Glycoconj J (2015) 32:635–641
DOI 10.1007/s10719-015-9610-x

ORIGINAL ARTICLE

Urine oligosaccharide pattern in patients with hyperprolactinaemia

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Received: 13 April 2015 / Revised: 15 June 2015 / Accepted: 9 July 2015 / Published online: 15 August 2015

Abstract Free milk-type oligosaccharides are produced during pregnancy and lactation and may have an impact on several cells in the immune system. Our aim was to investigate if patients with isolated hyperprolactinaemia, not related to pregnancy, also have increased synthesis and urinary excretion of milk-type oligosaccharides and to compare the excretion pattern with that found during pregnancy. Urine samples were collected as morning sample from 18 patients with hyperprolactinaemia, 13 healthy controls with normal prolactin levels and four pregnant women. After purification, lactose and free oligosaccharides were analysed and quantified by high-performance anion-exchange chromatography with pulsed amperometric detection. The identity of peaks was confirmed by exoglycosidase treatment and comparison with oligosaccharide standards. Prolactin was measured in serum collected between 09 and 11 a.m. by a standardized immunochemical method. Patients with hyperprolactinaemia had higher urinary excretion of lactose than normoprolactinemic controls and urinary lactose correlated positively to prolactin levels (r=0.51, p<0.05). Increased levels of the fucosylated oligosaccharides 2-fucosyl lactose and lacto-di-fucotetraose were found in urine from three and two patients, respectively. The acidic oligosaccharide 3-sialyl lactose was found in high amount in urine from two patients with prolactin of >10, 000 mU/l. However, pregnant women in their third trimester had the highest concentration of all these oligosaccharides and excretion increased during pregnancy. This study is first to show that both lactose and certain fucosylated and sialylated milk-type oligosaccharides are increased in some patients with hyperprolactinaemia. It remains to elucidate the functional importance of these findings.

Keywords Prolactin · Prolactinoma · Urine · Oligosaccharides · Lactose

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Introduction

Pituitary secretion of prolactin increases during pregnancy and is vital to the induction of lactation. Lactation starts when progesterone levels decrease during delivery. Prolactin specifically stimulate the synthesis of α-lactalbumin which alters the substrate specificity of β-galactosyltransferase towards glucose, thus stimulating the synthesis of lactose and larger oligosaccharides built on a lactose backbone [1–3]. Though lactose synthesis mainly starts after delivery, increased levels of lactose can be found in serum and urine throughout the whole pregnancy. Prolactin has also been found to increase the expression of β-galactosyltransferase in mammary tissue [4, 5]. It is unclear if prolactin also has these effects in other tissues or if prolactin has a regulatory role for other glycosyltransferases.

Small amounts of lactose and other oligosaccharides can be found in urine in healthy non-pregnant women and in men [6–8]. The origin of these oligosaccharides are however unclear. Some of them are probably derived from degradation, since oligosaccharides with corresponding blood group epitopes have been found in urine [9]. A small proportion of

lactose is probably derived from gastro-intestinal uptake and is to some extent dependent on intake, lactase activity and intestinal permeability [10]. Hyperprolactinaemia not related to pregnancy most commonly indicate a prolactin secreting pituitary adenoma or processes compressing the pituitary stalk [11, 12]. The concentration of prolactin also increases during conditions like stress, inflammation, hypothyroidism, renal failure, and treatment with antipsychotic drugs [12]. Macroprolactinemia is caused by a complex between immunoglobulin and prolactin which may interfere with immunochemical prolactin methods [13]. It is not known if hyperprolacinaemia in these cases is associated to an increased synthesis of lactose and milk-type oligosaccharides. However, galactorrhea is a common finding in patients with hyperprolactinaemia [11, 12]. Increased levels of lactose and milk-type oligosaccharides in blood and urine may have an impact on several cells in the immune system. Cells in the immune system express various glycan binding proteins (*e.g.*, lectins) such as C-type lectins, siglects and galectins. Binding to specific carbohydrate ligands may initiate or inhibit activation or adhesion of leukocytes, dendritic cells, monocytes, platelets and endothelial cells [14, 15]. An increase in free oligosaccharides in blood or alterations of oligosaccharide structures on membrane or plasma proteins may therefore affect these interactions and modulate the immune response. Our aim was to investigate if patients with isolated hyperprolactinaemia have increased synthesis and urinary excretion of milk-type oligosaccharides and to compare the excretion pattern with that found during pregnancy.

