study of asthma and bronchial cell dysfunction [73].

Hypoxia accelerates abnormal glycan expression in cancer cells trough initiating transcription of genes that control glycan synthesis [74,75]. Enhanced expression of sialyl and fucosyltransferases can be observed in hypoxia, which contributes to synthesis of abnormal sialyl Lewis A and sialyl Lewis X glycans [76,77]. Sialic acid residues in these structures mostly contain *N*-glycolyl neuraminic acid (Neu5Gc) instead of *N*-acetyl neuraminic acid (Neu5Ac) residues, which latter is typical for normal glycan structures [78]. Some genes; however, are commonly involved in the synthesis of glycans both in healthy and cancerous cells. Therefore, hypoxia-induced expression of disialyl Lewis A and sialyl 6-sulfo Lewis X glycans in normal cells can also be observed. On the other hand, ischemic conditions in normal cells induce an accumulation of *N*-glycolyl disialyl Lewis A and N-glycolyl sialyl 6-sulfo Lewis X glycans. Although glycans are synthetized by various genes, which reflect abnormalities in cancer, genetic aberrations are manifested at the glycoprotein level. Therefore, a glycoprotein that carries the effects of tumor hypoxia via abnormal gene expression has a potential to be a useful biomarker for screening hypoxia induced genes [74].

A comprehensive study of glycan structures of lung tumor tissues of 42 lung adenocarcinoma patients has shown that 29 glycan structures are differently expressed compared to non-malignant controls [79].

