

# Quantitative analysis of changes in endometrial gland morphology during the bovine oestrous cycle and their association with progesterone levels

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## Abstract

This study describes a digital technique for uterine morphometry and its application to endometrial structure during the bovine oestrous cycle. Neither the number nor the size of uterine gland ducts changed during the cycle but a reduction in total endometrial area from days 0 to 8 after oestrus led to an increase in the proportion of the endometrium occupied by gland ducts (gland duct density). This effect on day 8 was maintained to day 16. When endometrial morphology was related to circulating progesterone concentrations on days 5 and 8 of the luteal phase, no relationships were found on day 5, but on day 8, a high progesterone concentration was associated with an increased number of gland ducts. Furthermore, in animals slaughtered on day 8, a high progesterone concentration on day 5 was associated with decreased gland duct size, though a simultaneous decrease in endometrial area led to an increase in gland duct density. The results suggest that contrary to expectation, endometrial glands do not grow and regress during the oestrous cycle, although cyclic changes in endometrial area controlled by progesterone lead to changes in gland duct density.

*Reproduction* (2007) **134** 365–371

## Introduction

Morphometry has been successfully used to monitor pathological changes in the bovine endometrium (Gonzalez *et al.* 1985) and to study the effects of drugs and hormones on human endometrium (Johannisson *et al.* 1991). Recent developments in morphometrics have made the quantification of cyclic changes in endometrium feasible, and using these methods, it has been possible to identify the morphological changes occurring during the follicular and luteal phases in the human and ruminant uterus (Wahab *et al.* 1999, Dhaliwal *et al.* 2002). Furthermore, in human endometrium, glandular diameter during the proliferative and secretory phases has been correlated with oestrogen and progesterone levels (Johannisson *et al.* 1987). More recently in ruminants, morphometry has also been applied to the analysis of gland morphogenesis in animals depleted of uterine glands (Gray *et al.* 2001, Carpenter *et al.* 2003).

Changes in the cellular morphology of the bovine endometrium occurring during the oestrous cycle and in early pregnancy have been recorded in detail (Stinson *et al.* 1962, Marinov & Lovell 1968, Kojima & Selander 1970, Guillomot & Guay 1982). Broadly, these changes

reflect those observed in other species. There is an increase in the frequency of mitoses in the epithelium and stroma during the proliferative phase, when the epithelial cells become increasingly ciliated, and an increase in the branching of endometrial glands. This branching reaches a peak with the cessation of cell proliferation during the early secretory phase, when the basal cytoplasm of glandular epithelial cells becomes vacuolated with accumulating secretory material. Later, in the secretory phase, this material appears in the apical cytoplasm and fills the lumen of the glands. Despite an extensive knowledge of these changes, there is no automated technique for measurement of fluctuations in mean endometrial gland dimensions in whole uterine sections. Estimation of gland perimeter and area using digital imaging and image analysis software would represent an advance in terms of speed and accuracy (Wahab *et al.* 1999, Dhaliwal *et al.* 2002). The purpose of the present work was to provide such a technique and to use it to investigate the hormonal control of endometrial gland size.

An essential aspect of morphometric studies on endometrial glands is the selection of appropriate

parameters for measurement and estimation. Possible parameters include number, diameter and the area occupied by glands. However, while measurement of the number and characteristics of gland ducts is possible, it is not practical to quantify the size of individual glands as there is no way of determining which ducts are associated with which glands. Since numbers of gland ducts in whole endometrial cross sections can be determined by eye, without the assistance by digital image analysis techniques, changes in endometrial gland number are most frequently determined directly by counting. However, failure to assess the size of gland ducts may result in an error in estimating gland function and lead to inaccuracies in the assessment of relatively small changes in endometrial glands, for example, during the oestrous cycle. Furthermore, secretory function is presumed to depend on the number of glandular epithelial cells in each gland. Therefore, when compared with other parameters, the perimeter of each gland may serve as a more appropriate parameter to use when estimating morphological changes of glands during the oestrous cycle. The aims of the present study were to develop a digital method for the analysis of endometrial structure, to assess the perimeter of the gland as a novel parameter for morphometric evaluation of changes in endometrial gland structure, to clarify morphological changes in endometrial glands during the oestrous cycle and to investigate their association with circulating progesterone levels.

