# Impacts of fat mobilization on bovine endometrium function

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# Impacts of fat mobilization on bovine endometrium function


## Abstract

In cows with high milk production, negative relationships between negative energy balance (NEB), inducing non-esterified fatty acids (NEFAs) in the peripheral circulation, and reproductive performances are well documented. Three major NEFAs; oleic (OA), palmitic (PA) and stearic acids (SA) have adverse effects on reproductive cells. In this thesis, the effects of NEB on gene expression in specific endometrial cells were studied from an *in vivo* model. Full endometrial biopsies were collected from Swedish Red Breed cows with different NEB status at 80 days postpartum. The three types of endometrial cells (stroma; ST, glandular epithelium; GE, and luminal epithelium; LE) were isolated from a tissue sample by using laser microdissection. Cell type-specific gene expression was identified in those cell types by RNA sequencing. The effects of the severity of NEB on endometrial gene expression were identified and it was found that the response to NEB was specific in the different cell types. Changes in gene expression are more profound in ST in which genes encoding proteins involved in energy metabolism, cell structure and peri-implantation processes were affected. Over-expression of genes associated with inflammatory response was observed in GE, whereas under-expression of genes related to adaptive immunity was observed in LE. In an *in vitro* study, we investigated the effects of the three NEFAs on phenotypic and gene expression responses of bovine endometrial epithelial cells (bEECs) in culture. Cultured post-primary bEECs were exposed to different concentrations of OA, PA and SA or their mixture. Cell viability, proliferation, apoptosis, lipid accumulation, pro-inflammatory cytokines (IL-6 and IL-8) production were examined at 24 and 48 h. Elevated NEFA dosage decreased cell viability and proliferation, while apoptosis, lipid accumulation and cytokine production were increased. The negative effects of OA and PA were stronger than those induced by SA. Gene expression changes of bEECs induced by 300 µM of either OA or PA for 2 h were further determined by using RNA sequencing. Oleic acid induced stronger changes in gene expression than PA. The up-regulation of pathways related to energy metabolism, apoptosis and oxidative stress signaling were observed whereas significance was not reached following use of PA. In conclusion, these results indicate that metabolic stress can affect endometrial functions in various ways. The gene expression changes *in vivo* suggests that severe NEB have long term effects on endometrial cells and that the responses are specific to cell types. The results from *in vitro* studies clearly show that NEFAs, especially OA and PA, alters many functions of bEECs and are detrimental to the sub-population of epithelial cells. Taken together, the results document that metabolic imbalance during the post-partum period alters several functions in the endometrium in a specific way in the different cell types and strongly stimulate pro-inflammatory mechanisms especially in epithelial cells.



*Keywords:* cow, postpartum, endometrium, fatty acids, cell culture, laser microdissection, gene expression



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# Effekten av fettmobilisering på endometriets funktion hos ko


## Abstrakt

Hos kor med hög mjölkproduktion finns ett väldokumenterat negativt samband mellan negativ energibalans (NEB) och nedsatt reproduktiv förmåga. Biomarkörer för sådan NEB är ökade nivåer av icke-esterifierade fettsyror (non-esterified fatty acids; NEFA) perifert i blodcirkulationen. Tre huvudsakliga NEFA; oleinsyra (OA), palmitinsyra (PA) och stearinsyra (SA) har skadliga effekter på celler involverade i reproduktion. I denna avhandling studerades effekterna av NEB på genuttrycket i specifika celler i endometriet *in vivo*. Biopsier från endometriet hos SRB kor med olika grader av NEB togs 80 dagar postpartum. Tre olika celltyper (stroma; ST, glandulärt epitel; GE, och luminalt epitel; LE) isolerades från endometriet med laser mikrodissektion. Genuttrycket analyserades i dessa celltyper med RNA-sekvensering. Effekterna på genuttrycket i endometriet, beroende på graden av NEB, identifierades och en specifik respons kunde ses i de olika celltyperna. Förändringarna i genuttryck var mer framträdande i ST med en påverkan på energimetabolism, cellstruktur och processer involverade i implantationen. Ett ökat uttryck av gener involverade i inflammation detekterades i GE, medan minskat uttryck observerades för gener relaterade till adaptiv immunitet i LE. Vi undersökte effekterna av tre NEFA in vitro på fenotyp samt på genuttryck hos bovina epitelceller i endometriet (bEEC). Post-primära bEEC i cellkultur exponerades för olika koncentrationer av OA, PA och SA eller för en kombination av dessa. Cellviabilitet, proliferation, apoptos, lipidackumulering samt förekomst av proinflammatoriska cytokiner (IL-6 och IL-8) undersöktes 24 respektive 48 h efter exponering. Ökad koncentration av NEFA minskade cellviabiliteten och proliferationen, medan lipidackumulering och cytokinproduktion ökade. De negativa effekterna av OA och PA var mer uttalade än effekterna av SA. Förändringarna i genuttryck hos bEEC 2 h efter exponering för 300 µM OA eller PA studerades med RNA-sekvensering. Oleinsyra inducerade större förändringar i uttryck än PA. Aktivering av signaleringsvägar involverade i energimetabolism, apoptos och oxidativ stress observerades efter exponering för OA. Inga signifikanta resultat erhölls efter exponering för SA. Sammanfattningsvis indikerar resultaten att metabol stress påverkar olika funktioner i endometriet. Förändring i genuttryck *in vivo* tyder på att grav NEB ger långvariga effekter på celler i endometriet samt att responsen är cellspecifik. Resultaten från studierna in vitro visar tydligt att NEFA, framförallt OA och PA, förändrar många funktioner hos bEEC som är skadliga för subpopulationer av epitelceller. Sammantaget visar resultaten att en obalans i metabolism postpartum specifikt förändrar flera funktioner med olika mekanismer i de olika cellerna i endometriet samt ger en kraftig stimulering av proinflammatoriska mekanismer framför allt i epitelcellerna.



*Nyckelord:* ko, postpartum, endometriet, fettsyror, cellkultur, laser mikrodissektion, genuttryck



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# Dedication

To my beloved parents, family, teachers, friends and animals

*“The best preparation for tomorrow is doing your best today”*
การเตรียมการที่ดีที่สุดสำหรับวันพรุ่งนี้คือการทำวันนี้ให้ดีที่สุด

H. Jackson Brown, Jr.

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# List of publications

This thesis is based on the work contained in the following Papers, referred to by Roman numerals in the text:

I **Chankeaw, W.***, Lignier, S., Raliou, M., Ntallaris, T., Richard, C., Guo, Y.Z., Sandra, O., Andersson, G., Humblot, P. & Charpigny, G. Impact of negative energy balance on global transcriptomic profiles of three endometrial cell types isolated by laser capture microdissection in postpartum dairy cows. (manuscript)

II **Chankeaw, W.***, Guo, Y. Z., Båge, R., Svensson, A., Andersson, G., & Humblot, P. (2018). Elevated non-esterified fatty acids impair survival and promote lipid accumulation and pro-inflammatory cytokine production in bovine endometrial epithelial cells. *Reproduction, Fertility and Development, 30(12), 1770-1784.*

III **Chankeaw, W.***, Guo, Y. Z., Andersson, G., & Humblot, P. Gene expression changes induced by oleic acid and palmitic acid in bovine endometrial epithelial cells. (manuscript)

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* Corresponding author.

The contribution of **Wiruntita Chankeaw** to the Papers included in this thesis was as follows:

I Contributed to organize and performed sample collection, prepared RNA for RNA sequencing analysis, statistical analyses and data management, preformed downstream bioinformatics analysis, interpreted the results together with co-authors, and drafted the manuscript. Corresponded with the journal.

II Contributed to the conception and design of the study, performed all experiments and analyses, performed statistical analyses and data management, interpreted the results together with co-authors, drafted the manuscript and critical revised the manuscript together with co-authors. Corresponded with the journal.

III Contributed to the conception and design of the study, prepared RNA for RNA sequencing analysis, preformed downstream bioinformatics analysis, interpreted the results together with co-authors, and drafted the manuscript. Corresponded with the journal.

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of NEFAs increases the expression of many transcripts involved in lipid and carbohydrate metabolism which promotes lipid accumulation in epithelial cells. Dysregulation of energy metabolism may induce oxidative stress and lead to increased apoptosis, activates pro-inflammatory responses, lower cell survival and proliferation as well as alters cellular structures and cell adhesion and finally de-regulates networks of genes related to maternal recognition of pregnancy. Those changes may be favourable for the development of persistent inflammation and may be unfavourable for maternal recognition for pregnancy, embryo implantation and placentation. Red and blue arrows represent up- and down-regulation of cell processes respectively. 71

# Abbreviations

| | |
|---|---|
| µM | Micromolar |
| ANV | Annexin V |
| BCS | Body condition score |
| bEECs | Bovine epithelial endometrial cells |
| BHB | Beta-hydroxy butyrate |
| BOECs | Bovine oviductal epithelial cells |
| BP | Biological Process |
| BSA | Bovine serum albumin |
| CAMs | Cellular adhesion molecules |
| CAR | Caruncular |
| CC | Cellular Component |
| CCL2 | Chemokine (C-C motif) ligand 2 |
| CL | Corpus luteum |
| COCs | Cumulus-oocyte complexes |
| DEGs | Differentially expressed genes |
| EB | Energy balance |
| ECM | Energy-corrected milk |
| ECM | Extracellular matrix |
| EGFs | Epidermal growth factors |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| FA | Fatty acid |
| FABP | Fatty acid binding protein |
| FDR | False discovery rate |
| FGF | Fibroblast growth factor |
| GE | Glandular epithelial cell |

| | |
|---|---|
| GnRH | Gonadotropin-releasing hormone |
| GO | Gene ontology |
| ICAR | Intercaruncular |
| IFN-τ | Interferon-tau |
| IGF | Insulin-like growth factor |
| IGFBP | Insulin-like growth factor binding protein |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| ISG | Interferon-stimulated gene |
| JAK/STAT | Janus kinases/Signal Transducer and Activator of Transcription proteins |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LCM | Laser capture microdissection |
| LE | Luminal epithelial cell |
| LH | Luteinizing hormone |
| MAPK | Mitogen-activated protein kinase |
| MF | Molecular Function |
| min | Minute |
| MNEB | Mild negative energy balance |
| MUC | Mucin |
| NEB | Negative energy balance |
| NEFA | Non-esterified fatty acid |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| OA | Oleic acid |
| OCT | Optimal cutting temperature |
| OD | Optical density |
| PA | Palmitic acid |
| PBS | Phosphate buffered saline |
| PCA | Principle component analysis |
| PFA | Paraformaldehyde |
| PG | Prostaglandin |
| PI | Propidium Iodine |
| RFI | Residual feed intake |
| RIN | RNA Integrity Number |
| RNA-seq | RNA sequencing |
| ROS | Reactive oxygen species |
| S.E.M. | Standard error of the mean |
| SA | Stearic acid |

| | |
|---|---|
| SLB | Swedish Holstein Breed |
| SNEB | Severe negative energy balance |
| SRB | Swedish Red Breed |
| ST | Stromal cell |
| TAG | Triacylglyceride |
| TGF-β | Transforming growth factor beta |
| TNF-α | Tumor necrosis factor-alpha |
| TPM | Transcripts per million |

# 1 Introduction

In modern dairy cow farming, it is critical for dairy producers that cows express both the ability to produce high quantities of milk and a good longevity (Berglund, 2008). This may be compromised by many factors such as infectious and metabolic diseases which can affect production efficiency, reproductive performance, and life expectancy. The postpartum period put cows at risk to get diseases (Gröhn & Rajala-Schultz, 2000). In addition, in postpartum cows, reproductive organs need to recover physiologically and be ready for the establishment of next pregnancy. In high producing cows, the incidences of reproductive failure such as delayed resumption of ovarian activity (Wathes *et al.*, 2007), and subclinical endometritis (LeBlanc, 2008), which reduce pregnancy rates (Walsh *et al.*, 2007) are strongly associated with energy deficit and metabolic stress.

## 1.1 Energy metabolism and fat mobilization in postpartum cows

Around parturition, changes in energy metabolism occur due to the high demand of glucose, amino acids and fatty acids for milk synthesis (Butler, 2005b). The substantial increase in energy demand to satisfy high milk-yield production cannot be compensated by energy intake. This phenomenon is known as negative energy balance (NEB). The dairy cows are exposed to NEB starting usually two weeks before calving and can remain in this status for 1-2 months depending on nutrition management (Adewuyi *et al.*, 2005). When the cow requires energy for milk production, fat tissue mobilization occurs to compensate the limited supply of glucose. Non-esterified fatty acids (NEFAs) are released into the circulation and are another source of energy supply. They are subsequently oxidized in the liver to produce ketone bodies (Bowden, 1971;

Jean-Blain *et al.*, 1985). In addition, under NEB status, an increase of buta-hydroxy buterate (BHB) can be observed in peripheral blood circulation as a predominant form of ketone bodies (Loor *et al.*, 2007). Body condition score (BCS), which reflects the subcutaneous fat tissue deposits, is commonly used to determine the nutritional status of cows and changes in subcutaneous fat deposits over time (Edmonson *et al.*, 1989). Lowering of BCS has been related with metabolic and endocrine changes in the postpartum cows (Busato *et al.*, 2002). Many studies have shown that energy deficit predisposes to metabolic and infectious diseases such as milk fever, ketosis, displaced abomasum, endometritis, and retained placenta during the postpartum period (Mulligan & Doherty, 2008; Ospina *et al.*, 2010). Especially, reproductive diseases lowering fertility cause serious economic losses to the dairy industry (Lucy, 2001; Dubuc *et al.*, 2011). During NEB, circulating metabolic metabolites related to fat tissue mobilization such as NEFA and BHB are increased, but also a large number of molecules including adipokines, insulin, glucose and insulin-like growth factors (IGFs) are altered (Wathes *et al.*, 2012; Kasimanickam *et al.*, 2013). These molecules can directly influence the function of reproductive organs by local mechanisms, or indirectly by controlling the somatotrophic-gonadotrophic axis (Butler, 2005a; Wathes, 2012). Regarding the effects of NEB on uterine function, chronic uterine inflammation has been observed and related to altered immune responses and tissue remodeling in the endometrium (Wathes *et al.*, 2009; Wathes *et al.*, 2011; Swangchan-Uthai *et al.*, 2013). In addition, the decrease of plasma IGF-1 concentrations, which occurs under NEB, has been strongly associated with cytological endometritis in postpartum cow (Valdmann *et al.*, 2018). However, the mechanisms by which NEB alters endometrial cell functions are poorly understood. In most studies based on full endometrial tissue, responses of the endometrium to NEB have been difficult to interpret functionally due to possible confounding effects induced by the presence of different cell types such as endothelial cells, smooth muscle cells and leukocytes (Marchi *et al.*, 2016). Additional confusion may be results from differential effect of NEB on the three main different cells types which constitute the endometrium, *i.e.*, stromal cells (ST), glandular epithelial cells (GE) and luminal epithelial cells (LE). Therefore, it was anticipated here that studying single populations of endometrial cells would possibly help to decipher the negative impacts of NEB.

