Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Faculty of Veterinary Medicine and Animal Science**

# Uterine health in the postparturient period of the dairy cow

## Livmoderhälsa under postpartumperioden hos mjölkkor

*Linda Hulling*

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*Linda Hulling*

***Supervisor**: Patrice Humblot, Department of Clinical Sciences*
***Assistant Supervisor**: Renée Båge, Department of Clinical Sciences*
***Assistant Supervisor**: Theodoros Ntallaris, Department of Clinical Sciences*

***Examiner**: John Pringle, Department of Clinical Sciences*

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## SUMMARY

The main objective for dairy farmers is to produce the best amount of milk from healthy cows. When doing so the optimization of reproductive efficiency is a key variable. Uterine diseases in the postpartum period are among the main reasons for this goal not to be achieved. It is estimated that up to 90% of all dairy cows have bacterial contamination of the uterus during the first two weeks after calving, something that usually is taken care of by the process of uterine involution. However, the involution is not always fully effective and a large part of the cows show signs, clinically or microscopically, of ongoing bacterial infection even after the involution is completed.

The diversity and differences in composition between the microbiota of the healthy and diseased uterus in cows are not yet fully understood. Altough several potential causative pathogens have been identified, the complete comprehension of the pathogenesis of metritis and endometritis is still unknown.

The immune defence in the postpartum uterus is mainly depending on mucosal defence systems and innate immunity. It is shown that cows suffering from negative energy balance has an impaired total white cell count as well as a reduced efficiency of the innate immune response. This may prolong the bacterial contamination and is in many cases the cause of a subclinical chronic inflammation of the endometrium that remains undiagnosed.

Today there is no diagnostic method that is considered to be gold standard to evaluate subclinical inflammation of the endometrium. This study aims to investigate the certainty in one of the diagnostic methods available and also investigate the correlation between negative energy balance and persistence of inflammation in post-partum dairy cows.

## SAMMANFATTNING

Målet med mjölkproduktion är i de flesta fall att producera mjölk från friska kor utan att gå med ekonomisk förlust, för att åstadkomma detta är reproduktionen en mycket viktig faktor. Livmodersjukdomar under den första tiden efter kalvning är en orsak till att reproduktionen, och därmed också produktionen, inte optimeras. Uppskattade siffror från forskning visar att så många som 90% av alla mjölkkor har bakteriell kontamination av livmodern under de två första veckorna efter kalvningen. Detta i sig är inte sjukdomsorsakande, då detta är något som ofta tas om hand av involutionen, dock är det inte alltid 100 % effektivt och en stor andel av korna visar tecken på en pågående infektion även efter avslutad involution.

Det är ännu inte helt klarlagt hur den normala bakteriefloran i livmodern ser ut, det är heller inte helt klarlagt vilka av de bakterier som kan hittas i en sjuk livmoder som har förmågan att vara sjukdomsorsakande. Man vet inte heller helt hur patogenesen för metrit och endometrit ser ut.

Livmoderns försvar mot patogener är till största delen beroende av slemhinnans epiteliala skyddet i kombination med det medfödda immunsvaret. Forskning har visat att kor som lider av negativ energi balans både har en nedsatt effektivitet hos de vita blodkropparna samt ett minskat antal. Detta förlänger den bakteriella kontaminationen i livmodern efter kalvning och är i många fall en orsak till subklinisk sjukdom.

Idag finns ingen diagnostisk metod som anses vara gold standard för livmodersjukdomar hos mjölkkor, detta arbete syftar till att utvärdera specificiteten hos en av de metoder som används för diagnostik samt att ytterligare undersöka de samband som finns mellan negativ energibalans och livmodersjukdomar.

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# INTRODUCTION

One of the goals to achieve sustainable management in dairy herds is to optimize reproductive efficiency. Genetic selection for milk production has been the main source for decreased fertility, but infertility is multifactorial and can be atributed to nutrition, genetics, management as well as occurrence of diseases. The effects of these are not easy to decipher as most of the time they are confounded.

Metritis as well as clinical and subclinical endometritis are common diseases in postpartum (pp) dairy cows. It is proven that cows affected with any of these diseases have both prolonged days to first service as well as longer interval from calving to pregnancy (LeBlanc *et al*., 2002; Runciman *et al*., 2008). Due to the impaired reproductive performance and decreased milk yield in combination with an increased feed intake, culling rates are increased and these diseases cause severe economical losses (Borsberry & Dobson, 1989; LeBlanc *et al*., 2002; Kasimanickam *et al*., 2004).

In addition to this, there are knowledge gaps concerning what could be considered as the normal microbiota of the uterus (Williams *et al*., 2005; Sheldon *et al*., 2010) and also the exact pathophysiology behind metritis and endometritis (Knudsen *et al*., 2016). To add further complexity to the problem there is no sample method regarded as gold standard for uterine diseases, wich increases the difficulty when assessments of new test methods are performed.

One of the objectives of this study was to assess the repeatability of the results obtained when using different protocols for the counting of immune cells. The other focus was to study the relationship between persistent inflammation and NEB by using cytobrush evaluation.

# LITTERATURE REVIEW

## Physiology

### *Uterine involution*

Uterine involution is a physiological process leading to the restauration of uterine function. The pp uterus returns usually to a normal size within 25-35 days pp. The process of involution brings gross changes, and also microscopic and molecular changes associated with tissue remodelling. The uterus undergoes physical shrinkage, including muscular and glandular atrophy, followed by necrosis and sloughing that results in endometrial regeneration. The involvement of prostaglandins and specifically of PGF2α has been recognized, however, the exact mechanisms that controlls the involution still remains unclear (Noakes *et al*., 2009).

