



The patterns of the complex- and oligomannose-type glycans of uromodulin (Tamm-Horsfall glycoprotein) in the course of pregnancy

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Uromodulin was isolated from urine of three pregnant women. Urine of each donor was collected at subsequent stages of their pregnancy and at one month after gestation. Each batch of uromodulin was enzymatically N-deglycosylated and the released N-glycans were isolated, quantified and profiled by high-pH anion-exchange chromatography. In the course of pregnancy no significant changes were detected in the negative charge distribution stemming from sialic acid and sulfate residues on the complex-type carbohydrate chains of uromodulin. Furthermore, no significant changes in the molar ratio between Man₆GlcNAc₂ and Man₇GlcNAc₂ were found in the course of pregnancy, only uromodulin from non-pregnant periods showed small differences.

Keywords: Tamm-Horsfall glycoprotein, uromodulin, HPAEC profiling, N-glycans, pregnancy

Introduction

Uromodulin is a 90-kDa glycoprotein, which occurs in the urine of pregnant women in amounts of up to 100 mg/day. Its amino acid sequence is identical to that of urinary Tamm-Horsfall glycoprotein (THp), found in males and non-pregnant women [1,2]. Among the variety of biological functions reported for uromodulin, the immunomodulatory effects are remarkable. Uromodulin (THp) has been shown to be able to inhibit antigen-induced human T-cell proliferation, to stimulate the proliferation of human mononuclear cells, and to increase the arachidonic acid metabolism of polymorphonuclear neutrophils [3–9]. These immunomodulating effects have been ascribed to the carbohydrate chains displayed by uromodulin (THp) [10–12].

Analysis of the N-glycosylation sites of human THp from one healthy male donor has shown that seven out of the eight potential N-glycosylation sites are glycosylated [13], and detailed structural studies of the N-glycosylation pattern of

THp from individual male donors by ¹H NMR spectroscopy have resulted in the elucidation of 63 complex-type [14–16] and 4 oligomannose-type N-glycans (Man_{5–8}GlcNAc₂) [12,13]. Di-, tri-, and, most of all, tetraantennary structures (including dimeric *N*-acetylactosamine sequences) are present, which can be fucosylated, sialylated (including the Sd^a determinant) and/or sulfated (GalNAc4S and Gal3S). For one individual male donor the presence of over 150 different N-glycans could be demonstrated [14]. The occurrence of donor specificity in male THp has been demonstrated for dimeric *N*-acetylactosamine sequences with and without the Sd^a epitope (in molar percentages, Neu5Ac(α2-3)[GalNAc(β1-4)]Gal(β1-4)GlcNAc(β1-3)Gal : Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal = 50:50; 35:65; 41:59; or 28:72 [16]) and for oligomannose-type N-glycans (molar percentage oligomannose-type N-glycans: 20%, donor not specified and percentage based on peak heights [17]; 16%, donor not specified [18]; 5%, male donor [13]; 2%, male donor [19], and 0%, male donor [14]). For females a pregnancy-associated decrease (~38%) in the total amount of the oligomannose-type N-glycans of uromodulin (THp) has been reported [20]. Comparison of uromodulin of pregnant (third trimester) and non-pregnant females yielded typical shifts for the molar percentages of Man₅GlcNAc₂, Man₆GlcNAc₂ and

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Man₇GlcNAc₂ (6.2 versus 7.7%, 59.2 versus 66.4%, and 34.6 versus 25.9%, respectively) [20].

Mass spectrometric analysis of the O-glycosylation pattern of THp from males and non-pregnant females has shown the presence of core 1 type O-glycans terminated with either Neu5Ac or Fuc, but not with the sialyl Lewis x epitope. For pregnant females a series of unusual core 2 type O-glycans terminated with one, two or three sialyl Lewis x sequences have been established [21].

In this report we have analyzed the patterns of the complex- and mannose-type glycans of uromodulin (THp) of three pregnant females in the course of their pregnancies, using high-pH anion-exchange chromatography profiling.

Materials and methods

Materials

Recombinant peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* was purchased from Boehringer Mannheim (FRG).

Isolation and purification of uromodulin

Uromodulin was isolated from urine of three pregnant females. Approximately 2 l urine of each donor was collected at the beginning of each month, ranging from the fourth month of the pregnancy to one month after gestation (donor A), or from the sixth month of the pregnancy to one month after gestation (donors B and C). Uromodulin was isolated and purified using diatomaceous earth filters as described [22]. The total amount of uromodulin in each batch was determined by UV absorbance ($\epsilon_{280\text{ nm}} = 1.51$).