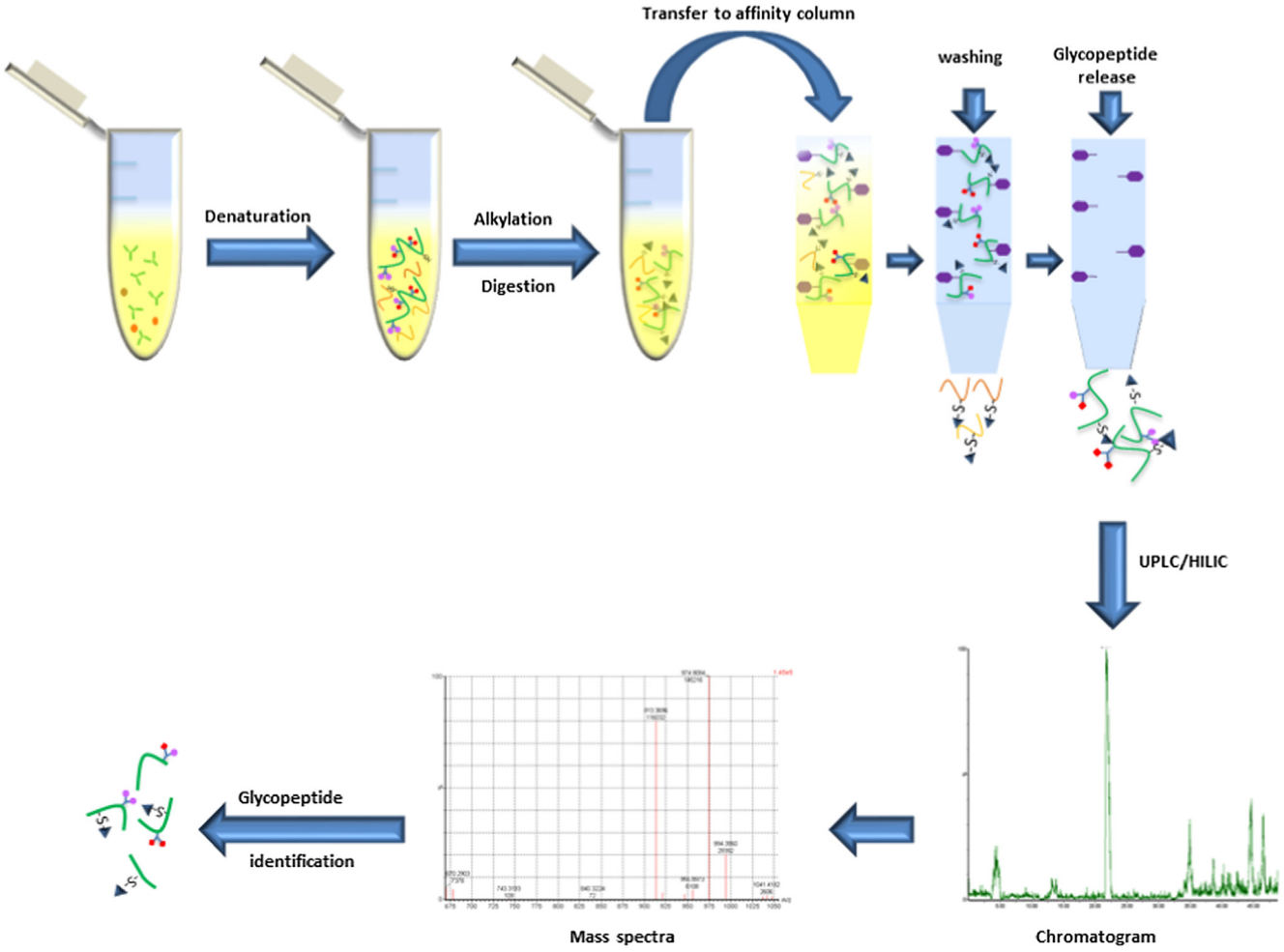

Fig. 2. Preparation of glycoprotein samples for LC-MS. – Intact IgG molecule. – various glycan structures, ▲ – iodoacetamide, – affinity ligand. Brown and orange circles and lines represent other intact and denatured proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Increased level of two fucosylated, lowly galactosylated tetraantennary glycans and several oligomannose type glycans together with non- or lowly galactosylated glycans mostly with core fucosylation were observed. Fully galactosylated glycans, including hybrid type and mostly without fucose, however, were downregulated in cancerous tissue. In lung cancer, increase in Sialyl Lewis X (SLeX), monoantennary glycans and highly sialylated glycans have been observed in the glycan structure of various proteins, such as the acute phase proteins of haptoglobin and α-1-Antitrypsin [11,80]. SLeX plays an important role in initiating inflammation that is characteristic in COPD, thus characterized by the local inflammatory response in the lungs [81]. α-1-Antitrypsin (A1AT) is a serum glycoprotein with three potential *N*-glycosylation sites on asparagin residues at positions 46, 83 and 247, and shows up-regulated glycosylation pattern in lung adenocarcinomas [82–84]. A number of A1AT glycovariants has been observed in lung cancer patients, particularly showing increase in the *N*-linked core-fucosylation [85].

6. IgG glycosylation in COPD, hypoxia and lung cancer

Changes in glycan structures associated with various diseases including cancer and COPD have been investigated in case of IgG subclasses as well. Normally, the biological activity and effector function of IgGs is modulated by covalently attached bi-antennary *N*-glycans at the highly conserved asparagine 297 residue at the C_H2 domains of the crystallizable Fc region on the heavy chain (Fig. 1) [86–93]. The main *N*-glycans are core-fucosylated and may carry sialic acid species or bisecting GlcNAcs [90].

IgG glycans show alternative glycosylation pattern in COPD compared to healthy patients. Pavic et al have completed the first comprehensive study of individual variation of plasma protein and IgG *N*-glycosylation involving 137 subjects with COPD and 95 matching controls [66]. They identified 39 peaks for *N*-glycans in plasma and 24 peaks for *N*-glycans for purified IgG followed by glycan release with PNGase F (Peptide *N*-glycosidase F) and separation with Hydrophilic Interaction Chromatography - Ultra High Performance Liquid Chromatography (HILIC - UHPLC). 16 out of 39 directly measured plasma glycans (including 4 out of 24 IgG glycans) differed significantly between the COPD and control groups. They presumed that the total plasma protein *N*-glycome reflected an overall trend of glycosylation changes, which could be attributed to changes in glycosylation of individual proteins as well as changes in their plasma concentration leading to enhanced sensitivity to the pathophysiological events.

Complexity of IgG *N*-glycans exhibit significant decrease in low branched mono and biantennary species and hypogalactosylation including decline in monogalactosylation, which is the most prominent change of the IgG *N*-glycome associated with COPD [66,94]. However, the decrease in galactosylated glycoforms of IgG has been observed in other autoimmune or inflammatory diseases, therefore it cannot be considered as a selective biomarker of COPD [95]. On the other hand, increase in more complex tri- and tetraantennary glycan structures as well as bisecting GlcNAc (N-acetylglucosamine) on IgG can also be observed with exacerbation of COPD.

