



Review

N-glycomic biomarkers of biological aging and longevity: A link with inflammaging

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ABSTRACT

Glycosylation is a frequent co/post-translational modification of proteins which modulates a variety of biological functions. The analysis of N-glycome, *i.e.* the sugar chains N-linked to asparagine, identified new candidate biomarkers of aging such as N-glycans devoid of galactose residues on their branches, in a variety of human and experimental model systems, such as healthy old people, centenarians and their offspring and caloric restricted mice. These agalactosylated biantennary structures mainly decorate Asn297 of Fc portion of IgG (IgG-G0), and are present also in patients affected by progeroid syndromes and a variety of autoimmune/inflammatory diseases. IgG-G0 exert a pro-inflammatory effect through different mechanisms, including the lectin pathway of complement, binding to Fc γ receptors and formation of autoantibody aggregates. The age-related accumulation of IgG-G0 can contribute to inflammaging, the low-grade pro-inflammatory status that characterizes elderly, by creating a vicious loop in which inflammation is responsible for the production of aberrantly glycosylated IgG which, in turn, would activate the immune system, exacerbating inflammation. Moreover, recent data suggest that the N-glycomic shift observed in aging could be related not only to inflammation but also to alteration of important metabolic pathways. Thus, altered N-glycans are both powerful markers of aging and possible contributors to its pathogenesis.

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1. Introduction

Enzymatic glycosylation (not to be confused with non-enzymatic glycosylation, referred to as glycation) represents one of the most frequently occurring co/posttranslational modification of proteins. A significant percentage of the human genes encode proteins related with biosynthesis, function and degradation of sugar chains. Protein-linked sugar chains play a variety of highly specific roles, acting as a “fine tuning” of the interactions between cells and between molecules (Hart and Copeland, 2010; Ohtsubo and Marth, 2006; Varki, 1993). A brief summary of some biological functions played by the glycans of glycoproteins is reported in Table 1. The sugar chains linked to glycoproteins are classified

mainly as N-linked chains, which are bound to the amidic nitrogen of asparagine, and O-linked chains which are bound to the hydroxyl group of serine or threonine. Glycosylation undergoes profound changes in a variety of pathological conditions, including cancer [reviewed in Dall'Olio et al., 2012a,b], inflammatory and autoimmune diseases and in the pathophysiological process of aging. Recently developed high-throughput methods of analysis have allowed investigating the whole spectrum of N-linked glycans (N-glycome) present in serum and body fluids of a large number of individuals, revealing characteristic aging-associated N-glycome changes which are reminiscent of those associated with inflammatory and autoimmune diseases. These glycosylation changes appear to be associated particularly with the sugar chains linked to Asn297 of immunoglobulin G. In turn, these aberrantly glycosylated antibodies might be responsible for the activation and up-regulation of the inflammatory response through different, although ill defined, mechanisms. In this review, we will describe briefly the basic biosynthetic and structural aspects of N-glycosylation and the methods of N-glycome analysis. Then, we will discuss the most relevant N-glycans associated with aging and inflammatory

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Table 1
Biological functions regulated by the sugar chains of glycoproteins.

Function	References
Activity of growth factors receptors	Lau et al. (2007)
Activity and stability of integrins	Chiricolo et al. (2006) and Shaikh et al. (2008)
Function of cadherins	Pinho et al. (2011)
Intercellular adhesion of neural cells	Kiss and Rougon (1997)
Apoptosis and cell survival	Lee et al. (2008) and Swindall and Bellis (2011)
Leukocyte adhesion to endothelia	Phillips et al. (1990)
Angiogenesis	Tei et al. (2002)
Antibody effector functions	Albert et al. (2008) and Nimmerjahn and Ravetch (2008a)
Anti-tumor surveillance	Ohyama et al. (2002)
Quality control of nascent proteins	Hebert et al. (2005) and Molinari (2007)
Sorting of lysosomal enzymes	Dahms et al. (1989) and Ghosh et al. (2003)
Fertilization	Pang et al. (2011)
Binding of viruses	Childs et al. (2009) and Yamada et al. (2006)
Binding of bacteria	Marcos et al. (2008)

Only a few representative references are provided for each of the indicated biological role of glycosylation. The regulation of biological phenomena by protein-linked carbohydrate structures is very complex. Frequently, slight modifications of the carbohydrate structures result in totally different biological effects. An example is provided by the interactions between IgG and Fc γ R, which are finely regulated by the N-glycan linked to Asn297 of IgG, as described in detail in Section 7.3.

diseases and their possible pathogenic role. Finally, we will propose that the N-glycomic changes observed in elderly are largely due to the low-grade pro-inflammatory status known as inflammaging (Franceschi, 2007; Franceschi et al., 2007).

2. Biosynthesis of N-linked chains

In the rough endoplasmic reticulum (RER), an oligosaccharide comprised of two N-acetylglucosamine, nine mannose and three glucose residues is assembled on a lipid molecule, the dolichol phosphate, through the successive action of specific enzymes: the glycosyltransferases. Then, this preassembled oligosaccharide is transferred to the Asn-X-Ser/Thr (where X is any amino acid except proline) motif on the nascent protein chain by the multi-meric enzyme complex oligosaccharyl transferase (Kornfeld and Kornfeld, 1985). The following steps of the biosynthesis are depicted in Fig. 1A. The oligosaccharide first undergoes trimming of the glucose and of some of the mannose residues. Then N-acetylglucosamine, galactose, sialic acid and fucose residues are added forming “complex type” glycans. The trisaccharide units comprised of sialic acid-galactose-N-acetylglucosamine are referred to as branches or *antennae* (Fig. 1). When a N-acetylglucosamine residue is β 1,4-linked to the innermost Man residue (Fig. 1B) is referred to as “bisecting GlcNAc” and is not elongated further. The presence of a fucose residue α 1,6-linked to the innermost N-acetylglucosamine is referred to as “core fucosylation” (Fig. 1B).