## 1.2 Physiological changes of the endometrium during oestrous cycle and pregnancy

The uterus is a reproductive organ that plays various biological roles. Changes in its function through the reproductive cycle are crucial for the establishment and maintenance of pregnancy until the birth of offspring. The endometrium is the inner layer of the uterus (Fig. 1A). This is an heterogeneous and complex tissue containing various cell types, constituting three key compartments; GE, LE and ST (Fig.1B) (Ohtani *et al.*, 1993; Espejel & Medrano, 2017). Changes in morphology and molecular patterns occur during oestrous cycle and pregnancy especially under steroid hormones regulation and embryo signaling (Fazleabas & Strakova, 2002; Bazer *et al.*, 2009). In ruminants, a simple LE which covers both caruncular (CAR) and intercaruncular (ICAR) areas, act as a barrier against pathogens and activate innate and adaptive immune functions (Wira *et al.*, 2005). During apposition and attachment processes occurring at time of implantation, the blastocyst establishes first contact with LE mainly at CAR areas (Chavatte-Palmer & Guillomot, 2007) which are mediated by adhesion molecules such as selectins, integrins and mucins (Spencer & Bazer, 2004; Mourik *et al.*, 2009). LE is transformed from a simplified epithelium to a tall columnar epithelium before blastocyst attachment (Fazleabas & Strakova, 2002). In human, changes of basal membrane and desmosomes of LE were also found during the window of implantation (Sarani *et al.*, 1999). In GE, the majority of glandular endometrial cells are found in the inner part of the ICAR area of the ruminant endometrium (Filant & Spencer, 2014). Both luminal and glandular epithelium can produce prostaglandin F2α (PGF2α) under up-regulation of oxytocin signaling during late dioestrus (McCracken *et al.*, 1999; Spencer *et al.*, 2006). During peri-implantation, GE produces histotrophs containing numerous cytokines, growth factors and nutrients which are essential for conceptus survival and growth (Gray *et al.*, 2001; Mullen *et al.*, 2012). The absence of endometrial glands affects the development of LE and activity of interferon-tau(IFN-τ)-stimulated genes which are crucial for maternal-conceptus recognition signaling (Gray *et al.*, 2002). In CAR areas of the ruminant endometrium, stromal cells are more abundant and this stromal part includes also endothelial and immune cells. These areas are the first sites of superficial implantation and placentation (Filant & Spencer, 2014). Under oestradiol and progesterone control, ST directly regulates underlying epithelial cell growth and differentiation (Donjacour & Cunha, 1991; Arnold *et al.*, 2001; Donofrio *et al.*, 2008). The function of ST on prostaglandin E2 (PGE2) production has been reported and is involved in the regulation of corpus luteum (CL) function during pregnancy (Fortier *et al.*, 1988; Danet-

Desnoyers *et al.*, 1994). In addition, IFN-τ from conceptus can stimulate PGE2 production from epithelial cells during peri-implantation as well (Asselin *et al.*, 1997). Recently, molecular changes of those three endometrial cell types in response to pregnancy have been described in sows (Zeng & Bauersachs, 2019) and mares (Scaravaggi *et al.*, 2018) by using laser capture microdissection (LCM). These studies confirmed that these three types of cells expressed specific responses to the presence of a conceptus in term of transcriptomic changes. However, so far there is no information on the specific gene expression changes of the three endometrial cell types possibly induced by energy deficit and metabolic imbalance in postpartum dairy cows.

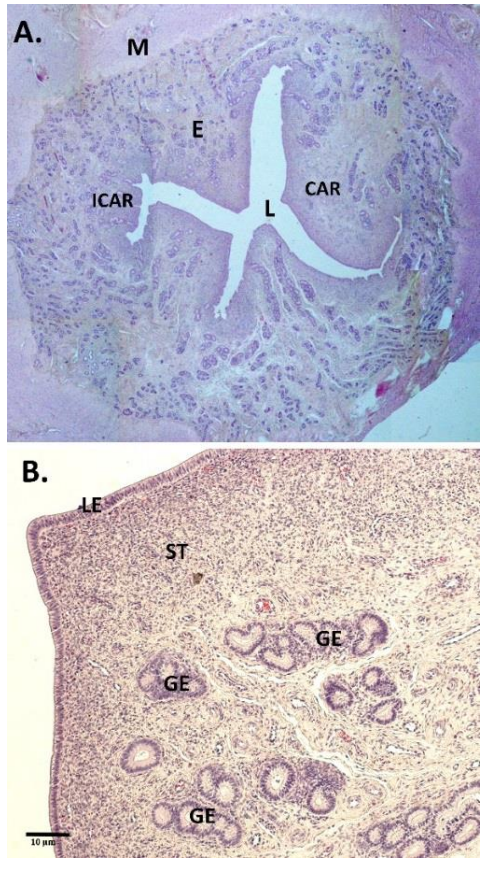


*Figure 1.* A.) Cross section of bovine uterine horn stained with haematoxylin and eosin, 25× magnifications. The abbreviations are myometrium (M), endometrium (E), intercaruncular area (ICAR), caruncular area (CAR) and lumen (L). B.) Cross section of the main three compartments of endometrial tissues; luminal epithelium (LE), glandular epithelium (GE) and stromal cells (ST), 100× magnifications, scale bar = 10 µm.

## 1.3 Molecular changes associated with conceptus-maternal interactions

The successful establishment of pregnancy relies on complex interactions between the embryo trophoblastic cells and the maternal endometrium which are regulated by ovarian steroid hormones (progesterone and oestrogen) and signaling from the conceptus inducing the maintenance of CL function (Bazer *et al.*, 2009). In addition to changes induced by these key signals, many molecules such as pro-inflammatory cytokines, growth factors and cell adhesion molecules are involved in implantation and have been used to assess endometrial receptivity to the conceptus (Achache & Revel, 2006; Spencer *et al.*, 2006).

### 1.3.1 Interferon-tau

In ruminants, IFN-τ is a key molecule secreted by the elongating conceptus that induce expression of interferon-stimulated genes (*ISGs*), by which the endometrium regulates maternal recognition of pregnancy and uterine receptivity (Gray *et al.*, 2002; Spencer & Bazer, 2004; Brooks *et al.*, 2014; Hansen *et al.*, 2017). IFN-τ also indirectly inhibits oxytocin signaling through silencing oestrogen receptor-α (*ESR1*) and stimulates IFN regulatory factor 2 (*IRF2*) gene transcription in LE and GE. This is followed by downregulation of PG receptors and lack of luteolysis (Fleming *et al.*, 2001). Moreover, IFN-τ dependent signaling activates several genes in a cell-specific manner within the endometrium during early implantation (Spencer *et al.*, 2006; Spencer *et al.*, 2008). For example, IFN-τ-inducible transcripts such as classical ISGs (*STAT1*, *STAT2*, *ISGF3G*), Guanylate Binding Protein 2 (*GBP2*), Interferon Alpha Inducible Protein 6 (*IFI6*), Interferon Induced Protein With Tetratricopeptide Repeats 1 (*IFIT1*), and *IRF2* were highly expressed in ST of pregnant ewes. In contrast, under expression of ISG15 Ubiquitin-Like Modifier (*ISG15*), major histocompatibility complex class I polypeptide-related sequence (*MIC*) and β2-microglobulin (*B2M*) mRNA expression were shown in LE during pregnancy (Spencer *et al.*, 2006). However, the precise roles of those IFN-τ-inducible mRNAs on uterine receptivity and in relation with fertility are still unclear.

### 1.3.2 Cellular adhesion molecules (CAMs)

A large number of cellular adhesion molecules such as integrins, cadherins, selectins, osteopontin, and immunoglobulins have been described in association with the implantation process in mammals (Achache & Revel, 2006; Raheem, 2018). More precisely, integrins and cadherins are well known as important

cellular adhesion molecules which play a part at an early stage, in the adhesion between the blastocyst and the uterine wall (Nose & Takeichi, 1986; Burghardt *et al.*, 2002). Integrins play a role in the interactions with the extracellular matrix (ECM) to induce cytoskeletal reorganization and mediate cell migration, proliferation and differentiation of LE and conceptus trophectoderm during the implantation process (Lessey, 1998; Burghardt *et al.*, 2002). A variety of integrins have been identified in LE and GE (Lessey *et al.*, 1994). In ruminants, the constitutive expression of integrin subunit α (v,4,5) and β (1,3,5) on apical surface of LE were observed in early pregnant ewes (Johnson *et al.*, 2001). Cadherins is a group of glycoproteins that are important the formation of cell-to-cell adhesion which is dependent on calcium-ion ($Ca^{2+}$) function (Maître & Heisenberg, 2013). However, only E-cadherin (CDH1) which is located on the lateral side of the endometrial plasma membrane has been shown to play a critical role for pregnancy establishment (Singh & Aplin, 2009). The under-expression of *CDH1* mRNA was observed in endometrial epithelial cells of pregnant rats and this change was necessary to the reorganization of epithelial cells allowing blastocyst invasion (Shih *et al.*, 2002).

### 1.3.3 Mucins (MUCs)

Mucins are glycoproteins that are expressed on the cell surface of endometrial epithelial cells. Mucin production is essential for hydration of the endometrial surface and protection from microorganisms (Brayman *et al.*, 2004). Mucin 1 (*MUC1*) has been extensively studied in the endometrium of mammals. Mucin 1 mRNA is over-expressed during oestrus and regulated by oestrogen (Meseguer *et al.*, 1998; Johnson *et al.*, 2001). Besides, increased in expression of *MUC1* mRNA has been reported in postpartum cows with uterine inflammation (Kasimanickam *et al.*, 2014). During implantation, the decreased expression of *MUC1* mRNA in LE has been suggested to contribute to increased uterine receptivity in sheep (Johnson *et al.*, 2001). On the contrary, the persistence of high expression endometrial *MUC1* mRNA may prevent the necessary interactions between trophoblast and the adhesion molecules from the apical luminal epithelium (Brayman *et al.*, 2004). For other *MUC* mRNAs such as *MUC4*, *MUC5*, *MUC6*, *MUC12* and *MUC16*, an increase in expression of these mRNAs has been related to uterine inflammation and repeat breeding in postpartum cows (Wagener *et al.*, 2017).

### 1.3.4 Growth factors

Several growth factors such as insulin-like growth factors (IGFs), epidermal growth factors (EGFs), fibroblast growth factor (FGF) and transforming growth factor beta (TGF-β) are known as key molecules that play crucial roles to promote endometrial and trophoblastic cell differentiation and maturation during implantation (Guzeloglu-Kayisli *et al.*, 2009). As reported in the dairy cow and in the human species, it is likely that the IGF system is involved in the link between nutrition and reproduction (Giudice *et al.*, 1998; Wathes *et al.*, 2011). IGF-I and IGF-II have both mitogenic and differentiation activities and are involved in early embryonic and placental development in several species (Wathes *et al.*, 1998; Giudice *et al.*, 2002). The localization of IGFs varies depending on oestrous cycle stage (Tang *et al.*, 1994; Robinson *et al.*, 2000). Also the expression of insulin-like growth factor binding protein (*IGFBP*)-*1, -2* and -*3* mRNAs are stimulated by the presence of an embryo (Robinson *et al.*, 2000), especially *IGFBP1* mRNA which has been identified as an endometrial marker of conceptus elongation in ruminant (Simmons *et al.*, 2009). Moreover, the member of EGF family which includes EGF itself, TGF-α (transforming growth factor α), HB-EGF (heparin-binding epidermal growth factor), AREG (amphiregulin) and BTC (betacellulin) have been shown to play important roles in embryo development and the implantation process in mammals (Lee & DeMayo, 2004; Guzeloglu-Kayisli *et al.*, 2009; Mucha *et al.*, 2013). Many proteins in the transforming growth factor-beta (TGF-β) family such as TGF-β1, 2 and 3 regulate uterine decidualization and have been associated to changes observed during implantation in various species (Godkin *et al.*, 1996; Ni & Li, 2017). In cattle, the activity of TGF-β family members is necessary for endometrial remodelling which is essential for implantation and placentation processes (Sugawara *et al.*, 2010).

It is now well established that the multiplicity of molecular mediators mentioned above exert complementary actions which are necessary for endometrial receptivity and embryo implantation. However, so far, a few studies observed effects of metabolic imbalance on the expression of those molecules such as IFN-τ-inducible mRNAs and cellular adhesion molecules in specific bovine endometrial cell types. The changes in expression patterns of those molecules due to metabolic imbalance may impair the maternal-conceptus crosstalk, and possibly be the source of alteration of embryonic development and/or increased embryonic loss in postpartum cows.

## 1.4 Non-esterified fatty acids (NEFAs) and their effects in the endometrium

Non-esterified fatty acids (NEFAs) are known as biological indicators of NEB, and their level starts to increase before calving (LeBlanc, 2014). In dairy cows, an increase of plasma NEFAs concentrations is observed approximately 2 weeks before calving and the levels are peaking at 1-10 days post-partum when NEB reaches a nadir (Adewuyi *et al.*, 2005; Contreras & Sordillo, 2011). The mean concentration of plasma NEFAs in positive energy balance cows was found to be around 200 µM, but was reported to be over 700 µM in severe NEB cows (Adewuyi *et al.*, 2005). High levels of plasma NEFAs are associated with increased incidences of periparturient diseases, including retained placenta and metritis (Melendez *et al.*, 2009; LeBlanc, 2014). In the bovine, NEFAs are mainly represented by three major fatty acids including the two saturated fatty acids, palmitic acid (PA; C16:0) and stearic acid (SA; C18:0), and the monounsaturated fatty acid, oleic acid (OA; C18:1cis 9) which are present in plasma, oviductal fluid and uterine fluid (Khandoker, 1997; Rukkwamsuk *et al.*, 2000; Leroy *et al.*, 2005). However, the composition of NEFAs in plasma and body fluids can vary depending on the diet and days in lactation (Leroy et al, 2005). NEFAs can be used as an energy source that help to preserve glucose for cellular metabolism used (Adewuyi *et al.*, 2005). In contrast, NEFAs have adverse effects on both somatic and reproductive cells; most particularly the saturated fatty acids (Vanholder *et al.*, 2005; Vanholder *et al.*, 2006). In addition, PA was shown to stimulate pro-inflammatory cytokine production in oviductal epithelial cells (Ohtsu *et al.*, 2017). Furthermore, different cells have different tolerance to the same fatty acid. For example, in bovine reproductive cells, PA when present at concentrations $\geq 150$ µM inhibits proliferation of granulosa cells and theca cells (Vanholder *et al.*, 2006), whereas concentrations lower than 50 µM of PA are able to decrease the development of cumulus-oocyte complexes (COCs) (Leroy *et al.*, 2005).

