Preface

The six kingdoms of life (Animalia, Plantae, Fungi, Protista, Archaea, and Bacteria) are composed of an enormous variety of organisms classified in different taxonomic groups, based on common traits. Despite all this diversity, there is a common trait present in all living organisms and even in biological entities such as viruses and prions: they have sugars attached to their surface. Sugars may be present as polysaccharides, as those composing the cell wall of organisms; and may be attached to lipid and protein cores, generating glycolipids and glycoproteins, respectively. Their great abundance and diversity make these molecules important for a variety of cell processes: since forming a though structural shield covering cells, such as the chitin exoskeleton of crustaceans, to play essential roles for cell viability. Glycobiology is a relative new disciple dedicates to study the structure and relevance of oligo- and polysaccharides for normal cell physiology. The last two decades have witnessed an increased interest in sugar biology, and this book tries to gather the most relevant information in some of the field's hot topics. The book contains contributions by world leaders in their fields and includes chapters dedicated to the structural analysis of glycans from different sources, and information related to the glycan functions in different biological process: the role of glycosaminoglycans in intercellular communication, polysaccharides as antiviral molecules, the immunomodulatory properties of helminth glycans, the role of glycans in *Drosophila* development, and the importance of mannoproteins for fungal biology. In addition, the use of glycans as potential diagnostic biomarkers is explored along with their importance for normal functions of human cells, as highlighted by chapters dealing with congenital disorders of glycosylation and hepatocellular carcinoma. Finally, the book includes more specific chapters on fungal lectins and dissection of amino acid requirements for enzyme activity, as examples of how the research in those files can be done.

Chapter I – The importance of glycosylation as one mode of post-translational modification is reflected by the large number of glycosylated proteins employed in a myriad of biological processes. Over the last decade, extensive studies have followed the evaluation of glycan changes in proteins during disease in a variety of biological samples and recently, attention has also been paid to glycosylation in association with treatment. Although a number of new methods have been developed for carbohydrate analysis and identification of glycans has reached a high degree of improvement, the introduction of new techniques to help elucidate glycoconjugate structures and their exact roles in living systems still presents tremendous challenges in the field of proteomics. In recent years, technologies amenable to the analysis of small amounts of glycoprotein oligosaccharides have improved considerably

in the areas of sample preparation, chromatographic and electrophoretic separations, but mass spectrometric (MS) methods are still among the most sensitive used for the characterization of biomolecules. For enhanced MS detection, carbohydrates are often derivatized. The type of labelling used can also influence the fragmentation pattern and simplify structural analysis. In 2003, we introduced the micro-scale reducing end tagging of glycans using phenylhydrazine (PHN) as a derivatizating reagent for carbohydrate analysis. PHN derivatized glycans showed enhanced detection and could be analyzed even without prior purification. Overtime, this simple approach of analysis with some modifications has helped to reveal new isomeric structures of glycans, including those of sialylated glycans with two types of acidic residues – NeuAc and NeuGc. The usefulness of this method has also been demonstrated successfully for the analysis of glycans isolated from serum samples and cancer cells. The comparison of glycan profiles of healthy versus cancerous samples indicated changes associated with diseases. The data presented for the analysis of a variety of samples have prompted attempts to use this simple technique for routine clinical diagnostic works.

Chapter II – Glycosaminoglycans (GAGs), such as heparan sulfate and hyaluronan, are present in the extracellular matrix (ECM) where they fulfill multiple functions, including modulation of intercellular communication and regulation of growth factor activities. As components of cell membrane and extracellular matrix proteoglycans either interact directly with growth factors and cytokines, or facilitate binding of growth factors to the actual binding or signaling portion of the receptor. Growth factors mediate cell to cell communication, and as such are inducers of a variety of cellular functions under physiological and pathophysiological conditions. Most growth factors are secreted into the ECM or extracellular fluids where they interact with numerous molecules, many of them glycosaminoglycans, proteoglycans or other glycoproteins as part of the communication process, for sequestration purposes and/or for transport to more distant cellular targets. In this chapter we review the roles of individual GAGs, and proteoglycans in receptor and ECM communication systems. They will be discussed on their own merit, and also in the framework of proteoglycans to which GAGs are attached, such as small-leucine rich proteoglycans, perlecan, and betaglycan.

