Abstract: The formation of new and functional structural components of several organs, such as parotid glands, can be influenced by the glycocode. This study analyzed the glycobiology of parotid salivary gland tissue during fetal development using specific biochemical probes (lectins and antibodies). Eleven parotid gland samples from human fetuses were obtained from spontaneous abortions at 14-28 weeks of gestation, and tissue sections were analyzed for lectin histochemistry and immunohistochemistry. From the 18th to 26th week, *Canavalia ensiformis* agglutinin, wheat germ agglutinin, *Ulex europaeus* agglutinin-I, peanut agglutinin, *Sambucus nigra* agglutinin, and *Vicia villosa* agglutinin lectin staining were predominantly observed in the apical and/or basement membranes of the ducts and tubulo-acinar units. Moreover, the presence of galectin-1 was found in the membrane, cytoplasm, and nucleus of both structures. Conversely, Gal-3 and mucin-1 were restricted to the glandular ducts. The lectin staining pattern changed during the weeks evaluated. Nevertheless, the carbohydrate subcellular localization represented a key factor in the investigation of structural distribution profiles and possible roles of these glycans in initial parotid gland development. These findings are defined by their high biological value and provide an important base for the development of subsequent studies. (J Oral Sci 58, 353-360, 2016)

Keywords: galectin; glycobiology; lectins; salivary gland.

Introduction

A wide range of carbohydrates cover the surfaces of almost all cells capable of differentiating and forming tissues in multicellular animals (1). Physiologically, many interactions at the cell surface require selective recognition of specific carbohydrate structures by cognate proteins known as lectins (2). These carbohydrates are often characteristic of a specific cell type and are expressed according to the particular stage of development of cells and tissues (3). Furthermore, human cells express a wide variety of glycans with structural and temporal specificity, which in turn affect the corresponding functions of these important molecules (1).

To date, little is known about the regulation and types of molecules involved in cell-cell and cell-mesenchyme interaction during human salivary gland development (4). However, in the recent decades, some studies have elucidated the involvement of different molecular components (extra- or intra-glandular) in several cross-talking signaling pathways important for salivary gland morpho-
genesis. Most of these have focused on their potential to promote salivary gland formation and branching, with special emphasis on the submandibular gland (5,6).

The development of the parotid gland begins in the 6th week of gestation when epithelial buds of ectodermal origin form the wall of the primitive mouth and invaginate into the surrounding mesenchyme. The epithelial buds of each gland enlarge, elongate, and branch to form solid structures initially (7), and eventually canalize to create a lumen. This ductal canalization process is completed before terminal bud or eventual acinar development. The lining epithelial cells of the ducts, tubules, and acini then differentiate morphologically and functionally, and the contractile myoepithelial cells arrange themselves around the acini (8,9). The mesenchyme is responsible for genesis of the capsule and connective parotid tissue (10,11).

During development and differentiation, human cells express a wide array of glycan structures whose amount and structure are controlled by temporal and lineage specificity. This carefully orchestrated modulation of glycan diversity during development implies corresponding and important functions for these molecules (1,12). There is evidence that glycan structures reflect their evolutionary backgrounds and functional aspects in various contexts, including differentiation, development, fertilization, inflammation, cell adhesion, and cell-cell recognition (13). Thus, there is an enormous capacity for storage of biological information inserted into these carbohydrates (14).

Lectins are molecules that interact with carbohydrates non-covalently in a way that is usually reversible and highly specific. In other words, while carbohydrates carry biological information, lectins act as a communication bridge and allow the interpretation of this “glycocode” (15). Thus, lectins are used in medical and biological fields (e.g., histochemistry), where they can provide a sensitive detection system for changes in glycosylation (16). As a result, the lectins, *Canavalia ensiformis* agglutinin (ConA), wheat germ agglutinin (WGA), *Ulex europaeus* agglutinin (UEA-I), peanut agglutinin (PNA), *Sambucus nigra* lectin (SNA), and *Vicia villosa* agglutinin (VVA), as well as the antibodies capable of binding to the glycoproteins, Thomsen Friedenreich antigen (TF antigen), mucin-1 (MUC-1), galectin-1 (Gal-1), and galectin-3 (Gal-3), were used in this study to determine the glycomic profile of the parotid gland during early fetal development.