Enzymatic release and isolation of N-glycans of uromodulin

The N-linked carbohydrate chains were enzymatically released from uromodulin according to a slightly modified version of a previously described protocol [14]. Batches containing approximately 80–90 mg glycoprotein were each dissolved in 8 ml H₂O. After addition of 0.8 ml 10% β -mercaptoethanol and 1.6 ml 10% SDS, the solution was boiled for 3 min. After cooling to room temperature, 8 ml 7.1% octyl α -D-glucopyranoside in 50 mM Tris-HCl, pH 7.1, containing 50 mM EDTA, was added, and the solution was incubated with 24 U PNGase-F for 24 h at room temperature. Then, the mixture was boiled for 3 min, cooled down to room temperature, and incubated again with 24 U of PNGase-F for 24 h. After boiling for 3 min, the N-deglycosylation was checked by SDS-PAGE on a 10% slabgel with Coomassie Brilliant Blue staining. Each batch was fractionated on a Superdex 75 column (60 $\times$ 2.6 cm, Pharmacia), eluted with 100 mM NH₄HCO₃, pH 7.0, at a flow rate of 4 ml min⁻¹. The effluent was monitored at 206 nm (Uvicord, LKB). Carbohydrate-positive fractions (orcinol/H₂SO₄) and fractions containing the N-deglycosylated protein were each pooled, lyophilized, desalted by HiTrap

(Pharmacia FPLC system; 4 columns connected, 4 $\times$ 5 ml; effluent, 5 mM NH₄HCO₃; flow rate, 3 ml min⁻¹; detection, 214 nm), and lyophilized. The N-deglycosylation of the various uromodulin samples was complete as was confirmed by monosaccharide analysis of the N-deglycosylated proteins. The total amount of released carbohydrate was determined by a phenol/H₂SO₄ assay [23].

Profiling of the enzymatically released carbohydrate chains by high-pH anion-exchange chromatography (HPAEC)

Small aliquots of the enzymatically released N-glycans of each batch of uromodulin were analyzed for oligosaccharide components by HPAEC, using a Dionex DX500 chromatography system, equipped with a CarboPac PA-100 column (4.6 $\times$ 250 mm, Dionex) [24,25]. Oligosaccharides were fractionated using a concentration gradient of NaOAc in 0.1 M NaOH as indicated in the figures, at a flow rate of 1 ml min⁻¹. Pulsed amperometric detection was performed using the following pulse potentials and durations: $E_1 = 0.05$ V (400 ms); $E_2 = 0.75$ V (200 ms); $E_3 = -0.15$ V (400 ms).

Results

Uromodulin was isolated from monthly collected urine of three pregnant women in periods ranging from the fourth month (donor A, A^{4th}) and sixth month (donor B, B^{6th}; donor C, C^{6th}) of pregnancy to one month after gestation (A^{AP}, B^{AP}, C^{AP}), affording 17 batches (A^{4th}–A^{9th} and A^{AP}, B^{6th}–B^{9th} and B^{AP}, and C^{6th}–C^{9th} and C^{AP}). All batches of isolated uromodulin displayed a single band on SDS-PAGE with an apparent molecular mass of 94 kDa; their purity was checked by N-terminal amino acid sequence analysis [1,2]. Each batch of uromodulin was N-deglycosylated by PNGase-F, the digests were fractionated by gel permeation chromatography, and the carbohydrate-containing fractions from each batch were pooled and quantified. The pools of enzymatically released N-glycans of each batch of uromodulin were profiled by HPAEC on CarboPac PA-100 [24,25], and the profiles of the batches A^{4th}–A^{9th} and A^{AP}, B^{6th}–B^{9th} and B^{AP}, and C^{6th}–C^{9th} and C^{AP} are displayed in Figures 1–3, respectively. The assignment of the HPAEC regions differing in negative charge, i.e. the charge fractions Q0–Q6, is based on a sequential anion-exchange chromatography approach applied to fraction A^{4th} [Resource Q (1 ml, Pharmacia) followed by CarboPac PA-100 (4.6 $\times$ 250 mm, Dionex); data not shown]. Charge fraction Q0 reflects the region of neutral N-glycans in the chromatogram and charge fractions Q1–Q6 reflect regions where N-glycans appear with 1–6 negative charges, respectively. Hereby, one negative charge is defined as one sialic acid equivalent, whereas a single sulfate group behaves as two sialic acid equivalents.

The relative amounts (%) of the N-glycans in the charge fractions Q0–Q6, reflected by the areas under the curves in the specific regions [25], are displayed in Figures 4a–c for the