Chapter III – Glycans are highly diverse carbohydrate moieties that have been selected in evolution to convey dynamic structural and functional properties to the macromolecules to which they are attached. For this reason, correct glycan synthesis is essential for various developmental and physiological processes, particularly in multicellular organisms that use them as communication pathways. The disruption of glycan synthesis frequently results in multisystemic disease with neurological involvement. Congenital disorders of glycosylation (CDGs) have been and will likely remain a rapidly growing group of genetic human diseases that involve different defects in the synthesis or remodelling of *N*- and *O*-linked glycans as well as defects in glycosphingolipid and glycosylphosphatidylinositol (GPI) anchor glycosylation. The molecular and clinical characterization of CDGs has enormously contributed to understanding the physiologic roles of the glycosylation machinery and its interaction with other cellular machineries. More than 50 different types of defects have been described and although the first type was described in 1984, CDGs remain widely under-diagnosed or misdiagnosed. This review describes the genetic and biochemical basis of CDGs, as well as the clinical phenotypes and current methods to diagnose them that are ultimately required to establish corrective treatments that are also discussed.

Chapter IV – Human hepatocellular carcinoma (HCC) is a malignancy of the liver which is among the most common and lethal cancers worldwide. Most cases of HCC are secondary

to viral infection or cirrhosis. The low survival rate of patients with HCC is due to its aggressiveness, late diagnosis, and lack of response to current surgical and medical treatments. Therefore, a better understanding of the cellular mechanisms that define these factors is necessary, including those related to the deregulation of key components involved in the biosynthesis of glycans. In HCC, as is the case with many other types of cancer, malignant transformation is accompanied by abnormal expression of certain glycan structures that in most cases play a fundamental role in providing cancer cells the means towards proliferation, migration and metastasis. This review aims to integrate the identified molecular mechanisms in HCC that pave the way towards a glycan-mediated malignant phenotype, as well as describing the clinical applications of screening for liver-secreted serum glycoproteins as HCC diagnostic markers.

Chapter V – Heparan sulfate proteoglycans are ubiquitously expressed at cell surfaces and in extra-cellular matrices. They are composed of a core protein and one or more heparan sulfate glycosaminoglycan chains (linear polysaccharides formed by alternating N-acetylated or N-sulfated glucosamine units and uronic acid) that interact with numerous proteins, including growth factors, morphogens and extra-cellular-matrix proteins. The heparan sulfate proteoglycans play crucial roles in physiology. On the other hand, under certain conditions, they also contribute to pathophysiology of some diseases. In this sense, the truncated HS chains within the HS proteoglycans cause the loss of binding to specific growth factors in chondrocytes, resulting in an abnormal signalization that leads to altered endochondral ossification. This produces a multiple osteochondroma formation leading to multiple osteochondromatosis / multiple hereditary exostosis, an autosomal dominant *O*-linked-glycosylation disorder recently classified as *EXT1/EXT2*-CDG in the new Congenital Disorder of Glycosylation (CDG) nomenclature. Products of the *EXT* genes are involved in heparan sulfate biosynthesis. Three loci have been identified, *EXT1* in chromosome 8q24, *EXT2* in chromosome 11p11-p13, and *EXT3*, in chromosome 19. The existence of this third locus is not clear, since most of the cases are caused by mutations in either *EXT1* (65%) or *EXT2* (35%). *EXT1* and *EXT2* genes code for two glycosyltransferases named exostosin 1 and 2, and both enzymes are part of a hetero-oligomeric complex localized within the Golgi complex. The *O*-linked xylosylation initiates the synthesis of a tetrasaccharide linker consisting of xylose-galactose-galactose-glucuronic acid (Xyl-Gal-Gal-GlcA) that links glycosaminoglycan moieties with the protein backbone in proteoglycans such as heparan sulfate. Recent studies have proposed a hypothetical model for hereditary exostoses formation, where a dominant germline mutation in either *EXT1* or *EXT2* in chondrocytes, inactivates the second copy of the same gene, leading to impaired heparan sulfate biosynthesis and expression. This will result in abnormal endochondral ossification, during the process of bone remodeling in childhood and early adolescence. Multiple osteochondromatosis has an estimated occurrence of about 1 in 50,000 in Western populations and is clinically characterized by cartilage-capped tumors developed from the growth plates of long bones or the surface of flat bones. Some common manifestations are orthopedic deformities of forearm, ankle, varus or valgus of knee, arthritis, vessels and nerves compression, but the most important complication is malignant transformation to chondrosarcoma. This review describes the clinical, biochemical and genetic basis of multiple osteochondromatosis (*EXT1/EXT2*-CDG), a congenital disorder of glycosylation.

