Acta Histochemica 113 (2011) 815–825

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Distribution of sugar residues in human placentas from pregnancies complicated by hypertensive disorders

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ARTICLE INFO

Article history:
Received 30 September 2010
Received in revised form
29 November 2010
Accepted 1 December 2010

Keywords:
Sugar residues
Lectins
Placenta
Hypertensive disorders
Human

ABSTRACT

The aim of the study was to investigate the content and distribution of sugar residues in placentas from pregnancies complicated by hypertensive disorders. Placentas from women with uncomplicated pregnancies (group 1), pregnancies complicated by gestational hypertension (group 2), pregnancies complicated by pre-eclampsia (group 3), pregnancies complicated by pre-eclampsia with HELLP syndrome (hemolysis, elevated liver enzymes and low platelets) (group 4) were collected. Lectins: ConA, WGA, PNA, SBA, DBA, UEA I, GNA, DSA, MAA, SNA, in combination with chemical and enzymatic treatments, were used. Data showed a decrease and/or lack of α-D-mannose, α-D-glucose and D-galactose-(β1-4)-N-acetyl-D-glucosamine in placentas from pre-eclampsia and pre-eclampsia with HELLP syndrome compared with control and hypertension cases. N-acetyl-D-galactosamine appeared and/or increased in placentas from hypertensive disorders. A different distribution of various types of sialic acid was observed in placentas from hypertensive disorders compared with the controls. In particular, placentas from pre-eclampsia, with and without HELLP syndrome, lacked the acetylated sialic acid side-chain. These findings demonstrate various alterations of the carbohydrate metabolism in the placentas from pregnancies complicated by different types of hypertensive disorders. This indicates correlation with the placental morpho-functional changes characteristic of these complications and with the degree of clinical severity.

Introduction

Glycoconjugate sugar residues play an important role in many biological processes such as: enzymatic activities, cell-to-cell adhesion, cellular recognition and proliferation, both in normal and pathological tissues (Damjanov, 1987; Zanetta et al., 1994). Some oligosaccharides of the proteoglycans, present in the protein 'core', and of the collagens are involved in the physical maintenance of tissue structure and integrity (Varki, 1993). Moreover, the oligosaccharide 'coating' on many glycoproteins has the function of protecting the protein chain from proteases or antibodies and to form an antithrombogenic barrier in the vascular lumen (Varki, 1993; Iozzo and Murdoch, 1996; Hascall et al., 1997). Lectins are proteins or glycoproteins that have a specific binding affinity for the sugar residues of glycoconjugates and are extensively used as histochemical reagents to investigate the distribution of sugar residues in various organs and tissues (Gabrielli et al., 2004; Gheri et al., 2004, 2007, 2009; Sgambati et al., 2007; Accili et al., 2008).

Studies on the sugar residue contents in the human placenta of normal growing pregnancies and those complicated by some types of pathologies have been studied using labelled lectins (Lee and Damjanov, 1984; Wells and Bulmer, 1988; Thrower et al., 1991; Sgambati et al., 2002, 2007; Konska et al., 2003; Serman et al., 2004; Masnikosa et al., 2006). However, no data are available on lectin-binding in placentas from pregnancies of women with hypertensive disorders.

In normal pregnancies, the invasion of the maternal decidua and transformation of the spiral arterioles in low resistance vessels by fetal trophoblast, are essential for successful placentation (Pijinenborg, 1994; Benirschke and Kaufmann, 1995). In pregnancies complicated by hypertensive disorders, trophoblast invasion of the spiral arterioles is inadequate, leading to poor placental perfusion and fetal hypoxia (Zhou et al., 1997; Damsky and Fisher, 1998). This in turn results in the release of factors in the maternal

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0065-1281/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.acthis.2010.12.001

circulation that may cause vasoconstriction, hypertension, proteinuria and endothelial dysfunction (Roberts and Redman, 1993; Zhou et al., 1997; Damsky and Fisher, 1998; Myatt, 2002; Campos et al., 2006; Gilbert et al., 2008; Hawfield and Freedman, 2009).

Several investigations on placentas from pregnancies complicated by hypertensive disorders, in particular pre-eclampsia, showed morpho-functional alterations of the various placental components such as the trophoblast, basement membrane and vessels (Jones and Fox, 1980; Soma et al., 1982; Bartl and Müller, 1985; Shanklin and Sibai, 1989; VanWijk et al., 2000; Wawrzycka et al., 2001; Ishihara et al., 2002; Myatt, 2002; Ockleford et al., 2004; Mayhew et al., 2004; de Luca Brunori et al., 2005; Kos et al., 2005; Stanek and Al-Ahmadie, 2005; Barden, 2006; Campos et al., 2006; Rampersad and Nelson, 2007; Corrêa et al., 2008; Romero Gutiérrez et al., 2008; Sazhina et al., 2008; Vinnars et al., 2008; Webster et al., 2008; Hawfield and Freedman, 2009).

In the present study we investigated the distribution of various types of sugar residues in human placental tissue from pregnancies complicated by hypertensive disorders with different degrees of clinical severity: gestational hypertension, pre-eclampsia and pre-eclampsia with hemolysis, elevated liver enzymes and low platelets (HELLP syndrome). The aim of this investigation was to evaluate if and how changes in content and distribution of saccharidic content could be related to morpho-functional placental alterations characteristic of these complications. In order to investigate a wide spectrum of different carbohydrate structures, we used a battery of six horseradish peroxidise (HRP) conjugated lectins (DBA, SBA, PNA, ConA, WGA, UEA I) and four digoxigenin (DIG) labelled lectins (GNA, DSA, MAA and SNA). The HRP conjugated lectins or DIG labelled were chosen on the basis of previous results obtained by us involving our experience in lectin histochemistry.

Materials and methods

Group classification

Four groups were included in the study: (Group 1) control group of women with uncomplicated pregnancies ($n = 25$); (Group 2) women with pregnancies complicated by gestational hypertension ($n = 34$); (Group 3) women with pregnancies complicated by pre-eclampsia ($n = 32$); (Group 4) women with pregnancies complicated by pre-eclampsia with HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) ($n = 27$). The gestational age was between 35 and 38 weeks.

Women with chronic hypertension, renal disease, diabetes or pregnancies complicated by IUGR (IntraUterine Growth Retardation) were not included in the study. In all the study groups, babies were delivered by Caesarean operation. In the control group, Caesarean section was carried out due to one of the following indications: breech presentation, cephalopelvic disproportion or for psychological reasons.