Materials and methods

Subjects

Eighteen patients (mean age 37 years, range 23–68, 15 women and 3 men) with hyperprolactinaemia defined as serum prolactin >470 mU/l were consecutively recruited among patients referred to the endocrine unit at Linköping University Hospital (Table 1). Five patients had a visible micro adenoma on MRI (magnetic resonance imaging), two patients had a macroprolactinoma and one patient had still prolactin hypersecretion after transphenoidal extirpation of a macroprolactinoma. In nine patents no adenoma was visible on MRI and of these one had a voluminous pituitary, one an arachnoidal cyst and one a corpus pineale cyst. One patient with macroprolactin as the dominating cause of hyperprolactinaemia also had a normal MRI. None of the patients were on dopamine agonists.

Thirteen healthy control subjects were recruited among laboratory personal and students for comparison (mean age 42 years, range 25–65, 10 women and 3 men). All controls were subjectively healthy and had normal prolactin values (<470 mU/l). Four pregnant women in their third trimester (gestation week 31–37) were included. Samples from one 35 year old pregnant woman were collected at six occasions during pregnancy (gestation week 5–38). All were healthy and their pregnancy was without complications.

Table 1 Anthropometric and basal hormone levels in 18 patients (3 men/15 women) with hyperprolactinaemia

Category	Results
Age (yrs.)	36.3±12.3
Height (cm)	166.7±9.6
Weight (kg)	72.6±19.0
BMI (kg/m^2)	26±6.0
Waist/Hip-ratio	0.85±0.09
Systolic BP supine	124±14
Diastolic BP supine (mm Hg)	76±5
Heart rate supine (beat/min)	68±13
Plasma-Creatinine (μmol/l)	70±8
Urine-Creatinine (mmol/l)	12.6±5.5
TSH (mU/l)	2.9±2.0
Free T4 (pmol/l)	14.8±4.0
Free T3 (pmol/l)	5.4±1.9
Prolactin 08.00 h (mU/l)	1225 (700–16,200)
Prolactin 10.00 h (mU/l)	1000 (620–15,900)

Data shown as mean (SD) except for prolactin levels, which are shown as median (min-max)

BP blood pressure

Blood and urine collection

Serum samples were drawn in to 4 ml Vacuette® Z Serum Sep Clot Activator tubes (Greiner Bio-One, Frickenhauser, Germany) and stored at −20 °C awaiting prolactin and creatinine measurement. All blood samples were collected between 9 and 11 a.m. Urine was collected as the midstream portion after one night fasting as the first morning sample. Within 3 h from the collection, urine was centrifuged at 1800 g for 10 min and transferred to a new vial containing 0.01 % Na N_3 as a preservative. The urine samples were kept frozen at −20 °C until analysis. Urine from the women followed during pregnancy were collected during 24-h in a can containing approximately 0.01 % Na N_3 and were thereafter mixed and transferred to vials stored at −20 °C.

Prolactin assay

Prolactin was analysed on Cobas e602 (Roche Diagnostics, Bromma, Sweden). The method is based on immunochemical detection using two monoclonal prolactin specific antibodies. The calibrator is traceable to the 3rd IRP WHO reference

standard 84/500. Results above 470 mU/l were regarded as hyperprolactinaemia.

All samples were checked for macroprolactin by precipitation with 25 % polyethylene glycol (PEG) v:v 1:1. The samples were centrifuged at 1800 g for 10 min. Prolactin was analysed in the supernatant and the value was multiplied by 2 to correct for dilution. A recovery after PEG precipitation of less than 40 % was considered positive for macroprolactin interference.

Creatinine and thyroid hormone assays

Creatinine was measured on Advia 1800 (Siemens Healthcare Diagnostics, Stockholm, Sweden) with reagents from the same manufacturer. The method was based on the Jaffe reaction. Creatinine in plasma was measured using a standardized method containing a rate-blanking measurement to compensate for interference from bilirubin and a correction for intercept due to pseudocreatinines. The reference intervals were 45–90 μmol/L for women and 60–105 μmol/L for men.