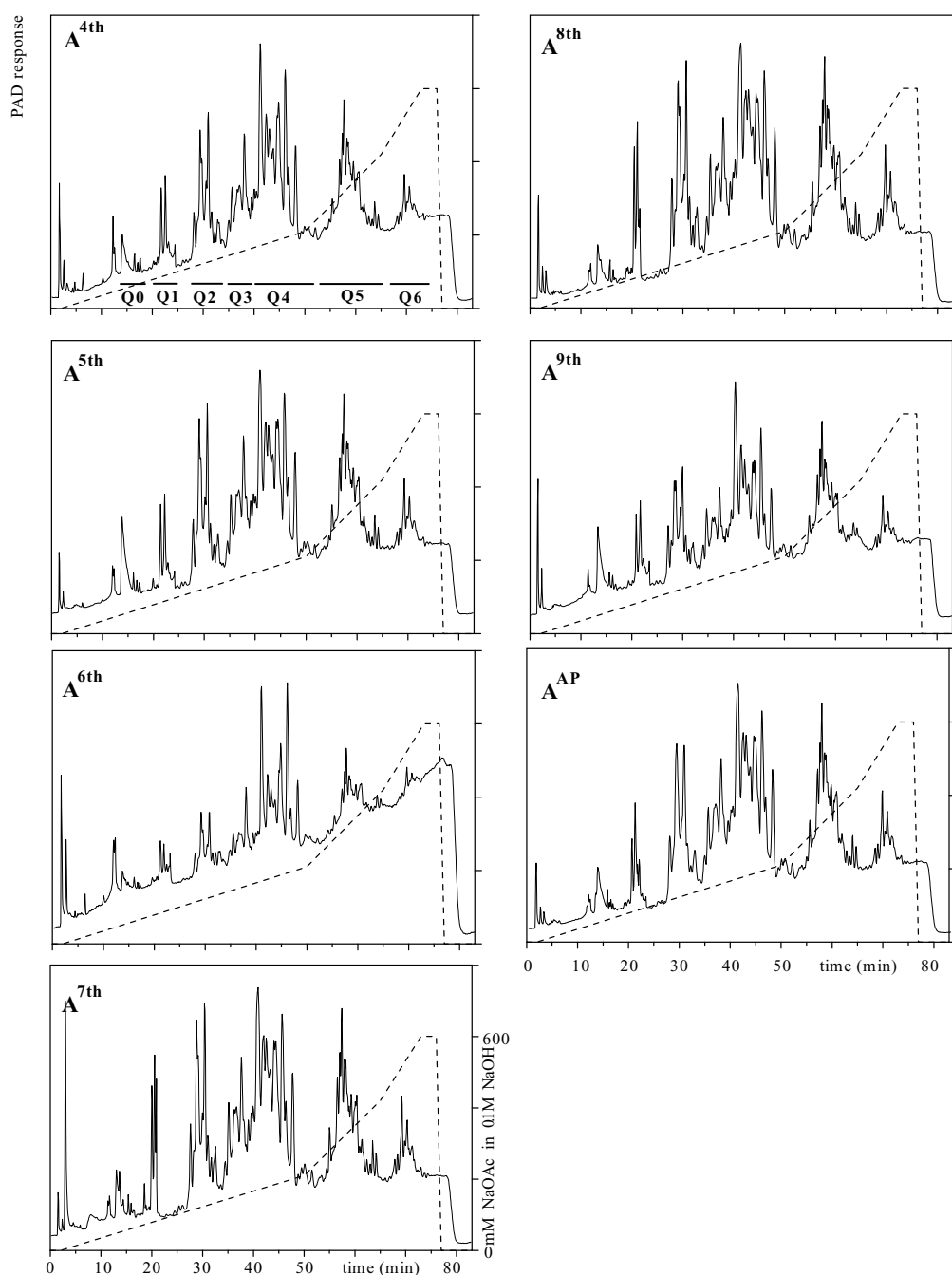


Figure 1. Elution profiles of uromodulin oligosaccharide fractions A^{4th}–A^{9th} and A^{AP} on CarboPac PA-100. Elutions were carried out with a concentration gradient of NaOAc in 0.1 M NaOH as indicated in the figures, at a flow rate of 1.0 ml min^{−1} with pulsed amperometric detection. Q0 to Q6 represent the charge fractions present in the carbohydrate pool of uromodulin, wherein one charge is defined as one sialic acid equivalent (N-glycans displaying no charge, Q0; N-glycans displaying one charge, Q1; etc.). The boundaries of the charge fractions were determined as follows: Separation of an aliquot of fraction A^{4th} on Resource Q followed by fractionation of the FPLC fractions on CarboPac PA-100 (data not shown).

samples of donors A, B, and C, respectively. Charge fraction Q4 is the main fraction containing structures with four charges, including tetrasialotetraantennary N-glycans and monosulfated disialotriantennary N-glycans [15].

To discriminate between batches of uromodulin of the same donor, a “hypothetical N-glycan charge” parameter, the charge number, earlier introduced for controlling lot-to-lot consistency of recombinant glycoprotein therapeutics [25], has been used.