## Materials and Methods

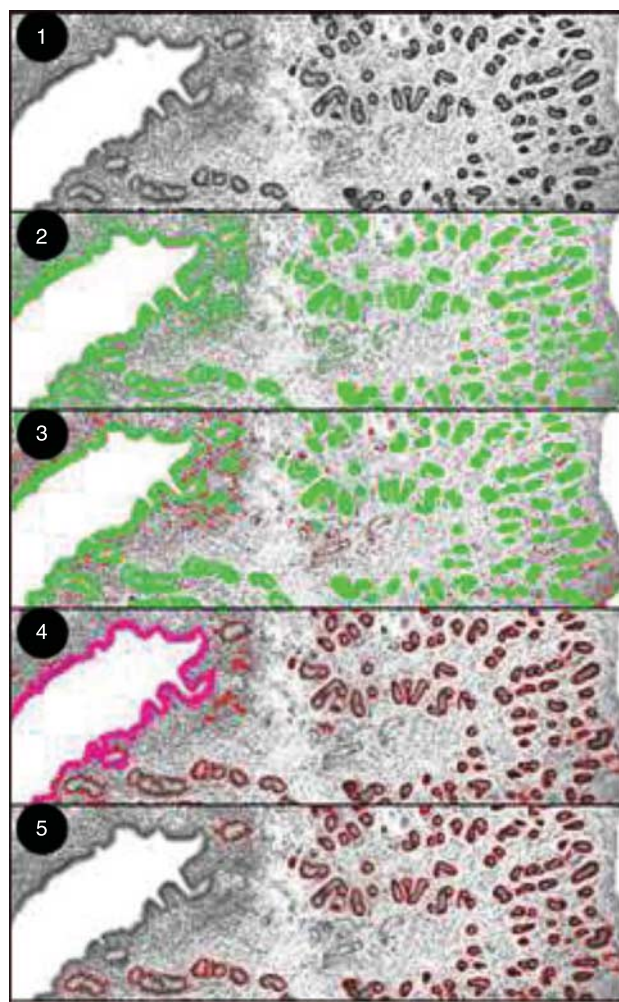
### Experimental animals

All studies were carried out in lactating multiparous Holstein/Friesian cows obtained from the University of Nottingham commercial dairy herd at the end of lactation. Oestrous cycles were synchronised by two injections of cloprostenol (estrumate; Schering-Plough Animal Health, Welwyn Garden City, UK) administered 12 days apart and the day of oestrus (day 0) was detected by 3 × daily observations. All procedures were conducted under the Animals (Scientific Procedures) Act 1986.

In study 1, uterine tissue was obtained from cows slaughtered a) on the morning that oestrus was observed, while still showing behavioural signs of oestrus (day 0;  $n=4$ ), b) during the mid-luteal phase (day 8;  $n=4$ ) and c) during the late luteal phase (day 16;  $n=4$ ). In study 2, uterine tissue was obtained from cows slaughtered on day 5 ( $n=9$ ) and day 8 ( $n=9$ ) after oestrus (day 0) and blood samples were collected on days 5 and 8 for progesterone analysis. Progesterone was measured in plasma samples by a direct RIA (Law *et al.* 1992). Assay sensitivity was 1 nmol/l, and intra- and inter-assay coefficients of variation were 13.5 and 12.2% respectively.

