DPhil thesis submitted to the University of Oxford

THE INFLUENCE OF METABOLIC HORMONES ON LACTATION

Hussam Rostom

Oxford Centre for the Endocrinology of Human Lactation,
Nuffield Department of Women's and Reproductive Health,
University of Oxford

St John's College,
University of Oxford

Word count: 29,097 (excluding contents pages, figures, tables and references)

Submission: Michaelmas Term 2024

Supervisors: Professor Fadil Hannan, Professor Joanna Poulton, Dr Taha Elajnaf

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ACKNOWLEDGEMENTS

This thesis would not be possible without my supervisors, Professor Fadil Hannan, Professor Joanna Poulton, and Dr Taha Elajnaf, as well as the Oxford Centre for the Endocrinology of Human Lactation (OCEHL) team, including Xin Meng, Michelle Ma, Bryony Davies, Amy Archer, Emily Hillier, Samantha Ewers, Emma Newcombe, Alexandria Fry, Helen Price and Isabella Honess. A particular thanks goes to Professor Hannan for his supervision, guidance and kindness throughout my DPhil.

I would also like to thank the University of Oxford and the Medical Research Council for provision of scholarship funds towards my fees and living costs, the Bioscientifica Trust for grant funding, and St John's College for funds towards travel and equipment.

DECLARATION

I declare that this thesis is entirely my own work and describes my own research, other than data incorporated by other members of the team as follows:

- Clinical samples obtained during the INSIGHT study by Amy Archer, Emily Hillier, Samantha Ewers, Alexandria Fry and Helen Price.
- Signalling studies performed in collaboration with Xin Meng.
- Imaging and flow cytometry of human mammary epithelial cells performed by Taha Elajnaf.
- Transcriptomic experiments performed by several members of the OCEHL team and data analysis undertaken by Taha Elajnaf and Isabella Honess.

ABSTRACT

Lactation is regulated by reproductive and metabolic hormones, and critical for infant and maternal health. However, the range of hormones acting on the mammary gland, their mechanisms of action, and concentrations required for milk secretion are unclear. The aims of my DPhil were to conduct clinical and cellular studies to define the normal serum hormone concentrations mediating lactation onset after childbirth, and to investigate how metabolic hormones act on mammary cells to promote lactation.

During my DPhil, I measured serum hormones in n=106 women at the onset of lactation and identified novel hormone changes during this period. Thus, maternal serum prolactin concentrations increase by approximately 20% following birth and are greater in multiparous women, who report earlier lactation onset. Thyroid stimulating hormone (TSH) increases on days 1-2 post-partum and is followed by a rise in free thyroid hormone concentrations. I have also demonstrated a novel post-partum increase in serum growth hormone (GH). Furthermore, I showed that insulin sensitivity increases post-partum, but this is impaired in women with increased BMI, a known risk factor for delayed lactation.

To investigate the mechanisms of hormone action of hormones, I used the HC11 mammary cellular model of lactation. My work showed that GH signals through the Janus kinase 2 (Jak2)- signal transducer and activator of transcription 5 (STAT5) pathway, while transcriptomic evaluation of GH-treated HC11 cells demonstrated that GH promotes synthesis of milk proteins and proteins associated with lipid synthesis. GH is therefore likely to augment the milk-synthesising actions of prolactin at lactation onset.

I also showed that insulin signals through the PI3K-Akt pathway in HC11 cells and promotes mitochondrial oxidative phosphorylation and glycolysis. In keeping with this,

transcriptomic assessment of insulin-treated HC11 cells showed upregulation of genes encoding proteins in all mitochondrial electron transport chain subunits, as well as of glycolytic and pentose phosphate pathway enzymes. Insulin therefore may support the bioenergetic requirements of lactation.

In summary, my clinical and basic scientific studies investigating the endocrine regulation of lactation have provided novel insights into serum hormone changes at the onset of lactation and advanced our understanding of hormone action in mammary cells.

ABBREVIATIONS

2DG	2-deoxyglucose
ACAD9	Acyl-CoA dehydrogenase
ACTH	Adrenocorticotrophic hormone
Akt	Akt kinase (also known as protein kinase B)
ALDO	Aldolase
AMPK	Adenosine monophosphate-activated protein kinase
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
ATPAF	ATP synthase mitochondrial F_1 complex assembly factor
BCS1L	Ubiquinol-cytochrome c reductase chaperone
BMI	Body mass index
CaSR	Calcium-sensing receptor
CMC	C-X9-C motif-containing protein
COA	Cytochrome c oxidase assembly factor
COQ	Coenzyme Q
COVID-19	Coronavirus disease 2019
COX	Cyclo-oxygenase
CPM	Counts per million
CREB	cAMP response element binding protein
CRH	Corticotropin-releasing hormone
CSN	Casein protein
CUZD1	CUB and zona pellucida-like domains-containing protein 1
CYC1	Cytochrome C1
CYCS	Cytochrome c
CYTB	Cytochrome B
D2R	Dopamine type 2 receptor
DAG	Diacylglycerol
DEG	Differentially expressed gene
DERA	Deoxyribose-phosphate aldolase
DMAC2L	Distal membrane arm assembly component 2 like

DMH	Dorsomedial nucleus of the hypothalamus
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein 1
E2F	E2F transcription factor
ECAR	Extracellular acidification rate
EDC	Endocrine disrupting chemical
EEF1A1	Eukaryotic translation elongation factor 1 α1
EGF	Epidermal growth factor
EHF	ETS homologous factor
ELF5	ETS transcription factor 5
ENO	Enolase
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinase
ESCIT	Evolutionarily conserved signalling intermediate in Toll pathway
ESR1	Oestrogen receptor 1
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
FBP	Fructose-1,6-bisphosphatase
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDR	False discovery rate
FOXO	Forkhead box O
FOXRED	FAD-dependent oxidoreductase domain-containing protein
G6PDX	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA3	GATA binding protein 3
GH	Growth hormone
GHR	Growth hormone receptor
GLUT	Glucose transporter
GnRH	Gonadotropin releasing hormone
GPI	Glucose-6-phosphate isomerase

GSEA	Gene set enrichment analysis
GSK3	Glycogen synthase kinase 3
H6PD	Hexose-6-phosphate dehydrogenase
HBP	Helix bundle peptide
HIGD1A	Hypoxia inducible gene 1 (HIG1) domain family member 1A
HIV	Human immunodeficiency virus
HK	Hexokinase
HLH	Helix-loop-helix
hMEC	Human mammary epithelial cell
HMO	Human milk oligosaccharide
hPL	Human placental lactogen
HRP	Horseradish peroxidase
ID4	Inhibitor of DNA binding 4
IGF	Insulin-like growth factor
ILK	Integrin-linked kinase
INSR	Insulin receptor
IP_3	Inositol-1,4,5-trisphosphate
IQ	Intelligence quotient
IRS	Insulin receptor substrate
Jak2	Janus Kinase 2
LALBA	Lactalbumin alpha
LC	Luminal cluster
LDH	Lactate dehydrogenase
LYRM	LYR motif-containing protein
MC4R	Melanocortin 4 receptor
Mcl-1	Myeloid cell leukaemia-1
MEK	Mitogen activated protein kinase
MFN2	Mitofusin 2
MSH	Melanocyte stimulating hormone
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease

ND	NADH dehydrogenase
NDUF	NADH:ubiquinone oxidoreductase
NDUFAF	NADH:ubiquinone oxidoreductase complex assembly factor
NRF	Nuclear respiratory factor
NTS	Nucleus of the tractus solitarius
NUBPL	Nucleotide-binding protein-like
OCR	Oxygen consumption rate
OPA1	Optic atrophy protein 1
OXTR	Oxytocin receptor
pAkt	Phosphorylated Akt
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCOS	Polycystic ovarian syndrome
PDK	Phosphoinositide-dependent kinase
PDSS	Decaprenyl diphosphate synthase
PET100	Cytochrome c oxidase chaperone
PFK	Phosphofructokinase
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PGAM	Phosphoglycerate mutase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PGD	Phosphogluconate dehydrogenase
PGK	Phosphoglycerate kinase
PGLS	Phosphogluconolactonase
PGM	Phosphoglucomutase
PHDA	Periventricular hypophyseal neurons
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PK	Pyruvate kinase
PLC	Phospholipase C
POMC	Proopiomelanocortin
PR	Progesterone receptor
PRLR	Prolactin receptor

PRPS	Phosphoribosyl pyrophosphate synthetase
PTHrP	Parathyroid hormone-related peptide
qRT-PCR	Real-time quantitative polymerase chain reaction
PVN	Paraventricular nucleus
RAC1	Ras-related C3 botulinum toxin substrate 1
RANKL	RANK ligand
RBKS	Ribokinase
REM	Rapid eye movement
rhGH	Recombinant human growth hormone
RIN	RNA integrity number
RNA	Ribonucleic acid
RPE	Ribulose-5-phosphate 3-epimerase
RPIA	Ribose 5-phosphate isomerase
SCO1	Synthesis of cytochrome c oxidase 1
SD	Standard deviation
SDH	Succinate dehydrogenase
SDHAF	Succinate dehydrogenase assembly factor
SEM	Standard error of the mean
SH2	Src homology 2
SMIM20	Small integral membrane protein 20
SOCS	Suppressor of cytokine signalling
SON	Supraoptic nucleus
SOX10	SRY-box transcription factor 10
SRC	SRC kinase
STAT	Signal transducer and activator of transcription
SURF1	Surfeit locus protein 1
T3	Triiodothyronine
T4	Thyroxine
TACO1	Translational activator of cytochrome c oxidase 1
TALDO1	Transaldolase 1
TBC1D4	TBC1 domain family member 4
TCA	Tricarboxylic acid

TDLU	Terminal ductal lobular unit
TEB	Terminal end bud
TFAM	Transcription factor A mitochondrial
THDA	Tuberohypophyseal neurons
THR	Thyroid hormone receptor
TIDA	Tuberoinfundibular neurons
TIMMDC	Translocase of inner mitochondrial membrane domain containing
TKT	Transketolase
TMEM	Transmembrane protein
TPI	Triosephosphate isomerase
TSH	Thyroid stimulating hormone
TTC19	Tetratricopeptide repeat domain-containing protein
TXNIP	Thioredoxin-interacting protein
UDP	Uridine diphosphate
UQCC	Ubiquinol-cytochrome c reductase assembly factor
UQCEC	Ubiquinol-cytochrome c reductase core protein
UQCR	Ubiquinol-cytochrome c reductase subunit
UQCRB	Ubiquinol-cytochrome c reductase binding protein
UQCRFS	Ubiquinol-cytochrome c reductase Rieske iron sulphur polypeptide
UQCRH	Ubiquinol-cytochrome c reductase hinge protein
VLM	Ventrolateral medulla
WAP	Whey acidic protein
WIP1	Wild-type P53-induced phosphatase 1

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1. INTRODUCTION

1.1. The importance of breastfeeding for mother and infant

1.1.1. Benefits for infant health

Human milk is a complex and diverse biofluid, containing both nutritional and bioactive components (1). In addition to macronutrients such as lactose, fat and protein, milk contains micronutrients and vitamins required for the developing infant. Bioactive substances are also present in milk, which do not have a direct nutritional role but do influence biological processes in the infant. They include components with an immune function, such as immune cells, immunoglobulins, cytokines, chemokines, and anti-microbial peptides, as well as other substances that influence tissue function and infant development, for example growth factors, hormones and human milk oligosaccharides (HMOs).

Breastfeeding reduces the risk of infection-related mortality in the first two years of life and decreases all-cause infant mortality in children up until the age of 11 (2, 3). Furthermore, breastfeeding confers protection to the infant from developing obesity and diabetes in later life and improves performance in intelligence test scores (table 1) (4-10).

Table 1. Infant benefits of breastfeeding.

Benefits	Relative risk reduction	Reference
Infant mortality		
↓All-cause mortality	93% [a,d]	(2)
↓Infection-related mortality	88% [a,d]	(2)
↓Diarrhoea-associated mortality	90% [a,d]	(11)
↓Respiratory infection-associated mortality	93% [a,d]	(12)
Infant health and development		
↑Intelligence test scores	IQ score ↑3.4 [b]	(5)
↓Obesity	13% [b,e]	(6)
↓Type 1 diabetes	71% [c,e]	(9)
↓Type 2 diabetes	35% [b,e]	(10)

[a]Exclusive breastfeeding versus no breastfeeding during first 5 months of life.
[b]Ever breastfed versus never breastfed.
[c]Breastfed > 4 months versus never breastfed.
Risk reduction estimated from available measures: [d] relative risk, [e] odds ratio

1.1.2. Benefits for maternal health

Breastfeeding is also associated with benefits for the mother. Maternal benefits of lactation include a decreased long-term risk of type 2 diabetes, cardiovascular disease and some cancers (table 2) (13-17).

Table 2. Maternal benefits of breastfeeding.

Benefits	Relative risk reduction	Reference
↓Type 2 diabetes	48% [b,f]	(14)
↓Cardiovascular diseases	11% [a,f]	(15)
↓Breast cancer	4% [c,d]	(13)
↓Ovarian cancer	23% [a,e]	(17)

[a]Ever breastfed versus never breastfed.
[b]6-12 months of breastfeeding versus no breastfeeding.
[c]Risk reduction for every 12 months of breastfeeding.
Risk reduction estimated from available measures: [d] relative risk, [e] odds ratio, [f] hazard ratio

Given the benefits of breastfeeding for both mother and infant, an understanding of the structure, function and regulation of the mammary gland is important when considering normal lactation, as well as the pathophysiology of insufficient lactation.

1.2. Mammary gland development and lactation

1.2.1. Structure of the mammary gland

1.2.1.1. Overall organisation of the human gland

At birth, the newborn breast consists of rudimentary ducts which end in short ductules and this remains the case throughout childhood (18). The onset of puberty is characterised by expansion of the mammary epithelium and the surrounding stroma. Proliferation arising from stem cells located at the tips of terminal end buds (TEBs), which are club-shaped structures at the ends of growing ducts, drives elongation of primary ducts and sprouting of secondary ducts (19). The result of pubertal mammary development is a highly organised tree-like branched ductal network, originating from the nipple and consisting of 15-20 lobes,

with each lobe containing 20-40 terminal ductal-lobular units (20). The organisation of the human mammary gland is shown in figure 1.

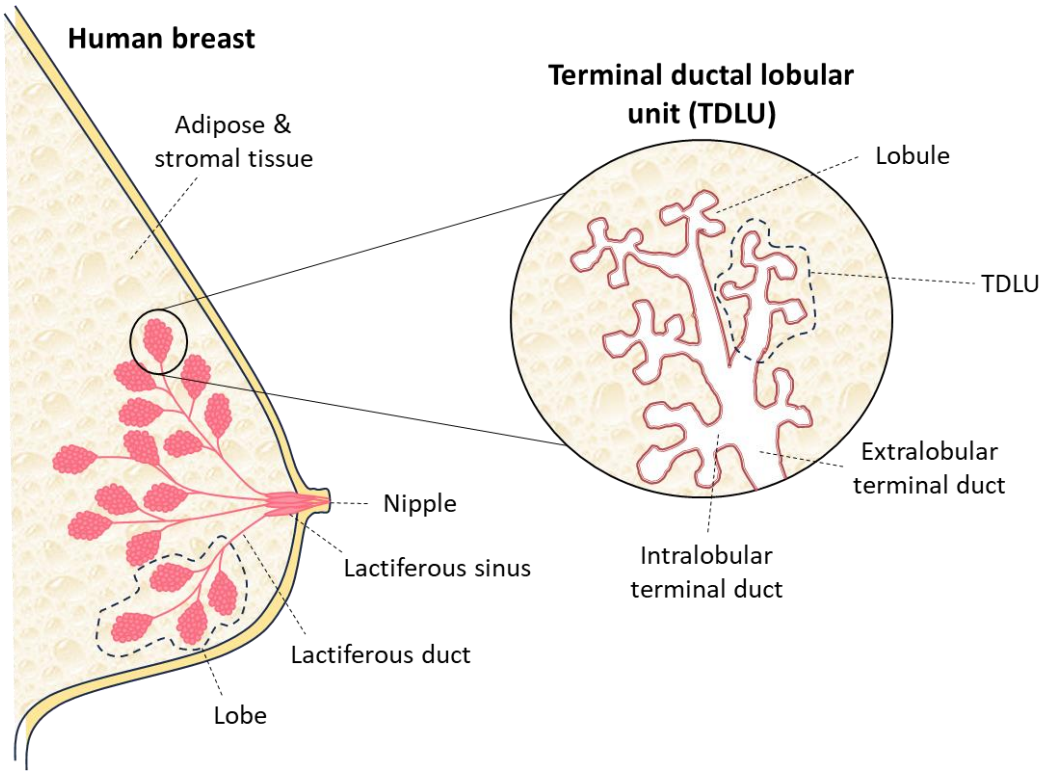


Figure 1. Organisation of the human mammary gland. The mammary gland is organised into 15-20 lobes which converge, via lactiferous ducts, at the nipple. Each lobe is comprised of terminal ductal lobular units (TDLUs).

TDLUs are considered the structural units of the human mammary gland, and each TDLU consists of a lobule and a draining terminal duct (18). This organisation of the mammary gland is species-specific and the human mammary gland differs from rodents, which do not have TDLUs, but instead have a single network of branched ducts (21). TDLUs are central to milk production and to lactation, yet understanding of their structure, composition and regulation is limited. Thus, most knowledge of human TDLUs to date has come from low-resolution images of histological specimens (22, 23). However, recent work by Paavolainen

and colleagues using 3-dimensional reconstruction histology and microscopy has improved understanding of TDLUs in human breast tissue (24). Their work has shown that TDLUs are composed of distinct cell types and there appears to be a main sub-tree that is predominantly responsible for bifurcation events (24). Furthermore, TDLUs have a similar organisation in different regions of a single human breast, as well as in different individuals (24). Interestingly, TDLUs in nulliparous women do not differ from those in multiparous women, confirming that structural reorganisation of TDLUs is not a major mechanism underlying greater breastfeeding success in multiparous women (25). Such structural insights are important for understanding normal mammary function, as well as mechanisms underlying disorders of lactation.

1.2.1.2. Cell types and their function

Epithelial cells

The human mammary epithelium is bilayered, consisting of an outer layer of contractile myoepithelial (basal) cells and an inner layer of luminal cells, with the latter further divided into alveolar and ductal subtypes (26). The alveolar luminal cells form the alveolar units that develop during pregnancy and are responsible for milk synthesis. In addition, there is a population of progenitor cells which are essential for mammary gland dynamics, particularly the expansion of the mammary gland during pregnancy. Most understanding of epithelial progenitors comes from mice, where there appear to be bipotent mammary stem cells that give rise to luminal and basal progenitors (27). These bipotent stem cells are important in maintenance and remodelling of the adult mammary gland (27).

Alongside progenitor and differentiated epithelial cells, a distinct population of epithelial-associated macrophages has been found in the mouse mammary gland (28-30). These ductal macrophages reside between the basal and luminal layers and constantly monitor the mammary epithelium, phagocytosing epithelial cells and contributing to mammary remodelling (31).

Adipocytes and connective tissue cells

Surrounding the TDLUs and outside the basement membrane are fibroblasts and a dense network of collagen (32, 33). This area also contains immune cells, including macrophages, dendritic cells and T cells, as well as peri-ductal blood vessels (29, 31, 34). The remainder of the adult human mammary gland consists of adipose tissue, blood vessels, extracellular matrix and immune cells (20). Rodent studies have established a dynamic role for mammary adipose tissue, interacting with the mammary epithelium and producing paracrine and endocrine factors, such as prolactin and oestrogen, during mammary development, pregnancy and lactation (35).

1.2.2. The lactation cycle

Mammary gland development and lactation are characterised by five hormonally-regulated stages termed the ‘lactation cycle' (figure 2) (36).

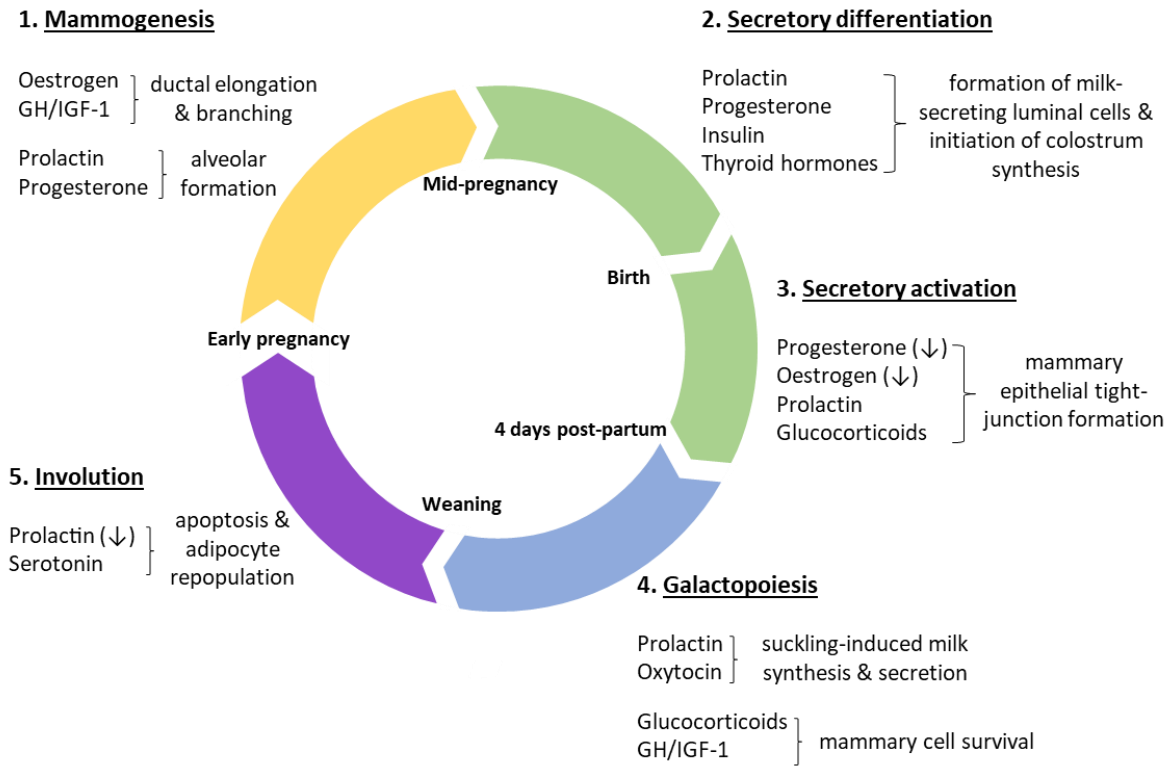


Figure 2. Hormonal regulation of the lactation cycle. GH, growth hormone; IGF-1, insulin-like growth factor 1.

Mammogenesis is characterised by epithelial proliferation, resulting in ductal elongation and branching from puberty to mid-pregnancy, and also in alveolar formation during pregnancy (36). Oestrogen and the growth hormone (GH)/insulin-like growth factor (IGF-1) axis promote ductal elongation and branching, while progesterone and prolactin mediate alveologenesis, in rodent models (37-41).

Secretory differentiation is characterised by the differentiation of alveolar mammary epithelial cells during pregnancy to milk-producing lactocytes. Rodent studies demonstrate that prolactin is the major hormonal driver of milk synthesis, and its actions are augmented by progesterone and thyroid hormones, which enhance prolactin-mediated signalling, and by insulin, which promotes lactose and lipid synthesis (42-47).

Secretory activation in humans is characterised by the onset of copious milk secretion 2-4 days after childbirth. This is predominantly mediated by elevations in prolactin and a decrease in progesterone and oestrogen following delivery of the placenta, while glucocorticoids are also important for mammary tight junction formation and closure (48-52). Delayed secretory activation is a major cause of early breastfeeding cessation and lactation insufficiency (53).

Galactopoiesis is characterised by the maintenance of milk production, and mediated by infant suckling, which causes increases in prolactin and oxytocin (54). During established lactation, glucocorticoids and GH/IGF-1 promote mammary cell survival (55-57).

Mammary gland involution occurs after weaning. A lack of suckling decreases prolactin secretion and increases concentrations of mammary paracrine factors associated with cell death, which promote mammary apoptosis and repopulation with adipocytes to return the breast to the pre-pregnant state (58, 59).

These stages of the lactation cycle are described in more detail below.

1.2.3. Mammary changes during pregnancy

1.2.3.1. Alveolar formation

Much of the existing knowledge surrounding mammary epithelial changes in pregnancy comes from studies in mice. Preparation of the mammary gland for lactation begins during early pregnancy with marked expansion of the mammary epithelium (60). In mice, ductal branching from the TEBs precedes alveologenesis, which is characterised by proliferation,

separation and differentiation of cells within alveolar buds (61). Thus, the alveolar cells become polarised and form a monolayer of cells around a central lumen, which is connected to the ductal network, while alveoli are surrounded by a discontinuous basket-like network of myoepithelial cells (62). Despite the extensive proliferation, the architecture of the mammary epithelial bilayer is maintained. Alveologenesis is mediated by prolactin, progesterone and oestrogens (63-65).

From mid-gestation, alveolar luminal cells undergo secretory differentiation (62). This is defined by changes in several cellular metabolic pathways relevant to lactation, although the production of milk is prevented by high circulating progesterone concentrations (60). Prolactin-mediated activation of transcription factors such as GATA binding protein 3 (GATA3) and E74-like ETS transcription factor 5 (ELF5) is considered central to secretory differentiation (66-68). In addition, a recent mouse knockout model has highlighted a non-redundant role for the transcription factor ETS homologous factor (EHF) in differentiation of alveolar cells (69). The DNA-binding domain of EHF shares high homology with that of ELF5, and they may act synergistically to promote differentiation (69, 70). Expression of EHF is reported to be oestrogen-responsive, suggesting a possible role for oestrogens in secretory differentiation, but understanding of EHF regulation at lactation onset remains incomplete (71).

1.2.3.2. Myoepithelial cell differentiation

In mice, basal cells also undergo differentiation during pregnancy to form smooth muscle-like myoepithelial cells, which are important for ejection of milk (72). Regulation of basal cell

differentiation remains poorly understood, although it appears that interactions between the helix-loop-helix (HLH) transcription factor inhibitor of DNA binding 4 (ID4) and the basic HLH protein HEB are important in this process, as demonstrated in mammary organoids and in ID4-knockout mice (73).

1.2.4. Secretory activation

1.2.4.1. Cellular changes

In humans, secretory activation refers to the onset of copious milk secretion occurring 2-4 days after birth, which precedes the maternal sensation of milk 'coming in' (74). It is characterised by several cellular changes in the mammary epithelium to support milk production, which are described below (figure 3).

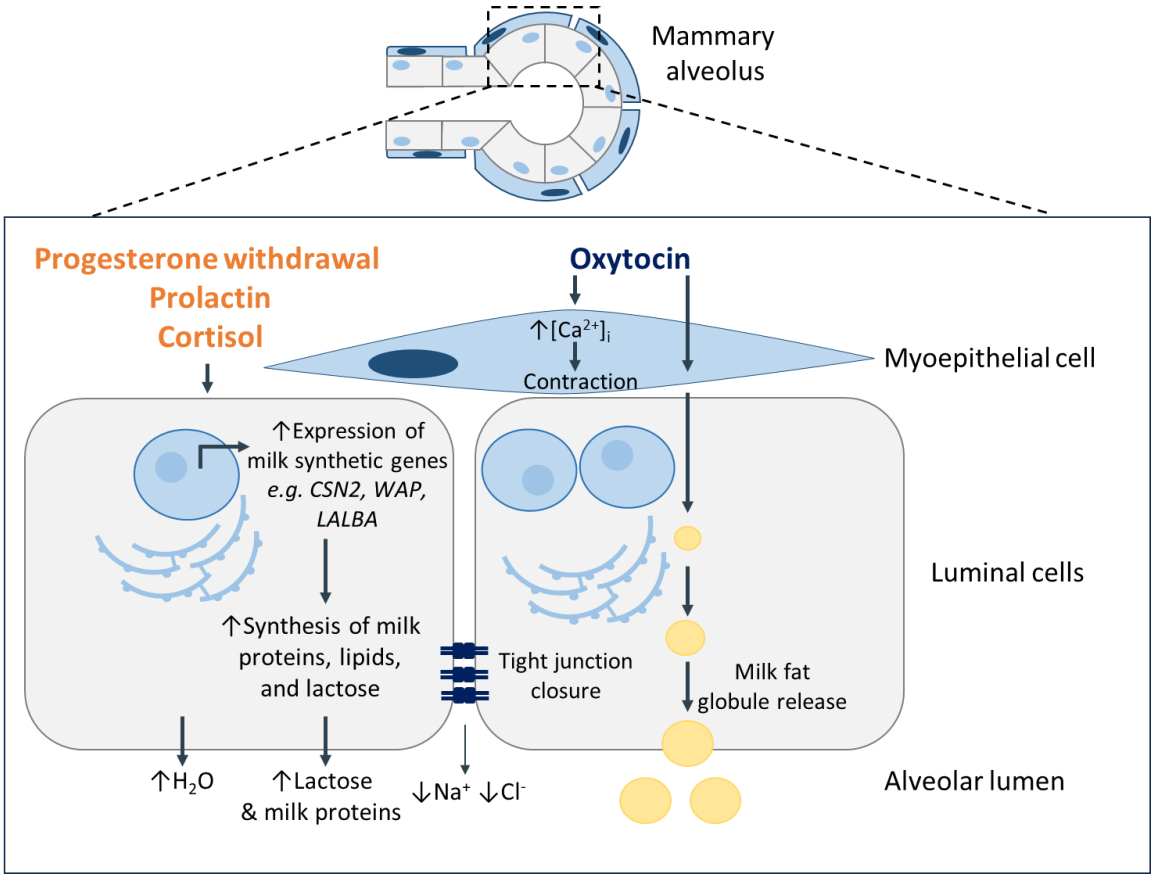


Figure 3. Summary of changes in mammary epithelial cells during secretory activation. Mammary epithelial cells undergo pronounced changes at lactation onset to support milk production. Luminal cells display binucleation, expansion of endoplasmic reticulum (ER) and Golgi, tight junction closure, and increased expression of milk-related genes. These changes require prolactin, cortisol and withdrawal of progesterone. Oxytocin induces contraction of myoepithelial cells, which promotes release of milk fat globules from luminal cells and expulsion of milk from alveoli. Adapted from Hannan et al (75).

Studies in mice have demonstrated that the majority of milk-producing luminal cells become binucleated in response to signals such as prolactin and epidermal growth factor (EGF), a finding also observed in human mammary epithelium (figure 3) (76). This may provide increased capacity for gene transcription. Furthermore, there is upregulated expression of genes required for protein synthesis, modification, transport and secretion (77). In addition, there is expansion of the endoplasmic reticulum and Golgi apparatus as milk production

increases (78). The endoplasmic reticulum is the site of milk protein lipid production. Synthesis of triacylglycerols occurs at the endoplasmic reticulum surface before packaging into lipid droplets and release into the cytoplasm (79). The Golgi compartment is the site of lactose synthesis, where the enzyme lactose synthase resides (60). Lactose synthase catalyses the conversion of glucose and UDP-galactose to lactose and, given the Golgi membrane is impermeable to lactose, this draws water into the Golgi (60).

Alongside these changes in cellular organelles, there is closure of intercellular tight junctions at the time of secretory activation (figure 3) (80). Lactose is the major osmotic component of milk, and its synthesis draws water into mammary epithelial cells (60). The combination of these processes results in increased volumes of milk secreted into the alveolar lumen, which are then ejected into the lactiferous ducts and towards the nipple by contraction of the myoepithelial cells.

1.2.4.2. Genetic changes mediating secretory activation

The transcriptional drivers for the changes observed during secretory activation in mouse studies include prolactin-mediated stimulation of ELF5, alongside the presence of glucocorticoids and the withdrawal of progesterone (52, 76). Furthermore, there is an increase of the pro-survival factor myeloid cell leukaemia-1 (Mcl-1) in luminal cells in mice, promoted by the >100-fold upregulation of epidermal growth factor (EGF) expression at lactation onset (81). Mcl-1 has been shown to be essential for survival of luminal cells and for repopulation of the mouse mammary epithelium during lactation (81).

