Immunosuppressive Glycodelin A is an independent marker for poor prognosis in endometrial cancer

Miriam Lenhard1,*, Sabine Heublein2, Christiane Kunert-Keil3, Thomas Vrekoussis2, Isabel Lomba2, Nina Ditsch1, Doris Mayr4, Klaus Friese1,2 and Udo Jeschke2

Abstract

Background: Knowledge on immunosuppressive factors in the pathogenesis of endometrial cancer is scarce. The aim of this study was to assess Glycodelin (Gd) and its immunosuppressive isoform Glycodelin A (GdA) in endometrial cancer tissue and to analyze its impact on clinical and pathological features and patient outcome.

Methods: 292 patients diagnosed and treated for endometrial cancer were included. Patient characteristics, histology and follow-up data were available. Gd and GdA was determined by immunohistochemistry and in situ hybridization was performed for Gd mRNA.

Results: Endometrial cancer shows intermediate (52.2%) or high (20.6%) expression for Gd in 72.8%, and GdA in 71.6% (intermediate 62.6%, high 9.0%) of all cases. The glycosylation dependent staining of GdA is tumour specific and correlates with the peptide-specific Gd staining though neither of the two is associated with estrogen receptor, progesterone receptor or clinic-pathological features. Also Gd protein positively correlates with Gd mRNA as quantified by in situ hybridization. Gd positive cases have a favourable prognosis (p = 0.039), while GdA positive patients have a poor outcome (p = 0.003). Cox-regression analysis proofed GdA to be an independent prognostic marker for patient survival (p = 0.002), besides tumour stage, grade and the concomitant diagnosis of hypertension.

Conclusion: Gd and GdA are commonly expressed in endometrial cancer tissue and seem to be of relevance in tumourigenesis. They differ not only in glycosylation but also in their biological activity, since only GdA holds prognostic significance for a poor overall survival in endometrial cancer patients. This finding might be explained by GdAs immunosuppressive capacity.

Keywords: Endometrial cancer, Glycodelin, Glycodelin A, Immunohistochemistry, In situ hybridization, Prognosis

Background

Endometrial cancer is the fourth common carcinoma in women following cancer of breast, colon and lung and accounts for 5.6% of all malignancies [1]. The diagnosis of endometrial cancer is typically made at postmenopausal age [2] and its 5-year survival ranges between 75 and 83% [2].

Some risk factors for the development of endometrial cancer have been described [3-8], though the exact mechanisms in tumourigenesis are by far not explained. A fast tumour progression is most likely favoured by local immunosuppression, which decreases the body’s own anti-tumour immunoreactivity. Until today little is known about tumour induced, local immunosuppression in endometrial cancer.

Glycodelin (Gd), also known progestagen-associated endometrial protein, is a glycoprotein with immunosuppressive capacity, which is mainly produced in reproductive tissue [9,10]. Four different isoforms have been described: GdS (in seminal vesicles and seminal plasma) [11], GdA (in endometrium/decidua, amniotic fluid, maternal serum) [12,13], GdF (in follicular fluid und oviduct) [14] und GdC (in the cumulus oophorus) [15].
The isoforms share a common protein backbone but differ in glycosylation and biological activity [16,17].

GdA holds several immunosuppressive abilities, which are best characterized in reproductive medicine [18]. These include the suppression of lymphocyte proliferation and inhibition of T- and B-cell activity [19-21]. Moreover, the induction of apoptosis via GdA has been investigated [22].

Recently, we found GdA to be of prognostic significance in ovarian cancer [23]. So far, there are very few results on endometrial cancer cells and Gd or GdA [24] and no clinical data on endometrial cancer. Therefore, the aim of this study was to assess the expression of Gd on mRNA and protein level. Further, we aimed to specify the proportion of the immunosuppressive glykomodification GdA in tissue samples of a large cohort of endometrial cancer patients by using an extensively validated anti GdA antibody. Finally, we aimed to analyse the impact of Gd/GdA positivity on clinical and pathological features including patient outcome.

Methods
Patients
Formalin fixed paraffin embedded (FFPE) tissue of 292 endometrial cancer patients (Table 1) was available. Most patients presented with early stage disease at primary diagnosis (Table 1). 72.6% of patients (n = 212) showed a Type I carcinoma with endometrioid histology. Among the remainder there were 7.9% with serous, 4.1% with mucinous, 1.7% with clear cell histology and 0.3% with squamous cell histology. 11.6% were classified as mixed and 1.7% as undifferentiated carcinomas. Patients were also evaluated for concomitant diseases and presented with hypertension in 39.7%, obesity in 30.5% and diabetes in 11.3% of all patients.