The effects of OA on cell functions are still controversial. Several previous studies suggested that OA protects from lipotoxic activity in COCs and myocytes (Coll *et al.*, 2008; Aardema *et al.*, 2011). In contrast, lipotoxic effects of OA have been observed at high concentrations while impairing cell viability and inducing apoptosis in hepatocytes (Vidyashankar *et al.*, 2013). However, in bovine endometrial cells, the information about the effects of those fatty acids on the phenotype and inflammatory responses of endometrial cells is scarce and changes in gene expression are poorly understood.

## 1.5 Alteration of adipokine profiles during fat mobilization

It is well documented that adipose tissue can produce some hormones and cytokines such as interleukin 6 (IL-6), chemokine (C-C motif) ligand 2 (CCL2), tumor necrosis factor-alpha (TNF-α) and more specific adipokines (or adipocytokines) leptin, adiponectin and resistin (Fantuzzi, 2005; Ouchi *et al.*, 2011). These adipokines have different functions, including pro-inflammatory or anti-inflammatory activities which contribute to pathogenesis of metabolic diseases in mammals (Ouchi *et al.*, 2011).

One of the most important is IL-6, a pro-inflammatory cytokine which is produced during the early stages of inflammation. In addition, concentrations of IL-6 are positively linked to chronic inflammation in case of obesity and insulin resistance (Kern *et al.*, 2001; Popko *et al.*, 2010), and also tumor growth by activating various pathways such as Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling (Neurath & Finotto, 2011). An increase of circulating IL-6 concentrations has been associated with plasma NEFA and BHB concentrations in postpartum cows suffering from NEB (Mansouryar *et al.*, 2018). Besides, postpartum cows which were diagnosed with endometritis showed higher expression of endometrial *IL6* mRNA compared with normal cows (Galvao *et al.*, 2011; Ghasemi *et al.*, 2012). CCL2 has also been found in adipose tissue and the over-expression of *CCL2* mRNA promoted recruitment of macrophages (Kamei *et al.*, 2006). In obese mouse, the endometrial *CCL2* mRNA was over-expressed and linked to ectopic lipid accumulation and inflammation in the endometrium (Shankar *et al.*, 2011). In addition, *CCL2* mRNA expression is increased during prostaglandin F-2α (PGF-2α)-induced luteal regression, and this is followed by activation of immune cells and impaired CL progesterone secretion (Townson, 2006). Tumor necrosis factor-alpha (TNF-α) is also a major pro-inflammatory cytokine which is secreted by activated macrophages and adipocytes. TNF-α regulates many cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism (Cawthorn & Sethi, 2008). In case of obesity in the human, TNF-α has been shown to promote lipolysis in mature adipocytes and directly interferes with the insulin signaling cascade while impairing insulin-stimulated glucose transport (Nieto-Vazquez *et al.*, 2008). In postpartum cows, an increase of circulating TNF-α concentrations was strongly related with reproductive diseases such as endometritis (Kasimanickam *et al.*, 2013), mastitis (Nakajima *et al.*, 1997) and metabolic disease (Ametaj *et al.*, 2005). Moreover, Hansen *et al.* (2004) reported that TNF-α activates prostaglandin secretion from bovine endometrial cells, and interfere with CL function and embryonic development in dairy cows.

Furthermore, in dairy cows, many studies found a relationship between several adipokines, metabolic imbalance and impairment of reproductive performances. Leptin is considered a permissive factor for reproduction, which plays an important role in the regulation of energy homeostasis and hormone secretions (Chilliard *et al.*, 2005). Decreased plasma leptin concentrations are associated to NEB in the periparturient period (Block *et al.*, 2001; Ingvartsen & Boisclair, 2001; Liefers *et al.*, 2003). Previous studies revealed the positive relationship between leptin and luteinizing hormones (LH) concentrations, and decreased leptin concentrations were associated with delayed first ovulation in postpartum cows (Kadokawa *et al.*, 2000; Kadokawa *et al.*, 2006). For the level of adiponectin, a nadir in blood circulation has been reported around calving, followed by increasing concentration during the postpartum period (Kabara *et al.*, 2014; Mellouk *et al.*, 2017). Also, the concentration of adiponectin was negatively correlated with NEFA concentrations (Kabara *et al.*, 2014) and BCS of dairy cows during the dry period (De Koster *et al.*, 2017), whereas positive correlation with insulin responsiveness to glucose and fatty acid metabolism were observed (De Koster *et al.*, 2017). On the contrary, increased concentrations of plasma resistin were found during the early postpartum and negative correlations with leptin and adiponectin concentrations were observed (Mellouk *et al.*, 2017). The negative relationship between increased resistin concentrations and follicular growth characteristic is consistent with results obtained in *in vitro* studies suggesting that resistin modulates negatively bovine granulosa cells proliferation and steroideogenesis by stimulating protein Kinase B (AKT) and p38/mitogen-activated protein kinase (MAPK) pathway in response to IGF-1 (Maillard *et al.*, 2011).

The information mentioned above show that adipocytokines and NEFAs are associated with metabolic imbalance and inflammatory responses both in humans and animals. However, so far, none of the previous studies were designed to observe direct and/or indirect effects of adipocytokines and NEFAs on molecular responses of bovine endometrial cells and how these mediators may contribute to the development of uterine inflammation in postpartum cows.

## 1.6 Comparative aspects

Elevated level of circulating NEFAs occurs both in dairy cows and humans. The NEB dairy cow is an example where molecules released from adipose tissue, including NEFAs play an important role on reproductive function (Wathes *et al.*, 2007; Mellouk *et al.*, 2017). In humans, lipolysis is related to various metabolic disorders such as obesity and diabetes (Kahn *et al.*, 2006). The consequences of

obesity associated with human infertility such as the polycystic ovarian syndrome and impaired embryonic and placental development have been documented (Jarvie *et al.*, 2010; Broughton & Moley, 2017). Effects of NEFAs on ovarian function in both women and cows are similar and in both cases, steroidogenesis is impaired (Vanholder *et al.*, 2005; Lavoie *et al.*, 2015). In the uterus, NEFAs also impaired human endometrial stromal cells (ESCs) decidualization (Mayer *et al.*, 2016) which is important for implantation and placentation. In the same way, NEFAs hampered bovine oviductal epithelial cells (BOECs) survival (Jordaens *et al.*, 2015) and induced the release of pro-inflammatory cytokines (Ohtsu *et al.*, 2017) which are associated to chronic inflammation. The similar effects of NEFAs and metabolic imbalance in the human and bovine species provide an excellent opportunity to give novel insights on the contribution of fat mobilization and NEFAs on endometrial functions, explore further their impacts in the cow and possible negative consequences for fertility in both species.

# 2 Aims and Hypotheses of the thesis

**Aims:**

- Investigate effects of negative energy balance on gene expression of three endometrial cells types harvested from endometrial tissue biopsies by using laser capture micro-dissection (**Paper I**).
- Determine effects of three single NEFAs and a mixture of NEFAs on cell viability, proliferation, apoptosis, lipid accumulation and pro-inflammatory cytokines production in bovine endometrial epithelial cells in culture (**Paper II**).
- Investigate effects of OA and PA used at high concentration on gene expression of bovine endometrial epithelial cells in culture (**Paper III**).

**Hypotheses:**

- The negative energy balance met in dairy cows would affect plasma NEFA concentrations in the early postpartum period, and these early changes would still affect specific endometrial cell types at the time planned for breeding (**Paper I**).
- NEFAs may have negative effects on the phenotype, inflammatory responses and transcriptomic profile of bovine endometrial epithelial cells in an *in vitro* model (**Paper II and III**).

# 3 Materials and Methods

## 3.1 Ethical permission

Endometrial samples were collected from living animals for the *in vivo* model. The experimental protocols were approved by the Uppsala Animal Experiment Ethics Board (application C329/12, PROLIFIC) and were followed and performed through agreements with the terms of the Swedish Animal Welfare act (Paper I). To establish endometrial cell culture followed by *in vitro* experiments, female genital organs of dairy cows were collected at slaughterhouse with permission of the local slaughterhouse, Uppsala, Sweden. No ethical permission was required (Paper II and III).

## 3.2 Study design

Two main studies, *in vivo* and *in vitro* were performed as following:

***In vivo* study**: We investigated long-term impacts of NEB on gene expression of three endometrial cell types measured from biopsies collected at planned time for breeding in postpartum dairy cows. Energy and metabolic changes during early postpartum that may influence later on uterine function and fertility were measured. Indicators of NEB were estimated from plasma NEFA concentrations and from BCS losses (Fig. 2). Two groups of cows experiencing differences in NEB were determined by energy balance profiles during the early postpartum period (Fig. 3). All biological materials were collected at the Swedish Livestock Research Centre, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. Isolation and harvesting of the three endometrial cell types from uterine biopsies by using LCM and total RNA extraction were performed at Biology of Development and Reproduction (BDR) platform, INRA, Jouy en Josas, France.

Library preparation and RNA sequencing were done at GenomEast Platform, IGBMC, Cedex, France. Bioinformatics analysis was accomplished at SLU in collaboration with BDR platform, INRA, Jouy en Josas, France.

***In vitro* study**: this study determined the effects of three NEFAs; *i.e.* oleic (OA), palmitic (PA), stearic (SA) acid and a mixture of NEFAs on the cell numbers, cell viability, cell apoptosis, lipid accumulation and pro-inflammatory cytokines (IL-8 and IL-6) production of bovine endometrial epithelial cells (bEECs) in culture. Three concentrations: 150, 300 and 500 µM of OA, PA or SA or a mixture of NEFAs (150 µM of each NEFA) were used in this experiment based on plasma NEFA concentrations found in early postpartum cows. Different responses were observed at different time points (2, 4, 6, 24 and 48 h) depending on type of variable measured (Fig. 4A, Paper II). We also observed the effects of OA and PA (at 300 µM) on gene expression of bEECs after treatment for 2 h (Fig. 4B, Paper III). All of the *in vitro* experiments, including RNA extraction and analysis of RNA quality were performed at the Department of Clinical Sciences and the Department of Animal Breeding and Genetics, Faculty of Veterinary Medicine and Agricultural Sciences, SLU. RNA sequencing was done at GenomEast platform, IGBMC, Cedex, France. Downstream analysis of transcriptomic data was carried out at SLU.

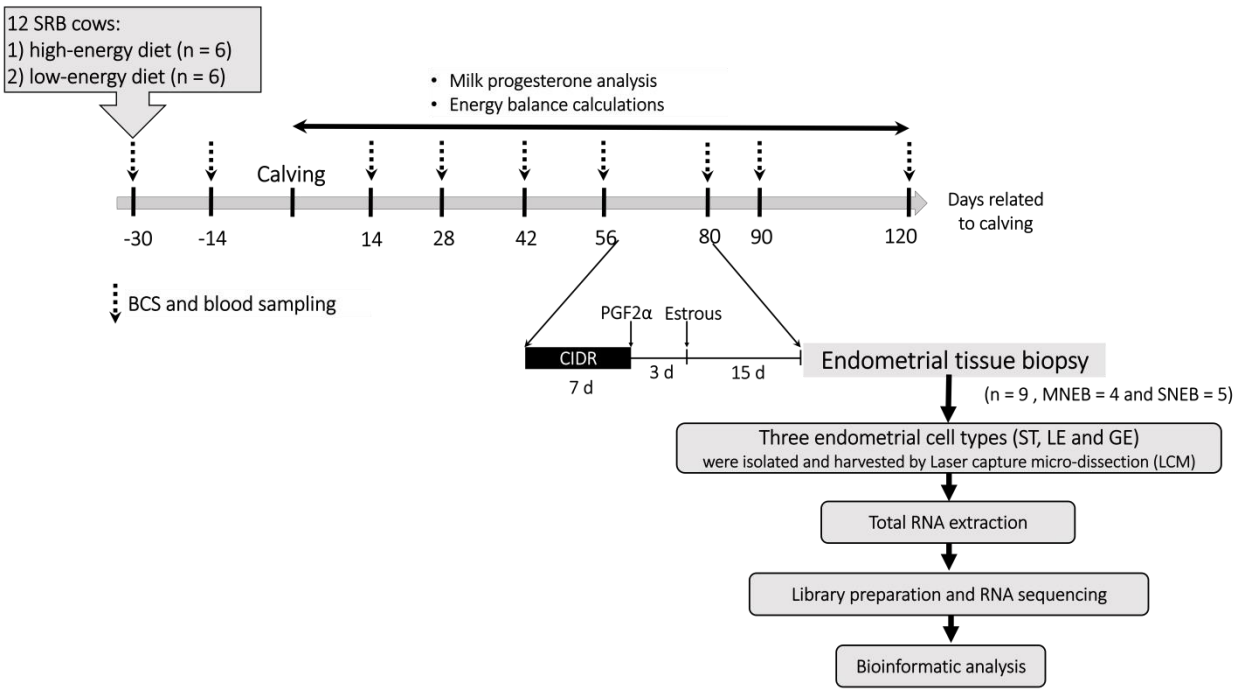


*Figure 2*. The experimental design for studying long-term impacts of negative energy balance on gene expression of three endometrial cell types. From energy balance profiles 9 cows were selected for LCM of endometrial tissue biopsies (5 mild NEB and 4 severe NEB cows). An arrow with dashed line indicates a timing for BCS measurement and blood sampling for NEFA measurement.