While most work evaluating mammary epithelial changes at the time of secretory activation has been done in rodents, insights in humans have come from RNA contained in milk fat globules (77, 82). These studies have demonstrated increased expression of genes related to lactose, lipid and protein synthesis. Furthermore, they have provided insights into regulation of these changes. Thus, the genes related to the prolactin-STAT5 pathway are upregulated, as are those related to insulin signalling (77, 82).

1.2.5. Galactopoiesis

1.2.5.1. Hormonal and local factors mediating galactopoiesis

Galactopoiesis, the maintenance of milk production, is dependent on lactogenic hormones and local factors. Infant suckling, alongside auditory and olfactory stimuli from the infant, are essential triggers for oxytocin and prolactin release (54, 83). In humans and mice, oxytocin stimulates coordinated contraction of myoepithelial cells, which causes milk transit from alveoli to the nipple, while prolactin is necessary for milk synthesis (84, 85). However, the rate of milk production does not correlate with blood concentrations of prolactin in breastfeeding mothers (85). In fact, milk synthesis in women appears to be correlated with the extent of breast emptying, suggesting that local factors are important for maintenance of established lactation (86). These local signals could include mechanical stretching of luminal mammary epithelial cells, and the autocrine, paracrine or intracrine release of factors such as insulin-like growth factors (IGFs), parathyroid hormone-related peptide (PTHrP) and the monoamine serotonin (87-89).

1.2.5.2. Genetic drivers of galactopoiesis

Further information about the role of alveolar luminal cells in human lactation has been obtained from single cell RNA sequencing of cells isolated from milk (90). This approach has identified two subgroups of luminal epithelial cells, luminal clusters 1 (LC1) and 2 (LC2), that share expression of luminal markers, milk protein genes and genes related to fat excretion (90). However, there appear to be functional differences, such that LC1 cells show elevated expression of genes involved in transcription, immune function and cell stress, while LC2 cells demonstrate high expression of genes required for synthesis of milk components. Furthermore, these subtypes show distinct regulation, with LC2 cells having high expression of *STAT5A* and LC1 cells expressing SRY-box transcription factor 10 (*SOX10)*, the latter being an important regulator of mammary development in mice (91). This highlights the functional heterogeneity of luminal cells during established lactation.

1.2.5.3. Metabolic adaptations supporting milk synthesis

Galactopoiesis is characterised by sustained metabolic adaptation of luminal mammary epithelial cells. Transcriptomic assessment of human milk fat globule RNA, which reflects RNA present in the cytoplasm of luminal secretory cells, highlights the high abundance of genes related to synthesis of milk proteins, lipid and lactose at 4-6 weeks post-partum (77). Thus, caseins are the most expressed proteins in mature milk, while there is also high expression of genes involved in cellular protein synthesis, such as eukaryotic translation elongation factor 1 α1 (*EEF1A1*). Moreover, genes mediating lipid synthesis, such as fatty

acid synthase, and α-lactalbumin, the regulatory subunit of lactose synthase, are highly expressed.

Rodent studies performed in the mid-20th century demonstrated that increased mammary mitochondrial function is required to support sustained lactation (92, 93). Later work in mice has revealed that mammary mitochondrial biogenesis increases approximately 5-fold and is associated with expansion of the inner mitochondrial membrane, changes that are coordinated by the transcription factor peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) (94, 95). This is associated with upregulation of multiple mitochondrial electron transport chain proteins, particularly in mid- and late-lactation (94).

Sustained lactation is associated with marked systemic changes in metabolism in order to support the synthetic and energetic requirements of the mammary gland. Assessment of energy expenditure and body composition in lactation has highlighted increased energy expenditure and a predominant use of carbohydrates in women (96). In keeping with this, infusion of isotopic tracers into breastfeeding women demonstrates that increased glucose production from glycogenolysis is the major means of meeting maternal energetic requirements during lactation (97). However, most understanding of the adaptations in extra-mammary tissues comes from non-human studies. Thus, mouse studies demonstrate decreased hepatic fatty acid oxidation and increased amino acid degradation (98). Rodent and bovine studies also show pronounced changes in adipose tissue, such that there is net fatty acid and glycerol export from white adipocytes and decreased activity of brown adipocytes (99-102). There is increased breakdown of muscle protein to provide a source of amino acids for lactation in rats and cows, while muscle use of fatty acids is reduced in rats (103, 104). In order to meet the increased demands, there is intestinal hypertrophy and

increased absorptive capacity in rodents (105-107). These adaptations are summarised in table 3. The overall effect of these systemic changes is to increase substrate availability for the lactating mammary gland.

Table 3. Maternal metabolic adaptations during lactation.

Adaptation	Species	Reference(s)
Mammary gland		(108, 109)
↑Glucose utilisation	Rat	
↑Fatty acid esterification (↑lipid synthesis)	Rat	
↑Lipid and amino acid uptake	Rat	
↑Protein synthesis	Various	
↑Substrate uptake (glucose, fatty acids, amino acids)	Mouse	
Liver		(97, 98, 110)
↑Glycolysis	Mouse	
↑Glycogenolysis	Human	
↑Gluconeogenesis	Human	
↑Amino acid degradation	Mouse	
↓Fatty acid oxidation	Mouse	
White adipose tissue		(99, 100)
↑Lipolysis	Rat	
↑Fatty acid and glycerol release	Rat	
↓Fatty acid synthesis	Cow	
↓Lipoprotein lipase activity	Cow	
Brown adipose tissue		(101, 102)
Hypotrophy	Mouse	
↓Mitochondrial content	Rat	
↓Thermogenesis	Mouse	
Skeletal muscle		(103, 104)
↓Fatty acid degradation	Rat	
↑Protein catabolism	Cow	
↑Glycolysis	Rat	
Intestine		(105-107)
↑Intestinal mass	Various	
↑Absorptive capacity	Rat	
↑Nutrient absorption	Rat	

1.2.6. Involution

Mammary involution marks the cessation of milk secretion and two stages to this process have been characterised in mice. The first stage is reversible and caused by stasis of milk within the alveoli, which leads to a change in luminal cell phenotype from a secretory to a phagocytic one (111). These cells begin phagocytosis and hydrolysis of milk fat globules, and the free fatty acids released trigger cell death (111). These changes are induced by expression of cytokines such as transforming growth factor-β3 isoform and leukaemia inhibitory factor, which activate STAT3 in mammary cells (112, 113).

In the subsequent, irreversible stage of involution, there is widespread apoptosis (114). Secretion of prolactin is reduced when suckling stops, and decreased prolactin concentrations lead to increased mammary release of the pro-apoptotic factor gonadotropin releasing hormone (GnRH) (115). GnRH may also promote mammary remodelling by attracting mast cells to the mammary epithelium (115). Furthermore, involution is associated with reduced glucocorticoid sensitivity during involution, which may increase STAT3 activation, and an increase in circulating oestradiol, which is reported to promote involution (116, 117).

1.3. Hormones with established roles in mammary development and lactation

1.3.1. Prolactin: the principal lactation hormone

1.3.1.1. Structure, release and regulation

The presence of a lactogenic hormone was first suggested by the work of Stricher and Greuter in 1928, who showed that injection of anterior pituitary extracts into castrated virgin rabbits induced lactation (118). The responsible hormone was isolated in 1933, and named prolactin due to its role in lactation, by Riddle and colleagues (119). However, it was not until 1971 that a radioimmunoassay was developed to detect human prolactin and used to demonstrate its presence in human plasma (120).

Human prolactin is a 23 kDa protein that contains 199 amino acids (121). It characterised by a three-dimensional structure consisting of four anti-parallel alpha helices, and shares structural homology with placental lactogen and GH (122). In the non-pregnant, non-lactating state, synthesis and secretion of prolactin by anterior pituitary lactotrophs is predominantly controlled by the inhibitory effect of dopamine (123). Dopamine is produced by neurons that are located in the arcuate nucleus of the hypothalamus (121). These neurons are the tuberoinfundibular (TIDA), tuberohypophyseal (THDA), and periventricular hypophyseal (PHDA) neurons, named according to their anatomy but with similar mechanisms of regulation (124). These dopamine-producing neurons are, in turn, regulated by a broad range of stimulatory and inhibitory substances (121).

In humans, prolactin concentrations increase throughout pregnancy and remain high during lactation (125). During pregnancy in women, the high concentrations of oestradiol produced by corpus luteum, and subsequently the placenta, cause a reduction in dopamine release by TIDA neurons and hypertrophy of pituitary lactotrophs (126). Both pregnancy and lactation are characterised by decreased dopamine release from TIDA neurons in rats, which had previously been considered to reflect insensitivity of these neurons to prolactin-mediated inhibitory feedback (127, 128). However, more recent work on these neurons in mice has

demonstrated that they maintain responsiveness to prolactin but instead uncouple their electrical activity from dopamine release (129). This facilitates high levels of prolactin secretion from the anterior pituitary during pregnancy and lactation.

Baseline prolactin concentrations in the first six post-partum weeks are higher than in non-pregnant, non-lactating women, and are increased by suckling (54, 130). Thus, prolactin concentrations are reported to rise across a breastfeeding episode and peak at approximately 45 minutes after initiation of feeding (130). After six post-partum weeks, a prolactin rise with suckling is still observed but the response is attenuated. The increase in blood prolactin concentration with suckling is reported to result from inhibition of dopamine release from the TIDA system in rats, although understanding of the neural circuits regulating this is incomplete (83).

Circulating prolactin concentrations in women are also influenced by a number of other factors in the non-pregnant, non-lactating state. Prolactin secretion shows circadian variation and is maximal during rapid eye movement (REM) sleep, typically peaking between 4am and 6am each day (131). Other physiological factors that increase prolactin concentrations include stress, nipple stimulation, chest wall stimulation, sexual intercourse, exercise, a high protein diet and hypoglycaemia (131). However, whether these factors influence prolactin secretion in lactation has not been investigated.

1.3.1.2. Prolactin signalling

Prolactin, like placental lactogen and growth hormone, is a member of group I of the helix bundle protein hormones (122). It binds to the prolactin receptor, a member of class 1 of

the cytokine receptor superfamily, and containing an extracellular, transmembrane and intracellular domain (132). Binding of prolactin to its receptor induces receptor dimerisation, and rapid transphosphorylation and activation of prolactin receptor-associated Jak2 (133-135). The activated Jak2 then acts to phosphorylate tyrosine residues on the intracellular domain of the prolactin receptor (136). The phosphorylated tyrosine residues are docking sites for proteins with Src homology 2 (SH2) domains, including the STAT proteins (137). STAT5 is particularly important in mediating the effects of prolactin in mammary epithelial cells. After its phosphorylation, STAT5 dimerises and translocates to the nucleus, where it promotes expression of genes related to lipid, protein and lactose synthesis (138, 139). The importance of the prolactin receptor-Jak2-STAT5 pathway is highlighted by mice with a knockout of one of these proteins, which have profound impairments in alveologenesis and milk production (140-142).

Signalling through the prolactin receptor-Jak2-STAT5 pathway is enhanced by interactions of luminal cells with the extracellular matrix. Interaction between basement membrane laminin and β1-integrins on the surface of luminal cells leads to activation of the integrin-linked kinase (ILK) and Ras-related C3 botulinum toxin substrate 1 (RAC1) proteins, which potentiate prolactin signalling (143, 144). Contact between mammary epithelial cells and the extracellular matrix promotes clathrin-mediated endocytosis of prolactin, which is required for maximal STAT5 activation, a process that is independent of Jak2 and RAC1 signalling (145). In addition, a number of other cellular proteins, including the tyrosine protein kinase SRC, the cell surface adhesion molecule nectin 4, and the intracellular protein CUB and zona pellucida-like domains-containing protein 1 (CUZD1), also appear to enhance Jak2-STAT5 signalling (146-148).

The prolactin signalling pathway is also under negative regulation. Thus, signalling is reduced by the plasma membrane protein caveolin 1 and the intracellular suppressors of cytokine signalling (SOCS) proteins (149, 150). The inhibitory action of these proteins is inhibited by Akt, which is activated by prolactin (151, 152). Furthermore, a recent article pre-print has demonstrated that prolactin-mediated activation of adenosine monophosphate-activated protein kinase (AMPK) leads to inhibition of STAT5 signalling (153). Prolactin signalling in mammary luminal epithelial cells, and its regulation, is summarised in figure 4.

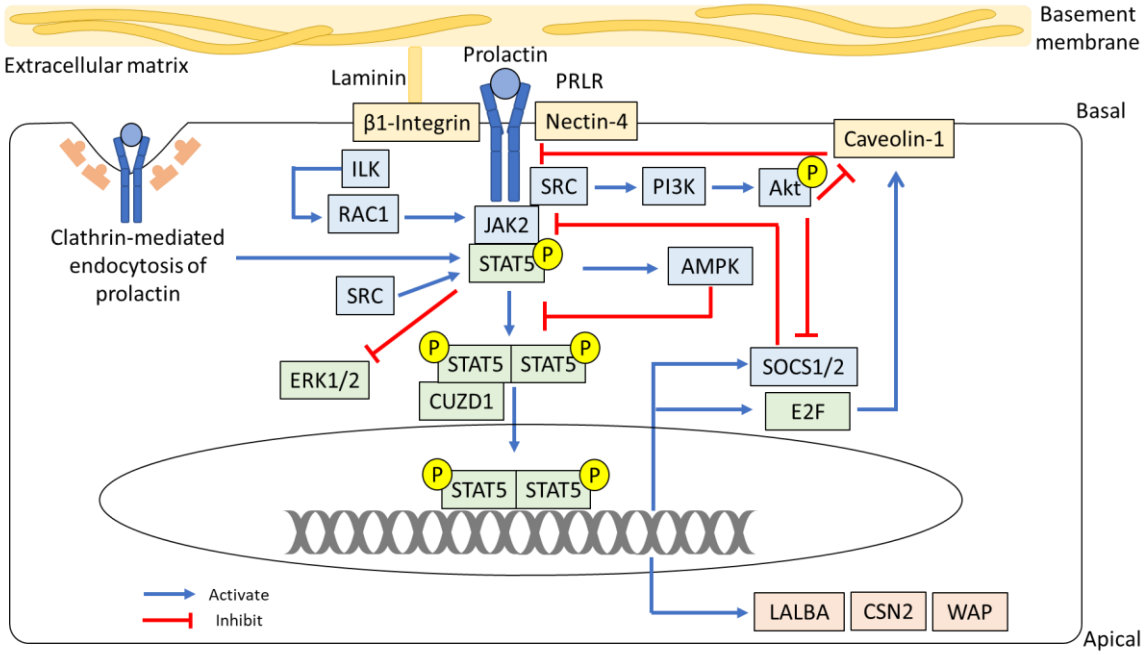


Figure 4. Prolactin signalling in mammary epithelial cells. Prolactin classically signals through the Jak2-STAT5 pathway to promote expression of milk protein genes (LALBA, CSN2, WAP). Cell interaction with the basement membrane potentiates prolactin signalling through ILK and RAC1. Clathrin-mediated endocytosis of prolactin is required for maximal STAT5 activation. Downstream effectors include AMPK, which downregulates prolactin signalling, and the PI3K-Akt pathway, which promotes prolactin signalling by inhibiting calveolin-1 and SOCS1/2 activity. Adapted from Hannan et al (75).

1.3.1.3. Effects of prolactin on the mammary epithelium

Prolactin plays a critical role in the mammary gland at several stages. Knockout of prolactin or the prolactin receptor in mice leads to reduced side branching of ducts during mammary development, and impaired alveologenesis and lactation (63, 141, 154, 155). The proliferative effects of prolactin on the mammary gland are largely indirect and mediated by RANK ligand (RANKL) (156-158). This is reflected in the phenotype of RANKL-knockout mice, which lack alveolar growth and fail to lactate (156). Prolactin-induced paracrine signalling through RANKL requires the serine/threonine protein phosphatase wild-type P53-induced phosphatase 1 (WIP1), and WIP1-knockout mice also display reduced alveolar development (159).

Prolactin-mediated activation of other transcription factors is also required for alveolar differentiation and lactation. Thus, signalling through transcription factors including ELF5 and GATA3 is essential for prolactin-induced differentiation and milk production in mice (66-68). Prolactin is also required for mammary tight junction formation, acting through STAT5 to increase expression of claudins in epithelial cells, and working in synergy with glucocorticoids (51, 160). Furthermore, prolactin promotes protein synthesis, glucose uptake, uptake of fatty acids, and synthesis of lipids in rodents (161-166).

1.3.1.4. Effects of prolactin on extra-mammary tissues

In addition to its effects on the mammary gland, prolactin influences several extra-mammary tissues. Prolactin has been demonstrated to contribute to the hyperphagia of lactation in rats, which is necessary to meet the metabolic demands of milk production

(167-169). This is mediated by prolactin receptor-expressing neuropeptide Y neurons in the dorsomedial nucleus of the hypothalamus (DMH), which project to the paraventricular nucleus (PVN) of the hypothalamus and stimulate food intake (169). Alongside promoting increased dietary intake, prolactin is also reported to increase intestinal mass and improve absorption of nutrients in rats (170-173).

In addition to its effects on appetite and intestinal absorption, prolactin has effects on systemic metabolism. Prolactin acts on the liver to improve insulin sensitivity (174). It also alters hepatic lipid handling, although effects vary according to the model used. Thus, mice injected with a short hairpin RNA to decrease prolactin receptor protein levels in hepatocytes demonstrate decreased hepatic triglyceride uptake (175). By contrast, mice lacking dopamine type 2 receptors (D2Rs), which have chronic high secretion of prolactin from lactotrophs, exhibit increased hepatic lipid uptake (176, 177). Similarly, while most studies in mice which assess the effect of prolactin on white adipose tissue support an adipogenic role, with inhibition of lipolysis, others report that prolactin reduces fat mass (178-183). Prolactin also promotes development and activity of brown adipose tissue, with prolactin receptor deficient mice having hypotrophic brown adipose tissue and decreased conversion of white adipose tissue to a brown adipose-like phenotype (184, 185).

The effects of prolactin on skeletal muscle are less-studied, but chronic hyperprolactinaemia is associated with reduced muscle mass in humans (131). Moreover, prolactin is reported to decrease insulin sensitivity of muscle in women (186). These effects of prolactin may be importing for diverting substrates away from skeletal muscle and to the mammary gland. Finally, evidence from rodent studies demonstrates that prolactin acts on the pancreas to promote β-cell development, proliferation and insulin secretion (187-190).

1.3.1.5. Disorders of prolactin

While the pathophysiology of delayed lactation onset is poorly understood in the majority of women, many of the described risk factors are associated with endocrine dysfunction. Genetic and acquired defects of prolactin production or signalling causing impaired lactation are perhaps the clearest example. Thus, germline mutations in the prolactin gene, as well as loss-of-function mutations of the prolactin receptor gene, are associated with alactogenesis in women (191, 192). Furthermore, autoantibodies against the prolactin-producing pituitary lactotrophs have been described in a woman with alactogenesis (193).

Defects of prolactin can also be observed as part of more widespread pituitary endocrine disruption. Pituitary insufficiency as a result of post-partum haemorrhage-induced pituitary necrosis (Sheehan's syndrome), which results in reduced prolactin, thyroid hormone and cortisol production, causes alactogenesis (194). Caesarean section impairs prolactin release and the pulsatility of oxytocin release on day 2 post-partum and is linked to delayed onset of lactation (195). Moreover, endocrine disrupting chemical (EDC) exposure is a risk factor for reduced breastfeeding duration (196-198). EDCs are reported to have oestrogenic actions and have been linked to decreased prolactin concentrations in mice (199-201).

1.3.2. Human placental lactogen

1.3.2.1. Structure, release and regulation

The existence of a lactogenic substance originating from the placenta was reported in various animals in the early 1900s, but the first report of a prolactin-like substance in the

human placenta came from the work of Erhardt in 1936, based on the ability of placental extracts to stimulate secretion from mammary gland explants (202-204). In 1953, Ito and Higashi isolated a substance from human placenta that was capable of inducing crop sac growth in pigeons (205). The presence of this substance in the serum of term women was demonstrated in 1962 by Josimovich and MacLaren (206).

Subsequent work has identified human placental lactogen (hPL) as a polypeptide hormone produced by the syncytiotrophoblast cells of the placenta. hPL is a 191 amino acid protein, which is structurally similar to GH and prolactin (86% and 13% homology to GH and prolactin respectively) and can bind to both the prolactin and GH receptors (207-210). hPL can first be detected in human plasma at around six weeks gestation. The plasma concentration of hPL then increases linearly until ~30 weeks gestation, with a concentration that is 25-fold greater than prolactin (211, 212). hPL is a pregnancy-specific hormone and rapidly disappears from maternal circulation following delivery of the placenta.

The regulation of hPL concentrations remains poorly understood. Maternal serum hPL concentrations positively correlate with placental mass and the number of foetuses carried (208, 212). Specifically, hPL production reflects syncytiotrophoblast mass (213, 214). Secretion of hPL is reported to be under metabolic control. Thus, intravenous glucose infusions decrease hPL concentrations, while a prolonged (84-90 hour) fast during pregnancy increases hPL concentrations (215-218). However, other studies have not observed an effect of glucose administration or insulin-induced hypoglycaemia, and the effect of glucose on hPL remains controversial (208, 219, 220). Epidermal growth factor is reported to increase hPL release by stimulating differentiation of cytotrophoblast cells to syncytiotrophoblast cells in placental organ culture (208), but not by increasing rate of

release. IGF-1 and angiotensin II may stimulate hPL release in human placental explants and trophoblastic cell suspensions, respectively (221, 222). In vitro studies suggest that high density lipoprotein may increase hPL synthesis and release in a dose-dependent manner, and this has been replicated in ewe studies (223, 224).

1.3.2.2. Effects of hPL on the mammary gland

Animal studies in the 1960s demonstrated a role of hPL in lactation. In addition to promoting crop sac growth in pigeons, hPL has been shown to induce lactation in pregnant rabbits (205, 206, 225). Subsequent co-culture of placental explants and mammary tissue demonstrated a lactogenic response in the latter in baboon, sheep, chinchilla, hamster, rat, mouse, and guinea pig tissue, but not in rabbit or dog tissue (226). Co-culture of mouse placental explants and mammary tissue resulted in mammotrophic activity, with secretion into the mammary alveolar lumen, while transplantation of placental fragments to mammary fat pads resulted in local lobuloalveolar development around the placental transplants (227).

Despite these animal studies, there is little understanding of the role of hPL in human mammary development, secretory activation or galactopoiesis (228, 229). Evidence from pregnancies with low or undetectable hPL suggests that it is not needed for a healthy pregnancy, and that lactation can occur normally in its absence. Multiple case studies report normal pregnancies and successful lactation in women producing no or minimal hPL (230-235). It is unclear whether this indicates that the role of placental lactogen is very minor

compared to other hormones in normal pregnancy, or if this indicates that other hormones are able to replace the normal role of placental lactogen.

Given its affinity for the prolactin receptor, it is assumed that placental lactogen exerts lactogenic effects via prolactin receptor-associated signalling pathways (161, 209, 212, 236, 237). There is variation between species in the relative affinities of prolactin and placental lactogen for the prolactin receptor (PRLR), and more research is needed on the relative affinities in a human context (238).

1.3.2.3. Effects of hPL on extra-mammary tissues

hPL has an important role in maternal metabolic adaptation. Beck and Daughaday (1967) found that overnight infusion of hPL resulted in increased blood glucose concentration after carbohydrate ingestion. Plasma insulin responses were also greater after overnight hPL treatment (239). Furthermore, hPL increases maternal prolactin receptors in human pancreatic β cells, promoting cellular adaptations to increased insulin requirements during pregnancy (236, 240, 241). hPL has a proliferative effect in rodent pancreatic beta cells, and this is also seen to a lesser degree in human beta cells *in vitro* (236, 242).

hPL has an inhibitory effect on leptin production in cultured human trophoblast cells (243, 244). In later stages of pregnancy, the increased leptin concentrations result in decreased maternal nutrient intake, although pregnant women develop a state of leptin resistance in order to maintain sufficient caloric consumption (236, 245, 246). The effects of hPL on leptin secretion have not been studied *in vivo*, nor are its effects on maternal food intake clear (236).

It has been suggested the hPL is also important for regulating foetal metabolic adaptations and growth (208, 236). This is based on observations of positive correlations between maternal hPL and birth weight (247, 248). A study involving 83 patients with insulin-dependent diabetes found that hPL levels among women who delivered macrosomic newborns were significantly higher compared to the mothers of newborns with a normal weight (249). One possibility is that hPL affects foetal growth by stimulating IGF production, and a positive correlation between IGF-1, IGF-2 and hPL levels has been described in serum samples collected at >33 weeks gestation, supporting this idea (236, 250).

1.3.3. Oxytocin

1.3.3.1. Structure, release and regulation

The presence of a pituitary substance capable of inducing contraction in the cat uterus was first reported by Sir Henry Dale in 1906, which he later named oxytocin (251). In 1910, Ott and Scott were the first to identify a galactagogic effect of oxytocin, demonstrating that pituitary extract caused mammary gland contraction and increased flow of milk in lactating goats (252). The role of oxytocin in humans was highlighted by MacKenzie in 1911, who observed a 1.6-fold increase in breast milk production in a woman injected with fowl pituitary extract (253).

The nine amino acid structure of oxytocin was described in 1953 (254, 255). Oxytocin is produced from the posterior pituitary and is evolutionarily well conserved across phyla (256, 257). Oxytocin is synthesised in magnocellular neurons, which are primarily located in the supraoptic nucleus (SON) and PVN regions of the hypothalamus (258). Noradrenergic

projections from the ventrolateral medulla (VLM) and nucleus of the tractus solitarius (NTS) form important inputs into the magnocellular neurons (259). Maternal neurons originating from the NTS are activated during birth and lactation, with noradrenaline acting to promote oxytocin secretion in rats (260-263). Moreover, oxytocin promotes activity of SON neurons in rats, which produces a local positive feedback loop to promote milk ejection during lactation (264).

Proopiomelanocortin (POMC) neurons from the arcuate nucleus also regulate oxytocin secretion (265). They secrete α-melanocyte stimulating hormone (αMSH), which acts on melanocortin 4 receptors (MC4R) in the SON and PVN (266). αMSH inhibits oxytocin secretion into the circulation and this effect may be mediated, at least in part, by endocannabinoids (267, 268). Inhibition of oxytocin secretion by αMSH is lost in mid-pregnancy, which may be important for oxytocin release during lactation (266).

Oxytocin also has an autocrine feedback role on magnocellular neurons. Thus, oxytocin activates oxytocin receptors on magnocellular neurons, which produces an inhibition of excitatory synaptic activity under basal conditions (269). This inhibition is partly mediated by endocannabinoid activity (269). Under some physiological states, including lactation, there is a shift from inhibitory to excitatory oxytocin feedback. This may result from a switch of endocannabinoid-mediated inhibition to excitation, perhaps due to increased expression of the endocannabinoid-activated excitatory transient receptor potential vanilloid-1 channels, changes in post-synaptic properties of magnocellular neurons, increased excitatory inputs, and/or a shift from inhibitory to excitatory GABA signalling (270-277).

1.3.3.2. Effects of oxytocin on the mammary gland

During lactation, plasma oxytocin increases in a pulsatile manner in response to suckling (278-281). In humans, maternal plasma oxytocin concentrations between day 3-5 post-partum significantly increase within two minutes of suckling, and peak after ten minutes (279). The plasma oxytocin response is significantly higher in established lactation compared to early and mid-lactation (280). Plasma oxytocin secretion can be initiated just before suckling commences in response to external stimuli such as infant crying (278), indicating that oxytocin is released in response to sensory inputs, such as visual, tactile, olfactory, and auditory stimuli (282).

Oxytocin acts through the G-protein coupled oxytocin receptor (OXTR), located on the surface of the myoepithelial cells surrounding the mammary alveoli and ducts (283, 284). *OXTR* gene expression is increased during lactation, increasing myoepithelial sensitivity to oxytocin (285). Oxytocin stimulates the hydrolysis of phospholipids, which in turn increases intracellular calcium and promotes contraction of myoepithelial cells (figure 5), and therefore expulsion of milk (286-290).

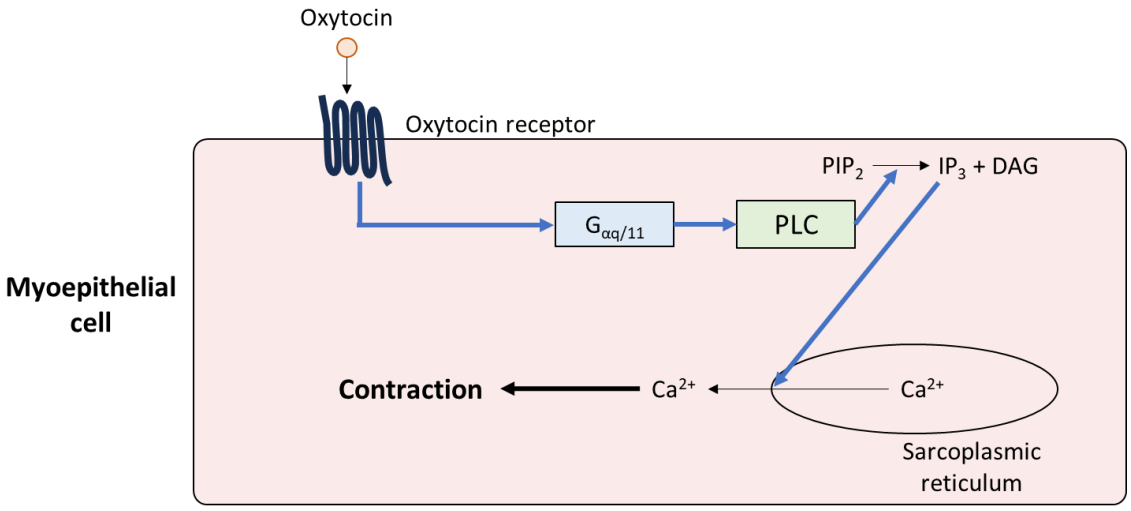


Figure 5. Oxytocin-mediated contraction of myoepithelial cells. Oxytocin signals through the oxytocin receptor, which leads to activation of phospholipase C (PLC) through the G protein, $G_{\alpha q/11}$. This results in hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP3 leads to calcium release from the sarcoplasmic reticulum, which triggers contraction of myoepithelial cells.

1.3.3.3. Effects of oxytocin on extra-mammary tissues

Oxytocin influences human mood by acting on the hypothalamus (291). Thus, studies in breastfeeding women have demonstrated that plasma oxytocin concentrations are inversely correlated with levels of anxiety and aggression (292-296). Furthermore, salivary oxytocin is inversely correlated with self-reported maternal fatigue and confusion (297). However, a relationship with mood is inconsistent and some studies have shown no associations between oxytocin and maternal mood (298, 299). Oxytocin also plays important roles in mother-infant bonding. Maternal circulating oxytocin increases in response to maternal–infant bonding events, skin-to-skin contact and sensory stimuli (300). Moreover, maternal skin temperature increases and conveys warmth to the infant via skin-to-skin contact, which leads to oxytocin release in the newborn brain (301).

Mouse studies have suggested potential roles for oxytocin in promoting maternal energy adaption during lactation, including adipose tissue lipolysis, fatty acid β-oxidation, and regulation of food intake (302). Potential metabolic roles of oxytocin during human lactation may therefore include food intake and weight regulation, improvements in insulin sensitivity in adipose tissue and increased glucose utilisation in adipose and skeletal muscle, regulation of maternal energy expenditure, and maternal bone metabolism and calcium homeostasis (303-306).

1.3.4. Progesterone

1.3.4.1. Role in mammary gland development

During pregnancy, progesterone plays an important role in promoting mammary growth while preventing milk production by antagonising the effects of prolactin.