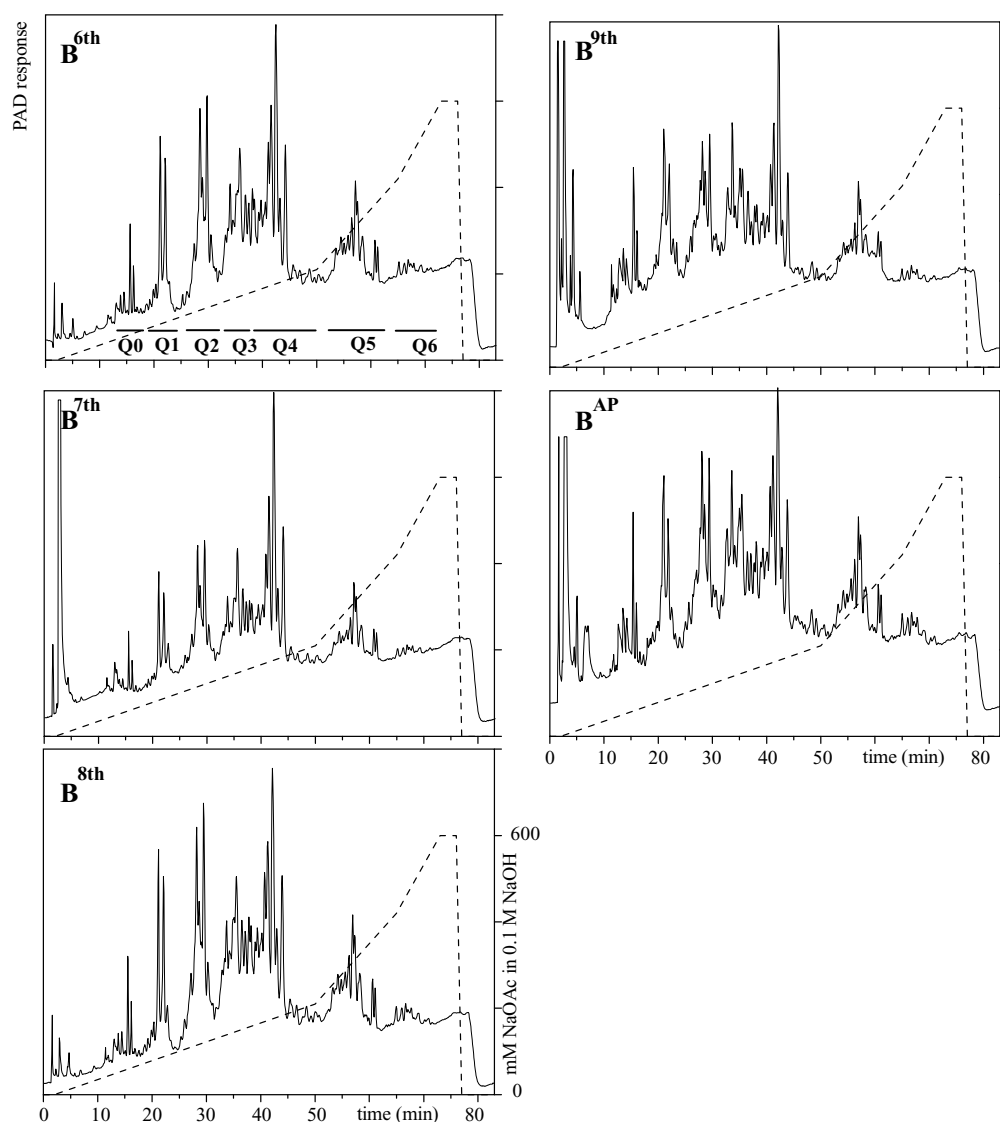


Figure 2. Elution profiles of uromodulin oligosaccharide fractions B^{6th}–B^{9th} and B^{AP} on CarboPac PA-100. For details, see Figure 1.

This parameter is defined as the sum of the percentages of the different charge fractions, each multiplied by the corresponding charge. In fact, the charge number describes the glycosylation status of a glycoprotein in terms of the distribution of charged groups over a chromatogram (here Neu5Ac and sulfate). The charge numbers of the batches A^{4th}–A^{9th} and A^{AP}, B^{6th}–B^{9th} and B^{AP}, and C^{6th}–C^{9th} and C^{AP} are depicted in Figures 4d–f, respectively.

Charge fraction Q0 contains oligomannose-type structures [13]. Using the oligomannose-type N-glycans obtained from RNase-B as standards, only Man₆GlcNAc₂ and Man₇GlcNAc₂ could be unambiguously identified in the HPAEC profiles of all donors. Man₅GlcNAc₂ could not be identified due to coelution with neutral complex-type structures. In the case of Man₈GlcNAc₂ the amount of material was too low for identification. The relative molar ratio (%) between Man₆GlcNAc₂

and Man₇GlcNAc₂, as calculated by integration, is presented in Table 1. Additionally, the relative molar amounts of Man₆GlcNAc₂ and Man₇GlcNAc₂ versus the total carbohydrate content were calculated.

Discussion

As is evident from Figures 1–3, the HPAEC profiles of the uromodulin-derived carbohydrate pools of donors A, B, and C show a very complex pattern of N-glycans. The profiles exhibit a large similarity to that obtained from THp of a male donor [13], except for the presence of charge fraction Q7 in male THp. The HPAEC profiles comprise di-, tri- and tetraantennary structures, containing GalNAc4S, Gal3S, Neu5Ac (including the Sd^a determinant) and/or Gal as terminal groups [14–16]. Only a small amount of the complex-type oligosaccharides of THp