### Materials and Methods

#### Specimens

Parotid gland samples from human fetuses (*n* = 11) at 18-26 weeks of gestation were obtained from Professor Barros Lima and Professor Bandeira Filho at the Children’s Hospital in Recife, State of Pernambuco, Brazil (Table 1). All specimens were fixed in 10% formalin and

### Table 1 Fetal clinical information and viability of samples

| Fetus   | Weight (g) | Gestational age (weeks) | Viability |
|---------|------------|-------------------------|-----------|
| P157/F8 | 34.7       | 16                      | Unviable  |
| P161/F12| 47.4       | 16                      | Unviable  |
| P126/F1 | 34.8       | 17                      | Unviable  |
| P129/F4 | 32.9       | 18                      | Viable    |
| P163/F14| 153.5      | 21                      | Viable    |
| P128/F3 | 227.4      | 23                      | Viable    |
| P127/F2 | 195.7      | 23                      | Viable    |
| P158/F9 | 302.2      | 24                      | Viable    |
| P132/F7 | 318.0      | 25                      | Viable    |
| P130/F5 | 295.0      | 26                      | Viable    |
| P131/F6 | 321.4      | 26                      | Viable    |

### Table 2 Lectin, sugar specificity, and working concentration in lectin histochemistry

| Marker       | Sugar specificity | Source                               | Work concentration |
|--------------|-------------------|--------------------------------------|--------------------|
| ConA         | Glucose/mannose   | Vector Labs, Burlingame, USA         | 80 µg/mL           |
| WGA          | N-acetylglucosamine| Vector Labs, Burlingame, USA         | 30 µg/mL           |
| UEA-I        | L-fucose          | Vector Labs, Burlingame, USA         | 80 µg/mL           |
| PNA          | N-acetylgalactosamine| Vector Labs, Burlingame, USA     | 80 µg/mL           |
| SNA          | Sialic acid       | Vector Labs, Burlingame, USA         | 80 µg/mL           |
| VVA          | Tn-antigen        | Vector Labs, Burlingame, USA         | 80 µg/mL           |

ConA, *Canavalia ensiformis* agglutinin; WGA, Wheat germ agglutinin; UEA-I, *Ulex europaeus* agglutinin; PNA, Peanut agglutinin; SNA, *Sambucus nigra* lectin; VVA, *Vicia villosa* lectin.
embedded in paraffin. Tissue sections were stained with hematoxylin-eosin for evaluation of the morphological and developmental phases. Adult samples obtained from tumor-free resection margins (n = 10) were used as an example of the fully developed salivary gland glycan pattern. This work was approved by the Ethical Committee of the University of Pernambuco, Brazil (CEP/UPE 084/2002), and conducted in accordance with the principles of the Declaration of Helsinki.

**Table 3** Antibody sources, clones, and working dilutions

| Marker | Source                | Clone | Work dilutions |
|--------|-----------------------|-------|----------------|
| TF     | Santa Cruz, Dallas, USA | STN320 | 1:100          |
| MUC-1  | Santa Cruz, Dallas, USA | N19   | 1:120          |
| Gal-1  | Santa Cruz, Dallas, USA | C8    | 1:100          |
| Gal-3  | Spring, Washington, USA | 9C4   | 1:150          |

TF, Thomsen Friedenreich antigen; MUC-1, Mucin-1; Gal-1, Galectin-1; Gal-3, Galectin-3.

**Lectin histochemistry**

Four-micrometer-thick sections of specimens were deparaffinized in xylene and rehydrated in graded alcohol (100%–70%). The slices were treated with 0.1% (v/v) trypsin solution for 2 min at 37°C, followed by a 0.3% (v/v) methanol-H₂O₂ solution for 30 min at 25°C. They were then incubated for 2 h with lectins conjugated to horseradish peroxidase (HRP) (Sigma Aldrich, St Louis, IL, USA) at 4°C (Table 2). All solutions and washings were carried out using a 10 mM phosphate buffer solution.

