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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 $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ 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 ^{1}H NMR spectroscopy have resulted in the elucidation of 63 complex-type [14–16] and 4 oligomannose-type N-glycans ($Man_{5\text{-}8}GlcNAc_2$) [12,13]. Di-, tri-, and, most of all, tetraantennary structures (including dimeric *N*-acetyllactosamine sequences) are present, which can be fucosylated, sialylated (including the Sd^a determinant) and/or sulfated (GalNAc4*S* and Gal3*S*). 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*-acetyllactosamine 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 $Man_5GlcNAc_2$, $Man_6GlcNAc_2$ and

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$Man_7GlcNAc_2$ (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 ($\varepsilon_{280\ nm} = 1.51$).

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 H_2O. 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 × 2.6 cm, Pharmacia), eluted with 100 mM NH_4HCO_3, 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/H_2SO_4) and fractions containing the N-deglycosylated protein were each pooled, lyophilized, desalted by HiTrap (Pharmacia FPLC system; 4 columns connected, 4 × 5 ml; effluent, 5 mM NH_4HCO_3; 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/H_2SO_4 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 × 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 × 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

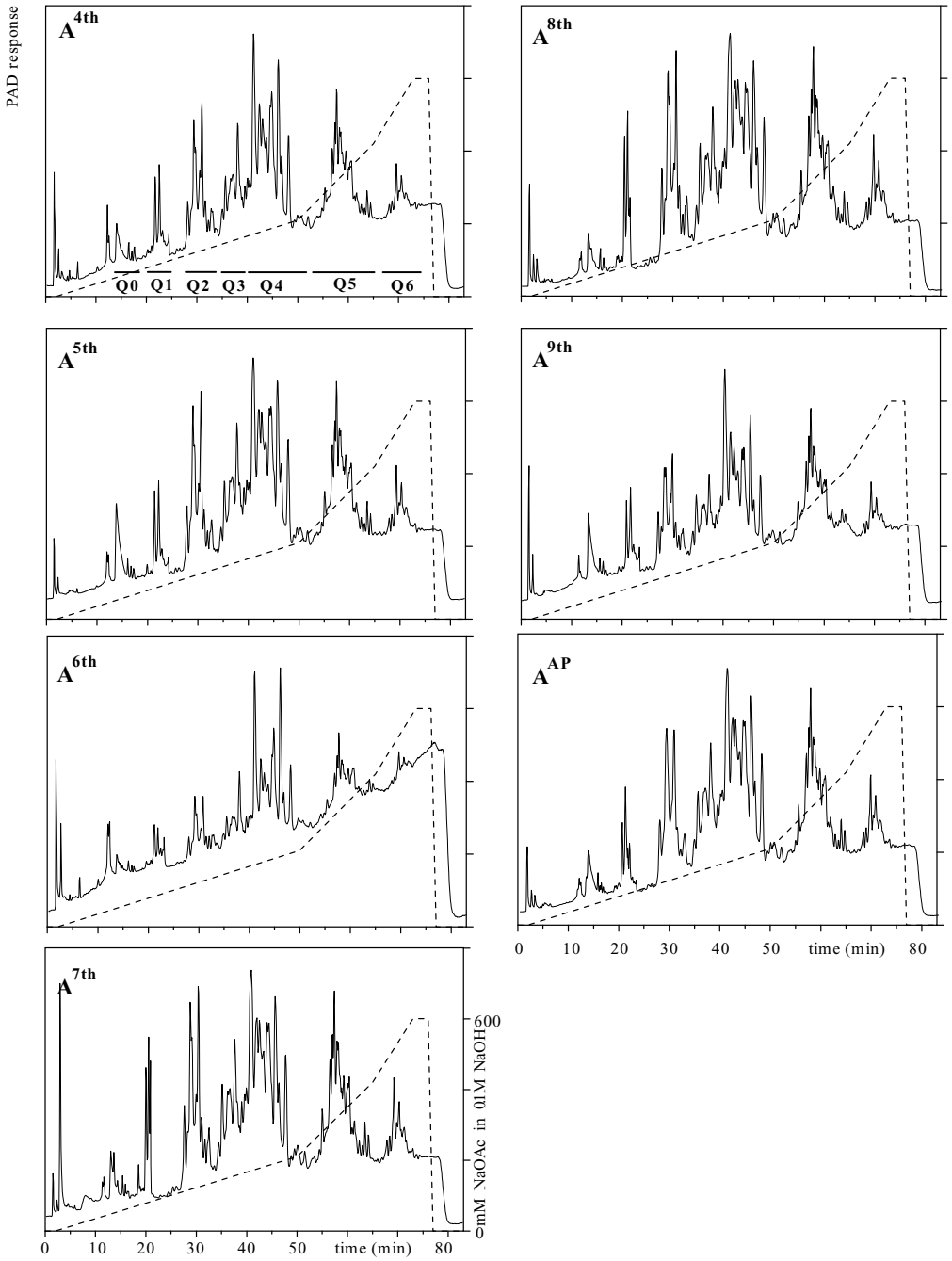

Figure 1. Elution profiles of uromodulin oligosaccharide fractions A^{4th}–A^{9th} and A^{AP} on CarboPac PA-100. Elutions were carried out with a concentration gradient of NaOAc in 0.1 M NaOH as indicated in the figures, at a flow rate of 1.0 ml min^{-1} with pulsed amperometric detection. Q0 to Q6 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.

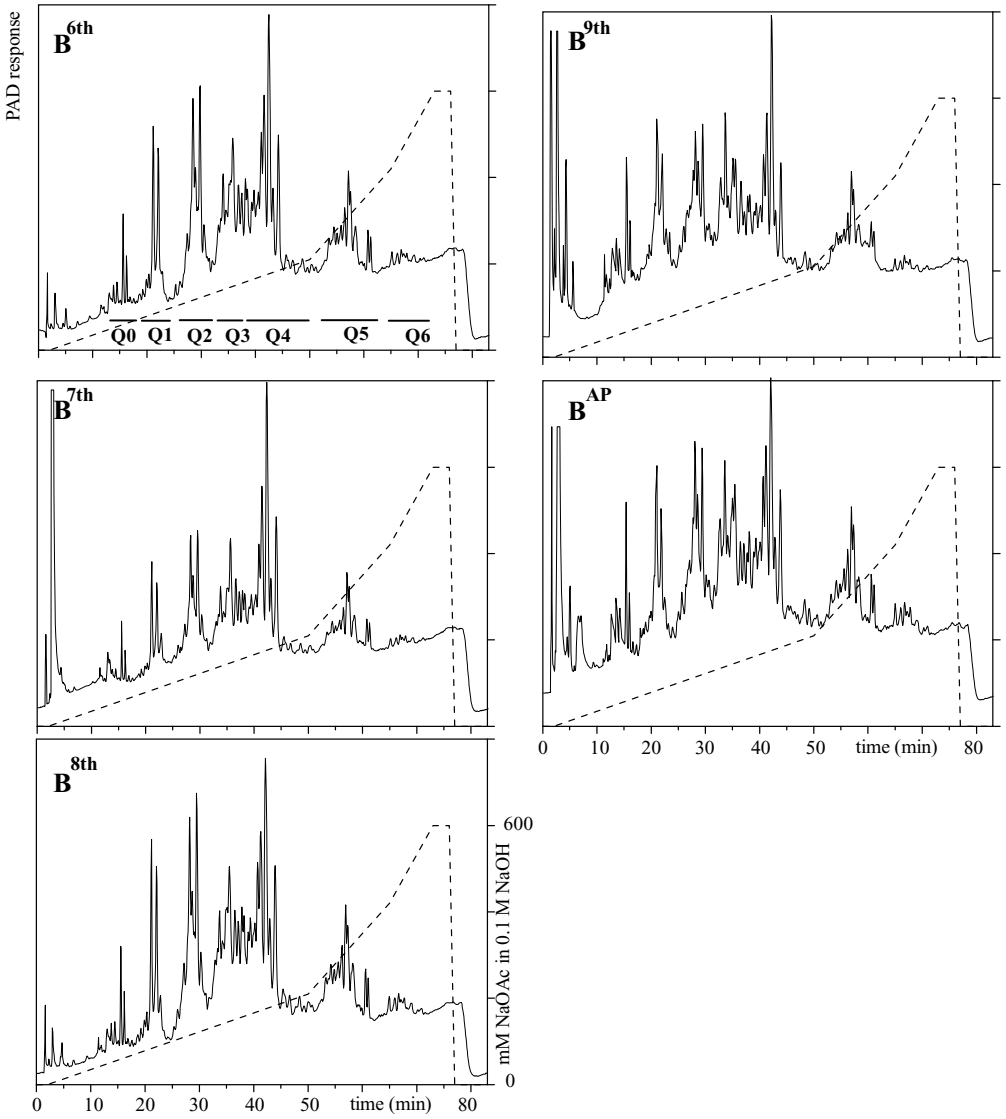

Figure 2. Elution profiles of uromodulin oligosaccharide fractions B^{6th}–B^{9th} and B^{AP} on CarboPac PA-100. For details, see Figure 1.