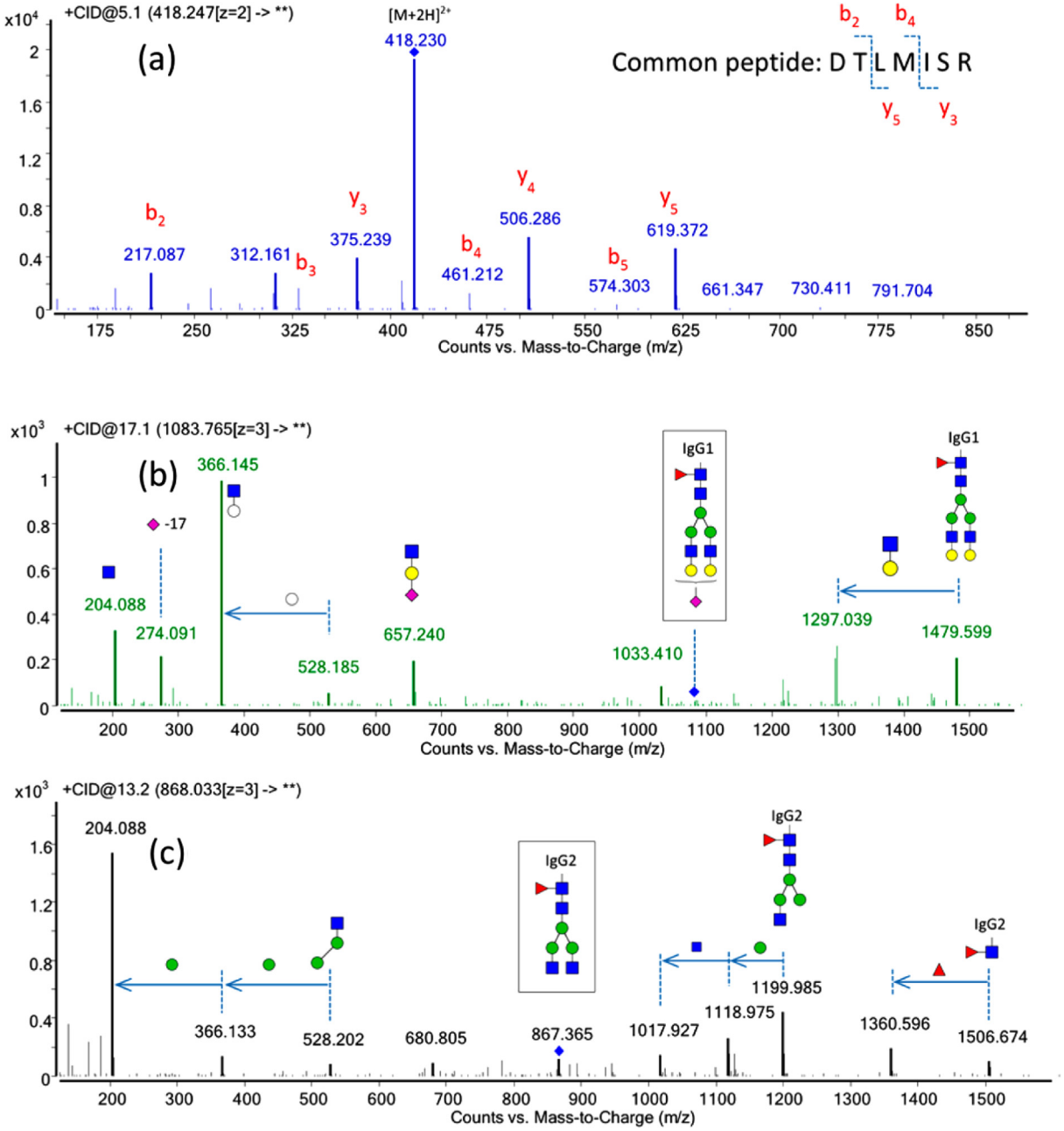

Fig. 3. Mass spectra of trypsinized IgG1-4. Adapted with permission from Hong et al, Absolute quantitation of immunoglobulin G and its glycoforms using multiple reaction monitoring. Anal. Chem. 2013; 85:8585-8593. Copyright 2013, American Chemical Society [121].