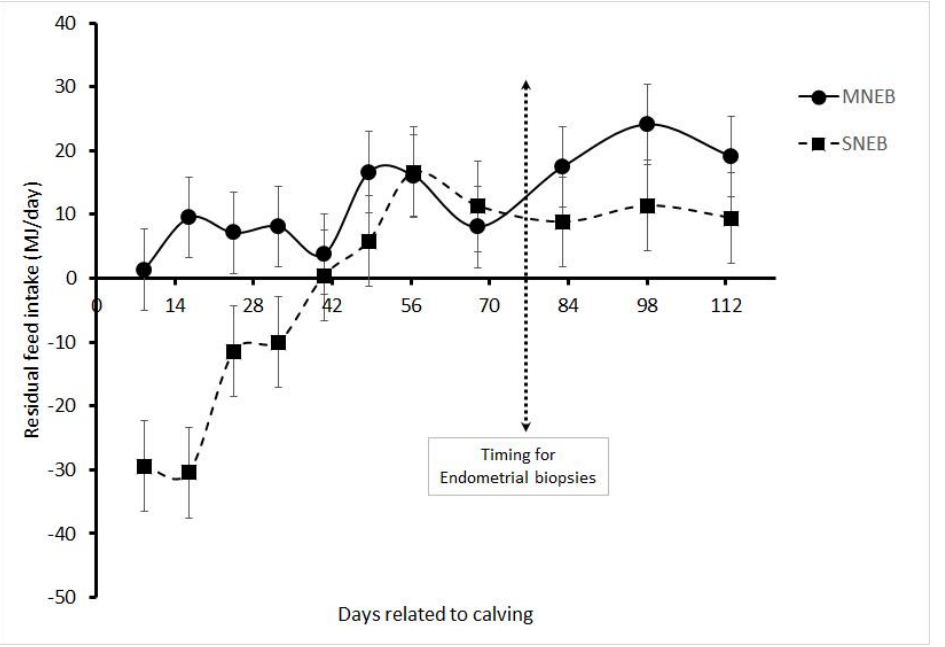


*Figure 3.* Energy balance profiles of mild negative energy balance (MNEB; n = 5) and severe negative energy balance (SNEB; n = 4) cows, which were selected for using LCM to isolate three endometrial cell types from endometrial tissue biopsies which collected during the postpartum period.

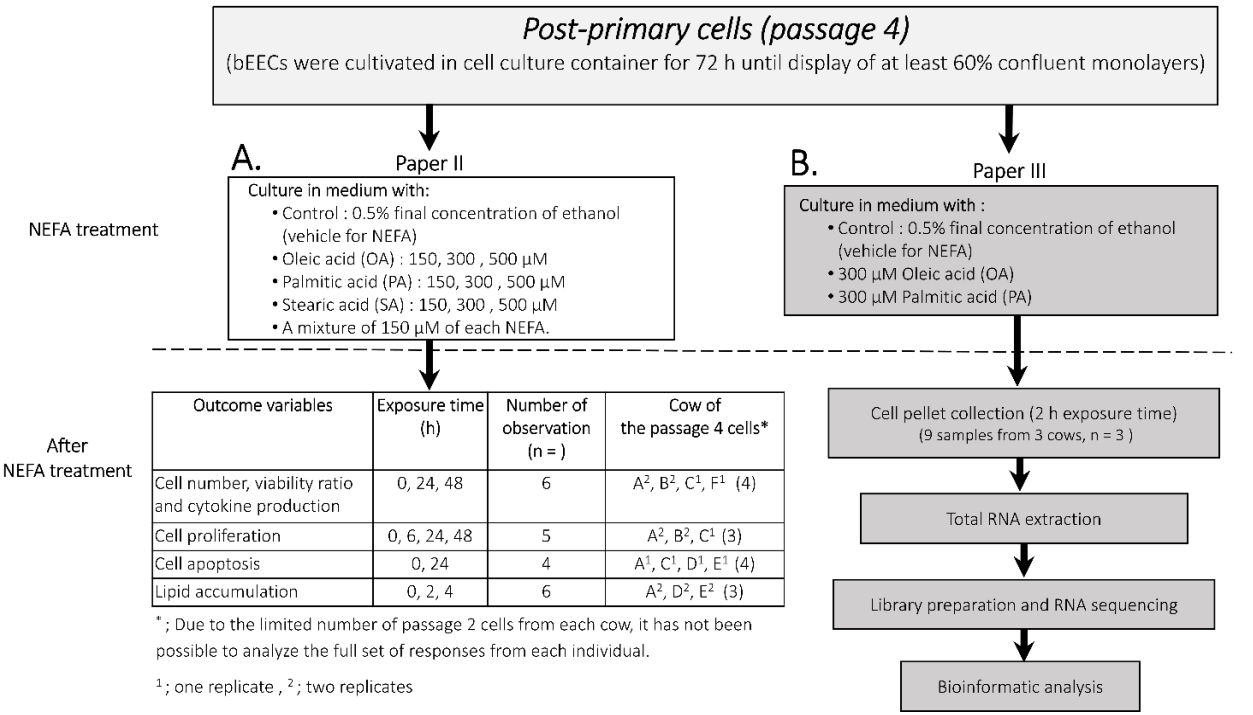


*Figure 4.* Experimental design for the *in vitro* study. Left panel A. refer to Paper II and right panel B. to Paper III.

## 3.3 Animals, sample collections and preparations (Paper I)

### 3.3.1 Animals

This study was carried out at the Swedish Livestock Research Centre in Lövsta, Uppsala, Sweden. In twelve Swedish Red breed cows (SRB; n = 12), the different energy balance profile were induced by feeding them with two diet regimens such as high energy diet and reduced energy diet. In the reduced energy diet, cows received 50% less concentrate than for the high energy diet group. An automatic feeding machine adjusted the amount of concentrate administered, while silage was given ad libitum in forage mangers for all cows. The period of experiment started from 30 days prepartum to 120 days postpartum. The nutrient composition of diets was previously described by Ntallaris *et al.* (2017). All cows were kept in a loose housing barn with a voluntary milking system (VMS, DeLaval, Tumba, Sweden), and had free access to water. At day 60 postpartum, oestrous synchronization was performed using a 7-d vaginal progesterone device (CIDR, Zoetis, NJ, USA). All cows received 500 µg of PGF-2α analog (Estrumate®, MSD animal health, Madison, NJ, USA) by intramuscular injection at time of vaginal progesterone device (CIDR, Zoetis, NJ, USA) removal. Endometrial tissue biopsies were collected 15 days after visual observation of oestrus under epidural anesthesia. The full experimental design is presented in Fig.2.

### 3.3.2 Energy balance calculation and classification

Individual energy balance (EB) was calculated as energy input minus output, requiring measurements of feed intake and energy output sources (milk, maintenance, activity, growth, and pregnancy) once per week from calving until 120 days postpartum. All data used for EB calculation were recorded routinely in the university herd and the calculations were performed using NorFor which is the reference system in the Nordic countries (Ntallaris *et al.*, 2017). Based on EB profiles construction, 9 of 12 cows were selected for endometrial transcriptomic study, following classification into mild negative energy balance (MNEB, n = 5) group and severe negative energy balance (SNEB, n = 4) group depending on the severity of residual feed intake value at first week postpartum. Residual feed intake (RFI) values in the first week postpartum of these nine cows ranged from -52.77 to 21.26 MJ/day and means (± S.E.M.) were 1.30 ± 6.35 and -29.48 ± 7.10 MJ/day in the MNEB and SNEB groups, respectively.

### 3.3.3 Assessment for body condition score (BCS), plasma NEFAs concentration, and milk progesterone

A single person performed body condition scoring every two weeks throughout the experimental period. The relative fatness of cow and loss of BCS were measured by using a five point BCS system (1.0-5.0) with values subdivided into 0.5 scores, 1 = very lean to 5 = fat, as described by Edmonson *et al.* (1989).

Blood samples were collected for plasma NEFAs measurement and taken once a week throughout the experimental period. NEFAs concentration was measured in duplicate by using a non-esterified fatty assay kit (Bioo Scientific Corporation, Austin, TX, USA).

Whole milk samples were collected by the automatic milking machine, VMS (DeLaval, Tumba, Sweden) for three times per week from Day 7 to Day 120 after calving for milk progesterone analysis. Progesterone concentrations were measured with a commercial enzyme-linked immunosorbent assay (ELISA) (Ridge way 'M' kit, Ridgeway Science, Gloucester, UK).

### 3.3.4 Endometrial tissue collection and tissue preparation

Endometrial biopsies were taken from ipsilateral uterine horn to the corpus luteum (CL) at 80 ± 12 days after calving. Biopsies were cut into three pieces (sizes ≈ 4×4 mm). One of them was frozen in cold isopentane (-80°C) and immediately embedded in ≈1 $cm^3$ optimal cutting temperature (OCT) compound. This procedure preserves both good morphology and RNA integrity in view of LCM. The biopsy samples issued from the nine cows were subsequently dissected by LCM. It was verified subsequently that all cows were in mid-luteal phase at time of biopsy while presenting progesterone concentrations > 5 ng/mL in milk samples collected that day or one day before or after. Tissue blocks were 8 µm sectioned with a cryostat.

### 3.3.5 Laser capture microdissection (LCM)

The tissue section was dehydrated with 75% ethanol (1 min), cresyl violet staining (15 sec), 75% ethanol (30 sec), 95% ethanol (1 min), 95% ethanol (1 min), 100% ethanol (1 min), 100% ethanol (1 min) and xylene (5 mins) respectively. Three endometrial cell types: luminal epithelial cell (LE), glandular epithelial cell (GE) and stromal cell (ST) were isolated from tissue sections using LCM (Applied Biosystems®, Arcturus, Maintain View, CA, USA) and harvested in sufficient numbers to obtain RNA quantities of at least 10 ng per

cell type. The histology of endometrial tissue before and after capture of the three cell types with LCM is presented in Fig. 3.

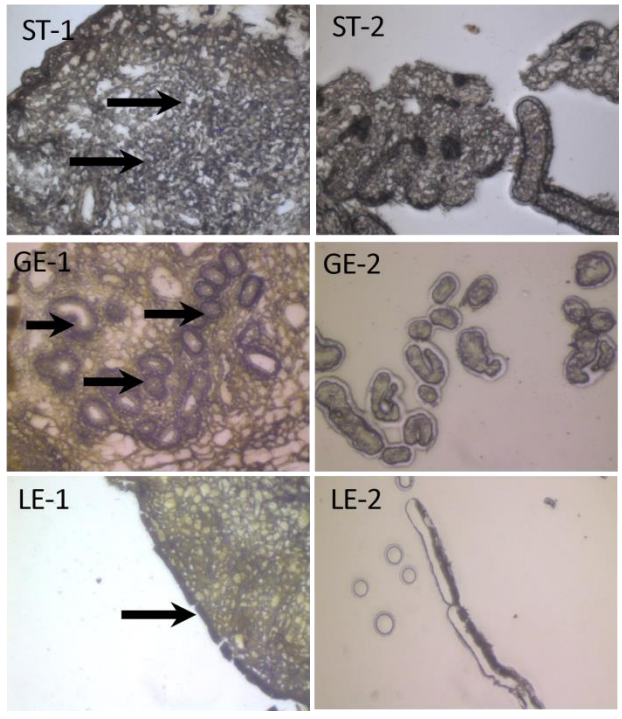


*Figure 5.* Isolation of the three bovine endometrial cell types by LCM: stromal cells (ST), glandular epithelial cells (GE) and luminal epithelial cells (LE), before (arrow) (1) and after (2) capture by LCM (100× magnification).

## 3.4 Cell culture preparation and NEFA treatment (Paper II, III)

### 3.4.1 Sample collection for cell culture

Bovine genital tracts from six non-pregnant dairy cows with normal morphological appearance and without apparent infection were randomly collected at the local Lövsta slaughterhouse in Uppsala, Sweden. Samples were immediately delivered to the KV laboratory at the Department of Clinical Sciences, SLU. Morphological and histological observations showed that all cows were in luteal phase at time of sampling following procedures described by Chanrot *et al.* (2017).

### 3.4.2 Bovine endometrial epithelial cells (bEECs) culture

The protocol for preparation, isolation and culture of bEECs was modified from Zhang *et al.* (1995) and Charpigny *et al.* (1999) studies. After cleaning uterus with distilled water, the right uterine horn was longitudinally opened and endometrial tissue was collected and tissue was minced until it become homogenous. Homogenous tissue was transferred into a solution containing digestive enzymes (collagenase IV and hyaluronidase) and incubated for 2.5 h. The suspension obtained from tissue homogenate was filtered to remove debris and mucus with sterile gauze, then passed through a cell strainer (mesh with 40 µm pores) to separate primary epithelial cells (retained) from fibroblast (passing through the mesh). Primary bEECs were cultured in the standard culture media, which is containing Dulbecco's modified Eagle's medium F-12, 10% fetal bovine serum (FBS), 0.5× ITS liquid media supplement, 2.5 mM L-glutamine, 50 U $mL^{-1}$ penicillin and streptomycin, 0.01 mg $mL^{-1}$ gentamycin and 100 U $mL^{-1}$ nystatin. Cell were cultured at 39°C with 5% $CO_2$ and passaged until passage 2. At the second passage, bEECs were cryopreserved at -80°C until start of the experiments.

### 3.4.3 Non-esterified fatty acid (NEFA) preparation and treatment

Frozen 2$^{nd}$ passage bEECs were sub-cultured until passage 4. Post-primary bEECs were cultured in standard media and incubated at 39 °C with 5% $CO_2$ for 72 h until displaying at least 60% confluence then new media containing vehicle of vehicle plus different concentrations of NEFAs. Oleic acid (C18:1, > 99%, Sigma-Aldrich), palmitic acid (C16:0, > 99%) and stearic acid (C18:0, > 98.5%) were dissolved in 95% ethanol to give a concentration of 25 mg/mL. Three concentrations (150, 300 and 500 µM) of OA, PA or SA were added to standard culture media and 0.5% of ethanol at final concentration was used as a control. For the mixture of NEFAs treatment, 150 µM of each fatty acid (OA+PA+SA; final concentration of NEFA concentration as 450 µM) in ethanol were added into the culture media. Solubility of fatty acid and pH were measured using osmometer and digital pH meter respectively. Cell numbers, survival, apoptosis, proliferation, lipid accumulation and inflammatory responses of bEECs were observed after different time of exposure time (Fig. 2).