Assay methods
Immunohistochemistry (IHC) staining has been described previously by us [23,26,27]. Glycosylation dependant staining differences were assessed using the polyclonal Gd and the monoclonal GdA antibody (A87-B/D2) [28]. Specificity of GdA binding was analyzed by Western blot analysis [29-31]. This antibody is suitable for the detection of GdA in endometrial tumour tissues [26]. Our former investigation showed that A87-B/D2 seems to be less restricted to GdA carbohydrate structures than other monoclonal antibodies made in our laboratories, although none of the three monoclonal antibodies recognize GDS or other pregnancy-related glycoproteins such as hCG or transferrin isolated from amniotic fluid [26].

Formalin fixed paraffin embedded tissue sections were dewaxed with xylol and endogenous peroxidase activity was quenched by dipping in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 min. Then sections were rehydrated in descending concentrations of alcohol. For GdA staining epitope retrieval was performed in a pressure cooker using sodium citrate buffer (5 min, pH 6.0). Following PBS washes samples were blocked as described in Table 2 and incubated with the primary antibodies (Table 2). Then samples were further processed as per manufacturer’s instructions. Finally, immunoreactivity was visualized using diaminobenzidine (Dako, Glostrup, Denmark), slides were counterstained using haematoxylin, dehydrated in ascending concentrations of alcohol, xylol treated and covered. Positive (placenta tissue) and negative (species matched pre-immune sera) controls were always included in the analysis (Additional file 1).

Preparation of riboprobes
Preparation of riboprobes was performed as described previously [27,33]. In short, a 227-bp fragment of the Gd cDNA (positions 41 to 268) was cloned into the EcoR1 restriction sites of pBluescript SK (Stratagene, Amsterdam, The Netherlands) and labelled with digoxigenin (DIG) by in vitro transcription using the DIG RNA labeling Kit (SP6/T7; Roche Biochemicals, Mannheim, Germany). The antisense cRNA probe binds in situ to Gd-mRNA and was utilized for Gd-mRNA detection. The sense cRNA probe was used as negative control.

In situ hybridization
Non-radioactive in situ-hybridization (ISH) analysis of Gd was performed on paraffin sections as described previously [27,28,34]. Briefly, paraffin sections were deparaffined, rehydrated and permeabilized by pepsin digestion (750 mg/ml pepsin in 0.2 M HCl, 37°C, 30 min). Postfixation (paraformaldehyde 4%, 20 min, 4°C) was followed by acetylation using 0.25% acetic anhydride in triethanolamine (0.1 M, pH 8.0, 15 min). After dehydration in an ascending series of alcohol, the sections were hybridized for 16 hr (56°C) in a solution containing 50% formamide, 50% solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0), 0.5% blocking reagent, 210 mg/ml t-RNA derived from E. coli MRE 600, and 125 ng DIG-labeled cRNA probe. After washing with decreased concentrations of SSC (203 SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.4), sections were incubated 1 hr with blocking reagent (all from Roche Biochemicals).

Bound riboprobe was visualized by incubation with alkaline phosphatase-conjugated anti-DIG antibody (Roche Biochemicals) and subsequent substrate reaction using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue-tetrazolium chloride [27,28,34].

Specimen characteristics
All tissue samples (n = 292) were gained at surgery in patients who had been treated for primary endometrial
Table 1 Patient characteristics: Immunohistochemical staining for oestrogen receptors (ER) (ER alpha, ER beta) and progesterone receptors (PR) (PR-A and PR-B) were performed and analysed as previously published by our research group [25]