Gestational hypertension was defined as persistent diastolic blood pressure above 90 mmHg and systolic blood pressure above 140 mmHg, in normotensive patients before 20 weeks of gestation. Criteria for diagnosis of pre-eclampsia were: diastolic blood pressure above 90 mmHg and systolic blood pressure above 140 mmHg, with edema and proteinuria greater than 0.3 g/24 h, in normotensive patients before 20 weeks of gestation. Criteria for the diagnosis of HELLP syndrome were: (1) hemolysis: abnormal peripheral blood smear, total bilirubin $\geq$1.2 mg/dL, lactic dehydrogenase (LDH) $\geq$ 600 U/L; (2) elevated liver enzyme(s): serum aspartate aminotransferase (AST) $\geq$ 70 U/L; (3) low platelets: platelets count $\geq$100,000/μL.

Table 1
Sugar residues binding specificity of lectin.

Lectin	Abbreviation	Sugar residues binding specificity
Dolichos biflorus agglutinin	DBA	α-D-GalNAc
Soybean agglutinin	SBA	α/β-D-GalNAc > D-Gal
Peanut agglutinin	PNA	D-Gal(β1 → 3)-D-GalNAc
Canavalia ensiformis agglutinin	ConA	α-D-Man > α-D-Glc
Wheat germ agglutinin	WGA	$(D\text{-GlcNAc})_n$ > Neu5Ac
Ulex europaeus agglutinin I	UEA I	α-L-Fuc
Galanthus nivalis agglutinin	GNA	$(Man)_n$
Datura stramonium agglutinin	DSA	$D\text{-Gal}(\beta1 \rightarrow 4)\text{-D-GlcNAc} > (D\text{-GlcNAc})_n$
Maackia amurensis agglutinin	MAA	Neu5Ac(α2 → 3)Gal
Sambucus nigra agglutinin	SNA	Neu5Ac(α2 → 6)Gal/GalNAc

Placenta collection

All tissue samples were obtained after receiving the approval of the Hospital Committee for Investigation in Humans (Azienda Ospedaliera Universitaria Careggi, Florence, Italy; protocol no. 6783-04) and after receiving informed consent from the patients.

At delivery, placentas were weighed and full thickness blocks were obtained. A random sampling procedure was used to obtain 12 blocks of full thickness tissue per organ. The specimens were immersed in 10% neutral buffered formalin solution for 12 h and processed in a standard manner for the preparation of paraffin blocks; 5 μm thick sections were cut.

Lectin histochemistry

Lectins and their sugar residue specificity are listed in the Table 1. HRP-conjugated lectins: DBA, SBA, PNA, ConA, WGA, UEA I were from Sigma–Aldrich, St. Louis; DIG-labelled lectins: MO, USA. GNA, DSA, MAA and SNA, were purchased from Roche Diagnostic GmbH, Mannheim, Germany.

HRP-conjugated lectins

After hydration, sections were treated with 3% hydrogen peroxide for 10 min to inhibit the endogenous peroxidase, rinsed in distilled water and treated with 0.1% bovine serum albumin (BSA) in TBS (0.05 M Tris–HCl, 0.15 M NaCl, pH 7.5) for 20 min to reduce the background labelling. Sections were then incubated for 30 min at room temperature in HRP-conjugated lectins solutions in TBS. The optimal concentration for each lectin, which allowed the maximum labelling with minimum background, was: DBA 25 μg/mL, SBA 20 μg/mL, PNA 25 μg/mL, ConA 50 μg/mL, WGA 25 μg/mL, UEA I 25 μg/mL. Then sections were rinsed three times in TBS. Labelling of the sites containing bound lectin-HRP was obtained by incubating the slides with 0.7 mg/mL 3,3′ diaminobenzidine (DAB) and 0.7 mg/mL hydrogen peroxide in 0.06 M TBS (Sigma Fast DAB tablet set, Sigma–Aldrich, St. Louis, MO, USA) for 10 min at room temperature.

DIG-labelled lectins

After hydration, sections were treated with 20% acetic acid for 15 min at 4 °C to inhibit the endogenous alkaline phosphatase, rinsed in distilled water and treated with 10% blocking reagent in TBS (Tris–HCl 0.05 M, NaCl 0.15 M, pH 7.5) for 30 min to reduce the background labelling. Then sections were washed two times in TBS for 10 min and rinsed in Buffer 1 (1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ in TBS, pH 7.5) for 10 min. Sections were then incubated for 1 h at room temperature in DIG-labelled lectins diluted in Buffer 1. Lectins were solutions (1 mg/1 mL) in 50 mM Tris–HCl, 0.05% sodium azide. The optimal dilution for each lectin, which

allowed the maximum labelling with minimum background, was: SNA 1 μL/1 mL, MAA 5 μl/1 mL, GNA 1 μL/1 mL and DSA 1 μL/1 mL. Sections were then rinsed three times for 10 min in TBS, incubated with anti-digoxigenin (0.3 mL polyclonal sheep anti-digoxigenin Fab fragments), conjugated with alkaline phosphatase (750 U/mL) (Roche Diagnostic GmbH, Mannheim, Germany) diluted in TBS (1 μL/1 mL) for 1 h and washed three times in TBS for 10 min. Labelling of the sites containing bound lectin-digoxigenin was obtained incubating the slides with Buffer 2 (0.1 M Tris–HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5), containing nitroblue tetrazolium (NBT)/X-phosphate (20 μL/1 mL), for 10 min at room temperature.

All the specimens were rinsed in distilled water, dehydrated using graded ethanol solutions, cleared in xylene and mounted in Permount™ (Fisher Scientific).

Enzyme and chemical treatments

Glucose oxidase

Glucose oxidase converts glucose residues into gluconic acid. ConA labels both glucose and mannose but after glucose oxidase pre-treatment, the labelling is only due to mannose. Sections were washed in 0.1 M acetate buffer, pH 5.0, and incubated overnight with 200 U/mL type VII glucose oxidase from *Aspergillus niger* at 37 °C, in a moist chamber, prior to labelling with ConA (Madrid et al., 2000).

Neuraminidase digestion, deacetylation and differential oxidation

In some experiments sialic acid was removed by pretreating the sections for 18 h at 37 °C in a solution of 0.25 M sodium acetate buffer, pH 5.5, containing 0.1 U/mL sialidase (neuraminidase Type X from *Clostridium perfringens* (Sigma–Aldrich), 50 mM $CaCl_2$ and 154 mM NaCl, prior to labelling with PNA. Deacetylation was performed by incubating sections with 0.5% KOH in 70% ethanol for 30 min at room temperature. This treatment, detaching all the acetyl substituents, renders sialic acid residues, containing also acetylic groups on C_4 of the pyranose ring, susceptible to sialidase digestion (Gabrielli et al., 2004; Gheri et al., 2009).