## 3.5 Evaluations of bEECs changes in response to NEFA treatment (Paper II)

### 3.5.1 Cell number and cell viability

At 24 and 48 h after NEFA treatment, floating cells and cell-free culture medium were collected for counting cell number and measuring pro-inflammatory cytokines concentration respectively. Attached cells were detached by adding trypsin for 10-12 min and the numbers of cells in suspension were counted. For cell number counting, both attached cells and floating cells in culture media were stained with trypan blue 0.4% in dilution 1:1. Stained cells were classified as follows: live (trypan blue negative; TB-) and dead (trypan blue positive; TB+) and numbers of live and dead cells were counted in counting chamber under light microscope. From attached cells only, the relative change in live cells compared to control (X; cell viability) at corresponding time points was calculated from:

$$\text{Cell viability (X)} = \frac{\text{Live cells number of NEFA treatment} - \text{Live cells number of Control}}{\text{Live cells number of Control}}$$

### 3.5.2 Cell proliferation

At 0, 6, 24 and 48 h post treatment, 20 µL WST-1/ECS solution (Quick cell proliferation test, Abcam, Cambridge, UK) was added in corresponding wells and cells were incubated for 4 h. Measurement of optical density (OD) at each time point was performed by using a microplate reader (MultiskanEX; Thermo Fisher Scientific) at 450 nm and at the reference wavelength 620 nm. The cell proliferation ratio of NEFA treatment compared to control (Z) at each time point was calculated by using the following formula.

$$\text{Cell proliferation ratio (Z)} = \frac{\text{OD. 450 of NEFA treatment} - \text{OD. 450 of Control}}{\text{OD. 450 of Control}}$$

### 3.5.3 Cell apoptosis

At 24 h after NEFA treatment, the detection of cell apoptosis was performed by labelling NEFA-treated and control cells with Annexin V (ANV) and Propidium iodine (PI), followed by flow cytometry analysis. Cells were classified into 4 groups as follows: non-apoptotic (ANV negative (-) and propidium iodide (PI) (-), early apoptotic (ANV positive (+) and PI (-), late apoptotic (ANV+ and PI+) or necrotic (ANV– and PI+), as illustrated in Fig. 4.

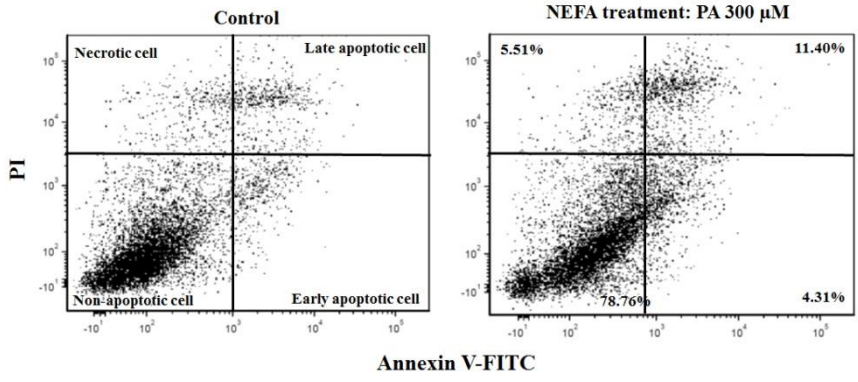


*Figure 6.* Representative dot plot showing Annexin V-FITC–PI double staining analysis of apoptosis of control and NEFA treatment (PA; 300 µM) using flow cytometry

### 3.5.4 Lipid accumulation

After NEFA treatment for 2 and 4 h, intracellular lipid accumulation was observed by Oil-red-O staining (BDH Laboratory Supplies, Poole Dorset, UK). Briefly, cells were washed with PBS twice, fixed with 4 % paraformaldehyde for 30 min, then washed with distilled water and kept in 60% isopropanol for 5 min in room temperature. After fixation, cells were stained with Oil-red-O in solution for 15 min at room temperature, and then washed with distilled water. The red vacuoles in the cytoplasm representing Oil-red-O accumulation were observed by light microscopy and photographed. For lipid accumulation quantification, intracellular Oil-red-O was eluted with 100% isopropanol and quantified using a microplate reader at OD 492 nm. The lipid accumulation concentration from NEFA treated cells was compared to value observed in controls (L) at each time point by using:

$$\text{Lipid accumulation concentration ratio (L)} = \frac{\text{OD. 492 of NEFA treatment} - \text{OD. 492 of control}}{\text{OD. 492 of control}}$$

### 3.5.5 Pro-inflammatory cytokine production

The measurement of interleukin-8 (IL-8) and IL-6 production in cell-free culture media of NEFA treated cells and in control cells at 24 and 48 h post-treatment were performed by ELISA test kit. The optical density was determined using a microplate reader at 450 nm. Pro-inflammatory cytokines concentration ratio (per total cell number ($\times 10^6$ cells)) was subsequently calculated.

## 3.6 Transcriptomic analyses

### 3.6.1 Cell collection and RNA isolation

Following LCM isolation, specific-endometrial cells on plastic cap were eluted with 50 µL extraction buffer provided in PicoPure$^{TM}$RNA isolation kit (KIT0202, Arcturus, Mountain View, CA, USA) and were subsequently incubated for 30 min at 42°C. After removing the cap, isolated cells in extraction buffer solution were frozen at -80°C for RNA isolation procedure. Total RNA was extracted using PicoPure$^{TM}$RNA isolation kit (KIT0202, Arcturus) following manufacturer's protocol recommendation. RNA amount was quantified and quality evaluated by using Pico RNA chip on the Agilent 2100 Bioanalyzer (Aligent technologies, Santa Clara, CA, USA). Number of samples used for RNA-seq in each cell type and NEB group, means value (± S.E.M.) of RNA Integrity Number (RIN) and number of tissue sections used in each endometrial cell type and full endometrial tissue section are presented in Table 1.

Table 1. *Number of samples used for RNA sequencing for each cell type and NEB group , means value (± S.E.M.) of RNA Integrity Number (RIN) and number of tissue sections used to obtain at least 10 ng of total RNA in each endometrial cell type and full endometrial tissue section.*

| **Sample type** | **Number of sample for RNA-seq** | | **RIN** | **Number of tissue section per sample** |
|---|---|---|---|---|
| | **MNEB** | **SNEB** | | |
| Full tissue section | - | - | 7.39 ± 0.13 | 1 ± 0 |
| Stroma | 5 | 4 | 7.23 ± 0.13 | 9 ± 3 |
| Glandular epithelium | 5 | 4 | 7.44 ± 0.13 | 13 ± 3 |
| Luminal epithelium | 5 | 1 | 7.75 ± 0.15 | 35 ± 3 |

Cell pellet of bEECs following each treatment was collected at 2 h post-treatment. Briefly, attached cells were detached by trypsinization method, then centrifuged at 120 × g for 5 min and the supernatant discarded. Cells were washed with PBS twice, and then centrifuged. Total RNA extraction from cell pellet was performed using AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's recommendations. The quantity and quality of RNA were measured using an Agilent RNA 6000 Nano Kit with the Agilent 2100 bioanalyzer.

### 3.6.2 Library preparation and RNA sequencing

cDNA libraries prepared from **24 samples in Paper I,** as shown in Table 1 and **9 samples in Paper III,** as illustrated in Fig. 2B**,** were processed for RNAsequencing at the GenomEast Platform (IGBMC, Cedex, France; http://genomeast.igbmc.fr/). The libraries were prepared from samples containing between 4 ng to 1 µg of total RNA by using the TruSeq RNA Sample Prep Kit including polyA selection. RNA sequencing was then performed using the Illumina HiSeq 4000 system. Samples were sequenced in 50 bp paired-end reads. Quantification of gene expression was performed using HTSeq with annotations from Ensemble. Data were normalized by calculating transcripts per million (TPM) values from gene counts and transcript lengths in Paper I and by using the median-of-ratios method proposed by Anders and Huber (2010) in **Paper I and III**. Principal component analysis (PCA) was performed to analyze clustering and differential gene expression (DEGs) was determined using DESeq2 (R package, Version 1.1.3) with the statistical method proposed by Love et al. (2014). *P*-values were adjusted using the Benjamini-Hochberg correction method for multiple testing. **In Paper I,** individual cell type specific signatures were identified followed by the identification of DEGs of specific-endometrial cell samples (SNEB vs MNEB groups). **In Paper III,** DEGs of three pairwise contrast as 1) OA vs Control, 2) PA vs Control, and 3) OA vs PA were identified with an adjusted $p$-value < 0.05. Volcano plot was applied to gene lists of each comparison in both **Paper I and III**. The Volcano plot identified genes with equal variance, and genes which showed equal or higher than log2 fold change and an adjusted $p$-value < 0.05 in the Student's t-test.

### 3.6.3 Bioinformatics analysis

**Paper I,** the functional annotation analysis was performed with each separate data sets of over- and under- differentially expressed genes (DEGs) of three endometrial epithelial cells when comparing SNEB versus MNEB groups using DAVID (Database for Annotation, Visualization and Integrated Discovery version, version 6.8, https://david.ncifcrf.gov/) and also PANTHER (Protein Analysis Through Evolutionary Relationships, version 13.1, http://www.pantherdb.org/) database for determining the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) classification respectively. In the GO and KEGG analyses, FDR $p$-value < 0.25 or adjusted $p$-value < 0.05 were considered to indicate significant enrichment. For the molecular interaction network analysis, a set of all DEGs of each endometrial cell types, including DEGs which are involved in significant KEGG pathways were generated from STRING database (version 10.5, http://string-db.org/) at

low confidence level (0.15) for giving an overview of the genes network and their interactions.

**Paper III,** GO classification and PANTHER pathway enrichment were analyzed using PANTHER database (version 13.1, http://www.pantherdb.org/) with separate transcript sets with increased or decreased expression from each comparison. In addition, the STRING database (version 10.5, http://string-db.org/) was used to analyze KEGG pathways with the same gene list as applied with PANTHER database. To identify the significant processes or pathways, FCR *p*-value was set as lower than 0.25 with PANTHER or lower than 0.05 with STRING database. For the molecular interaction network analysis, set of over- and under-expressed DEGs from each comparison was generated by using STRING database at medium confidence level (0.04) for giving an overview of the genes network and their interactions.

## 3.7 Statistical analysis

All statistical analyses on conventional phenotypic responses/variables were performed with SAS (Version 9.4) and all data were analyzed by using mixed models. The normality of data was tested by using a normal Q-Q plot and log10 transformation were performed in case of deviation from normality. Effects of the cow were considered as random when running the models.

**Paper I,** all continuous data was analyzed by mixed procedure with repeated measurement model. The model included NEB groups, diet groups, time for sampling as fixed effects and their second order interactions. For each variable in the final models, the effects and interactions which were not significant were excluded. For multiple comparisons and pairwise comparison, Scheffe's post hoc test was performed and "estimate" and "contrast" SAS statements were used respectively. The lowest BCS postpartum value and a nadir of RFI value after calving were recorded as well as BCS losses from start of experiment. Pearson correlation coefficients between these different variables were calculated using the Proc CORR function. **Paper II,** the model included the effect of NEFA treatment group (NEFA type × concentration), exposure time and their interactions as fixed effects. Non-significant interactions were also removed from the final models. Dunnett's post hoc test was used for multiple comparisons and Satterwaite's approximation was applied for correction of degrees of freedom. For variables X, Z and L which were calculated referring to control, only Scheffe's test was used.

All results from both studies are presented as least square means (LSmeans) ± standard error of the mean (S.E.M.) and $p$-value < 0.05 was considered to be statistically significant.

# 4 Results

The results in Papers I-III are summarized as follows;

## 4.1 Response of three endometrial cell types to SNEB in the *in vivo* model (Paper I)

### 4.1.1 Indicators of fat mobilization in NEB cows

Body condition score (BCS) was examined as a measurement of the degree of fat mobilization. There was no significant effect of NEB group on BCS, however BCS of SRB cows in both NEB groups tended to decrease ($p = 0.08$) between the antepartum and the postpartum period. The concentration of plasma NEFA was measured for a biomarker of energy balance. Overall plasma NEFAs concentrations did not differ between NEB groups. However, plasma NEFAs concentrations of SNEB cows were greater than MNEB at specific times; *i.e.* at 14 days before and 14 days after calving ($p < 0.05$). A strong negative relationship between BCS loss and EB nadir values was observed ($r = -0.68$, $p < 0.05$), in addition, the correlation between NEFA concentrations and the residual feed intake values was close to be significant ($r = -0.28$, $p = 0.06$).

### 4.1.2 Transcriptomic profiles of three endometrial cell types in postpartum cows

The RNA-Seq analysis of endometrial stromal (ST), glandular epithelium (GE) and luminal epithelium (LE) cells by LCM revealed that the highest number of detectable transcripts (over than 10 transcript per million (TPM)) was observed for ST cells (8,242) whereas the lowest number was detected in LE cells (6,622). The Venn diagram formed from these sets of genes (Fig. 7A)

shows that a relatively high number of genes (5,672) are common for the three cell types. In addition, the highest number of specifically expressed genes over than 10 TPM was obtained for ST (1,236) whereas relatively low numbers of genes are uniquely observed for GE (330) and LE (551) respectively.

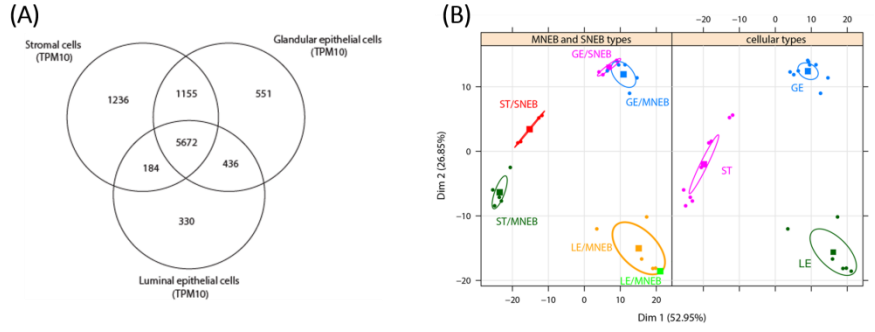


*Figure 7.* A.) Venn diagram of expressed genes detected (over than 10 transcripts per million (TPM)) in specific endometrial cell types (stromal cells; ST, glandular epithelial cells; GE and luminal epithelial cells; LE) and B.) PCA analysis illustrating differences in expression between cell types (right panel) and differential effects of NEB status (mild NEB; MNEB and severe NEB; SNEB) in these cell types (left panel).