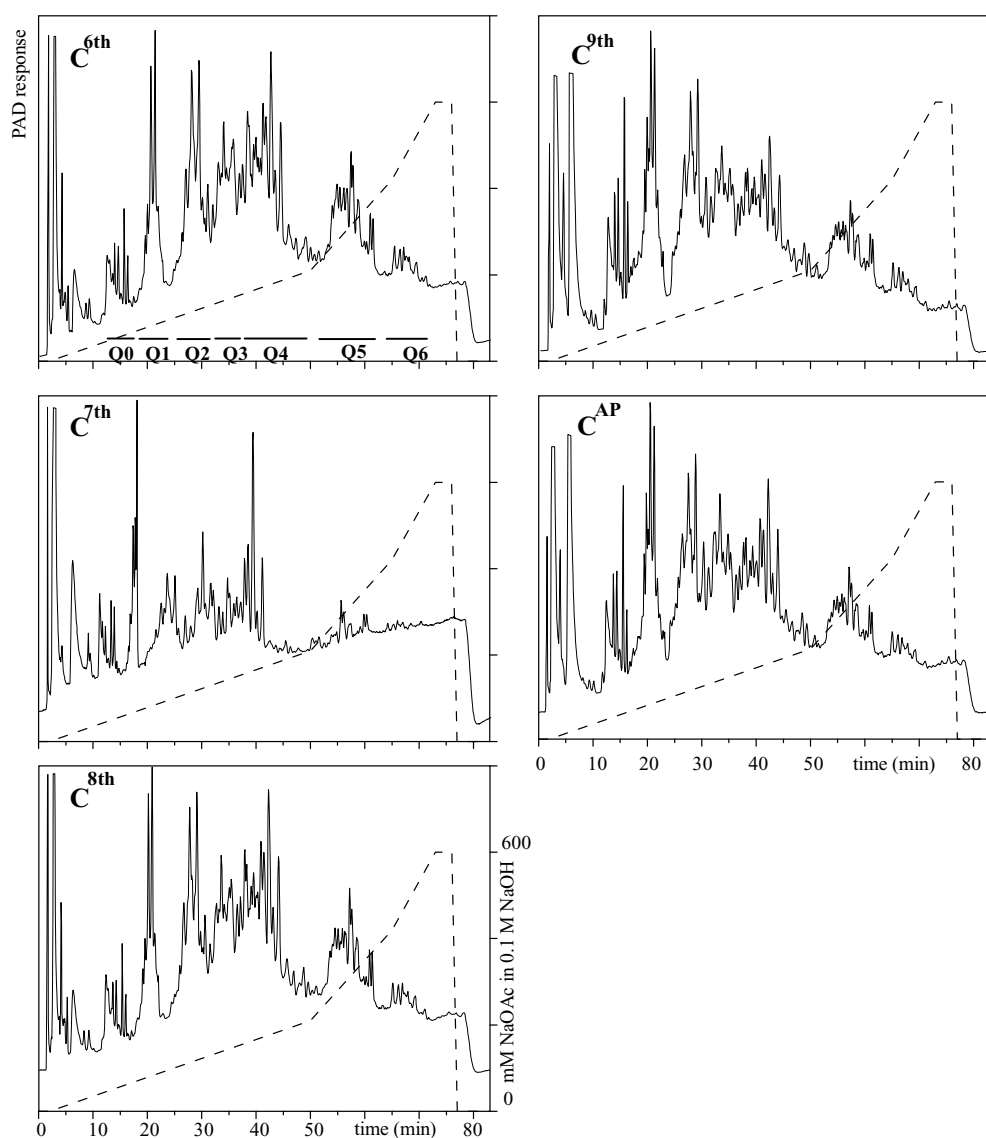


Figure 3. Elution profiles of uromodulin oligosaccharide fractions C^{6th} – C^{9th} and C^{AP} on CarboPac PA-100. For details, see Figure 1.

display non-substituted terminal Gal residues, indicating an almost complete capping of the $\text{Gal}(\beta 1-4)\text{GlcNAc}(\beta 1-)$ element by Neu5Ac or sulfate [14,15]. Changes in the Neu5Ac/sulfate distribution in the oligosaccharides of uromodulin in the course of pregnancy should become visible in the relative percentages of the charge fractions. The results indicate that for each donor (A, B or C) the relative amounts of the charge fractions Q0–Q6 of the uromodulin samples from the various periods, including that collected at one month after gestation, did not change significantly (Figures 4a–c). This is also reflected by the charge numbers of these samples per donor (Figures 4d–f). Therefore, it is likely that no changes occur in the Neu5Ac/sulfate distribution in the N-glycans of uromodulin in the course of pregnancy.

Several examples have been reported of human glycoproteins displaying changes in their N-glycan moieties as pregnancy

proceeds. Especially, increased branching of complex-type structures has been mentioned for serum transferrin, serum transcortin, amniotic fluid fibronectin, α_1 -acid glycoprotein, and chorionic gonadotropin α subunit [26–30]. These changes in carbohydrate chains can result in an enhanced protection against proteolytic attack or changed binding properties of the glycoproteins. Both features can be favorable during pregnancy. An increase in branching would generate the possibility for the attachment of an extra sialic acid residue or a sulfate group (q), which will cause an increase of the relative amount of one charge fraction at the cost of another charge fraction ($Q_x \rightarrow Q_x + q$). Since no significant shifts in any of the charge fractions were observed during pregnancy (Figures 4a–c), and taking into account the low amount of oligosaccharides containing terminal $\text{Gal}(\beta 1-4)\text{GlcNAc}(\beta 1-)$ elements [14], it