For differential oxidation, 1 mM aqueous periodic acid (1 mM PO, mild oxidation) and 44 mM aqueous periodic acid (44 mM PO, strong oxidation) were used for 15 min at room temperature. Mild oxidation abolishes the labelling with sialidase/PNA or KOH/sialidase/PNA when sialic acid does not contain C_7-and/or C_8-and/or C_9-O-acetyl groups in the side chain. Strong oxidation blocks the subsequent labelling with sialidase/PNA or KOH/sialidase/PNA except for C_9 acetylated sialic acids linked via α2,3 bound to the penultimate β-galactose (Gabrielli et al., 2004; Gheri et al., 2009).

Controls

Controls for lectin specificity included substitution of the lectin-conjugates with the respective unconjugated lectins, or preincubation of lectins with the corresponding hapten sugars (concentration of 0.1–0.5 M in TBS). Controls of glucose oxidase and sialidase digestions were made by incubating sections with enzyme-free buffer. The efficacy of sialidase digestion was tested treating adjacent sections with the enzyme solution, with and without prior deacetylation, prior to MAA and SNA labelling. Some control sections were treated with desulphation procedure, i.e. placing them in 0.15 N HCl in methanol for 5 h at 60 °C and then in KOH in 70% ethanol for 15 min at room temperature (Gabrielli et al., 2004; Gheri et al., 2009). This procedure eliminates sulphated groups present on the carbohydrate chains that could interfere with lectin binding.

All the slides were stained with the same batch, for each lectin, to eliminate inter-batch variation.

Evaluation of reactivity location

A 1 mm × 1 mm grid, divided in 100 squares, was used to evaluate the reactivity location. An investigator, blinded to the tissue identity, examined 20 fields, selected at random, for each section (5 sections for each specimen). The trophoblast, the trophoblast basement membrane and/or plasma membrane (BM/BPM) and the vessels of the chorionic intermediate and terminal villi were examined.

Evaluation of reactivity intensity

In each field of each section examined for the reactivity location, using a 40× objective, a semi-quantitative analysis, using a computerized image analyzer program (Image-Pro Plus v. 4.5, Media Cybernetics, Bethesda, MD, USA), was also performed to evaluate the intensity of reactivity in ten tracts of the trophoblast, in ten tracts of the BM/BPM and in 10 vessels of the chorionic intermediate and terminal villi. Using this measurement program, the optical density (O.D.) was determined. The reactivity intensity was measured and expressed in arbitrary units standardized from 0 to 256, with 0 representing the maximum labelling and 256 no labelling. The data were expressed as the mean ± SD of values obtained from densitometric analysis in the 20 fields of the 5 sections of each case from each group.

Statistical analysis

To compare clinical data among the study groups, analysis of variance, performed by ANOVA with post hoc test (Fisher's protected least significant difference procedure), was used.

Statistical analysis of the data obtained from the semi-quantitative analysis of the reactive components was performed using Student's *t*-test for paired samples and analysis of variance, performed by ANOVA with post hoc test. The following differences were evaluated: (1) differences in lectin reactivity intensity with and without treatments (ConA, PNA) in specimens of the same group (Student's *t*-test for paired samples); (2) differences in lectin reactivity intensity between specimens of the three study groups (ANOVA with post hoc test).

Probability of being due to chance alone of less than 5% ($P < 0.05$) for Student's *t*-test and 1% ($P < 0.01$) for ANOVA with post hoc test was considered statistically significant.

Reproducibility of the measurements was assessed comparing the measurements made by one observer at different times and measurements of two observers. Intra-observer coefficient of variation was 1.5% and inter-observer was 5.2%.

Results

Clinical details

Clinical details of the women whose placentas were used for this study are shown in Table 2. Mean weeks of gestation and mean birth weight at delivery were significantly lower in groups 3 and 4 with respect to groups 1 and 2. Mean placental weight was significantly lower in groups 3 and 4 with respect to groups 1 and 2, and in group 2 compared to group 1. Mean pressure (systolic and diastolic) was statistically higher in groups 2, 3 and 4 when compared with group 1. The other clinical data did not show significant differences between the study groups.

Lectin histochemistry

It should be pointed out that 24 cases of group 2, 19 of group 3 and 11 of group 4 showed a different reactivity with regard to some lectins compared to the other cases of the same group.

Table 2
Clinical details of the study groups. Values shown are mean ± SEM.

	Group1 (n = 25)	Group2 (n = 34)	Group3 (n = 32)	Group4 (n = 27)
1) Maternal age (years)	31 ± 8	30 ± 7	31 ± 4	31 ± 6
2) Gestation at delivery (week)	38 ± 2	37 ± 1	34 ± 7*	35 ± 4*
3) Birth weight (g)	3475 ± 617	3340 ± 701	2755 ± 520*	2641 ± 616*
4) Placenta weight (g)	620 ± 77	530 ± 55*	444 ± 61*	439 ± 49*
5) Primigravidae (no.)	14	11	11	12
6) Smokers (no.)	5	14	6	4
7) Pregnancy BMI	24 ± 4	24 ± 3	25 ± 2	23 ± 4
Blood pressure at delivery (mmHg)				
8) Systolic	113 ± 8	155 ± 8*	157 ± 8*	154 ± 9*
9) Diastolic	65 ± 9	98 ± 5*	100 ± 8*	99 ± 7*

BMI: body mass index; 2) and 3) groups 3, 4 *vs* groups 1, 2; 4) groups 3, 4 *vs* groups 1, 2 and group 2 *vs* group 1; 8) and 9) groups 2, 3, 4 *vs* group 1. * $P < 0.01$.

Lectin reactivity location (Table 3)

ConA

In all the study groups, reactivity was observed in the trophoblast, in the BM/BPM and in the vessels (Fig. 1A, B, C and D).

Glucose oxidase/ConA

In all the cases of groups 1 and 2, 19 cases of group 3 and 11 of group 4 the placental components showed reactivity (inserts Fig. 1A, B, C and D).

WGA

In all the study groups, WGA reacted with all the placental components.

DBA

No reactivity in any placental component of group 1 was observed. On the other hand, all the placental components of groups 2, 3 and 4 showed reactivity (Fig. 2A, B, C and D).