The importance of progesterone for mammary development during pregnancy is highlighted by a study of 91 mothers, demonstrating a positive association between antenatal serum progesterone concentrations and milk output volume at weeks 1 and 4 post-partum (307). In mice, progesterone induces proliferation of mammary epithelium through cyclin D1-dependent and RANKL-dependent mechanisms (64). The presence of RANKL is important for the formation of lobulo-alveolar mammary structures in mice during pregnancy (156). In addition, progesterone-mediated control of RANKL and associated cell proliferation has been demonstrated in healthy non-pregnant, non-lactating human breast tissue (308). This is under classical progesterone receptor (PR)-mediated transcriptional effects. Furthermore, serum progesterone levels correlate with RANKL protein expression in a subset of human

luminal epithelial cells that express progesterone receptor (308). Progesterone also drives ductal side-branching morphogenesis in mice via a progesterone-dependent activation of Rac-DTPase signalling (309).

During pregnancy, progesterone plays an important role in inhibiting milk production. Thus, the human progesterone receptor has been shown to inhibit prolactin-mediated induction of β-casein gene transcription in mammary epithelial cells (310). High progesterone levels have an inhibitory action on secretory activation and progesterone antagonises the actions of prolactin receptors in rabbit mammary tissue (311). Moreover, progesterone represses the synthesis of major milk proteins (α-lactalbumin and caseins) and sugars (lactose) in animal studies (312, 313). The effects of progesterone in pregnancy are outlined in figure 6.

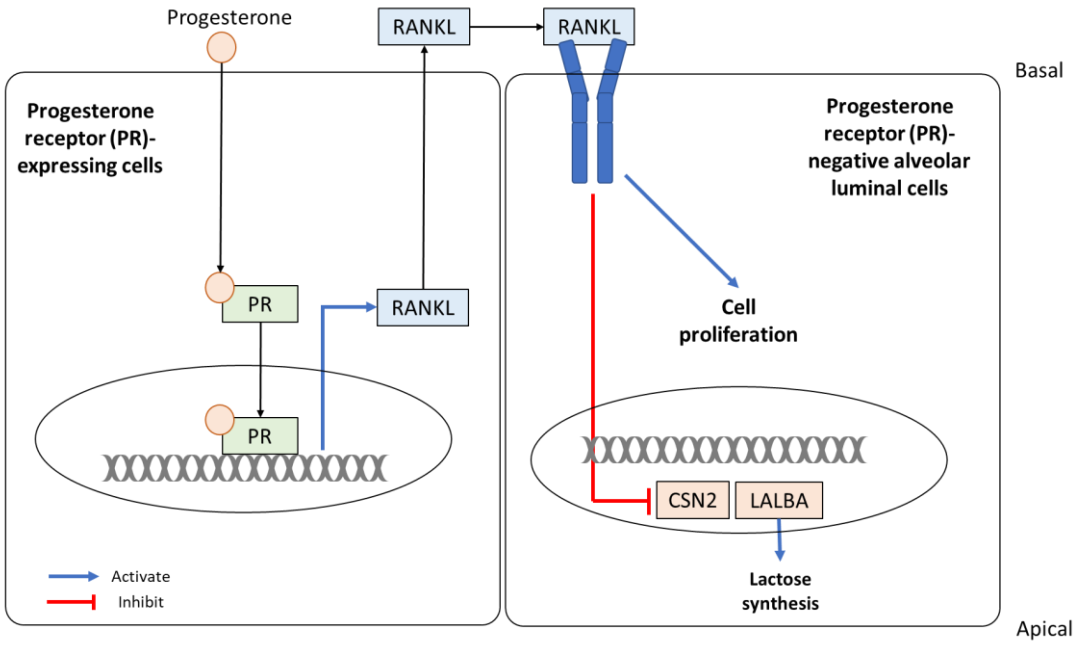


Figure 6. Summary of progesterone effects on mammary epithelial cells during pregnancy. Progesterone signals through the intracellular progesterone receptor (PR) in PR-expressing mammary cells. This inhibits expression of milk proteins (including caseins and α-lactalbumin) and production of lactose. Furthermore, progesterone acts through RANKL to promote cell proliferation in PR-negative alveolar luminal cells (314).

1.3.4.2. Role in secretory activation

Rapid progesterone withdrawal during complete delivery of the placenta is necessary for the onset of secretory activation in humans (figure 2). Retained placental fragments in women produce progesterone, which inhibits secretory activation, and women fail to lactate until retained placental fragments are removed (50, 315). Furthermore, the rate of excretion of lactose in urine was related to the rate of decline of progesterone in women (316).

In mice, withdrawal of progesterone promotes secretory activation by facilitating closure of mammary gland tight junctions (52). Additionally, inhibitory effects of progesterone on prolactin are removed following delivery of the placenta. Specifically, progesterone may inhibit prolactin-induced increases in mammary prolactin receptor expression, as well as prolactin-mediated gene expression, in mice (317).

1.3.5. Glucocorticoids

1.3.5.1. Role in mammary gland development

The importance of glucocorticoids in mammary development during pregnancy is demonstrated by mammary epithelial cell-specific knockout of the glucocorticoid receptor in mice (318). The mammary gland in these mice exhibits decreased cell proliferation and reduced infiltration of the mammary fat pad, although there was no defect in alveolar differentiation or milk production in this study (318).

Hydrocortisone treatment of mammary explants from mid-pregnant mice highlights a role for glucocorticoids in organelle development (319). Addition of hydrocortisone increases the volume of rough endoplasmic reticulum and Golgi apparatus in mammary cells, and this correlates with increased casein synthesis. Furthermore, it appears that hydrocortisone must be present in media before or at the same time as prolactin in order for prolactin to exert its effects on mammary cell differentiation (320).

1.3.5.2. Role during lactation

Glucocorticoids play an essential role in the formation of mammary tight junctions at the time of secretory activation (figure 2). Plasma lactose, a marker of tight junction permeability, increases in cows after stopping milking for 17 hours, and this is prevented by injection of adrenocorticotrophic hormone (ACTH), which increases circulating cortisol concentrations (321). Furthermore, the synthetic glucocorticoid dexamethasone promotes tight junction formation in mouse mammary cell lines when combined with prolactin, but only when cells are pre-treated with dexamethasone (51). Subsequent work on a mammary epithelial tumour cell line has shown that this effect requires downregulation of the RhoA GTPase, as well as Akt-mediated phosphorylation and degradation of glycogen synthase kinase 3 (GSK3) (322, 323). As GSK3 phosphorylates and destabilises β-catenin, increased unphosphorylated β-catenin supports maintenance of mammary tight junctions (323).

Work in the 1950s on rats also demonstrated a role for glucocorticoids in preventing involution (324). Moreover, corticosterone implants prevent the decline in milk volume in late-lactation rats, increasing mammary RNA content and lipoprotein lipase activity, while

also decreasing lipid storage in adipose tissue (325). The effect in mammary epithelial cells is mediated by reducing the degradation of STAT5, which is required for milk protein synthesis, and through delayed activation of the involution-promoting transcription factor STAT3 (116).

Adrenal insufficiency in women is reported to be a cause of lactation insufficiency, although cases described typically have pituitary insufficiency rather than isolated glucocorticoid deficiency (326, 327).

1.3.6. Oestrogens

1.3.6.1. Role in mammary gland development

A role for oestrogens in mammary development was first demonstrated through implantation of pellets, coated with oestrogen or oestrogen antagonists, directly into the mammary gland in mice (328). These studies demonstrated the oestrogens are necessary for mammary ductal growth during puberty (329, 330). The mammary effects of oestrogens are mediated by oestrogen receptor 1 (ESR1), and their effects are synergistic with the mammary ductal effects of IGF-1 (38, 39). Subsequent work has demonstrated that oestrogens exert their effects on the stromal cells adjacent to terminal end buds, which then signal through paracrine factors to basal mammary stem cells (41, 331). In adult mice, cyclical changes in oestrogen promote lateral ductal branching (332).

1.3.6.2. Role during lactation

Oestrogens promote alveologenesis during pregnancy (figure 2). Thus, ablation of the oestrogen receptor during pregnancy in mice results in reduced tertiary branching, decreased alveolar numbers and impaired lactation (65). Moreover, a study involving breastfeeding women demonstrated that antenatal oestrogen deficiency is associated with reduced serum prolactin concentrations and impaired secretory activation in the post-natal period (333). However, during the post-partum period, oestrogens are reported to exert an inhibitory effect upon lactation. Thus, animal studies, including mammary administration of oestrogen to rats and injection of 17β-oestradiol into cows, showed that oestrogens inhibit milk production (49, 334, 335). Furthermore, in breastfeeding women, there is a negative correlation between plasma oestradiol concentrations and milk output at 4 weeks post-partum (307). These observations may be due to the involution-inducing effects of oestrogens. Oestrogens are reported to mediate mammary cell death, neutrophil-dependent inflammation, and adipocyte repopulation of the mammary gland (117, 336). Therefore, loss of oestrogens following delivery of the placenta may promote lactation.

1.3.7. Parathyroid hormone related peptide (PTHrP)

1.3.7.1. Regulation and effects on the mammary gland

PTHrP was first described as the factor causing humoral hypercalaemia of malignancy (337). The mammary gland was subsequently identified as one of the first tissues to produce PTHrP physiologically, with large amounts of PTHrP mRNA expressed in the mammary

epithelium during lactation and plasma concentrations being approximately 1000-fold higher than seen in humoral hypercalcaemia of malignancy (338, 339).

Mammary synthesis of PTHrP is controlled by the calcium-sensing receptor (CaSR), whose expression increases six-fold in mouse luminal epithelial cells between mid-pregnancy and mid-lactation (340). Activation of the CaSR, triggered by an increase in extracellular calcium concentrations, suppresses mammary epithelial release of PTHrP (340, 341). The mammary gland thus functions in an analogous fashion to the parathyroid glands by sensing the prevailing circulating calcium concentration, and secreting PTHrP when this decreases, in order to maintain a sufficient supply of calcium for lactation. These effects are summarised in figure 7.

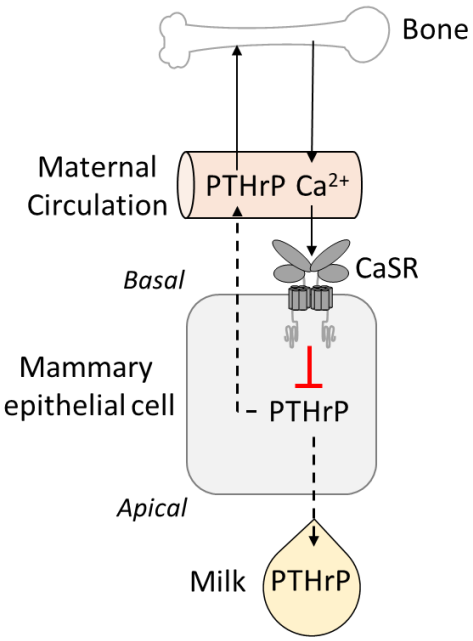


Figure 7. Role of PTHrP in ensuring calcium supply for milk. PTHrP is produced by mammary epithelial cells during lactation. Its synthesis is inhibited by extracellular calcium, which acts through the cell-surface CaSR. PTHrP is released into milk, as well as into the maternal circulation, where is acts to promote mobilization of calcium into the circulation. Adapted from Hannan et al (75).

PTHrP is also regulated by hormonal and paracrine factors. PTHrP expression is increased by serotonin stimulation in cultured mouse and bovine mammary epithelial cells (342). Mammary serotonin synthesis increases in mice during lactation, which may help to promote PTHrP synthesis (343). Prolactin increases mammary serotonin production, which may be due to increased expression of the serotonin-synthesising enzyme tryptophan hydroxylase (342, 343). Thus, prolactin may act via serotonin to promote PTHrP secretion, which may account for the positive correlation reported between serum prolactin and PTHrP concentrations (344, 345). By contrast, progesterone inhibits PTHrP production in a dose-dependent manner in cultured mammary epithelial cells obtained from lactating rats, suggesting that the fall in circulating progesterone with delivery of the placenta may contribute to the increased PTHrP observed in lactation (344).

In the mammary gland, PTHrP acts in an intracrine manner to activate STAT5 and ELF5 and this is associated with increased expression of milk proteins and mammary cell proliferation (87). Furthermore, overexpression of PTHrP in luminal epithelial cells in mice leads alveolar hyperplasia, secretory differentiation and delayed involution (87).

1.3.7.2. Effects in extra-mammary maternal tissues

The PTHrP released into the maternal circulation plays an important role in mobilising maternal calcium stores to ensure an adequate supply to the mammary gland during lactation (340). In mice with selective mammary deletion of PTHrP during pregnancy and lactation, reduced bone loss during the lactation period was observed (340). PTHrP-

mediated mobilisation of calcium from bone may occur through increased osteoclast numbers, osteoclast-mediated bone resorption and perilacunar remodelling (346).

1.3.7.3. Secretion into milk

PTHrP is secreted into milk at concentrations 1000-10000-fold higher compared with maternal blood (347). Concentrations of PTHrP are lowest in colostrum and increase by day 7 post-partum, with concentrations being higher in women who exclusively breastfeed (348, 349). The relevance of high milk PTHrP concentrations is uncertain, although administration of the N-terminal PTHrP (1-40) fragment promoted calcium uptake and increased expression of calcium transporter proteins in rat enterocytes, suggesting a possible role for PTHrP in neonatal calcium uptake (350). However, there is no correlation between milk PTHrP concentrations and infant serum calcium concentrations (347).

1.4. Lactation disorders and endocrine causes

Low milk supply leading to suboptimal infant weight gain affects around 15% of breastfeeding women (351). Causes include reduced mammary glandular tissue and delayed secretory activation. Delay in secretory activation, defined as maternal perception of milk "coming in" more than 72 hours post-partum, affects >20% of breastfeeding women (53, 352). Women who experience delayed onset of lactation are at increased risk of reduced breastfeeding duration and lactation insufficiency (53, 353-355). Furthermore, delayed secretory activation is associated with excessive neonatal weight loss and an increased rates

of infant infection and mortality (352, 356-358). Despite this, the underlying causes remain poorly characterised in affected women. Many causes of low milk supply are associated with endocrine abnormalities, which are summarised in table 4.

Table 4. Endocrine disorders associated with impaired lactation. Adapted from Hannan et al (75).

Condition	Effect on lactation	Endocrine change	Reference(s)
Pituitary disorders			
Autoantibodies against lactotrophs	Absent lactation	↓Prolactin	(193)
Pituitary necrosis	Absent/delayed onset	↓Multiple pituitary hormones	(194)
Caesarian section	Delayed onset	↓Prolactin ↓Oxytocin pulsatility	(195)
Extra-pituitary disorders			
Endocrine disrupting chemicals	Reduced duration	Oestrogenic effects ↓Prolactin	(196-201)
Polycystic ovary syndrome	Delayed onset/impaired	↑Oestrogens ↓Prolactin	(359, 360)
Type 1 diabetes	Delayed onset	↓Insulin	(361)
Obesity	Delayed onset	Prolactin resistance Insulin resistance	(362, 363)
Thyroid dysfunction	Insufficiency	↓Thyroid hormones	(364, 365)
Pre-term birth	Delayed onset	↓Lactation hormones	(366)
Placental retention	Absent/delayed onset	↑Progesterone	(50)

Disorders affecting prolactin are well described and outlined in section 1.3.1.5. However, conditions impacting on other hormones involved in milk synthesis are also associated with negative lactation outcomes. Polycystic ovary syndrome (PCOS) is characterised by insulin

resistance and hyperandrogenism, excess circulating oestrogens and reduced prolactin concentrations. PCOS is associated with delayed lactation onset and lactation insufficiency, which may in part be due to aberrant mammary gland development during pregnancy (367, 368). Mothers of pre-term infants are more likely to experience delayed onset of lactation, likely due to decreased exposure to lactation hormones and impaired mammary gland development during pregnancy (366), while retention of placental fragments delays lactation due to persistence of progesterone-mediated inhibition of milk production (50).

Metabolic disorders are also associated with lactation disorders, highlighting the importance of metabolic hormones. Both hypothyroidism and hyperthyroidism have been linked to lactation insufficiency in women (364, 365). Women with type 1 diabetes mellitus, a state of insulin deficiency, have delayed lactation onset and reduced milk volumes (361). Moreover, obesity is a state of insulin resistance and obese women are at greater risk of delayed lactation, while mouse models of obesity have suggested a role for leptin in inducing mammary prolactin resistance (362, 363). However, little work has been done to define concentrations of hormones needed for successful lactation, and the mechanisms by which endocrine disorders may affect the lactating mammary gland.

1.5. Existing knowledge gaps

1.5.1. Full range of hormones involved in lactation is unclear

In addition to the hormones described above, several other factors likely influence human lactation. Thyroid hormones may have a role, demonstrated by data showing that both triiodothyronine (T3) and thyroxine (T4) concentrations positively correlate with the amount

of milk produced in women (369). Furthermore, in women with a history of gestational diabetes mellitus, free T3 correlates with lactation duration (370). In addition, women with thyroid disease have impaired lactation. In a study of n=178 women with thyroid disease, 31% of women with hypothyroidism suffered from lactation insufficiency compared to 17% of women without (364). In another study of n=26 Indian women with hypothyroidism, 19.2% demonstrated insufficient lactation (371). Moreover, individual case reports exist of hypothyroidism associated with impaired lactation (372). However, there is little evidence on the effects of hyperthyroidism on lactation, and only a single case report exists of a 33 year old woman identified to have lactation insufficiency associated with Graves' disease, an autoimmune cause of hyperthyroidism (365). How thyroid hormones act on the mammary gland is uncertain, although proteomic analysis of colostrum in women with and without gestational hypothyroidism shows that women with gestational hypothyroidism have altered composition of whey protein (373). Thus, proteins involved in carbohydrate metabolism, cell structure and mammary integrity were downregulated in mothers with hypothyroidism, while immune protein fragments were increased (373).

GH represents another candidate lactation hormone, although existing studies are conflicting. Acromegaly, caused by GH-secreting pituitary tumours, is well known to induce milk release in non-pregnant women (374). Moreover, transgenic female mice that overexpress human GH produce milk and can successfully raise foster litters to weaning without having been pregnant (375). There is also data from interventional studies to support the role of GH. Thus, injection of growth hormone releasing factor stimulates milk production in cows, while GH injection into healthy women or women of pre-term infants

increases milk volume (376-378). However, studies of GH in breastfeeding women are small and not all studies confirm a role for GH in milk production (379).

Evidence for a role for insulin in lactation came from cellular, mouse and human studies. Mice with a mammary-specific insulin receptor knockout have reduced expression of genes related to milk proteins, lipid synthetic enzymes and lactose synthesis (45). These mice also display impaired mammary development. Furthermore, treatment of the HC11 mammary epithelial cell line with insulin has previously been reported to increase expression of β-casein and whey acidic protein (WAP) (380). Data from humans is indirect and comes from studies of women with insulin resistance. Thus, overweight and obese women (body mass index 25-29.9 and ≥30, respectively) are at risk of delayed lactation onset and early cessation of breastfeeding (381, 382).

1.5.2. Hormone concentrations required for lactation are unknown

Delayed secretory activation is a major cause of early breastfeeding cessation and lactation insufficiency (53). Secretory activation is hormonally mediated and is dependent on the actions of several hormones including prolactin (48-52). However, the concentrations of hormones required at the onset of lactation is unknown. Furthermore, it is unclear if the concentration of hormones such as prolactin are altered in women with delayed secretory activation.

1.5.3. Influence of maternal factors on lactation hormones remain to be elucidated

Several maternal factors are associated with delayed onset of lactation. These include advanced maternal age (≥30 years old), increased body mass index (BMI) (≥25), delivery by Caesarean section and primiparity (381, 383, 384). However, despite the presence of epidemiological data demonstrating the likely involvement of these factors, the physiological basis by which they impair lactation is unknown. Specifically, how risk factors for delayed lactation onset influence hormones required for lactation has not previously been assessed. A mechanistic understanding of lactation disorders is an essential first step in identifying strategies for the diagnosis and management of impaired lactation.

1.5.4. Actions of hormones on mammary epithelial cells are poorly understood

While several hormones have been described to influence lactation, their role in mammary cell function and milk synthesis remain poorly understood. Mammary epithelial cells shed into human milk represent a potential tool for characterising the cellular basis of lactation. However, these cells may be phenotypically different from mammary epithelial cells *in situ*. Milk also contains non-epithelial cell types including immune and stromal cells (385). Use of surrogate approaches have therefore been explored, such as using milk fat globule RNA for assessing gene expression in lactating mammary epithelial cells. However, a lactating human cell model for assessing the effects of hormone stimulation is lacking (77, 386). Immortalised human mammary epithelial cell lines exist, but these are not representative of primary cells, while establishing a lactogenic phenotype in primary cells has proved challenging (387, 388).

For these reasons, much of the knowledge about lactation comes from animal studies. While important insights have been gained from such work, there are key differences between human and non-human lactation. For example, mice, commonly used in lactation studies, have five pairs of mammary glands with each consisting of only a single lobe (389). In addition, the physiology of milk production and milk composition vary according to the species studied (389). Thus, although animal studies have improved understanding to lactation biology, a complete understanding of human lactation requires dedicated work in humans and the use of appropriate models where no human option exists.

2. AIMS AND OBJECTIVES

My work encompasses three related studies, the aims and objectives of which are listed below.

2.1. Study 1: Hormonal changes at the onset of lactation

Aim: To evaluate serum concentrations of lactogenic hormones during secretory activation in breastfeeding women.

Objectives:

1. Define normal serum concentrations for key lactation hormones in breastfeeding women, specifically those related to:
    i. Synthetic function: prolactin, GH and IGF-1, cortisol, progesterone and oestradiol
    ii. Mammary bioenergetics: insulin and thyroid hormones
2. Understand how maternal factors influencing lactation onset, such as parity and BMI, may alter these key hormones.

2.2. Study 2: Influence of growth hormone on mammary cell metabolism

Aim: To investigate the mechanisms by which GH may contribute to milk production in mammary cells during lactation.

Objectives:

1. Evaluate mammary GH sensitivity (receptor expression) at lactation onset using human milk fat globule RNA.

2. Determine whether GH signals through STAT5 and/or Akt signalling pathways.

3. Understand the effects of GH in a lactogenic mammary cell model, specifically on:

    i. Synthesis of milk components (β-casein).

    ii. Cell metabolism (glycolysis and mitochondrial activity).

2.3. Study 3: Influence of insulin on mammary cell metabolism

Aim: To investigate the mechanisms by which insulin may contribute to milk production in mammary cells during lactation.

Objectives:

1. Evaluate how mammary insulin sensitivity (receptor expression) changes during lactogenic differentiation.

2. Determine whether insulin signals through the PI3K-Akt signalling pathway in mammary cells.

3. Understand the effects of insulin in a lactogenic mammary cell model, specifically on:

    i. Cell metabolism (glycolysis and mitochondrial activity).

    ii. Production of intermediates required for milk synthesis.

3. METHODS

3.1. INSIGHT study: sample collection and hormone measurements

The INSIGHT clinical study (REC reference 20/EE/0172) aims to:

1. Define normal blood hormone concentrations associated with secretory activation.
2. Assess the effect of risk factors for delayed lactation on hormone concentrations during secretory activation.
3. Evaluate hormone responsiveness of mammary epithelial cells at lactation onset.

An overview of the study protocol is shown in figure 8 (390).

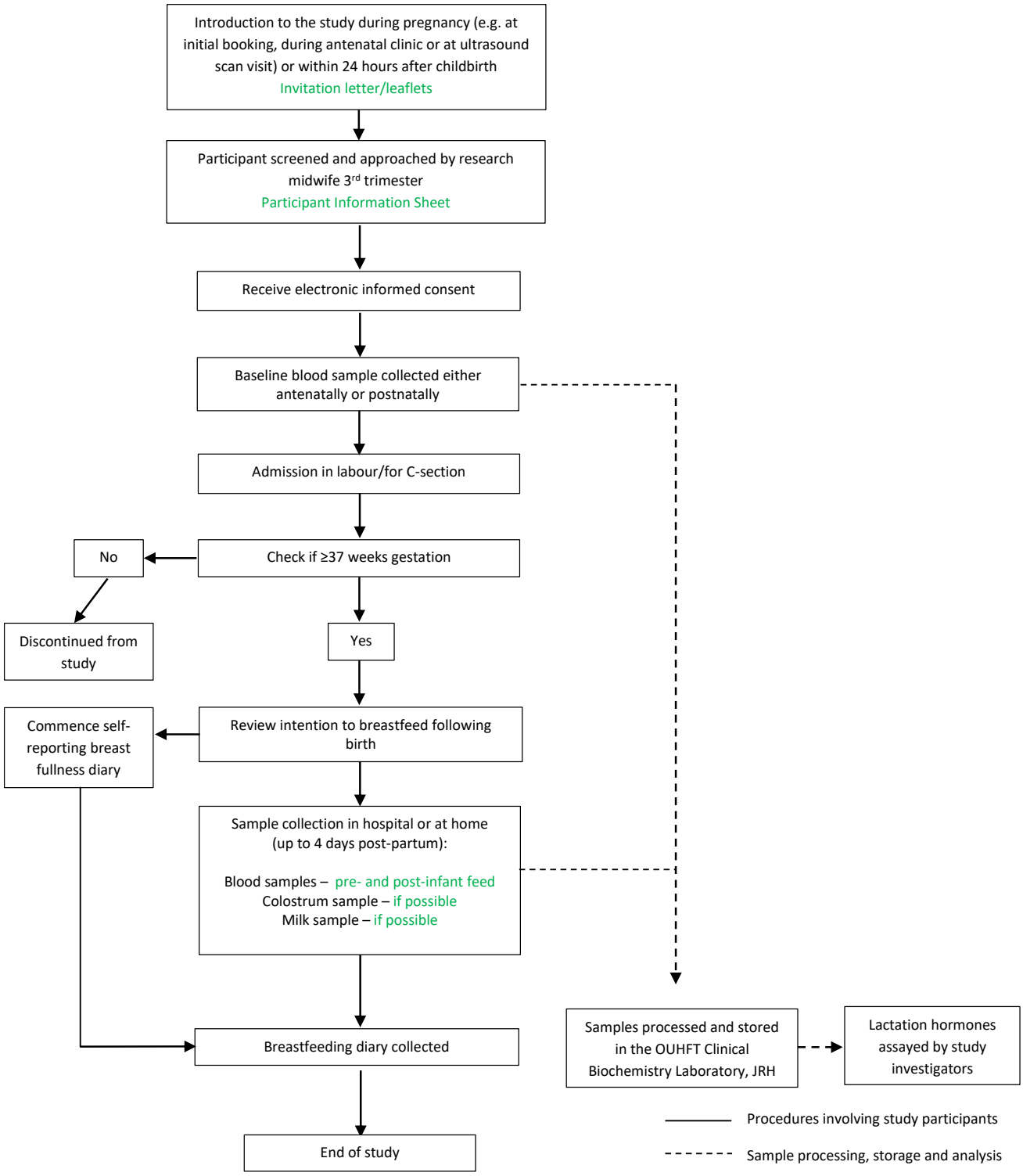


Figure 8. Overview of INSIGHT study.

Women were recruited from the Maternity Unit at John Radcliffe Hospital in Oxford or through self-referral from study advertising materials, and were assessed against the following criteria to determine eligibility:

Inclusion criteria:

- Pregnant women, aged ≥18 years
- Term singleton pregnancy (37-43 weeks' gestation)
- Intention to fully or partially breastfeed
- Willing and able to give informed consent for participation in the study

Exclusion criteria (women excluded if any of these apply):

- Severe maternal illness including diagnosis of postpartum depression or psychosis
- Severe infant illness including major congenital abnormalities and infants who are only expected to live for a short period of time
- Prolonged separation of infant from mother e.g. due to admission to the neonatal unit
- Major COVID-19 symptoms e.g. pyrexia and continuous cough
- Mother or infant infected with blood borne viruses such as HIV
- Resides outside of Oxfordshire
- Safeguarding issues that may impede the safety of research staff carrying out home visits
- Current participation in another research study which involves investigational medicinal products

Eligible women who provide informed consent are recruited in the third trimester of pregnancy, where blood samples are collected and a baseline assessment is performed. Post-partum samples are collected on days 1-4 after delivery, reflecting the days on which secretory activation typically occurs (352). Day 1 post-natal study visits are undertaken on the post-natal ward, with subsequent visits (days 2-4) taking place in the participants' homes. At these visits, further clinical information is collected, participants donate breast milk, and pre- and post-feed blood samples are collected to capture hormonal changes across a breastfeeding episode. All samples are transported in refrigerated sample bags, to be processed within two hours of collection. The sample collection schedule for the INSIGHT study is outlined in figure 9, and samples provided by each woman are in appendix 1.

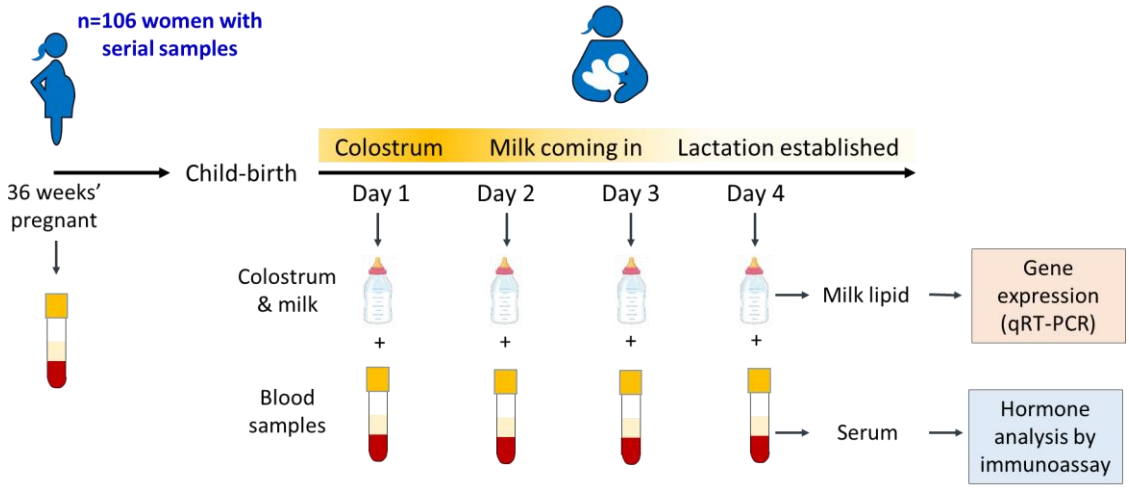


Figure 9. INSIGHT study sample collection schedule. n=106 women recruited to the INSIGHT study had blood samples collected at 36 weeks' gestation and blood and milk samples collected on days 1-4 post-partum.

The clinical information collected at baseline and during the post-partum visits is given in table 5. Information collected relates to the characteristics of the women recruited, and risk factors for delayed lactation, including primiparity, pregnancy co-morbidities and delivery

complications (351, 352, 391-393). Collection of this information is to enable assessment of the impact of these factors on lactation onset and on serum hormone concentrations.

Table 5. Clinical information collected from participants.

Time point	Clinical information collected
Baseline (36 weeks' gestation)	Participant details: • Age • Ethnicity • Body mass index (BMI) • Parity Gestational age Co-morbid conditions, including: • Diabetes mellitus • Obesity • Hypothyroidism Medications Pregnancy complications
Days 1-4 post-partum	Method of delivery Delivery complications Peri-partum medications: • Syntocinon • Anaesthesia • Analgesia Infant birthweight Infant feeding details: • Type of feeding (breast only vs mixed) • Use of breast pump • Time of feeding • Duration of breastfeed • Strength of infant suckling

Participants are also asked to complete a breastfeeding diary to record breast fullness on each post-partum day, and the onset of lactation (when milk 'comes in'). Breast fullness is

assessed using the validated breast fullness scale developed by Dewey and colleagues (352). Thus, women self-report breast fullness on a scale from 1-5, where 1 = no change following delivery, 3 = noticeably fuller and 5 = uncomfortably full. Using this scale, a score of 3 correlates most closely with milk volume exceeding 15g per feed, while the timing of the first reported score of 3 correlates with milk volume at day 5 post-partum (352).