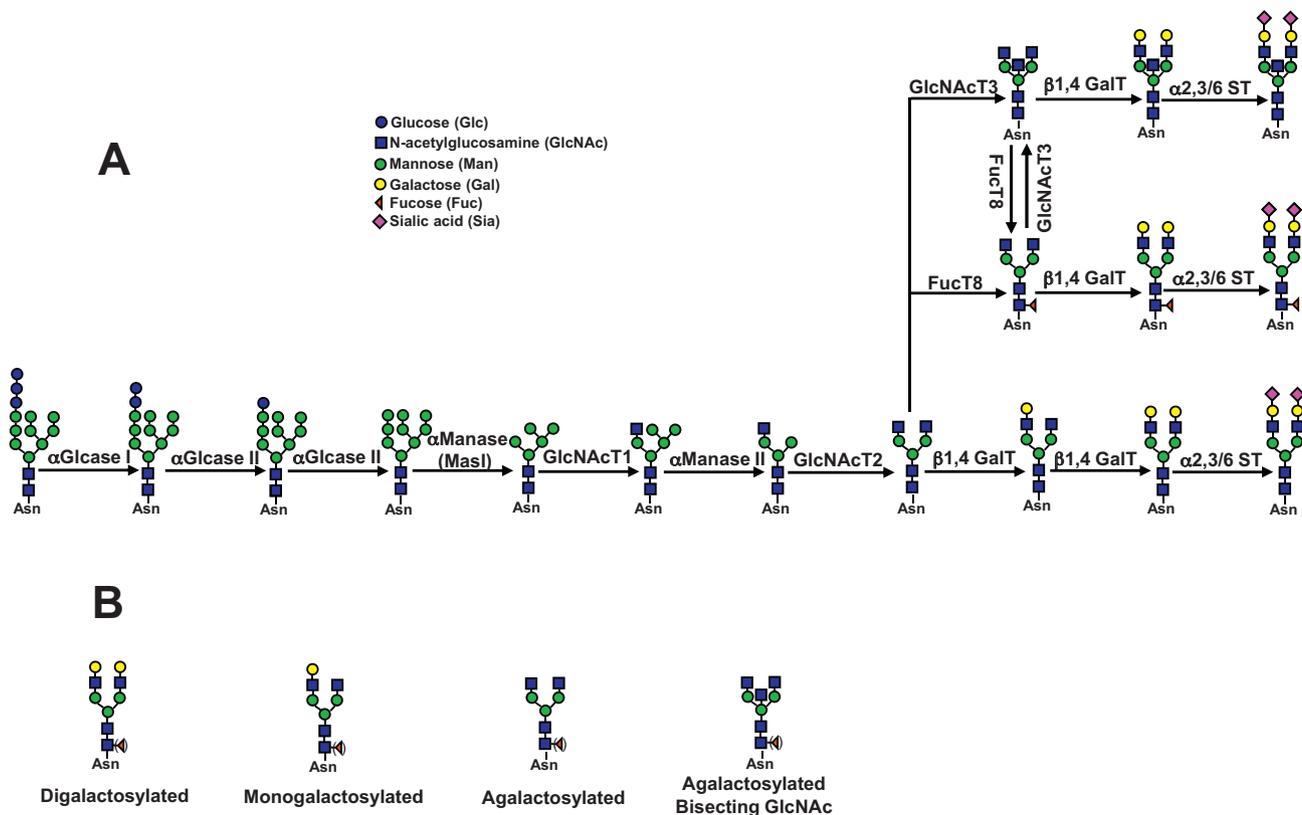


Fig. 1. (A) Biosynthesis of N-glycans in mammals. A preassembled oligosaccharide comprised of 2 GlcNAc, 9 Man and 3 Glc residues (left) is co-translationally transferred to an asparagine residue of a nascent protein chain. This structure undergoes processing by the sequential removal of 3 Glc residues, mediated by α -glucosidases (α Glcase) I and II, and 4 Man residues, mediated by α -mannosidases (α Manase). Then, a first antenna GlcNAc is added to the Man5GlcNAc2 structure by the action of GlcNAcT1. Subsequently, 2 other Man residues are removed by α -mannosidase II (α Manase II) and a second GlcNAc residue is added by GlcNAcT2. The resulting structure can be further elongated by the addition of galactose residues, mediated by the β 1,4GalT family and/or by core-linked fucose (mediated by FucT8) and/or bisecting GlcNAc (mediated by GlcNAcT3). The addition of these modifications is not mutually exclusive. After the addition of galactose, the sugar chains are frequently terminated by sialic acids which can be linked either via α 2,3 or α 2,6 to galactose. (B) Structure of N-glycans relevant in aging and inflammatory processes mentioned in the text. IgG molecules carrying on Asn297 digalactosylated, monogalactosylated or agalactosylated sugar chains are referred to as IgG-G2, IgG-G1 and IgG-G0, respectively. The presence of core-linked fucose, which is usually present on IgG, is indicated in parenthesis.

Unlike the biosynthesis of nucleic acids and proteins, which are deterministic processes, glycosylation is a stochastic process, regulated mainly by the relative abundance of the specific glycosyltransferases and nucleotide-activated sugar donors and by that of the catabolic enzymes (glycosidases) which remove single monosaccharide units. The stochastic nature of the glycosylation process is at the basis of the phenomenon known as microheterogeneity, which means that the structure of the sugar chains attached to a specific glycosylation site in a given glycoprotein displays a certain degree of variability (for example, the core fucose or the bisecting GlcNAc can be present on some but not all molecules).

3. Methods of glycan analysis

Lectins are invaluable tools for the study of glycosylation in normal and pathologic conditions, including inflammatory diseases (Tsuchiya et al., 1998). In particular, the sugar epitopes most relevant in inflammatory diseases and aging have been investigated in western blot or enzyme-linked assays using the following lectins: for α 2,6-linked sialic acid, the lectin from *Sambucus nigra* (SNA) (Shibuya et al., 1987); for terminal galactose, *Ricinus communis* (RCA) (Barker et al., 1999; Parekh et al., 1985; Wong et al., 1993) or *Erythrina cristagalli* (ECL) (Nimmerjahn et al., 2007) lectins; for terminal GlcNAc, *Bandeiraea simplicifolia* II (BSA II) (Alavi et al., 2000; Croce et al., 2007; Sumar et al., 1991) or *Psathyrella velutina* (PVL) (Tsuchiya et al., 1993); for core fucosylation, *Ulex europaeus* I (UEA I) (Flogel et al., 1998; Gornik et al., 1999), *Aspergillus oryzae* (AOL) (Vanhooren et al., 2011) or *Lens culinaris* agglutinin (LCA) (Shinkawa et al., 2003); for bisecting GlcNAc, *Phaseolus vulgaris* E4 (PHA-E4) (Shinkawa et al., 2003).

In recent years, methods allowing the high-throughput analysis of free N-glycans or of tryptic glycopeptides have been developed. Free N-glycans were obtained by treatment with peptide N-glycanase F (PNGase F), an enzyme which releases any kind of N-linked chain irrespective of the presence of core fucose, and then fluorescently labeled with 8-amino-1,3,6-pyrenetrisulfonic acid (Laroy et al., 2006), 2-aminobenzamide (Knezevic et al., 2010) or with aminobenzoic acid (Ruhaak et al., 2011). A method for high-throughput glycan analysis combines hydrophilic interaction liquid chromatography (HILIC) HPLC using amide-based columns with exoglycosidase digestion and weak anion exchange columns for the separation of branched sialylated N-linked glycans (Knezevic et al., 2010). Structure assignment of eluted peaks was made on the basis of a relational database (Royle et al., 2008). HILIC HPLC with fluorescence detection has been used in several investigations (Knezevic et al., 2009, 2010; Pucic et al., 2010, 2011; Ruhaak et al., 2011, 2008; Wuhler et al., 2009). Analysis with MALDI-TOF mass spectrometry of tryptic peptides of IgG has also been applied (Ruhaak et al., 2010b).

Electrophoretic separation of fluorophore-linked carbohydrates has been shown to provide reliable results, compared with lectin analysis (Martin et al., 2001). An adaptation of carbohydrate electrophoresis to high-throughput analysis has been obtained by the use of a genome sequencer (DNA sequencer-adapted-fluorophore assisted carbohydrate electrophoresis, DSA-FACE) (Callewaert et al., 2001; Laroy et al., 2006). The original version of this technique required the removal of sialic acids from PNGase F-released oligosaccharides, yet recently a variant of this procedure has been described that allows the profiling of sialylated glycans (Ruhaak et al., 2010a). The technique has been utilized for the analysis of human and mice glycome in aging (Vanhooren et al., 2007, 2008, 2009, 2010, 2011). The main advantages of this technique are its robustness, high throughput, high sensitivity and reliable quantification. Also this technique can be performed on any kind of biofluid (serum, plasma, urine, semen, saliva, etc.) and

homogenized tissue. N-glycans are removed from their carrier protein, making it impossible to state which protein is differently N-glycosylated. This can be overcome by firstly isolating a fraction of proteins of interest (e.g. antibodies) and prepare N-glycans from this subfraction of proteins (Vanhooren et al., 2007) or by combining the DSA-FACE results with lectin blotting (Vanhooren et al., 2011). A comparison of the advantages and limitations of the different analytical approaches is reported in Table 2.

