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UDP-sugar accumulation drives hyaluronan synthesis in breast cancer

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Abstract

Increased uptake of glucose, a general hallmark of malignant tumors, leads to an accumulation of intermediate metabolites of glycolysis. We investigated whether the high supply of these intermediates promotes their flow into UDP-sugars, and consequently into hyaluronan, a tumor-promoting matrix molecule. We quantified UDP-N-Acetylglucosamine (UDP-GlcNAc) and UDP-glucuronic acid (UDP-GlcUA) in human breast cancer biopsies, the levels of enzymes contributing to their synthesis, and their association with the hyaluronan accumulation in the tumor. The content of UDP-GlcUA was 4 times, and that of UDP-GlcNAc 12 times higher in the tumors as compared to normal glandular tissue obtained from breast reductions. The surge of UDP-GlcNAc correlated with an elevated mRNA expression of glutamine-fructose-6-phosphate aminotransferase 2 (GFAT2), one of the key enzymes in the biosynthesis of UDP-GlcNAc, and the expression of GFAT1 was also elevated. The contents of both UDP-sugars strongly correlated with tumor hyaluronan levels. Interestingly, hyaluronan content did not correlate with the mRNA levels of the hyaluronan synthases (HAS1-3), thus emphasizing the role of the UDP-sugar substrates of these enzymes. The UDP-sugars showed a trend to higher levels in ductal vs. lobular cancer subtypes. The results reveal for the first time a dramatic increase of UDP-sugars in breast cancer, and suggest that their high supply drives the accumulation of hyaluronan, a known promoter of breast cancer and other malignancies. In general, the study shows how the disturbed glucose metabolism typical for malignant tumors can influence cancer microenvironment through UDP-sugars and hyaluronan.

Abbreviations

BMI, body mass index; GFAT1-2, glutamine-fructose-6-phosphate aminotransferase1-2; GNPDA1-2, glucosamine-6-phosphate deaminase 1 and 2; HBS, hexosamine biosynthesis; HAS1-3, Hyaluronan synthase1-3; UDP-GlcNAc, UDP-N-Acetylglucosamine; UDP-Glc, UDP-glucose; UDP-GlcUA, UDP-glucuronic acid.

Keywords: UDP-sugar, breast cancer, UDP-N-Acetylglucosamine, hyaluronan, Warburg effect

Introduction

One of the hallmarks of malignant tumors is metabolic reprogramming. Despite the fact that cancer cells are genetically divergent, with varied losses of tumor suppressors, and activation of oncogenes, they often share certain metabolic changes. These include increased uptake of metabolites such as glucose, glutamine and fatty acids. These changes satisfy the demands for ATP, maintenance of redox balance and generation of biomass [1]. Recent data have suggested that hypoxia, activated oncogenes (such as Ras and Myc) and mutated tumor suppressors (such as P53) can drive this metabolic reprogramming [2].

The most common metabolic feature of cancer cells is their increased glucose uptake, a property widely utilized in the clinical detection of tumors and their metastases by the ^{18}F-deoxyglucose uptake and PET imaging [3]. Most malignant tumors prefer to utilize glycolysis and limited oxidative phosphorylation, also when oxygen is abundant. This so-called Warburg effect generates ATP less efficiently, but the low yield of ATP is compensated by increased glucose uptake and utilization of alternative metabolites to drive ATP synthesis [4]. There are also tumor types, which have a normal rate of oxidative phosphorylation, but still show the high glucose uptake and lactate production [5]. The high glucose uptake and aerobic glycolysis increase the levels of glycolysis intermediates and hence enhance their flux into biosynthesis pathways that utilize these intermediates as substrates (Fig. 1). One of these synthesis routes is the pentose phosphate pathway that provides nucleotides and NADPH [6]. Glucose-6-phosphate can also be converted into UDP-glucose (UDP-Glc), which can serve the production of glycogen and UDP-glucuronic acid (UDP-GlcUA). Likewise, fructose-6-phosphate can flux into the hexosamine biosynthesis (HBS) pathway that produces UDP-N-acetylglucosamine (UDP-GlcNAc).

UDP-sugars function as substrates for the synthesis of numerous conjugated glycans, mostly constructed in the Golgi apparatus. The supply of UDP-sugars for these products is considered to stay relatively constant due to the specific membrane transporters that maintain their level in the Golgi apparatus. In contrast, production of glycoconjugates that depend on the highly variable cytosolic concentrations of UDP-sugars can experience large fluctuations. These include hyaluronan, and O-GlcNAc modification of intracellular proteins. Both of them have profound effects on tissue microenvironment and cell signalling, respectively, and are closely linked to fundamental aspects of cancer development and progression [7,8].

The HBS producing UDP-GlcNAc utilizes fructose-6-phosphate, an intermediate of glycolysis, as a starting point, but also requires the input of glutamine, acetyl-CoA and UTP, thus combining four major metabolic systems. Hence, UDP-GlcNAc is considered as a metabolic sensor. The rate-limiting step of UDP-GlcNAc synthesis is the generation of glucosamine-6-phosphate from glutamine and fructose-6-phosphate, catalysed by the glutamine-fructose-6-phosphate aminotransferase (GFAT1 and GFAT2). Another enzyme pair, glucosamine-6-phosphate deaminase 1 and 2 (GNPDA1 and GNPDA2), can catalyse the reaction of glucosamine-6-phosphate back to fructose-6-phosphate and NH_3 [9,10]. Interestingly, data in the international mouse phenotyping consortium (IMPC, http://www.mousephenotype.org/) show that homozygous knockout of GFAT1 or GNPDA1 are embryonically lethal, emphasizing the importance of UDP-GlcNAc.