| Grade (%) | (n = 287) | 1 | 147 (51.2) |
|-----------|-----------|---|-------------|
|           | 2         | 93 (32.4) |
|           | 3         | 47 (16.4) |
| FIGO stage (%) | (n = 292) | I | 219 (75.0) |
|           |           | II | 21 (7.2) |
|           |           | III | 44 (15.1) |
|           |           | IV | 8 (2.7) |
| Histology (%) | (n = 292) | Endometrioid | 212 (72.6) |
|           |           | Serous | 23 (7.9) |
|           |           | Clear cell | 5 (1.7) |
|           |           | Mucinous | 12 (4.1) |
|           |           | Squamous cell | 1 (0.3) |
|           |           | Mixed | 34 (11.6) |
|           |           | Undifferentiated | 5 (1.7) |
| Patient age ± sem [y] (range) |             | 65.1 ± 0.6 (35.6-88.1) |
| Deaths (%) |             | 160 (54.8) |
| Survival ± sem [y] (95% CI) |             | 13.6 ± 0.5 (12.6-14.6) |
| Follow up ± sem [y] (95% CI) |             | 13.8 ± 0.3 (13.1-14.5) |
| Glycodelin (%) (n = 291) | Low | 79 (27.1) |
|           | Intermediate | 152 (52.2) |
|           | High | 60 (20.6) |
| Glycodelin A (%) (n = 289) | Low | 82 (28.4) |
|           | Intermediate | 181 (62.6) |
|           | High | 26 (9.0) |
| ER alpha (%) (n = 292) | Positive | 133 (45.5) |
| ER beta (%) (n = 292) | Positive | 40 (13.7) |
| PRA (%) (n = 292) | Positive | 121 (41.4) |
| PRB (%) (n = 292) | Positive | 134 (45.9) |
| Co-morbidities | Hypertension (%) | 116 (39.7) |
|           | Diabetes (%) | 33 (11.3) |
|           | Obesity (%) | 89 (30.5) |
| Lymphangiosis (%) (n = 292) | Positive | 27 (9.2) |
|           | Negative | 263 (90.1) |
|           | Unknown | 2 (0.7) |
| Hemangiosis (%) (n = 292) | Positive | 8 (2.7) |
|           | Negative | 281 (96.2) |
|           | Unknown | 3 (1.0) |
| Radiotherapy (%) (n = 292) | Yes | 116 (39.7) |
|           | No | 170 (58.2) |
|           | Declined | 6 (2.1) |

Statistical analysis methods

Statistical analysis was performed using SPSS 20.0 (PASW Statistic, Ehningen, Germany). The non-parametric Kruskal-Wallis rank-sum test and for pairwise comparisons the non-parametric Mann–Whitney-U rank-sum test were used to test for differences between groups. Correlation analysis was performed using Spearman correlation. For the comparison of survival times, Kaplan-Meier curves were drawn. The chi-square statistic of the log-rank test was calculated to test differences between survival curves for significance. Multivariate analysis for prognostic value was performed using the Cox-regression model. Mean values are displayed ± standard error and p values below 0.05 were considered statistically significant.

Immunohistochemical staining was assessed using a semiquantitative immunoreactive score (IRS) according to Remmele and Stegner [35]. The IRS, ranging from 0 to 12.
Gd mRNA was neither statistically associated with histological types (Figure 1). In the current study positivity for Gd expression was detected among histological tumour subtypes (Figures 2, 3 and Additional file 3). The most common histological subtypes (endometrioid and serous) show median GdA expression of IRS 6.0, and the mixed cell type (median IRS 6.0; mean IRS 5.74 ± 0.76) and the serous (median IRS 6.0; mean IRS 5.74 ± 0.76) and the undifferentiated histological subtype (median IRS 8.0; mean IRS 7.80 ± 0.49, Additional file 3), followed by the serous (median IRS 6.0; mean IRS 5.9 ± 0.23), the serous (median IRS 6.0; mean IRS 5.74 ± 0.76) and the mixed cell type (median IRS 6.0; mean IRS 4.97 ± 0.60), though differences among Gd expression and the histological subtypes did not reach statistical significance (p > 0.05) (Figures 2 and 3). The most common histological subtypes (endometrioid and serous) show median GdA expression of IRS 6.0. Also, no statistically significant differences in GdA expression were observed among the different histological subtypes (p > 0.05) (Figures 2, 3 and Additional file 3). Interestingly, there is a significant reduction in Gd expression observed from FIGO III to FIGO IV (p = 0.044) (Figure 3). However, overall Gd/GdA immunoreactivities comparing cases of low vs. high FIGO stage were not significantly different (Additional file 4). There were no significant differences in Gd and GdA expression between different tumour grades (Figure 3). Immunoreactivity of Gd or GdA staining was not significantly different comparing cases being negative vs. positive for ERs, PRs or co-morbidities.

**Prognostic value**

Statistical analysis was also performed to test for a prognostic value of Gd or GdA expression. Univariate Kaplan
Meier analysis revealed a good prognosis for intermediate and high Gd expression (p = 0.039) (Figure 4A). In contrast, highly positive GdA endometrial cancer patients had a poor outcome compared to intermediate and low GdA expression (p = 0.003) (Figure 4B). Gd mRNA expression was not significantly associated with patients’ outcome.