Thyroid stimulating hormone (TSH), free triiodothyronine (T3) and free thyroxine (T4) were measured in serum by an immunochemical method on Advia Centaur (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The reference intervals were 0.4–4.0 mU/l, 3.0–6.5 pmol/l and 9–22 pmol/l, respectively.

Purification of urine samples

The urine samples were thawed at RT, mixed carefully and 20 μl of internal standard was added to 980 μl urine. Stachyose (2 mg/ml, Sigma-Aldrich) was used as internal standard for neutral oligosaccharides and galacturonic acid (1 mg/ml, Sigma-Aldrich) for acidic oligosaccharides. The samples were then ultracentrifuged using Amicon Ultra-4 (Millipore, Billerica, MA, USA) with a 3 kDa molecular cut off. The tubes were centrifuged at 4000 g for 30 min and the filtered samples were collected. For neutral oligosaccharides, the filtrate was further applied to an anion exchange column (LC-SAX, 1 ml, Sulpeco, Bellefonte, PA, USA) preconditioned according to instructions. The eluate was collected and osmolality measured on Mikro-Osmometer Typ 15 (Labex AB, Helsingborg, Sweden). The sample was diluted with ultraclean water to a final osmolality of 200 mOsm/kg and then further desalted by adding 50 mg AG 501-X8 resin (20–50 mesh, Bio-Rad, Hercules, CA, USA). The exchange of stachyose and lactose after these purification steps was similar and typically 70–75 %.

For acidic oligosaccharides the ultrafiltrate was applied to an ISOSEP C18 column (Sorbent AB, Västra Frölunda, Sweden) and the eluate was collected for further analysis.

Analysis by HPAEC-PAD

The purified urine samples were further analysed by High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, ICS-3000, Dionex) equipped with a CarboPac PA200 column. The flowrate was 0.5 ml/min and the injection volume 20 μL. Two different gradient programs were used. For neutral oligosaccharides a constant concentration of 20 mM NaOH and a gradient with sodium acetate (NaOAc) from 0 to 25 mM at 7 to 30 min were used. For acidic oligosaccharides a constant concentration of 0.1 M NaOH and a two step gradient of NaOAc from 5 mM to 20 mM at 0 to 10 min and from 20 mM to 150 mM at 10 to 30 min were used. Several milk oligosaccharide standards were analysed and retention time and response factors were recorded (Table 2). All oligosaccharides standards were from Dextra (Reading, UK). Lactose was from Sigma-Aldrich. A mixture of lactose and stachyose at different concentrations were analysed to get a standard curve for quantitative measurement of lactose. A standard curve for 3-SL and galacturonic acid was analysed several times. For 2-FL and LDFT linearity was checked once, whereafter one standard at a fixed concentration was used to calculate the response factor compared to the internal standard stachyose.

Exoglycosidase digestion

To verify the identity of the peaks recorded by HPAEC-PAD, exoglycosidases were used to cleave the oligosaccharides. To 100 μl purified urine sample and 100 μl of phosphate buffer (0.05 M, pH 7.8) 5 μl (15 U) β-Galactosidase from Echerichia coli (Sigma-Aldrich) was added. To 30 μl urine and 30 μl of

Table 2 List of abbreviated trivial names compared to structure of mono- and oligosaccharides found in urine or used as internal standards

Trivial name	Oligosaccharide name/structure
Fuc	Fucose
Gal	Galactose
Glc	Glucose
GlcNAc	N-acetyl glucose amine
Neu5Ac	5-acetyl neuraminic acid
S	Stachyose
L	Lactose
2-FL	Fucα(1-2)Galβ(1-4)Glc
LDFT	Fucα(1-2)Galβ(1-4)[Fucα(1-3)]Glc
LNT	Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glc
G	Galacturonic acid
3-SL	Neu5Acα(2-3)Galβ(1-4)Glc
6-SL	Neu5Acα(2-6)Galβ(1-4)Glc

actetate buffer (0.05 M, pH: 5.5) 5 μl (25 mU) α-fucosidase from bovine kidney (Sigma-Aldrich) was added. The samples were incubated at 37 °C for 24 h and desalted by AG 501-X8 resin (10 and 5 mg respectively). The presence of acidic oligosaccharides was confirmed by using neuraminidase from Arthrobacter Ureafaciens (Calbiochem). To 50 μl of purified oligosaccharides in actetate buffer (0.05 M, pH: 5.5) 10 mU of neuraminidase was added and the mixture was incubated at 37 °C for 24 h. The sample was then applied to an ISOSEP C18 column eluted with water before analysis by HPAEC.