### 4.1.3 Gene expression of three endometrial cell types in response to SNEB in postpartum cows

The clustering of individual RNA-Seq data sets from all cows and three cell types using PCA analysis as illustrated in Fig. 7B. PCA shows that the three endometrial cell types have differential expression profiles. Additionally, there is a clear separation between gene expression patterns in MNEB and SNEB cows, especially in ST. In contrast, overlapping gene expression patterns were evident in GE and LE. Overall, the number of differentially expressed genes (DEGs) in relation with the severity of NEB obtained from ST was the highest (1,058) when compared to numbers observed in LE (111) and GE (27) (Table 2). The majority of DEGs in ST and GE were over-expressed (72.31% and 62.96% respectively), whereas a large majority of DEGs in LE were under-expressed (98.19%). Only Zinc finger protein 36 (*ZFP36*) mRNA was found to be differentially expressed for all three cell types in SNEB cows. The analysis of the gene sets of DEGs from the three endometrial cell types in cows revealed that many functional pathways were affected by SNEB (Fig. 8). For ST cells, most of the functional terms related to metabolic process (including lipid metabolic process), cytoskeleton, calcium ion binding and receptor activity were significantly enriched with over-expressed DEGs, whereas enriched biological processes with under-expressed DEGs were related to cell adhesion, viral

infection process and protein binding. In GE cells, many over-expressed DEGs were involved in cytokine signaling, whereas cellular component genes related to microvillus were under-expresse. In luminal epithelium, under-expressed DEGs were enriched for biological processes (BP) and molecular functions (MP) associated with complement activation, B-cell mediated immunity, defense response to bacteria, cell differentiation, as well as for cellular component (CC) linked with membrane and organelle, while no significant enriched functional term was revealed for over-expressed DEGs.

Table 2. *Number of differentially expressed genes (DEGs) which were identified as being over- or under-expressed in specific endometrial cell types (ST, GE and LE) between SNEB and MNEB cows*

| **DEGs** | **Cell types** | | |
|---|---|---|---|
| | **ST** | **GE** | **LE** |
| Over-expressed | 765 | 17 | 2 |
| Under-expressed | 293 | 10 | 109 |
| **Total** | **1058** | **27** | **111** |

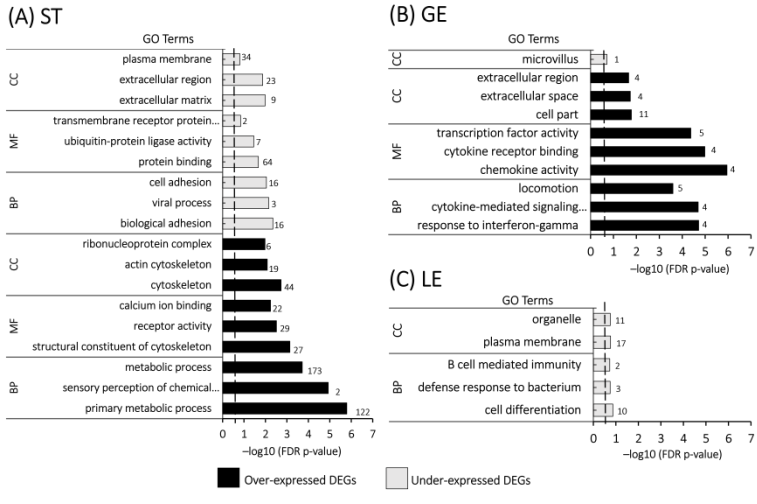


*Figure 8.* Top 3 significant GO processes in three categories: biological process (BP), cellular compartment (CC) and molecular function (MF) of over- or under-expressed DEGs (black and grey bars represent significant GO Terms corresponding to set of over- and under-expressed DEGs respectively) of A) stromal cells (ST) , B) glandular epithelial cells (GE) and C) luminal epithelial cells (LE) in SNEB. GO enrichment analysis was performed using PANTHER database with FDR cut off $p < 0.25$ (dash line). The significantly enriched GO Terms are presented as –log10 (FDR *p*-value) and number of related genes in over- or under-expressed DEGs were indicated for each column.

## 4.2 Response of endometrial epithelial cells to NEFA treatment in culture (Paper II, III)

### 4.2.1 Changes in phenotypes and pro-inflammatory cytokines production (Paper II)

*Cell number, viability and proliferation*

Increase of single NEFA concentrations had negative effect on cell number and cell viability, especially the highest dosage (500 µM) of OA. At 24 h exposure, supplementation with 300 and 500 µM of PA and OA was followed by a decrease in numbers of live cells ($p < 0.05$), but no effect of SA was observed at equal dosages. After longer exposure time (48 h), a significant decrease in cell viability was found with 300 and 500 µM of the three single NEFA treatments ($p < 0.05$), whereas treatments with the lower concentration (150 µM) of each single NEFA and a mixture of NEFAs did not reduce cell viability compared to control at 24 and 48 h post-treatment. Moreover, a significant decrease in proliferation was observed when cells were treated with 300 µM PA and 500 µM of single NEFA at 24 and 48 h. Only 500 µM OA strongly decreased bEECs proliferation at 6 h after treatment. However, 150 µM of single NEFA treatment and a mixture of NEFA did not influence the proliferation of bEECs when compared to control at any time points.

*Cell apoptosis*

At 24 h after NEFA treatment, the population of late apoptotic and necrotic cells was significantly increased in cells treated with 300 µM PA, 500 µM OA, PA or SA and with a mixture of NEFAs ($p < 0.05$). With single NEFA treatments, at concentrations $\leq$ 300 µM, early apoptotic events were not more frequent when compared to the control condition, whereas an increase in the percentage of early apoptotic cells was observed in cells treated with 500 µM OA and PA treatment ($p < 0.0001$).

*Intracellular lipid accumulation*

An increase in the amount of intracellular lipid droplets was visually observed when media was supplemented with either 300 and 500 µM of OA or a mixture of NEFAs. These findings were confirmed by the quantitative analysis of lipid accumulation following exposure to NEFAs for 2 and 4 h. The mean ratio of lipid accumulation with all NEFA treatments was significantly higher

than in control after 4 h exposure ($p < 0.05$). Especially all dosages of OA or a mixture of NEFAs treatment promoted lipid accumulation at 2 h when compared to the control condition ($p < 0.01$).

The above results suggest elevated concentrations of single NEFA impaired cell survival, proliferation and promoted cell apoptosis and lipid accumulation depending on fatty acids and exposure times.

*Pro-inflammatory cytokines production*

Changes in the production of pro-inflammatory cytokines IL-6 and IL-8 were detected following exposure of bEECs to NEFAs. The total concentration and IL-6 and IL-8 concentration ratio per $10^6$ bEECs were significantly higher when compared to control, and response differed depending on which fatty acids and their dosages ($p < 0.05$). Especially, total concentration and ratio of IL-6 concentration were significantly increased for all dosages of OA and PA ($p < 0.05$), whereas neither SA nor a mixture of NEFA treatment increased IL-6 concentration ratio. A significant increase of IL-8 concentration was found with the highest dosage of all single NEFAs ($p < 0.05$).

### 4.2.2 Changes in transcriptomic profiles induced by OA or PA (Paper III)

The strongest changes of phenotypes and pro-inflammatory cytokine production of bEECs were seen in response to OA and PA concentrations $\leq$ 300 μM. Lipid accumulation occurred also early after start of exposure. Therefore, we investigated molecular changes of bEECs after being exposed to 300 μM of OA or PA for 2 h and compared the results with the control condition.

Overall, results from the RNA-seq analysis show that 151, 5 and 49 genes were differentially expressed in OA vs Control, PA vs Control and OA vs PA at 2 h post-treatment respectively. Most of these DEGs were over-expressed following OA (74.17%) and PA (100%) treatment when compared to control or when comparing OA and PA (65.30%).

The most relevant pathways associated with the over-expressed DEGs for OA treatment were MAPK signaling pathway, JAK/STAT signaling pathway, apoptosis signaling pathway, Oxidative stress response, gonadotropin-releasing hormone receptor pathway, and biological process link to cancer. In contrast, some pathways such as Wnt signaling, integrin signaling and TNF signaling were found enriched from the set of under-expressed DEGs. When comparing between OA and PA treatments, over and under-expression of genes involved in apoptosis signaling pathway and TNF signaling pathway were found respectively (Fig. 9 and Table 3).

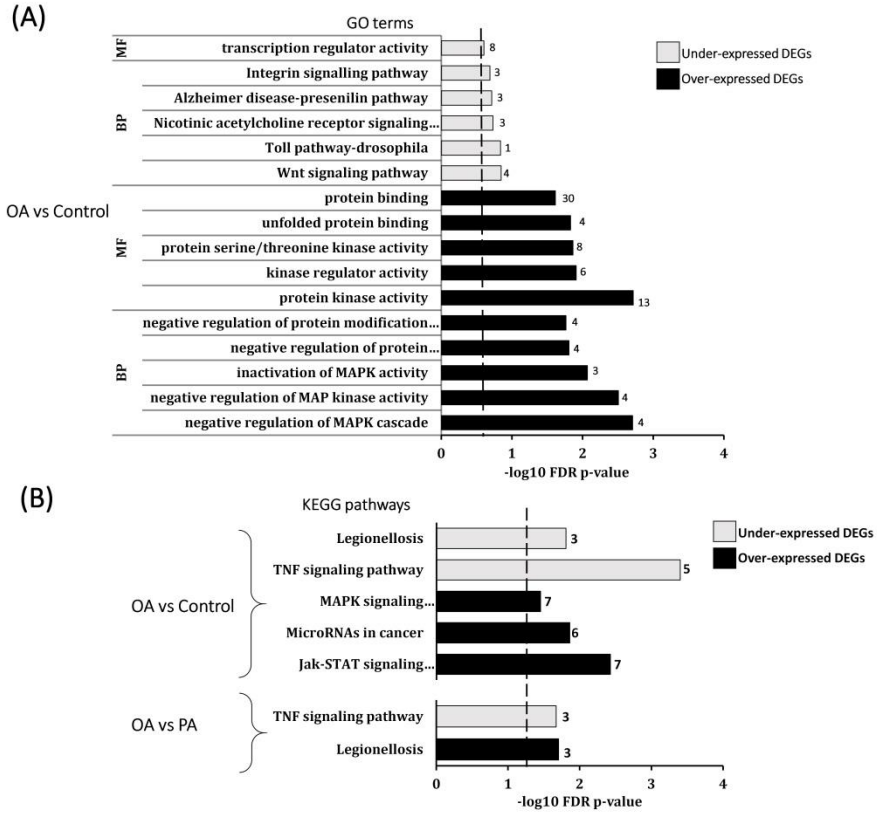


*Figure 9.* A) Top 5 significant GO processes in two categories: biological process (BP) and molecular function (MF) of over- or under-expressed DEGs following OA treatment when compared to control at 2 h post-treatment from PANTHER database with FDR cut off $p < 0.25$ (dash line) showing the significantly enriched GO Terms are presented with the –log10 (FDR *p*-value) and corresponding numbers of related DEGs (Black and grey bar represent significant GO Terms from over- and under-expressed DEGs sets respectively). B) Significant KEGG pathway associated with over- or under-expressed DEGs from two comparisons: OA vs Control and OA vs PA at 2 h post-treatment obtained from STRING database with FDR cut off $p < 0.05$ (dash line).

Table 3. *Significant PANTHER pathways from over- or under-expressed DEGs of OA vs Control and OA vs PA at 2 h post-treatment (FDR p < 0.25).*

| Comparision | DEGs set | PANTHER pathway | Gene count | Fold enrichment | FDR | Genes involved |
|---|---|---|---|---|---|---|
| OA vs Control | Over-expressed | P00006: Apoptosis signaling pathway | 7 | 10.28 | 6.19E-04 | *HSPA6, HSPA1A , BAG3, JUN, CREM, MCL1, ENSBTAG00000007662* |
| | | P00046: Oxidative stress response | 5 | 16.52 | 7.75E-04 | *DUSP5, DUSP1, DUSP4, MYC, JUN* |
| | | P06664: Gonadotropin-releasing hormone receptor pathway | 9 | 6.28 | 9.42E-04 | *FST, SLC2A1, PRKAG2, PTGER4, JUN, HSPA1A, DUSP1, INHBA, TGIF1* |
| | | P06959: CCKR signaling map | 7 | 6.97 | 2.70E-03 | *IER3, PLAU, JUN, CREM, MYC, MCL1, HBEGF* |
| | | P00050: Plasminogen activating cascade | 2 | 16.52 | 2.05E-01 | *PLAU, MMP13* |
| | Under-expressed | P00057: Wnt signaling pathway | 4 | 6.99 | 1.42E-01 | *ACTA2, ACTG2, EDN1, LRP6* |
| | | P06217: Toll pathway-drosophila | 1 | > 100 | 1.45E-01 | *NFKBIA* |
| | | P00044: Nicotinic acetylcholine receptor signaling pathway | 3 | 14.98 | 1.85E-01 | *STX19, ACTA2, ACTG2* |
| | | P00004: Alzheimer disease-presenilin pathway | 3 | 11.52 | 1.93E-01 | *LRP6, ACTA2, ACTG2* |
| | | P00034: Integrin signaling pathway | 3 | 8.1 | 2.04E-01 | *ACTA2, ACTG2, COL1A1* |
| OA vs PA | Over-expressed | P00006: Apoptosis signaling pathway | 4 | 20.38 | 3.89E-03 | *CREM, HSPA1A, BAG3, HSPA6* |

# 5 Discussion

## 5.1 Isolation of specific endometrial cells using LCM (Paper I)

Most studies aiming at evaluating the effects of negative energy balance on the gene expression in the endometrium have so far been based on full endometrial tissue lysates (Wathes *et al.*, 2009; Wathes *et al.*, 2011). However, for transcriptomic and proteomic studies, poor feature identification and higher interference from surrounding cells such as leukocytes, red blood cells and endothelial cells have been reported from entire tissue lysates when compared to material obtained by single cell isolation techniques (Marchi *et al.*, 2016). Laser capture microdissection (LCM) allows single cell type isolation. This technique was used to isolate specific-endometrial cells such as stroma (ST), glandular epithelium (GE) and luminal epithelium (LE) cells from uterine biopsy tissue and the cells were successfully separated in most samples. This method is reliable and efficient to isolate target cells from heterogeneous tissue via microscopic visualization (Hu *et al.*, 2016). However, several limitations of LCM were found such as the time taken for tissue preparation, the need of special skill and the risk for contamination by neighboring cells (Bidarimath *et al.*, 2015; Hu *et al.*, 2016). Specific difficulties were encountered here to isolate cells from the luminal epithelium in sufficient quantities. Also, the time to prepare the specimen and the amount of full tissue needed was ten times higher when compared to the others cell types.