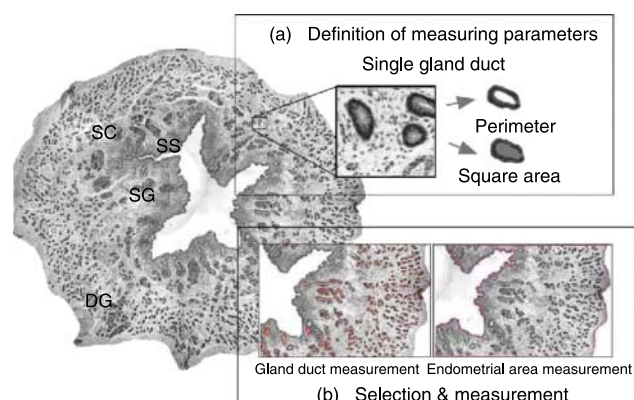
### Preparation of histological sections

Uterine horn cross sections were collected from the horn contralateral to the corpus luteum at a point 5 cm from the uterotubal junction and fixed in 10% (v/v) formalin in PBS for 24 h followed by 70% (v/v) industrial methylated spirit (IMS, Fisher, Leicestershire, UK). The contralateral horn was chosen, rather than the ipsilateral horn, in order to exclude possible local effects of ovarian hormones on endometrial structure. Tissue was dehydrated and embedded in 70% IMS for 2 h, 90% IMS for 2 h twice, 100% IMS for 2 h twice, toluene for 2 h twice and paraffin wax for 2 h twice in an automatic tissue



**Figure 1** Use of SimplePCI software to quantify endometrial gland ducts in bovine endometrium. (1) A digital image of the endometrial section is uploaded; (2) threshold ranges of staining signal are set in order to automatically select the gland ducts, indicated in green; (3) threshold ranges are set to clean background noise, indicated in red, which is automatically deleted; (4) the selected gland duct staining is assessed and any inappropriate selections marked (indicated in magenta) and removed (in the figure, the luminal epithelium has been selected for deletion); (5) the corrected selected regions are then automatically analysed to determine parameters such as the number of selected gland ducts, the perimeter of gland ducts and the area of gland ducts.





**Figure 2** Processing of an image of a cross section of bovine uterus. (a) Definition of gland ducts and parameters measured; (b) selection and measurement of total gland duct and total endometrial area. Measurements were performed automatically using SimplePCI software. SC, stratum compactum; SS, stratum spongiosum; DG, deep glands; SG, superficial or shallow glands.

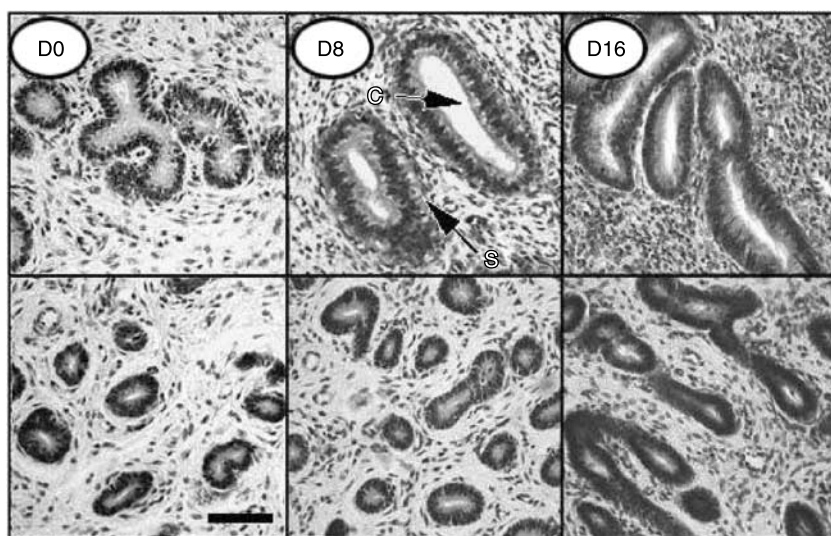
processor (TP1020, Leica, Bucks Milton Keynes, UK). Embedded uterine horn tissue was sectioned at 6–8  $\mu\text{m}$  using a microtome (HM355, Microm, Walldorf, Germany). Sections were stained by the haematoxylin and eosin method. They were rehydrated by washing in xylene for 5 min twice to remove excess paraffin followed by 100% IMS for 2 min twice, 95% IMS for 1 min, 70% IMS for 1 min and running tap water for 2 min. Rehydrated sections were then stained in Harris's haematoxylin for 5 min and excess stain removed by washing under running tap water. After staining, sections were differentiated in 1% (v/v) acetic acid/IMS for 2–4 s and washed in running tap water. The sections were then coloured blue with ammoniated water and transferred to 1% (v/v) eosin/acetic acid solution for 5 min for eosin staining, before washing under running tap water again to remove excess eosin solution. Stains were then differentiated in 70% IMS for 2–4 s, 100% IMS