3.2. Processing of blood samples

At each visit, blood is collected into serum and lithium heparin tubes (Becton Dickinson, USA). Lithium heparin samples underwent centrifugation at 1600g for 20 minutes at 4^{o}C, and plasma was aliquoted. Lithium heparin plasma was used for leptin and adiponectin analysis at the Core Biochemical Assay Laboratory in Cambridge. Serum samples were taken directly to Clinical Biochemistry at the John Radcliffe Hospital, Oxford, where they were centrifuged at ~1600g for 10 minutes at room temperature before undergoing analysis for the remaining hormones shown in table 6.

Table 6. Hormones analysed in INSIGHT study.

Hormone	Analysis platform and location
Hormones with known or likely roles in milk synthesis	
Prolactin	Abbott Architect, John Radcliffe Hospital, Oxford
Progesterone	Abbott Architect, John Radcliffe Hospital, Oxford
Oestradiol	Abbott Architect, John Radcliffe Hospital, Oxford
Cortisol	Abbott Architect, John Radcliffe Hospital, Oxford
Growth hormone (GH)	Siemens Immulite, John Radcliffe Hospital, Oxford

Table 6 (continued).

Hormones with potential mammary metabolic roles	
Insulin	Abbott Architect, John Radcliffe Hospital, Oxford
Insulin-like growth factor-1 (IGF-1)	IDS-iSys, John Radcliffe Hospital, Oxford
Thyroid hormones (TSH, T4, T3)	Abbott Architect, John Radcliffe Hospital, Oxford
Leptin	PerkinElmer AutoDELFIA, CBAL, Cambridge
Adiponectin	PerkinElmer AutoDELFIA, CBAL, Cambridge

3.3. RNA extraction from milk lipid fraction

While assessment of mammary epithelial cells at lactation onset in women can be undertaken from cells shed into milk, there are also immune and stromal cells in milk (385). Furthermore, the phenotype of cells may change during shedding, milk collection, processing and cell sorting. Milk fat globule RNA has therefore been established as a means of assessing gene expression in mammary epithelial cells. Milk fat is rich in RNA, derived from cytosolic RNA as milk fat globules are released from mammary epithelial cells, and is representative of RNA in these cells (77, 386).

The isolation of milk fat globule RNA was performed based on the protocol reported by Maningat and colleagues (386). Thus, milk collected from post-natal study visits was immediately diluted 1:1 with cold Dulbecco's phosphate-buffered saline (Gibco, USA). The diluted sample was spun at 400g for 10 minutes at 4^{o}C, with the top (lipid) layer removed with a spatula and transferred to a clean 1.5mL tube (figure 10).

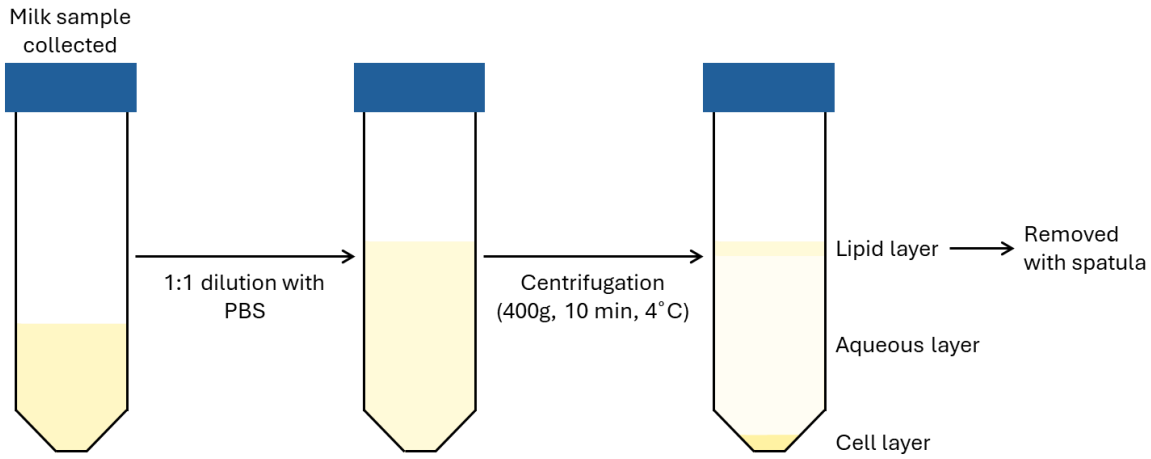


Figure 10. Milk processing protocol and layers obtained. Following collection and transport of milk, samples are diluted with phosphate buffered saline (PBS). Centrifugation yields 3 layers: an upper lipid layer containing milk fat globules, a middle aqueous layer, and a cell pellet at the bottom.

RNA was extracted from the milk fat using the RNeasy Universal Kit (Qiagen, Germany). The concentration and purity of collected RNA was measured using the Nanodrop spectrophotometer (ThermoFisher Scientific, USA) and frozen at -80^{o}C until required for quantitative reverse transcription polymerase chain reaction (qRT-PCR) or bulk RNA-seq.

3.4. Culture of human mammary epithelial cells

Human mammary epithelial cells (hMECs) are commercially-obtained primary cells derived from benign breast tissue obtained from non-pregnant and non-lactating women. Four batches of hMECs were used for experiments: two batches from Lonza (Switzerland) and one each from Merck (Germany) and InnoProt (Spain). The details of cells used are shown in table 7.

Table 7. Details of hMEC batches used for experiments, and characteristics of donors (where available).

Supplier	Lot number	Height (m)	Weight (kg)	Age (years)
Merck	2049	-	-	31
InnoProt	P10891	-	-	-
Lonza	22TL115626	65	65.8	24
Lonza	21TL195759	65	70.3	29

hMECs were maintained in complete HuMEC media (Gibco, USA), comprised of HuMEC basal media added to a supplement mix containing epidermal growth factor (EGF), hydrocortisone, insulin, isoproterenol, transferrin and bovine pituitary extract, as well as 1% penicillin/streptomycin solution (Sigma-Aldrich, USA). The supplement mix is designed to promote survival and growth of hMECs, and ensure they maintain an epithelial phenotype (394). Cells were cultured in at 37^{o}C in an incubator containing 5% carbon dioxide. Media was replaced every 2-3 days, and cells were subcultured at 70-80% confluency. Only cells with a passage number below 10 were used for experiments.

For hormone stimulation, hMECs were at 0-4 weeks post-confluency. The morphology of hMECs in culture as they transition from sub-confluent to confluent to post-confluent states is shown in figure 11. These cells transition from an elongated shape in the sub-confluent state to a cuboidal morphology after reaching confluency.

Flow cytometry of hMECs was undertaken using the LSRII flow cytometer (BD, USA), using EpCAM-AF647 and CD49f-PE antibodies (BioLegend, USA).

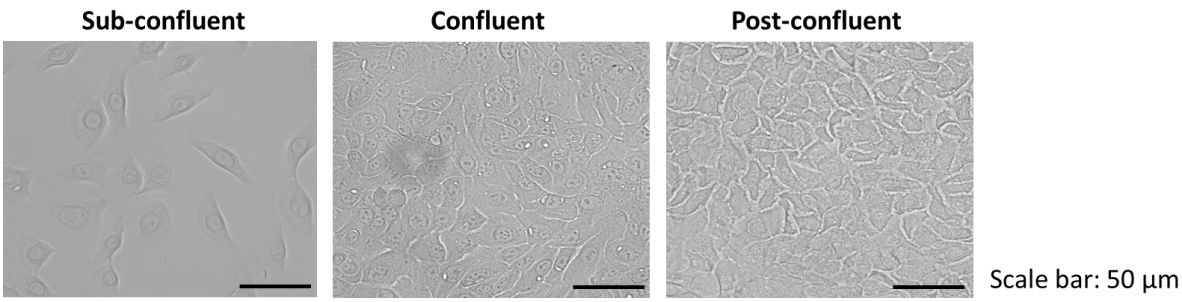


Figure 11. Appearances of hMECs. Light microscopy appearances of sub-confluent, confluent and post-confluent hMECs.

3.5. Culture and lactogenic differentiation of HC11 cells

The HC11 mouse mammary epithelial cell line (ATCC, USA; catalogue number CRL-3062, lot number 70041959) is capable of lactogenic differentiation with appropriate treatment and represents a model for studying the effect of hormones in cells with a lactogenic phenotype (395). Lactogenic differentiation in this context refers to expression of milk proteins such as beta-casein, rather than production of milk per se, and the extent of differentiation is less than that of milk-producing mammary cells *in vivo* (396). In keeping with manufacturer protocol, cells were cultured in complete media, consisting of ATCC-formulated RPMI-1640 medium (ATCC, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), 10ng/mL recombinant human epidermal growth factor (EGF; Gibco, USA), 5µg/mL recombinant human insulin (Sigma-Aldrich, USA) and 1% penicillin/streptomycin solution (Sigma-Aldrich, USA). Cells were cultured in at 37^{o}C in an incubator containing 5% carbon dioxide. Media was replaced every 2-3 days, and cells were subcultured at 70-80% confluency. Only cells with a passage number below 15 were used for experiments.

Lactogenic differentiation of HC11 cells was performed using the protocol previously established by Berlato and Doppler (380). Cells were grown for approximately three days, until confluent. The culture media was then changed to complete media without EGF for a further two days, as EGF has been shown to inhibit differentiation of HC11 cells by inhibition of prolactin signalling (397, 398). Finally, cells were stimulated in serum-free RPMI-1640 containing 10 nM insulin, 0.1 µM dexamethasone (Sigma-Aldrich, USA) and 1 µg/mL recombinant mouse prolactin (PeproTech, USA) for two days to achieve a lactogenic phenotype. This combination of hormones is well-established to induce lactogenic differentiation in HC11 cells (380, 395). Lactogenic differentiation was confirmed by increased β-casein expression on qRT-PCR. This protocol is outlined in figure 12.

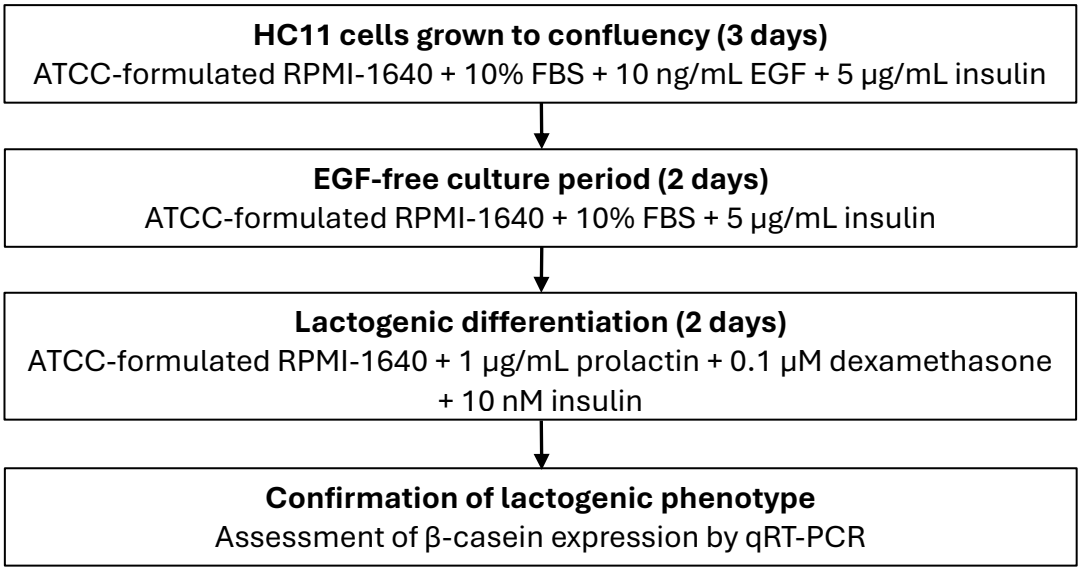


Figure 12. Protocol for differentiation of HC11 cells.

For hormone signalling studies, cells were cultured on Transwell inserts (Sarstedt, Germany). These inserts are permeable supports, meaning that both apical and basal aspects of cells are exposed to media, and therefore to the hormone of interest, during stimulation. For all other experiments, cells were cultured in two-dimensional monolayer culture in plastic wells. These approaches to culture are shown schematically in figure 13.

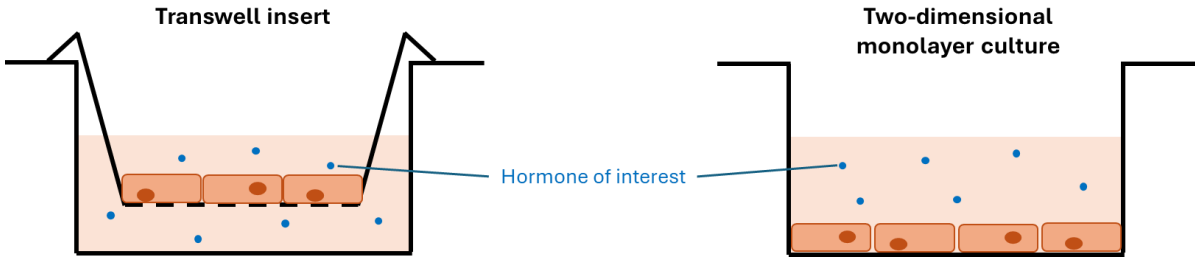


Figure 13. Approaches for culture of mammary cells. Cells were cultured on Transwell inserts for signalling studies, which are a permeable support that expose cells to media on both basal and apical aspects. For other studies, cells were cultured in plastic wells in standard two-dimensional monolayer culture.

3.6. Cellular phosphoprotein signalling assays

Capillary protein electrophoresis provides an automated method for protein separation, immunoprobing, detection and analysis, obviating some of the problems of traditional Western blotting (399). Lysates, primary and secondary antibodies, and detection reagents are loaded on to assay-specific plates (Bio-Techne, USA). Following insertion of the plate, protein lysate is loaded into capillaries and voltage is applied to separate proteins by molecular weight. Once separation is complete, ultraviolet light is used to immobilise proteins within the capillary. After fixation of proteins, incubation with primary antibody followed by horseradish peroxidase (HRP)-linked secondary antibody is performed. Finally,

chemiluminescent substrate is added and detection is undertaken. The assay principles are outlined in figure 14.

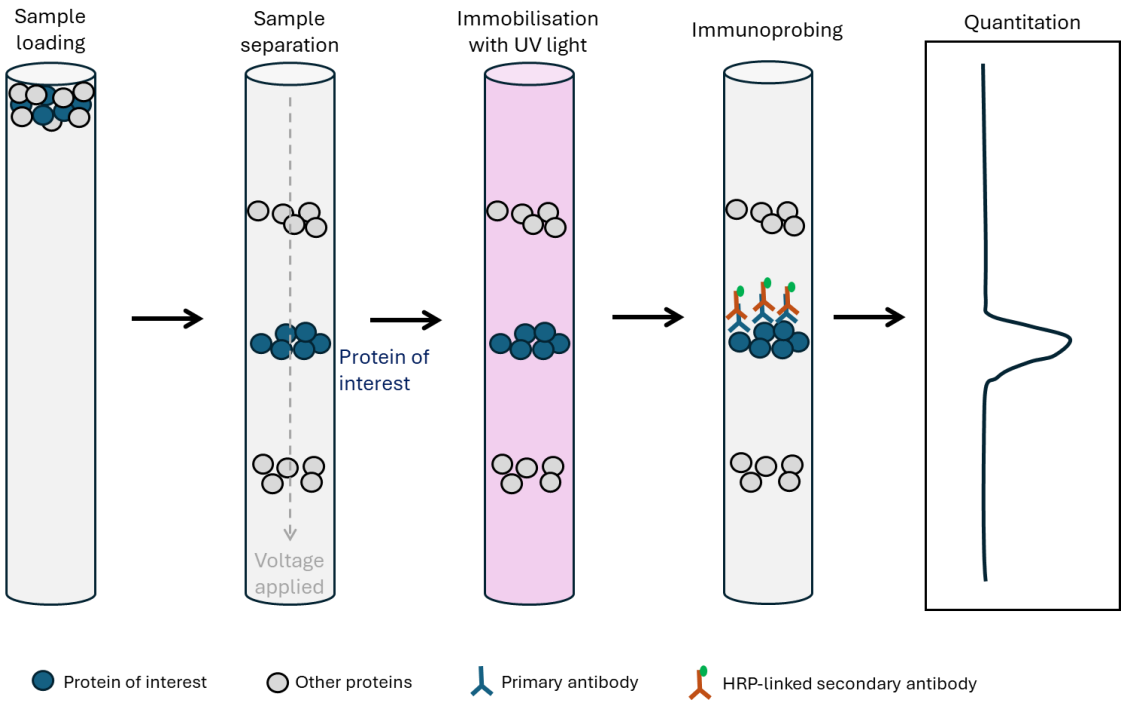


Figure 14. Principles of capillary protein electrophoresis. Following sample loading, proteins are separated through application of voltage to capillaries. Proteins are immobilised using ultraviolet (UV) light, after which primary and HRP-linked secondary antibodies are sequentially added for detection. Figure adapted from Harris (399).

The workflow for capillary protein electrophoresis is shown in figure 15. For phosphoprotein assays, stimulated HC11 cells were placed on ice and washed once with cold PBS. Cells were then immediately lysed in RIPA buffer (ThermoFisher Scientific, USA) supplemented with protease inhibitor (ThermoFisher Scientific, USA) and phosphatase inhibitors (Merck, Germany). Lysis was performed for five minutes in the culture dish, before all cellular material was scraped and transferred to a 1.5mL Eppendorf tube on ice. The lysate was vortexed every 10-15 minutes for at least 30 minutes. At the end of this period, the lysate

was centrifuged at maximum speed for 10 minutes at 4^{o}C and the supernatant was collected and frozen at -80^{o}C until required.

Protein concentration in the stored lysate was measured using the BCA assay (ThermoFisher Scientific, USA), and capillary protein electrophoresis was performed using the Jess for ProteinSimple platform (Bio-Techne, USA) according to the manufacturer's protocol for chemiluminescent detection. The primary antibodies used were against phospho-Akt S473 (catalogue number 4060; Cell Signalling Technology, USA), total Akt (catalogue number 58295; Cell Signalling Technology, USA), phospho-STAT5 (catalogue number 9359; Cell Signalling Technology, USA) and total STAT5 (catalogue number AF2168; R&D systems, USA). The secondary antibody used was a goat anti-rabbit or anti-mouse antibody linked to HRP (Bio-Techne, USA).

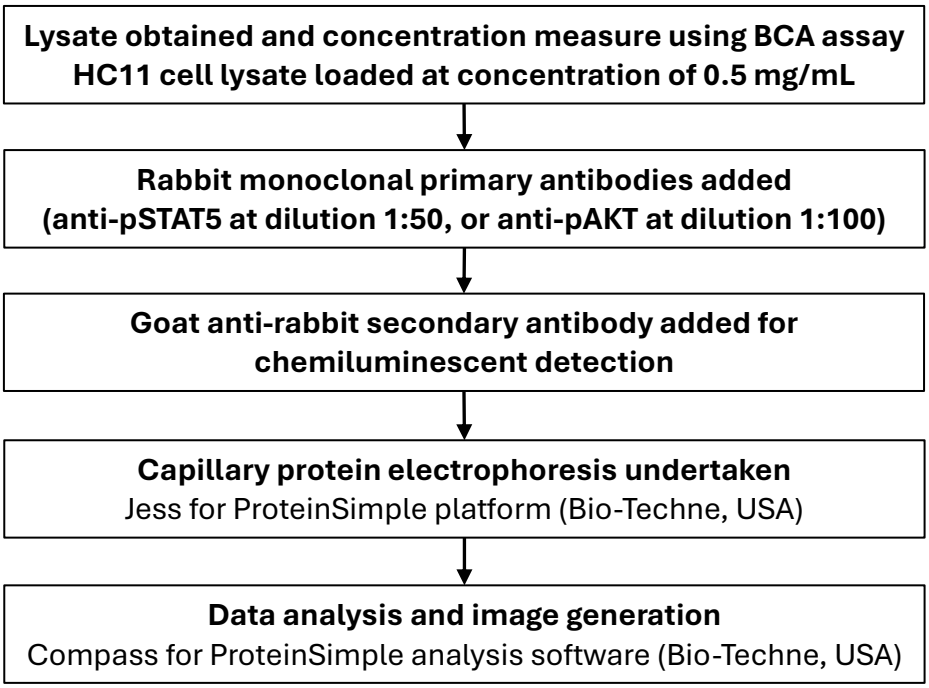


Figure 15. Workflow for capillary protein electrophoresis.

The chemiluminescent signal generated is visualised on the Compass for ProteinSimple analysis software (Bio-Techne, USA). The area of the peak at the molecular weight of interest, corresponding to the target protein, is quantified and compared between conditions. The output can also be displayed as a 'virtual' Western blot, where the intensity of the band is proportional to the peak area. Example outputs of the software are shown in figure 16.

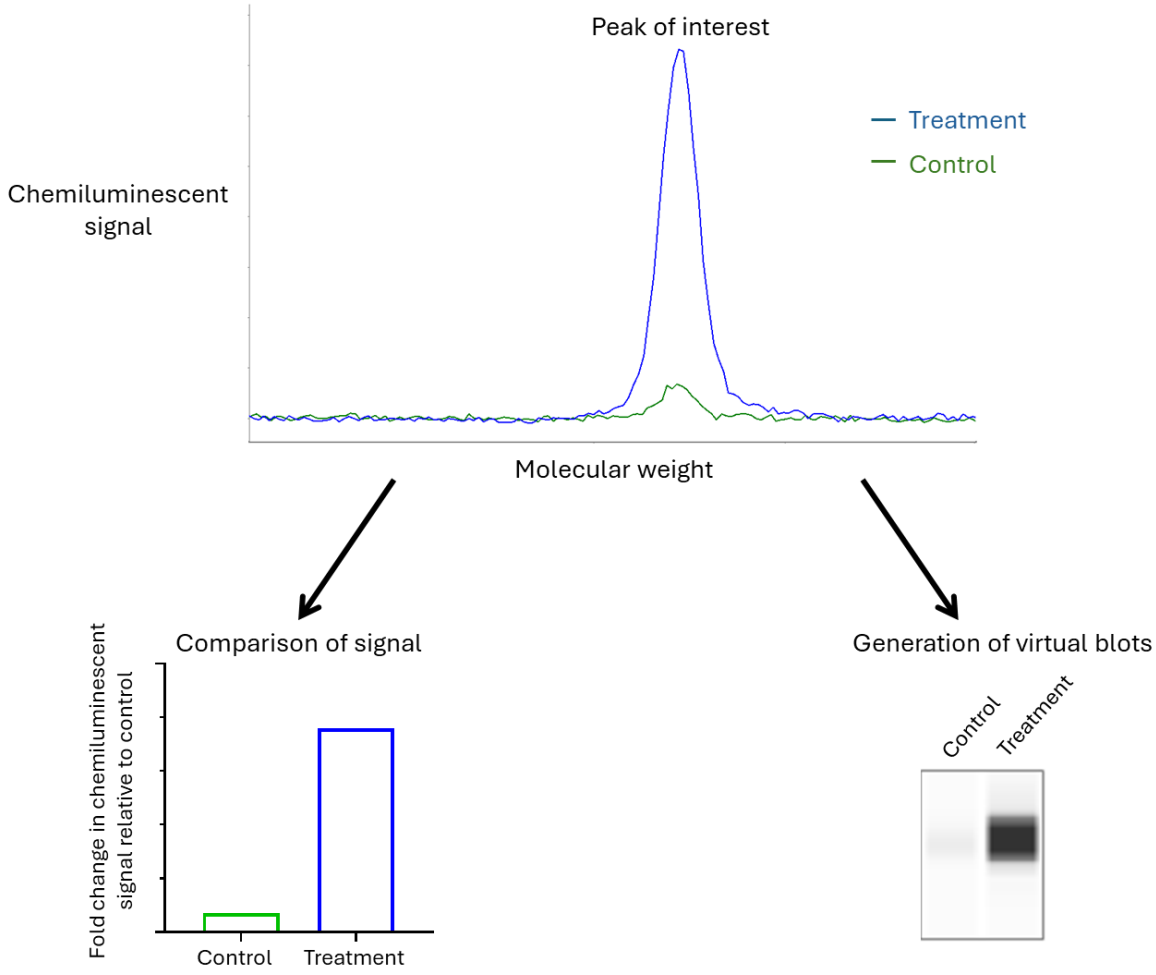


Figure 16. Representative data generated from capillary protein electrophoresis. The chemiluminescent signal obtained from capillary protein electrophoresis is displayed as a graph in the Compass for ProteinSimple software (top). The peak area can be quantified and used for numerical comparison of signals or used to generate 'virtual' blots.

3.7. Extraction of RNA from cultured cells

RNA from cultured cells was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany). RLT Plus buffer was supplemented with 1% β-mercaptoethanol, and 350µL of the supplemented RLT Plus was used to lyse cells. The culture dish was scraped and RNA was extracted from the lysate as per the manufacturer's kit instructions. The concentration and purity of collected RNA was measured using the Nanodrop spectrophotometer and frozen at -80^{o}C until required for qRT-PCR.

3.8. Non-invasive monitoring of O_2 in cultured cells

The two major adenosine triphosphate (ATP)-generating pathways in cells are mitochondrial oxidative phosphorylation and glycolysis, with the former typically producing the most ATP (400). Assessment of both basal and maximal mitochondrial respiration provides insights into the mitochondrial function of cells (401). Basal mitochondrial oxidative phosphorylation is that required to meet the ATP demands of cells and can be assessed by measuring cellular oxygen consumption rate (OCR) without addition of mitochondrial inhibitors or activators. Maximal mitochondrial oxidative phosphorylation reflects the total capacity of the mitochondria to respire, which can be altered by changes mitochondrial biogenesis and expression of mitochondrial protein genes. Maximal respiration is therefore the rate of oxygen consumption when mitochondria are maximally activated, and can be determined through addition of a mitochondrial uncoupling compound such as carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; Sigma-Aldrich, USA) (401). Uncoupling abolishes the mitochondrial proton gradient, driving mitochondrial respiration to regenerate the

gradient. FCCP is reconstituted in dimethylsulfoxide (DMSO) and added to media to achieve a final concentration of 1µM (with a final DMSO concentration of 1%). A diagrammatic explanation of the determination of basal and maximal respiration is shown in figure 17.

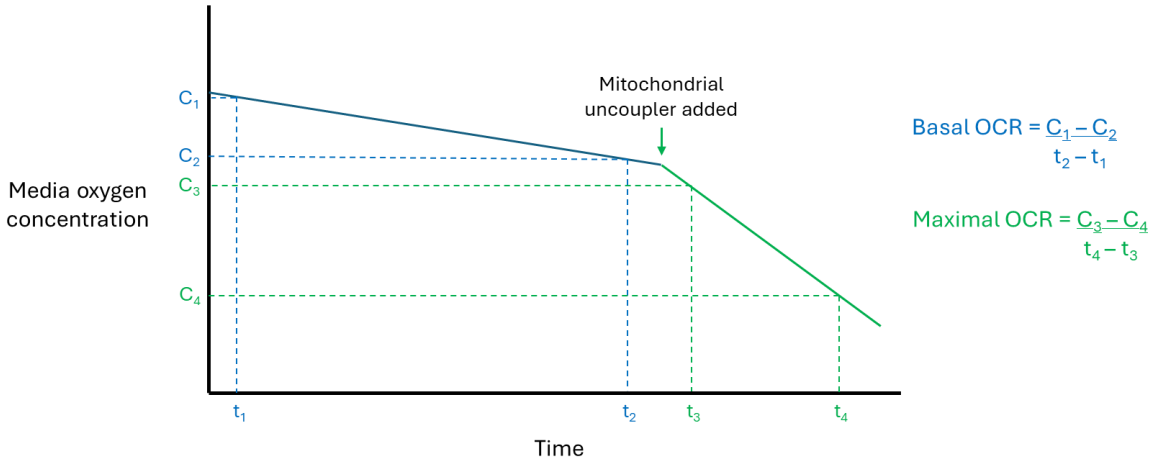


Figure 17. Determination of basal and maximal oxygen consumption rate (OCR). Media oxygen concentration is measured continuously throughout the recording period. The basal OCR, reflecting basal mitochondrial respiration, is the rate of oxygen consumption without mitochondrial inhibitors or activators. The maximal OCR, reflecting maximal mitochondrial respiration, is the rate of oxygen consumption after a mitochondrial uncoupler is added.

Non-invasive monitoring of oxygen in HC11 cells was undertaken in black-walled 96-well plates, and the workflow is summarised in figure 18.

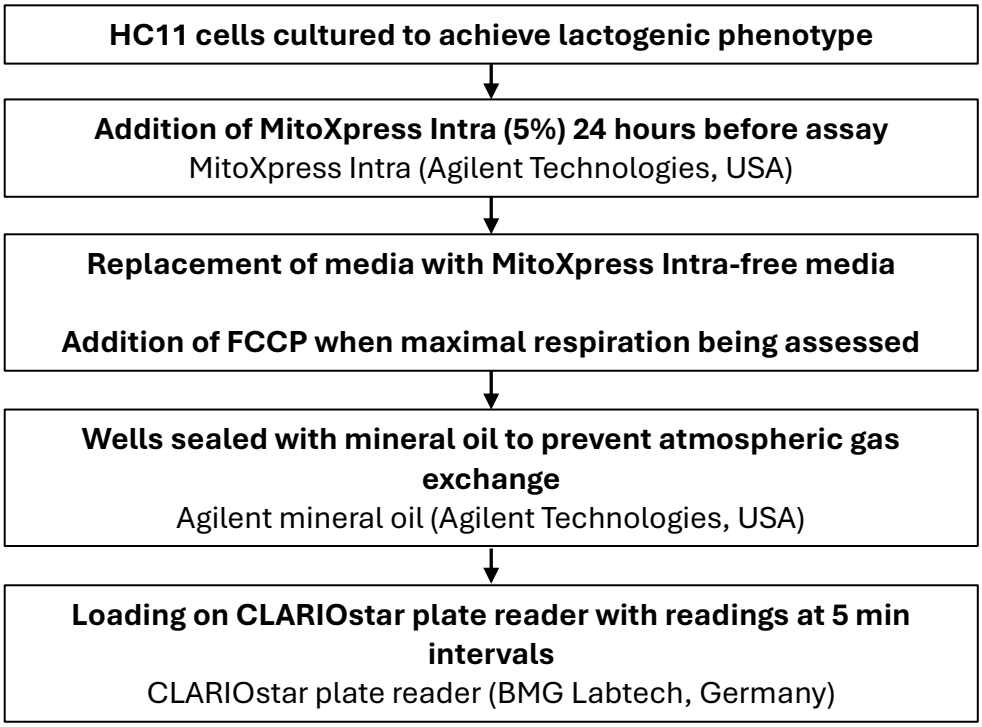


Figure 18. Workflow for MitoXpress Intra assays.

Monitoring of intracellular oxygen concentrations was performed using the MitoXpress Intra intracellular oxygen probe (Agilent Technologies, USA). An intracellular oxygen probe allows measurement of the oxygen concentration that cells are actually experiencing in culture, and is more direct than an extracellular probe (402). MitoXpress Intra is taken up by endocytosis and its phosphorescent emission is quenched by oxygen, meaning that its signal decreases as intracellular oxygen increases (figure 19). MitoXpress Intra was reconstituted in sterile water as per manufacturer protocol and added to media 24 hours before the assay, at a concentration of 5%. The media was replaced immediately prior to plate loading with media containing no MitoXpress Intra, and each well was sealed with mineral oil (Agilent Technologies, USA) to prevent exchange of oxygen between media and

atmosphere. Plates were immediately loaded on the CLARIOstar plate reader (BMG Labtech, Germany).