The greatest size reduction takes place between 10-14 days pp in a healthy cow (Bajcsy *et al*., 2006). A bacterial contamination of the uterus is believed to be an important part in the process (Azawi, 2008). The contamination is believed to activate the innate immune response in the uterus which then induces inflammation and may clear, more or less quickly, the uterus from infection. It is generally hypothesized that if the immune response is inadequate the inflammation will continue and may cause uterine disease. If the inflammation persists it will in turn impair fertility and reduce the milk yield (Chapwanya *et al*., 2009).

### ***Bacterial contamination***

The uterine environment is generally considered to be sterile, with one exception, the pp period (Sheldon & Dobson, 2004; Dubuc, 2011). However, Knudsen *et al*. (2016) expressed the hypothesis that this still remains unproven by using culture-independent methods.

An estimated 90% of all dairy cows have bacterial contamination in the uterus during the first 10-14 days pp (Sheldon & Dobson 2004; Sheldon *et al*., 2008). The process of involution will then take place and restore the sterile environment in the healthy uterus (Noakes *et al* 2009).

The process of involution however is not always effective. In up to 20% of the cows the bacterial contamination of the uterus will progress into an infection and may instead cause metritis, or endometritis in 5-25% of the cows. The percentage of cows developing a subclinical infection may reach even 30-50% within 4-8 weeks pp (Dubuc, 2011; LeBlanc, 2014).

## Uterine inflammation

### ***Innate immunity***

The uterine immune defence rely mostly on mucosal defence systems and innate immunity. When a uterine infection occurs, cell surface receptors detects pathogens and initate an immune response. By activating transcription factors that, in turn, activates genes encoding for cytokines and chemokines a proinflammatory immune response is induced (Bonizzi & Karin, 2004). Neutrophils and epithelial cells then produces PAMs, an important part of the innate immune response which makes it possible for the neutrophils to detect the pathogens to ingest them (Tizard, 2009).

Chapwanya *et al*. (2009) found evidence for an increased expression of genes encoding for cell surface receptor markers CD45 that mirrored the severity of the uterine inflammation. CD45 is primarily expressed by neutrophils and the increased expression indicates recruitment of immune cells to the pp uterus. This supports previous findings proving that neutrophils are the primarily innate immune cells responding to the uterus in early pp (Gilbert *et al*., 2007; LeBlanc *et al*., 2011).

Neutrophils play an important part in the cellular defence of the uterus as they ingest opsonized particles and then kill the bacteria. The neutrophil killing ability can be measured by cytochrome c reduction and myeloperoxidase activity in the blood. By doing so it has been shown that the killing ability of neutrophils only decreased slightly prior to parturition in cows with normal uterine health and then remained relatively stable (Figure 1a). In contrast a drastic decline in myeloperoxidase activity was seen prior to parturition in cows with puerperal metritis or subclinical endometritis (Hammon *et al*., 2006). The cytochrome c reduction also declined in the same period in the cows with puerperal metritis but not in the cows with subclinical endometritis or normal uterine health (Figure 1b).

### ***Risk factors***

#### *Negative energy balance*

The fact that the dry matter intake (DMI) is decreased before parturition is well established. Despite DMI usually increases after calving, this is not sufficient to cover the energy demand due to lactation. In most of the cows, this causes a mobilization of fat tissue during the postpartum period which in turn results in the release of non-esterified fatty acids (NEFA) from adipose tissue. This tissue mobilization is in part genetic and in part environmental (Friggens *et al.*, 2007).

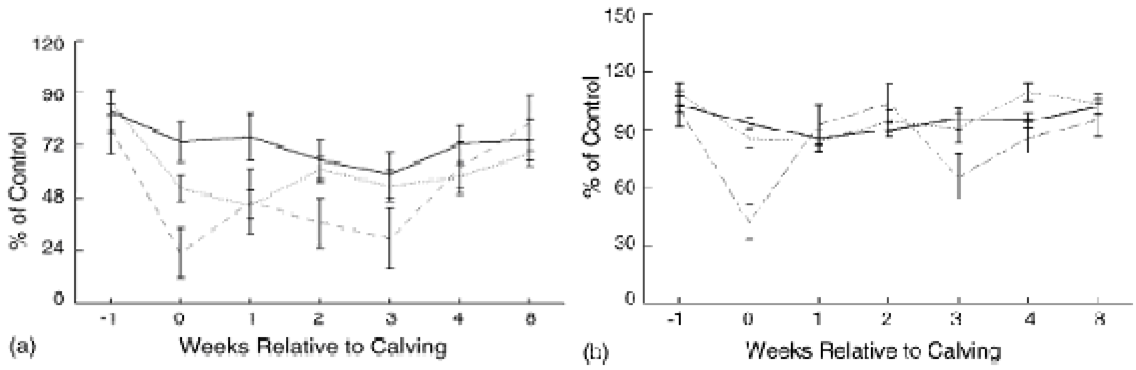


Figure 1. *Myeloperoxidase activity in blood neutrophils (a) and cytochrome c reduction in blood neutrophils (b). From dairy cows diagnosed with: (—) normal uterine health; (···) subclinical endometritis; and (--) puerperal metritis. Cytochrome c reduction and myeloperoxidase activity is shown as the percentage of response from neutrophils (Hammon et al., 2006).*

Increased levels of NEFA in combination with decreased DMI has been shown to cause a temporary immune suppression 3-5 weeks prior to calving due to a supression of myelo-peroxidase activity (Hammon *et al.*, 2006). The function of the peripheral blood neutrophils is then impaired until approximatly 4 weeks pp (Rukkwamsuk *et al*., 1999; Hammon *et al*., 2006).