Besides tumour stage, grade and the concomitant diagnosis of hypertension (each p < 0.05), Cox-regression analysis (Table 3) showed GdA to be an independent prognostic marker for patient survival (p = 0.002, 95% CI 1.362-3.943).

**Figure 1** Gd mRNA (PAEP) was detected in endometrial cancer tissue by in situ hybridization. Representative microphotographs of Glycodelin (Gd) mRNA (PAEP, progestagen-associated endometrial protein) as detected by in situ hybridization in different histological subtypes (A-C) of endometrial cancer tissue are shown. Samples were treated with an antisense riboprobe recognizing Gd mRNA (A-C) or with the complementary sense riboprobe as a negative control (insert in A), respectively. Mean optical density of Gd mRNA signal has been quantified in a semi-automated manner and Gd positive pixels were determined by KSRun software. Gd mRNA positivity in dependence of histological subtype (D), FIGO stage (E) and grading (F) is illustrated using box plot diagrams. Scale bar in C represents 100 µm and refers to A-C.

**Discussion**

Endometrial cancer can be subdivided into two histological subtypes, the estrogen-associated Type I and the estrogen-independent Type II carcinoma [36,37]. The most common cause for endometrial Type I carcinoma is thought to be an excess of estrogens, which are inadequately antagonized by gestagens [38]. Therefore obesity, polycystic ovarian syndrome, menopausal hormone use are associated with a higher risk for endometrial cancer [3-5]. The Type II carcinoma, which comprises mostly the serous and clear cell histological subtypes, is

**Figure 2** Glycodelin and Glycodelin A protein was detected in endometrial cancer tissue by immunohistochemistry. Representative microphotographs of Glycodelin (Gd, A-C) and its immunosuppressive glyco-variant Glycodelin A (GdA, D-F) as detected by immunohistochemistry in different histological subtypes of endometrial cancer tissue are shown. Pan-Glycodelin as well as its immunosuppressive glyco-variant Glycodelin A was found to be predominantly produced by epithelial components of endometrial carcinomas. Scale bars in F represent 100 µm and refer to A-F.
known to metastasize more often and to have a worse survival. In contrast to endometrial Type I carcinomas estrogen dominance does not seem to be causally linked to this type of the disease, rather higher age and previous radiation therapy of the uterus [39].

The majority of cases are classified as Type I carcinoma and comprise the endometrioid adenocarcinomas. In literature it accounts for 75-85% of all adenocarcinomas [36,37,40], which is in accordance with our study population of 72.6% endometrioid tumours.

Figure 3 Glycodelin as well as its immunosuppressive glyco-variant Glycodelin A protein was analysed and quantified in endometrial cancer tissue. Quantification of Glycodelin (Gd; A, C, E) and its immunosuppressive glyco-variant Glycodelin A (GdA; B, D, F) by immunohistochemistry is shown. Gd/GdA was visualized in endometrial carcinoma tissue of different histological subtypes (A, B), FIGO stages (C, D) or tumour grades (E, F). Gd and GdA were detected by immunohistochemistry and quantified employing an immunoreactive score (IRS) ranging from 0 (lowest) to 12 (highest). Significant differences (p < 0.05) as determined by Mann–Whitney Test are indicated by #.

Figure 4 Kaplan Meier survival analyses were performed for Glycodelin and Glycodelin A in endometrial cancer patients. Overall survival of patients with low, intermediate and high Glycodelin A (A) and Glycodelin (B) protein expression as detected by immunohistochemistry is shown.
Interestingly, we found the concomitant diagnosis of hypertension to be a negative predictor in patients diagnosed with endometrial cancer. This finding is in accordance with newly published data by Nicholas et al., who reported diabetes and hypertension to adversely affect survival and demanded to give more attention to comorbidities, since they are gaining more influence on current health care and policy [41].

Though Gd has been identified in a range of different tissue types, not all of them do indeed synthesize the protein, which is made evident by the presence and absence of Gd mRNA [14,15,42,43]. Our immunohistochemistry results were confirmed by in situ hybridization showing not only the presence of Gd in endometrial cancer but also its synthesis and thus underline its role in carcinogenesis. To our best knowledge this is the first study reporting Gd to be present on both mRNA and protein level in endometrial cancer. Moreover, existence of Gd mRNA and its close correlation to Gd protein immunoreactivity suggests that endometrial cancer cells themselves possess the ability to synthesize the Gd protein. Interestingly, no significant association of Gd mRNA and the immunosuppressive Gd glyo-epitope GdA was observed, implying that GdA positivity marks a subfraction of endometrioid cancer that cannot be predicted by sole presence of Gd mRNA. Unfortunately, due to the very limited amount of tissue available protein extraction and western blot analysis, which would allow direct quantification of Gd/GdA of the same sample, was not possible.