4. Carbohydrate structures associated with inflammatory diseases

The N-linked chains attached to Asn297 in the Fc portion of IgG are of the complex biantennary type (Arnold et al., 2007). Antennae are terminated by a variable number of sialic acid and galactose residues (Tsuchiya et al., 1998), while the core structure can be substituted by bisecting GlcNAc and/or core-linked fucose (Fig. 1B). Unlike in IgA, where more than 90% of the sugar chains are sialylated, in IgG approximately only 10% of the chains are terminated by sialic acid (Mattu et al., 1998). The sugar chain linked to Asn297 is peculiar because it is structurally constrained within the cavity formed by the polypeptide chains of the IgG Fc (Krapp et al., 2003). Moreover, one of the two branches of the N-linked chains can shift between a protein-bound and an unbound state, regulating the access to glycan modifying enzymes and glycan recognition molecules (Barb and Prestegard, 2011). Thus, it is not surprising that glycans bound to Asn297 play key roles in mediating IgG stability (Yamaguchi et al., 2006) and effector functions (Raju, 2008).

Many investigations published in the past 25 years have revealed that the N-linked chains attached to Asn297 of IgGs undergo characteristic glycosylation changes in a variety of inflammatory and autoimmune conditions and in aging. In particular, it has been observed: (i) a reduced presence of galactose and sialic acid on the antennae, leaving GlcNAc in terminal position; (ii) an increased expression of bisecting GlcNAc and (iii) an increased core fucosylation. The first of these three features is by far the most relevant. Depending on the presence of galactose on one or both arms of the molecule, three subfamilies called IgG-G0 (no galactose), IgG-G1 (galactose on one arm) and IgG-G2 (galactose on both arms) have been defined (Jefferis et al., 1990; Wormald et al., 1997). The structures of the two N-linked chains attached to the Asn297 residues of the two heavy chains of the IgG molecule are not necessarily identical, further increasing the heterogeneity. In a 1985 study (Parekh et al., 1985), it was shown for the first time that IgG from both rheumatoid arthritis (RA) and primary osteoarthritis patients exhibited an increased level of short N-linked oligosaccharides, chains terminated by N-acetylglucosamine [reviewed in Parekh et al., 1989; Rademacher et al., 1988; Rademacher, 1991]. Successively, the presence of agalactosylated N-linked chains was found to be associated with different autoimmune diseases (Axford et al., 2003; Isenberg, 1995; Pilkington et al., 1995b; Martin et al., 2001), as summarized in Table 3.

Likewise, the presence of IgG-G0 is associated with inflammatory conditions in animal models. For example, arthritis induced by pristane administration in CBA/Igb mice (Rook et al., 1991a), or adjuvant treatment in Lewis rats (Yagev et al., 1993) was accompanied by a rise of IgG-G0. Moreover, in arthritis-prone MLR-lpr/lpr, disease was accompanied by an increase of IgG-G0 (Kuroda et al., 2001a), while in other arthritis prone mice strains arthritis and IgG-G0 rise were induced in parallel by the mere administration of complete Freund adjuvant (Bodman et al., 1994). The relationship between arthritis and IgG-G0 production was observed also in arthritis-prone HTLV-1 transgenic mice (Endo et al., 1993).

In recent years, the presence of agalactosylated structures has been associated also with epithelial cancers [reviewed in Arnold

Table 2
Advantages and limitations of analytical techniques used for glycomic analysis.

Technique	Advantages	Limitations
Lectin analysis (Parekh et al., 1985; Fogel et al., 1998; Tsuchiya et al., 1998; Alavi et al., 2000; Vanhooren et al., 2011; Shinkawa et al., 2003).	<ul style="list-style-type: none"> • Unexpensive • Easy • Does not require pre-treatment of the samples • Does not require sophisticated equipments 	<ul style="list-style-type: none"> • Provides information only on the presence or absence of some structural motifs. • Unable to provide information on the overall structure of glycans • Poor sensitivity
DNA-sequencer-adapted fluorophore-assisted electrophoresis (DSA-FACE) (Callewaert et al., 2001; Laroy et al., 2006; Vanhooren et al., 2007, 2008, 2009, 2011).	<ul style="list-style-type: none"> • Does not require a dedicated equipment • Easy interpretation of the patterns • High-throughput sensitivity 	<ul style="list-style-type: none"> • Relatively expensive • Requires the enzymatic release of glycans and their derivatization • In its basic version requires the removal of sialic acids • No information on the carrying proteins
Hydrophilic interaction liquid chromatography (HILIC) (Ruhaak et al., 2008, 2011; Royle et al., 2008; Wuhrer et al., 2009; Knezevic et al., 2010; Pucic et al., 2011).	<ul style="list-style-type: none"> • High-throughput • Sensitivity Analyzes sialylated compounds	<ul style="list-style-type: none"> • Relatively expensive • Requires the enzymatic release of glycans and their derivatization • Difficult interpretation of the data because many peaks represent mixtures of different compounds. • No information on the carrying proteins
MALDI-TOF analysis of tryptic glycopeptides (Ruhaak et al., 2010b).	<ul style="list-style-type: none"> • High-throughput • Provides information on the carrying proteins • High sensitivity 	<ul style="list-style-type: none"> • Expensive equipment

et al., 2008]. Agalactosylated N-linked glycans are increased in lung (Kanoh et al., 2006) and ovarian (Saldova et al., 2007) cancers, while in breast cancer correlated with lymph node metastasis (Pierce et al., 2010). Recently, a rise of core-fucosylated IgG-G0 and complement activation were described in stomach cancer patients as a result of the immune response to tumor (Bones et al., 2011). The association of agalactosylated N-linked chains with cancer progression and metastasis is confirmed by other studies (Kanoh et al., 2008, 2004a,b). Next to IgG glycosylation changes, triantennary N-glycan from total plasma/serum glycoproteins have been found to be associated with tumor stage in hepatocarcinoma patients as revealed by DSA-FACE (Vanhooren et al., 2009).

The increased expression of IgG-G0 in inflammatory conditions is reversible and closely associated with disease status. This is particularly clear in RA patients in which the IgG-G0 level correlated with the inflammatory process of the synovial tissues (Parekh et al., 1988b), with a more aggressive disease course (Van Zeben et al.,

Table 3
Human inflammatory and autoimmune diseases associated with serum IgG-G0.

Disease	References
Rheumatoid arthritis	Parekh et al. (1985)
Primary osteoarthritis	Parekh et al. (1985)
Juvenile onset rheumatoid arthritis	Parekh et al. (1988c) and Sumar et al. (1991)
Spondyloarthropathy	Leirisalo-Repo et al. (1999)
Childhood tuberculosis	Pilkington et al. (1996a)
Adult tuberculosis	Parekh et al. (1989) and Rademacher et al. (1988)
Erythema nodosum leprosum	Filley et al. (1989)
Systemic vasculitides associated with ANCA ^a	Holland et al. (2002) and Holland et al. (2006)
Systemic lupus erythematosus	Pilkington et al. (1996b)
Lambert–Eaton myasthenic syndrome	Selman et al. (2011)
Myositis	Perdivara et al. (2011)
Crohn disease and ulcerative colitis	Dube et al. (1990) and Shinzaki et al. (2008)
HBV-associated fibrosis	Gui et al. (2010)
HCV-associated fibrosis	Mehta et al. (2008)
Epithelial cancers	Arnold et al. (2008), Bones et al. (2011), Kanoh et al. (2004a,b, 2006, 2008), Pierce et al. (2010) and Saldova et al. (2007)

^a ANCA: antineutrophil cytoplasm antibodies.