Statistical methods

Mann–Whitney *U*-test was used to examine the significance of difference between groups. Correlation between different parameters was examined by calculating Spearman rank order correlation coefficient (r).

Results

Morning urine samples were collected from 18 patients with hyperprolactinaemia (range: 620–16,000 mU/l), 13 healthy individuals with normal levels of serum prolactin (range: 85–420 mU/l) and four pregnant women. The pregnant women were in their third trimester and had prolactin values between 4000 and 5600 mU/l. Eight of the patients had a diagnosis of pituitary adenoma and ten patients had no distinct tumor on MRI, demographic data are presented in Table 1. Results from analysis of thyroidal hormones and creatinine indicated normal thyroidal status and normal renal function. After purification, the oligosaccharides were quantified by HPAEC-PAD. The individual oligosaccharides were identified by comparing retention times with standards and by using specific exoglycosidases for cleavage. Figure 1a–c shows examples of chromatograms before and after digestion with fucosidase, galactosidase and neuraminidase.

The excretion pattern of urine oligosaccharides during pregnancy was followed in one individual from the 5th to 38th week of gestation together with the serum level of prolactin. A number of pregnancy specific oligosaccharides were identified and found to increase in concentration during pregnancy (Fig. 2). These oligosaccharides together with lactose are also found in human milk. Lactose increased in parallel with prolactin, but seemed to reach a maximum in the 31th week when prolactin was approximately 4000 mU/l.

Lactose was also found in small amount in all samples from normoprolactinaemic controls. Patients with hyperprolactinaemia had significantly higher lactose excretion in urine compared to controls ($p<0.01$), but in most cases lower levels than pregnant women (Fig. 3a). Samples from one of the patients contained macroprolactin as the dominant form of prolactin, as judged by a low recovery of prolactin after PEG-precipitation. Urinary lactose was low in urine from this patient (0.3 g/mol creatinine). After excluding this sample, prolactin showed a significant correlation to urinary lactose ($r=0.51$, $p<0.05$, $n=30$).

Besides lactose, urine from pregnant women also contained clearly detectable amounts of LDFT, 2-FL and LNT and the acidic oligosaccharides 3-SL and 6-SL (Table 2). All these oligosaccharides increased during lactation (Fig. 2). In urine from healthy controls and patients with hyperprolactinaemia small amounts of the di-fucosylated oligosaccharide LDFT was found in most cases (Fig. 3b). The levels of LDFT in both groups were considerably lower than for pregnant women, but the patients had more variable levels and two patients had relatively high urinary excretion of LDFT (>1 g/mol creatinine). However, the difference between patients and controls was not statistically significant ($p=0.17$). The mono-fucosylated oligosaccharide 2-FL was also found in urine from three patients but not in urine samples from the controls. A small peak which may represent LNT was found in most samples, however the galactosidase failed to cleave LNT and presence of LNT could therefore not be verified. The area of the peak representing LNT showed no correlation to prolactin concentration neither in samples from controls or patients.

Sialylated oligosaccharides were analyzed separately. The mono-sialylated oligosaccharide 3-SL was found in all urine samples with higher values in pregnant women. There was no significant difference between patients and the group of healthy controls ($p=0.63$, Fig. 3c), but two of the patients with the highest prolactin levels (14,000 and 16,000 mU/L) also had the highest urinary excretion of 3-SL (1.17 and 1.30 g/mol creatinine). A small peak representing 6-SL was found in urine from all pregnant women. However, 6-SL was not detected in samples from patients with hyperprolactinaemia or controls.