for 1 min twice and xylene for 2 min twice. Sections were finally mounted in DPX (Raymond A LAMB, East Sussex, UK).

### Morphometric analyses

Uterine endometrial sections stained with haematoxylin and eosin were photographed using a Leica DMR microscope with a Hamamatsu ORCA-ER C4742-95 digital camera (Hamamatsu, Hamamatsu city, Japan), which was controlled by SimplePCI software (Compix Inc., Cranberry Township, PA, USA). Captured digital image files were assembled and edited using Adobe Photoshop 5.0 (Adobe) and analysed by SimplePCI software as follows. After capture, digital image files were uploaded into the SimplePCI software. In the Identify Objects window, a threshold range of signal was selected to identify gland ducts and a threshold range was set to remove background noise. The selected noise signals were then deleted automatically and their deletion checked manually. Gland duct numbers, areas and perimeters were then estimated automatically in the Select Measurements window. To determine endometrial area, each endometrial section was selected manually and the area determined in the Select Measurements window. Outputs were then saved in SimplePCI as a data file. The procedure is described in Fig. 1.

When endometrial glands are transected histologically, a number of gland ducts are observed which may or may not represent the same gland. For this reason in this study, we refer to gland ducts rather than glands. For each uterine cross section, analysed endometrial gland ducts were counted and the perimeter ( $\mu\text{m}$ ) and area ( $\mu\text{m}^2$ ) of individual gland ducts were measured (Fig. 2). Total endometrial area ( $\text{mm}^2$ ) was also determined, allowing calculation of gland duct density (number of gland ducts per  $\text{mm}^2$ ) and percentage area of endometrium occupied by gland



**Figure 3** Sections of superficial and deep glands on day 0, 8 and 16 of the oestrous cycle. Superficial glands in the stratum spongiosum are shown in the upper row and deep glands in the stratum compactum are in the lower row. C, ciliated epithelium cells and S, the accumulation of secretory material. Scale bar = 100  $\mu\text{m}$ .

**Table 1** Morphometric characteristics of endometrial glands in bovine endometrium during the oestrous cycle in study 1.

Parameters	Follicular phase Day 0	Luteal phase	
		Day 8	Day 16
Total number of gland ducts per uterine cross section	1829±149	1366±191	1515±209
Mean gland duct perimeter (µm)	279±19	339±37	300±42
Mean gland duct area (µm <sup>2</sup> )	3056±318	3638±619	3400±682
Total endometrial area (mm <sup>2</sup> )	72.1±5.8 <sup>†</sup>	35.7±2.5	40.6±2.1
Gland duct density (ducts/mm <sup>2</sup> endometrium)	25.3±5.3*	39.9±8.6	38.0±6.4
Gland duct area (% of total endometrial area)	7.8±0.9 <sup>†</sup>	13.0±1.3	12.0±1.8

\* $P<0.05$ ; <sup>†</sup> $P<0.001$  when compared with the luteal phase;  $n=4$  animals on each day.

ducts (%). Total endometrial area described the whole uterine endometrium including stratum compactum and stratum spongiosum, but excluded the myometrium and uterine lumen.

### Statistical analyses

All data are presented as means±S.E.M. In study 1, endometrial characteristics on days 0, 8 and 16 were compared by one-way ANOVA. In study 2, differences between days 5 and 8 were analysed by unpaired Student's *t*-test. Relationships between progesterone concentrations and endometrial characteristics were analysed by linear regression analysis. All statistical tests were carried out using Genstat version 8 (Lawes Agricultural Trust; obtained through VSN International Ltd, Hemel Hempstead, UK).