Hypoxia substantially decreases the levels of *N*-glycosylation of various integrins leading to abnormal trafficking to the cell surface [96]. Regoetzi and coworkers have also confirmed that fucosylation of IgG is significantly less in hypoxic liver cells (10.4 ± 2.1%) than in normal cells (20.1 ± 2.2%) but no evidence has been found in relation to abnormal IgG glycosylation due to hypoxia in COPD or lung cancer [96,97].

Aberrant glycosylation can be observed in essentially all types of human cancers [98,99]. Significantly increased level of SLeX, monoantennary and trisialylated glycans have been identified in SCLC [100]. Altered branching of *N*-glycans in conjunction with aberrant glycosylation of mucins have also been observed, which are accompanying phenomenon of carcinogenesis and cancer metastasis [101–103]. Further, Ruhaak and coworkers have found four glycans (Hex3HexNAc4Fuc1, Hex5HexNAc5Sia1, Hex5HexNAc5Sia2, and Hex5HexNAc5Fuc1Sia2), which differed in IgG fractions of lung adenocarcinoma patients compared to healthy controls [104]. It has also been revealed that in lung cancer a marked decrease in sera IgG1 Fc - galactosylation occurred together with galactosyl-related Fc-glycosylation, which is not typical for healthy individuals [105].

It is possible that alternative IgG glycosylation might have similar function(s) with the progress of COPD and lung cancer. This is because inflammation leads to abnormal *N*-glycan processing and thus aberrant glycosylation pattern of proteins can be observed in patients with COPD [87]. The IgG glycovariants lacking of core fucose have increased affinity to activating Fc-receptors (FcγRs) resulting in drastic enhancement of antibody-dependent cellular cytotoxicity, whereby IgG glycovariants with terminal sialic acid residue have strong anti-inflammatory activity [106–108]. However, the link between COPD and lung cancer considering abnormal IgG glycosylation has been poorly investigated.

Table 1
Mass spectrometric analysis of trypsinized IgG subclasses. Adapted with permission from Hong Q et al, Absolute quantitation of immunoglobulin G and its glycoforms using multiple reaction monitoring. Anal. Chem. 2013; 85:8585-8593. Copyright 2013, American Chemical Society [121].

Compound Name	Precursor ion (*m/z*)	Product ion (*m/z*)
H3N5F1-IgG1	946.5	204.1
H4N4-IgG1	884.1	204.1
H4N4F1-IgG1	932.8	204.1
H3N4F1-IgG1	878.8	204.1
H5N4-IgG1	938.1	366.1
H5N4F1-IgG1	986.8	366.1
H4N5-IgG1	951.7	204.1
H4N5F1-IgG1	1000.5	204.1
H5N5F1-IgG1	1054.5	366.1
H4N4F1S1-IgG1	1029.8	204.1
H5N4F1S1-IgG1	1083.8	366.1
H3N4F1-IgG3/4	873.4	204.1
H4N4F1-IgG3/4	927.4	204.1
H3N5F1-IgG3/4	941.1	204.1
H4N5F1-IgG3/4	995.1	204.1
H5N4F1S1-IgG3/4	1078.4	366.1
H3N5F1-IgG2	935.8	204.1
H4N4F1-IgG2	922.1	204.1
H4N5F1-IgG2	898.9	204.1
H5N4F1-IgG2	976.1	366.1
H3N4F1-IgG2	868.1	204.1
H4N5-IgG2	941.1	204.1
H4N4-IgG2	873.4	204.1
H5N4F1S1-IgG2	1073.1	366.1
H5N5F1-IgG2	1043.8	366.1
H4N4F1S1-IgG2	1019.1	204.1
Peptide IgG1234 (DTLMISR)	418.2	506.3
		619.4
Peptide IgG3	472.9	697.4
		534.3
Peptide IgG1	839.4	968.5
		1067.6
Peptide IgG2	970.1	1100.6
		839.5
Peptide IgG4	635.0	1217.6
		425.2