1994; Van Beneden et al., 2009) and is predictive of the development of RA (Young et al., 1991; Ercan et al., 2010). Moreover, conditions resulting in an amelioration of the disease status, such as treatment with anti-TNF monoclonal antibody (Van Beneden et al., 2009; Croce et al., 2007; Pasek et al., 2006), pregnancy (Rook et al., 1991b; Alavi et al., 2000; van de Geijn et al., 2009; Pekelharing et al., 1988) and fasting (Kjeldsen-Kragh et al., 1996) resulted in a reduction of IgG-G0 levels. The same relationship between IgG-G0 and status of the inflammatory disease was observed in tuberculosis (Rook et al., 1994), in juvenile chronic arthritis (Fogel et al., 1998) as well as in arthritis-prone DBA/1 mice (Van Beneden et al., 2009).

The reduced galactosylation of N-linked chains appears to be specific for the heavy chain Asn297 of IgG, while it does not involve the light chains of IgG (Mimura et al., 2007; Holland et al., 2006; Youings et al., 1996), or IgA (Field et al., 1994). Consistently, it was recently shown that the effect of different agonists on glycosylation was restricted to IgG, among B-lymphocyte glycoproteins (Wang et al., 2011). These data suggest that disease-associated glycosylation changes of Fc portion might be due to a different accessibility of the acceptor molecule more than to an alteration of the glycosylation machinery. Nevertheless, reduced galactosylation of N-linked chains in RA patients is detectable even in whole serum (Nakagawa et al., 2007).

A relevant role for the cytokine IL-6 in IgG-G0 production is suggested by studies in both humans and animal models. In fact, in Castelman disease (a rare condition characterized by non-cancerous growth of B-lymphocytes in lymph nodes), in which there is hypersecretion of IL-6, IgG-G0 are increased (Nakao et al., 1991). Moreover, IL-6 administration to mice resulted in the appearance of IgG-G0 (Hitsumoto et al., 1992), while the administration of pristane to DBA-1 mice induced an increase of IL-6 which correlated with that of IgG-G0 (Thompson et al., 1992). However, a recently published study has shown that *in vitro* the level of galactosylation of IgG was not altered by IL-6 treatment but rather was increased by treatment with all-*trans* retinoic acid and reduced by IL-21 treatment (Wang et al., 2011). These data suggest that the effect of IL-6 *in vivo* is not direct and requires the presence of other mediators.

In the mouse model MLR-1pr/lpr, the level of IgG-G0 closely paralleled the development of arthritis. However, the depletion of CD4+ lymphocytes inhibited the development of arthritis but not that of IgG-G0 (Kuroda et al., 2001a), indicating that this

glycosylation change was not dependent on T-cell function (Kuroda et al., 2001b). Other investigations suggest that the production of IgG-G0 can be a mere feature of the normal immune response. In fact, when mice kept in a sterile environment were moved to normal housing conditions, it was observed a relative increase of IgG-G0 (Lastra et al., 1998). Moreover, the normal antibody response to bovine serum albumin was accompanied by a relative reduction of IgG galactosylation (Lastra et al., 1998).

In RA and juvenile arthritis it was observed also an increase of core fucosylation of IgG (Gornik et al., 1999), even though the level of fucosylation was not affected by the activity of the disease (Flogel et al., 1998).

5. Carbohydrate structures associated with aging

5.1. Aging in humans

A relationship between galactosylation of the N-linked chains of IgG and age [reviewed by Kobata, 2003] was formerly observed in a 1988 study (Parekh et al., 1988a) reporting that in a group of 151 individuals of both sex (age 1–70 years), the percentage of IgG-G0 reached a minimum at the age of 25 and then increased with age. IgG-G2 showed a concomitant inverse relationship with age, while the level of IgG-G1 remained constant (Parekh et al., 1988a). Successively, in a study on 403 individuals (age 0–85 years) the increased expression of IgG-G0 in old individuals and especially in females was confirmed (Yamada et al., 1997). In male individuals also an increased expression of bisecting GlcNAc on the N-linked chains of human IgG was reported (Yamada et al., 1997). In a study published one year later, the glycosylation of IgG was investigated by HPLC in association with sequential exoglycosidase treatment (Shikata et al., 1998) in men and women age 18–73. It was found an increased age-dependent incidence of agalactosylated structures in females only, while an age-dependent increase of structures containing a bisecting GlcNAc was detected in both genders (Shikata et al., 1998).

More recently, three groups of investigators have studied the age-dependent N-glycomic changes in serum of large cohorts of people from different European countries, by different high-throughput methods of analysis. Some studies were restricted to the IgG fraction of serum glycoproteins whereas other investigations involved the whole serum N-glycome.

A first group of investigators analyzed the age-dependent changes of serum N-glycome by DSA-FACE in Italians and Belgian individuals (Vanhooren et al., 2007). In both populations, it was observed a strong increase of core-fucosylated, agalactosylated biantennary N-linked chains with or without a bisecting GlcNAc, in people over 60. This change was accompanied by a concomitant decrease of core-fucosylated digalactosylated structures (Vanhooren et al., 2007, 2008, 2009). This pattern was common to IgG and IgG-depleted serum glycoproteins, indicating that the hypogalactosylation was not restricted to the antibody fraction of serum glycoproteins. Analysis of an Italian populations of healthy centenarians and of people aged 60–90, revealed that in people above 90 the tendency to increase of agalactosylated structures and the decrease of digalactosylated structures was even more dramatic than in people aged 60–90. Interestingly, a 45-old individual affected by the progeroid Werner syndrome and 6 patients with progeroid Cockayne syndrome aged less than 20 displayed the same feature of centenarians, strongly reinforcing the linkage between physiological or pathological aging and alterations of serum glycome (Vanhooren et al., 2007, 2010).

A second group of investigators established the Leiden Longevity Study which consists of nonagenarian sibling pairs, their offspring, and partners of the offspring which serve as control. This cohort

allows evaluating not only the association of given parameters with calendar age, but also their incidence in the offspring, revealing markers of longevity. MALDI-TOF mass spectrometry analysis of tryptic peptides from IgG Fc glycopeptides confirmed the association with age of agalactosylated and monogalactosylated N-linked structures with and without a bisecting GlcNAc (Ruhaak et al., 2010b). The level of galactosylation was similar in the two genders above the age of 60, whereas it was higher in younger females. Interestingly, in the younger offspring (age <60), the level of agalactosylated structures with a bisecting GlcNAc was lower than in their partners, indicating that a low level of a marker of aging in young people associates with propensity to long life (Ruhaak et al., 2010b). In a successive study, N-glycans from total serum glycoproteins, released by N-glycanase, were analyzed by HPLC separation (Ruhaak et al., 2011). This study confirmed the association of agalactosylated structures with calendar age. Moreover, two glycan features were found to be more highly expressed in the offspring than in controls, indicating their association with longevity. While the corresponding glycans have not been fully characterized, preliminary information points toward two non-fucosylated biantennary glycans (Ruhaak et al., 2011). Although one of these markers was positively associated with parameters of cardiovascular health, such as low BMI and high HDL-cholesterol, their association with familial longevity was independent on these parameters. Interestingly these markers correlated negatively with the C-reactive protein levels which is a marker of inflammation (Ruhaak et al., 2011).