## Results

### Study 1

At a cellular level, the histological characteristics of the endometrium changed qualitatively during the oestrous cycle in the manner expected based on previous reports (Fig. 3; see the Introduction for references). Secretory material accumulated in the basal cytoplasm of the epithelial cells between days 0 and 8. Cilia were clearly evident on both superficial and deep gland surfaces on day 8, and by day 16, all glandular epithelial cells were columnar, ciliated and fully developed and stromal cells appeared at their most dense.

Application of the automated morphometric method described here showed that these changes were not accompanied by changes in gland duct perimeter or area between the three stages of the oestrous cycle (Table 1). Total number of gland ducts was also similar though total endometrial area was significantly higher in samples collected during the follicular phase ( $P<0.001$ ). Since number of gland ducts was unchanged, while endometrial area increased, both gland duct density ( $P<0.05$ ) and total area occupied by gland ducts ( $P<0.001$ ) were lower in the follicular phase than at other stages of the cycle.

### Study 2

Mean circulating progesterone levels on days 5 and 8 were  $8.5\pm0.81$  and  $18.6\pm1.85$  nmol/l respectively ( $P<0.01$ ). There were no differences between days 5 and 8 in total endometrial area, average gland duct size (perimeter or area) or gland duct density per mm<sup>2</sup> endometrium (Table 2). However, there was a trend towards a higher gland duct density (number per mm<sup>2</sup> endometrium) on day 8 ( $P<0.2$ ).

On day 5, there were no associations between plasma progesterone concentration and any of the morphometric parameters measured (Table 3). On day 8, total gland duct number increased with increasing progesterone concentration ( $R=0.71$ ;  $P<0.05$ ) though no other parameters were associated with progesterone concentration at the time of slaughter. In the animals slaughtered on day 8, a high day 5 progesterone concentration was associated with decreased total endometrial area and gland duct perimeter and area and with increased gland duct density (Table 3). Representative endometrial cross sections on day 8 from animals with high or low progesterone levels on day 5 (Fig. 4) reflect this relationship, and show that the association with progesterone level was particularly marked in deep glands. There were no significant correlations between endometrial morphology and the change in progesterone level between days 5 and 8.

**Table 2** Plasma hormone concentrations and morphometric characteristics endometrium in study 2 in animals killed on day 5 ( $n=9$ ) and day 8 ( $n=9$ ) of the luteal phase.

Parameters	Day 5	Day 8
Plasma progesterone on day 5 (nmol/l)	9.43±0.92	7.48±0.70
Plasma progesterone on day 8 (nmol/l)	–	18.63±1.85
Total number of gland ducts per uterine cross section	1202±171	1313±151
Mean gland duct perimeter (µm)	358±29	315±19
Mean gland duct area (µm <sup>2</sup> )	3961±454	3457±339
Total endometrial area (mm <sup>2</sup> )	34.9±2.5	31.4±2.9
Gland duct density (ducts/mm <sup>2</sup> endometrium)	33.7±3.4	43.3±4.5
Gland duct area (% of total endometrial area)	12.5±1.1	13.8±0.7

**Table 3** Correlation coefficients between progesterone concentrations on day 5 and 8 and morphometric parameters.

	Day 5	Day 8	
	Correlations with day 5 progesterone	Correlations with day 5 progesterone	Correlations with day 8 progesterone
Total number of gland ducts per uterine cross section	0.05	0.12	0.71*
Mean gland duct perimeter ( $\mu\text{m}$ )	0.15	-0.76*	-0.46
Mean gland duct area ( $\mu\text{m}^2$ )	0.16	-0.68*	-0.33
Total endometrial area ( $\text{mm}^2$ )	-0.34	-0.70*	0.21
Gland duct density (ducts/ $\text{mm}^2$ endometrium)	0.22	0.67*	0.45
Gland duct area (% of total endometrial area)	0.10	0.35	0.45

For animals slaughtered on day 8, both day 8 and day 5 progesterone concentrations are available (\* $P < 0.05$ ).