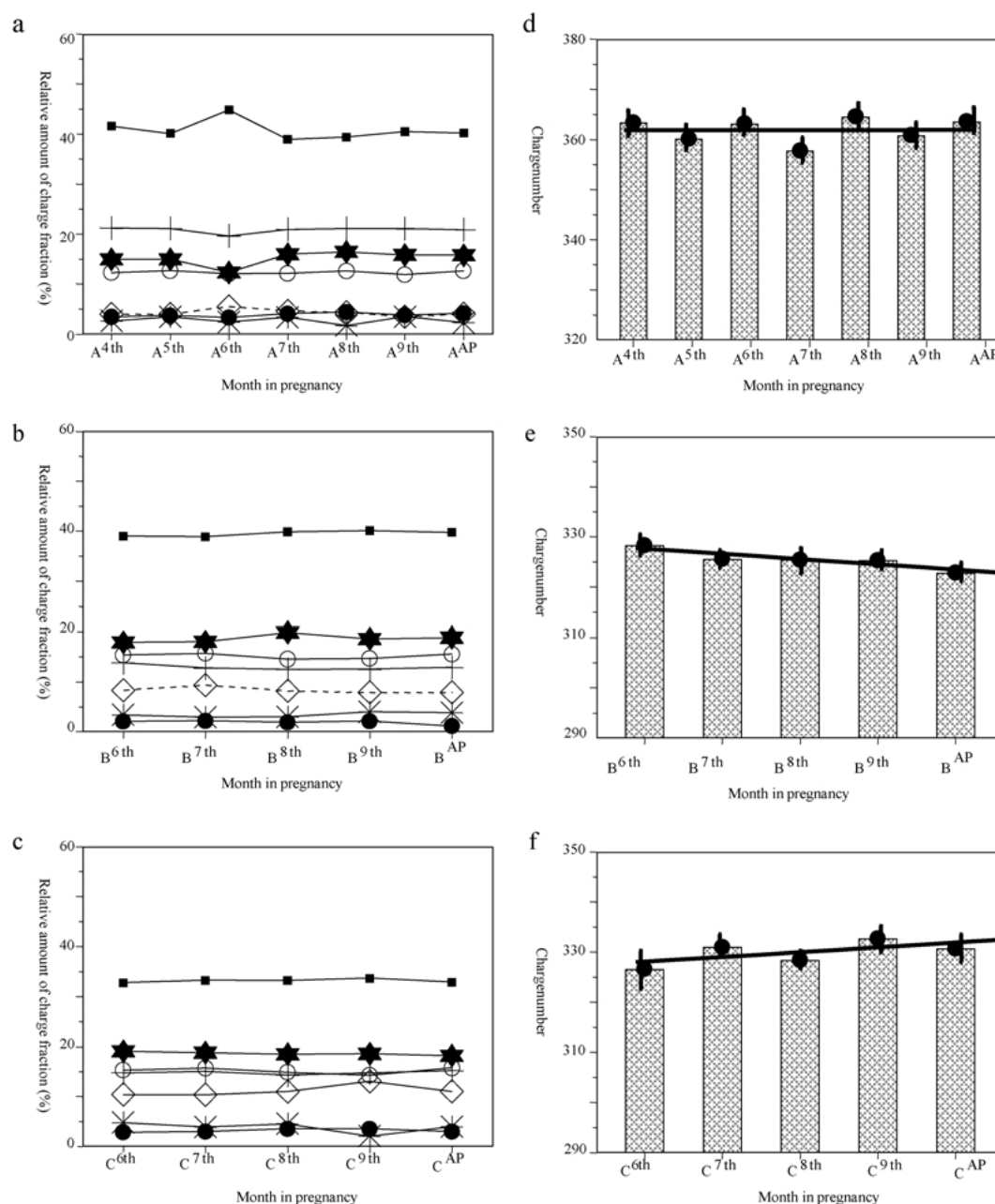


Figure 4. Relative amount (%) of charge fractions Q0 to Q6, and the charge numbers of uromodulin glycan samples of donors A, B, and C during pregnancy. The relative amount (%) of the charge fractions as well as the charge numbers are calculated from the profiles of the oligosaccharide pools of donors A, B, and C, displayed in Figures 1–3, respectively, as described [25]. The numbers of the charge fractions correspond to the following symbols: Q0, ✱; Q1, ◇; Q2, ★; Q3, ○; Q4, ■; Q5, +; Q6, ●. (a) relative amount of charge fractions of uromodulin glycan samples A^{4th}–A^{9th} and A^{AP} of donor A; (b) relative amount of charge fractions of uromodulin glycan samples B^{6th}–B^{9th} and B^{AP} of donor B; (c) relative amount of charge fractions of uromodulin glycan samples C^{6th}–C^{9th} and C^{AP} of donor C; (d) charge numbers of uromodulin glycan samples A^{4th}–A^{9th} and A^{AP} of donor A; (e) charge numbers of uromodulin glycan samples B^{6th}–B^{9th} and B^{AP} of donor B; (f) charge numbers of uromodulin glycan samples C^{6th}–C^{9th} and C^{AP} of donor C.

is suggested that no changes in the branching pattern of the complex-type carbohydrate chains of uromodulin occur in the course of pregnancy. It should be noted that in contrast to the glycoproteins mentioned above, uromodulin is a kidney-derived

glycoprotein, which may explain the deviating behaviour with respect to branching.