In a study from a third group of investigators on 1008 individuals from the Croatian Adriatic island of Vis, the N-glycome was analyzed by HPLC before and after sialidase treatment. This experimental approach gave rise to 33 peaks comprised of different carbohydrate structures. Owing to the high heterogeneity of the chromatographic peaks, individual glycan structures were not measured. This study confirmed the previously reported tendency to age-dependent increase of biantennary agalactosylated or mono-galactosylated structures with or without bisecting GlcNAc and/or core fucosylation (Knezevic et al., 2009). Consistently, digalactosylated structures sometimes terminated by sialic acid were reported to decrease with age. In addition, a tendency to down-regulation of some, but not all, peaks containing core-fucosylated glycans was observed. The increased presence of agalactosylated biantennary glycans and the concomitant decrease of digalactosylated and sialylated diantennary glycans was further confirmed in a more recent study from the same group in 1914 individuals from the Croatian islands of Vis and Korcula (Knezevic et al., 2010). In addition, in the same study a reduced age-dependent core-fucosylation was reported, but only in females. Interestingly, risk factors for cardiovascular diseases, such as high body fat and blood pressure, were also associated with increased biantennary agalactosylated structures and decreased digalactosylated structures (Knezevic et al., 2010). In a genome wide association study, the presence of agalactosylated sugar structures in human blood was found to be associated with a single nucleotide polymorphism (SNP) in an intron of the FUT8 locus (Lauc et al., 2009), encoding for the fucosyltransferase which mounts core-linked fucose (Yanagidani et al., 1997; Lauc et al., 2010). No associated SNPs were found in the genes encoding other glycosyltransferases potentially involved in the biosynthesis of the above mentioned structure, such as GnT3 or β 4GalT1. Linkage with agalactosylated biantennary N-linked chains was displayed also by the locus encoding estrogen receptor β (ESR2). These findings can explain the individual variability of N-glycome (Pucic et al., 2010) on a genetic basis, but hardly the age- or disease-dependent modulation of N-glycome. The same group of investigators showed that the glycome of healthy individuals undergoes very little or no changes over short periods of time (about 5 days), while minor changes can be observed in the same

healthy individuals analyzed over a 1 year long period (Gornik et al., 2009). A study on the individual variability of human N-glycome has revealed the existence of a normal pattern, which is shared by the majority of individuals and of a limited number of outliers with remarkably different N-glycomic patterns (Pucic et al., 2010). Among them, some individuals with reduced galactosylation of N-linked chains and some with increased core-fucosylation were identified and found to be healthy (Pucic et al., 2010).

Altogether, the studies summarized above indicate that the human serum glycome is strongly influenced by the genetic background even though environmental conditions are likely to play a relevant role. Importantly, plasma glycan features reflect age as well as the health status of a person and represent a reservoir of disease biomarkers.

5.2. Aging in experimental model systems

Although N-glycosylation is very different among eukaryotes, there are clear links between N-glycosylation and aging in eukaryotic model-organisms. α 1,2-mannosidase I (Mas1), a member of the class I glycosidases, is expressed in the ER, the Golgi and the lysosome and is involved in N-linked glycosylation (Herscovics, 2001). Several lines of evidence indicate that Mas1 is important during the aging process. In fact, its expression is decreased in aging and oxidatively stressed *Drosophila* (Zou et al., 2000) as well as in the livers of aging mice and humans (Cingle et al., 1996; Zhu et al., 2006). A causal relationship between Mas1 and longevity is supported by the recent finding that in both *Drosophila melanogaster* and *Caenorhabditis elegans*, when Mas1 expression was reduced via RNA interference the longevity of the organisms was extended (Liu et al., 2009).

Sato et al. (1998) investigated the alteration of N-linked glycan pattern of expression in the brain as a function of age. N-glycoproteins in the soluble fraction and in the membrane-bound fraction of various portions of the brain and spinal cords, obtained from 9-week-old rats and 29-month-old rats, were comparatively analyzed by SDS-polyacrylamide gel electrophoresis and lectin staining. The lectin staining pattern of each brain part showed marked differences by the age of the donors. These results show that the glycosylation state of some, but not all, proteins are changed during aging.

A correlation between agalactosylated IgG and aging was observed also in mouse models. In fact, a marked tendency to age-dependent increase of IgG-G0 was observed in all seven mice strain examined between 2–8 months of age (Bodman et al., 1994). A more recent study described a clear age-related increase of agalactosylated N-glycans in full serum in inbred mice housed in a specific pathogen free environment examined between 3–26 months of age (Vanhooren et al., 2011). In the same study it was shown that caloric restriction (CR) could reduce and even invert this age-related increase of agalactosylated N-glycans in serum. In mammals, CR is an effective way to delay age-related diseases and extend good health into old age. Mice that consume 30–40% fewer calories survive 20–50% longer than mice fed *ad libitum* (AL) (Weindruch et al., 1988). A clear anti-aging inversion of the glycan profile was seen in CR mice in comparison to AL control mice. While agalactosyl N-glycans increased during aging in the AL group, they decreased in CR mice demonstrating that agalactosyl structures are really related with the health status of the mouse (Vanhooren et al., 2011). This observation paves the way for using N-glycosylation and especially agalactosyl N-glycans in serum, as biomarkers of good health in the search for CR mimics and aging-delaying interventions and compounds.

Although the wealth of data point to a strong link between altered glycosylation and inflammation, it should also be considered that some of the above summarized observations in both

Table 4

Conditions associated with the presence or absence of digalactosylated or agalactosylated N-glycan structures.

Conditions	Asn	Asn
Human conditions		
Age below 60	High	Low
Age above 60	Low	High
Age above 90 (centenarians)	Very low	Very high
Offspring of centenarians	High	Low
Cockaine syndrome	Low	High
Werner syndrome	Low	High
RA and various inflammatory diseases	Low	High
Some epithelial cancers	Low	High
Pregnancy	High	Low
Animal models		
Young mice	High	Low
Old mice	Low	High
Mice under caloric restriction	High	Low
Arthritis in MLR-1lpr/lpr mice	Low	High

For simplicity, it was reported only one digalactosylated N-glycan structure, with or without core-linked fucose (left) and one agalactosylated structure with or without core-linked fucose and bisecting GlcNAc (right). The monosaccharide composition of the two N-linked chains is explained in Fig. 1.

humans and animal models point also to a direct link between altered glycosylation and metabolic pathways in an inflammation-independent manner.

Data reported in Table 4 make clear how similar are the N-glycosylation changes associated with aging and inflammatory conditions in both human and animal models.

6. Molecular basis of N-glycosylation changes

Lymphocytes from RA patients are able to produce IgG-G0 *in vitro* (Bodman et al., 1992), suggesting that the reduced galactosylation of IgG in pathological conditions is dependent on an alteration of the glycosylation machinery in antibody-producing cells. A possible explanation of the reduced antibody galactosylation would be a reduction of β 1,4galactosyltransferase expression in antibody-producing cell. In testing this hypothesis, it should be kept in mind that the addition of Gal in β 1,4-linkage to glycoproteins can be mediated by at least 6 galactosyltransferases (β 4galactosyltransferases1–6, product of genes B4GALT1–6) (Guo et al., 2001). Investigations on the level of β 1,4galactosyltransferase (β 4GalT) activity in both T and B lymphocytes of healthy volunteers and RA patients revealed that in the latter group the mean β 4GalT activity was 40% reduced (Axford et al., 1987) and the presence of a significant inverse relationship between lymphocyte-associated β 4GalT activity and IgG-G0 (Axford et al., 1992; Alavi and Axford, 1995b). According to other studies, however, the level of galactosyltransferase activity (Furukawa et al., 1990) as well as the level of galactosyltransferase enzyme protein (Keusch et al., 1998a) were found to be the same in B lymphocytes of RA patients and healthy controls. These data, together with the finding that the β 4GalT1 mRNA level appeared to be the same in B lymphocytes of RA patients, compared with control population (Delves et al., 1990; Jeddi et al., 1996), suggest that the regulation of enzyme activity might occur at a post-translational level (Keusch et al., 1998a). This notion is supported by the finding that in RA patients, but not in other inflammatory diseases, an increased percentage of more acidic serum galactosyltransferase isoforms was detected (Alavi et al., 2004). This change was at least in part due to increased

sialylation of galactosyltransferase molecules, a type of modification that is associated with decreased enzyme activity (Alavi et al., 2004). Thus, it can be hypothesized that a regulatory network exists in which increased sialyltransferase activity results in increased sialylation and a concomitant reduced activity of GalT enzyme proteins which, in turn, would be responsible for decreased galactosylation of IgG (Alavi and Axford, 1995a).