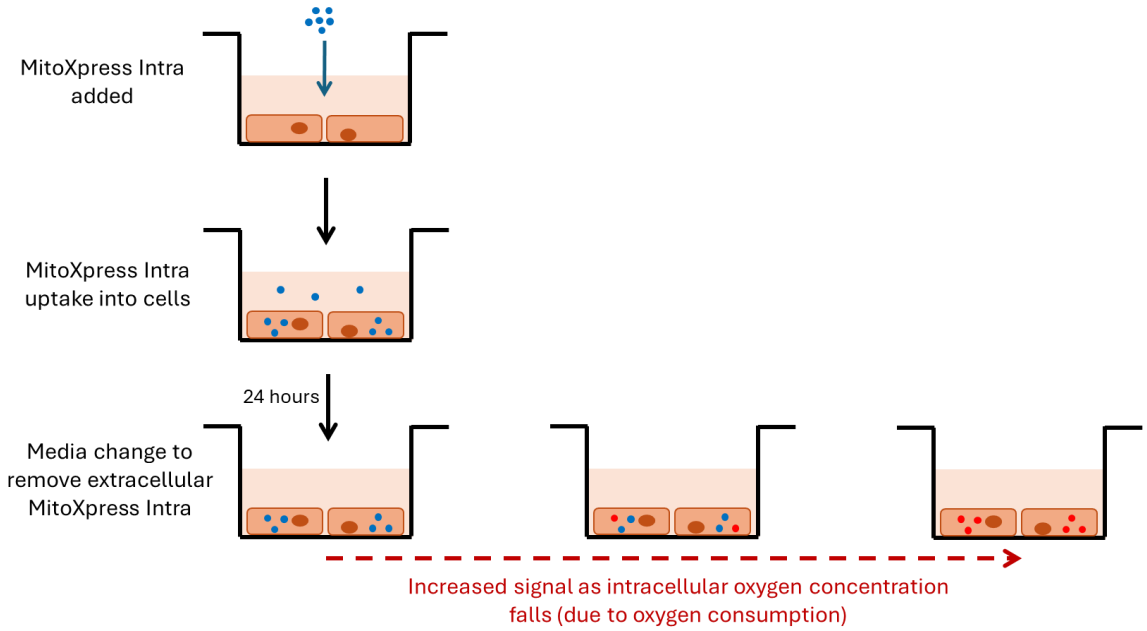
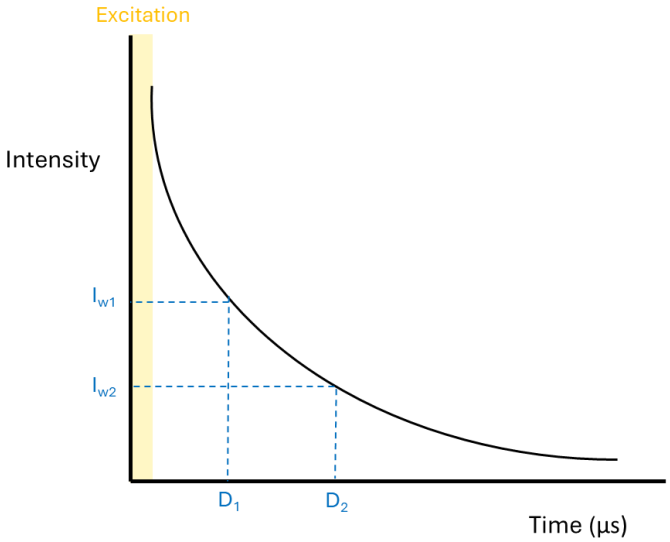


Figure 19. Principles of the MitoXpress Intra assay. MitoXpress Intra is added to the media 24 hours before the assay, allowing endocytosis into cells. After 24 hours, the media is replaced in order to remove extracellular probe and reading is commenced. Over time, the MitoXpress Intra signal increases as intracellular oxygen concentration falls.

Lifetime measurements were undertaken every five minutes for the duration of the assay. The use of lifetime-based approaches is favoured because it allows direct correlation with oxygen concentrations, and is independent of variations in probe loading, probe intensity and scattering, which can influence standard point fluorescence measurements (402). Lifetime is calculated by measuring probe intensity at two delay times after excitation, and using the ratios of these intensities to determine lifetime according to the calculation shown

in figure 20. The lifetime values can be used to generate a calculated oxygen concentration (%).



$$\text{Lifetime (T)} = \frac{D_2 - D_1}{\ln(I_{w1}/I_{w2})}$$

$$O_2\ (\%) = 659.3 \times \text{Exp}\ \frac{-T}{8.475}$$

Figure 20. Determination of lifetime values and intracellular oxygen (O_2) concentration. Following excitation, lifetime (T) is calculated using intensity values at two delay times. The lifetime value can then be used to calculate the oxygen concentration (%).T represents emission lifetime and I_{w1} and I_{w2} represent signals measured at window 1 (30µs delay; D_1) and window 2 (70µs delay; D_2).

The oxygen consumption rate (OCR) was calculated as the change in oxygen concentration (%) per minute, during the monitoring period where oxygen concentration showed a linear decrease (figure 21).

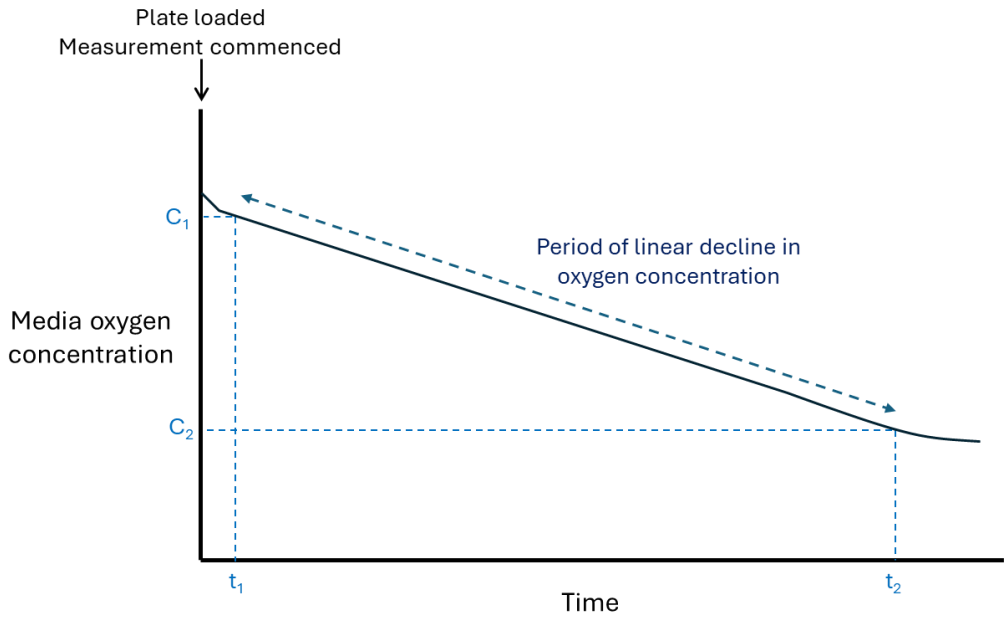


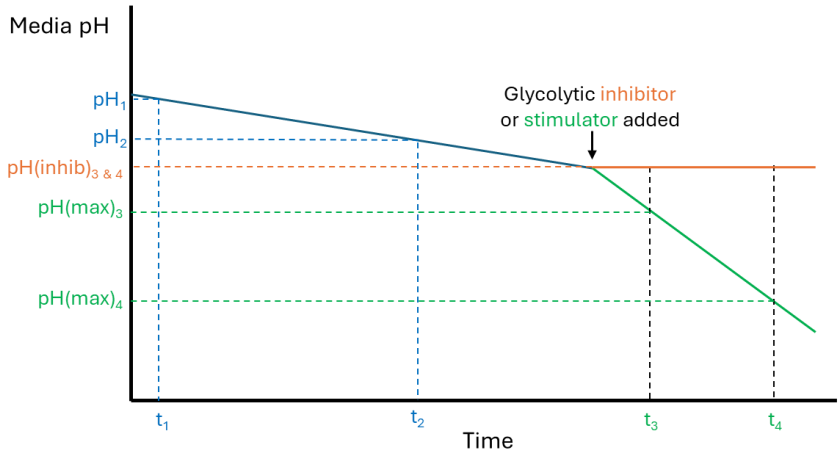
Figure 21. Calculation of oxygen consumption rate. Oxygen consumption rate (OCR) is calculated as the rate of decline in media oxygen during the period of linear decline in oxygen concentration. This occurs after a period of equilibration after plate loading (before time t_1) and before any cell dysfunction or death (after time t_2).

3.9. Non-invasive monitoring of pH in cultured cells

Monitoring of media pH can be used to assess glycolytic activity of cells, as glycolysis results in production of lactic acid. Lactic acid is exported from cells and acidifies the surrounding media, and the extracellular acidification rate (ECAR) therefore reflects the rate of glycolysis. Lactic acid production accounts for the majority of media acidification in glucose-containing media, with carbon dioxide generated by the tricarboxylic acid (TCA) cycle being a minor contributor (403, 404).

Evaluation of glycolysis should involve assessments of both basal glycolysis and glycolytic capacity (403). Measurement of basal glycolysis is the glycolytic rate without any stimulation or inhibition of glycolysis. However, cells often operate at sub-maximal rates of glycolysis to ensure they can respond to any increases in demand for ATP (403). Therefore, to assess the

maximal ability of cells to generate energy through glycolysis, an assessment of glycolytic capacity is required. Glycolytic capacity is defined as the rate of maximal glycolysis, determined through inhibition of the mitochondrial ATP-synthase inhibitor oligomycin, minus the rate of glycolysis when inhibited using the glycolytic inhibitor 2-deoxyglucose (403). Glycolytic rate is measured as the rate of proton generation from the end-product of glycolysis, lactic acid, and expressed as the extracellular acidification rate (ECAR) (405). The calculation of basal glycolysis and glycolytic capacity is outlined in figure 22.



$$\text{Basal ECAR} = \frac{pH_1 - pH_2}{t_2 - t_1}$$

$$\text{Maximal ECAR} = \frac{pH(max)_3 - pH(max)_4}{t_4 - t_3}$$

$$\text{Inhibited ECAR} = \frac{pH(inhib)_3 - pH(inhib)_4}{t_4 - t_3}$$

Glycolytic capacity = maximal ECAR – inhibited ECAR

Figure 22. Determination of basal glycolysis and glycolytic capacity. Extracellular acidification rate is the rate of change in media pH over time. Basal ECAR is used as an assessment of basal glycolysis, and is the ECAR without inhibitors or stimulators of glycolysis added. Glycolytic capacity is the rate of maximal glycolysis (following addition of a glycolytic stimulator such as oligomycin) minus the rate of inhibited glycolysis (following addition of a glycolytic inhibitor such as 2-DG, which should approximate to zero).

Monitoring of media pH was performed using pH Xtra (Agilent Technologies, USA), which assesses the pH of the media. Extracellular pH monitoring is an established approach for assessing glycolysis, and reflects the fact that cells intensively export lactic acid to maintain a near-neutral intracellular pH, which is required for cell function (406). The pH Xtra probe can be used to assess media acidification in cultured cells, and its signal increases with increased acidification (lower pH) of the media (407). An outline of the pH Xtra assay is shown in figure 23.

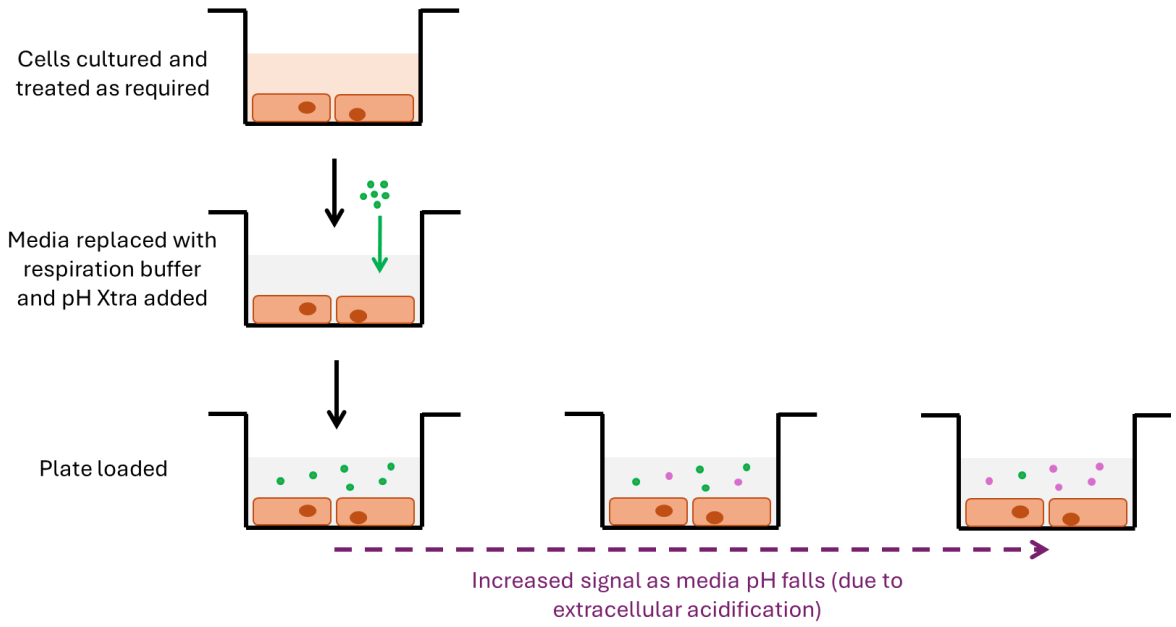


Figure 23. Principles of the pH Xtra assay. Following culture and treatment of cells, media is replaced with respiration buffer and pH Xtra is added. The plate is loaded and the signal produced by pH Xtra is monitored over time, with acidification of media increasing the signal.

Prior to the assay, respiration buffer (Agilent Technologies, USA) is made according to manufacturer protocol, by adding deionised water to tablets, adjusting the pH to 7.4, and making the volume up to 50mL. Respiration buffer is used instead of media because of its

low buffering capacity, meaning that changes in media pH can be more easily detected. pH Xtra was reconstituted in 1mL of respiration buffer. For the assay, treated cells were washed twice with respiration buffer before being left in respiration buffer. 10uL of reconstituted pH Xtra was added to each well and plates were immediately loaded on the CLARIOstar plate reader. Lifetime measurements were undertaken every five minutes for the duration of the assay. Lifetime values were calculated from the intensity data using the same equation used for MitoXpress Intra, with lifetime values inversely correlating with media pH.

The extracellular acidification rate (ECAR) was calculated as the change in lifetime per minute (in µs/min), during the period where lifetime showed a linear change (408), as outlined in figure 24.

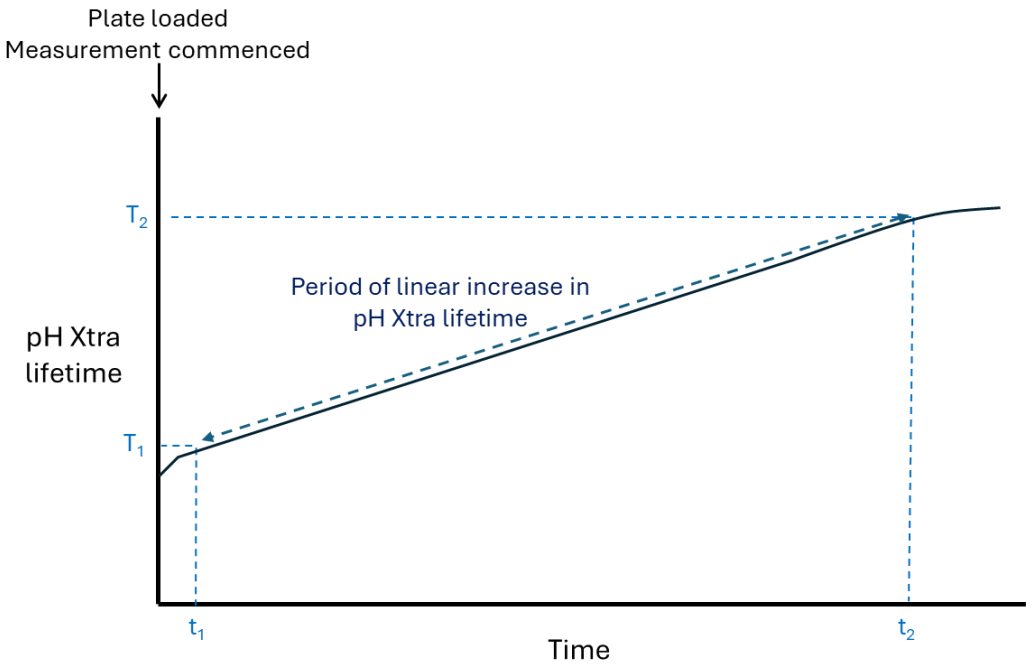


Figure 24. Calculation of extracellular acidification rate. Extracellular acidification rate (ECAR) is calculated as the rate of increase in pH Xtra lifetime during the period of linear increase in lifetime. This occurs after a period of equilibration after plate loading (before time t_1) and before any cell dysfunction or death (after time t_2).

Basal glycolysis represents ECAR without addition of any stimulators or inhibitors of glycolysis. Maximal glycolysis was determined following the addition of the mitochondrial ATP synthase inhibitor oligomycin (Sigma-Aldrich, USA) reconstituted in DMSO and added to media to achieve a final concentration of 1µM (with a final DMSO concentration of 1%). Inhibition of glycolysis was achieved through addition of 2-deoxyglucose (2DG; Sigma-Aldrich, USA) at a final concentration of 50mM. The workflow for pH Xtra assays is shown in figure 25.

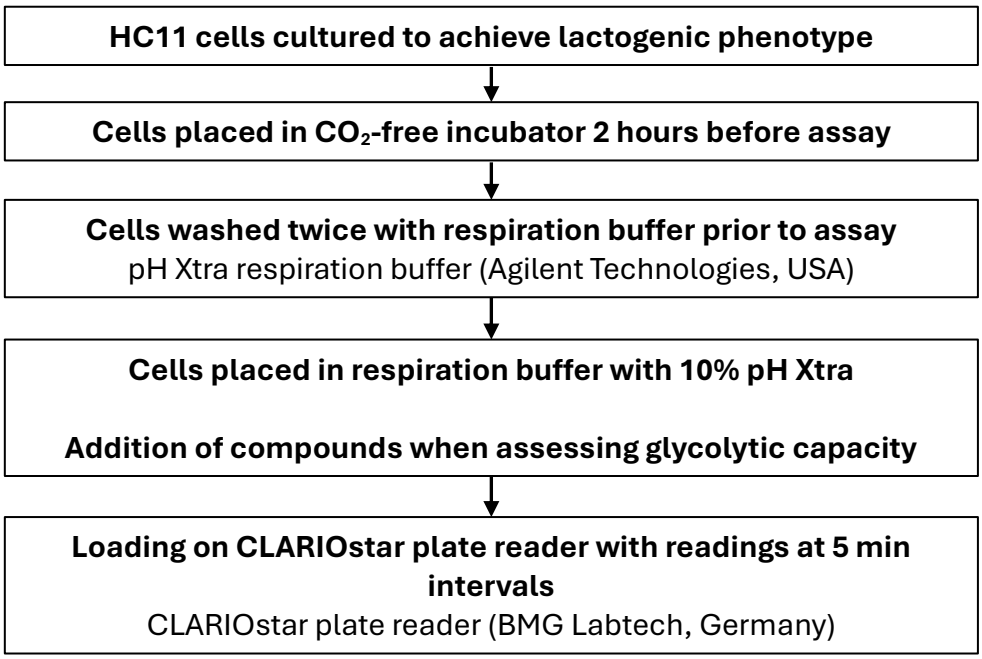


Figure 25. Workflow for pH Xtra assays.

3.10. Assessment of gene expression with qRT-PCR

RNA extracted from milk fat or from cultured cells was thawed on ice prior to qRT-PCR. Reverse transcription to cDNA was performed using the High Capacity Reverse Transcription

kit (Applied Biosystems, USA), according to the manufacturer's protocol. 1µg of RNA was used for each reaction.

The cDNA produced was diluted 1 in 15, aliquoted, and frozen at -20^{o}C until the time of PCR. For the PCR reaction, 5µL of diluted complementary deoxyribonucleic acid (cDNA) was added to 4.55µL of SYBR Green master mix (ThermoFisher Scientific, USA) and 0.225µL of each of the forward and reverse gene primers (Sigma-Aldrich, USA). qRT-PCR was performed on the Quantstudio3 platform (Applied Biosystems, USA) in a 0.2mL 96-well plate (Applied Biosystems, USA), and each reaction was undertaken in triplicate. The workflow for qRT-PCR is summarised in figure 26.

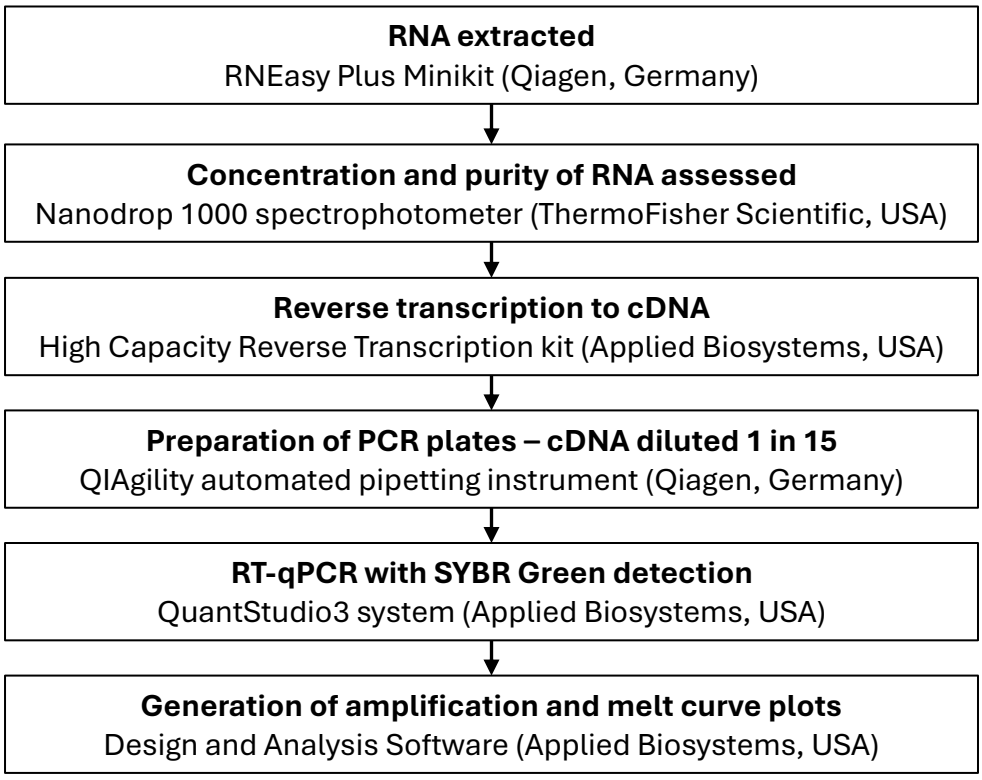


Figure 26. Workflow for RNA extraction and RT-qPCR.

For each triplicate, the mean Ct value (the cycle number at which the qRT-PCR reaction crosses the fluorescence threshold) was calculated using the Design and Analysis software (ThermoFisher Scientific, USA). Gene expression was assessed using the 2(-ΔΔCt) method after normalisation to the geometric mean of housekeeping genes (409). The genes for β-actin and β2 microglobulin are well-established housekeeping genes for HC11 cells (410-412). To determine the best housekeeping genes to use for assessment of RNA obtained from hMECs and milk fat globule RNA, eight candidate genes were evaluated in two samples of isolated milk cells and two batches of hMECs (figure 27). These were selected based on previous work on housekeeping genes in the human mammary setting (413). The three genes with the least variability (lowest standard deviation) were selected. These were *FAM130B, SYMPK* and *PRDX1*.

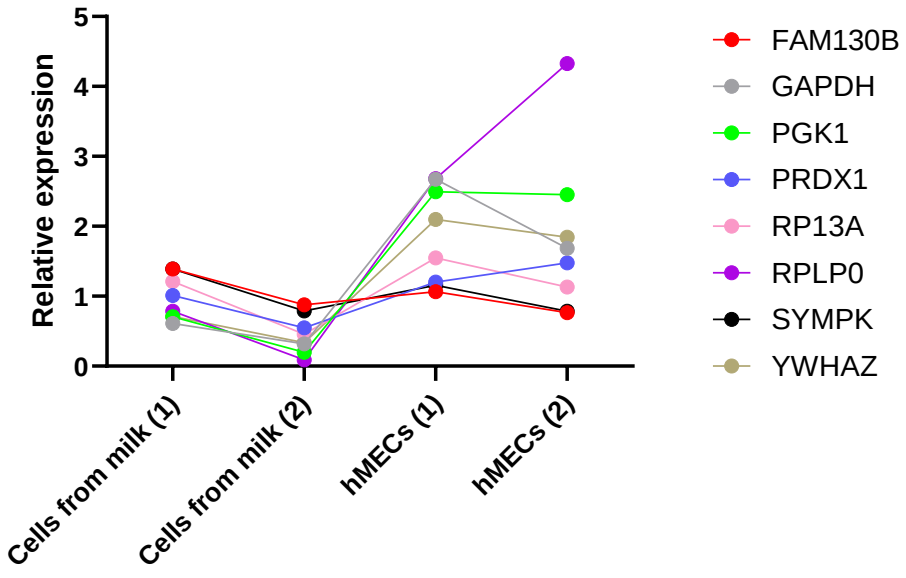


Figure 27. Assessment of housekeeping genes. Eight housekeeping genes (*FAM130B, GAPDH, PGK1, PRDX1, RP13A, RPLP0, SYMPK, YWHAZ*) were assessed in four samples: two sets of cells obtained from human milk, and two batches of cultured hMECs. The relative expression of each housekeeping gene in each sample is shown.

3.11. Transcriptomic analysis of HC11 cells

For transcriptomic analysis, RNA was extracted from hormone-treated HC11 cells using the protocol outlined in section 3.7 and frozen until analysis. RNA sequence analysis (RNASeq) was performed by Azenta Life Sciences using the Illumina HiSeq X platform (Illumina, USA), generating raw counts, which represent the number of reads of each gene. To provide relative expression values on a comparable scale, and to account for differences between samples, raw counts were normalised to counts per million (CPM) using Omics Playground (BigOmics Analytics, Switzerland). CPM is a simple measure of read abundance that can account for different library sizes, and is determined by dividing each read count by the corresponding library size (in millions) (414). Assessment of quality involved comparison of $\log_2$-transformed CPM values to ensure similarity between samples, while RNA integrity was assessed using an RNA integrity number (RIN) provided by Azenta Life Sciences. A sample with a RIN value of ≥8 is considered to be of good quality.

To evaluate the effect of treatment (compared to control conditions), and to ensure the effects of treatment were consistent among replicates, cluster analysis was performed using Omics Playground. This is done using principal component analysis (PCA), a method for visualising high-dimensional data by giving each data point a location in a two-dimensional plot (415).

Differentially expressed genes (DEGs) with a fold-change ≥2, using a false discovery rate (FDR) of 0.05, were determined and plotted on volcano plots. To increase the statistical reliability, differential gene expression analysis was undertaken by merging the results of three commonly accepted methods in the literature, namely, limma (trend), edgeR (QLF), and DESeq2 (Wald). To identify relevant gene sets which are enriched, gene set enrichment

analysis (GSEA) was performed in Omics Playground, using a fold-change threshold of 1.4 and a FDR of 0.05. Genes were aggregated into sets according to shared biological or functional properties. Omics Playground has >50,000 gene sets and pathways in total which are divided into 30 gene set collections such as Hallmark, MSigDB, KEGG and GO. The workflow for transcriptomics is outlined in figure 28.

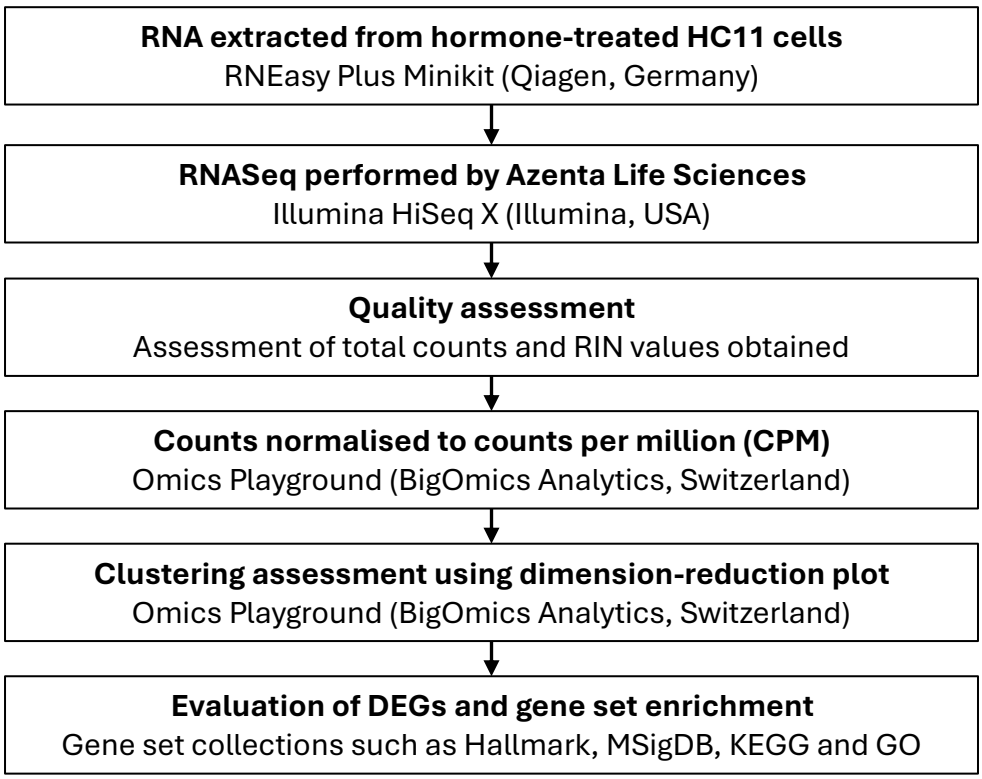


Figure 28. Workflow for transcriptomic assessment of hormone-treated HC11 cells.

3.12. Statistical testing

Given the importance of prolactin for secretory activation and lactation (74), the participant sample size has been determined using reported prolactin concentrations obtained from healthy breastfeeding mothers at the onset of lactation (416). The sample size has been

calculated to detect a 20% difference in mean prolactin levels at 95% power. Thus, using a 20% mean difference in prolactin of 779 mU/L and a pooled standard deviation (SD) of 681 mU/L, an effect size (d; calculated as mean difference between two groups divided by pooled SD) is calculated to be 1.14. G*Power statistical software (Kiel University, Germany) was used to determine sample size, using an effect size of 1.14, an alpha error probability of 0.05, a power of 0.95 and an allocation ratio of 1:1. This gives a total sample size of 44 participants. Assuming that participants provide samples on 2 of 4 post-natal days on average, at least 88 participants will be required for comparative hormone assessment.

Following collection of plasma and serum hormone data, an initial assessment is made using descriptive statistics (mean, range, standard deviation), while normality of data at each collection time point is assessed using the Anderson-Darling test. Plasma and serum hormone results are displayed as mean ± standard error of the mean (SEM) at each sampling time point. Outliers were removed using the ROUT method with a maximum false discovery rate of 1% (417). Statistical comparison of single variables in multiple (≥3) groups was performed using one-way ANOVA, with only statistically significant comparisons indicated on the graphs with asterisks. Statistical comparison of multiple variables in two or more groups was performed using a mixed effects model. Comparison of two unrelated groups was performed using an unpaired t-test.

For cell data, statistical comparison of multiple groups was performed using one-way ANOVA, with only statistically significant comparisons indicated on the graphs with asterisks. Comparison of two unrelated groups was performed using an unpaired t-test. Assessment of the phenotype of hMECs, which are primary cells that vary according to batch, was made using n=4 batches (each obtained from a different woman). Studies with

HC11 cells used n=4 biological replicates per condition, to allow statistical comparison between treatment and control conditions, unless otherwise specified.

All statistical comparisons were performed using GraphPad Prism (GraphPad, USA).

3.13. Materials used

The materials used for cell experiments are summarised in table 8.

Table 8. Materials used in experiments.