Discussion

This study is the first attempt to elucidate if hyperprolactinaemia alone may increase production and excretion of lactose based oligosaccharides. Increased urinary excretion of lactose [16, 17] and oligosaccharides [18, 19] have previously been found during pregnancy, though lactose synthesis is mainly suppressed by progesterone [20]. Patients with pituitary adenomas have often increased prolactin, but normal levels of progesterone, which then hypothetically could lead to a high production of lactose and milk-type oligosaccharides. However, it is unclear if and to what extent the mammary gland or other tissues is capable of oligosaccharide synthesis in these cases. In our study, we found an increased urinary excretion of lactose and the fucosylated oligosaccharides 2-FL and LDFT in some of the patients with hyperprolactinaemia. However, compared to samples from

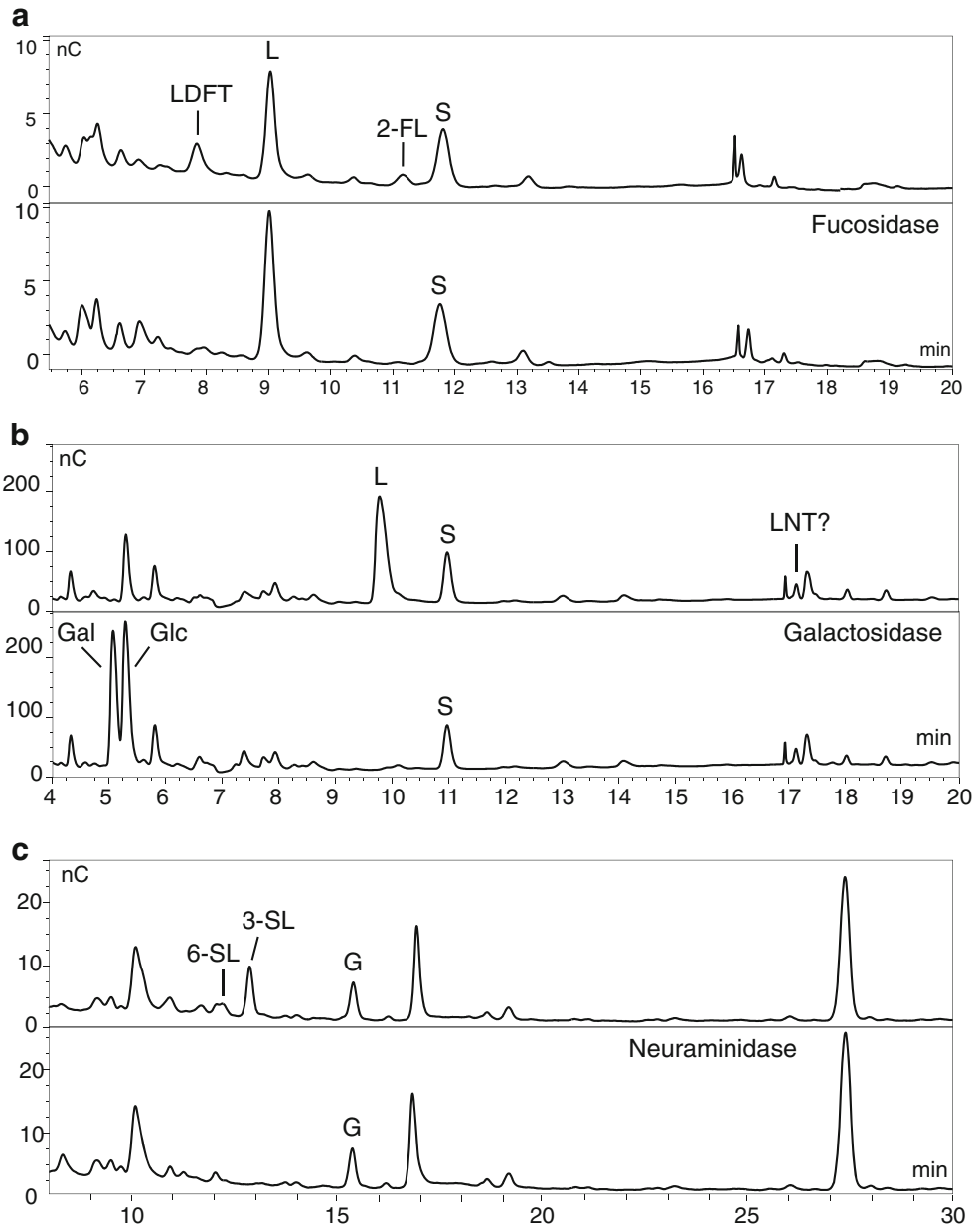

Fig. 1 Purified urine samples before and after exoglycosidase digestion analysed by HPAEC-PAD. **a** shows a sample from a patient with hyperprolactinaemia (prolactin 1800 mU/l) and fucosidase treatment; **b** a patient with hyperprolactinaemia (prolactin 1780 mU/l) and galactosidase treatment; **c** a pregnant women (prolactin 4960 mU/l) and neuraminidase treatment. S and G denote standards used for quantification. Peaks representing lactose (L) and milk-type oligosaccharides are indicated in the figures