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 $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ could be unambiguously identified in the HPAEC profiles of all donors. $Man_5GlcNAc_2$ could not be identified due to coelution with neutral complex-type structures. In the case of $Man_8GlcNAc_2$ the amount of material was too low for identification. The relative molar ratio (%) between $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$, as calculated by integration, is presented in Table 1. Additionally, the relative molar amounts of $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ 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 GalNAc4*S*, Gal3*S*, 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

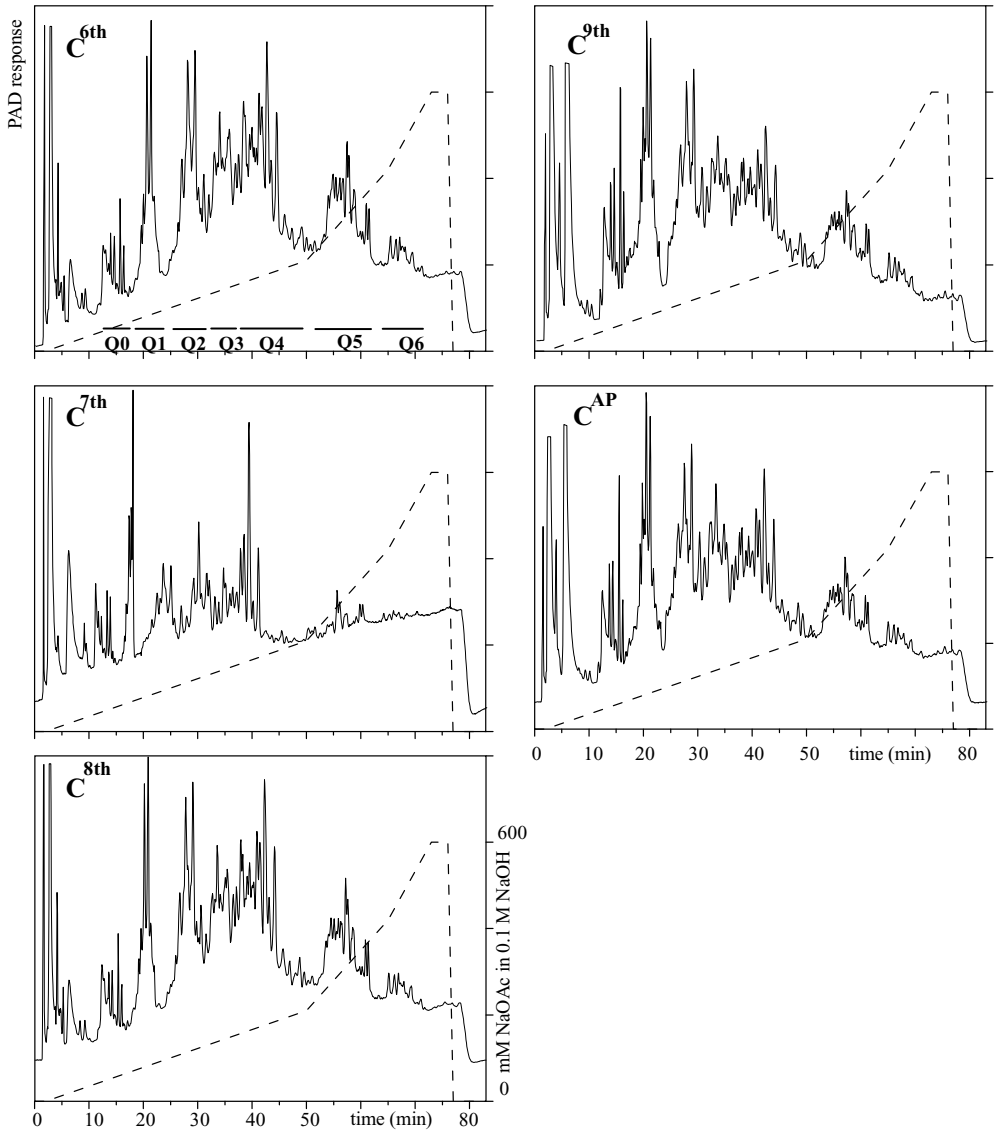

Figure 3. Elution profiles of uromodulin oligosaccharide fractions C^{6th}–C^{9th} and C^{AP} on CarboPac PA-100. For details, see Figure 1.

display non-substituted terminal Gal residues, indicating an almost complete capping of the Gal(β1-4)GlcNAc(β1-) 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 ($Qx \rightarrow Qx + q$). 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 Gal(β1-4)GlcNAc(β1-) elements [14], it