SBA

Group 1 showed reactivity only in some vessels. All the placental components reacted in 10 cases of group 2, 13 of group 3 and 16 of group 4 (Fig. 3A, B, C and D). In 24 cases of group 2, 19 of group 3 and 11 of group 4, reactivity was seen only in some vessels.

UEA I

Trophoblast and vessels reacted in placentas of all the study groups.

GNA

Reactivity was observed in all the placental components of all the cases of groups 1 and 2, 19 cases of group 3 and 11 of group 4.

DSA

The apical border of the syncytiotrophoblast reacted in placentas of all groups.

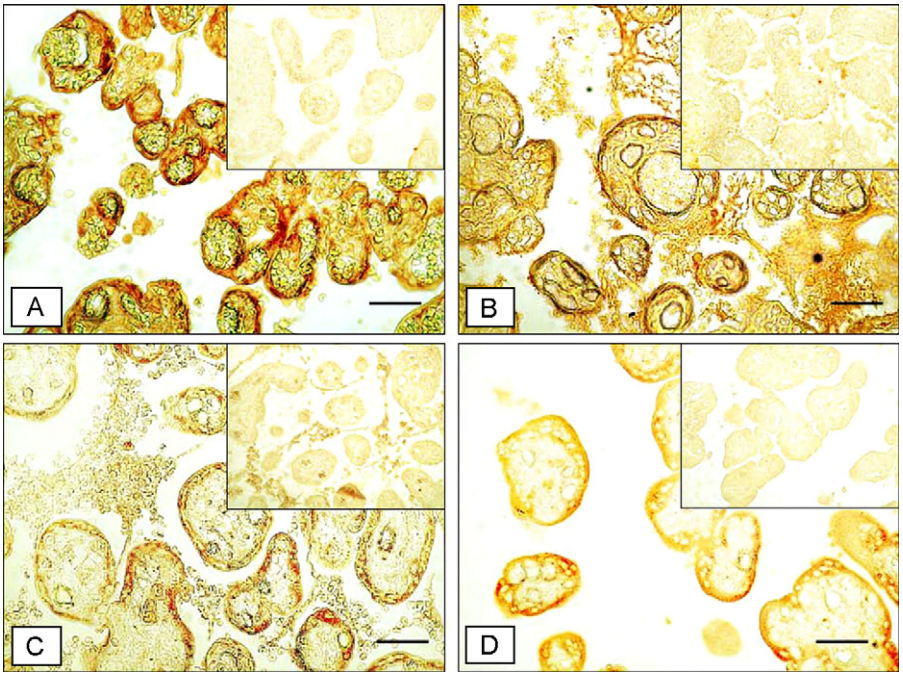

Fig. 1. ConA reactivity in placenta (37 weeks). Reactivity is detectable in all placental components of the four study groups; groups 1 (A) and 2 (B) show stronger reactivity with respect to groups 3 (C) and 4 (D). Scale bar = 25 μm. Inserts: Glucose oxidase-ConA reactivity in placenta (37 weeks). Reactivity was weaker in all placental components of all study groups. Scale bar = 100 μm.

Table 3
Lectin reactivity location and intensity in the groups 1, 2, 3, 4. Data are expressed as the mean ± SD of values obtained from densitometric analysis (O.D.).

	Trophoblast				BM/BPM				Vessels			
	Group1	Group2	Group3	Group4	Group1	Group2	Group3	Group 4	Group1	Group2	Group3	Group4
ConA	(S C) 130 ± 19	(S C) 128 ± 21	(S C) 181 ± 15	(S C) 185 ± 17	131 ± 11	129 ± 12	183 ± 15	182 ± 20	127 ± 11	130 ± 16	180 ± 13	179 ± 10
GlucOx/ConA	(S C) 225 ± 12	(S C) 223 ± 20	(S C) 222 ± 13*	(S C) 224 ± 15*	216 ± 10	220 ± 9	218 ± 5*	221 ± 19*	227 ± 21	223 ± 18	225 ± 17*	226 ± 12*
WGA	(S C) 127 ± 9	(S C) 130 ± 17	(S C) 131 ± 7	(S C) 128 ± 10	134 ± 11	137 ± 15	133 ± 12	135 ± 14	124 ± 11	120 ± 13	122 ± 18	120 ± 10
DBA	–	(S C) 144 ± 11	(S C) 146 ± 22	(S C) 150 ± 13	–	140 ± 12	137 ± 14	142 ± 19	-	151 ± 15	158 ± 12	155 ± 18
SBA	–	(S C) 190 ± 13**	(S C) 187 ± 20**	(S C) 189 ± 19**	–	185 ± 9**	187 ± 10**	184 ± 16**	176 ± 10#	174 ± 12§	123 ± 8§	125 ± 9§
UEA I	(S C) 119 ± 18	(S C) 121 ± 17	(S C) 118 ± 20	(S C) 121 ± 18	–	–	–	–	123 ± 10	127 ± 19	124 ± 15	122 ± 17
GNA	(S C) 228 ± 10	(S C) 224 ± 13	(S C) 226 ± 20*	(S C) 227 ± 19*	217 ± 10	216 ± 19	220 ± 13*	218 ± 7*	223 ± 8	227 ± 10	224 ± 11*	223 ± 9*
DSA	a (S) 122 ± 13	a (S) 120 ± 17	a (S) 123 ± 19°	a (S) 121 ± 18°	–	–	–	–	–	–	–	–
MAA	a (S) 191 ± 10	a (S) 189 ± 13	a (S) 192 ± 10	a (S) 190 ± 7@*	186 ± 13@	184 ± 20@	185 ± 19@	–	–	–	–	–
SNA	–	–	–	a (S) 189 ± 10@	–	–	–	–	–	181 ± 15#**	179 ± 13#	182 ± 20#
PNA	–	–	–	–	–	–	–	–	–	–	–	–
Neu/PNA	a (S) 177 ± 15	a (S) 179 ± 11	a (S) 178 ± 13	a (S) 175 ± 9	–	168 ± 9**	170 ± 11**	166 ± 7	171 ± 10	174 ± 8	172 ± 13	175 ± 9
KOH/Neu/PNA	a (S) 121 ± 10	a (S) 124 ± 13	a (S) 122 ± 15	a (S) 120 ± 9^	130 ± 7	167 ± 12	169 ± 10	165 ± 11	125 ± 6	129 ± 11^	127 ± 17^	126 ± 12
1 mMPO/Neu/PNA	a (S) 211 ± 10	a (S) 215 ± 8*	–	–	–	–	–	–	219 ± 17	212 ± 11*	–	–
1 mMPO/KOH/Neu/PNA	a (S) 214 ± 7	a (S) 209 ± 10*	–	–	–	–	–	–	220 ± 12	215 ± 8*	–	–
44 mMPO/Neu/PNA44 mM/KOH/Neu/PNA	–	–	–	–	–	–	–	–	–	–	–	–