Conflicting results have been published on the relationship between IgG-G0 and galactosyltransferase expression in mouse models. Splenic B lymphocytes have reported to be the major source of IgG-G0 (Jeddi et al., 1996). In apparent agreement, the level of β 4GalT1 transcript in total splenic lymphocytes (B+T) was reduced in the arthritis-prone MLR *lpr/lpr* mice (Jeddi et al., 1994, 1996). However, no difference in β 4GalT1 mRNA expression was detected among isolated B lymphocytes of different mouse strains, regardless whether from spleen or peripheral blood (Jeddi et al., 1996). On the contrary, in mouse models of arthritis, the galactosyltransferase activity was found to be lower in peripheral lymphocytes than in splenic lymphocytes (Axford et al., 1994) and reduced in peripheral, but not in splenic B lymphocytes (Alavi et al., 1998). Moreover, structural studies on antibodies of monoclonal origin suggest that high levels of IgG-G0 might result from the expansion of specific subsets of plasma cells (Endo et al., 1989; Omtvedt et al., 2006), while the β 4GalT activity was found to be lower in hybridomas producing rheumatoid factors (RF) than in hybridomas secreting irrelevant antibodies (Axford et al., 1994). Altogether, the above mentioned studies indicate that big differences exist between the galactosyltransferase levels of lymphocyte populations of the same organism and that the reduced galactosyltransferase activity affects only specific antibody-producing cell clones.

As mentioned above, the transfer of galactose to N-linked chains could be mediated by at least six different β 4GalTs. However, the fact that β 4GalT1 KO mice exhibited a dramatic downregulation of β 1,4-galactosylated glycoproteins (Kido et al., 1999; Kotani et al., 2001) indicates that β 4GalT1 is the major. In an attempt to establish the role of β 4GalT1 in IgG galactosylation, a B cell line was transfected with either sense or antisense β 4GalT1 constructs and the effect on IgG galactosylation was determined (Keusch et al., 1998b). It was demonstrated the role of β 4GalT1 overexpression in increasing the galactosylation of IgG, while it was not clear whether its inhibition by antisense construct resulted in a reduction of basal IgG galactosylation.

It can be hypothesized that hypo-galactosylation is a mere consequence of increased antibody production. According to this view, the level of β 4GalT in antibody-secreting cells would be not sufficient to ensure efficient galactose transfer in the presence of increased IgG production. However, this does not appear to be the case. In fact, the disruption of the CD4 gene in MLR-*lpr/lpr* mice resulted in a reduced IgG production but in unchanged IgG-G0 level (Jeddi et al., 1999). In addition, in an *in vitro* culture of naïve human B-cells no correlation was found between the IgG production rate and the degree of galactosylation of IgG Fc N-glycans (Wang et al., 2011). Although many data suggest that the abnormal glycosylation of IgG is regulated at the biosynthesis level, it has also been proposed that galactose of N-linked chains of IgG is destroyed by free radicals generated by phagocytes during inflammation (Griffiths and Lunec, 1989).

In considering the increased level of bisecting GlcNAc associated with aging, it should be kept in mind that the agalactosylated biantennary N-linked chains which accumulate in aging are a preferred substrate for GnT3 (Narasimhan, 1982), which is the only enzyme mediating the addition of bisecting GlcNAc (Ihara et al., 1993). Thus, the biosynthesis of this structure can be favored simply by the increased presence of its precursor substrate.

Altogether, clinical and experimental studies suggest that the altered Asn297 glycosylation affects autoreactive IgG produced by a limited number of B cell clones. The phenomenon is probably caused by a decreased β 1,4GalT1 activity which is not due to down-regulation of B4GALT1 transcription but rather to postranslational modifications of the β 1,4GalT1 enzyme protein.

7. Pathogenic effects of altered IgGs

A first indication on the pathogenic effect of IgG-G0 came from a study showing that arthritis induced in mice by inoculation of IgG from affected mice was enhanced when IgG were previously treated with galactosidase to increase the percentage of IgG-G0 (Rademacher et al., 1994). A differential glycosylation of IgGs could modulate their effector function through at least four mechanisms: (1) interaction with the mannose binding lectin (MBL), the first component of the lectin pathway of complement activation; (2) interaction with lectin receptors on the membrane of APC, namely macrophages and dendritic cells; (3) interaction with Fc γ receptors (Fc γ R) of phagocytes and natural killer cells; (4) formation of antibody/antibody aggregates.

7.1. Interaction with the mannose binding lectin

It has been shown that IgG-G0 possess a five fold higher complement activation activity than normally glycosylated IgG, because of a much stronger interaction with MBL, a molecule homologous to complement C1q which activates complement through the lectin pathway (Malhotra et al., 1995; Ezekowitz, 1995) [reviewed in Rudd et al., 2001; Arnold et al., 2006]. Interestingly, enzymatic removal of galactose enables MBL binding also of IgA (Terai et al., 2006). However, *in vivo* studies indicate that the role of MBL in RA is complex (Garred et al., 2000). On the one hand, the risk of developing heart disease is higher in RA patients with high levels of MBL, but only if they concomitantly express high levels of IgG-G0 (Troelsen et al., 2007). On the other hand, in both MBL-deficient RA patients (Stanworth et al., 1998) and mice with MBL deficiency, the pathogenic activity of IgG-G0 antibodies was not impaired (Nimmerjahn et al., 2007). Further investigations are necessary to clarify the role of the complement activation through the lectin pathway in IgG-G0-mediated pathogenesis.

7.2. Interaction with lectin receptors of APC

The exposure of terminal GlcNAc residues by IgG-G0 has been shown to increase their uptake by macrophages and dendritic cells through lectin receptors, such as the mannose-binding receptor (Dong et al., 1999) and DC-SIGN (Yabe et al., 2010). As detailed below, the administration of high doses of human IgG confers anti-inflammatory activity in a variety of autoimmune settings (Baerenwaldt et al., 2010). This phenomenon involves the interaction of IgG with DC-SIGN and requires the α 2,6-sialylation of the sugar chain linked to Asn297 (Anthony et al., 2008b). It is conceivable that the lack of galactose, which obviously results in a lack of sialic acid, also inhibits this IgG-mediated anti-inflammatory response.