The synthesis of hyaluronan is particularly sensitive to changes in the concentration of available UDP-sugars, fluctuations in the sizes of the substrate pools have rapid and marked influences on the activity of all three hyaluronan synthezising enzymes (HAS1-3) [11]. This glycosaminoglycan is involved in complex interactions underlying inflammation [12], invasion and metastasis [7]. This is likely due to its unique physicochemical properties, position on cell surfaces, signalling functions through its plasma membrane receptors, and interactions with the stromal matrix. Hyaluronan synthesis is also known to support epithelial to mesenchyme transition (EMT) and cellular stem cell properties [13]. Accordingly, hyaluronan levels are increased in numerous tumor types [7] including breast cancer, in which its accumulation strongly correlates with poor outcome of the disease [14].

There is very little previous information on the levels of UDP-sugars in human cancers. Here we analysed breast cancer biopsies and control benign breast tissues for their UDP-sugars, hyaluronan contents, and mRNA expression of key enzymes of HBS and hyaluronan synthesis. Our results show that breast cancers have highly increased levels of UDP-sugars, their surplus probably supported by upregulation of enzymes involved in their synthesis, and the Warburg effect that provides ample supply of glycolysis intermediates for these pathways. Importantly, the data suggest that the increase of UDP-sugars, especially UDP-GlcNAc, drives the synthesis of hyaluronan, and thus promotes tumor progression.

Results

The clinicopathological data of the 28 cancer cases are presented in Table 1. Of the samples only one was triple-negative. The 12 control samples were obtained from patients undergoing bilateral reduction mammoplasty because of excessive breast weight. The mean body mass index

(BMI) of these patients was 30.3 (range 25.0 – 37.1) compared to 26.7 (range 18.7 – 34.6) of those with cancer; this difference was statistically significant (p=0.046). However, BMI showed no correlation with any of the biochemical parameters assayed. The median age of the carcinoma patients was 59.6 years (39-86 years) and 37 years (20-61 years) for the controls. This difference was statistically significant (p=0.005) but there was no correlation with levels of the UDP-sugars or hyaluronan with the age.

Breast cancers contain increased levels of UDP-sugars

The analyses of UDP-GlcNAc, UDP-GlcUA and UDP-Glc showed that the breast cancers had significantly higher levels of all three UDP-sugars, compared with the benign breast tissue ($p<0.001$, Fig. 2A-C). Interestingly, while the amounts of UDP-Glc and UDP-GlcUA showed approximately a four-fold increase, there were over 12 times higher levels of UDP-GlcNAc in the tumors. Furthermore, the levels of UDP-GlcNAc in cancer patients did not overlap at all with those of control samples. Although there was a clear difference in the increase of UDP-sugars, the levels of UDP-GlcNAc and UDP-GlcUA correlated with each other (Pearson $r =0.794$, $p<0.0001$).

Increased expression of the HBS enzymes GFAT1-2 in breast cancer

The higher accumulation of UDP-GlcNAc suggested that there might be more than increased supply of glycolysis intermediates behind the increase. We determined the mRNA levels of the four HBS enzymes functioning in the assumed rate-limiting step, i.e. GFAT1-2 and GNPDA1-2. The tumors showed significantly increased expression levels of both GFAT1 and GFAT2, whereas those of GNPDA1-2 remained unchanged (Fig. 3A-D). Furthermore, the increased expression of GFAT2 correlated with the content of UDP-GlcNAc (Spearman's rho = 0.568, $p=0.006$) (Fig. 3F). The correlation of GFAT1 with UDP-GlcNAc was also significant, but the level of correlation was lower (Pearson $r=0.447$, $p=0.019$, Fig. 3E). LThe levels of GNPDA1-2 did not correlation with UDP-sugars (Fig. 3G and H). This suggests that upregulation of GFAT1 and GFAT2 contributed to the high content of UDP-GlcNAc. The mRNA levels of these genes showed no correlation with any of the clinical factors listed in Table 1.

UDP-sugars and clinical parameters

Ductal and lobular, the two main histological types of breast cancer, show different growth patterns and biological properties. *Interestingly, the highest levels of UDP-sugars were found in the ductal type, resulting in almost 3 times more UDP-GlcNAc as compared with the lobular type, but the significance of the difference could not be reliably determined as the number of the lobular*

samples remained so low. A trend towards decreased levels in lobular type was also found in UDP-GlcUA (Figure 4D). The other clinical factors listed in Table 1 showed no significant correlation with UDP-sugar contents, although PR-status showed a trend to decreased levels of UDP-GlcNAc in patients with a PR percentage of 5 or under (p=0.053) (Figure 4).

UDP-GlcNAc and UDP-GlcUA correlate with the increased hyaluronan levels

The mean hyaluronan content in the tumors, quantified with an ELISA-like assay was more than twice that in the control tissue (p=0.0055, Fig. 5A). These results corresponded to those obtained with the histological assays; the staining of hyaluronan both in the stroma and tumor cells was stronger than that of controls (Fig. 5B-D). The results of biochemically assayed and immunohistochemically scored hyaluronan levels correlated with each other (p = 0.018 for tumor cells and 0.014 for stroma). The results thus confirm the reliability of the histochemical scorings.

To investigate the possible dependence of hyaluronan synthesis on the contents of the UDP-sugar substrates we correlated the levels of hyaluronan with UDP-GlcNAc and UDP-GlcUA. The results indicated a positive correlation between UDP-GlcNAc and hyaluronan (Spearman's rho=0.585, p=0.0001), and a similar result was seen for UDP-GlcUA (Spearman's rho=0.594, p=0.0001, Fig. 5E,F). The correlation between hyaluronan and UDP-GlcUA persisted within the cancer only group (0.475, p=0.016), while that between hyaluronan and UDP-GlcNAc did not reach significance (0.354, p=0.083). Similar significant correlations with hyaluronan were found in the immunohistochemical scorings of the whole material (UDP-GlcNAc: p=0.005 and p<0.001 for tumor cell and stromal hyaluronan, respectively; UDP-GlcUA: p=0.003 and p=0.001 tumor cell and stroma, respectively). As equimolar amounts of the two UDP-sugars are needed for hyaluronan production, and the concentration of UDP-GlcUA was markedly lower, we performed the partial correlation to test whether UDP-GlcUA supply can become the limiting factor. When the effect of the other substrate is neglected, the correlations remained significant only with UDP-GlcUA (r=0.455; p=0.005). Linear regression analysis supported the results, demonstrating that 34.6% of the variation in hyaluronan level can be explained by changes in UDP-sugar contents. Of the UDP-sugars only UDP-GlcUA showed a significant effect (coefficient=1.719, p=0.005). The results of partial correlations and regression analyses were similar when cancer cases only were analysed.