S: syncytiotrophoblast; C: cytotrophoblast; a: apical border; –: no reactivity, 250 O.D.; *: in some cases—(n = 10 group 2, n = 13 group 3, n = 16 group 4); **: in some cases—(n = 24 group 2, n = 19 group 3, n = 11 group 4); #: some vessels -; §: in some cases only some vessels (n = 24 group 2: 177 ± 8 O.D.; n = 19 group 3: 172 ± 10 O.D.; n = 11 group 4: 175 ± 13 O.D.); °: in some cases of the groups 3 (n = 13) and 4 (n = 16), respectively 180 ± 17 O.D. and 178 ± 14 O.D.; @: some tracts -; ^: in some cases: a (S) 177 ± 12 O.D and vessels 173 ± 9 in the group 2 (n = 10), a (S) 174 ± 14 and vessels 176 ± 10 in the group 3 (n = 13), a (S) 178 ± 13 and vessels 176 ± 7 in the group 4 (n = 16).

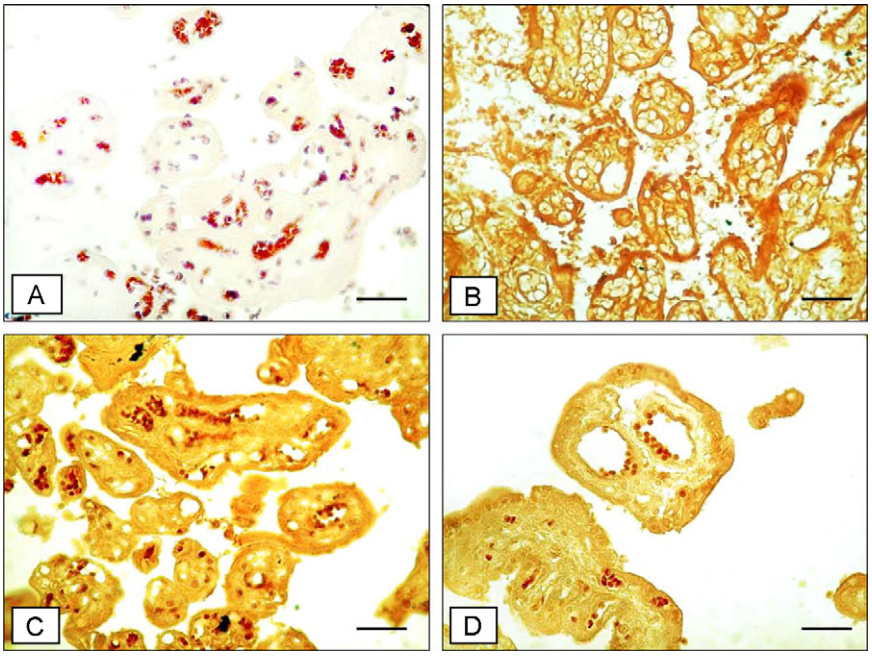

Fig. 2. DBA reactivity in placenta (38 weeks). No reactivity is observable in placental tissue of group 1 (A). In groups 2 (B), 3 (C) and 4 (D), all placental components are lectin reactive. Scale bar = 25 μm.

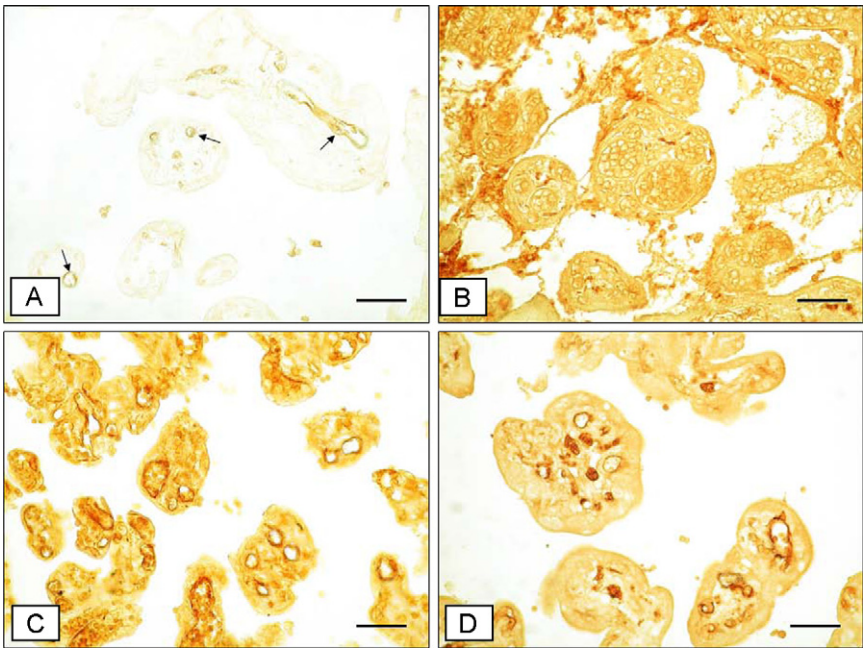

Fig. 3. SBA reactivity in placenta (38 weeks). In group 1 (A) only some vessels show reactivity (arrows). In groups 2 (B), 3 (C) and 4 (D), all placental components show reactivity. Scale bar = 25 μm.

MAA

The apical border of the entire syncytiotrophoblast and some tracts of the BM/BPM in all the cases of groups 1, 2 and 3 reacted with the lectin (Fig. 4A, B and C). The apical border of some tracts of the syncytiotrophoblast in 11 cases of group 4 also reacted. No reactivity was observed in any placental components in 16 cases of group 4 (Fig. 4D).

SNA

Reactivity was observed in the apical border of some tracts of the syncytiotrophoblast in group 4, in some vessels of 10 cases in group 2, and of all the cases in groups 3 and 4.

PNA and PNA with enzymatic and chemical treatments

No reactivity with PNA was observed in any placental component in all the study groups. After neuraminidase digestion, PNA reactivity was observed in the apical border of the syncytiotrophoblast and in the vessels in all the study groups; in the BM/BPM of 10 cases in group 2, 13 in group 3 and of all cases in group 4 (Fig. 5A, B, C and D). After deacetylation-neuraminidase treatment, PNA reacted with the apical border of the syncytiotrophoblast, BM/BMP and vessels of all the study groups (inserts Fig. 5A, B, C and D). After mild oxidation-neuraminidase treatment, with and without deacetylation, reactivity was observed in the apical border of the syncytiotrophoblast and in the vessels of all the cases in group 1 and of 24 cases in group 2 (Fig. 6A, B, C and D).