Material	Supplier	Species
Cells		
hMECs	Merck, Germany (Lot no. 2049)	Human (primary)
	InnoProt, Spain (Lot no. P10891)	
	Lonza, Switzerland (Lot no. 22TL115626)	
	Lonza, Switzerland (Lot no. 21TL195759)	
HC11 cells	ATCC, USA (Lot no 70041959)	Mouse (cell line)
Cell culture		
HuMEC basal media	Gibco, USA	
HuMEC supplement mix	Gibco, USA	
RPMI-1640 (ATCC)	ATCC, USA	
Fetal bovine serum (FBS)	Sigma-Aldrich, USA	Bovine
Epidermal growth factor (EGF)	Gibco, USA	Human
Insulin	Sigma-Aldrich, USA	Human
Penicillin/streptomycin solution	Sigma-Aldrich, USA	
Dexamethasone		
Prolactin	Peprotech, USA	Mouse
Growth hormone	Peprotech, USA	Human

Table 8 (continued).

Signalling studies		
RIPA buffer	ThermoFisher Scientific, USA	
Protease inhibitor	ThermoFisher Scientific, USA	
Phosphatase inhibitors	ThermoFisher Scientific, USA	
BCA assay reagents	ThermoFisher Scientific, USA	
Capillary protein electrophoresis plates, capillaries and reagents	Bio-Techne, USA	
Phospho-Akt S473	Cell Signalling, USA (cat. no. 4060)	Rabbit
Total Akt	Cell Signalling, USA (cat. no. 9359)	Mouse
Phospho-STAT5	Cell Signalling, USA (cat. no. 9359)	Rabbit
Total STAT5	R&D systems, USA (cat. no. AF2168)	Rabbit
Anti-rabbit secondary	Bio-Techne, USA (cat. no. 042-206)	Goat
Anti-mouse secondary	Bio-Techne, USA (cat. no. 042-205)	Goat
RNA processing & qRT-PCR		
RNeasy Plus Mini Kit	Qiagen, Germany	
High Capacity Reverse Transcription Kit	Applied Biosystems, USA	
SYBR Green master mix	ThermoFisher Scientific, USA	
Gene primers	Sigma-Aldrich, USA	
Cell metabolism studies		
FCCP	Sigma-Aldrich, USA	
Oligomycin	Sigma-Aldrich, USA	
2DG	Sigma-Aldrich, USA	
Respiration buffer	Agilent Technologies, USA	
MitoXpress Intra	Agilent Technologies, USA	
pH Xtra	Agilent Technologies, USA	

4. STUDY 1: HORMONAL CHANGES AT THE ONSET OF LACTATION

4.1. Background

4.1.1. Serum hormone changes during breastfeeding

Synthesis of milk requires a number of hormone-controlled changes in the mammary gland (figure 2). Prolactin plays important roles during pregnancy, where it promotes alveolar formation and induces secretory differentiation of alveoli (63, 66-68, 418). Importantly, prolactin is considered essential for secretory activation. Studies in mice demonstrate that prolactin upregulates genes promoting milk synthesis and induces closure of tight junctions between mammary epithelial cells, the latter being necessary for large-volume milk secretion (52, 419). Serum prolactin concentrations in women progressively rise during pregnancy and are approximately 10-fold higher than non-pregnant concentrations by the third trimester (420). The concentration of prolactin correlates with urinary lactose secretion, a surrogate for mammary synthetic activity, during pregnancy (316). However, there is no correlation between prolactin concentrations and milk production in the post-partum period (85, 421). These findings imply that prolactin is necessary for milk production, but that concentrations do not directly correlate with milk synthesis during lactation. As such, other factors may be important.

Like prolactin, GH signals through the Jak2-STAT5 pathway, while GH is also capable of activating the prolactin receptor (422, 423). Given the importance of the Jak2-STAT5 pathway for lactation, this suggests a potential role for GH. However, data on GH concentrations in the post-partum period in women are limited and conflicting, with one study reporting increasing GH concentrations after birth, and another reporting decreased

pituitary GH secretion (424, 425). In addition, GH promotes expression of IGF-1 through the Jak2-STAT5 pathways, predominantly in the liver (426). IGF-1 is considered to promote alveolar budding in early gestation (427), but whether IGF-1 has a role in milk synthesis remains unclear (428).

Glucocorticoids are also important for milk production at lactation onset (see background section 1.3.5). Cortisol concentrations are reported to increase approximately two-fold by the end of pregnancy, and decline by day 5 after birth, although existing data is from a small study of 12 women (429).

In contrast, progesterone and to a lesser extent oestradiol, have an inhibitory effect on milk synthesis, and withdrawal of progesterone following delivery of the placenta is essential for initiating milk synthesis (see background section 1.3.4).

4.1.2. Potential involvement of additional metabolic hormones in lactation

Milk production is energy-consuming and requires ≥500 kcal/day (430). In addition, metabolic and bioenergetic changes in mammary cells support lactation. Thus, mammary mitochondrial biogenesis increases ~5-fold in lactating mice, and is associated with expansion of the inner mitochondrial membrane and increases in mitochondrial proteins generating ATP (94, 95). Furthermore, there are alterations in several metabolic pathways in mammary epithelial cells to support lactation. There is increased glucose transporter 1 (GLUT1)-mediated glucose uptake, increased production of glycolytic intermediates that support lipid synthesis, and increased amino acid uptake to support protein synthesis (94,

95, 108, 431). However, the hormonal drivers underlying these changes remain poorly understood.

Insulin is an essential metabolic hormone with important effects on glucose, lipid and protein metabolism, and is considered to play a direct role in milk production (432). Insulin signals predominantly through the phosphoinositide 3-kinase (PI3K)-Akt pathway, and promotes glucose uptake, glycolysis, protein synthesis and lipogenesis in non-mammary tissues (433). Insulin also increases mitochondrial biogenesis and oxidative capacity in muscle, liver and pancreatic β-cells (434). Mammary studies in mice demonstrate that insulin promotes amino acid uptake and protein synthesis, while also being important for expression of the key lactogenic transcription factors STAT5a and ELF5 (435). However, whether insulin mediates mammary mitochondrial changes at lactation onset remains uncertain. Insulin resistance increases as pregnancy develops due to factors produced by the placenta, including progesterone, oestradiol and hPL (436). Following delivery of the placenta, leading to declining concentrations of these factors, and with increased mammary glucose uptake for milk production, insulin sensitivity increases post-partum (437).

Thyroid hormones are also considered important for lactation, as evidenced by the increased prevalence of lactation insufficiency in women with hypothyroidism (364). Rat models of hypothyroidism demonstrate that thyroid hormones are important for maintaining mammary prolactin receptor expression and for preventing mammary involution (438). In addition, thyroid hormones are well established to have important effects on mitochondrial energetics which include promotion of mitochondrial biogenesis (439).

A number of hormones known to be important in lactation therefore have potential roles in milk synthesis and mammary bioenergetics. However, how the concentrations of these hormones change at lactation onset in women remains poorly understood. We sought to investigate this through the INSIGHT study, which measures serum hormone concentrations in breastfeeding women at 36 weeks' gestation and during days 1-4 post-partum (section 3.1).

4.2. Study aims

The aims of this chapter are to:

1. Define normal serum concentrations for key lactation hormones in breastfeeding women, specifically those related to:
    i. Synthetic function: prolactin, GH and IGF-1, cortisol, progesterone and oestradiol
    ii. Bioenergetics: insulin and thyroid hormones
2. Understand how maternal factors influencing lactation onset, such as parity and BMI, may alter these key hormones.

4.3. Results

4.3.1. Participant characteristics

Eligible women were recruited to the INSIGHT study at 36 weeks' gestation, and their characteristics are shown in table 9. A total of 106 women were recruited, with a mean age of 35.0 years. The majority (88.7%) gave their ethnicity as white, while just over half (54.7%) were primiparous. 70.0% of women had a healthy weight, with the remainder being overweight or obese, and most (86.8%) had no comorbidities. All women delivered at term, with the modes of delivery and medications during labour/delivery described in table 9.

Table 9. Baseline characteristics of INSIGHT participants.

Characteristic	Value
Age (years)	Mean 35.0 (range 26-46)
Ethnicity	
•White	94/106 (88.7%)
•Asian/Asian British	9/106 (8.5%)
•Other	2/106 (1.9%)
Parity	
•Primiparous	58/106 (54.7%)
•Multiparous	48/106 (45.3%)
BMI	
•Healthy weight (BMI 18.5-24.9)	71/106 (70.0%)
•Overweight (BMI 25-29.9)	23/106 (21.7%)
•Obese (BMI ≥30)	12/106 (11.3%)
Comorbidities	
•None	92/106 (86.8%)
•Gestational diabetes	5/106 (4.7%)
•Anaemia	3/106 (2.8%)
•Hypothyroidism	2/106 (1.9%)
•Hyperthyroidism	1/106 (0.9%)
•Pregnancy-induced hypertension	2/106 (1.9%)
•Other*	6/106 (5.7%)
Gestational age at delivery (weeks)	Mean 39.9 (range 37.0-42.7)

Table 9 (continued).

Mode of delivery	
•Spontaneous vaginal delivery	45/106 (42.5%)
•C-section (elective or emergency)	38/106 (35.8%)
•Assisted delivery (forceps or ventouse)	23/106 (21.7%)
Medications during labour/delivery	
•None	16/106 (15.1%)
•Synthetic oxytocin or ergometrine	62/106 (58.5%)
•Epidural or spinal pain medications	45/106 (42.5%)
•Other**	8/106 (7.5%)
•Unknown	13/106 (12.3%)

*Other comorbidities are polycystic ovarian syndrome (PCOS), scoliosis, asthma, chronic fatigue syndrome, fibromyalgia, anxiety and ulcerative colitis. **Other medications are tranexamic acid and diamorphine.

Maternal and infant characteristics on days 1-4 post-partum are shown in table 10. Around 95% of women reported onset of copious milk secretion by day 4 post-partum, and most (87.7%) were exclusively breastfeeding.

Table 10. Maternal and infant characteristics assessed on days 1-4 post-partum.

Characteristic	Value
Onset of copious milk secretion	
•Post-partum day 1	5/106 (4.7%)
•Post-partum day 2	17/106 (16.0%)
•Post-partum day 3	53/106 (50.0%)
•Post-partum day 4	17/106 (16.0%)
•Later than post-partum day 4	5/106 (4.7%)
•Unknown (incomplete breastfeeding diary)	9/106 (8.5%)
Type of infant feeding	
•Exclusive breastfeeding	93/106 (87.7%)
•Mixed breast and pumping	9/106 (8.5%)
•Exclusive pumping	1/106 (0.9%)
•Mixed artificial formula and breastfeeding	1/106 (0.9%)
•Not recorded	2/106 (1.9%)
Effectiveness of infant feeding during breastfeeding episodes	
•Good breast attachment and suckling	259/414 (62.6%)
•Problems attaching to breast and/or suckling	90/414 (21.7%)
•Not assessed	65/414 (15.7%)
Mean duration of breastfeed during study visit	23 min (range 0-212 min)

4.3.2. Hormones influencing synthesis of milk components

4.3.2.1. Prolactin concentrations increase following delivery and are greater in multiparous women

Serum prolactin concentrations at 36 weeks' gestation, and pre- and post-breastfeeding on days 1-4 post-partum, are shown in figure 29. As previously described, prolactin concentrations are high in late pregnancy when compared to non-pregnant women (420), while our data shows a significant rise in prolactin concentrations after delivery. Thus, mean prolactin concentrations increased from 4128 mU/L at 36 weeks' gestation (range 1514-9005 mU/L) to 5369 mU/L in pre-feed day 1 post-partum samples (range 1804-12288 mU/L;

p<0.01). However, there is no significant increase in mean prolactin concentrations across a breastfeeding episode (figure 29).

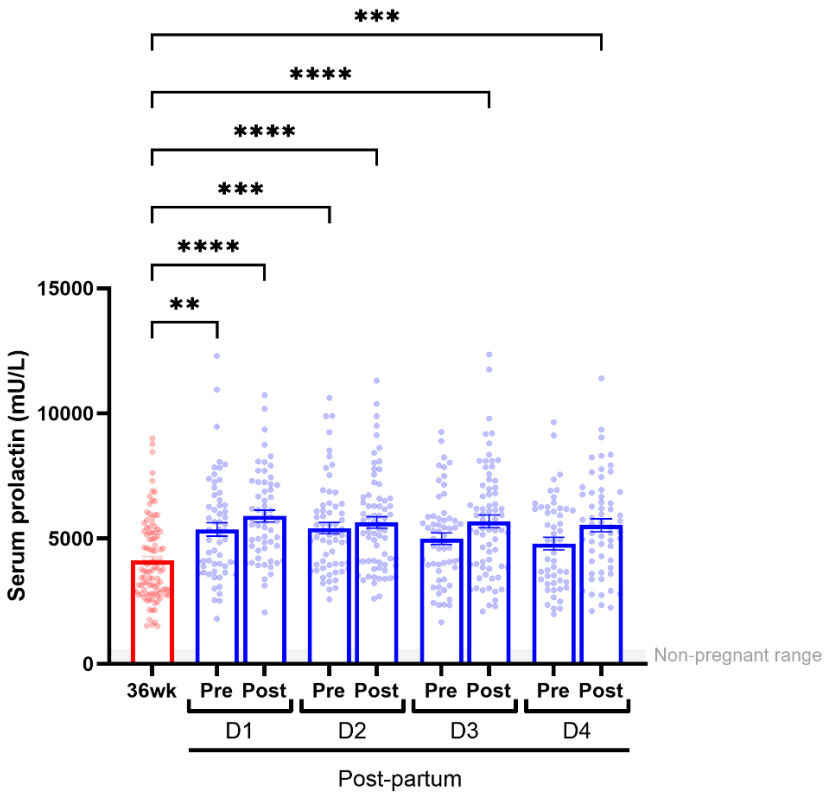


Figure 29. Prolactin. Prolactin concentrations in the third trimester (36wk, red) and pre- and post-feed on days 1-4 post-partum (blue) are shown for n=106 women, and a non-pregnant reference range is highlighted (110-560 mU/L). Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; ** p<0.01, *** p<0.001, **** p<0.0001.

Given the large spread of prolactin values (>4-fold difference between largest and smallest values at each time point), we next assessed whether any maternal characteristics linked to lactation onset or success of breastfeeding had an impact on prolactin concentrations. Increased maternal age (>35 years) and being primiparous are known to be associated with

lower breastfeeding success (25). We sought to evaluate these factors, and found no significant association between older age (>35 years) and prolactin concentrations but found that multiparous women had significantly greater prolactin concentrations in the post-partum period (figure 30). Notably, multiparity was associated with earlier onset of milk coming in (p=0.0057 using Fisher's exact test). We also assessed whether the duration of gestation correlated with prolactin concentration but found no association (p=0.4 when comparing gestation of <40 weeks versus ≥40 weeks using a mixed effects model).

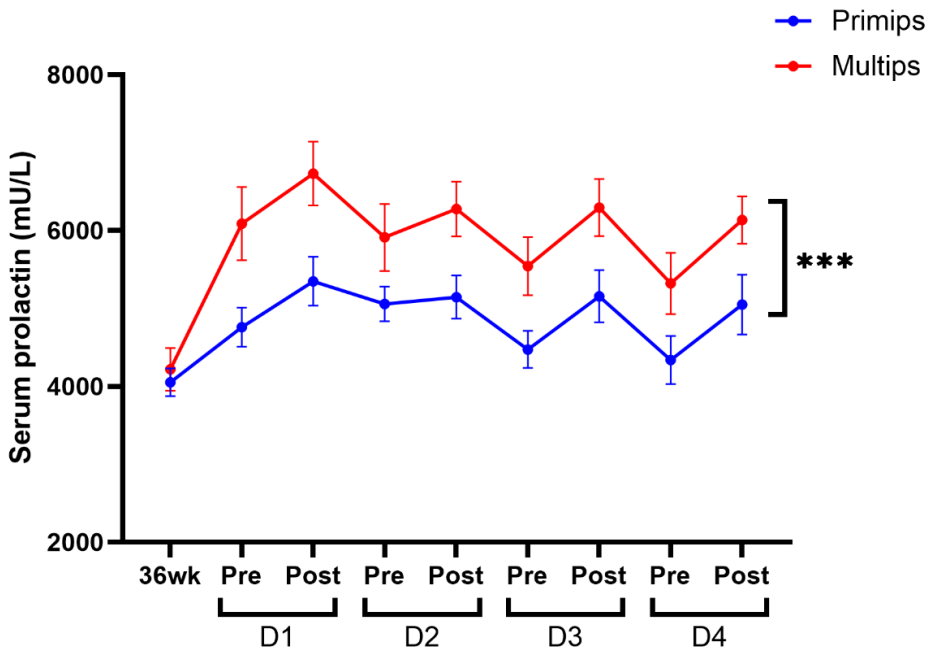


Figure 30. Prolactin concentrations in primiparous and multiparous women. Serum prolactin concentrations in n=58 primiparous women (blue) and n=48 multiparous women (red). Data analysed using a mixed-effects model; *** p<0.001.

Prolactin concentrations have not previously been shown to correlate with milk production in the post-partum period, although published work uses prolactin measurements from 1 month post-partum (85). We therefore assessed prolactin concentrations by the day on which milk came in, in our cohort (figure 31). We found significantly higher prolactin concentrations in women whose milk came in on days 1-2 compared to those whose milk came in on days 3-4, particularly on day 1 post-partum.

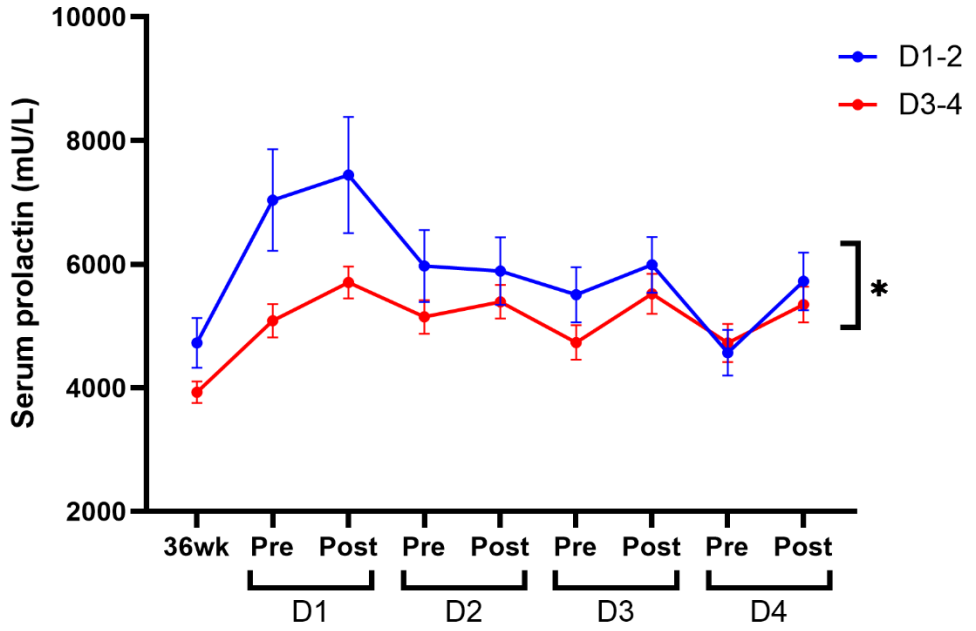


Figure 31. Prolactin concentrations according to day of milk coming in. Serum prolactin concentrations in women whose milk came in on day 1-2 (n=22, blue) versus day 3-4 (n=70, red). Data analysed using a mixed-effects model; * $p<0.05$.

4.3.2.2. Growth hormone concentrations increase post-partum

Serum GH concentrations at 36 weeks' gestation and on days 1-4 post-partum are shown in figure 32. Pituitary GH secretion is suppressed in late pregnancy, in keeping with existing

literature demonstrating a predominance of the placental GH variant as pregnancy proceeds (440). There is, however, a lack of reported data on GH concentrations after delivery, and our findings demonstrate an increase in GH concentrations on post-partum days 1-3. Furthermore, there is a significant increase in GH across a breastfeeding episode on day 3 post-partum.

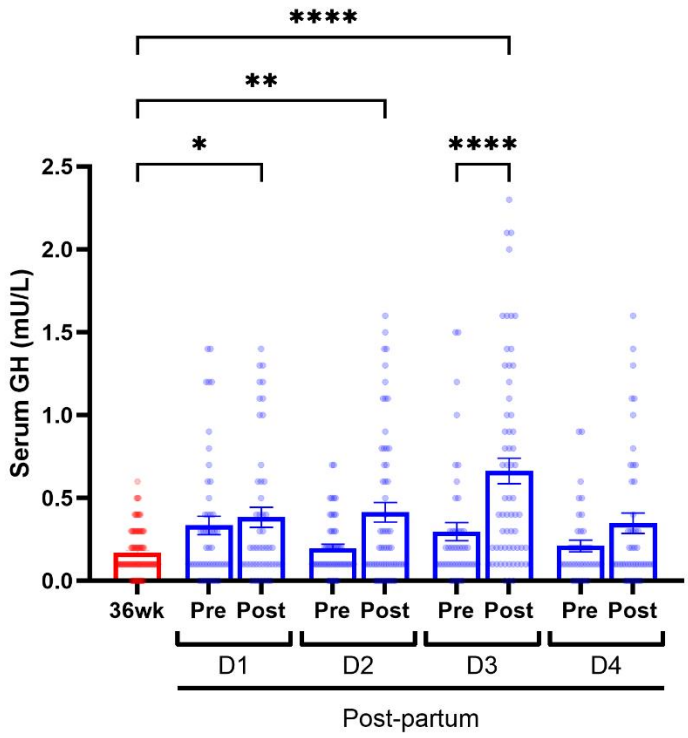


Figure 32. Growth hormone. Growth hormone concentrations in the third trimester (36wk, red) and pre- and post-feed on days 1-4 post-partum are shown for n=106 women. Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

A role for GH in milk production is indicated by studies reporting that recombinant GH injection augments milk production (377, 378), and by the ability of GH to signal through the Jak2-STAT5 pathway. We therefore next assessed whether the increases in GH

concentration across a feed on day 3 post-partum had any link to milk coming in. Specifically, we demonstrated that women whose milk came in early (≤day 2 post-partum) had a significantly greater increase in GH concentration across a breastfeed compared to women whose milk came in later (≥day 3 post-partum) (figure 33).

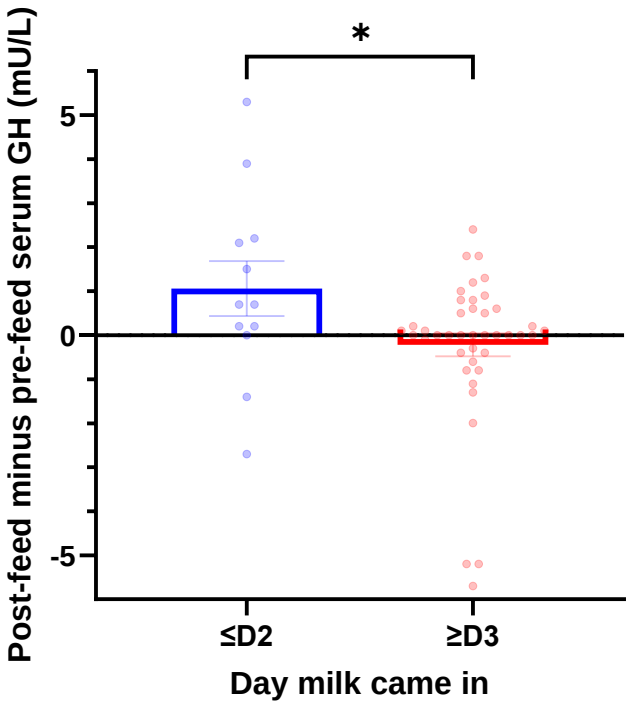


Figure 33. Change in GH concentration on day 3 according to when milk came in. Post-feed minus pre-feed GH concentrations on day 3 post-partum in women whose milk came in on or before day 2 (n=12, blue) and women whose milk came in on or after day 3 (n=36, red). Data analysed using unpaired t-test; * $p<0.05$.

4.3.2.3. IGF-1 concentrations fall in post-partum period

By contrast to GH, IGF-1 concentrations are high at 36 weeks' gestation and fall in the post-partum period (figure 34). Moreover, IGF-1 concentrations do not change significantly across a breastfeeding episode.

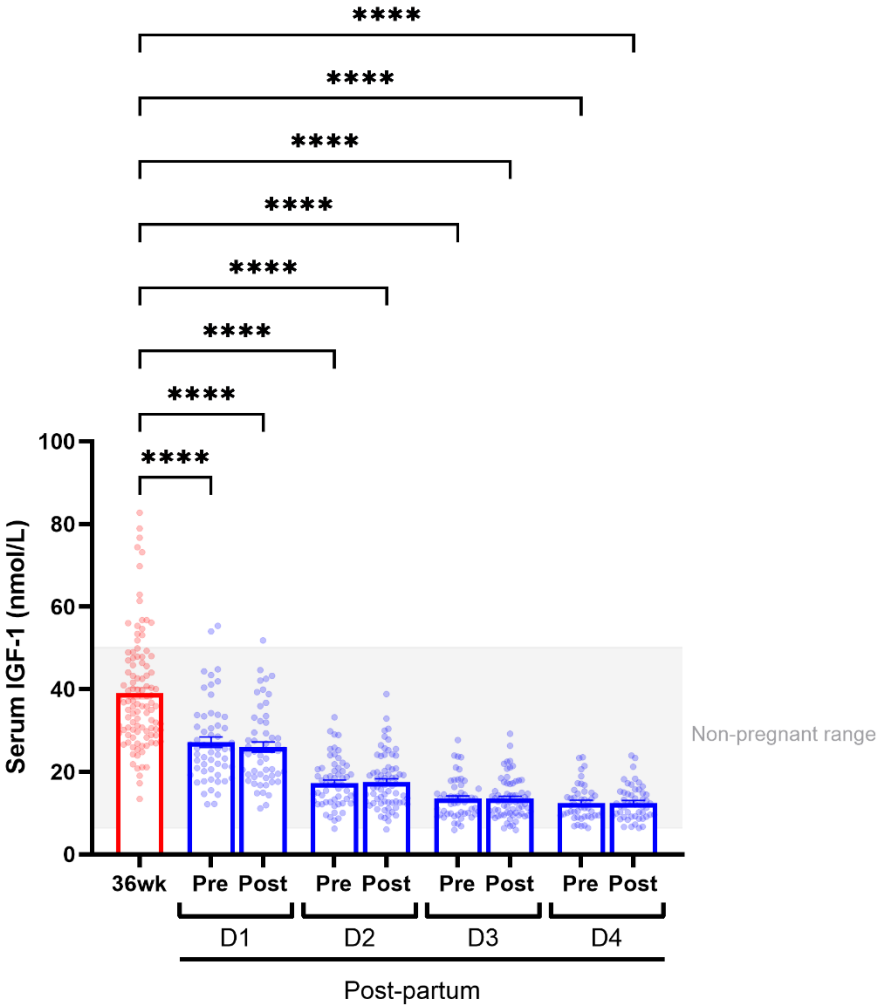


Figure 34. IGF-1. Insulin-like growth factor 1 (IGF-1) concentrations in the third trimester (36wk, red) and pre- and post-feed on days 1-4 post-partum (blue) are shown for n=106 women, and a non-pregnant reference range is highlighted (6.4-50.1 nmol/L). Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; **** p<0.0001.

4.3.2.4. Serum cortisol concentrations fall in post-partum period

Serum cortisol concentrations decrease in the post-partum period (figure 35). There is no fall in concentrations immediately post-partum, but there is a fall on days 2-4 post-partum. There is no significant decrease in cortisol across a breastfeeding episode, although it is notable that cortisol values show substantial inter-individual variability at all sampling time points, in keeping with what is known about cortisol in the non-pregnant, non-lactating setting (441).

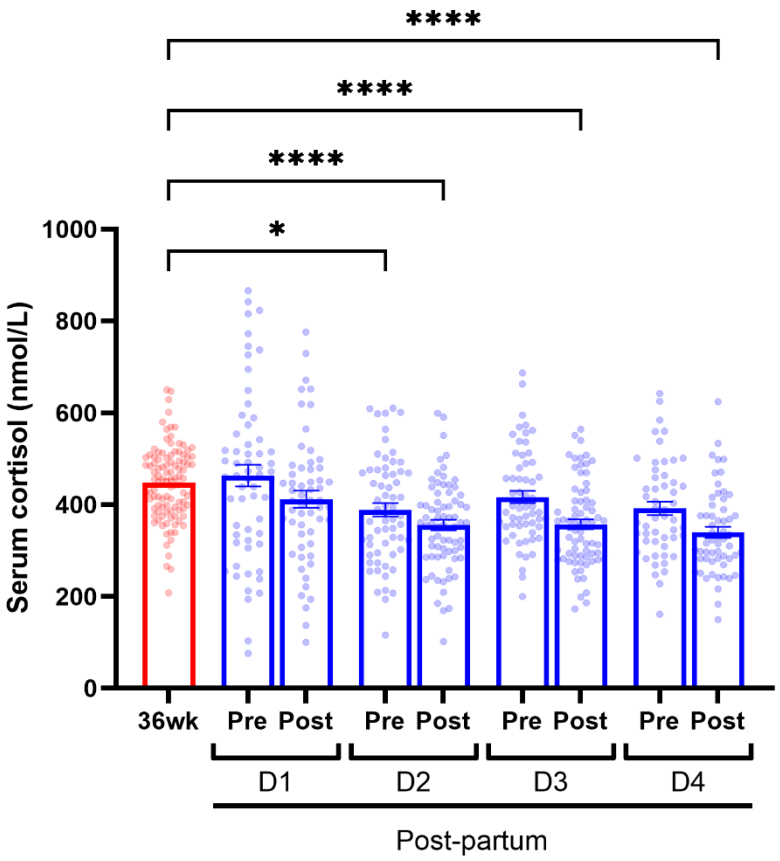


Figure 35. Cortisol. Cortisol concentrations for participants in the third trimester (36wk, red) and pre- and post-feed on days 1-4 post-partum (blue) are shown for n=106 women. Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; * $p<0.05$, **** $p<0.0001$.

4.3.2.5. Progesterone and oestradiol fall with delivery of placenta

Progesterone and oestradiol are produced by the placenta during pregnancy, and their serum concentrations are high at 36 weeks' gestation (figure 36). Following delivery of the placenta, there is a rapid fall in the concentrations of both of these hormones.

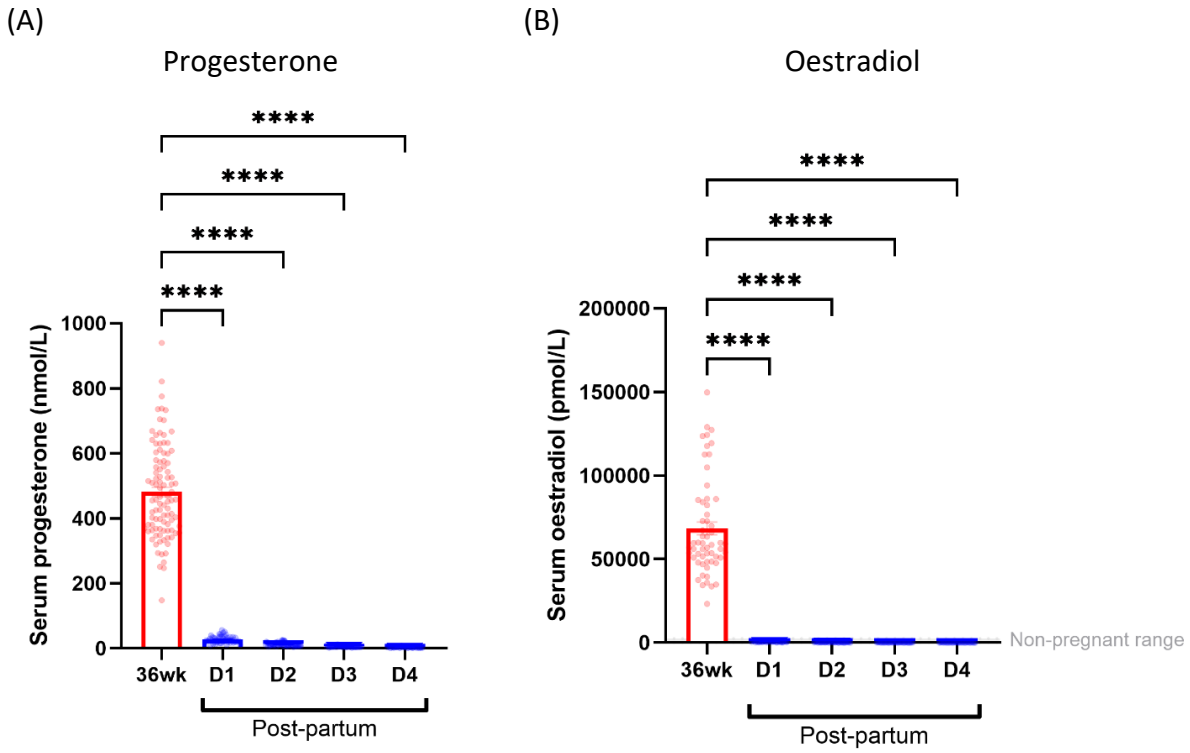


Figure 36. Hormone withdrawal initiating secretory activation. (A) Progesterone and (B) oestradiol concentrations are shown for n=106 women. Serum hormone concentrations in the third trimester (36wk, red) and days 1-4 post-partum (blue) are plotted. Data shown as mean ± SEM after removal of outliers using the ROUT test, and a non-pregnant reference range is highlighted (77-2400 pmol/L for oestradiol, none available for progesterone). Data analysed using one-way ANOVA; **** p<0.0001.