Changed *hyaluronan synthase (HAS)* gene expression does not account for the increased hyaluronan content

The data above suggest that the markedly increased UDP-sugar contents account for the high level of tumor hyaluronan, but it could also be due to upregulation of the *hyaluronan synthase (HAS)* genes. We therefore quantified the *HAS1-3* mRNAs in the samples. The mean levels of *HAS2-3* mRNAs showed no significant change. But the *HAS1* mRNA levels were significantly reduced (Fig. 6A-C), indicating that increased transcription of the *HAS* genes was not responsible for the accumulation of hyaluronan.

Interestingly, a trend to negative correlation existed between UDP-GlcNAc content and *HAS1* mRNA (Pearson correlation -0.385, P=0.065) (Fig. 6D), a finding suggesting that UDP-GlcNAc excess might downregulate *HAS1* expression, a response shown previously for *HAS2* [15].

Discussion

The present results show for the first time that UDP-sugar levels are markedly elevated in breast cancer. The increase is likely due to an increased glucose uptake that provides glycolysis intermediates feeding their biosynthesis. The content of UDP-GlcNAc was increased three fold more than the other UDP-sugars. This was associated with the upregulation of GFAT1 and 2, both key HBS enzymes. We further demonstrate that the excess of the UDP-sugars is associated with the accumulation of stromal hyaluronan, a strong indicator of poor prognosis, and a recently introduced target of therapy. These data demonstrate how a metabolic trait common in cancers can influence the microenvironment, important in tumor progression.

While unequivocal demonstration of increased UDP-GlcNAc contents in breast cancer has been missing, several previous papers are consistent with a metabolic shift to enhanced UDP-GlcNAc synthesis. The breast cancer data in the Oncomine database shows increased expression of HBS enzymes (Oncomine Research Edition, https://www.oncomine.org), a notion consistent with the GFAT1-2 upregulation in our material. Another recent study showed that the level of UDP-GlcNAc is increased in primary prostate cancer, together with the expression of HBS enzymes [16,17]. Likewise, in a mouse model of pancreatic ductal adenocarcinoma, kRAS mutation stimulates the activity of HBS through increased GFAT1 mRNA and protein levels, while knock-down of GFAT1 inhibits the clonogenic and soft-agar growth of tumor cells [6].

Glucose supply increases the cellular content of UDP-GlcNAc in non-malignant cells such as adipocytes and COS-1 cells [11,18]. Hence, it is not surprising that HBS is activated also in hyperglycaemic conditions like diabetes [19,20,21]. In A549 cancer cells induction of EMT elevates glucose uptake, which is specifically directed to UDP-GlcNAc production [22].

Interestingly, in all the three cancers (breast, prostate, pancreatic) in which there is information suggesting the activation of HBS, the content of hyaluronan in the stromal matrix is also increased, and the level of the increase indicates progressively worse prognosis [7]. This suggests that high UDP-GlcNAc supply stimulates hyaluronan production. It has been shown in numerous *in vitro* studies that UDP-GlcNAc availability can suppress or enhance hyaluronan production [10,11,23]. UDP-GlcNAc has a dual role in hyaluronan synthesis: First, it is a substrate of the HAS enzymes. Second, its content regulates the O-GlcNAc modification of HAS2 and HAS3, a posttranslational modification which increases the stability and activity of the enzymes [23,24]. Our previous studies on breast cancer, showing increased tumor O-GlcNAcylation [25] and a higher immunohistochemical signal of HAS isoenzymes [26], are in line with increased HAS enzyme stability by O-GlcNAc modification. Thus, while either UDP-GlcNAc or UDP-GlcUA alone can be a limiting substrate in hyaluronan synthesis, UDP-GlcNAc has a special role increasing the posttranslational modification of HAS. Since UDP-GlcNAc and UDP-GlcUA contents correlated with each other, it was very difficult to distinguish the contributions of the individual UDP-sugars in hyaluronan synthesis in the patient material.

The idea that UDP-sugars drive the rate of hyaluronan synthesis in breast cancer was supported by the strong correlation between tumor UDP-sugars and hyaluronan content. This notion becomes even more obvious by the finding that there was no such positive correlation between hyaluronan content and the mRNA levels of the HAS enzymes. Although HAS mRNAs were not dramatically changed, HAS proteins can be increased since UDP-GlcNAc increases their stability and enzymatic activity through O-GlcNAc modification, making the role of this compound even more interesting [23,24]. High breast cancer incidence is associated with the western life style, obesity and consumption of dietary sugars. The increased sugar intake, activation of the HBS, accumulation of UDP-sugars, and hyaluronan, and the resulting inflammation may induce or promote breast cancer. A recent study indicated that diabetic hyperglycaemia accelerates tumor growth in a mouse xenograft model in a hyaluronan dependent manner. Furthermore, *in vitro* experiments in the same paper indicate that the hyaluronan surplus depends on increased levels of glycolysis intermediates [27]. Western life style and obesity are associated with increased BMI. Although our control and breast cancer patients had a significant difference in their BMI, it did not show any correlation with the biochemical parameters analysed. This indicates that cancer related changes are more profound.