7.3. Interactions with Fc γ R

Fc γ R belong to a family of membrane proteins expressed by leukocytes, acting as receptors for the Fc portion of IgG. The different Fc γ R possess either activating or inhibitory activity for antibody-mediated cellular functions, including antibody-dependent cell cytotoxicity (ADCC) and phagocytosis (Nimmerjahn and Ravetch, 2006, 2008b). Asn297 glycosylation plays a major role in regulating the binding of IgG to Fc γ R (Nimmerjahn and Ravetch,

2006, 2008b; Albert et al., 2008). Owing to the peculiar position of the sugar chain linked to Asn297, the absence of galactose would induce a conformational change of the IgG molecule which is crucial for the binding to Fc γ R (Krapp et al., 2003). The relevance of Asn297 glycosylation in Fc γ R-mediated phenomena [reviewed in Nimmerjahn and Ravetch, 2008b] is further supported by the fact that ADCC was negatively modulated by the α 2,6-sialylation (Krapp et al., 2003; Scallon et al., 2007) and by core fucosylation (Niwa et al., 2005; Shields et al., 2002; Shinkawa et al., 2003; Satoh et al., 2006; Iida et al., 2009; Shibata-Koyama et al., 2009; Iida et al., 2006) and positively modulated by bisecting GlcNAc (Umana et al., 1999; Davies et al., 2001) on this sugar chain. However, low fucose appears to increase ADCC mediated by NK cells but not that mediated by granulocytes (Peipp et al., 2008). Very recently, crystal structure studies have shown that the presence of core-linked fucose prevents the interaction of the Fc glycans of IgG1 with an N-glycan of the Fc γ RIIIa (Ferrara et al., 2011), while hemi-glycosylated (*i.e.* with only one of the two heavy chains glycosylated) IgG1 displayed reduced binding affinity with all types of Fc γ R (Ha et al., 2011). A key role for the interaction with Fc γ R in IgG-G0-mediated pathogenesis was supported by the observation that in inflammatory bowel diseases, IgG-G0 appeared to be associated with increased antibody-dependent phagocytosis, rather than with complement activation (Nakajima et al., 2011). However, the impact of IgG glycosylation on the Fc γ R function is far from clear. In fact, a study reported that the ability of IgG-G0 to interact with low affinity Fc γ RII is undistinguishable from that of normally glycosylated antibodies (Groenink et al., 1996), while other studies reported that the binding to Fc γ R of asialyl- (Adler et al., 1995) or agalactosyl IgG (Adler et al., 1995; Kumpel et al., 1995) was impaired.

The therapeutic administration of high doses of IgG to patients with a variety of hematological and immunological disorders (the so called intravenous IgG, IVIG) results in a suppression of the immune response (Nimmerjahn and Ravetch, 2007, 2008a). Although this phenomenon has already for a long time been exploited in the clinical practice, its mechanism of action remained obscure until it was shown that the immunosuppressive activity of IgG is restricted to 1–3% of the molecules, those bearing α 2,6-sialylated N-linked chains on Asn297 (Kaneke et al., 2006; Anthony et al., 2008a). Mouse experiments indicated that upon binding to DC-SIGN, these α 2,6-sialylated antibodies triggers IL-33 production which induces the expansion of IL-4 producing basophils in a novel Th2 pathway, leading to the expression by phagocytes of the inhibitory receptor Fc γ RIIB (Anthony et al., 2008a, 2011). Moreover, it has been proposed that IgG lacking terminal sialic acid show increased binding to activating Fc γ R, supporting inflammation (Nimmerjahn et al., 2007). Owing to the fact that IgG-G0 obviously lack sialic acid, their differential biological effect would not be mediated by the exposure of terminal GlcNAc but rather by the absence of sialic acid (Nimmerjahn et al., 2007).

7.4. Formation of autoantibody aggregates

Rheumatoid arthritis and other autoimmune diseases are characterized by the presence of anti-IgG antibodies which, in some cases, are directed against IgG-G0 (Mimura et al., 2004, 2005a,b; Nishijima et al., 2001; Das et al., 2004; Maeno et al., 2004). According to some studies, a low galactose content would promote the IgG ability to form aggregates with RF. In fact, the galactose content of IgG in the aggregates from the synovial fluid of RA patients was lower than that of IgG found in non aggregated form (Leader et al., 1996), while the level of galactosylation of IgGs with rheumatoid factor activity (*i.e.* IgG binding activity) was lower than that of IgGs not displaying RF activity from the same patients (Matsumoto et al., 2000). According to other studies, however, the relationship is less

clear (Soltys et al., 1994; Imafuku et al., 2003). Anti-citrullinated protein antibodies (ACPA) are highly specific autoantibodies for a subgroup of RA patients who have severe erosive disease. Many lines of evidence point to a role for ACPA in disease pathogenesis, although through incompletely understood mechanisms. An analysis of Fc glycosylation of IgG1 ACPA in the serum and synovial fluid of patients with early arthritis revealed that IgG1-G0 ACPA were higher in the synovial fluid than in blood of RA patients, suggesting their possible role in the pathogenesis of the disease (Scherer et al., 2010). Moreover, the level of galactosylation was much lower among ACPA than in IgG repertoire (Ercan et al., 2010).

Galactosylation could influence the placental transport of antibodies, in that the level of IgG-G0 is always lower in the fetus than in its mother (Williams et al., 1995; Kibe et al., 1996). Nevertheless, IgG-G0 antibodies appear to be involved in the transmission from mother to child of autoimmune diseases, such as systemic lupus erythematosus (SLE) (Pilkington et al., 1996b) and myasthenia gravis (Pilkington et al., 1995a).

A large body of evidences supports the pathogenic role of IgG-G0 in inflammation through different non mutually exclusive mechanisms. However, the relative contribution of these mechanisms to the pathogenesis of inflammatory diseases and aging is far from clear.

8. Are the alterations of N-glycome in aging a consequence of inflammaging? A possible unifying theory

A variety of stimuli of microbial or endogenous origin can trigger the local or systemic defense response known as inflammation. The recognition of microbial products (pathogen-associated molecular patterns, PAMPs) by the receptors of the innate immune system (pathogen recognition receptors, PRR) is the prerequisite for the activation of the adaptive immune system. However, also a variety of molecules of endogenous origin released by damaged and necrotic tissues (danger-associated molecular patterns, DAMPs) activate the receptors of the innate immune system, resulting in an inflammatory response (Kono et al., 2008). The “advanced glycation end products” (AGEs), which result from proteins modified by glucose in a process known as glycation (non-enzymatic glycosylation) are typical products increased in serum of elderly individuals and type 2 diabetes patients and are among the most important DAMPs. The life-long exposure of the immune system to stimuli of microbial (PAMPs) or endogenous (DAMPs) origin provokes an imbalance between pro-inflammatory networks and anti-inflammatory networks, leading a low-grade systemic inflammatory status which characterizes elderly, known as inflammaging (Franceschi et al., 2000, 2007; Franceschi, 2007). A correct balance between pro-inflammatory and anti-inflammatory networks, which avoids either a too strong or a too weak reaction to inflammatory stimuli, is crucial for successful aging. This notion fits with recent data showing that among different polymorphisms of the mannose binding lectin (MBL, a well known PRR), only those with intermediate levels of activity were associated with extreme longevity (Tomaiuolo et al., 2012).