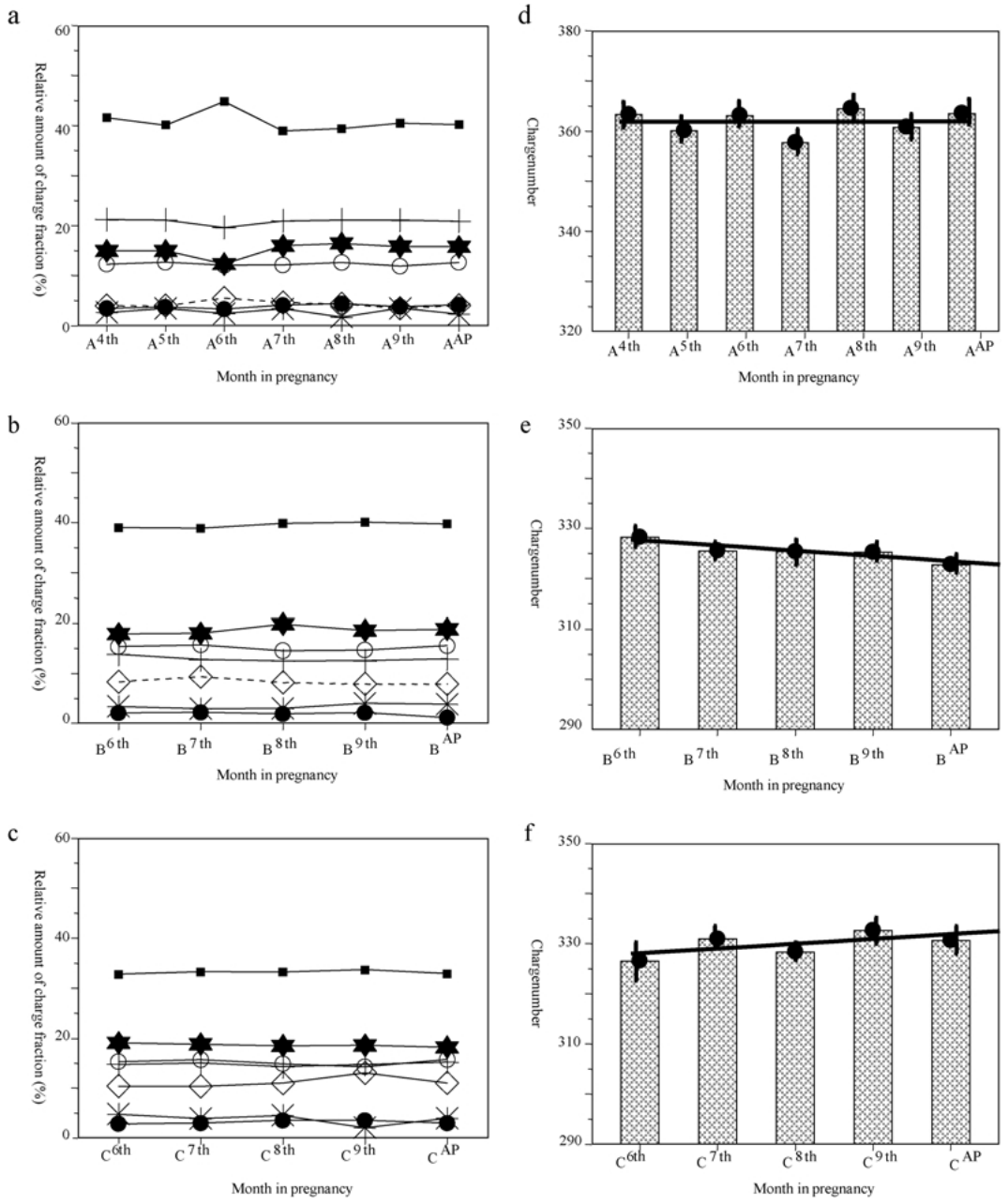

Figure 4. Relative amount (%) of charge fractions Q0 to Q6, and the charge numbers of uromodulin glycan samples of donors A, B, and C during pregnancy. The relative amount (%) of the charge fractions as well as the charge numbers are calculated from the profiles of the oligosaccharide pools of donors 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 $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ of uromodulin glycan samples of donors A, B, and C in the course of the pregnancy. $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ 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 $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ was calculated by integration

	$Man_6GlcNAc_2$	*$Man_7GlcNAc_2$*
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 $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ does not significantly change in the course of pregnancy. However, one month after the pregnancy, the molar amount of $Man_7GlcNAc_2$ slightly decreases, relative to the molar amount of $Man_6GlcNAc_2$ (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.

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