The importance of the present findings comes from the growing evidence indicating that the metabolism of hyaluronan in the tumor microenvironment is one of the key factors in the invasive growth and metastasis of certain tumors. CD44 and RHAMM/CD163, plasma membrane receptors of hyaluronan, and versican, the hyaluronan binding partner in the matrix, have also been associated with an unfavourable prognosis in human breast cancer [13,28,29]. Likewise, KIIAA99/CEMIP, a protein associated with hyaluronan degradation has recently turned out to be an indicator of tumor progression [30,31]. The latest findings indicate that high expression of TMEM2, a cell surface hyaluronidase, induces fragmentation of matrix hyaluronan [32] and promotes pro-invasive, pro-migratory and metastatic activity, and associates with an unfavourable prognosis in breast cancer [33]. Constant synthesis and degradation of hyaluronan thus promotes the malignant phenotype, likely by maintaining a type of inflammation characterized by type two macrophages [12,34].

This study shows that the glucose metabolites UDP-GlcNAc and UDP-GlcUA are markedly increased in human breast cancer, and contribute to the tumor-promoting microenvironment through enhanced hyaluronan synthesis. This finding may turn out to be important since treatments targeting hyaluronan show promising results in animal experiments [35,36], including models of breast cancer [37], and a recent report suggests that removing hyaluronan enhances the efficacy of standard cytostatic regimen in the treatment of patients with pancreatic cancer [38]. Depletion of hyaluronan by blocking the excessive supply of UDP-sugars could thus offer a new dimension in the current set of therapeutic options. For example, angiogenesis inhibitors, important in the current practice, have faced tolerance in cancers adapting to anaerobic conditions [39,40]. Thus, inhibition of both angiogenesis and induced glucose metabolism might have stronger clinical impact than either one alone.

Experimental procedures

Patient material

This study consists of 40 women treated in Kuopio University Hospital between the years 2013-2015. Control breast glandular tissue was collected from 12 patients during bilateral reduction mammoplasty operations due to benign indications. The remaining 28 patients were clinically diagnosed with invasive breast cancer. Samples were obtained during the preoperative ultrasound-guided core needle biopsy, avoiding any cystic or possible necrotic areas. The samples were snap frozen in liquid nitrogen, and stored in -70°C until analysis. The biopsies were not microdissected for individual cells and hence contained both tumor and stromal cells. Paraffin-

embedded samples were taken for immunohistochemical analysis during tumor removal. The permission for this study was provided by the Ethics Committee of the University of Eastern Finland and a written informed consent was obtained from all the patients prior to any procedures.

UDP-sugar determination

UDP-sugars were determined as published before [41], except that the results were normalized to tissue weight. In short, samples were weighed and homogenized in 80% ethanol in PBS with Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA, USA) with FastPrep® homogenizer (Savant, Thermo Fisher Scientific Inc., Waltham, MA, USA). Non-dissolved material was removed by centrifugation for 20 min at 8000 g. The supernatant was evaporated, and lipids extracted by butanol. The samples were further purified using Supelclean Envi-Carb SPE columns (Sigma-Aldrich, St. Louis, MO, USA), evaporated and dissolved in water for HPLC with a CarboPac™ PA1 column (Dionex, Thermo Fisher Scientific). To confirm the identity and purity of the peaks, each UDP-sugar peak was collected, salts removed by Supelclean Envi-Carb SPE columns, evaporated, and dissolved in 0.1 M formic acid-50% methanol (Sigma Aldrich). The samples were analysed by direct syringe infusion (10 µl/min) using a Q-STAR XL Quadrupole-TOF-mass spectrometer (Applied Biosystems/MDS Sciex, Toronto,Canada) fitted with an Ion spray ion source and using negative polarity. Ion spray voltage was - 4.5 kV and nitrogen as a curtain- and collision gas. De-clustering and focusing potentials were set at -150 and -200 volts, respectively. Pulser frequency was 9.99 KHz and pulse duration 1 µs. Ion release time and ion release width were 6 and 5 µs, respectively. Control compounds with known m/z were used for instrument calibration. One second TOF MS survey scans were recorded for mass range m/z 100–1000 until at least 200 scans were obtained. Data was processed using Analyst QS v1.1 software.

Hyaluronan measurements

Hyaluronan contents were measured from the same samples as UDP-sugars. The pellet from the ethanol extraction of UDP-sugars was dissolved in PBS and re-centrifuged at 8000 g for 20min. The supernatants were collected for hyaluronan determinations using an ELISA-like method [42] and normalized to tissue weight.

Hyaluronan was also analysed histochemically from paraffin-embedded tissue samples, using a biotinylated hyaluronan-binding complex as described previously [25,43]. The specificity of the bHABC signal was controlled by digesting the sections with Streptomyces hyaluronidase to remove

hyaluronan. The intensity of hyaluronan staining in the stroma was graded as: weak, moderate or strong. The hyaluronan staining in breast carcinoma cells was graded according to the percentage of the hyaluronan-positive breast carcinoma cells: negative (0-5%), weak (6-25%), moderate (26-50%) or strong (51-100%).

mRNA isolation and quantitative PCR

mRNA was isolated from tissue samples with TRI Reagent© (Molecular Research Center Inc., Cincinnati, OH, USA). Lysing Matrix D tubes and FastPrep® homogenizer were used for homogenization. The cDNA was synthesized with a Verso cDNA kit (Thermo Scientific), and the qRT-PCR was performed with FastStart Universal SYBR Green mix (Roche, Basel, Switzerland) using Stratagene Mx300P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). The primers used are indicated in the supplementary Table 1. The representative capacity of the control samples was confirmed by analysing mRNA levels of the gene SCGB2A2 (secretoglobin, family 2A, member 2) which is specific for breast glandular tissue [44]. Only samples with strong expression of SCGB2A2 mRNA were used. Results were calculated using the formula $2^{-(\Delta\Delta Ct)}$ with HUWE1 (HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase) as a reference gene. The functionality of HUWE1 as a normalizer was confirmed by comparing the results to those normalized to Ribosomal protein, Large, P0 (Rplp0) and ribosomal protein S23 (RPS23).