To our knowledge, this is the first time that the specific effects of NEB on the three main types of cells of the endometrium are reported. Recently, the populations of three endometrial cell types (ST, GE and LE) have been isolated by using LCM from pregnant mares (Scaravaggi *et al.*, 2018) and sows (Zeng & Bauersachs, 2019). Our result is consistent with these studies showing that a

number of identified mRNA obtained from specific-endometrial cells was higher than from entire endometrial tissue. In addition, our results fully confirm that ST, GE and LE display specific molecular signatures as previous reported in other species such as horse, sheep and human (Yanaihara *et al.*, 2004; Brooks *et al.*, 2016; Scaravaggi *et al.*, 2018). We did find a higher number of mRNA with a strong constitutive expression in stromal cells compared with epithelial cells (either glandular or luminal). Our results obtained from biopsies collected in the luteal phase, show different pattern of gene expression when compared to previous studies that were performed at the beginning of pregnancy (Scaravaggi *et al.*, 2018).

## 5.2 Impacts of fat mobilization on gene expression in three endometrial cell types from SRB cows (Paper I)

For over two decades, a large number of studies aimed at deciphering the impacts of negative energy balance and metabolic imbalance on bovine reproductive performances and most of them have often used Holstein cows. However, the degree of fat mobilization is different between dairy cow breeds. Ntallaris *et al.* (2017) reported that SRB cows had less mobilization of body fat reserves than Holstein cows during the beginning of lactation. Swedish Red breed is the second major dairy breed found in Scandinavian countries. Bendixen *et al.* (1987) reported that SRB cows had a higher incidence of ketosis than Swedish Holstein breed (SLB). However, their reproductive performances is often reported as better than SLB cows (Löf, 2012). Therefore, in our *in vivo* model, we decided to investigate gene expression of the three endometrial cell types in response to severe negative energy balance in SRB cows. The severity of negative energy balance during the early postpartum period of SRB cows is effectively lower than in previous studies performed in the Holstein cows. This may influence molecular changes and this should be taken into consideration when comparing results to other studies (Wathes *et al.*, 2009; Wathes *et al.*, 2011). Nevertheless, we could identify groups of cows with different NEB profiles during the early postpartum period.

Previous studies revealed that alterations of endometrial gene expression associated with NEB involved genes encoding proteins with functions related to immune response, pro-inflammatory cytokines and activation of the IGF-insulin signaling pathway, susceptible to delay endometrial tissue remodeling during the early postpartum period (Wathes *et al.*, 2009; Swangchan-Uthai *et al.*, 2013) and 80 days postpartum (Valour *et al.*, 2013). Our results confirm the impact of NEB on changes in gene expression of endometrial cells and we observed that some of the effects of NEB still exist at time for breeding. Our results suggest that

SNEB induced the alterations of endometrial function in a different way depending on cell types (Fig. 10). Abundant specific changes in gene expression were found in ST demonstrating dysregulation of metabolic processes especially of lipid and carbohydrate metabolism, cytoskeleton and cell adhesion abilities. For epithelial cells, strong up-regulation of pro-inflammatory responses via chemokine pathway existed for GE, whereas down-regulation of adaptive immunity and defense mechanism pathways were found in LE. In addition, molecular changes related to inflammatory response were less important in ST. Despite the animals from both groups presented similar energy balance values when biopsies were collected, the former effects of NEB (at time of nadir; first two weeks after calving) are still present at time planned for breeding. On top of the major changes in the expression of gene involved in immunity, we found a strong alteration in the expression of many transcripts involved in prostaglandin synthesis and maternal-conceptus recognition such as prostaglandin F receptor (*PTGFR*) and IFN-τ inducible genes found mainly in ST and in GE. Considering the critical role of IFN-τ for embryo implantation and maintenance pregnancy (Brooks *et al.*, 2014), we may speculate that the under-expression of IFN-τ inducible genes linked to the over-expression of genes involved in oxytocin and PGF2α signaling may be unfavorable to the establishment of pregnancy in SNEB cows. Other type of alterations have been found such as those reported for growth factors, but it is difficult today to interpret their real impact in a consistent way.

These results provide novel insights into mechanisms regulating endometrium physiology in the postpartum dairy cow. They indicate that stromal, glandular and luminal epithelial cells have specific and differential responses to metabolic disturbance. Unfortunately, due to the quality and orientation of some of the biopsies from the SNEB group the information related to the impact of SNEB on LE needs confirmation. However, the specific responses of ST and GE to NEB were well documented here and pave the way for functional studies targeting the changes of key molecules for the success of pregnancy establishment.

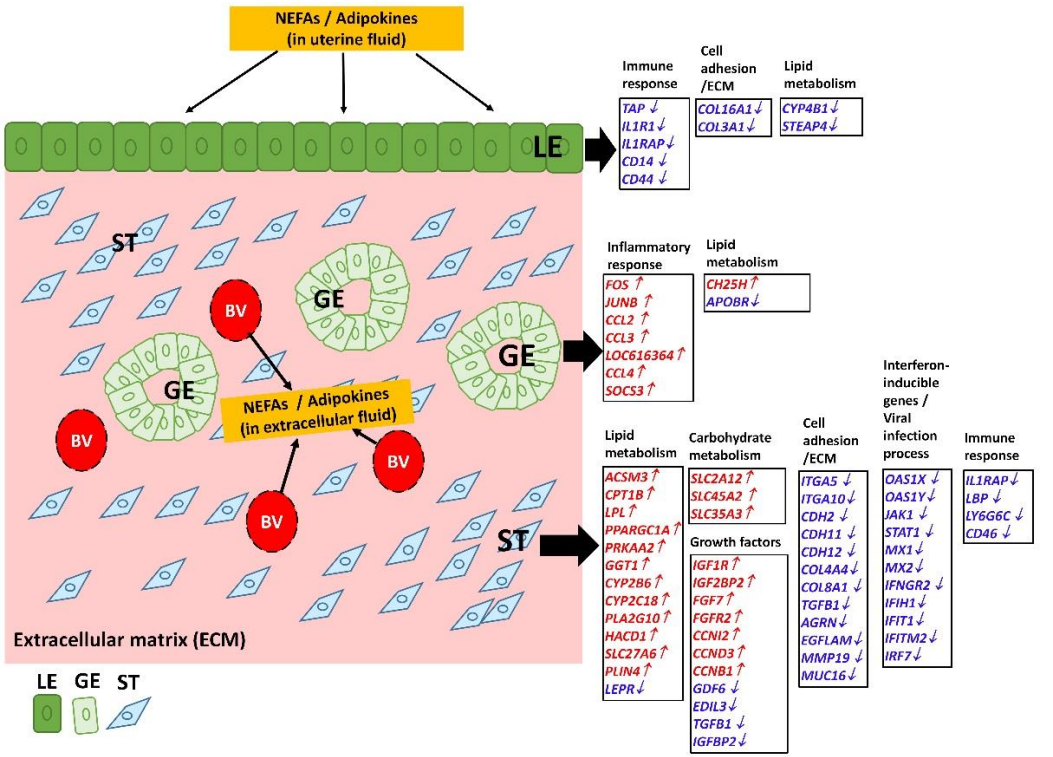


*Figure 10.* Long-term effects of severe negative energy balance through NEFAs and/or adipokines on gene expression of three endometrial cell types (luminal epithelium; LE, glandular epithelium; GE and stroma; ST) of Swedish red cows at time planned for breeding. Differentially expressed genes (DEGs) with red colour are over-expressed and DEGs with blue colour are under-expressed. Abbreviation; BV: Blood vessels, ECM: Extracellular matrix.

## 5.3 Types, concentrations of NEFA and cell culture system used in the *in vitro* model (Paper II,III)

It is well established that oleic (OA), palmitic (PA) and stearic (SA) acids are the three main NEFAs found in bovine plasma and reproductive organ secretions (Khandoker, 1997). Especially, an increase of OA and PA proportions was detected during the early postpartum period (Rukkwamsuk *et al.*, 2000; Leroy *et al.*, 2005). In human and animal studies, an adverse effect of those fatty acids on various somatic and reproductive cells have been reported, in particular for ovarian functions and blastocyst development (Mu *et al.*, 2001; Vanholder *et al.*, 2005; Vanholder *et al.*, 2006; Valckx *et al.*, 2014; Aardema *et al.*, 2016). However, the current knowledge related to possible impact of NEFAs on bovine endometrial cell responses is limited, therefore we decided to approach studies in endometrial epithelial cells by using NEFA concentrations used in *in vitro* studies for granulosa and theca cells (Vanholder *et al.*, 2005; Vanholder *et al.*, 2006) and consistent with the level of plasma NEFAs in early postpartum cows as documented by Rukkwamsuk *et al.* (2000) and Leroy *et al.* (2005). However, those fatty acids have different solubility characteristics that caused slightly lower concentration of PA and SA than expected in our culture system. In spite of these limitations, PA and SA had still strong negative effect on endometrial epithelial cell responses. Regarding the cell culture system used in this study, a non-polarized monolayer or two-dimension (2D) cell culture system was used and cells were exposed to NEFAs only on their surface. However, this system allowed the study for various types of responses in a specific type of endometrial cells with no bias or unspecific response from other cell types. On the other hand, those conditions are different from the *in vivo* situation. *In vitro* 2D-cells have been purified to obtain a single population of endometrial epithelial cells, but interactions between the cellular, extracellular environments as well as cell morphology and polarity are not available (Kapałczyńska *et al.*, 2018). However, despite differences from other culture systems, especially regarding polarity, similar results in terms of survival were found. The results of Jordaens *et al.* (2015) showing that bovine oviductal epithelial cells exposed to NEFAs under different conditions (either mono- or bi-direction in 2D culture system) expressed similar responses such as decreased cell viability and cell migration capacity are consistent with our results. Moreover, we used ethanol as a vehicle to dissolve fatty acids, which may cause differences in cell responses when compared to more physiological vehicles to transport NEFAs in cells such as a complex with albumin (Aardema *et al.*, 2011). The limitations mentioned above should be taken into consideration in further studies to sort out the specific adverse effects of NEFAs on cells.

## 5.4 Impacts of NEFAs on endometrial epithelial cell responses in culture (Paper II)

The results obtained in this thesis showing that the highest dosage (500 µM) of OA, PA and SA had adverse effects on cell number, viability and proliferation 24 and 48 h after NEFA treatments. Most of our findings confirm results from previous studies observed in various cell types (Cury-Boaventura *et al.*, 2006; Vanholder *et al.*, 2006; Jordaens *et al.*, 2015). However, OA had the strongest negative effect in our model, which is in disagreement with results from Vanholder *et al.* (2005) showing that PA had a more detrimental effects than SA and OA. Based on apoptosis results, our study showed that the highest dosage of the three single NEFAs, and also 300 µM of PA and mixture of NEFAs increased late apoptosis and necrosis events at 24 h post-treatment. This is consistent with the reduced cell viability and proliferation in our investigations and with results of previous studies obtained in other cell types (Mu *et al.*, 2001; Leroy *et al.*, 2005; Vanholder *et al.*, 2005). Moreover, we found that all NEFA treatments promoted intracellular lipid accumulation in bEECs 4 h after exposure. Most particularly, OA had the strongest effect in promoting intracellular lipid accumulation which was related to a reduction of cell viability and proliferation and increased apoptosis. Our results seem to disagree with previous studies showing that lipid accumulation induced by OA alone or co-incubation of OA and saturated FA did not increased cell death via the apoptosis pathway (Coll *et al.*, 2008; Henique *et al.*, 2010). In terms of pro-inflammatory cytokine responses, an increase of IL-6 concentration was only observed with OA or PA treatments, whereas IL-8 production was stimulated with the highest dosage of single NEFAs. However, the effects of SA and a mixture of NEFAs seem to be limited in our model. These findings are in partial agreement with observations in human pancreatic cells showing that only PA induced both the released and increased corresponding mRNA expression of IL-6 and IL-8 through NF-κB activation (Igoillo-Esteve *et al.*, 2010). In addition, it has been found that excess of OA promoted IL-8 and TNF-α production in hepatocytes (Vidyashankar *et al.*, 2013). The detrimental effects on bEECs survival and proliferation observed *in vitro* especially in the presence of OA and PA may be due to a lipotoxic effect of NEFAs that activate apoptosis mechanisms, promote lipid accumulation and release of pro-inflammatory cytokines. However, activation of this cascade may be different for each of these NEFAs when considering the differences observed between them in relation with exposure time and dosage.

## 5.5 Changes in gene expression of endometrial epithelial cells induced by Oleic or Palmitic acid (Paper III)

The results from the transcriptomic study made from the *in vitro* model revealed that the bEECs treated with either OA or PA at 300 µM for a short exposure time (2 h) induced gene expression changes when compared to controls. Oleic acid had more pronounced effects on gene expression than palmitic acid. Multiple pathways were affected by OA including a large number of DEGs belonging to lipid and carbohydrate metabolism, apoptosis signaling, oxidative stress signaling and pro-inflammatory cytokine signaling which were mostly over-expressed. Our results showed changes in expression of several mRNAs coding for proteins associated with energy metabolism when cells were treated with OA. For example, under expression of insulin induced gene (*INSIG1*) mRNA has been associated with lipogenesis and insulin resistance in obese mouse (Carobbio *et al.*, 2013) which is consistent with our findings. In addition, we observed an increased expression of pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*) which plays an important role on metabolic flexibilty by adjusting glucose and fatty acids oxidation and also promotes gluconeogenesis to compensate the deficiency of glucose supply (Majer *et al.*, 1998; Zhang *et al.*, 2014). The above result is very consistent with what was reported in the endometrium of SNEB cow presenting high NEFA concentrations (Wathes *et al.*, 2011). Besides, the increase in expression of perilipin 2 (*PLIN2*) and *IL6* mRNA induced by OA confirm our results from Paper II where OA promoted lipid accumulation and pro-inflammatory cytokine release. This information indicated that increased lipid metabolism possibly induce reactive oxygen species (ROS) production and contribute to dysregulation of carbohydrate metabolism possibly leading to insulin resistance (McGarry & Dobbins, 1999; Kusminski *et al.*, 2009). Moreover, over-expression of transcripts related to oxidative stress responses due to increase of ROS production may contribute to activate pro-inflammatory responses and the apoptosis cascade. Interestingly, we found that several transcripts associated to TNF-signaling, especially the NF-Kappa-B Inhibitor Alpha (*NFKBIA* or *IKBA*) were under-expressed. This change potentially activates the NF-κB pathway and upregulates pro-inflammatory processes (Oeckinghaus & Ghosh, 2009). Regarding the molecular changes related to cell migration and tissue remodeling, an increase in expression of Matrix Metallopeptidase 13 (*MMP13*) mRNA was observed following OA treatment. This finding is consistent with the observations previously made in the endometrium of SNEB cows during early postpartum (Wathes *et al.*, 2011). In contrast, transcripts encoding proteins of the Integrin and Wnt signaling pathway which are involved in cellular structure and cell adhesion were under-expressed. In addition, follistatin (*FST)* and

Inhibin-β A (*INHBA*) mRNAs which codes for proteins belonging to the TGF-β superfamily, play essential roles on female fertility (Li, 2014) and are associated with systemic inflammation (Jones *et al.*, 2004), were over-expressed following OA treatment. However, the possible impacts of those molecular alterations regulated by nutritional stress on pregnancy success are still questionable and would deserve further investigation.