In some individuals the negative energy balance (NEB) will be aggravated compared to others, meaning that cows suffering from an increased NEB prior to, or around the time of, calving are predisposed to periparturient immune suppression (Hammon *et al.*, 2006). LeBlanc *et al*. (2004) showed that cows in greater NEB were 80% more likely to suffer from retained placenta.

Wathes *et al*. (2009) found differences in the genetic expressions regarding genes activating parts of the innate immune response when comparing cows with mild and severe NEB. They showed that the total white cell count as well as lymphocyte numbers were reduced in individuals suffering from more severe NEB, thus preventing an effective immune response to the pp bacterial contamination. These differences could also be seen in the endometrium where the cows in severe NEB still had an active immune response two weeks pp, whereas the endometrium in cows with milder NEB showed signs of more advenced stages of repair. Similar changes showing the lower expression of immune genes in models inducing a more severe NEB have also been documented in endometrial tissue by Valour *et al*. (2013). This also corresponds with other studies showing that worse pp NEB can be connected to more prolonged and severe uterine inflammation and an impaired tissue repair capacity (LeBlanc *et al*., 2012).

### *Management*

Proper feeding during the late gestation is of utmost importance. Body condition score (BCS) is a five grade scale for easy assessment of the overall weight and fat deposition of the cow.

The optimal BCS around the time for calving is 3-3.25 (Roche *et al*., 2009). A deviation from the optimized BCS at calving in either direction will result in decreased milk yield and impaired fertility (Roche *et al*., 2009). A too high BCS at time of calving has been associated with reduced appetite and an aggravated NEB pp (Rukkvamsuk *et al*., 1999).

Other risk factors for uterine inflammation have also been identified, for example retained placenta (Ghavi Hossein-Zadeh *et al*., 2011).

### ***Bacterial infection***

The diversity and composition of the normal microbiota of both the healthy and diseased uterus in dairy cows are not yet fully understood. Altough several potential causative pathogens have been identified for these diseases (Williams *et al*., 2005; Sheldon *et al*., 2010), the complete comprehension of the pathogenesis of metritis and endometritis is still unknown (Knudsen *et al*., 2016).

The presence of *E. coli* and *T. pyogenes* in the uterus have long been considered important for the development of metritis and endometritis, this since they are the predominantly isolated bacteria from diseased animals (Williams *et al*., 2005; Földi *et al*., 2006; Sheldon *et al*., 2008; Ordell *at al*., 2016). *T. pyogenes* and other gram negative bacteria often follows infection with E. coli and this is thought to be connected to the development of endometritis (Sheldon *et al*., 2010; Williams *et al*., 2007), however these bacteria are sometimes also present in healthy cows (Williams *et al*., 2005).

Machado *et al*. (2012) observed *T. pyogenes* using DNA-profiling. This is in contrast to other culture independent studies that did not find *E. coli* in significant numbers in cows with uterine disease (Knudsen *et al*., 2016; Santos & Bichalo, 2012). Knudsen *et al*. (2016) could only assign less than 1% of the sequences read to either *E. coli* or *T. pyogenes*, in fact, they did not note any bacteria that occurred more frequently in diseased cows compared to healthy. However, Santos *et al*. (2011) found a significantly larger amount of *Fusobacterium* spp. in the diseased cows compared to the healthy ones.

Several studies have linked virulence factors from *E. coli* and *T. pyogenes* to uterine disease. Sheldon *et al*. (2010) found an association between a phylogenic group of *E. coli B2* and metritis. In that study the cows with metritis had *E. coli* that was more adherent and invasive to endometrial cells, this despite their lack of 16 genes typically associated with these virulence factors in *E. coli*. Bichalo *et al*. (2010) found that metritis was 6 times more likely to occur in cows with *E. coli* that expressed genes for fimH than in cows culture negative for *E. coli*. fimH is a gene encoding for adherence capability. They also showed that the risk for metritis was even higher when *E. coli* expressed fimH together with one of five other genes associated with adherence and invasion, astA, cdt, kpsMIII, ibeA and hlyA. These cows had an increased risk of purulent vaginal discharge at 4 weeks pp.

When it comes to *T. pyogenes* no virulence factors or specific high pathogenic strains that can be associated with uterine disease has been found (Silva *et al*., 2008). But it is also suggested that it is not the bacteria itself that is responsible for inducing uterine infection but rather a heat labile component, and an intact endometrium in healthy cows is thought be protective against this component (Miller *et al*., 2007).

### *Metritis*

Acute puerperal metritis (APM) occurs within 21 days after perturition and is a systemic illness cause by a profound infection situated in the uterine wall (Sheldon *et al*., 2006). APM is characterised by the presence of fever and a watery red-brown to mucopurulent discharge detectable in the vagina in combination with an abnormally enlarged uterus (Sheldon *et al*., 2006).

### *Endometritis*

Endometritis and subclinical endometritis occurs 21 days pp or later and is defined as an inflammation in the inner lining, the endometrium, of the uterus. The most common symptom is a purulent or mucopurulent discharge in the vagina originating from the uterus (Sheldon *et al*., 2006). However, in most of the cases, the infection and/or persistence of inflammation remains silent and consequently undiagnosed.

## Diagnostic methods

There is no diagnostic method that is considered to be gold standard for uterine diseases, therefore the use of multiple diagnostic methods to obtain a more certain result is commonly accepted.