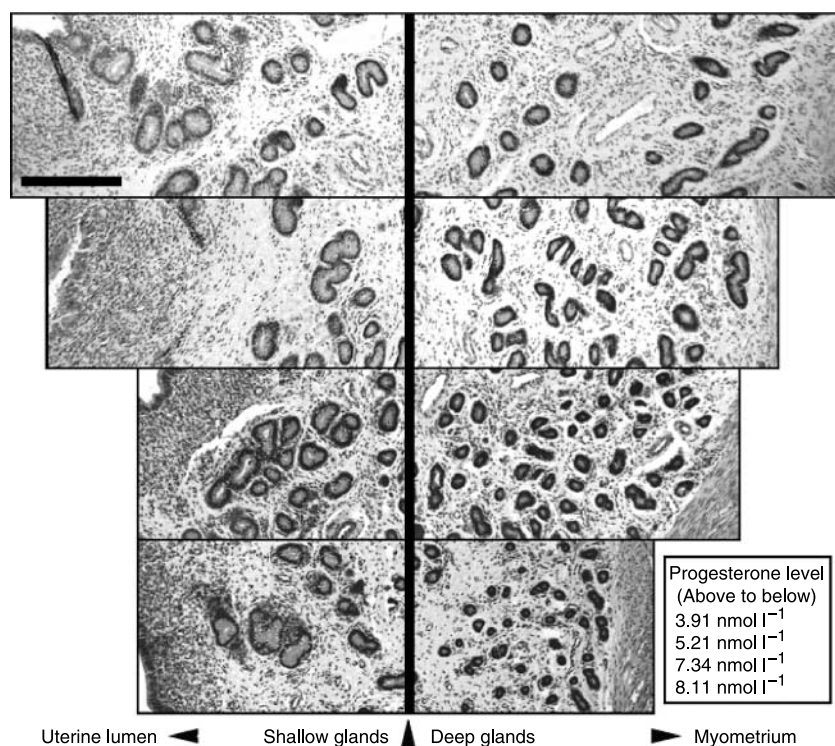
## Discussion

When assessing endometrial morphology, gland duct number has most frequently been used to quantify changes for diagnostic purposes or following clinical treatments in both humans and farm animals (Gonzalez *et al.* 1985, Johannisson *et al.* 1987, Wahab *et al.* 1999, Gray *et al.* 2001, Carpenter *et al.* 2003). However, some authors have reported that numbers of endometrial glands do not change during the natural cycle (Johannisson *et al.* 1987, 1991, Wahab *et al.* 1999).

Other morphological parameters including glandular area, diameter and volume have been used to evaluate changes in the structure and function of endometrial glands more accurately. Wahab *et al.* (1999) reported that gland area and diameter differ between the follicular and luteal phases in the human endometrium. In cattle, Dhaliwal *et al.* (2002) used two-dimensional and three-dimensional analyses to evaluate endometrial gland

changes between the follicular and luteal phases. In heifers, a number of parameters differed between the follicular and luteal phase, while in cows, gland area was the only parameter that differed. However, as endometrial gland secretion is a function of epithelial cells lining the lumen of glands, the perimeter of an individual duct rather than its cross-sectional area may be more closely associated with a gland's secretory capacity.

In the present study, the total number of endometrial gland ducts was similar in the follicular and luteal phases. Furthermore, neither the perimeter nor area of individual gland ducts changed between the follicular and luteal phases. Since total endometrial area was greater during the follicular phase, the density of gland ducts (number per  $\text{mm}^2$ ) was reduced. The decrease in endometrial area during the transition from the follicular to the luteal phase presumably reflected a reduction in the oedema associated with oestrus. This is inconsistent



**Figure 4** Endometrial samples obtained on day 8 from cows with high or low progesterone levels on day 5. All four cross sections are to the same scale (bar = 250  $\mu\text{m}$ ).



with the assumption that the endometrium proliferates to thicken at the secretory luteal phase (Roberts 1971).