The fact that more DEGs were found following exposure of cell to OA when compared to PA and the lack of effect of PA on enrichment of specific pathways may be due in part of the differential oxidation and ketogenesis rate of these fatty acids. Unsaturated FAs tend to be oxidized more rapidly than saturated FAs (Bruce & Salter, 1996; Leyton *et al.*, 2007) and this could explain why OA may affect cells faster than PA.

Molecular changes induced by PA included over-expression of a few transcripts such as insulin induced gene 1 (*INSIG1*), *PLIN2* and DNA damage inducible transcript 4 (*DDIT4*) which relates to lipid metabolism and apoptosis. Although limited in number, these are consistent with the increase in expression of caspase 3 (CASP3), Toll-like receptor 4 (TLR4), NF-κB and p62 protein expression related to apoptosis, inflammatory response and autophagy which were reported following exposure of bovine oviductal epithelial cells to PA (Ohtsu *et al.*, 2017).

To our knowledge, these effects of NEFAs, especially OA as discussed above have not been reported previously for the bovine endometrium. This new information provides knowledge on the mechanism by which NEFAs potentially contribute to the development of uterine diseases and inflammation in early postpartum cows.

Taken together, these molecular changes mentioned above may influence the uterine environment, increase pro-inflammatory mechanisms and may be unfavorable for embryonic implantation and maintenance of pregnancy.

# 6 Conclusions

Based on the results obtained from Paper I-III, the conclusions can be drawn as follows:

*In vivo* study:

- The impacts of the severity of NEB during the first week postpartum on endometrial gene expression are still present at 80 days postpartum in SRB cows.
- Strong differences exist in the transcriptomic profile signatures of the three main types of cells (ST, GE and LE) which constitute the endometrium.
- The impacts of the severity of NEB on endometrial gene expression are different in the ST, GE and LE cells. They are more numerous in stromal cells and relate mainly to lipid and carbohydrate metabolism, cell structure and maternal-conceptus recognition. The upregulation of pathways associated with immune responses and pro-inflammatory responses were identified for glandular epithelial cells, whereas genes involved in adaptive immunity and defence mechanism were down-regulated in luminal epithelial cells.

*In vitro* study:

- High NEFAs concentrations have detrimental effects on bEECs viability and proliferation and increases apoptosis, lipid accumulation and pro-inflammatory cytokines production.
- OA and PA present stronger negative effects than SA on cell viability, proliferation and apoptosis and also induced a stronger stimulation of pro-inflammatory cytokine production.

- The difference in response to the individual NEFA suggests that their negative effects on epithelial cell functions are mediated by different mechanisms.

- OA had a much stronger effect than PA on bEEC gene expression changes after a short time of exposure. Treatment of bEECs with OA induced the over-expression of many transcripts involved in lipid and glucose metabolism, apoptosis signaling, oxidative stress and pro-inflammatory cytokine response, whereas, PA had little effects on gene expression changes.

To our knowledge, these effects of NEB and NEFAs, especially the specific changes in gene expression in the three main types of cells constituting the bovine endometrium have not been report before. Taken together the results from this thesis give new insights on the different mechanisms by which fat mobilization affects endometrial function (Fig. 11). Increased inflammation and apoptosis associated to a decrease in cell adhesion and tissue remodelling may affect negatively the dialogue between the embryo and the endometrium as well as the various processes controlling the interaction between embryonic cells and maternal ones at the beginning of placentation.

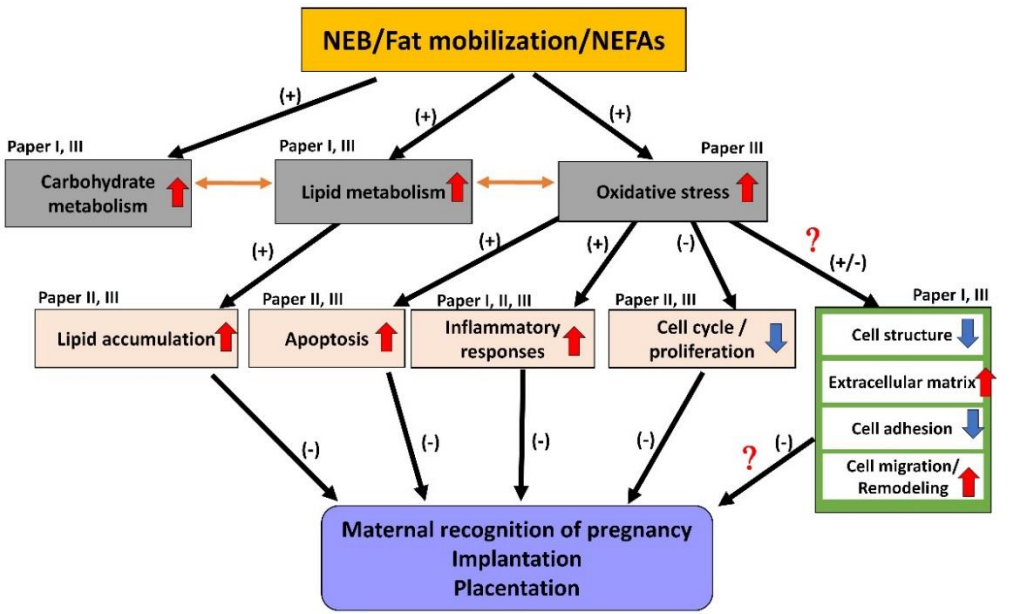


*Figure 11.* Schematic diagram built from integrating the information of the three manuscripts showing the roles of negative energy balance (NEB), fat mobilization and non-esterified fatty acids (NEFAs) on bEECs' phenotypes and gene expression in the bovine endometrium. Excess of NEFAs increases the expression of many transcripts involved in lipid and carbohydrate metabolism which promotes lipid accumulation in epithelial cells. Dysregulation of energy metabolism may induce oxidative stress and lead to increased apoptosis, activates pro-inflammatory responses, lower cell survival and proliferation as well as alters cellular structures and cell adhesion and finally de-regulates networks of genes related to maternal recognition of pregnancy. Those changes may be favourable for the development of persistent inflammation and may be unfavourable for maternal recognition for pregnancy, embryo implantation and placentation. Red and blue arrows represent up- and down-regulation of cell processes respectively.

# 7 Future Perspectives

In this thesis, we provide new information about the impacts of negative energy balance (NEB) and non-esterified fatty acids (NEFAs) on phenotypes, pro-inflammatory responses, and gene expression of single population of bovine endometrial cell types both from *in vivo* and *in vitro* models. The most obvious link between *in vivo* and *in vitro* results is the activation of pro-inflammatory mechanisms found in GE (Paper I) and in epithelial cells (Paper II and III) which are issued mainly from GE due to the preparation procedure. This paves the way for continuing studies targeting specifically the effect of NEFAs on stromal cells and luminal epithelial cells. Challenging stromal cells would be easy with our protocol of separation. The study of luminal epithelial cell looks more difficult but this work could be considered through use of a 3D culture system that would be able to generate them. In addition, developing a 3D system possibly based on the three types of cells would be of interest to approach the changes induced by NEFAs in a more physiological way.

From results of both in *in vivo* and *in vitro* studies, some key molecules such as zinc finger protein 36 (*ZFP36*) and amphiregulin (*AREG*) were identified which may be used later on for diagnostic proposes to detect a persistent uterine inflammation. Moreover, a possible additional outcome from these results is to use the information to further evaluate the impact of treatments and/or nutritional strategies. Additional, markers such as adipokines would be of interest to determine the intensity of the impact of NEB on the endometrium. Due to differential fat mobilization between SRB and Holstein cows, it would be interesting to investigate and compare the effects of metabolic imbalance on gene expression of three endometrial cell types between those two breeds. Other methods could be used in the future to expose cells to NEFAs including use of a complete culture system but also other vehicles that may deliver NEFAs in different ways.

Finally, this thesis paves the way for functional studies aiming at evaluating the long term consequences of fat mobilization on uterine function and embryo-maternal interactions.

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# Popular science summary

High milking dairy cows suffer from energy deficit (so called negative energy balance (NEB)) which occurs after calving due to a high energy demand for milk production. This is associated with the mobilization of fat tissue and the release of non-esterified fatty acids (NEFAs) into blood circulation.

The endometrium is the part of the uterus establishing the contact with the embryo at time of pregnancy. Its sensitivity to molecules such as NEFAs is critical for pregnancy success which impacts dairy farmer economy.

Therefore, this thesis aimed to determine the impacts of NEB and NEFAs on the functions of the endometrial cells both from *in vivo* and *in vitro* models.

The impact of NEB on gene expression controlling the function of the endometrium was studied in the second lactation cows of the Swedish Red Breed. Changes in cell survival, cell death, the release of pro-inflammatory molecules and corresponding changes in gene expression were studied in the *in vitro* model. Results obtained *in vivo* show that *i)* the NEB status as observed early after calving influences the function of the endometrium long after, by expected time of breeding, *ii)* the response of the three main types of cells which constitute the endometrium, to NEB is very specific to each cell type. The *in vitro* study demonstrated that NEFAs strongly impacts negatively the survival of endometrial cells and exerts pro-inflammatory mechanisms susceptible to impair the establishment of pregnancy.

Both types of studies revealed that the function of the endometrial cells is strongly perturbed by NEB status and/or NEFAs. Mechanisms altered relates to pro-inflammatory mechanisms but also to specific signals and cell function associated to maternal recognition of pregnancy and establishment of placentation. Overall the results clearly demonstrate the link between metabolic imbalance and the inflammatory status of the endometrium. A new finding

relates to additional negative impacts of NEB on specific signals related to the establishment of pregnancy. These observations strike out the importance of controlling NEB. The identification of targets such as those found here in the endometrium may contribute to lower its negative impact on dairy cow fertility.

# Populävetenskaplig sammanfattning

Högmjölkande mjölkkor drabbas av energibrist, så kallad negativ energibalans, efter kalvningen när energibehovet för mjölkproduktionen är högt. När korna inte kan äta så tillräckligt tar de istället energi från sina kroppsreserver, de bryter ner fettvävnad, och fria fettsyror (NEFA) cirkulerar i blodomloppet.

Livmoderslemhinnan har nära kontakt med det tidiga embryot när dräktigheten etableras. Livmoderns funktion påverkas av kons näringstillstånd, och NEFA kan påverka chanserna att behålla dräktigheten. Om dräktigheten äventyras blir det negativa, ekonomiska konsekvenser för mjölkföretaget.

Syftet med denna avhandling var att studera vilken effekt negativ energibalans och NEFA har på livmoderns slemhinna, endometriet, både hos den levande kon och i en cellodlingsmodell i laboratoriet.

Effekten av negativ energibalans på genuttrycket som reglerar livmoderslemhinnans funktion studerades hos kor av rasen svensk röd boskap, SRB, som fått sin andra kalv (under andra laktationen).

I cellodlingsmodellen studerades livmodercellers överlevnad och celldöd samt frisättningen av pro-inflammatoriska molekyler och hur detta var kopplat till förändringar i cellernas genuttryck.

Studierna på levande kor visade att a) den negativa energibalans som drabbar kon kort efter kalvning påverkar livmoderslemhinnans funktion långt senare, under den period då kon ska insemineras och förväntas bli dräktig, och b) de tre dominerande celltyper som livmoderslemhinnan är uppbyggd av har väldigt specifika sätt att reagera på negativ energibalans. Tillsats av NEFA i cellodlingen påverkade cellernas överlevnad kraftigt och gav upphov till en immunreaktion som kan minska chanserna till dräktighet.

Båda typerna av studier visade att funktionen hos cellerna i livmoderslemhinnan störddes kraftigt vid negativ energibalans och/eller

förekomst av NEFA. De cellfunktioner som ändrades hör till generella mekanismer i cellerna och till pro-inflammatoriska mekanismer, men också till specifika signaler och cellfunktioner kopplat till dräktighetssignalering från embryot till modern, och till bildandet av placentan, moderkakan.

Avhandlingen visar hur de använda forskningsmodellerna kan nyttjas för att utvärdera hur obalans i näringsförsörjningen påverkar livmoderslemhinnans funktion, och hur det i sin tur påverkar kons fruktsamhet. Resultaten visar att det finns en tydlig länk mellan kons näringsstatus och livmoderns inflammationsstatus. Det är dessutom en ny upptäckt att negativ energibalans påverkar specifika signaler som är nödvändiga för etablering av dräktigheten. Dessa fynd visar hur viktigt det är att styra utfodringen runt kalvning och undvika alltför grav eller långvarig negativ energibalans. Det är viktigt att i tidigt skede hitta markörer, som dem som projektet har identifierat i livmodern, för att kunna minska negativa effekter på mjölkkornas fruktsamhet.

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**สุดท้ายนี้ขอขอบพระคุณคุณพ่อ (คมกฤษ จันทร์เขียว), คุณแม่ (ละม้าย จันทร์เขียว) และน้องแอน (สุชารัตน์ จันทาพูนธยา) รวมทั้งสมาชิกในครอบครัวจันทร์เขียวและครอบครัวทองศรีอันเป็นที่รัก ที่คอยเป็นกำลังใจให้เสมอมาทั้งในยามทุกข์หรือสุข ความสำเร็จในการจบการศึกษาระดับปริญญาเอกครั้งนี้เกิดขึ้นได้จากแรงผลักดันและกำลังใจจากทุกคนในครอบครัว**