4.3.3. Hormones influencing mammary metabolism

4.3.3.1. Insulin sensitivity increases in the post-partum period

Pregnancy, particularly the third trimester, is known to be a period of insulin resistance due to factors produced by the placenta, including progesterone, oestradiol and human placental lactogen (436). In keeping with this, and given lactation is a state of relative insulin sensitivity as compared to pregnancy (437), we have observed high insulin concentrations at 36 weeks' gestation which fall in the post-partum period (figure 37). We have also noted that insulin concentrations fall during a breastfeeding episode, which is significant on days 1 and 2 post-partum.

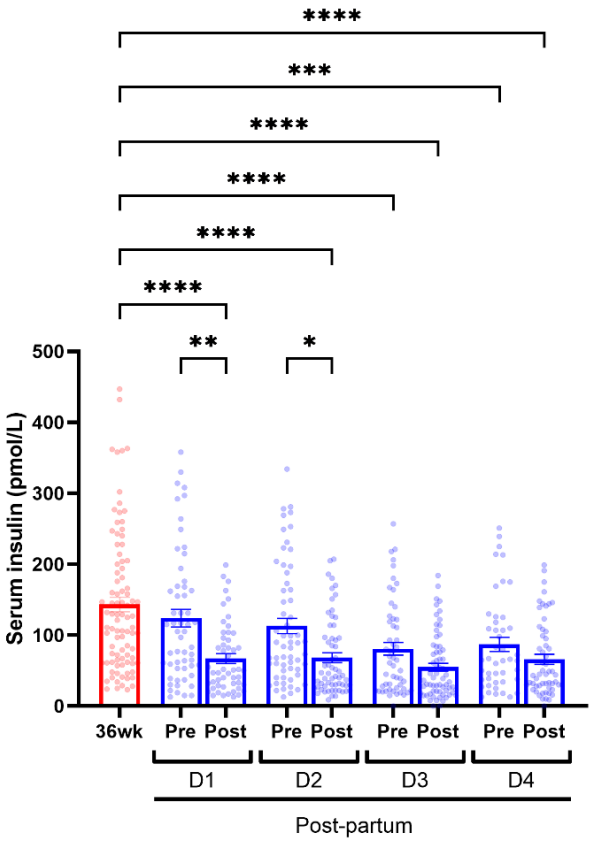


Figure 37. Insulin concentrations. Insulin concentrations for all participants in the third trimester (36wk, red) and pre- and post-feed on days 1-4 post-partum (blue) are shown for n=106 women. Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Importantly, we have demonstrated higher insulin concentrations in women with a high BMI (BMI ≥25), in keeping with insulin resistance in these women (figure 38).

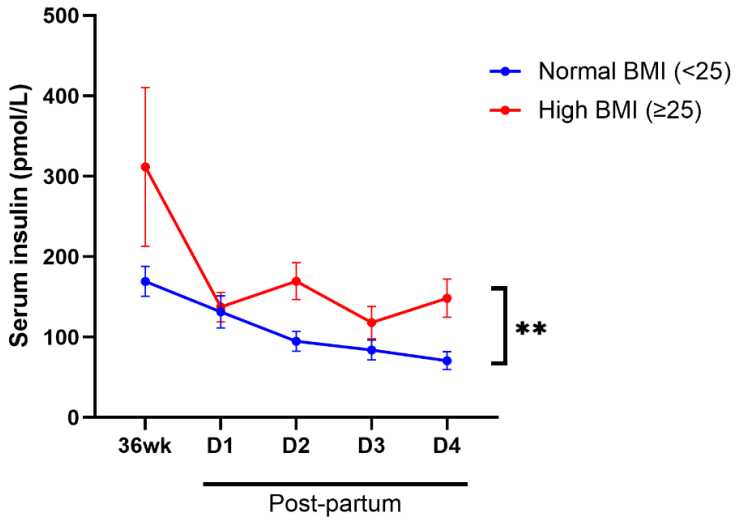


Figure 38. Insulin concentrations by BMI. Insulin concentrations in n=71 women with normal BMI (BMI <25, blue) and n=35 women with high BMI (BMI ≥25, red). Data analysed using a mixed-effects model; ** p<0.01.

Insulin levels vary throughout the course of a day, being low during fasting periods and high after meals. Given it is not practical or ethical to take fasting samples or control for meals in the early post-partum period, we have observed a large degree of variability in serum insulin concentrations. We therefore assessed the non-fasting leptin:adiponectin ratio in a subset of 36 women. Leptin concentrations are positively correlated, and adiponectin concentrations negatively correlated, with the degree of insulin resistance (442). Variations in fasting and post-prandial leptin and adiponectin concentrations tend to be small, and the leptin:adiponectin ratio is considered a useful measure of insulin resistance (443). Our data demonstrate that leptin concentrations fall in the post-partum period (figure 39(A)) while adiponectin concentrations remain stable (figure 39(B)). Importantly, the leptin:adiponectin ratio is greater in women with a high BMI on all post-partum days (figure 39(C)).

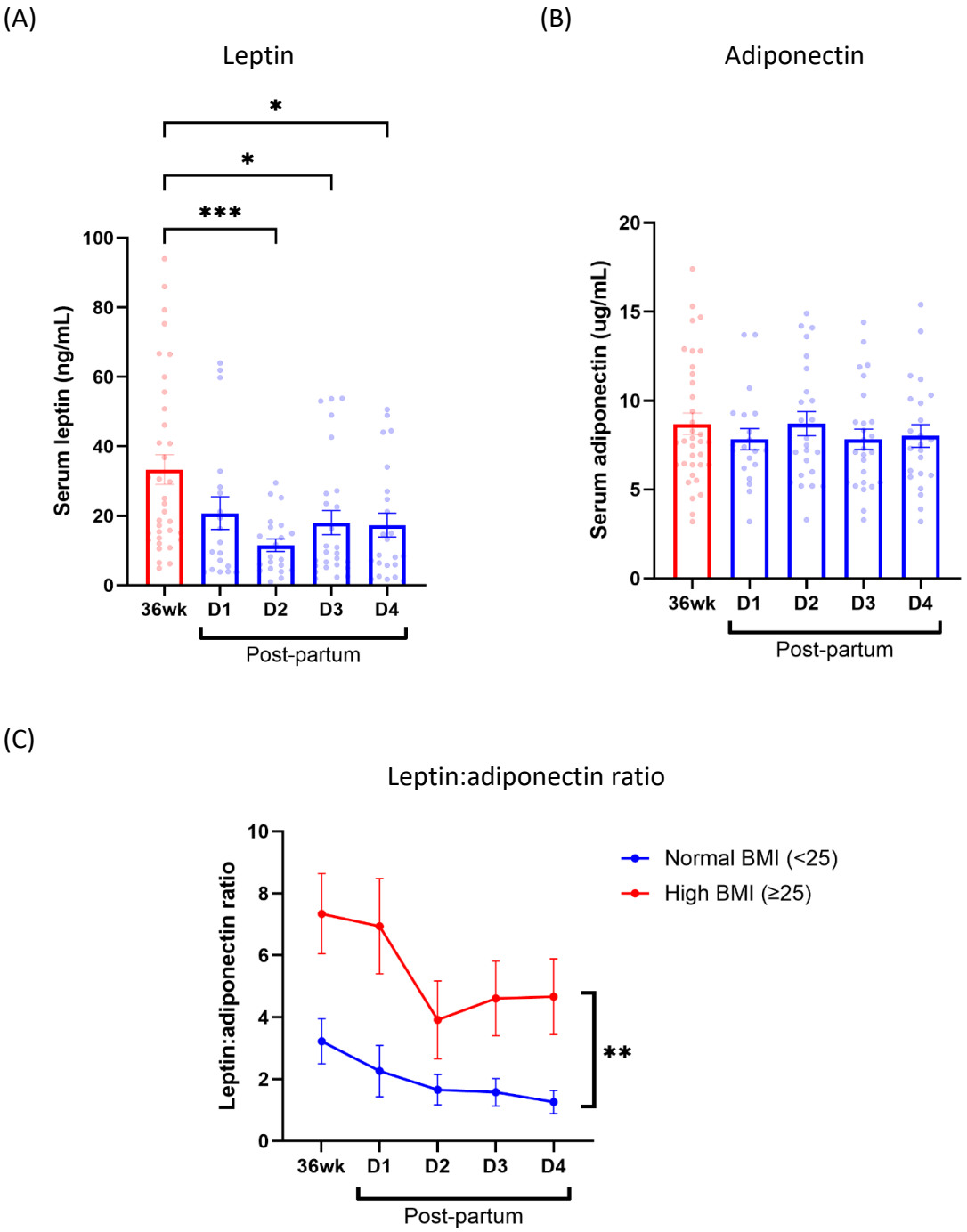


Figure 39. Leptin and adiponectin concentrations. (A) Leptin and (B) adiponectin concentrations for participants in the third trimester (36wk, red) and on days 1-4 post-partum (blue) are shown for n=36 women. Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; * $p<0.05$, *** $p<0.001$. (C) Leptin:adiponectin ratios in n=23 women with normal BMI (BMI <25, blue) and n=13 women with high BMI (BMI ≥25, red). Data analysed using a mixed-effects model; ** $p<0.01$.

4.3.3.2. TSH and free T4 increase in the early post-partum period

TSH concentrations at 36 weeks' gestation tend towards the lower part of the non-pregnant TSH range and increase soon after delivery, on days 1 and 2 post-partum (figure 40).

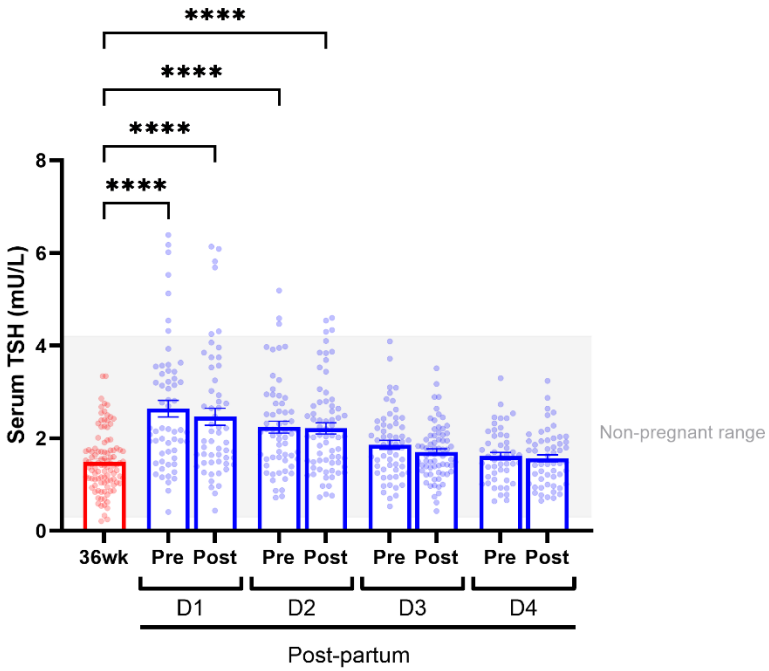


Figure 40. Thyroid stimulating hormone. TSH for participants in the third trimester (36wk, red) and pre- and post-feed on days 1-4 post-partum (blue) are shown for n=106 women, and a non-pregnant reference range is highlighted (0.3-4.2 mIU/L). Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; **** p<0.0001.

Having observed this early rise in TSH, we sought to evaluate changes in free thyroid hormones on days 1-4 post-partum. We observed that the rise in TSH is followed by an increase in free T4 concentrations on days 3 and 4 post-partum (figure 41(A)), whereas free T3 concentrations were decreased on postpartum day 1 (figure 41(B)). Thyroid hormones showed no change during breastfeeding episodes (figure 41).

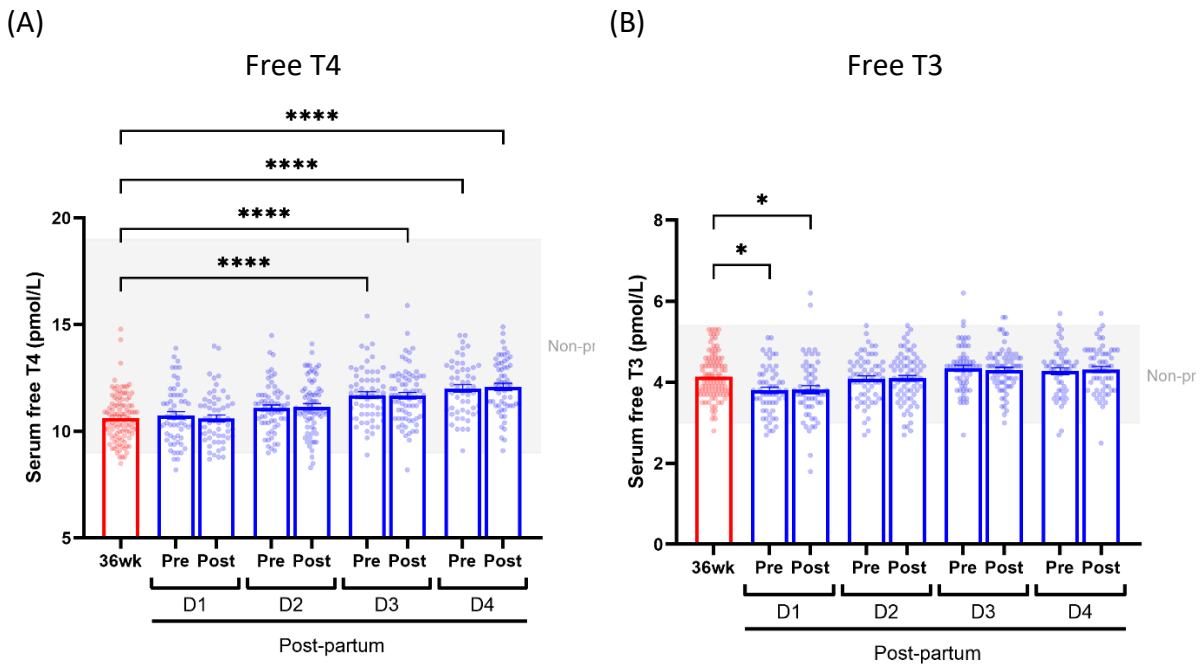


Figure 41. Free thyroid hormones. (A) Free T4 and (B) free T3 concentrations for participants in the third trimester (36wk, red) and pre- and post-feed on days 1-4 post-partum (blue) are shown for n=106 women, and a non-pregnant reference range is highlighted (9.0-19.0 pmol/L for free T4, 3.0-5.4 pmol/L for free T3). Data shown as mean ± SEM after removal of outliers using the ROUT test. Data analysed using one-way ANOVA; * $p<0.05$, **** $p<0.0001$.

The mean concentration, range and standard deviation for all hormones at each sampling timepoint are displayed in tables S4 and S5.

4.4. Discussion

In summary, our assessment of serum hormone concentrations during secretory activation showed increases in prolactin, GH and thyroid hormones; whilst insulin, cortisol, IGF-1, oestradiol and progesterone decreased in the early post-partum period. These findings are discussed below.

4.4.1. Reproductive hormones show well-characterised changes

4.4.1.1. Prolactin concentrations are high post-partum and greater in multiparous women

Serum prolactin concentrations are reported to increase throughout pregnancy, being approximately ten-fold higher than non-pregnant concentrations by the end of pregnancy (420). We have demonstrated similarly high levels of prolactin at 36 weeks' gestation in the INSIGHT study, and have also shown that prolactin significantly increases after birth, particularly in multiparous women (figure 30). However, in contrast to published data demonstrating that maternal prolactin concentrations increase with suckling (444), we have not observed significant increases in prolactin concentrations across a breastfeeding episode. It is notable that our sampling time points (1-4 days post-partum) are earlier than those previously assessed (4-40 weeks post-partum), and it may be that suckling-induced increases in prolactin only occur later in the post-partum period. The underlying cause is unclear but may be due to infant suckling being less effective soon after birth.

Prolactin is essential for milk production (445), but it remains unclear whether the absolute prolactin concentration is important. While prolactin concentration correlates with urinary lactose secretion during pregnancy (316), there is no correlation with milk production in the post-partum period (85, 421). We evaluated whether maternal characteristics associated with successful breastfeeding correlated with prolactin concentrations. Our findings showed the influence of parity, with multiparous women having higher prolactin values than primiparous women (figure 30), in addition to an earlier onset of lactation. This is in contrast to the limited existing data from a study of 21 breastfeeding mothers, which showed lower prolactin concentrations in multiparous women (446). Furthermore, prolactin

concentrations were significantly higher in women whose milk came in earlier, particularly on day 1 post-partum (figure 31).

Progesterone, and to a lesser extent oestradiol, are well-established to have an inhibitory effect on milk synthesis (52, 307, 310, 447). Our data confirm that the concentrations of these placenta-derived hormones decrease substantially soon after birth in all women (figure 36), which is required for establishing milk production following birth.

4.4.1.2. Novel changes in anabolic hormones at lactation onset

Like prolactin, GH promotes signalling through the Jak2-STAT5 pathway, while GH is also capable of activating the prolactin receptor (422, 423). Furthermore, a potential role for GH in lactation is highlighted in studies which show increased milk production in women following administration of recombinant GH (377, 378). We therefore sought to determine whether GH might have a role in initiating lactation and increase in the early post-partum period. Indeed, our data demonstrates that GH concentrations do increase after delivery, and also acutely increased during a breastfeed on day 3 post-partum (figure 32).

There are a number of potential explanations for the post-partum increase in serum GH. The most likely is a shift from placental to pituitary GH secretion after childbirth. The Siemens Immulite 2000 GH immunoassay used in this study does not provide information on isoform specificity, similar to other commercial assays (448). However, it is unlikely that placental GH is detected by this assay given the absence of detectable serum GH concentrations in the third trimester of pregnancy (figure 32). Secondly, pregnancy is known to be a period of insulin resistance (436), and the relative increase in circulating glucose concentrations may

inhibit pituitary GH release during the third trimester (449). A third possible explanation is that suckling promotes GH release through a direct neuroendocrine mechanism. In keeping with this, suckling has been shown to induce a rapid (within 30 minutes) rise in GH in cows and rats (450, 451).

Given GH exerts many of its effects through IGF-1, we next sought to evaluate IGF-1 concentrations on days 1-4 post-partum. IGF-1 has been demonstrated to rise during pregnancy due to the production of placental GH (452). IGF-1 concentrations fall after delivery (figure 34), in keeping with delivery of the placenta and a post-partum decline in placental GH (453). This suggests that any post-partum effects related to increased pituitary GH production are independent of IGF-1, and GH is reported to have direct effects on protein, lipid and glucose metabolism in human liver, muscle and adipose tissue (426).

Cortisol is also important for lactation and promotes tight junction formation and synthesis of milk components in cows and goats (74, 321, 454). We observed a decline in maternal cortisol concentrations after birth (figure 35). Cortisol concentrations in women are known to increase approximately two-fold by the end of pregnancy, compared to the non-pregnant state, and decline soon after birth (429). This reflects placental production of corticotropin-releasing hormone (CRH), concentrations of which fall after delivery (455). Despite this, post-partum cortisol concentrations only decreased slightly and likely remain sufficient for lactation onset. Furthermore, evidence from cows shows an increased number of cortisol binding sites in mammary tissue during lactation (456), which may reflect a greater number of cortisol receptors in lactating mammary epithelial cells. Increased mammary sensitivity to cortisol may therefore reflect a local mechanism for ensuring adequate glucocorticoid activity despite a decrease in systemic concentrations.

4.4.1.3. Insulin sensitivity at lactation onset varies according to BMI

Placenta-derived factors induce a state of insulin resistance during pregnancy, which is considered to be important for diversion of substrates to the developing foetus (436, 457). Following delivery, insulin sensitivity increases in women, both systemically and in the mammary gland (77, 437). This is reflected in our serum insulin data, which shows a decline in insulin concentrations on days 1-4 post-partum compared to the third trimester. Furthermore, there is an acute fall in serum insulin concentration across a breastfeeding episode on days 1 and 2 postpartum. The cause is unclear and may reflect a fall in maternal blood glucose during breastfeeding, although previous work has shown no change in glucose during breastfeeding (458). Another possible explanation is that women are unlikely to eat while breastfeeding and a fall in insulin concentration may reflect increasing time since the previous meal, given that serum insulin concentration peaks and declines rapidly after a meal (459).

Mouse studies demonstrate an important role for insulin in lactation, through increasing substrate uptake and expression of the key lactogenic transcription factors such as STAT5a and ELF5 (435), while states of insulin resistance are a known risk factor for delayed lactation onset and unsuccessful lactation in women (460, 461). We therefore assessed whether serum insulin concentrations differ between women with a normal BMI (<25) and women with a high BMI (≥25), and observed significantly greater insulin concentrations in women with a high BMI (figure 38). However, insulin concentrations vary markedly throughout the day and in relation to meals (459), something that is difficult to control for in the early post-partum period. We therefore used the leptin:adiponectin ratio as a stable measure of insulin resistance (443), and demonstrated a significantly higher ratio in women

with a high BMI. This confirms that women with increased BMI have greater insulin resistance in the post-partum period. It is notable that we found no significant correlation between BMI and the day on which milk came in. However, the number of obese women recruited to the study is small (n=12) and this warrants further evaluation as the number of study participants increase.

4.4.1.4. An early TSH rise is followed by a rise in free T4

Thyroid disorders are associated with reduced milk supply in women, while serum T3 and T4 are reported to correlate with human milk production (369, 462). Furthermore, rodent and bovine models of hypothyroidism exhibit lactation insufficiency (463, 464). Yet, despite the apparent role of thyroid hormones in lactation, there is no existing data on maternal thyroid hormone concentrations at lactation onset and this is something we have sought to address.

Our data demonstrates that serum TSH concentrations increase on days 1-2 post-partum (figure 40). This may reflect the loss of placental human chorionic gonadotrophin after delivery, which is known to stimulate TSH receptors and may therefore have decreased pituitary TSH secretion during pregnancy (465, 466). The rise in TSH is followed by a rise in free T4, but not free T3, on days 3-4 post-partum (figure 41). T4 has little biological activity and undergoes peripheral deiodination to the active hormone T3 (467). Deiodinase expression data from cows and rats demonstrates that the mammary gland increases its capacity to convert T4 to T3 in the post-partum period (468, 469). Thus, the systemic increase in free T4 but not free T3 that we have observed may promote intracellular thyroid hormone activity without inducing systemic hyperthyroidism. In the mammary context,

thyroid hormones appear to act with prolactin to prevent involution in rats, while thyroid hormones promote mitochondrial biogenesis in various tissues (438, 439). Increased mammary thyroid hormone activity may therefore help to prevent involution and drive mitochondrial changes needed for lactation, although further work is required to understand these effects.

4.4.1.5. Limitations

The INSIGHT study represents the largest study to-date that aims to systematically assess changes in lactation-relevant hormones during the early post-partum period (390). However, ongoing recruitment to increase the number of participants is essential to fully characterise hormonal changes at lactation onset, particularly given the intra- and inter-individual variation we have seen for several hormones. A larger cohort would also allow more comprehensive assessment of lactation hormones in participants at risk of delayed secretory activation such as primiparous women, women who have had prolonged labour, and those with comorbidities such as obesity, diabetes mellitus and polycystic ovaries syndrome that are associated with insulin resistance.

A number of hormones that we have evaluated are susceptible to maternal factors that we have not controlled for. Thus, both insulin and GH are influenced by changes in blood glucose and meal intake (449, 459, 470, 471). Cortisol, prolactin and GH show diurnal variation (472, 473), and are altered also by physical exertion (474). Multiple hormones, including cortisol, thyroid hormones, prolactin, GH and insulin may be influenced by maternal stress (475). Prolactin can form physiologically inactive complexes with

immunoglobulins in some individuals, called macroprolactin, which can elevate measured prolactin concentrations (476). Many of these factors are difficult to control for, given that we study mothers immediately after birth and study resources are finite. However, future work could consider incorporating meal and/or exercise diaries, self-reported stress scores, blood glucose measurements and macroprolactin measurements.

Another important limitation is the study population. The participants included represent women residing in Oxfordshire who are willing to participate in this study. As shown in table 9, the majority of women are of white ethnicity, >30 years of age, with a normal BMI and no comorbidities. The data obtained will therefore not be characteristic of all groups. Some groups may achieve adequate representation as the study expands, but it would be important to consider undertaking similar work elsewhere in the UK or abroad. An important consideration is that very few women included in the study had a delayed onset of lactation (after day 4 post-partum), and it is therefore very difficult to evaluate hormonal determinants of delayed lactation at this stage.

5. STUDY 2: INFLUENCE OF GROWTH HORMONE ON MAMMARY CELL METABOLISM

5.1. Background

Prolactin is essential for mammary development and milk production and often considered the principal lactogenic hormone (121). However, while the composition and volume of human milk vary considerably between women, rates of milk synthesis do not correlate with circulating prolactin concentrations (85, 421). Therefore, although prolactin is essential for lactation, other hormones may be important in dictating the amount of milk produced and the success of lactation, and one such candidate is GH.

Prolactin and GH are members of group I of the helix bundle peptide (HBP) hormones, alongside hPL (122). The genes encoding these hormones originated from a common ancestral gene by duplication and share sequence homology (477, 478). Binding of these hormones to their cognate receptors leads to activation of the Jak2-STAT5 pathway (422, 479). Phosphorylated STAT5 proteins then dimerise and translocate to the nucleus, where they act as transcriptional modulators, including of genes encoding milk proteins such as β-casein (480).

Through the Jak2-STAT5 pathway, GH induces transcription of IGF-1, which mediates many of the anabolic effects of GH (426). However, IGF-1-independent actions of GH have also been described (422). For example, GH signals via the Jak2 kinases, which phosphorylate Shc adaptor proteins, leading to activation of the Shc-grb2-SOS-Ras-Raf-MEK-ERK pathway (481). This pathway has a key role in the regulation of processes related to proliferation, differentiation and cell survival (482).

Furthermore, Jak2 induces tyrosyl phosphorylation of insulin receptor substrate 1 and 2 (483, 484), the first step in the PI3K-Akt pathway (485). Akt is a central mediator of cellular metabolism, promoting glucose uptake via GLUT transporters through inhibition of thioredoxin-interacting protein (TXNIP)-mediated endocytosis of GLUT1 (486). Akt also increases activity of the key glycolytic enzymes hexokinase 2 (HK2) and phosphofructokinase 1 (PFK1), as well as enhancing the production of intermediates for lipid, protein and nucleotide synthesis (487). GH therefore has the potential to promote glycolysis via growth hormone receptor (GHR)-Jak2 activation of the PI3K-Akt pathway (422, 488, 489).

Growth hormone may also influence mitochondrial function via the actions of Akt as this signalling factor is reported to promote mitochondrial biogenesis by activating the nuclear respiratory factor 1 (NRF1) transcription factor, which in turn increases expression of mitochondrial transcription factor A (mtTFA) (490). Akt also stimulates expression of a number of mitochondrial biogenesis genes via the cAMP response element-binding protein (CREB)/CREB-binding protein (CBP) pathway (491). In support of this, GH is reported to promote mitochondrial function and oxidative phosphorylation capacity in non-mammary tissues in both human and non-human settings (492-495).

Given these cellular effects (figure 42), GH represents a candidate mediator of the increased mammary synthetic and bioenergetic requirements seen at lactation onset (430). We therefore hypothesise that the increases in serum GH concentration that we have described across a breastfeeding episode may exert synthetic and/or metabolic effects on mammary cells.

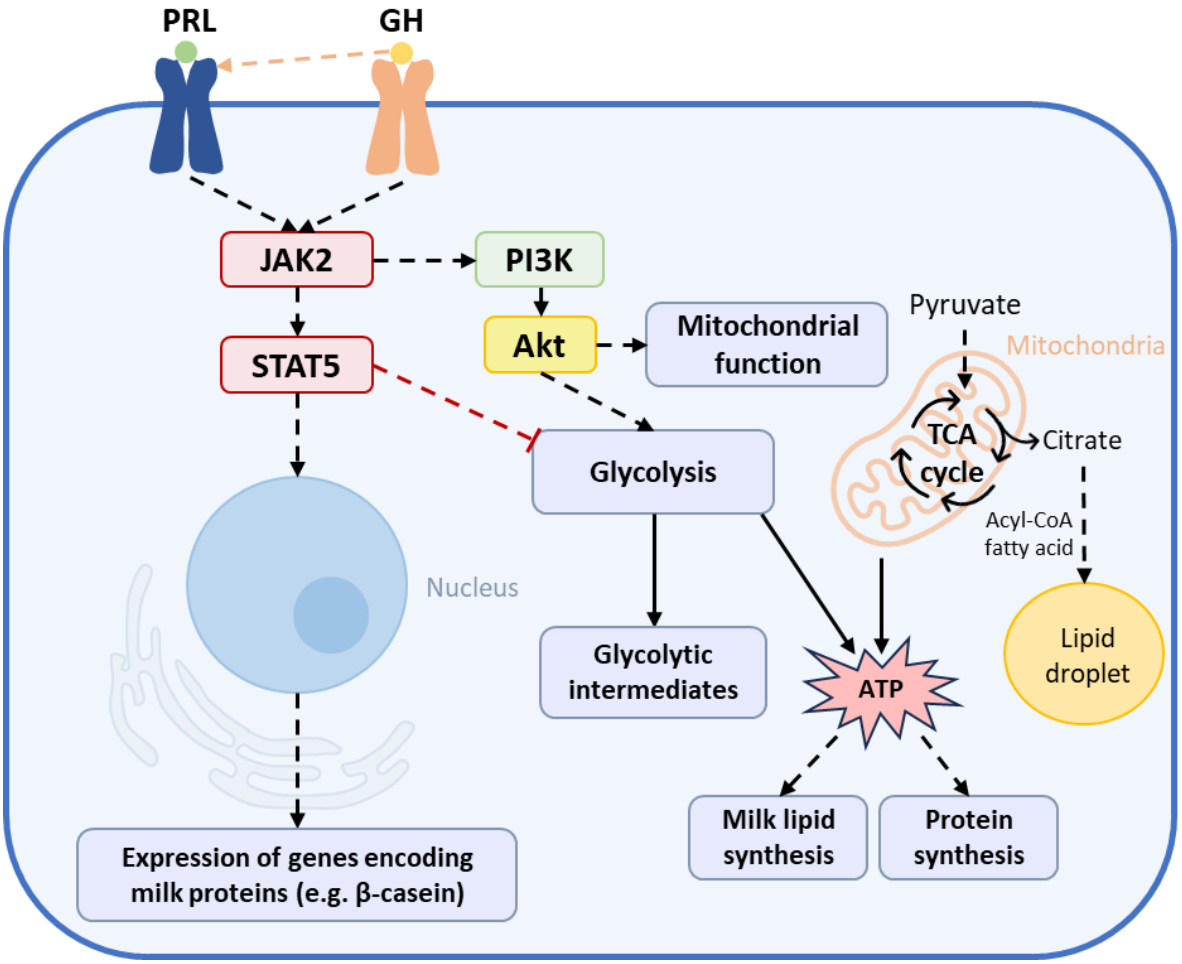


Figure 42. Schematic showing potential effects of growth hormone in mammary cells. GH binds and activates the growth hormone receptor (GHR) which, like prolactin (PRL), may signal through the Jak2-STAT5 pathway. GH can also bind the PRL receptor. STAT5 translocates to the nucleus and induces transcription of target genes, including milk components such as β-casein, while potentially mediating the inhibitory effect of GH on glycolysis. In addition, GHR, via Jak2, may activate the PI3K-Akt pathway. Akt is a central cellular metabolic regulator and its roles include promoting mitochondrial function, glucose uptake, glycolysis and lipid synthesis.