In hormone-dependent tumours, Gd has been described to have various effects through reduced expression of oncogens and raised expression of tumour suppressor

### Table 3 Multivariate COX regression analysis: Patient survival was analysed by multivariate COX regression analysis

| Covariate                  | Coefficient (b) | [HR Exp(b)] | Lower | Upper | P-value |
|----------------------------|-----------------|-------------|-------|-------|---------|
| FIGO stage                 |                 |             |       |       |         |
| I                          | (0.000)         | (1.00)      |       |       | <0.001  |
| II                         | −3.064          | 0.047       | 0.11  | 1.92  |         |
| III                        | −3.025          | 0.049       | 0.10  | 2.33  | <0.001  |
| IV                         | −1.810          | 0.164       | 0.40  | 0.669 | .012    |
| WHO grade                  |                 |             |       |       | .023    |
| 1                          | (0.000)         | (1.00)      |       |       |         |
| 2                          | −.820           | .440        | .246  | .790  | .006    |
| 3                          | −.561           | .571        | .325  | 1.002 | .051    |
| Histology                  |                 |             |       |       | 0.068   |
| Endometrioid               | (0.000)         | (1.00)      |       |       |         |
| Serous                     | −.297           | .743        | .233  | 2.372 | .616    |
| Clear cell                 | −.069           | .934        | .260  | 3.349 | .916    |
| Mucinous                   | −1.423          | .241        | .024  | 2.403 | .225    |
| Squamous cell              | .074            | 1.077       | .260  | 4.470 | .918    |
| Mixed cell                 | 3.167           | 23.742      | 2.245 | 251.079 | .008 |
| Undifferentiated           | −1.163          | .850        | .255  | 2.828 | .790    |
| Lymph node metastasis      | −.732           | .481        | .209  | 1.109 | .086    |
| Age (≤50 y vs. >50 y)      | 1.939           | 6.953       | .909  | 53.204 | .062 |
| Diabetes                   | .451            | 1.570       | .880  | 2.800 | .127    |
| Obesity                    | −.065           | .937        | .600  | 1.462 | .774    |
| Hypertension               | .454            | 1.575       | 1.043 | 2.380 | .031    |
| Lymphangiosis              | .216            | 1.241       | .631  | 2.443 | .532    |
| Hemangiosis                | .681            | 1.975       | .476  | 8.187 | .348    |
| ER alpha                   | −.031           | .970        | .652  | 1.442 | .880    |
| PRA                        | −.156           | .855        | .572  | 1.279 | .446    |
| GdA                        | .840            | 2.317       | 1.362 | 3.943 | .002    |
| Gd                         | −.298           | .743        | .456  | 1.209 | .232    |

Significant results are shown in bold.
genes. Among these Gd induced chances are reduced tumour growth, decreased metastatic properties or decreased chemoresistance [24,44]. Hautala et al. showed glycodeolin to reduce breast cancer tumour growth in vivo [44]. Koistinen et al. transfected endometrial adenocarcinoma HEC-1B cells with Gd CDNA in both antisense and sense orientations [24]. They observed sense-transfected, Gd-producing carcinoma cells to have a reduced proliferation, morphologic changes, and altered expression of cancer-related genes in comparison to native and antisense-transfected carcinoma cells [24]. These results illustrate some aspects of Gd’s potential in gynecological cancers. In some hormone-depending tumours, Gd expression has been shown to go along with a favourable outcome, like in breast and ovarian cancer [45,46]. Results on ductal carcinoma in situ and invasive breast revealed that Gd positivity is inversely correlated with the occurrence of metastasis [45]. These data are in line with our findings, though Gd expression reached only univariate prognostic significance in Kaplan Meier analysis.