7. Glycoprotein, glycopeptide and glycan analysis

Mass spectrometry (MS) is a powerful technique for profiling the human glycome leading to discovery of several potentially promising biomarkers for several types of diseases [57,109–113]. MS-based techniques are key for the recent expansion of glycomics capabilities and they are widely used in glycoprotein analysis. For example, Wu and co-workers investigated the structure of fucosylated corticosteroid-binding globulin, histidine-rich glycoprotein, complement factor B, N-acetylmuramoyl-L-alanine amidase and thyroxine-binding globulin in ovarian cancer with MS and found significant differences in the glycan structure of the trypsynized glycoproteins [114,115]. Other authors have currently reported decreased high-mannose, hybrid-type, complex-type asialo, bi, tri- and tetraantennary sialylated structures in blood samples of ovarian cancer patients. On the other hand, increased tri, tetraantennary fucosylated, afucosylated, and fucosylated triantennary structures were observed in MS analysis [84,116].

MS analysis of glycoproteins in most instances requires thorough sample preparation by exposure the glycoproteins to reducing agents such as dithiothreitol, which reduce disulfide bonds in the intact proteins, heat denaturation and alkylation with organic compounds, such as iodoacetamide to prevent re-formation of disulfide bonds in the denatured protein, and proteolytic digestion, enrichment of the glycopeptides, however, losses cannot be avoided (Fig. 2) [117]. Proteolytic digestion is mostly carried out with trypsin and in combination with reversed-phase high-performance liquid chromatographic (RP-HPLC) separation allowing characterization of the proteins of interest and monitoring for minor alterations in a population of molecules [118]. Recently HILIC is the most frequently applied technique for glycopeptide separation and analysis. It employs traditionally polar silica stationary phases rendered with various hydrophilic organic groups, such as amino, amide, diol or sulfoalkyl betaine groups and a hydrophobic, mostly organic mobile phase. Retention increases with the hydrophilicity of solutes [119]. HILIC can be considered as a variant of normal-phase liquid chromatography (NP-LC), however, its separation mechanism is more complicated than that of in NP-LC [120]. HILIC has numerous advantages over NP-LC or RP-HPLC, for example it is suitable for analyzing compounds in complex matrices that normally elute in the dead volume of RP-HPLC. Furthermore, no expensive ion pair reagents are required in HILIC and it can be conveniently coupled to mass spectrometry with electrospray ionization (ESI) mode [120].

Hong et al have developed a method using Multiple Reaction Monitoring (MRM) to monitor protein glycosylation in serum/plasma to examine quantitative changes in glycosylation at a site-specific level [121]. They identified 26 glycopeptide fragments from tryptic digested IgG samples (Fig. 3), which corroborated the findings of other researchers [122]. However, the use of MRM for such a complex glycan and glycoconjugate analyses is limited, mostly because MRM is performed on triple quadrupole instruments that are often optimized for small molecules and thus large glycopeptides are outside of the mass range of the instrument.

Reducing the sample complexity by exposure the glycoprotein or glycopeptide to glycosidases and the analysis of the glycan structures themselves may provide a better understanding of glycosylation mechanisms in COPD and lung cancer with the opportunity to detect even more subtle differences in the carbohydrate patterns characteristic of the two diseases. Increased resolution of separation techniques allows more sophisticated analysis and identification of the oligosaccharide structures. Capillary electrophoresis (CE) is a high performance separation technique, which offers high resolution, short analysis time and minimal consumption of samples making it an attractive technique for glycoprotein and glycan screening [123]. CE applies a very high potential (10–30 kV) in a narrow bore (typically 50–100 μm ID) glass capillary and separation occurs based on the different electrophoretic mobility of the analytes. The majority of CE instruments are equipped with UV, LIF detectors or MS. Meszaros and coworkers used CE-LIF to perform glycoprotein analysis of human serum acquired from healthy, COPD and patients with comorbidity of COPD lung cancer. They have identified 13 *N*-glycan structures and their subclasses, such as fucosylated, mono-, bi-, tri- and tetra-sialylo, as well as mono-, bi-, tri- and tetra-antennary glycans providing the opportunity for development of promising biomarkers for early detection of COPD and lung cancer [124].

Furthermore, some other biomarkers, such as CA-125 (Cancer Antigen 125), CD44 (Cluster of Differentiation 44, a cell surface adhesion receptor that is highly expressed in many cancers), CD166 (Cluster of Differentiation 166, a transmembrane glycoprotein), lysosome-associated membrane glycoprotein 2 (LAMP-2), and pulmonary surfactant protein A were found to be associated with lung cancer and tumor progression [125]. Comprehensive glycomic analysis of these biomarkers may open a new avenue in early diagnosis of lung diseases and contribute to the discovery of new medicines.