**Fig. 1** Histochemistry of fetal salivary gland samples. (A) ConA, glucose/mannose, 23rd week; (C) UEA-I, L-fucose, 23rd week; (D) PNA, N-acetylgalactosamine, 23rd week; (E) SNA, sialic acid, 26th week, and (F) VVA, Tn-antigen, 21st week. Staining was observed on the basement membrane of the ducts and TAUs. In contrast, (B) WGA, N-acetylglucosamine, 25th week, shows apical staining as well as basement staining. In all panels, black arrows indicate basement membrane staining. In panels B and E, red arrows indicate apical membrane staining. All the pictures are in ×100 magnification.
(PBS; pH 7.2) containing 150 mM NaCl. Peroxidase was visualized with a solution of diaminobenzidine (DAB) and H₂O₂ (DAKO, São Paulo, Brazil). Hematoxylin was used for counter-staining nuclei, and the tissues were evaluated using light microscopy. Lectin binding inhibition assays were developed by incubating lectins with their corresponding specific sugar (100-500 mM) prior to sample incubation. Negative controls were obtained by replacing lectins with PBS, and tumor samples were used as positive controls.

**Immunohistochemistry**

Four-micrometer-thick deparaffinized sections of specimens were incubated in methanol-hydrogen peroxide (1:1, v/v) and assayed with a streptavidin-biotin peroxidase kit (DAKO). Antigenic sites were thermally recovered in a steamer with 10 mM citrate buffer at pH 6.0 for 30 min at 60°C. Primary antibodies, dilutions, incubation times, and sources have been shown in Table 3. The sections were then incubated with DAB and counterstained with Mayer’s hematoxylin. Negative controls were obtained by replacing the primary antibodies with non-immune serum, and adult normal and neoplastic parotid glands were included as positive controls.

**Histological evaluation**

All slides were evaluated by an experienced oral pathologist. The glandular structures were considered positive when at least one of the ten areas analyzed were positive for the marker studied, and the signal was regarded as strong when at least 70% of the examined tissue area was positive.

**Results**

Expression of glucose/mannose (ConA), N-acetylgalactosamine (WGA), L-fucose (UEA-I), sialic acid (SNA), and Tn-antigen (VVA) was observed on the extracellular matrix and vascular endothelium of the samples (Fig. 1a, b, c, e, f). A strong staining for ConA, UEA-I, and PNA (Fig. 1d, N-acetylgalactosamine) was observed in the ductal basement membrane and tubulo-acinar units (TAU), and this was also associated with some stromal cells. Conversely, the presence of N-acetylgalactosamine and sialic acid was found in the basement membranes as

![Fig. 2 Immunohistochemistry of fetal salivary gland samples. Nuclear, cytoplasmic, and apical membrane staining only on the ducts with Gal-3 (C, Galectin-3, 25th week), and in ducts and TAU's (A and B, Galectin-1, 26th week) for Gal-1. MUC-1 staining is presented in (D) (Mucin-1, 25th). All pictures are in ×100 magnification.](image-url)
As well as the apical membranes of ducts, Goblet cells, and TAU. SNA and ConA staining was present in striated muscle cells. The temporal pattern of lectin staining has been shown in Table 4.

Tn antigen expression was heterogeneous with nuclear and basement lamina staining, but was predominantly observed in ductal and TAU basement membrane (Fig. 1f). The nuclear staining was observed only in the 21st and 26th weeks (data not shown).

There was no expression of Gal-3, MUC1, or TF antigen related to stromal cells, extracellular matrix, and vascular endothelium. Gal-1 and Gal-3 immunostaining was associated with the nucleus, cytoplasm, and membrane of duct cells (Fig. 2a, b, c). Moreover, Gal-1 presented the same staining pattern on TAU structures, and nuclear staining occurred only in samples from the 26th week (Fig. 2a, b). Meanwhile, Gal-3 was present between the 21st and 26th week (data not shown). MUC-1 expression was limited to ductal cells (18th to 26th weeks), as was Gal-3 (Fig. 2d). The spatial and temporal staining for each glycomarker can be found in Tables 4 and 5.

**Discussion**

The findings of this study demonstrated that an increase in the glycomic component complexity in fetal parotid glands occurred during and after the 18th week of development. It was also noted that expression of the carbohydrates analyzed was associated with cell membrane structures, demonstrating their likely involvement in the glyocalyx composition. The antibody staining was more specific to glandular structures, demonstrating participation in its construction and/or function.