Recently histone deacetylase inhibitors (HDACIs) have been highlighted as promising new anti-cancer agents. In 2006 the HDACI suberoylanilide hydroxamic acid (SAHA, Vorinostat (rINN), Zolinza*) has been approved by the FDA for the treatment of cutaneous T-cell lymphoma and has further been evaluated in patients suffering from e.g. glioblastoma multiforme [47], non-small-cell lung [48] cancer or myelodysplastic syndroms [49]. Uchida et al. [50-52] demonstrated that SAHA is capable of up-regulating Gd in endometrial cancer and chorio- carcinoma cell lines and further that SAHA induced Gd in fact influences cell differentiation and migration in the model system employed. Since we found that Gd is significantly associated with prolonged overall survival in endometrial cancer, it remains challenging to investigate whether endometrial cancer patients might also benefit from the application of SAHA. Of course randomized and properly powered clinical trials are indispensable in order to validate this hypothesis on a clinical basis.

Depending on Gd glycosylation status, it can induce apoptosis in T cells and monocytes. These in vivo results on Gd and GdA may explain the partially contradictory results in clinical studies. In contrast to Gd, we observed a poor outcome in patients expressing the immunosuppressive isoform GdA. This result was made not only on the basis of univariate but also multivariate survival analysis and in concordance with a recently published study on ovarian cancer and GdA, where we report GdA to be a prognostic marker for poor outcome in advanced stage ovarian cancer [23]. Nevertheless, there are controversial results on Gd expression and patient survival [23,45,46,53]. These may be attributable to various mono- and polyclonal antibodies being either peptide-specific or glycosylation specific. Bearing in mind that differently glycosylated Gd isoforms may exert opposing actions may at least partially explain the conflicting research results published on this issue [54]. Functional analysis e.g. employing an endometrial cancer animal model is thus needed to further clarify the immunomodulatory actions of Gd/GdA.

**Conclusion**

In conclusion, Gd and GdA are commonly expressed in endometrial cancer tissue and seem to be of relevance in tumourigenesis. They differ not only in glycosylation but also in their biological activity, since Gd is associated with a better survival, whereas GdA holds prognostic significance for a poor outcome in endometrial cancer patients. Therefore, Gd and especially GdA might help to select patients for a more individualized tumour therapy.

**Consent**

As stated above the current study has been approved by the ethics committee of the Ludwig-Maximilians University Munich (approval number: 063–13) and has been carried out in compliance with the guidelines of the Helsinki Declaration of 1975. All specimens included in this study were left over samples collected during routine clinical diagnostics. Patient data were fully anonymised and the current study has been approved by the ethics committee of the LMU Munich.

**Additional files**

Additional file 1: Representative microphotographs of positive (A, B) and negative controls (C, D) for Gd (A, C) and GdA (B, D) are shown. Placental tissue was either incubated with the respective antibodies detecting Gd (A) or GdA (B) or with the respective species matched pre-immune sera (C, D). Scale bar in A equals 100 μm and applies to A-D.

Additional file 2: Representative microphotographs of Gd (A, C) and GdA (B, D) in strongly (A, B; high IRS) and weakly/negatively (C, D; low IRS) stained tissue samples are shown. Scale bar in A equals 100 μm and applies to A-D.

Additional file 3: Representative microphotographs of Gd (A) and GdA (B) in endometrial cancer samples of undifferentiated histology are presented. Scale bar in A equals 100 μm and applies to A, B.

Additional file 4: Representative microphotographs of Gd (A, C) and GdA (B, D) in advanced (A, B; high stage) and early (C, D; low stage) staged cases are shown. Scale bar in A equals 100 μm and applies to A-D.

**Competing interest**

All authors declare to have no financial or non-financial competing interests. There is no funding source to be disclosed.

**Authors’ contributions**

ML, CKK, IL made substantial contributions to conception, design and acquisition of data. SH and DM have made substantial contributions to analysis and interpretation of data. ND has been involved in drafting the manuscript and revising it critically for important intellectual content. KF and UJ have given final approval of the version to be published. In addition, KF and UJ have made substantial contributions to conception and design of the study. All authors read and approved the final manuscript.
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Author details
1 Department of Obstetrics and Gynecology, Ludwig-Maximilians-University Munich, Campus Grosshadern, Marchioninistr. 15, 81377 Munich, Germany.
2 Department of Obstetrics and Gynecology, Ludwig-Maximilians-University Munich, Campus Innenstadt, Maistr. 11, 80337 Munich, Germany.
3 Department of Orthodontics, Technische Universitat Dresden, Fetscherstr. 74, 01309 Dresden, Germany.
4 Department of Pathology, Ludwig-Maximilians-University Munich, Thalkirchner Str. 36, 80337 Munich, Germany.

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