### *Transrectal examination*

Transrectal palpation of the uterus is a common diagnostic technique for reproductive diseases such as metritis and endometritis (Heuwiser *et al*., 2000). The purpose of the transrectal palpation is to detect pathological changes in the uterus consistent with endometritis, such as a general enlargement, fluctuating contents and a hardened uterine wall (Callahan & Horstman, 1993). The method is commonly used in clinical practice, but the findings are subjective and it is uncertain if an actual connection exists between the symptoms and an effected reproductive ability (Sheldon *et al*., 2006). LeBlanc *et al*. (2002) showed that the only thing possible to evaluate via transrectal palpation that has any value in the diagnosis of endometritis is a cervical diameter of more than 7.5 cm after 20 days pp. Runciman *et al*. (2008) showed that if the visible status of the vagina is known, using a speculum, the findings of a transrectal palpation has none or little additional value.

To combine the transrectal palpation with an ultrasound evaluation of the uterus can improve the reliability. The ultrasound examination can provide an objective evaluation of the uterine wall and the uterine contents, and abnormal findings can therfore be indicative of uterine disease. However, the correlation between clinical and ultrasound findings is low (Sheldon *et al*., 2006).

### *Examination of vaginal discharge*

It is commonly known that purulent vaginal discharge is associated with an impaired reproduce-tive performance (Dubuc *et al*., 2011; McDougall *et al*., 2001; Runciman *et al*., 2008; Pleticha *et al*., 2009). Evaluating the discharge concerning odour, color and texture can therefore partly reflect the bacterial status of the uterus (Willams *et al*., 2005). Simply evaluating discharge visible on the outside of vulva or the tail is however not considered reliable. Using a speculum, a Metricheck device or simply a gloved hand to evaluate the discharge found in the vagina increases the reliability (Sheldon *et al*., 2006; Runciman *et al*., 2008).

Platicha *et al*. (2009) found that more cows were diagnosed with endometritis using metricheck compared to using a gloved hand. Metricheck is a device that allows evaluation of secretions found in the uterine lumen, even in small amounts. However, treating the diagnosed cows for endometritis did not result in an improved reproductive performance, suggesting that the cows in question should be regarded as false positives or that treatment is inefficient due to misdiagnosis.

The examination of vaginal discharge tends to be subjective regardless of the method used, and may therefore not be adequate in clinical use. Keeping in mind the health and welfare for the animal, but also the responsible use of antibiotics, the diagnostic method used in clinical practice should be objective and reliable (Sannmann & Heuwiser, 2014).

### *Cytology*

Uterine cytology makes it possible to quantify inflammatory cells (Dubuc *et al*., 2010; Sheldon *et al*., 2006). The proportion of neutrophils is counted and then used to assess the degree of uterine inflammation. Different cut-off values (Table 1) for inflammation have been calculated for different pp periods and sampling techniques (Dubuc *et al*., 2010; Kasimanickam *et al*., 2004; Galvão *et al*., 2009).

Cytological samples can be collected either with uterine lavage or a cytobrush. The cytobrush gives an in situ sample wich may represent the inflammatory status of the endometrium. It has also been found that cytobrush samples results in less cell distortion compared to samples collected through lavage (Kasimanickam *et al*., 2005). The uterine lavage is not useful in early pp period due to the size of the uterus wich makes the recovery of the lavage fluid difficult. The sample collected represents a dilution of the contents in the uterine lumen (Kasimanickam *et al*., 2005).

Table 1. *Cut-off values for the proportion of neutrophils in uterine cytology for diagnosis of endometritis in dairy cows*

| Number of sampled animals | Days in milk | Sampling technique | Cut-off value | Reference |
|---|---|---|---|---|
| 228 | 20-33 | Endometrial cytobrush | 18% | Kasimanickam *et al*., 2004 |
| 228 | 34-47 | Endometrial cytobrush | 10% | Kasimanickam *et al*., 2004 |
| 1044 | 35 | Endometrial cytobrush | 6% | Dubuc *et al*., 2010 |
| 1044 | 56 | Endometrial cytobrush | 4% | Dubuc *et al*., 2010 |
| 445 | 21 | Uterine lavage | 8,5% | Galvão *et al*., 2009 |
| 445 | 35 | Uterine lavage | 6,5% | Galvão *et al*., 2009 |
| 445 | 49 | Uterine lavage | 4% | Galvão *et al*., 2009 |

## MATERIAL AND METHODS

### Study layout

The study has been done in the framework of the EU project "PROLIFIC" as part of a workpackage aiming to define the impact of diet on various fertility phenotypes.

The animals included in the study were second parity cows in the Swedish Livestock Research Centre, Lövsta, in Uppsala. The cows were held in two different groups, one group on a low energy diet and one control group. The low energy group had a 50% reduction of concentrate supply aiming at a reduction in milk production by 10 kg/day. The two groups both included cows of the Swedish red breed (SRB) and Holstein which makes it possible to compare the outcome based on both diet and breed. In total 23 animals were sampled for the purpose of this study (Table 2).

The cows included started their respective diets one month before expected calving and then remained in their diet group until four months pp. Weekly assessments of DMI, body weight, BCS and milk yield was made to calculate energy balance. Blood sampling with analyses for glucose, insulin and NEFA was made every two weeks from prior to the diet start untill two months pp, then monthly untill day 120 pp. All cytobrush samples were collected at day 42 ±5 pp.

Table 2. *Distribution of Swedish red breed (SRB, n=12) and Holstein (n=11) cows in two diet groups*

| | Holstein | SRB | **Total** |
|---|---|---|---|
| Low energy | 5 | 7 | **12** |
| Control | 6 | 5 | **11** |
| **Total** | **11** | **12** | **23** |

### Cytobrush sampling

The samples in this study were collected by using a single use and sterile cytology brush (Minitube, Berlin, Germany). Before sample collection, vulva was washed and then dried with paper towels. A cytobrush in double protective sheets was used to avoid vaginal and cervical contamination. The instrument was passed through the vagina to the external cervical os; the sanitary sleeve was punctured and the instrument was advanced through the cervix into the uterine body, at which point the plastic tube was retracted far enough to expose the cytobrush. Endometrial cytology samples were collected by rotating the cytobrush in a clockwise direction against the uterine wall. The cytobrush was retracted into the plastic tube prior to removal from the uterus.