Glycoproteins frequently undergo post-translational and post-transcriptional modifications related to elongation, which includes O- and N-glycosylations. These events can be histochemically characterized through lectin binding to specific terminal or internal sugar sequences of glycoproteins in several tissues (17). Although little is known about the molecular mechanisms that regulate the development of the human salivary gland (18), it is known that variations in the glycosyl components of the cell surface and cytoplasm play a significant role in normal development and pathology (15,19).

ConA, one of the lectins used in this study, is capable of specifically recognizing many cells including striated muscle cells commonly found associated with stroma (1). The histochemical findings (Fig. 1a, Table 5) showed a strong association between ConA staining and all tissues, suggesting ubiquitous N-glycosylation distribution, which is a necessary step in normal development (19).

Distinct cellular sites showed WGA staining, suggesting that as with ConA, the sugar target (N-acetylglucosamine) was present in all tissues analyzed (Fig. 1b). This staining disappeared around the 24th and 25th gestational weeks (Table 4), indicating a reduced content of Glc-NAc. This phenomenon was also reported by

### Table 4 Temporal distribution of glycomarkers

| Weeks of development | ConA | WGA | UEA-I | PNA | SNA | VVA | TF | MUC | Gal-1 | Gal-3 |
|----------------------|------|-----|-------|-----|-----|-----|----|-----|-------|-------|
| 18                   | +    | +   | +     | +   | +   | +   | −  | +   | +     | +     |
| 21                   | +    | +   | +     | −   | +   | +   | −  | +   | +     | +     |
| 23                   | +    | +   | +     | +   | +   | +   | +  | +   | +     | +     |
| 24                   | +    | +   | +     | +   | +   | +   | +  | −   | +     | +     |
| 25                   | +    | +   | +     | +   | +   | +   | +  | +   | +     | +     |
| 26                   | +    | +   | +     | +   | +   | +   | +  | +   | +     | +     |

+, positive staining; −, there is no lectin staining. The general tissue staining was analyzed. ConA, *Canavalia ensiformis* agglutinin; WGA, Wheat germ agglutinin; UEA-I, *Ulex europaeus* agglutinin; PNA, Peanut agglutinin; SNA, *Sambucus nigra* lectin; VVA, *Vicia villosa* lectin; TF, Thomsen Friedenreich antigen; MUC-1, Mucin-1; Gal-1, Galectin-1; Gal-3, Galectin-3.

### Table 5 Qualitative expression of lectin and antibody staining in the parotid gland tissue

| Parenchyma | ConA | WGA | UEA-I | PNA | SNA | VVA | TF | MUC | Gal-1 | Gal-3 |
|------------|------|-----|-------|-----|-----|-----|----|-----|-------|-------|
| Ducts      |     |     |       |     |     |     |    |     |       |       |
|            | +   | +   | +     | +   | +   | +   | +  | +   | +     | +     |
|            |     |     |       |     |     |     |    |     |       |       |
| TAU        |     |     |       |     |     |     |    |     |       |       |
|            |     |     |       |     |     |     |    |     |       |       |
| Stroma     |     |     |       |     |     |     |    |     |       |       |
| Cells      |     |     |       |     |     |     |    |     |       |       |
| EM         |     |     |       |     |     |     |    |     |       |       |
| Vascular endothelium |     |     |       |     |     |     |    |     |       |       |

TAU, Tubulo-acinar units; EM, extracellular matrix; AM, apical membrane; BM, basement membrane; Ct, cytoplasm; N, nucleus, and A, all analyzed structures (AM + BM + Ct + N).
Chisholm et al. (20) who performed a histochemical analysis of human sublingual salivary glands, and by Pedini et al. who examined adult dog submandibular glands (21). In this context, there is evidence that transmembrane proteins in the endoplasmic reticulum and Golgi apparatus are O-GlcNAc branched on their cytosolic domains, and those monosaccharides modify and modulate their membrane proteins (22).

Additionally, L-fucose expression (UEA-I target) was also investigated and found to be temporally and spatially broad, possibly as it frequently exists as a terminal modification on glycan structures (23). The study analyzes this glycan in different situations with distinct expression patterns. Sobral et al. (23) found a weak UEA-I staining associated with the adult normal parotid salivary gland, while Adi et al. (24) observed a continuous and embracing L-fucose expression during minor salivary gland development. In this study, L-fucose expression was found to be associated with basement membranes and cytoplasm (Fig. 1c, Table 5). Therefore, the presence of L-fucose may influence tissue function in distinct ways under different conditions.