Statistical Analyses

The significance of differences between the groups was tested using Mann-Whitney or two-tailed students t-test. Welch's correction was used in t-test when samples had significantly different variances. When comparing categorical and continuous variables independent samples Kruskal-Wallis test was used. Pearson's and Spearman's correlation coefficients were used for samples with normally and non-normally distributed samples, respectively. The interdependency of UDP-sugars and hyaluronan was tested with partial correlations and linear regression. The statistical analyses were performed with IBM SPSS statistics 21 for windows (IBM Corporation, Armonk, NY, USA) or GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. *Position of glycolysis intermediates in the synthesis pathways of UDP-sugars and hyaluronan.* Major metabolic pathways utilizing glycolysis intermediates are indicated. TCA = Tricarboxylic acid cycle.

Figure 2. *UDP-sugar concentrations in human breast cancer and normal glandular tissue*. UDP-GlcNAc (A), UDP-Glc (B) and UDP-GlcUA (C) were measured with anion exchange HPLC from breast cancer biopsies and control tissue from breast reductions. The dots represent individual patients, the mean values are indicated as a line. ***p<0.001 (student's t-test).

Figure 3. *Expression of the enzymes in the first step of HBS, and their correlations with UDP-GlcNAc contents in breast cancer and control tissue.* The mRNA levels of GFAT1 (A), GFAT2 (B), GNPDA1 (C) and GNPDA2 (D) were measured with qRT-PCR. The relative expressions of the enzymes were plotted against the contents of UDP-GlcNAc (pmol/mg tissue) in (E-H). Significances of the differences between cancer and control groups were calculated with student's t-test for samples showing normal distribution (A), and with Mann Whitney test for (B,C,D) which was not normally distributed. p-values are indicated in the figure. Pearson correlation coefficients (r) were calculated for samples with normal distribution (E), while Spearman's correlation coefficient (ρ) was used for non-normally distributed samples (F,G,H).

Figure 4. *Differences in UDP-sugar contents according to clinical parameters.* UDP-GlcNAc and UDP-GlcUA contents of patients are shown in (A-F). Each point represents an individual patient, means are indicated by a horizontal line. The significances of the differences were tested with the independent samples Kruskal-Wallis test. The histological type and PR status show a trend to changed levels between groups. For histological type a significance for the difference is reached (P=0.02 for UDP-GlcNAc and p=0.033 for UDP-GlcUA). The PR status does not reach significance (p=0.053 for UDP-GlcNAC). The data for ER status are not presented, as there was only one ER negative sample.

Figure 5. *Hyaluronan content and its correlations with UDP-sugars in breast cancer.* Hyaluronan (HA) was assayed with an ELISA-like method (A) and immunohistochemistry (B,C,D). (B) The staining of the control sample with weak staining of the connective tissue surrounding the

glandular epithelia. Cancer samples with moderate (C) and strong staining (D) of the tumor stroma. Each patient is represented by a dot, and means as a line. The significances of the differences were tested using Mann-Whitney test, p-value indicated in the figure. Hyaluronan contents of all samples were plotted against those of UDP-GlcNAc (E) and UDP-GlcUA (F), and samples from cancer only in (G) and (H), respectively. The Spearman's correlation coefficients (ρ) and their p-values are indicated in the figure.

Figure 6. *Expressions of the enzymes producing hyaluronan, and their correlations with UDP-sugar contents.* The mRNA levels of *HAS1* (A), *HAS2* (B) and *HAS3* (C) are shown in control and cancer tissues. The mRNA levels of the *HASs* were plotted against those of UDP-GlcNAc (D,E,F) and UDP-GlcUA (G,H,I). Each patient is represented by a point, and means indicated as lines. Significances between control and cancer were tested using Mann-Whitney test and Spearman's correlation coefficient (ρ) to calculate correlations. The coefficients and significances are indicated in the figure.

Table 1. Clinicopathological data of the patients**.**

BMI	
Mean	26,7
Range	18.7- 34.6
Tumor stage, n (%)	
pT1	14 (51.9)
pT2	12 (44.4)
pT3	1 (3.7)
pT4	-
Nodal classification, n (%)	
pN0	16 (59.3)
pN1	6 (22.2)
pN2	3 (11.1)
pN3	2 (7.4)
Histological grade, n (%)	
1	5 (18.5)
2	13 (48.2)
3	9 (33.3)
Tumor histology, n (%)	
Ductal	24 (85.7)
Lobular	3 (10.7)
Other	1 (3.6)
HER2 status, n (%)	
Positive	5 (17.9)
Negative	23 (82,1)
ER status, n (%)	
Positive	27 (96.4)
Negative	1 (3.6)
PR status, n (%)	
Positive	23 (82,1)
Negative	5 (17,9)