Slides for cytological examination were then prepared by rolling the cytobrush against several clean glass microscope slides, thus creating several slides per animal (from 2 to 3 per cow). The samples were air-dried and brought back to the laboratory within 2 h were they were stained with Papanicolaou stain.

## Calculation of NEB

Individual negative energy balance calculations have been performed from calving to 120 days post-partum according to methods described by Ntallaris *et al.*, 2016. From this, mean values between days 5-12 pp and days 13-20 pp was calculated giving NEB8 and NEB16 respectively.

## Cytological assessment

Initiallly the microscope slide was divided in four sections, after this each section was assessed and a representative area of each section was then selected for counting. The cytological assesment was then performed by counting 200 cells in 400x magnification in each section to determine the percentage of neutrofils. To improve the accuracy a minimum of two slides per animal were counted. All of the slides were assessed by the same person and the counting was blinded to which animal was tested.

## Statistical analysis

All statistical analyses were performed with SAS® software version 9.3.

Initally, for the statistical analysis of the cytobrush samples, the individual animals were grouped toghether in classes based on their mean neutrophil cell count obtained from the counting procedure, as described previously. This resulted in four groups, where class 1 could be regarded as healthy, class 2 could be both interpreted as suffering from subclinical endometritis or healthy depending on the reference used to determine the cut-off value for disease. Class 3 and 4 could be diagnosed as suffering from subclinical endometritis regardless of cut-off value used. A comparison was then made between the findings from each glass section against the supposed findings correlating with the respective class.

The effects of diet, breed and NEB on number of cells counted were analysed further by using the GLM procedure; backward elimination was used to build the final models, excluding non-significant ($p>0.20$) main effects or interactions.

The residuals from the observations generated from the models were tested for normal distribution. Total number of immune cells deviated from a normal distribution and was log-transformed. However, to improve clarity and to avoid redundancy, the respective log-transformed values are referred to as total immune cells throughout the remainder of this paper.

## RESULTS

### Overall description of cytological assessment

The numerical findings from the cytological assessment varied greatly between individuals as shown in Table 3. It was also clear that there were substantial numerical differences between the two slides taken from the same individual (Figure 2).

Table 3. *Raw data from the cytological assessment presented as mean number of neutrophils per 200 counted cells from two different slides per individual. The mean of each slide was calculated from 4 sectors per slide and the full count consisted in a total of 1600 cells per animal.*

| Animal ID | Mean slide 1 | Mean slide 2 | Animal ID | Mean slide 1 | Mean slide 2 |
|---|---|---|---|---|---|
| 17 | 2.3 | 1 | 61 | 3.75 | 5.5 |
| 52 | 2 | 0.75 | 55 | 4.5 | 4.75 |
| 67 | 2 | 1.25 | 95 | 5.75 | 2.5 |
| 14 | 2.5 | 1 | 32 | 7 | 1.5 |
| 18 | 1.25 | 2.25 | 40 | 7.75 | 4.5 |
| 26 | 0.5 | 1 | 63 | 8.25 | 5.25 |
| 60 | 2.75 | 1.5 | 66 | 4.5 | 9.25 |
| 96 | 2 | 1.75 | 37 | 6.25 | 9 |
| 50 | 2.75 | 1.5 | 10 | 13.5 | 11 |
| 33 | 4.25 | 3.5 | 57 | 46.75 | 12.5 |
| 74 | 6 | 2.5 | 23 | 42.5 | 52.25 |
| 36 | 4 | 5.25 | | | |

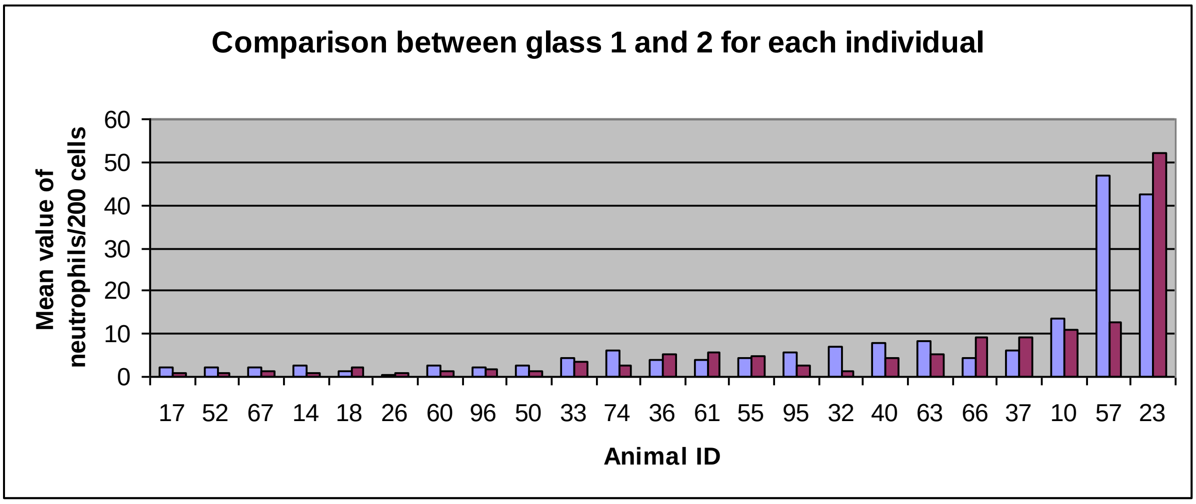


Figure 2. *Mean numbers of neutrophils identified per 200 counted cells in two glass slides per individual cow (n=23). Blue bars, glass 1; Purple bars, glass 2. The mean of each slide was calculated from 4 sectors per slide and the full count consisted in a total of 1600 cells per animal.*

## Comparisons of results obtained from different protocols of assessment

Close to 70% (16/23) of the cows were classified in Class 1, with 2.5-5 neutrophils per 200 counted cells, as presented in Table 4.