8. Analysis of IgG in COPD and lung cancer

LC-MS is a rapid and sensitive technique for proteomics and therefore emerged as a unique tool in glycopeptide analysis, for instance investigation of IgG subclasses [111,121,122,126]. Glycopeptides are exposed to mass spectrometric analysis followed by proteolytic cleavage of the IgG molecule and chromatographic separation of the resulting glycopeptides [127]. Tryptic digestion generates a large number of glycopeptides with different molecular masses and structures. Separation and mass spectrometric detection of all IgG

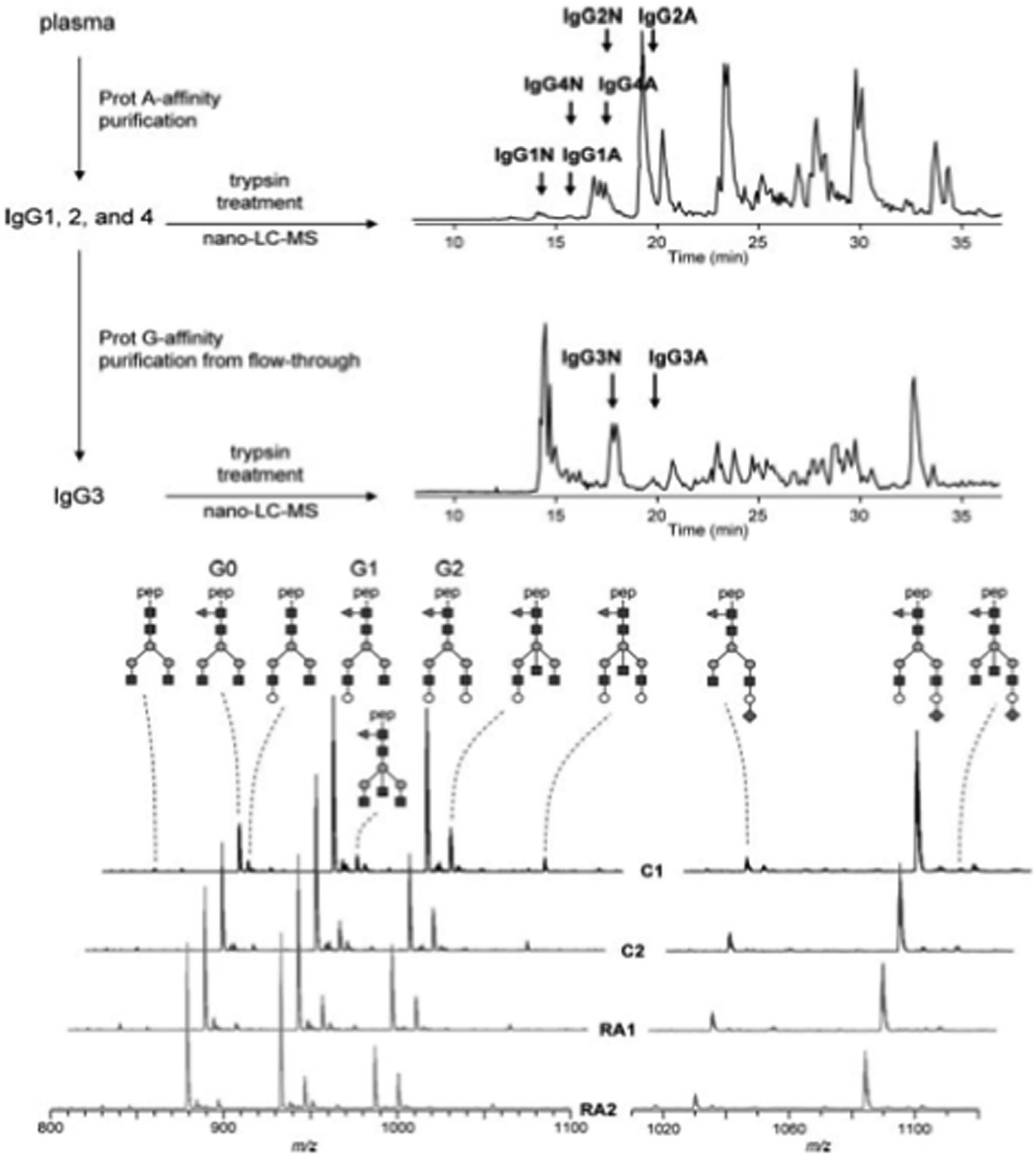

Fig. 4. HPLC chromatogram of trypsinized IgG and mass spectra of IgG1. C1 and C2: healthy controls, RA1 and RA2: patients with rheumatoid arthritis. With permission from Wuhrer et al, Glycosylation profiling of immunoglobulin G (IgG) subclasses from human serum. Proteomics 2007, 7, 4070–4081. Copyright 2007,WILEY-VCH Verlag GmbH & Co. [122].

glycopeptides can be performed with the use of modern UPLC technique with high separation capacity and in conjunction with high resolution MS systems, which allows generating large and complex data sets. However, some characteristic structures of IgG with well-defined *m/z* values have been identified (Table 1) contributing to more simple data review. Typically the characteristic ions with *m/z* values of 204.1 (HexNAc), 366.1 (Hex1-HexNAc1), 292.09 (Neu5Ac) and 657.24 (Hex1HexNAc1Neu5Ac1) are searched in the mass spectra to identify glycopeptides followed by tryptic digestion of IgG [121].

Mass spectrometric analysis has revealed abnormal glycosylation of the IgG family members associated with various diseases, such as rheumatoid arthritis, inflammatory bowel disease [128], multiple sclerosis [59], ankylosing spondylitis, primary Sjögren's syndrome, psoriatic arthritis [129] and systemic lupus erythematosus (SLE) [95]. In general, the degree of galactosylation is decreased together with elevated levels of truncated *N*-glycans in these diseases, just like in lung cancer [90], however, in rheumatoid arthritis, the lowered degree of galactosylation is associated with the relative abundance of the bisected species and it is well expressed in IgG1. Similar tendencies can be observed with the other IgG subclasses (Fig. 4).