pregnant women in the last trimester, the lactose excretion was smaller. This could be explained by most patients having lower prolactin values than the pregnant women (17 out of 19 patients). Indeed, a significant correlation between prolactin and urinary lactose was found for all patients and controls. This indicates that even small increases in serum prolactin may affect lactose production. Even though 2-FL and LDFT also were increased in urine from some patients, the correlation with prolactin levels was more unclear. These two oligosaccharides are not produced in secretions from individuals with a genetically inactive α (1-2)-fucosyltransferase II, so called non-secretors [21, 3]. Approximately 20 % of the population is non-secretors. Thus, most likely some of the patients did not have the ability to produce 2-FL and LDFT, which partly explain the variability in concentrations. The increased synthesis of fucosylated milk-type oligosaccharides may indicate that prolactin has a stimulatory role of some fucosyltransferases. All four pregnant women were secretors as judged by clearly detectable amounts of 2-FL and LDFT.

The urinary excretion of sialyllactoses has previously been studied in a small group of healthy men [8]. The measurements were done in urine collected for 6 h and the concentrations were not corrected for diuresis by creatinine as in our study. They found four times more 3-SL than 6-SL and a mean excretion of 3-SL of 6.7 mg/6 h, as compared to our study were the controls had a mean excretion of 3-SL of 6.7 mg/L.

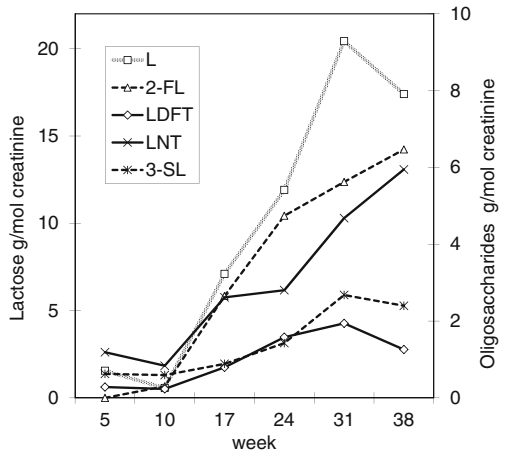

Fig. 2 Urinary excretion of lactose and milk-type oligosaccharides during pregnancy. Urine was collected from one healthy pregnant woman for 24-h on six occasions. Oligosaccharide concentrations are expressed as g/mol creatinine

Sialyllactose excretion in urine has been found to increase in response to tissue injury and inflammation, that is in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and during myocardial infarction [22–25]. The cause of this is unknown. Interestingly, increased serum prolactin has been associated to both non-organ-specific and organ-specific autoimmune diseases like SLE, RA, Sjogren's syndrome, Hashimoto's thyroiditis, multiple sclerosis, psoriasis, hepatitis C, Behcet's disease, peripartum cardiomyopathy and active celiac disease [26]. Prolactin has been shown to modulate the Th1 and Th2 type cytokine production, *e.g.*, increasing the production of IFN-gamma, IL-2 and IL-6 by Th1 lymphocytes, and enhancing immune globulin production in Th2 lymphocytes [26, 27]. Furthermore, studies have implicated that prolactin increases the production of Th17-cytokines [26, 28].

This study shows that sialyllactoses also increase during pregnancy with approximately 3–4 times higher values in late pregnancy. However, there were no significant difference between urine 3-SL in the group of patients with hyperprolactinaemia compared to controls. Interestingly, two patients with prolactin levels above 10,000 mU/l showed a substantially higher urinary excretion of 3-SL (approximately two times higher) than patients with lower serum prolactin concentration and controls. This indicates that prolactin at higher levels may have a regulatory role in the synthesis of 3-sialyllactose.