Table 4. *Cut off used for the number of neutrophils required for each class and the number of animals in each class respectively*

| Class | Number of neutrophils | Number of animals |
|---|---|---|
| 1 | 2.5-5 | 16 |
| 2 | 5-10 | 4 |
| 3 | 10-20 | 1 |
| 4 | >20 | 2 |

From the comparison made between the findings from each section of the slide against the supposed findings correlating with the respective class it could be seen that in class 1 (Table 5), 79.7% of the counted sectors was concordant with the supposed findings. 18.7% of the counted sectors in class 1 cows was instead attributed a score corresponding to class 2, and in 1.6% of the sectors wrongly diagnosed as class 3. Depending on the reference used to determine cut-off levels, this gives a 1.6-20.3% risk of overestimating the presence of uterine disease if only one sector per glass is counted compared to eight from 2 different slides (Kasimanickam *et al*., 2004; Dubuc *et al*., 2010).

Table 5. *Comparison between results from full counting (all slides, all sectors for each cow) and counting only one sector in cows from class 1. The full concordant results are seen in 1:1. In 1:2, 1:3 and 1:4, number of cases misdiagnosed from a single sector evaluation is visible, where :2, :3 and :4 refers to the wrong class attributed*

| | | 1:1 | 1:2 | 1:3 | 1:4 |
|---|---|---|---|---|---|
| Slide 1 | | | | | |
| | Sector 1 | 12 | 4 | | |
| | Sector 2 | 12 | 4 | | |
| | Sector 3 | 13 | 3 | | |
| | Sector 4 | 10 | 5 | 1 | |
| Slide 2 | | | | | |
| | Sector 1 | 15 | 1 | | |
| | Sector 2 | 14 | 2 | | |
| | Sector 3 | 13 | 2 | 1 | |
| | Sector 4 | 13 | 3 | | |
| Total | | 102 | 24 | 2 | 0 |
| % | | 79.7% | 18.7% | 1.6% | 0% |

The classes 2 and 3 were merged due to the fact that only one individual existed in class 3 wich otherwise made the analysis impossible. In this new 2-3 class (Table 6), 80% of the sectors were consistent with the supposed findings for class 2. Only 2.5% of the sectors were consistent with the supposed findings of class 3 (i.e. only one sector). From all the individual sector results that should correspond to class 2-3, 17.5% gave instead results corresponding to findings of class 1, giving a 17.5% risk for underestimating the presence of subclinical endometritis regardless of the cut-off value used (Kasimanickam *et al*., 2004; Dubuc *et al*., 2010).

Table 6. *Comparison between results from full counting (all slides, all sectors for each cow) and counting only one sector in cows from class 2-3. The full concordant results are seen in 2-3:2 and 2-3:3. In 2-3:1 and 2-3:4, number of cases misdiagnosed from a single sector evaluation is visible, where :1 and :4 refers to the wrong class attributed*

| | | 2-3:1 | 2-3:2 | 2-3:3 | 2-3:4 |
|---|---|---|---|---|---|
| Slide 1 | | | | | |
| | Sector 1 | | 5 | | |
| | Sector 2 | 1 | 4 | | |
| | Sector 3 | 1 | 4 | | |
| | Sector 4 | 1 | 4 | | |
| Slide 2 | | | | | |
| | Sector 1 | 3 | 2 | | |
| | Sector 2 | 1 | 4 | | |
| | Sector 3 | | 5 | | |
| | Sector 4 | | 4 | 1 | |
| Total | | 7 | 32 | 1 | 0 |
| % | | 17.5% | 80% | 2.5% | 0% |

In class 4 (Table 7), 75% of the results from these sectors correspond well with the supposed findings of the class. In this group of results, 12.5% were instead with the supposed findings of classes 2 and 3 respectively; giving a 25% risk of underestimating the severity of the subclinical endometritis.

The risks of misinterpreting the findings from the cytological assessment is further clarified in Figure 3, where the bars symbolise the classes obtained from the full count and the different colours of the bars shows the findings observed within each class proportionately.

Table 7. *Comparison between results from full counting (all slides, all sectors for each cow) and counting only one sector in cows from class 4. The full concordant results are seen in 4:4. In 4:1, 4:2 and 4:3, number of cases misdiagnosed from a single sector evaluation is visible, where :1, :2 and :3 refers to the wrong class attributed.*

| | | 4:1 | 4:2 | 4:3 | 4:4 |
|---|---|---|---|---|---|
| Slide 1 | | | | | |
| | Sector 1 | | | | 2 |
| | Sector 2 | | | | 2 |
| | Sector 3 | | | | 2 |
| | Sector 4 | | | | 2 |
| Slide 2 | | | | | |
| | Sector 1 | | | 1 | 1 |
| | Sector 2 | | 1 | | 1 |
| | Sector 3 | | 1 | | 1 |
| | Sector 4 | | | 1 | 1 |
| Total | | | 2 | 2 | 12 |
| % | | | 12.5% | 12.5% | 75% |