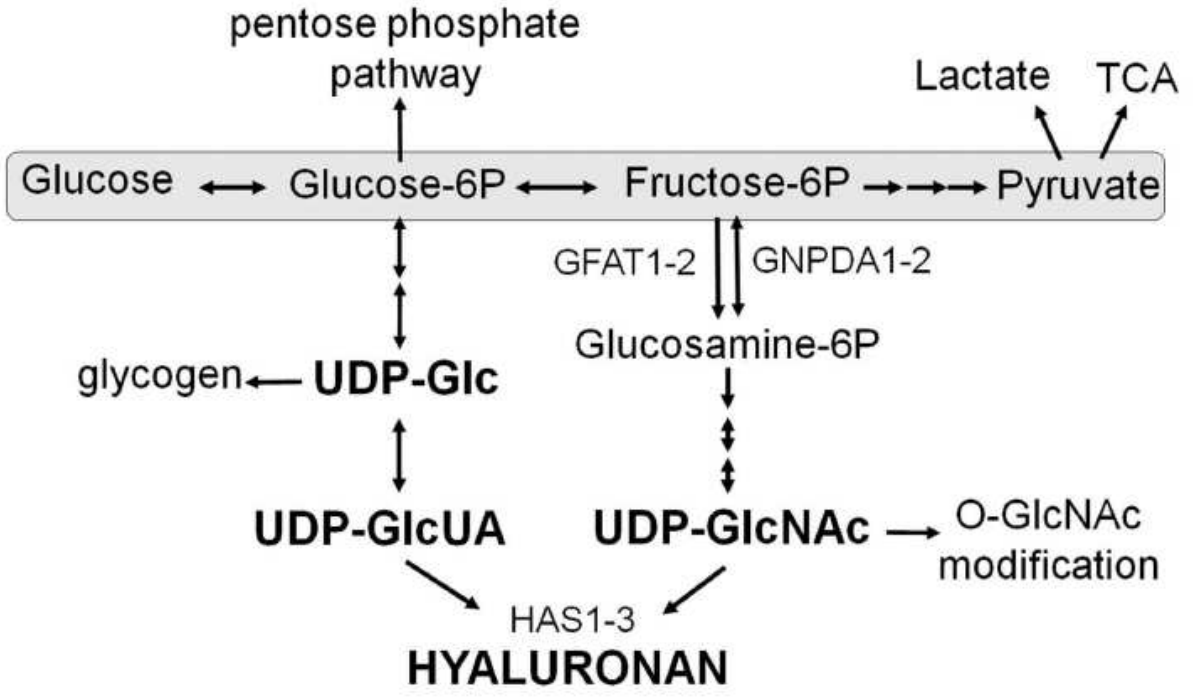

Figure 1

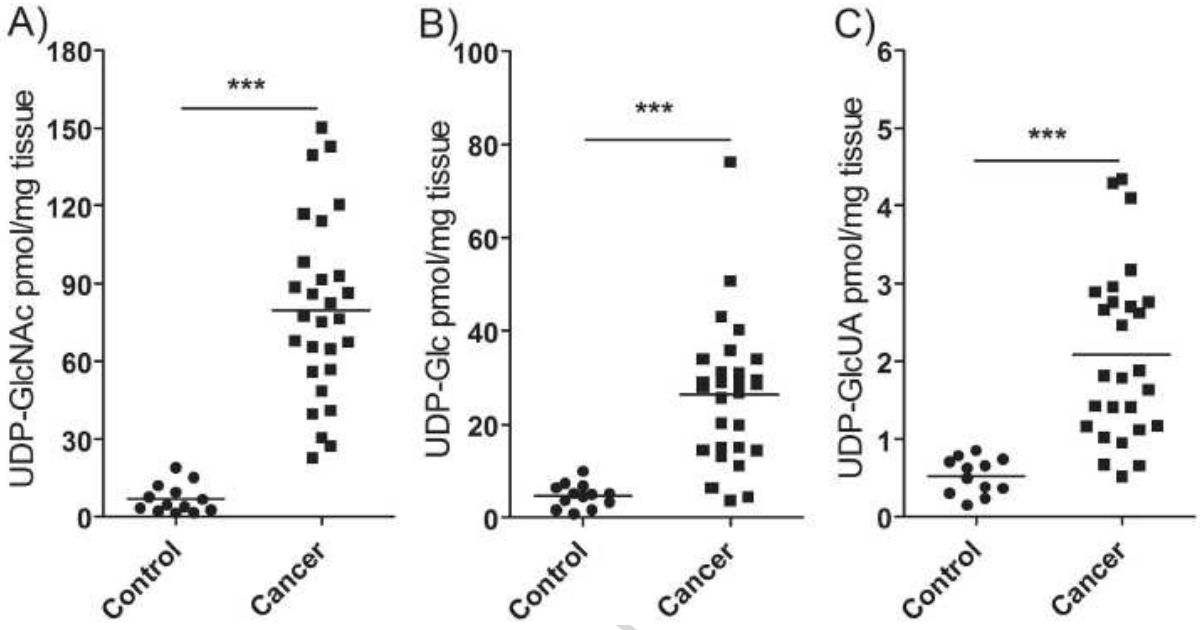

Figure 2

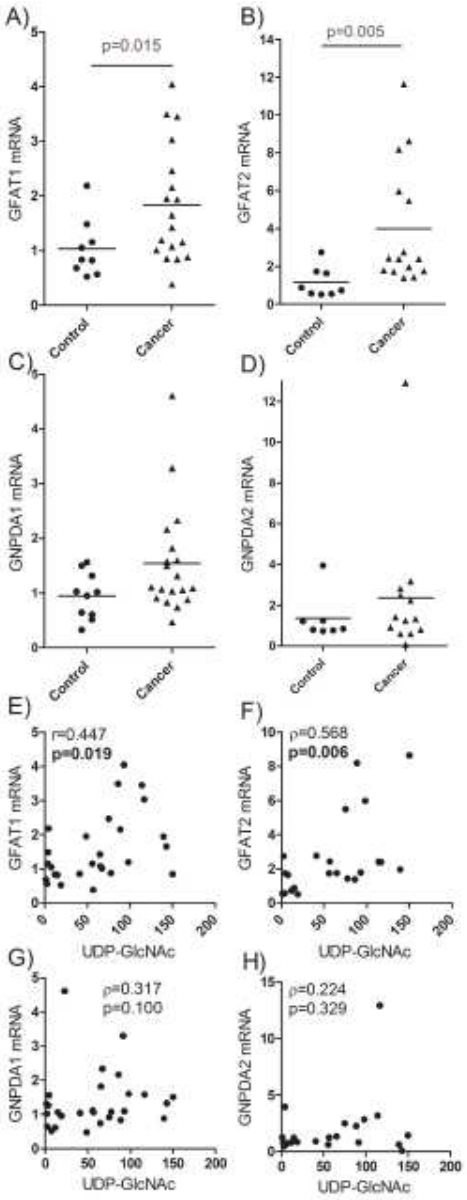

Figure 3

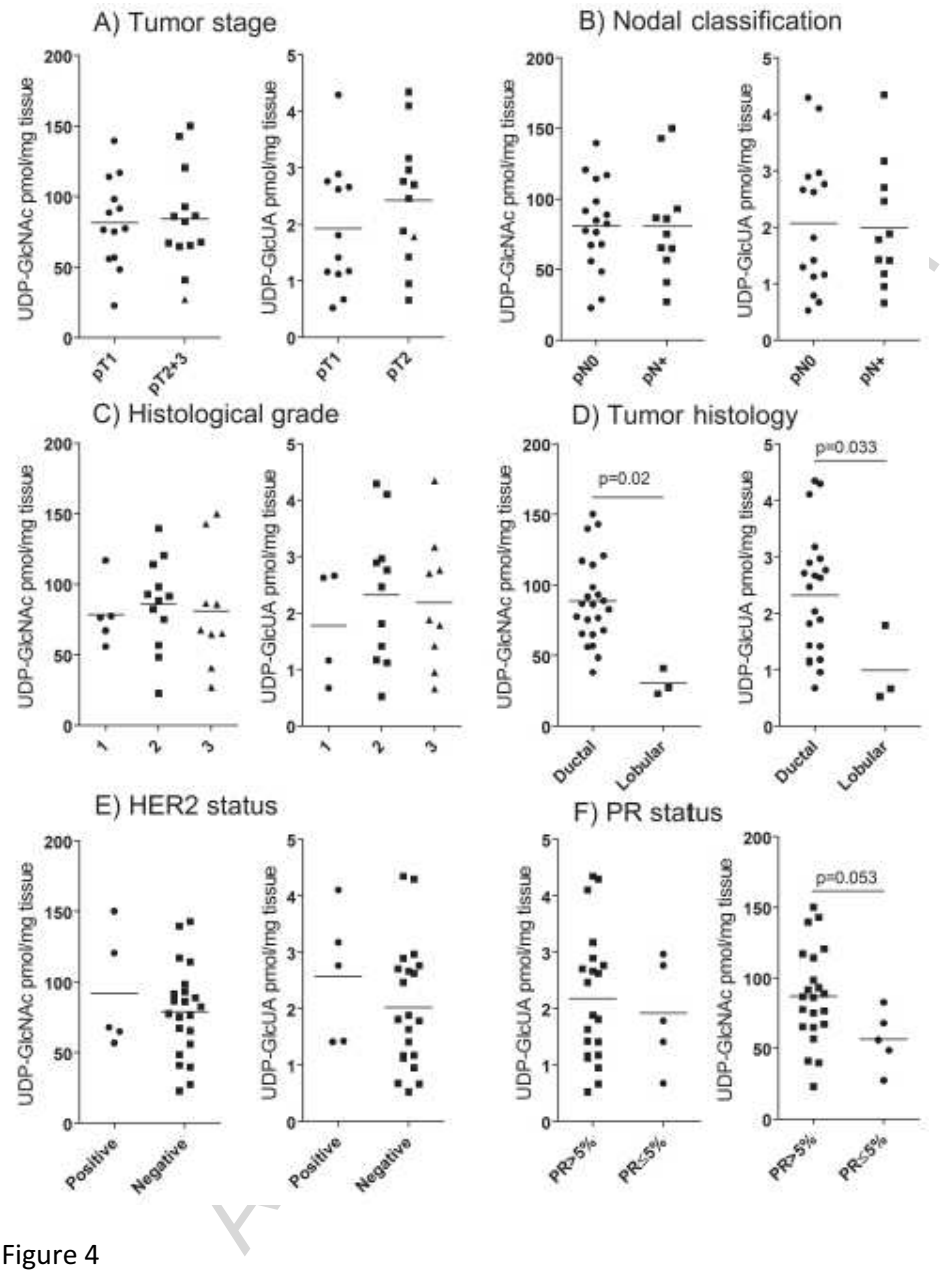

Figure 4

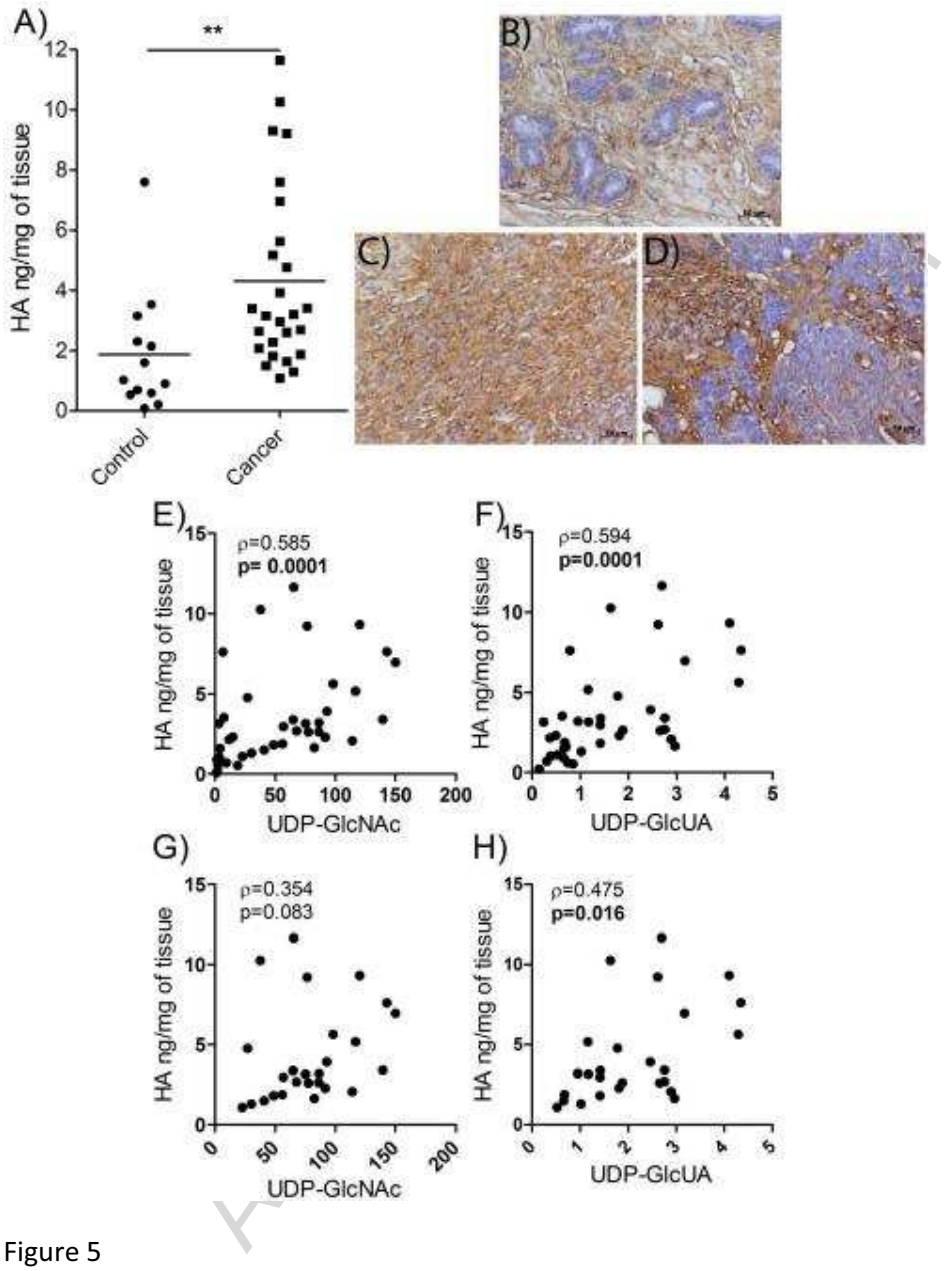

Figure 5

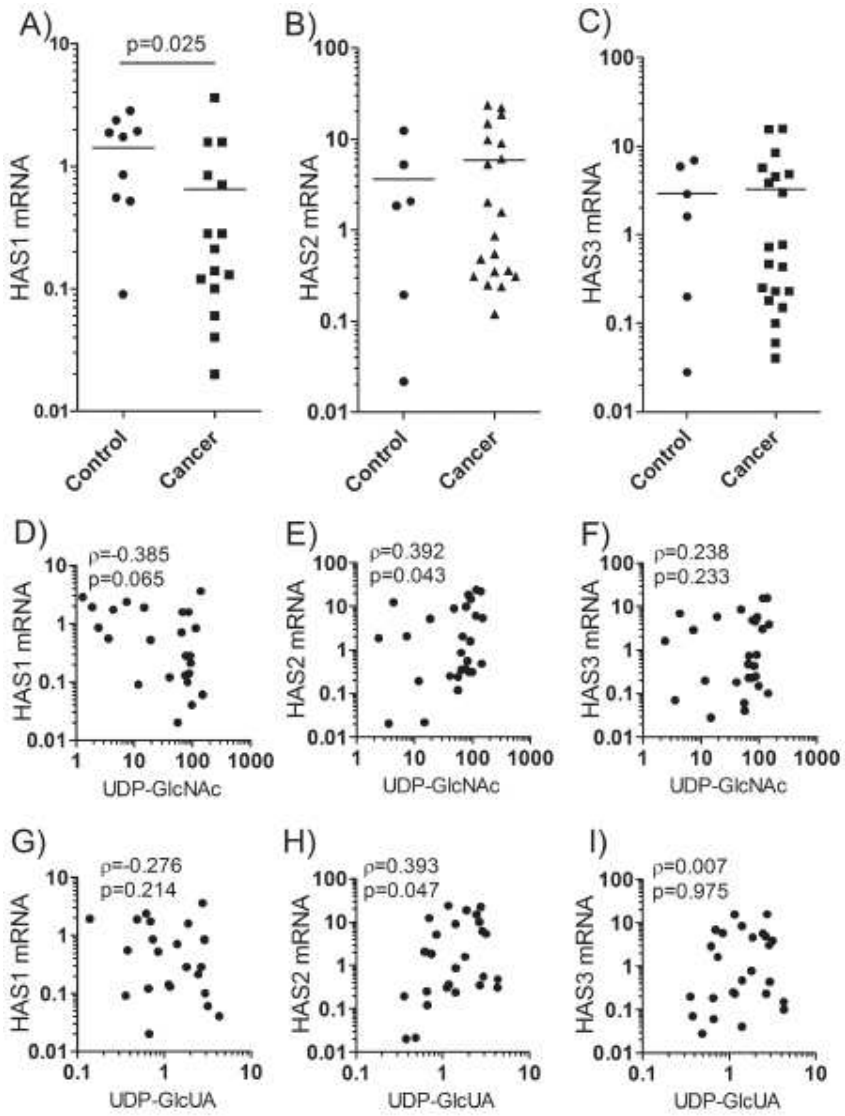

Figure 6

“UDP-sugar accumulation drives hyaluronan synthesis in breast cancer”

by S. Oikari, T. Kettunen, J. Häyrinen, A. Masarwah M. Sudah, A. Sutela, R. Vanninen, M. Tammi and P. Auvinen

Highlights:

- This is the first quantitative assay of UDP-sugars in malignant human breast tumors
- Several-fold increases of UDP-sugars take place in breast cancer
- UDP-GlcNAc increase is most drastic, likely due to the elevated expression of GFAT2
- Hyaluronan content correlates with UDP-sugars but not with HAS1-3 mRNA levels
- Glucose flux into UDP-sugars offers a new mechanistic insight into tumor hyaluronan accumulation