No reactivity was detected in any placental component of the four study groups after strong oxidation-neuraminidase treatment with and without deacetylation.

Statistical analysis of lectin reactivity intensity

Data of the lectin reactivity intensity obtained from densitometric analysis are presented in Table 3. As previously reported in materials and methods, a 0–256 scale intensity was used (being 0 the maximum labelling and 256 no labelling).

Statistically significant differences in lectin reactivity intensity, with and without treatments (ConA, PNA), in specimens of the same group ($P < 0.05$)

Statistical analysis showed that ConA reactivity intensity was higher in all placental components of all the study groups compared to ConA reactivity after glucose oxidase treatment. After deacetylation-neuraminidase treatment, PNA reactivity was higher in the apical border of the syncytiotrophoblast and in the vessels of all the cases in group 1, 24 cases in group 2, 19 in group 3 and 11 in group 4 compared to neuraminidase-PNA reactivity without deacetylation. After mild oxidation-neuraminidase treatment, with and without deacetylation, PNA reactivity intensity was lower in the apical border of the syncytiotrophoblast and in the vessels of group 1 and in 24 reactive cases of group 2 compared to neuraminidase-PNA reactivity and deacetylation-neuraminidase PNA reactivity.

Statistically significant differences in lectin reactivity intensity between specimens of the three study groups ($P < 0.01$)

Comparing the measurements of the lectin reactivity intensity of group 1 with those of groups 2, 3 and 4 the statistical analysis showed that ConA reactivity intensity was higher in all the placental components of group 1 compared to groups 3 and 4. SBA reactivity intensity was higher in some vessels of 13 cases of group 3 and 16 of group 4 with respect to all the cases of group 1. DSA reactivity intensity was higher in the apical border of the syncytiotrophoblast of all the cases in group 1 with respect to 13 cases in group 3 and 16 in group 4. After deacetylation-neuraminidase treatment PNA reactivity was higher in the apical border of the syncytiotrophoblast and in the vessels of all the cases in group 1 compared to 10 cases in group 2, 13 in group 3 and 16 in group 4, and in the BM/BPM of all the cases in group 1 with respect to the all reactive cases in the other groups.

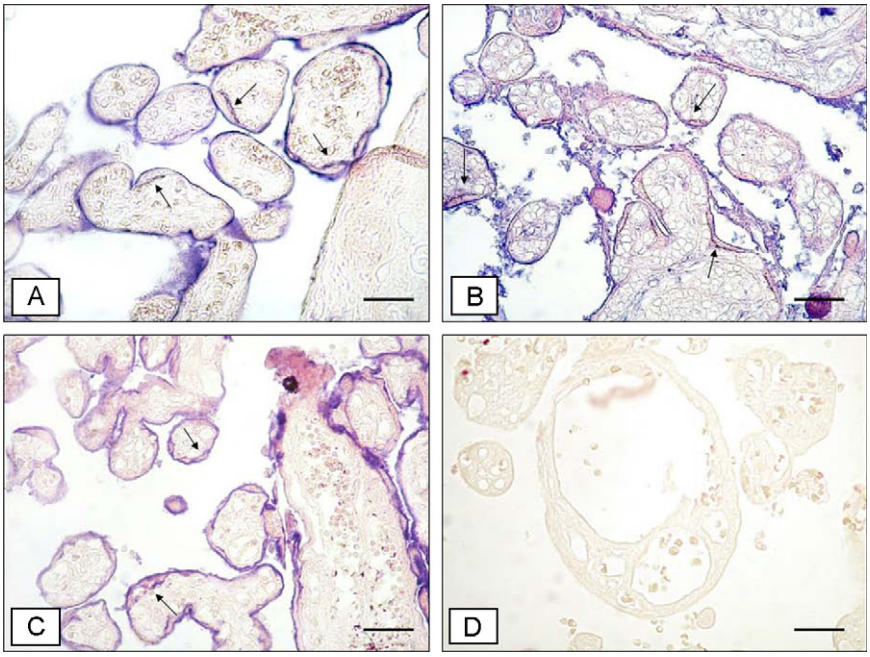

Fig. 4. MAA reactivity in placenta (36 weeks). The apical border of the syncytiotrophoblast and some tracts of the BM/BPM of groups 1 (A), 2 (B) and 3 (C) (arrows) show reactivity. In group 4 (D) no reactivity is seen. Scale bar = 25 μm.

The comparison of the measurements among groups 2, 3 and 4 showed that ConA reactivity intensity was higher in all the placental components of group 2 with respect to groups 3 and 4. SBA reactivity intensity was lower in the vessels of 10 cases in group 2 compared to 13 in group 3 and 16 in group 4. DSA reactivity intensity was higher in the syncytiotrophoblast apical border of all the cases in group 2 compared to13 cases of group 3 and 16 of group 4. After deacetylation-neuraminidase treatment, PNA intensity was lower in the apical border of the syncytiotrophoblast and in the vessels of 13 cases in group 3 and 16 in group 4 with respect to 24 cases in group 2, and of the 13 cases in group 3 compared to the 11 in group 4.

Controls

Sections incubated with lectins and their corresponding hapten sugars and sections incubated with unconjugated lectins, were unstained. Sections incubated with enzyme-free buffer, did not show any change in lectin binding. Results of the efficacy of