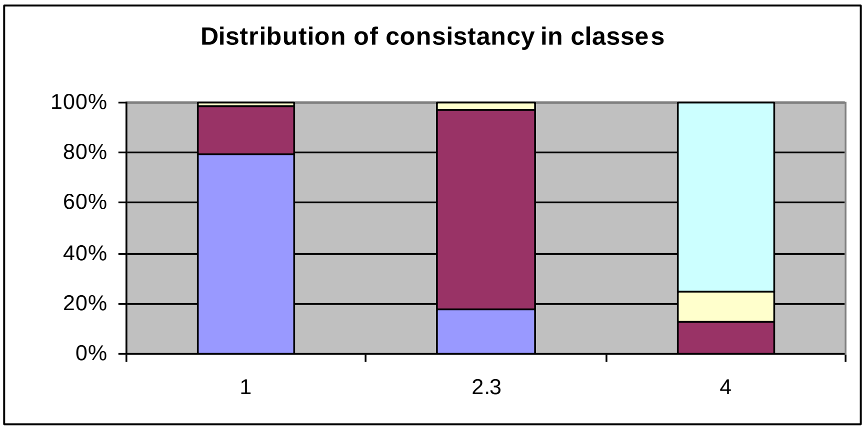


Figure 3. *The bars show the distribution of the findings from the comparison between full counting (all slides, all sectors for each cow) and only counting one sector for each cow in class 1, 2-3 and 4 respectively. Where 1, 2.3 and 4 on the x-axle refers to the classes obtained from the full counting. The colours within the bars shows the distribution between the classes, both the correct and wrongly attributed within each class: (Blue) class 1; (purple) class 2; (yellow) class 3; (green) class 4.*

### *Negative energy balance*

As large individual variations existed in response to diet, individual negative energy balance calculations have been performed from calving to 120 days post partum. No correlation could be found between breed and the total neutrophil count, neither between diet and total neutrophil count. However, the generated data from the cytobrush samples and the energy balance calculations (by days 5-12 pp, Day8) shows a significant ($p \leq 0.05$) positive ($r=0.63460$) correlation between the total number of neutrophils and the severity of the NEB. Figure 4 shows that cows with more severe NEB in early pp has a lower total neutrophil count in their endometrial samples. There was also a similar significant correlation between these two variables when comparing the total neutrophil count to the NEB between days 5-35 pp. However, in both cases it could be noted that the correlation relates only to 2 to 3 individual cows showing results above cut-off chosen to diagnose subclinical inflammation.

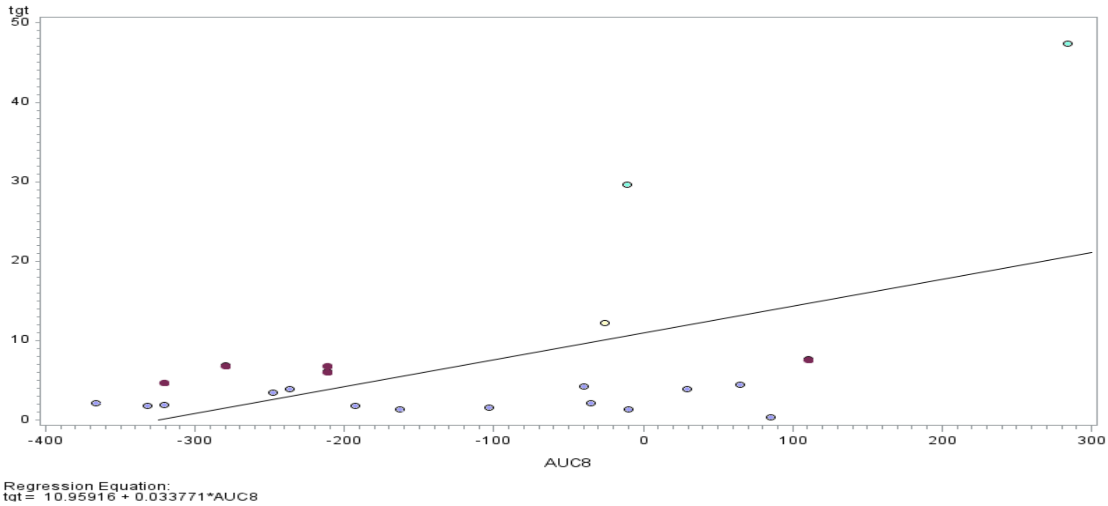


Figure 4. T*he correlation between total uterine neutrophil count and NEB at 5-12 days pp. The severity of the NEB is shown by the x-axis and the uterine neutrophil count is shown on the y-axis. The colours shows the distribution between classes based on neutrophil count as describes earlier: (Blue) class 1; (purple) class 2; (yellow) class 3; (green) class 4.*

A significant ($p \leq 0.05$) correlation between the DMI and the NEB was also found (r=0.37003). Cows in more severe NEB days 5-12 pp had a lower mean DMI at 5-35 days pp (Figure 5).