As with L-fucose, sialic acid molecules can promote co-translational and post-translational modifications and participate in the formation of many glycoproteins that serve as recognition determinants in intercellular communication during tissue growth and differentiation (25). Additionally, changes in expression of this sugar are found in other situations such as cancer and inflammation (26).

Recently, it was found that ppGalNAc-T1 is the most expressed N-acetylgalactosaminyltransferase isoform in the submandibular salivary gland (27). This transferase controls the production of key molecules for the dynamic basement membrane, an important aspect of cleft initiation and formation during glandular epithelial development, through protein O-glycosylation (28). The expression of GalNAc, the sugar target of PNA and utilized by ppGalNAc-T1 for the production of glycans, is associated with the formation of secreted and membraneous glycoproteins, particularly the mucin-type (29,30).

Mucins correspond to a major glycoprotein component of saliva and other mucous secretions, whose expression is organ, tissue or cell type specific, including major and minor salivary glands (31,32). Although MUC-1 is one of the most well characterized mucins in the salivary glands, there is little information about its expression in the development of these organs (33). Our results indicate that the presence of MUC-1 occurred only after the 18th week of gestation (Table 4), characterized by the appearance of the ducts and TAU, and was expressed in the cytoplasm and apical membrane of ductal cells (Fig. 2d, Table 5). According to Liu et al. (33), MUC-1 may facilitate the secretion flow through the ductal system, and it is believed that this function occurs because of decreased cell-cell interactions via the cellular apical domain (33-35). Kirkeby et al. studied the MUC-1 and mucin-type O-glycan expression in adult human submandibular glands, confirmed ductal presence for MUC-1, and laid emphasis on the role of this molecule in tissue physiology (36).

Cellular carbohydrate antigens may vary antigenically and biochemically between different cells and organs, as well as between cells in different stages of differentiation and maturation (37). Therefore, this specific expression and organization of glycans represents part of the mechanism responsible for influencing the individual formation of organs at different stages of development (38).

In epithelial cells, the TF and Tn antigens are O-glycans, which are mainly carried by mucins (39). Despite this, the TF antigen has been found in samples of adult major salivary glands by some studies, in contrast to the present study which observed complete absence, suggesting that this molecule was not expressed at the human parotid development stage (40). Unlike the TF antigen, the Tn antigen was seen in all samples on the plasma membrane of ductal and glandular cells at all fetal ages (Tables 4, 5). This finding is consistent since this antigen is a precursor of the O-glycan complex found in several membranes and glycoproteins and involved in cell signaling, and metabolism, and could, therefore, act as a receptor (41).

Another important family of proteins is the galectins, an ancient family of carbohydrate-binding proteins with affinity for β-galactosides (42). Many galectins can be found both intra and extracellularly in normal or neoplastic tissues where they stimulate cell-cell and cell-extracellular matrix adhesion, induce cell proliferation and motility, and also myogenic differentiation (43). Possibly, some or all of these functions are exercised by Gal-1 during ductal cell development. Gal-1 staining was confirmed by Xu et al., who reported its expression in the ducts and associated stromal cells, including striated muscle cells, of the three types of salivary glands (44).

Gal-3 showed a peculiar staining with a predominant presence in the luminal layer of ductal cells, mainly situated in the apical membrane but also in the cytoplasm and nuclei (Fig. 2c, Table 5). Its cytoplasmic presence has been related to its anti-apoptotic activity, while its nuclear staining indicates functions in mRNA splicing. Furthermore, it is also involved in cell-cell and cell-extracellular matrix interactions (45). The same Gal-3
profile was also observed by Xu et al. in the ducts of adult salivary glands (44).

Histochemical and immunohistochemical studies found morphological evidence that the glycomic profile changes during parotid gland development and, therefore, can be used as an early marker of differentiation since the cytodifferentiation markers are negative. Moreover, analysis of sugar moieties of glycoconjugates will help understand the biological importance of glycosylation.

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Conflict of interest
The authors have no conflicts of interest to declare.

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