Endometrial morphometry is widely used to quantify endometrial changes after hormone replacement treatment in women (Casanas-Roux *et al.* 1996, Habiba *et al.* 1998, Wahab *et al.* 1999). However, few investigations have been carried out on the relationship between steroid hormone levels and endometrial changes in the natural cycle, especially in ruminants. Though morphological changes in endometrial glands and stroma such as oedema and epithelial hyperplasia are considered to be controlled by steroid hormones, the relationship between cyclic endometrial changes and progesterone levels remains unclear. The present study clarifies several such relationships between plasma progesterone levels and endometrial changes during the secretory phase.

On day 8, total endometrial area as well as endometrial gland perimeter and area were all negatively correlated with plasma progesterone concentration measured in the same animals on day 5. This indicates that a higher concentration of progesterone on day 5 is associated with a lower total endometrial area on day 8 and suggests that endometrial thickness and gland size during the early secretory phase may be regulated by the rate at which progesterone levels rise during formation of the corpus luteum. In contrast, gland duct density on day 8 was positively associated with progesterone level on day 5. This response probably reflects the decrease in endometrial area in response to a high level of progesterone on day 5, the same number of gland ducts occupying a reduced area. Interestingly, on day 8, gland ducts in endometrium exposed to a high progesterone level on day 5 appeared to be smaller and more condensed in deep glands than in shallow ones, possibly also reflecting the reduction in endometrial area through compression by the regressing stroma. This observation, which may indicate that progesterone affects deep and shallow glands differentially, needs further investigation. Levels of progesterone receptor are low in both deep and shallow glands on day 16 (Robinson *et al.* 1999) and as far as we are aware there is no other evidence to suggest a differential sensitivity to progesterone. Together, these results suggest that endometrial gland morphology is associated with circulating progesterone concentrations during the early luteal phase.

The morphological changes observed in endometrial cells reflect the differentiation, proliferation and migration of different cell types during the proliferative and early secretory phases of the cycle (Gray *et al.* 2001). These cellular changes are controlled by steroid hormones, growth factors and the extracellular matrix (Ohtani *et al.* 1993, Johnson *et al.* 1997, Rider *et al.* 1998, Kimmins & MacLaren 1999). The extracellular matrix is involved in cell–cell interactions during cell proliferation and migration (Juliano & Haskill 1993, Gille & Swerlick 1996) and therefore plays a key role in

endometrial cellular regulation. *In vivo* and *in vitro* experiments have shown that some extracellular matrix factors such as integrin receptor  $\alpha_v\beta_3$  are regulated by progesterone and oestradiol (Bowen *et al.* 1996, Castelbaum *et al.* 1997, Somkuti *et al.* 1997, Kimmins & MacLaren 1999). Various growth factors that are regulated by gonadal steroid hormones, such as transforming growth factor- $\beta$ , influence the expression of integrin  $\alpha_v\beta_3$  in the endometrium (Ignatz *et al.* 1989, Yang *et al.* 1996, Somkuti *et al.* 1997, Flores *et al.* 1998). In some cases, these compounds are differentially expressed in various endometrial cell types (Kimmins & MacLaren 1999), providing the opportunity for differential morphological changes, such as that suggested here in shallow and deep glands. The morphological changes in endometrial thickness and gland ducts may reflect interactions between gonadal steroid hormones, growth factors and extracellular matrix components in endometrial epithelial and stromal cells.

The cyclic changes in total endometrial area observed here show that cyclic changes in endometrial gland duct density may be driven by changes in total endometrial area rather than by the proliferation and regression of glandular epithelial cells. The morphometric method described can be used to detect endometrial changes during the early secretory phase that appear to be associated with progesterone concentration. This analytical method is therefore a potentially useful tool with which to evaluate endometrial gland function.

## Acknowledgement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work

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Received 4 August 2006

First decision 1 September 2006

Revised manuscript received 12 April 2007

Accepted 20 April 2007