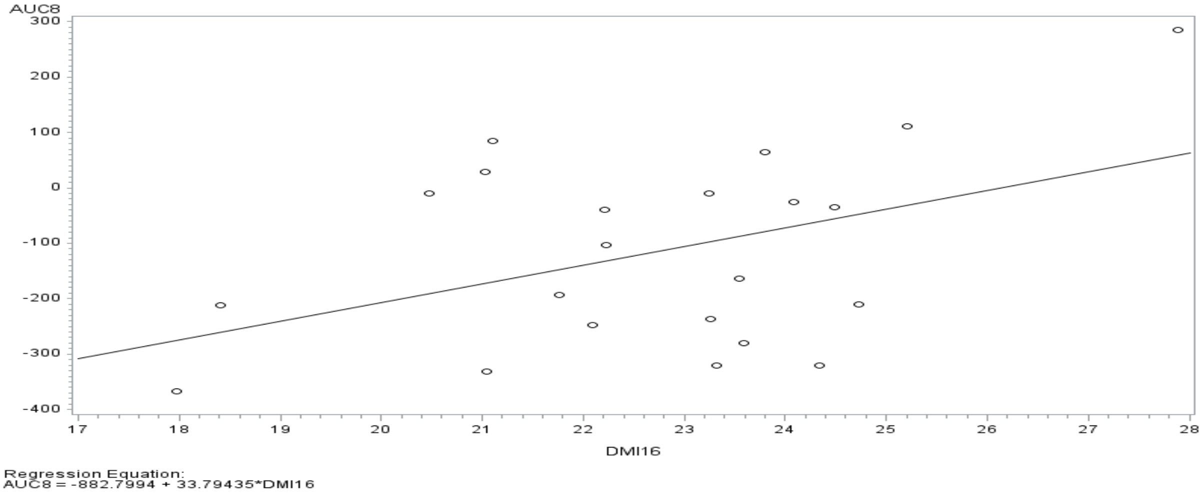


Figure 5. *The correlation between DMI and NEB. Cows in more severe NEB at days 5-12 pp have a lower mean DMI at 5-35 days pp.*

A tendency ($p>0.10$) could be seen between the total neutrophil cell count and the kg energy corrected milk (ECM), where a high total neutrophil count corresponded to a lower kg ECM than the average for the cows included in the study.

## DISCUSSION

This study focused on finding possible correlations between NEB, breed and endometrial neutrophils in pp dairy cows by comparing data from Holstein and SRB on two different diet regimes. The purpose was to investigate if breed and/or diet could be regarded as risk factors for the development of uterine disease. The intent was also to evaluate the method of cytological assessment of cytobrush samples.

### Negative energy balance

The results show that a significant correlation can be seen between the total number of neutrophils and the severity of the NEB, where the total neutrophil count declines when the NEB worsen. Although this observation is based on very few animals deviating from background noise, this is consistent with previous findings (Hammon *et al*., 2006; LeBlanc *et al*., 2004; Wathes *et al*., 2009). On the contrary, it should be noted also, that these results do not fit well with those obtained in a more recent study (Kasimanickam *et al.,* 2013). These discrepancies, could be related to the relatively low number of animals used in this study to establish such correlations. The relative level of negative energy balance, differing between studies, and which was not extremely pronounced in our study should be considered when interpreting the results.

Even though an association between NEB and uterine disease has been previously documented, there is still uncertainties regarding the determinant of disease risk between individuals within a herd or between herds held under similar conditions (LeBlanc *et al.,* 2011). However, in this study no correlation was found between the total neutrophil count and variables such as breed or diet, meaning that the determinant of disease risk is still unknown. Again, attention should be paid also to the fact that our results have been obtained from relatively few cows. From those also, only two presented high number of cells and results from these two cows have a very strong weight in the correlation observed. Due to this, these finding should be regarded as preliminary and further studies are necessary to support them.

Keeping in mind that several studies points out severe NEB as a factor for disease risk, the management ante partum is of utmost importance. Keeping the cows in a good BCS before calving and having a good feeding regime both before and after calving will reduce the risk of severe NEB pp.

### Cytology

The results from the statistical analysis of the cytobrush samples indicates that it is easy to misinterpret the findings from the cytological evaluation and thereby also easy to misdiagnose subclinical endometritis especially if results are obtained from a single measurement. When comparing counting 1600 cells distributed on four sectors and 2 glasses per animal to only counting 200 cells in one sector it was found that, depending on the cows uterine health class, the risk of the risk of misinterpreting the findings was quite high (from 17,5-25%). When using cytological tests in research regarding the prevalence of subclinical endometritis, and the treatment thereof, the most common protocol is based on counting 100 cells in one sector per individual (Kasimanickam *et al*., 2004; Dubuc *et al*., 2010). Possible erroneous findings may result from this and be even greater than in this study.

There are relatively large differences in the consistency of the findings between classes. This can probably be attributed to the small amount of animals used considering that the class with the largest deviation only was composed of two animals. One of these two animal was also the one that showed the largest individual difference between glasses in the entire study. A larger test base that made it possible to exclude extreme values would perhaps reduce the difference between classes.

In Sweden these cows would probably not be treated for uterine infections, as they often do not show any signs of disease apart from an impaired reproduction. A prolongation to this study would be to investigate the reproductive performance of these cows depending on number of cell classes. Unfortunately, the realization of samples made at later stages post-partum do not allow such studies. However, possible relationships between these results and proper uterine function will be possible from molecular analyses performed both on uterine fluid and uterine biopsies. From these it will be interesting to investigate if high number of immune cells are really pejorative for future reproduction or if they are a response to proper stimulation of the immune system as suggested from the negative relationships observed between the numbers of immune cells and the severity of negative energy balance.

## CONCLUSIONS

Within this project we have assessed the repeatability of the measurements obtained by using different protocols for counting immune cells. We found that there is a risk of misinterpreting the findings if not enough cells and/or sectors per slide are counted and that the size of the risk varies with the cow’s uterine health status.

We have also seen a correlation between persistant uterine inflammation and the severity of NEB in the early pp period. Although findings look consistent with some of previous studies in the field (Hammon *et al*., 2006), discrepancies between studies still exists while interpreting the sense of this relationship and our results obtained from very few cows with high number of immune cells/with subclinical inflammation would deserve complementary investigations.

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