Chapter VI – Recently, there are many risks of viral infections because emerging and re-emerging viruses have appeared and spread worldwide. Under these situations, management

of viral infectious diseases becomes a relevant subject for public health. For this purpose, both of development of novel antiviral drugs and stimulation of host immunity such as innate and adaptive immune systems are promising strategies. Polysaccharides are the most abundant natural resources, and possess various biological effects. During the last decade, polysaccharides have been paid attention to as important medicinal and functional food resources, and many studies on their chemical structures and biological effects have been conducted. Especially, natural polysaccharides might be important molecules because of their useful biological activities such as antiviral and immunomodulating effects. In this chapter, we will introduce the nature of polysaccharides and their useful biological effects on the control of viral infectious diseases.

Chapter VII – Parasitic helminths continuously adapt to their complex biological niche and in the case of humans this has been for tens of thousands of years. Far from being a friendly environment, the host immune system works actively to eliminate the parasites. To survive within their host for long time periods, parasitic worms have thus co-evolved with the host immune system to effectively regulate or suppress it. Among many survival strategies, worms use glycans to modulate host immune responses, exposing glyco-conjugates on their surfaces or secreting glycosylated products. Historically, the importance of glycans has been attributed to metabolism and structural properties, but due to their complex structure, glycans added to protein and lipid backbones can be considered a source of biological information. This information is decoded by Carbohydrate Binding Proteins (CBPs) in the host, which play an essential role in the initiation and maintenance of the immune response. The glycan repertoire employed by the parasites involves host-like glycans, including *N*- and *O*-linked sugars, and in addition, many helminths express unusual glycans that are not found in the host, such as polyfucose, tyvelose, phosphorylcoline and methyl group-containing glycans. It has been shown that these helminth glycans can interact and modulate host immune responses thanks to their ability to target some CBPs (C-type lectins, Dectin-2, galectins) and also other pattern recognition receptors in the host (Toll Like Receptors). This suggests that these molecules have potential as immunomodulators and this has started to be explored in a number of laboratories. In this article, we summarize the recent advances regarding helminth glycan structure and their ability to modulate host immune responses, since understanding these glycan-dependent interactions could be of paramount importance in finding new strategies for modulating the immune response in many inflammatory and autoimmune diseases.

Chapter VIII – Glycosylation of proteins shows two striking features: first, there are a large number of glycan structures that are involved in various biological events (diversity) and, second, certain structures are selectively added to specific sites within proteins (specificity) in a context-dependent manner. However, the mechanisms that regulate diversity and specificity are unknown. Over the last few decades, genetic studies in *Drosophila* have been used to examine the functions mediated by glycosylation *in vivo*. Recent improvements in the combined use of biochemical/biophysical, genetic, and cell biology approaches has allowed the rapid and detailed analysis of the structure, function and formation of glycans in *Drosophila*. This chapter aims to provide an overview of the different types of glycans and their biological function(s) in *Drosophila*. The regulation of glycan synthesis in *Drosophila* and mammals is also discussed.

Chapter IX – The fungal cell wall is a robust, but dynamic structure that covers the fungal cell and protects it from changes in the extracellular environment. The main wall components

are sugars, which contribute with about 75-90% of the wall dry weight. Glucans, chitin, and chitosan are the main polysaccharides conforming the wall skeleton, provide strength and work as scaffold molecules to attach lipids and proteins to the cell wall. The latter are synthesised in the endoplasmic reticulum and transported to the wall by the secretory pathway, and in most of the cases are modified either by addition of the glycolipid glycosylphosphatidylinositol to the C-terminus and/or with oligosaccharides covalently attached to asparagine (*N*-linked glycosylation) or serine/threonine (*O*-linked glycosylation). Despite these glycoproteins are not as abundant as the cell wall polysaccharides, they play an important role in the cell wall integrity pathway, some of them are sensors for changes in the extracellular environment, have adhesion properties and hydrolytic activities. Thus, special attention has been given to the cell wall glycoproteins of fungal pathogens, as some of them are considered virulence factors.