The microbial and endogenous stimuli resulting in inflammaging are originated by a variety of tissues and systems, as recently reviewed (Cevenini et al., 2010a,b). A good example of how a microbial infection can differentially induce an inflammatory status in young and old people is provided by cytomegalovirus (CMV), which chronically infects a large proportion of humans, from newborns to centenarians. In centenarians, the T cell response to CMV antigens, necessary to cope with the infection, results in a seven fold higher IFN- γ production than in young people, providing an obvious contribution to inflammaging (Vescovini et al., 2007). Examples of inflammatory stimuli of tissue origin are provided by adipokines

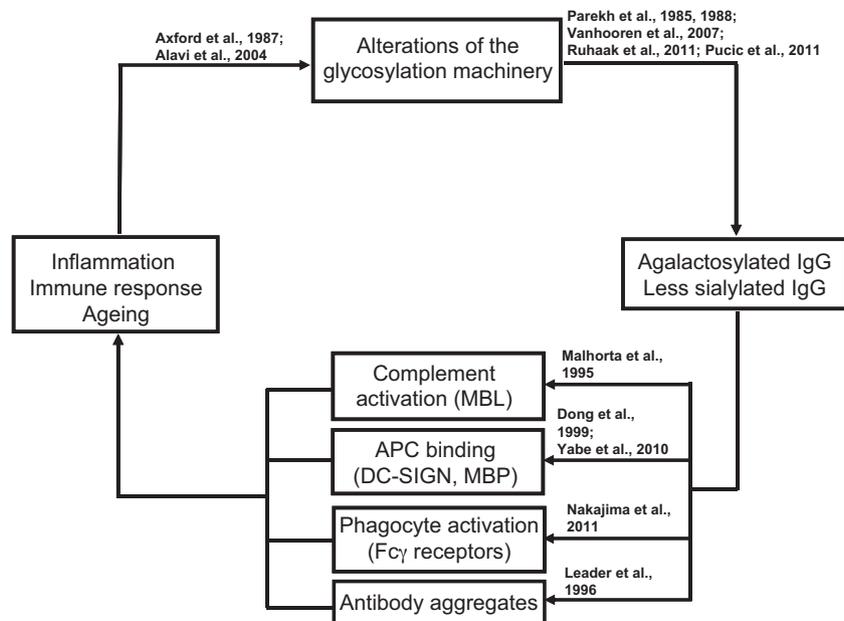


Fig. 2. Proposed model of IgG-G0 in the pathogenesis of inflammatory conditions and aging. A systemic inflammatory condition is common to a variety of autoimmune diseases infectious diseases, cancers and aging. It is thought that this condition alters the glycosylation machinery of antibody-producing cells, resulting in the increased expression of agalactosylated and poorly sialylated IgG. These aberrantly glycosylated IgG activate different effector branches of the immune system (complement, APC, phagocytes), resulting in the amplification of inflammatory signals. According to this model, IgG-G0 are a key factor in the establishment of a self-amplifying inflammatory loop and not merely a marker of inflammation and aging. Moreover, a concomitant lower expression of sialylated IgG is observed, resulting in phagocyte activation. For space limitations, only a few relevant citations are present.

associated with obesity or by the neuroinflammation associated with Alzheimer disease. The age-related deeply rearranged gut microbiota, enriched in opportunistic, and possibly pathogenic, bacterial groups offers a good example of a systemic contribution to inflammaging (Biagi et al., 2010, 2011). Finally, a robust set of data in a variety of human and animal models indicate that DNA damages, which accumulate in elderly, and the consequent DNA-damage response are among the strongest inducers of the release of inflammatory cytokines in the different microenvironments, thus contributing to inflammaging and to the progression of a variety of cancers in the elderly (Bonafe et al., 2012). Indeed, animals knock out for DNA repair, showing accelerated accumulation of DNA damages and an accelerated aging phenotype, show highly increased expression levels of immune/inflammatory genes (Schumacher et al., 2008).

Within such a general scenario, that suggests a profound pathogenetic link between chronic inflammation and the aging phenotype, and taking into account that N-glycomic changes occur in aging and in a variety of chronic inflammatory conditions, we hypothesize that the age-related alterations of the N-glycome are not only a strong biomarker of biological age but that they are also causally interconnected with inflammaging. According to this view, the common condition underlying autoimmune diseases, cancer and aging would be a peculiar low grade chronic inflammatory status depending on a variety of stimuli and sources, including the production of aberrantly glycosylated IgG. As depicted in Fig. 2, such an aberrant production is capable of fuelling an inflammatory vicious loop which includes the activation of the lectin pathway of complement, the interaction with lectin receptors of APC and with Fc γ R_s, as well as the formation of antibody aggregates.

In this light, the predictive value of successful aging, which is associated with a low level of agalactosylated IgG with bisecting GlcNAc in the offspring of nonagenarians and of long lived siblings in the Leiden Longevity Study [LLS, Ruhaak et al., 2010b] would be explained by a milder inflammatory status in these groups of people. This points at the link between metabolic health and

inflammaging since a major driver of longevity in the LLS study is a healthy metabolic profile. Also the N-glycomic shift between the offspring from nonagenarians and their partners depended on metabolic conditions (Ruhaak et al., 2011) as is the case in animal models (Vanhooren et al., 2011). Thus, healthy aging is strongly influenced by metabolism and a comprehensive, fundamental connection between inflammaging (Franceschi et al., 2000) and the chronic, metabolically driven low-grade inflammatory status recent conceptualized as “metaflammation” (Hummasti and Hotamisligil, 2010) can be hypothesized, also considering that adipose tissue is a major source of inflammatory cytokines and the complex role that it plays in aging (Tchkonina et al., 2010). Accordingly, we predict that N-glycomic shifts similar to those considered in this review will be found in a variety of other, so far unsuspected, pathological conditions where chronic inflammation and affected metabolic health play a major role.

9. Concluding remarks

Many questions regarding the age-related N-glycomic shift are still open. A summary of the most relevant open questions and of their possible answers is reported in Table 5. Although the biochemical basis and the pathogenetic implications of altered N-glycosylation in aging remain largely obscure, there is no doubt that some of the age-related glycans are among the most potent markers of both calendar aging and premature aging conditions as well as of successful aging. This indicates that, regardless of the molecular mechanisms regulating their expression, they are intimately linked with the process of aging and metabolic health. The glycome stability is mainly maintained by a balance between the activity of anabolic enzymes (glycosyltransferases) and exoglycosidases (of RER and Golgi origin). It can be hypothesized that, with aging, this balance shifts toward a different equilibrium because of a slight but constant modulation of the expression of anabolic and catabolic enzymes, resulting in the N-glycomic shift. It is reasonable to speculate that genes regulating glycosylation and epigenetic

Table 5
Open questions and perspectives.

Questions	Perspectives
Is inflaming the only or the main underlying condition at the basis of the production of agalactosylated N-linked chains?	It is necessary to compare the presence of agalactosylated structures with markers and/or mediators of inflammation. The role played by metabolic pathways should be further investigated.
Which is the contribution of glycoproteins other than IgG to serum agalactosylated structures?	It is necessary to characterize further the glycoproteins bearing diantennary N-linked chains after depletion of the IgG fraction.
Which is the tissue of origin of these glycoproteins and which is the effect of aging on membrane glycoproteins of the tissue(s) of origin.	Liver is the most likely candidate. The FUT8 gene of mice liver has been recently shown to undergo age-dependent modulation. Studies on liver glycome in aging humans are required.
Is the mechanism leading to hypogalactosylation of IgG and of other serum glycoproteins the same?	Not necessarily. Many investigations support the notion that among B-lymphocytes glycoproteins, the change is specific for Asn297 of IgG. In other tissues different mechanisms may take place.
Which is the enzymatic basis of the age- and inflammation-dependent “N-glycomic shift”?	Investigations on galactosyltransferase expression in B lymphocytes of RA patients failed to provide conclusive evidences. Studies on glycosyltransferase expression in aging humans are lacking. Also the contribution of glycosidases is underinvestigated.
Which are the most relevant pathogenic mechanisms through which IgG-G0 support inflammation?	It is possible that the relative contribution of different mechanisms varies in different settings.
Which is the contribution of epigenetic mechanisms of gene regulation to the age-dependent “N-glycomic shift”.	It can be hypothesized that epigenetic mechanisms, including promoter methylation, progressively alter the expression of glycosyltransferases and/or glycosidases. High-throughput studies on the methylation status of key glycosyltransferases and glycosidases are required.

mechanisms influencing such genes would play a major role in determining this life-long process.

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