All three donors display, relative to the total molar amount of N-glycan structures, comparable low molar amounts of

Table 1. The molar ratio (%) between Man₆GlcNAc₂ and Man₇GlcNAc₂ of uromodulin glycan samples of donors A, B, and C in the course of the pregnancy. Man₆GlcNAc₂ and Man₇GlcNAc₂ were identified in the HPAEC profiles of the oligosaccharide pools of donors A (Figure 1), B (Figure 2), and C (Figure 3), using oligomannose standards obtained from RNase-B. The ratio (%) between Man₆GlcNAc₂ and Man₇GlcNAc₂ was calculated by integration

	Man ₆ GlcNAc ₂	Man ₇ GlcNAc ₂
A ^{4th}	64	36
A ^{5th}	60	40
A ^{6th}	63	37
A ^{7th}	61	39
A ^{8th}	62	38
A ^{9th}	60	40
A ^{AP}	65	35
B ^{6th}	65	35
B ^{7th}	66	34
B ^{8th}	65	35
B ^{9th}	67	33
B ^{AP}	71	29
C ^{6th}	66	34
C ^{7th}	64	36
C ^{8th}	68	32
C ^{9th}	70	30
C ^{AP}	74	26
Uro ¹ _{preg}	63	37
THP ¹ _{nonpreg}	72	28

¹ Taken from Ref. 20.

oligomannose-type structures (Donor A: ~0.3%; donor B: ~1.8%; and donor C: ~1.0%), so the donor specificity is here not as diverse as reported earlier (c.f. [13,14,17–19]). As can be concluded from the data in Table 1, the molar ratio between Man₆GlcNAc₂ and Man₇GlcNAc₂ does not significantly change in the course of pregnancy. However, one month after the pregnancy, the molar amount of Man₇GlcNAc₂ slightly decreases, relative to the molar amount of Man₆GlcNAc₂ (Donor A: A^{4th}–A^{9th}, mean 38%, A^{AP}, 35%; Donor B: B^{6th}–B^{9th}, mean 34%, B^{AP}, 29%; Donor C: C^{6th}–C^{9th}, mean 33%, C^{AP}, 28%), a finding in agreement with earlier data (Uro_{preg}, 37%; THP_{nonpreg}, 28%) [20].

Summarizing, during pregnancy none of the donors exhibited significant changes in the charge numbers of the batches of the uromodulin N-glycans. This observation implies that no significant changes in the negative charge distribution stemming from sialic acid and sulfate residues occur; it suggests that the branching patterns of the complex-type carbohydrate chains remain fairly constant. The composition of the oligomannose-type glycans does not undergo significant alterations during pregnancy. However, comparison of uromodulin samples from pregnant and non-pregnant periods of each donor indicated small shifts in the molar ratio of the various oligomannose-type glycans.

References

- Hession C, Decker JM, Sherblom AP, Kumar S, Yue CC, Mattaliano RJ, Tizard R, Kawashima E, Schmeissner U, Heletky S, Chow EP, Burne CA, Shaw A, Muchmore AV, Uromodulin (Tamm-Horsfall glycoprotein): A renal ligand for lymphokines, *Science* **237**, 1479–84 (1987).
- Pennica D, Kohr WJ, Kuang W-J, Glaister D, Aggarwal BB, Chen EY, Goeddel DV, Identification of human uromodulin as the Tamm-Horsfall glycoprotein, *Science* **236**, 83–8 (1987).
- Muchmore AV, Decker JM, Uromodulin: A unique 85-kilodalton immunosuppressive glycoprotein isolated from urine of pregnant women, *Science* **229**, 479–81 (1985).
- Horton JK, Davies M, Topley N, Thomas D, Williams JD, Activation of the inflammatory response of neutrophils by Tamm-Horsfall glycoprotein, *Kidney Int* **37**, 717–26 (1990).
- Yu CL, Lin WM, Liao TS, Tsai CY, Sun KH, Chen KH, Tamm-Horsfall glycoprotein (THG) purified from normal human pregnancy urine increases phagocytosis, complement receptor expression and arachidonic acid metabolism of polymorphonuclear neutrophils, *Immunopharmacology* **24**, 181–90 (1992).
- Thomas DBL, Davies M, Peters JR, Williams JD, Tamm-Horsfall protein bind to a single class of carbohydrate specific receptors on human neutrophils, *Kidney Int* **44**, 423–9 (1993).
- Thomas DBL, Davies M, Williams JD, Release of gelatinase and superoxide from human mononuclear phagocytes in response to particulate Tamm Horsfall protein, *Am J Pathol* **142**, 249–60 (1993).
- Yu CL, Tsai CY, Lin WM, Liao TS, Chen HL, Sun KH, Chen KH, Tamm-Horsfall urinary glycoprotein enhances monokine release and augments lymphocyte proliferation, *Immunopharmacology* **26**, 249–58 (1993).
- Toma GJ, Bates Jr JM, Kumar S, Uromodulin (Tamm-Horsfall glycoprotein) is a leukocyte adhesion molecule, *Biochem Biophys Res Commun* **200**, 275–82 (1994).
- Muchmore AV, Shifrin S, Decker JM, *In vitro* evidence that carbohydrate moieties derived from uromodulin, an 85,000 dalton immunosuppressive glycoprotein isolated from human pregnancy urine, are immunosuppressive in the absence of intact protein, *J Immunol* **138**, 2547–53 (1987).
- Muchmore AV, Sathyamoorthy N, Decker JM, Sherblom AP, Evidence that specific high-mannose oligosaccharides can directly inhibit antigen-driven T-cell responses, *J Leukoc Biol* **48**, 457–64 (1990).
- Dall'Olio F, Chiricolo M, Malagolini N, Franceschi C, Serafini-Cessi F, Immunosuppressive activity of Tamm-Horsfall glycoprotein oligosaccharides: Effect of removal of outer sugars and conjugation with a protein carrier, *Cell Immunol* **137**, 303–15 (1991).
- Van Rooijen JJM, Voskamp AF, Kamerling JP, Vliegthart JFG, Glycosylation sites and site-specific glycosylation in Tamm-Horsfall glycoprotein, *Glycobiology* **9**, 21–30 (1999).
- Hård K, van Zadelhoff G, Moonen P, Kamerling JP, Vliegthart JFG, The Asn-linked carbohydrate chains of human Tamm-Horsfall glycoprotein of one male. Novel sulfated and novel N-acetylgalactosamine-containing N-linked carbohydrate chains, *Eur J Biochem* **209**, 895–915 (1992).
- Van Rooijen JJM, Kamerling JP, Vliegthart JFG, Sulfated di-, tri- and tetraantennary N-glycans in human Tamm-Horsfall glycoprotein, *Eur J Biochem* **256**, 471–87 (1998).