Here, we present an overview of the different pathways involved in the biosynthesis of cell wall glycoproteins in *Saccharomyces cerevisiae* and *Candida albicans,* as these routes have been extensively studied in these organisms. In addition, evidences of their importance for the cell integrity and virulence in *C. albicans* are shown.

Chapter X – Galactofuranosyl units (Gal*f*), mainly in the β-configuration, are constituents of infectious microorganisms, such as the *Mycobacteria,* trypanosomatids like *Trypanosoma cruzi* and *Leishmania*, and fungi like *Aspergillus fumigatus.* The metabolic pathways involved in the biosynthesis of the microbial glycoconjugates containing Gal*f* are attractive targets for the development of therapeutic agents, because Gal*f* is absent in mammalian cells. The enzymes involved in the biosynthesis of Gal*f*-containing molecules are the UDP-galactopyranosyl mutase, which catalyzes the interconversion of UDP-Gal*p* into UDP-Gal*f*, the donor of Gal*f* units, and the galactofuranosyltransferases, which are responsible for the incorporation of the sugar into the glycoconjugates. In some species, β-D-galactofuranosidases have been identified as responsible for the degradation of the D-Gal*f* containing glycoconjugates.

Although the metabolism of β-D-galactofuranosides has been extensively studied, the α-D-Gal*f* biosynthetic and metabolic machinery still remain unclear. The description of the enzymes involved in its metabolism is important, considering that glycoconjugates containing α-D-Gal*f* residues have been also described in several pathogenic microorganisms including bacteria, such as *Escherichia coli* and *Streptococcus pneumoniae*, and fungi, such as *Paracoccidioides brasiliensis,* the causative agent of paracoccidioidomycosis. It is interesting that in the latter Gal*f* is present in both anomeric configurations. Recent methods for the synthesis of galactofuranosides are included in this chapter.

Chapter XI – The search and analysis of literature devoted to mushroom's lectins testify to an apparent lack of information regarding the demonstration of extracellular lectin activity in fungal cultures, the preparation of purified samples of extracellular fungal lectins, and the study of their properties and supposed functions.

Fungi are a group of living organisms very interesting in theoretical and practical respect; however, extracellular fungal lectins have not been described. Initially we paid attention to the lectin activity of culture liquid and submerged mycelium of *Lentinula edodes* (shiitake). This activity was found, the carbohydrate-binding specificity of hemagglutinins established; and the lectin activity in relation to selected physicochemical factors of cultivation explored.

The activity of the culture liquids proved to be much higher than that of mycelia. The considerable changes were revealed also in the agglutinating activity of different morphogenetic structures of *L. edodes*, including mycelium, brown mycelial film (MF), primordia, and fruiting bodies. The hemagglutination titers increased as the morphology changed from mycelium to MF, and then decreased as the MF produced the primordia and the fruiting body. The involvement of lectins in MF formation was confirmed. The relationship between the process of MF formation and the extracellular lectin activity, in the presence of divalent metal cations (M^{2+}) and natural amino acids, during cultivation of *L. edodes* F-249 in liquid medium was established.

Another phenomenon revealed at the different morphogenesis steps was the relationship of the carbohydrate composition of *L. edodes* mycelium with the lectin activity and carbohydrate specificity of lectins. The lectins of *L. edodes* on solid-phase cultivation manifest their carbohydrate specificity mainly to the sugars found in mycelium at the MF stage. Both the lectin activity and the level of carbohydrates, to which the lectins display their specificity, increase before fruiting. The relationship of fatty-acid composition of shiitake total lipids and the lectin activity at different morphogenesis steps was examined. When the abnormal fruit bodies form in the absence of MF, the lectin activity of mycelium does not increase.

All the above experiments at the level of crude lectins were followed by the isolation, purification, and characterization of preparations of *L. edodes* extracellular lectins. Two lectins unique in respect to their extracellular nature, basidiomycete origin, and carbohydrate specificity of one lectin to arabinose have been isolated and purified from the culture liquid of *L. edodes*. This fungus biosynthesizes a glycolipid associated with the arabinose-specific extracellular lectin. Galactose glycan residue, as well as the lipid pool composition, which includes nonhydroxylated short-chain fatty acids, is uncommon for basidiomycetes. Development-dependent galactolipid production is capable of changing the lectin activity of shiitake. The glycolipid is therefore assumed to be an endogenous regulator of fungal lectin activity.