One sample contained macroprolactin as the dominating form. Macroprolactin is a complex between immunoglobulin and prolactin and may interfere with immunochemical prolactin methods. Macroprolactin is often associated with lack of clinical symptoms and has a lower bioactivity than monomeric prolactin [13]. The low levels of oligosaccharides and lactose in urine from the patient with macroprolactin in this study

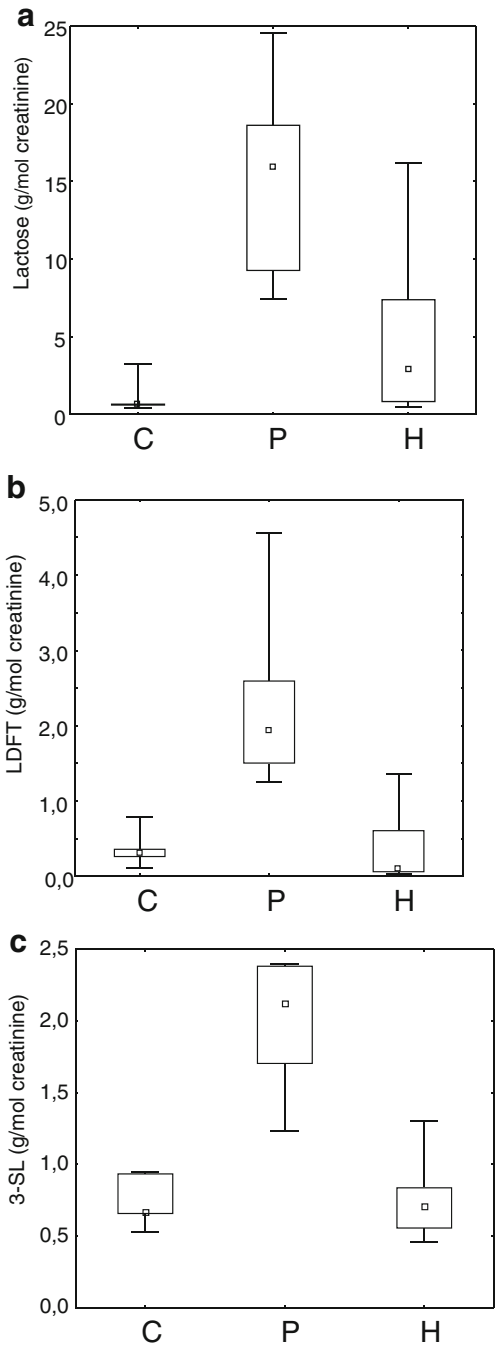

Fig. 3 Urinary excretion of lactose (**a**), LDFT (**b**) and 3-SL (**c**) in a group of healthy controls (*C*), pregnant women in gestation week 31–37 (*P*) and patients with hyperprolactinaemia (*H*). One sample with macroprolactin was excluded. This sample contained low levels of lactose (0.3 g/mol creatinine), LDFT (0.08 g/mol creatinine) and 3-SL (0.4 g/mol creatinine). The point label indicates the median. The *box* indicates the 25th to the 75th percentiles and the *bars* indicate range

is thus in accordance with other findings regarding macroprolactin.

The findings of increased synthesis of lactose and certain oligosaccharides in patients with hyperprolactinaemia may have impact on the immune system by modulating

interactions between cells. However, if the prolactin level in blood is important for expression of certain glycosyltransferases as indicated, this may also have impact on glycosylation of proteins and lipids and thereby lead to widespread effects on several physiological processes.

This study shows that patients with hyperprolactinaemia have increased urinary excretion of lactose, fucosylated oligosaccharides and to some extent 3-sialyllactose. These findings indicate that prolactin levels in blood affect glycosylation in certain ways, but it remains to evaluate if protein- and lipid glycosylation also is affected by prolactin and if these alterations have physiological and clinical implications.

Acknowledgments The authors thank Emmilie Jonsson for technical assistance. This study was funded by FORSS (Medical Research Council of Southeast Sweden, no. 4065) and the Faculty of Health and Sciences, Linköping University, Sweden.

Compliance with ethical standards The study was approved by the local Ethics Committee at Linköping University Hospital and performed in accordance with the Declaration of Helsinki. The patients were informed about the purpose and procedure of the study and gave their written informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

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