- 16 Van Rooijen JJM, Kamerling JP, Vliegthart JFG, The abundance of additional *N*-acetylglucosamine units in N-linked tetraantennary oligosaccharides of human Tamm-Horsfall glycoprotein is a donor-specific feature, *Glycobiology* **8**, 1065–75 (1998).
- 17 Serafini-Cessi F, Dall'Olio F, Malagolini N, High-mannose oligosaccharides from human Tamm-Horsfall glycoprotein, *Biosci Rep* **4**, 269–74 (1984).
- 18 Dall'Olio F, de Kanter FJJ, van den Eijnden DH, Serafini-Cessi F, Structural analysis of the preponderant high-mannose oligosaccharides of human Tamm-Horsfall glycoprotein, *Carbohydr Res* **178**, 327–32 (1988).
- 19 Afonso A-MM, Charlwood PA, Marshall RD, Isolation and characterisation of glycopeptides from digests of human Tamm-Horsfall glycoprotein, *Carbohydr Res* **89**, 309–19 (1981).
- 20 Smagula RM, van Halbeek H, Decker JM, Muchmore AV, Moody CE, Sherblom AP, Pregnancy-associated changes in oligomannose oligosaccharides of human and bovine uromodulin (Tamm-Horsfall glycoprotein), *Glycoconjugate J* **7**, 609–24 (1990).
- 21 Easton RL, Patankar MS, Clark GF, Morris HR, Dell A, Pregnancy-associated changes in the glycosylation of Tamm-Horsfall glycoprotein; expression of sialyl Lewis^x sequences on core 2 type *O*-glycans derived from uromodulin, *J Biol Chem* **275**, 21928–38 (2000).
- 22 Serafini-Cessi F, Bellabarba G, Malagolini N, Dall'Olio F, Rapid isolation of Tamm-Horsfall glycoprotein (uromodulin) from human urine, *J Immunol Methods* **120**, 185–9 (1989).
- 23 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F, Colorimetric method for determination of sugars and related substances, *Anal Biochem* **28**, 350–6 (1956).
- 24 Hermentin P, Witzel R, Vliegthart JFG, Kamerling JP, Nimtz M, Conradt HS, A strategy for the mapping of N-glycans by high-pH anion-exchange chromatography with pulsed amperometric detection, *Anal Biochem* **203**, 281–9 (1992).
- 25 Hermentin P, Witzel R, Kanzy E-J, Diderrich G, Hoffmann D, Metzner H, Vorlop J, Haupt H, The hypothetical N-glycan charge: A number that characterizes protein glycosylation, *Glycobiology* **6**, 217–30 (1996).
- 26 Zhu BC, Fisher SF, Pande H, Calaycay J, Shively JE, Laine RA, Human placental (fetal) fibronectin: Increased glycosylation and higher protease resistance than plasma fibronectin. Presence of polylactosamine glycopeptides and properties of a 44-kilodalton chymotryptic collagen-binding domain: Difference from human plasma fibronectin, *J Biol Chem* **259**, 3962–70 (1984).
- 27 Avvakumov GV, Strel'chyonok OA, Properties of serum levels of pregnancy-associated variant of human transcortin, *Biochim Biophys Acta* **925**, 11–6 (1987).
- 28 Leger D, Campion B, Decottignies JP, Montreuil J, Spik G, Physiological significance of the marked increased branching of the glycans of human serotransferrin during pregnancy, *Biochem J* **257**, 231–8 (1989).
- 29 Biou D, Bauvy C, N'Guyen H, Codogno P, Durand G, Aubery M, Alterations of the glycan moiety of human α_1 -acid glycoprotein in late-term pregnancy, *Clin Chim Acta* **204**, 1–12 (1991).
- 30 Blithe DL, Iles RK, The role of glycosylation in regulating the glycoprotein hormone free α -subunit and free β -subunit combination in the extraembryonic coelomic fluid of early pregnancy, *Endocrinology* **136**, 903–10 (1995).

Received 5 March 2002; revised 19 April 2002;
accepted 19 April 2002