In order to get insights on the physiological role played by shiitake lectins, one should remember on their feasible interrelations with the most abundant enzymes of the mushroom. We explored laccase and lectin activities produced in a submerged culture of shiitake with respect to their mutual influence. *L. edodes* produced *D*-melibiose-specific lectins and two laccase forms in a lignin-containing medium. The maxima of laccase and lectin activities coincided, falling within the period of active mycelial growth. The enzymes and lectins were isolated and purified. The *L. edodes* lectins were found to be able to stabilize the activity of the fungus's own laccases. Lectin activity during the formation of lectin–enzyme complexes remained unchanged.

To reveal proteins intrinsic to definite fungal morphological structures, a comparative study of a series of glycoproteins and their properties on going from one morphogenic step to another was attempted. A number of glycoproteins with lectin activity varying in polypeptide composition and carbohydrate specificity were isolated from *L. edodes* at different stages of its morphogenesis: three lectins were identified in the non-pigmented mycelium, including two dimers and one tetramer. The fractions with lectin activity obtained at the MF stage contained three polypeptides characteristic of this morphological structure. The fruiting body was shown to contain two lectins. All of the isolated lectins expressed the highest affinity towards *D,L*-melibiose, *D*-lactose, and *D*-galactose.

Representatives of another ecological niche were also examined for lectins. A dimeric membrane-bound 68 kDa-lectin was isolated from the surface of the dikaryotic mycelium of the xylotrophic basidiomycete *Grifola frondosa*. The lectin was a hydrophilic glycoprotein with the protein : glycan ratio of 3:1, *D*-glucose and *D*-glucosamine being detected in the carbohydrate part. The lectin exhibited high affinity to native rabbit erythrocytes and to human erythrocytes of the *O* blood group, but not to trypsin-treated ones. The hemagglutination caused by lectin was not blocked by any of the 25 tested mono-, di-, and amino sugars; it was also not blocked by some of glyco derivatives. Only 13.9 μg/ml of the homogeneous preparation of a polysaccharide, a linear D-rhamnan with the definite structure of the repeated component blocked hemagglutination completely.

The comparative studies comprising both mushrooms were performed. Quantitative and qualitative estimations of the basidiomycetes *G. frondosa* and *L. edodes* lectins binding to the specific and non-specific polyclonal rabbit antibodies were attempted. The *G. frondosa* lectin complexation with homological antibodies was shown to be characterized by greater binding constants as compared to non-homological antibodies. Therewith the values of changes in standard free energy ΔG^0 of both complexes were essentially the same. As for the two extracellular *L. edodes* lectins, *L*1 and *L*2, the anti-lectin *L*1 antibodies bound crude *L*2 with ten-fold-greater ELISA results compared to crude *L*1, but showed only limited reaction with *L*2 in the course of this lectin purification. The data obtained could give new insight into the phenomenon of these biospecific interactions. Obviously, the actual specificity in the above mentioned processes of bio-recognition should be discussed in the especial case of the lectins and antibodies incorporation into a living system.

Chapter XII – GlcAT-P is a glucuronyltransferase that biosynthesizes the HNK-1 (Human Natural Killer-1) carbohydrate involved in synaptic plasticity. Previously, we purified the catalytic domain of recombinant human GlcAT-P and determined its X-ray crystal structure. In the GlcAT-P catalytic domain, a disordered region called a flexible loop, that showed no electron density, was identified; but not function for it has been established. Many glycosyltransferases have a similar flexible loop, and it was speculated that upon donor-substrate binding, the loop was ordered to partially facilitate acceptor binding, although it was unclear whether this notion was applicable to all glycosyltransferases. Here, we examined the role of the GlcAT-P flexible loop for its activity. A mutant GlcAT-P lacking the loop (GlcAT-PΔloop) hardly exhibited glucuronyltransferase activity in cells, and exhibited *in vitro* activity of less than 3% of wild-type, but Golgi-localization and dimerization were not influenced. Moreover, we revealed that GlcAT-PΔloop could bind with both acceptor- and donor-substrate same as wild-type. Finally, we demonstrated that the presence of acidic amino acids, but not amino acid length or sequence order within the GlcAT-P loop, was essential for enzyme activity. These results suggest that the flexible loop of GlcAT-P may have a specific function different from substrate binding.