IgG glycosylation in COPD and lung cancer with LC-MS is relatively poorly investigated. Chen and coworkers employed Fourier Transform Ion Cyclotron Resonance Mass Spectrometery FTICR-MS to evaluate IgG glycosylation in 259 lung cancer patients. They reported significant differences in Fc-glycosylation features, such as agalactosylation, galactosylation, and digalactosylation between lung cancer patients and healthy controls. A marked increase in IgG1 Fc *N*-linked-agalactosylation and decrease in galactosylation and digalactosylation were observed compared to 410 control individuals, which were associated with increased susceptibility of lung cancer [130].

9. Conclusion

Abnormal glycosylation patterns of numerous glycoproteins associated with different diseases, such as inflammation or cancer have been discovered and subjected to high sensitivity bioanalytical screenings. Global glycan analyses has revealed similar glycosylation patterns of proteins in COPD and lung cancer patients indicating that the response of cells to inflammation and malignant transformation processes may occur via similar pathological routes. Generally, decrease of the complexity of *N*-glycans regarding low branched simple glycans and monogalactosylation is typical in both diseases. On the other hand, more complex, highly branched tri and tetra antennary glycans also with sialylation exhibit increase in COPD and lung cancer. Furthermore, patients diagnosed with lung cancer have increased level of IgG1 Fc-agalactosylation. Despite of the discovery of these abnormal IgG glycosylations in COPD and lung cancer, no detailed and comprehensive mass spectrometric study of aberrant IgG peptidoglycans has been provided until now. Therefore, further development of LC-MS and CE methods is desirable to detect tiny variations in the glycan structures shedding insight into differences or similarities as well as key points of IgG glycosylation that might have critical role in formation and consequences of progression of both diseases. UPLC-MS has excellent peak resolution and detection capabilities to separate the glycopeptide fragments of trypsinized IgG molecules and could serve as a discovery tool for new and unknown IgG glycosylation patterns specific to COPD and lung cancer. CE-MS offers a unique combination of high sensitivity and resolution as well as minute sample requirement and short analysis time providing the opportunity for selective diagnosis of the two diseases. Utilization of IgGs as abundant glycan carrying biomarkers can considerably contribute to better understanding of COPD and lung cancer on the molecular level.

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