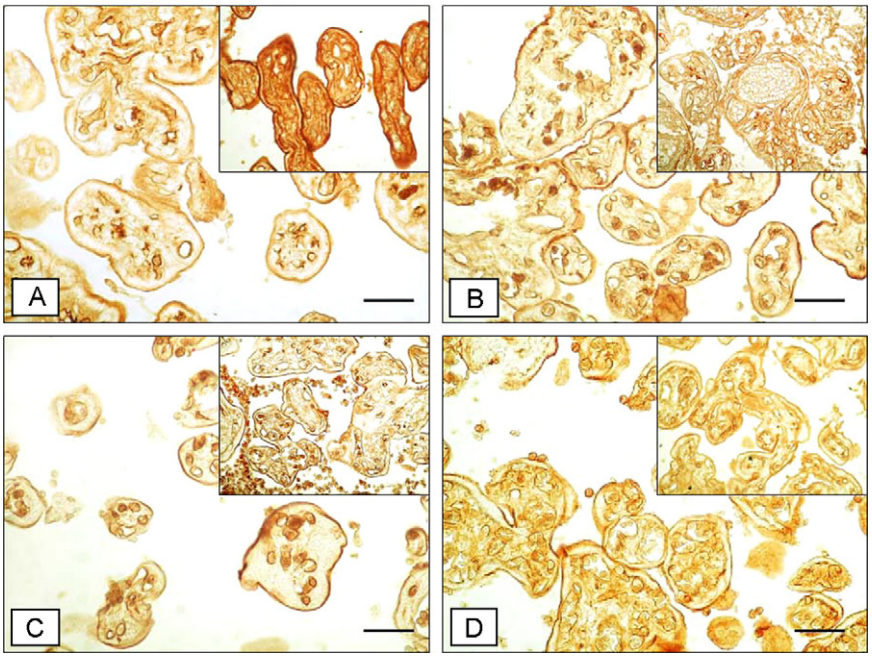

Fig. 5. Neuraminidase-PNA reactivity in placenta (37 weeks). Reactivity is detectable in the apical border of the syncytiotrophoblast and in the vessels of groups 1 (A), 2 (B), 3 (C) and 4 (D). BM/BPM is reactive in group 4 (D). Scale bar = 25 μm. Inserts: KOH-neuraminidase-PNA reactivity in placenta (37 weeks). Reactivity is observable in all placental components of all study groups; stronger reactivity is detectable in group 1 (A). Scale bar = 100 μm.

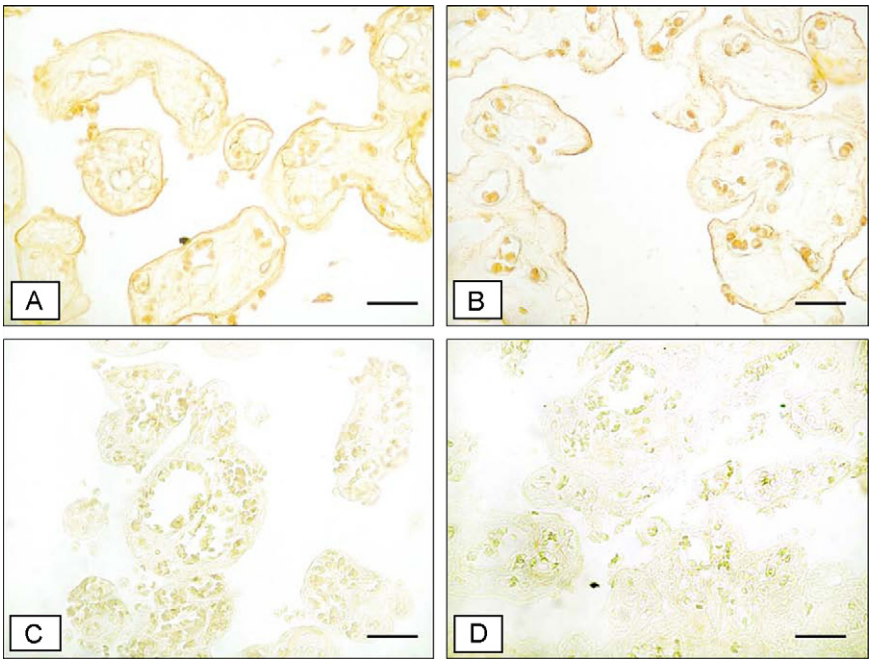

Fig. 6. Mild oxidation-KOH-neuraminidase-PNA reactivity in placenta (38 weeks). The apical border of the syncytiotrophoblast and the vessels show reactivity in groups 1 (A) and 2 (B). No reactivity was observed in placental tissue of groups 3 (C) and 4 (D). Scale bar = 25 μm.

enzymatic digestion were as expected. Desulphation procedure did not prove to affect the lectin-binding.

Discussion

Our study demonstrated a different distribution and/or content of various sugar residues in placentas from normal pregnancies compared to those from pregnancies complicated by hypertensive disorders. Differences were also observed among pathological groups. Carbohydrate α-glucose was in less amount in all the cases, α-mannose was lacking in some cases, in all the placental components derived from pregnancies complicated by pre-eclampsia and pre-eclampsia with HELLP syndrome with respect to the normal and hypertension ones (ConA, Glucose oxidase/ConA, GNA). α-N-acetyl-D-galactosamine appeared in all the placental components of all the cases in the pathological groups. β-N-acetyl-D-galactosamine appeared in the trophoblast and BM/BMP of some cases in the pathological groups; it was in major amount in the pathological groups vessels, particularly in pre-eclampsia and pre-eclampsia with HELLP syndrome. The findings regarding the presence of α and/or β anomers of N-acetyl-D-galactosamine are evidenced by the differential binding of DBA and SBA (Schulte et al., 1985). D-galactose-β(1,4)-N-acetyl-D-glucosamine (DSA) was in less amount in the apical border of the syncytiotrophoblast in some cases of pre-eclampsia and pre-eclampsia with HELLP syndrome. PNA with treatments, indirect method to detect sialic acid, demonstrated that it was present in the apical border of syncytiotrophoblast, BM/BMP and vessels in all the cases of study groups, but it was not always acetylated in the pathological ones, particularly in the pre-eclampsia and pre-eclampsia with HELLP syndrome. It is noteworthy that different reactivity with MAA and SNA (direct methods for sialic acid detection) was found with respect to PNA after treatments both in the normal and in the pathological groups. One explanation, purely speculative, as reported in other studies by us, might be the presence of long chains of polysialic acid (Gheri et al., 2009; Mencucci et al., 2009) creating a steric hindrance for MAA and SNA binding, which recognize sialic acid and its linkages α(2,3) and α(2,6) to the penultimate sugar residues galactose and/or galactosamine. Another hypothesis may be the presence of acetylated sialic acid, here evidenced by the indirect method, as well as the eventual presence of other sialoderivatives, such as methylated sialic acid, that might cause a steric hindrance (Accili et al., 2008). Obviously, one or more of these steric hindrances could be present. On the basis of these hypotheses, the findings obtained with direct and indirect methods for sialic acid detection indicate different types of sialoderivatives in the pathological groups, in particular in the pre-eclampsia with HELLP syndrome, with respect to the control one.

All these findings indicate that almost all the sugar residues investigated were in lower amounts in the placentas from hypertensive disorders, particularly in the more severe clinical groups, demonstrating a decrease in glycosylation metabolism. The exception was represented by N-acetyl-D-galactosamine that appeared as anomer α in the placenta of all the cases of hypertensive disorders and as anomer β in some cases. This feature could be due to addition of these carbohydrates in the saccharidic chain or, more probably, to the lack of other sugar residues in the chain displaying the carbohydrate N-acetyl-D-galactosamine. Nevertheless, these two lectins, in particular DBA, can be considered as "markers" of these complications, independently from clinical severity. Another noteworthy feature arising from our data is that placentas of some cases in each pathological group showed a more similar glycosylation to normal, independently from the clinical severity.

As reported in previous studies, sugar residues seem to have an important role in the development and maintenance of the normal structure of placenta and in its functionality (Sgambati et al., 2002, 2007; Jones et al., 2007). Therefore, changes in placental tissue glycosylation in the hypertensive disorders study groups might be related to some morpho-functional alterations characteristic of these complications, observed especially in pre-eclampsia. Reduction of villous surface, increase in number of syncytial knots, abnormal shaped microvilli, areas of focal necrosis, release of STBM (syncytiotrophoblast microvillous membrane particles), dilatation of rough endoplasmic reticulum, decreased syncytial pinocytotic droplets, hyperplasia and degeneration of cytotrophoblast, increase of apoptosis, mitochondrial damage and

altered enzymatic activity are some of the important alterations, often related to clinical severity, that occur in trophoblast (Jones and Fox, 1980; Soma et al., 1982; Bartl and Müller, 1985; VanWijk et al., 2000; Wawrzycka et al., 2001; Ishihara et al., 2002; Myatt, 2002; Ockleford et al., 2004; Kos et al., 2005; Barden, 2006; Campos et al., 2006; Rampersad and Nelson, 2007; Corrêa et al., 2008; Romero Gutiérrez et al., 2008; Hawfield and Freedman, 2009). In addition, investigations on pre-eclampsia showed that functions such as coagulation, materno-fetal communication and internalisation of lipoproteins, so important in fetal nutrition, are impaired (Myatt, 2002). Proteoglycans, such as syndecan-1 and glypican-3 present in the apical border of the syncytiotrophoblast seem to have an important role in these functions. Their expression appears altered in pre-eclampsia (Jokimaa et al., 2000; Ogawa et al., 2007). Other studies showed altered expression of adhesion molecules, receptors, transporters and enzymes in the hypertensive disorders' trophoblasts, that seem to be related to altered placental development and materno-fetal exchanges (Neudeck et al., 1996; Myatt, 2002; Li et al., 2003; Zenclussen et al., 2003; Marini et al., 2007; Abe et al., 2008; Ozkan et al., 2008; Webster et al., 2008). Trophoblast basement membrane is thickened and changes in collagens content are observable; these might be responsible for alteration of permeability and exchange properties of the villi (Jones and Fox, 1980; Soma et al., 1982; Wawrzycka et al., 2001; Sazhina et al., 2008). Presence of narrow vessels and structural changes of endothelium were observed (Jones and Fox, 1980; Soma et al., 1982; Shanklin and Sibai, 1989; VanWijk et al., 2000; de Luca Brunori et al., 2005). Studies on the expression of receptors, transporters, enzymes and junction molecules have demonstrated that their changes are responsible for alteration of endothelium structure and functionality (Myatt, 2002; Mayhew et al., 2004; Liévano et al., 2006; Marini et al., 2007; Escudero et al., 2008; Webster et al., 2008). Many complex molecules investigated in the various placental components (proteoglycans, adhesion molecules, receptors, transporters, enzymes and collagens) are glycosylated. Therefore, changes in glycosylation could contribute to affect expression and then function. However, other glycosylated complex molecules important for correct functionality of placenta even if not showing changes in expression may, nevertheless, exhibit alteration in function due only to glycosylation changes.

On the basis of our findings and all these observations, it would result that functionality of placenta is gradually impaired from hypertension to the more severe types of disorders, such as pre-eclampsia and pre-eclampsia with HELLP syndrome. However, it is noteworthy that, according to our findings, not all cases in each studied pathological group, independently from clinical severity, would seem to present the same placental impairment. On the other hand, it has been seen that not all women who develop pre-eclampsia have placental pathology (Barden, 2006).

An interesting feature is the presence of sialic acid differently linked to penultimate sugar residue and/or the different distribution of sialoderivatives in the placentas of the pre-eclampsia with HELLP syndrome with respect to the other study groups, in particular pre-eclampsia. It is to be noted that different underlying pathogenetic mechanisms and courses can occur in cases with pre-eclampsia and HELLP syndrome (Vinnars et al., 2008). This would lead to a histopathologic profile and a range of placental lesions partially different in the pre-eclampsia with and without HELLP. Therefore, also sialic acid metabolism could be partially different in the two types of hypertensive disorders, contributing to lead to their different histopathologic aspects.

Another interesting point is the acetylation of sialic acid that in the placental tissue of the more severe hypertensive disorders cases is reduced or lacking. One of the important functions of acetylation of sialic acid seems to be masking linkage sites for various molecules (Schauer, 2009). This could attribute to the acetylated sialoderivatives a defensive role to the attachment of various molecules such as sialidase (bacterial) (Accili et al., 2008) and proteases. It might also be hypothesized that acetylated sialic acid in side chain, observed in the apical border of the syncytiotrophoblast in normal placentas and in some cases of hypertensive ones, can be a constituent of oligosaccharides present in the protein 'core' of syndecan-1 and glypican-3 and might contribute to maintain their important functions. However, acetylation could also be necessary for sialic acid to maintain the normal physical structure and integrity of the placental components (Varki, 1993; Gheri et al., 2007, 2009; Mencucci et al., 2009). Therefore, in the more severe hypertension disorders these functions of sialic acids might be seriously impaired.

In conclusion, this study demonstrated change in the glycosylation metabolism in placentas from pregnancies complicated by hypertensive disorders, related to clinical severity, with respect to physiological ones. These changes seem to be related to morphology and functionality alterations of the placenta. Therefore, changes in the expression of sugar residues could have an important role in the pathophysiology of the placenta. An interesting feature arising from our findings is that not all the pathological cases show the same drastic change in glycosylation, independently from clinical severity. It is noteworthy that several maternal factors have been suggested to be involved in defective placentation in pre-eclampsia (VanWijk et al., 2000). Our findings could be due to individual different expression of all or some of these factors, influencing differently glycosylation in the placenta. Also a different individual placental sensibility to factors could occur. On the basis of these observations, our findings could contribute to demonstrate that the role of placenta may be more determinant in the pathogenesis of hypertensive disorders in some cases more than in others (Barden, 2006).

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