5.2. Aims

The aims of this chapter are to:

1. Evaluate mammary GH sensitivity (receptor expression) at lactation onset using human milk fat globule RNA.

2. Determine whether GH signals through STAT5 and/or Akt signalling pathways in cultured mammary cells.

3. Characterise the effects of GH in a lactogenic mammary cell model, specifically on:

    i. Synthesis of milk components (β-casein).

    ii. Cell metabolism (glycolysis and mitochondrial activity).

5.3. Results

5.3.1. Mammary cell growth hormone sensitivity at lactation onset

5.3.1.1. Growth hormone receptor expression at lactation onset

Serum hormone data from breastfeeding women demonstrates acute increases in GH concentrations following a breastfeeding episode (section 4.3.2.2). By contrast, prolactin concentrations, although consistently high, do not show such a marked post-feed rise. Given the overlap between GH and prolactin signalling, this supports a hypothesis that GH may augment the actions of prolactin between feeds. To investigate this further, we first sought to confirm human mammary epithelial cell receptor expression for both GH and prolactin at

the onset of lactation. RNA in milk fat globules is considered to be representative of gene expression in mammary epithelial cells (77), and was used for this analysis (section 3.3).

We isolated RNA from the lipid fraction of colostrum and milk and assessed expression of prolactin and growth hormone receptors from day 2 to day 5 post-partum (figure 43). GHR expression is greater than PRLR expression on all post-partum days assessed (p=0.01).

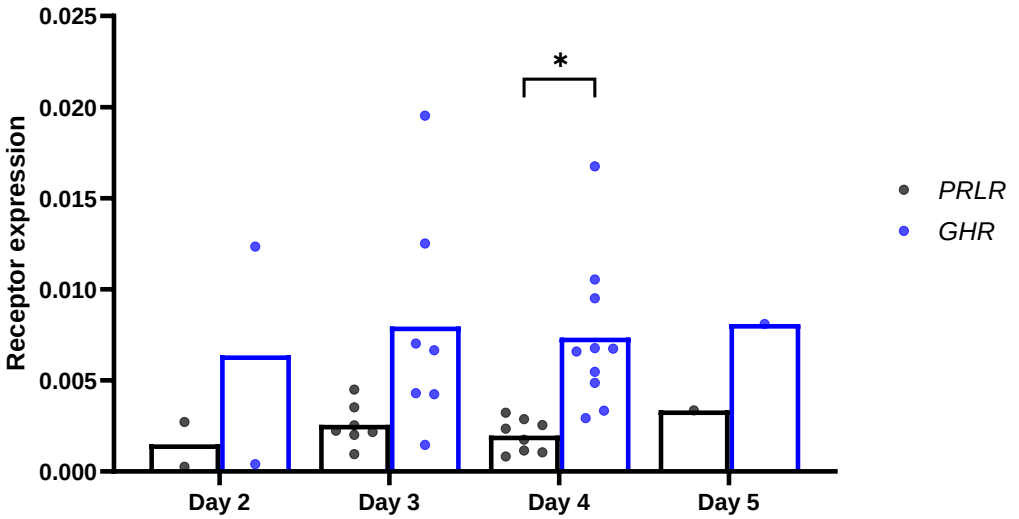


Figure 43. Prolactin receptor (PRLR) and growth hormone receptor (GHR) expression in milk fat globule RNA. *PRLR* expression in n=19 milk fat globule RNA samples (black) and *GHR* expression in n=20 milk fat globule RNA samples (blue) on days 2-5 post-partum. Individual data points are represented by filled circles, and bars represent the mean on each post-partum day. Outliers excluded using ROUT test. Data analysed using mixed-effects model; * $p<0.05$.

5.3.2. Signalling effects of growth hormone in mammary epithelial cells

5.3.2.1. GH-STAT5 signalling in HC11 cells

The increased mammary expression of GHR compared to PRLR supports a potential role for GH during secretory activation. We next assessed whether GH activates the STAT5 pathway in cultured mammary cells. We initially used the HC11 mouse mammary cell line, which exhibits lactogenic features such as β-casein expression, with appropriate hormone treatment (section 3.5) (395).

To assess the effect of GH on STAT5 phosphorylation, proliferating, confluent and differentiated HC11 cells were treated with 1 μg/mL recombinant human GH (rhGH) for five minutes. Cell lysates from control and treated cells were obtained and evaluated for phosphorylated STAT5 (pSTAT5) using capillary protein electrophoresis (section 3.6).

Relative to untreated cells, rhGH induced a robust STAT5 phosphorylation at all HC11 cell stages (figure 44). However, the degree of phosphorylation was greatest in cells that had undergone differentiation to a lactogenic (β-casein expressing) phenotype, with a greater than 400-fold increase in STAT5 phosphorylation relative to control cells. This suggests that GH may induce the greatest activation of the STAT5 pathway in lactogenic cells.

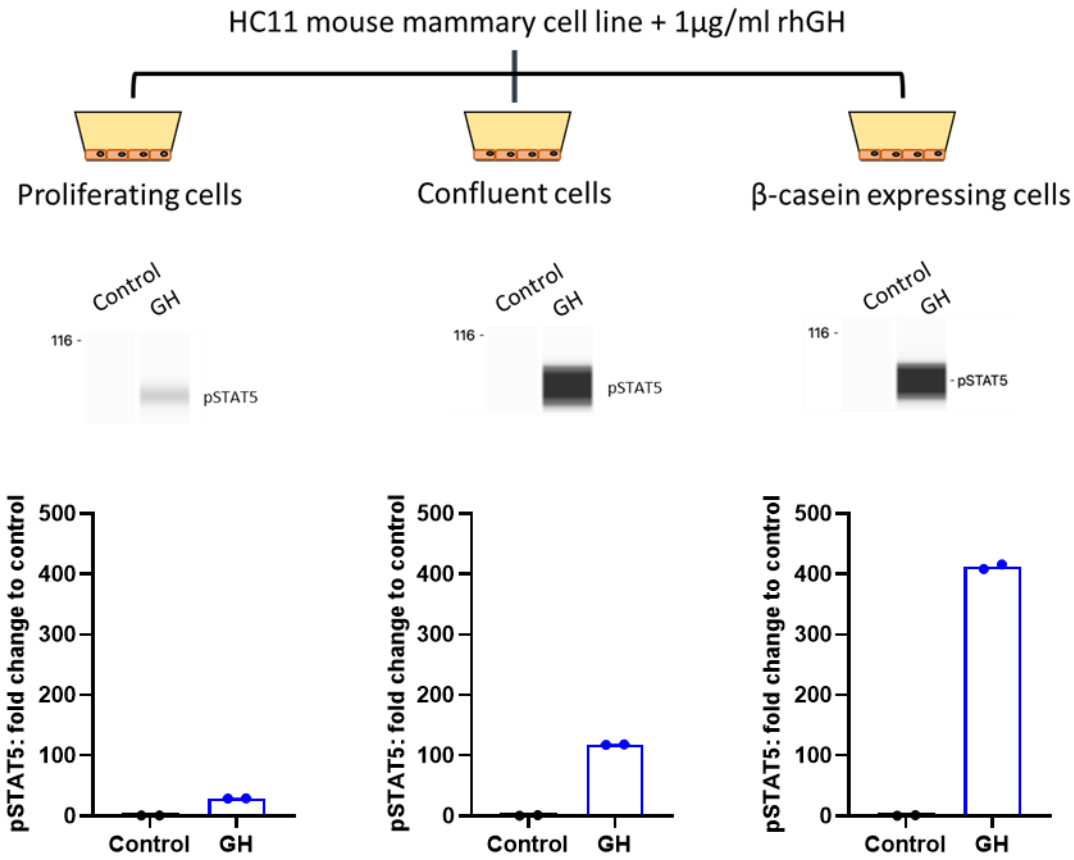
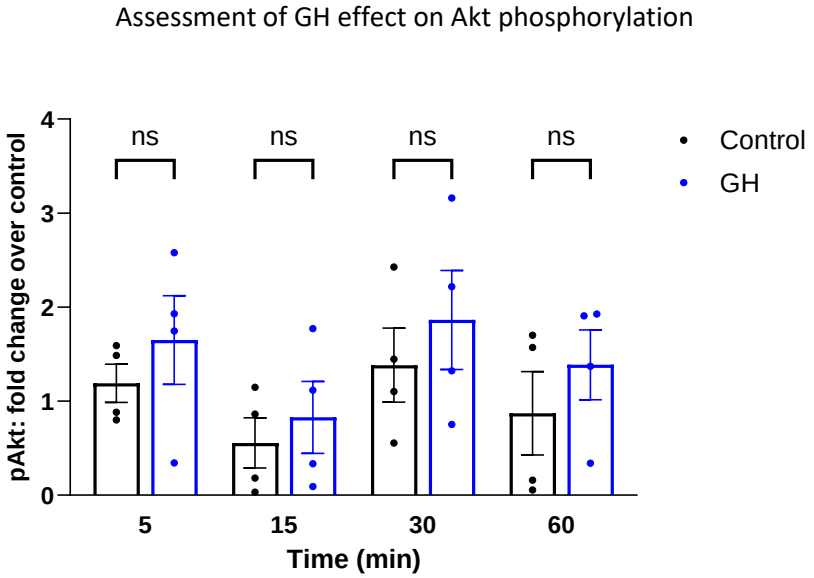


Figure 44. STAT5 phosphorylation in response to growth hormone in HC11 cells. Cultured HC11 cells at three stages – proliferating (left), confluent (centre), and differentiated to a β-casein expressing phenotype (right) – treated with 1 µg/mL recombinant human GH (rhGH). STAT5 phosphorylation is assessed by capillary protein electrophoresis, with representative virtual blots shown above each graph. STAT5 phosphorylation was evaluated in control and GH-treated cells for n=2 replicates, with pSTAT5 shown as fold change relative to the matched control.

By contrast, GH stimulation of HC11 cells for 5, 15, 30 or 60 minutes did not induce phosphorylation of Akt (control versus GH-treated cells with n=4 each time point, figure 45). Activation of the PI3K-Akt pathway is therefore unlikely to mediate effects of GH in the mammary context.

(A)



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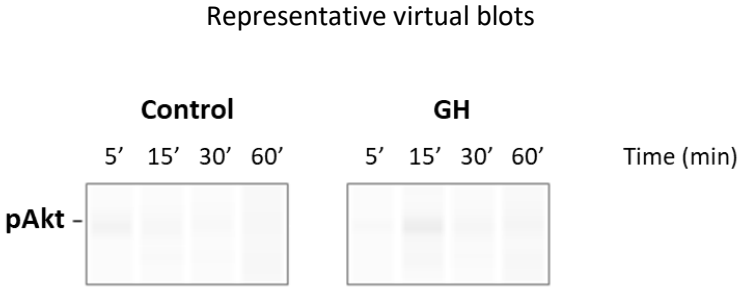


Figure 45. Effects of GH on Akt phosphorylation in confluent HC11 cells. (A) Effects of 1 µg/mL recombinant human GH (rhGH) on Akt phosphorylation, assessed following 5, 15, 30 and 60 minutes GH stimulation. Plotted as mean ± SEM for n=4 replicates. (B) Representative virtual blots for pAkt (n=1 replicate per condition shown). The molecular weight of Akt is approximately 60 kDa. Data analysed using two-way ANOVA with Sidak's multiple comparisons test; ns, non-significant.

5.3.2.2. GH-STAT5 signalling in human mammary cells

Given HC11 cells are a non-human mammary cell model, we also investigated whether GH signals through the STAT5 pathway in primary hMECs. Although not expressing milk components, these cells provide a means to assess GH-mediated mammary activation of the Jak2-STAT5 pathway in the human setting. Initial experiments evaluating treatment of confluent hMECs with rhGH demonstrated no STAT5 phosphorylation in these cells, unlike in HC11 cells (figure 46).

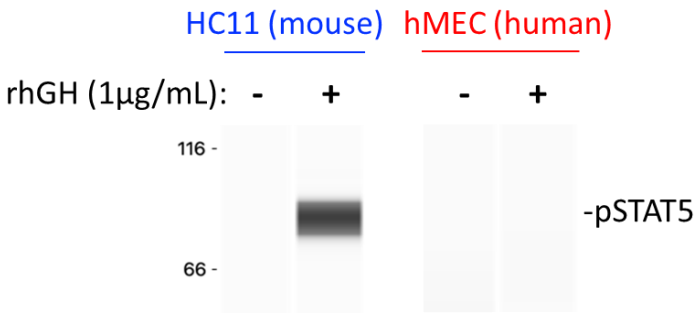


Figure 46. STAT5 phosphorylation in response to rhGH treatment in HC11 cells and hMECs. STAT5 phosphorylation in HC11 mouse mammary cells and human mammary epithelial cells (hMECs), in the presence (+) or absence (-) of 1 µg/mL rhGH.

We next assessed whether hMECs express GHR by qRT-PCR. This showed low expression of GHR compared to other metabolic hormone receptors (figure 47).

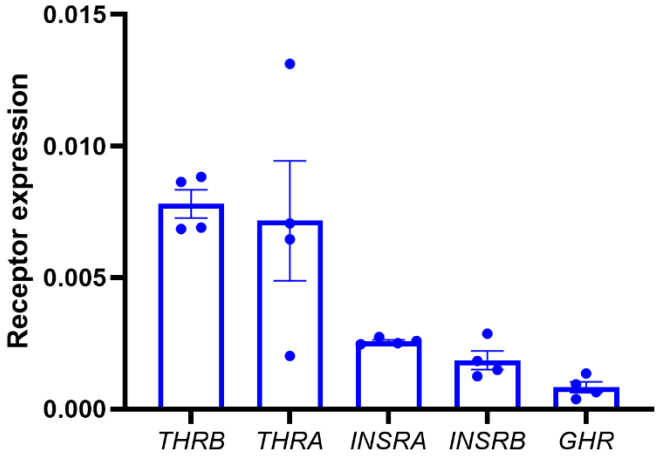


Figure 47. Expression of metabolic hormone receptors in hMECs. Expression of thyroid hormone receptor isoforms A and B (THRA and THRB), insulin receptor isoforms (INSRA and INSRB) and the growth hormone receptor, in n=4 hMEC batches. Data shown as mean ± SEM, with filled circles representing individual data points.

We postulated that this may be due to commercially obtained hMECs being obtained from non-lactating benign breast tissue, which may contain undifferentiated or progenitor mammary epithelial cells. We assessed the mammary cell subtypes present in commercially available hMECs by flow cytometry using antibodies against epithelial cell adhesion molecule (EpCAM), an epithelial marker, and CD49f, a stem cell marker, as reported (496). This approach categorises mammary cells into: mature luminal cells; progenitor luminal cells, stromal cells and basal and mammary stem cells. Flow cytometry conducted by Dr Elajnaf (post-doc) showed that most hMECs have a luminal progenitor phenotype (figure 48).

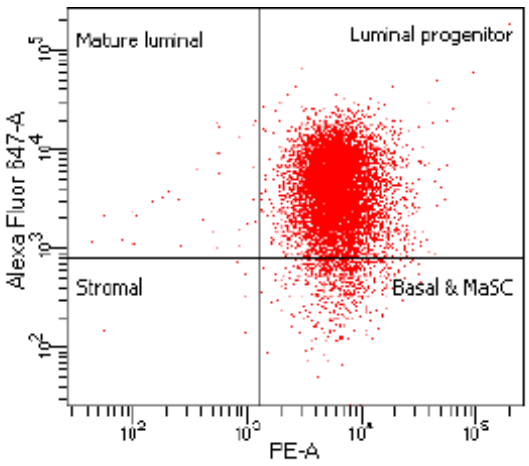
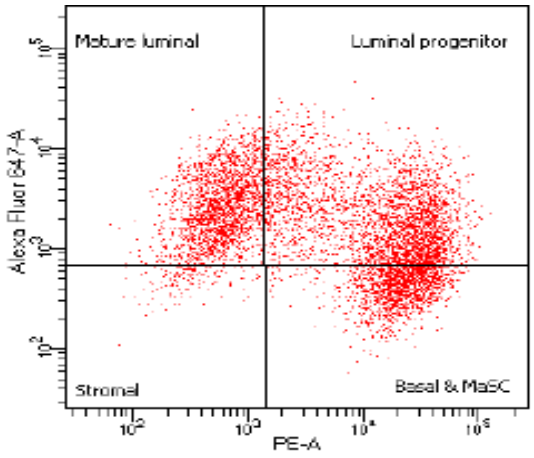


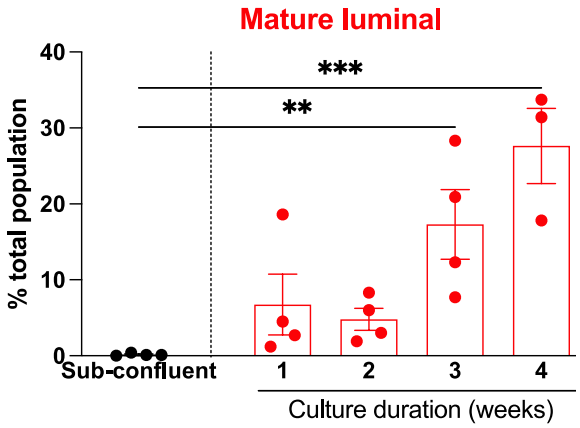
Figure 48. Flow cytometric analysis of cell populations in cultured hMECs. Representative flow cytometric analysis of sub-confluent hMECs (Lonza lot 22TL115626). Cells are stained with anti-EpCAM-AF647 and anti-CD49f-PE to enable separation into mature luminal, luminal progenitor, stromal and basal/mammary stem cell (MaSC).

We next investigated whether prolonged culturing of hMECs may facilitate differentiation of luminal progenitor cells to mature cells. Cells were cultured over a 4-week period, with flow cytometry performed at weekly intervals. This demonstrated that the proportion of mature luminal cells increased after 2 weeks of culture and at 4 weeks accounted for ~30% of all hMECs, while the proportion of progenitor luminal cells decreased (figure 49).

(A)



(B)



(C)

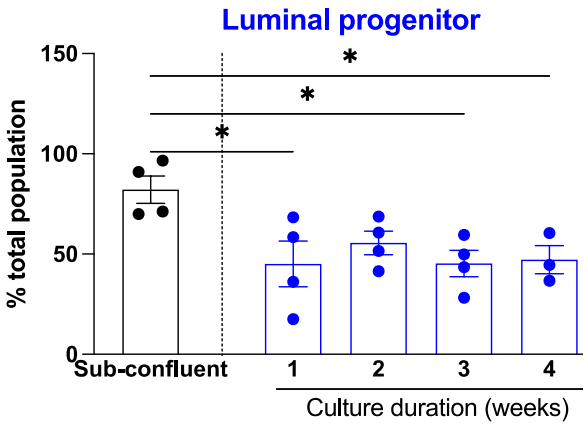


Figure 49. Flow cytometric analysis of cell populations in cultured hMECs. Representative flow cytometric analysis of week 4 post-confluent hMECs (Lonza lot 22TL115626) (A). Cells are stained with anti-EpCAM-AF647 and anti-CD49f-PE to enable separation into mature luminal, luminal progenitor, stromal and basal/mammary stem cell (MaSC). (B) Proportion of mature luminal and (C) luminal progenitor cells in sub-confluent hMECs and hMECs. Data shown for n=4 hMEC batches. Analysis performed using one-way ANOVA; * p<0.05, ** p<0.01, *** p<0.001.

Having established an increase in mature luminal cells with increasing culture duration, we next evaluated whether this led to an increase in *GHR* expression. RNA from sub-confluent hMECs was compared with that from hMECs at weeks 1-4 post-confluency (figure 50). From this work, it is evident that *GHR* expression is low in all four hMEC batches used when cells

are sub-confluent, but expression is increased in weeks 1-3 post-confluency, and then may decrease by week 4. This suggests that cells may initially be poorly responsive to GH but their *GHR* expression, and therefore GH sensitivity, increases as the proportion of mature luminal cells increases.

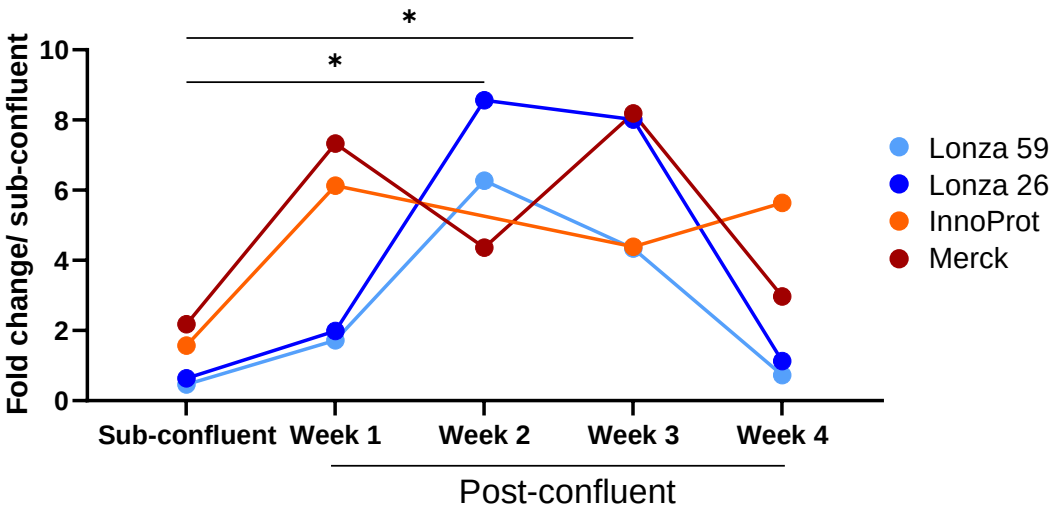


Figure 50. Growth hormone receptor (GHR) expression in hMECs at different culture durations. GHR expression in sub-confluent hMECs and cells at weeks 1-4 post-confluency, using n=4 cell batches (n=2 from Lonza, n=1 from InnoProt, n=1 from Merck). Data shown as fold-change relative to the mean of sub-confluent cells. Differences between time points analysed using one-way ANOVA; * $p<0.05$.

After confirming increasing *GHR* expression with increased culture duration, we next evaluated whether this correlated with a greater GH-induced STAT5 phosphorylation (figure 51). As shown in figure 51, STAT5 phosphorylation in response to rhGH treatment was consistently poor in sub-confluent cells. Prolonged culture increased the GH-mediated STAT5 response, which was greatest at week 4 post-confluency. These findings therefore

confirm that primary hMECs are capable of STAT5 activation following GH treatment, but only when cultured in a manner that selects for mature luminal cells.

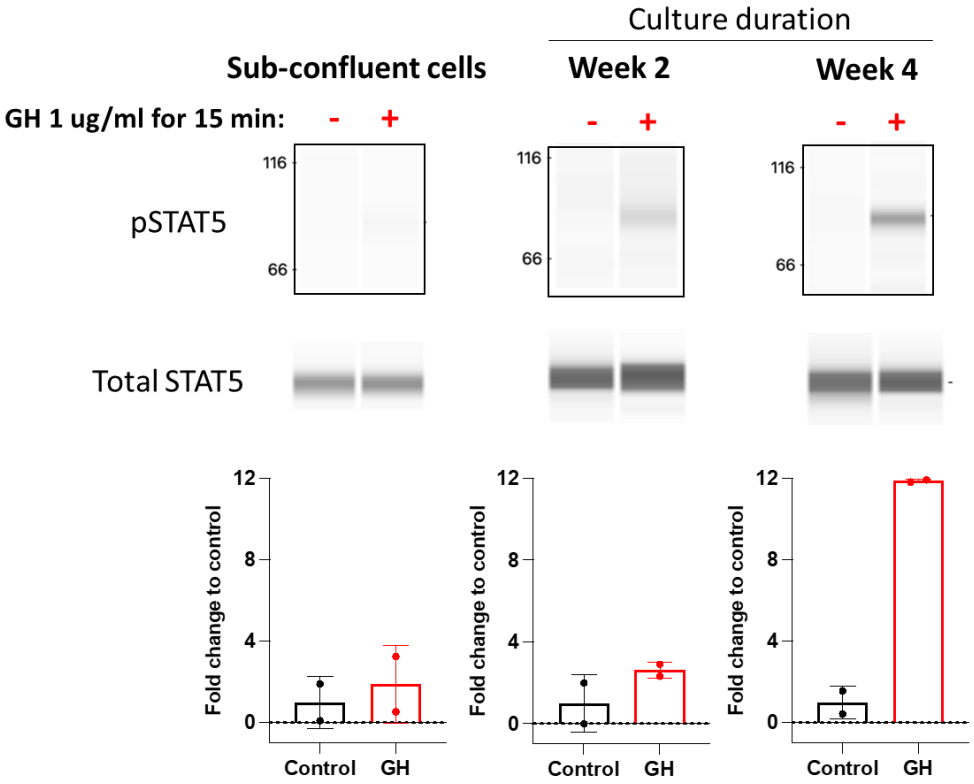


Figure 51. STAT5 phosphorylation in response to GH in hMECs. STAT5 phosphorylation in sub-confluent hMECs and cells at weeks 2 and 4 post-confluency, treated with or without 1 µg/mL rhGH. pSTAT5 and total STAT5 were assessed using capillary protein electrophoresis and representative virtual blots are shown. Column graphs are shown for n=2 replicates, with columns representing the mean pSTAT5/total STAT5 relative to the mean of control cells.

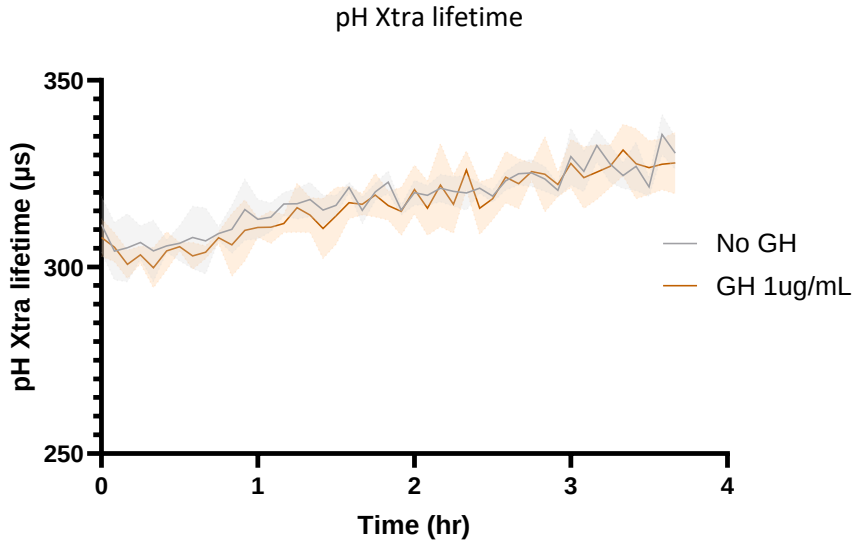
5.3.3. Metabolic effects of growth hormone in mammary epithelial cells

5.3.3.1. Effects of growth hormone on glycolysis

The initial steps of glycolysis are inhibited at the onset of lactation, which likely partition glucose towards lactose synthesis (108). Mouse studies in non-mammary tissues demonstrate an inhibitory effect of GH on glycolysis, through a reduction in expression of the rate-limiting glycolytic enzyme glucokinase (497, 498). Inhibition of glycolysis supports an anabolic role for GH and, in the mammary context, may increase availability of glucose for lactose synthesis. We therefore sought to assess whether GH inhibits glycolysis in HC11 cells with a lactogenic phenotype, through assessment of basal glycolysis and glycolytic capacity (section 3.9).

Differentiated HC11 cells were treated with 1µg/mL rhGH for 24 hours prior to being assayed, which is in keeping with reported *in vitro* work which has demonstrated an effect of GH on transcription and glycolysis (493, 499), and maintained in rhGH-containing buffer during the assay. As shown in figure 52, HC11 cells utilise glycolysis at baseline but GH does not alter the rate of basal glycolysis.

(A)



(B)

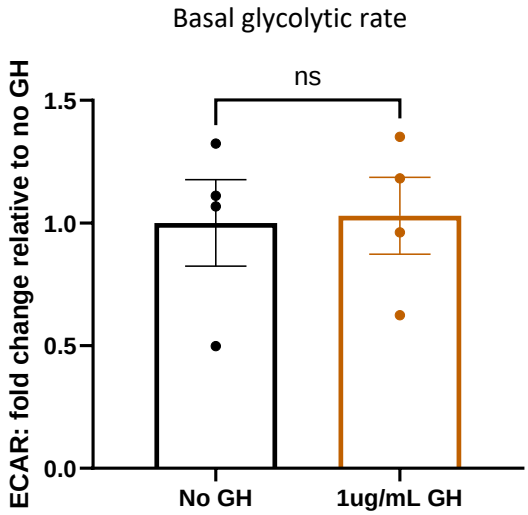
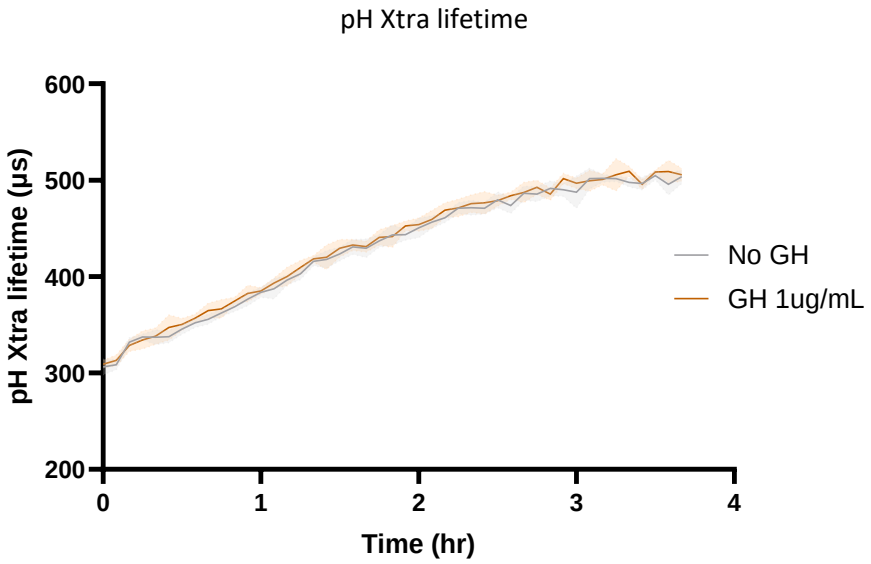


Figure 52. Effect of GH on basal glycolytic rate in lactogenic HC11 cells. (A) pH Xtra lifetime in lactogenic HC11 cells treated with (orange) or without (grey) GH 1µg/mL, shown as mean ± SEM for 4 replicates. (B) Extracellular acidification rate (ECAR) determined over recording period, plotted as mean ± SEM. Data analysed using unpaired t-test; ns, non-significant.

Furthermore, 1µg/mL GH does not alter the rate of maximal glycolysis or glycolytic capacity (figure 53). These results therefore show that GH treatment over 24 hours does not affect glycolysis in HC11 cells with a lactogenic phenotype.

(A)



(B)

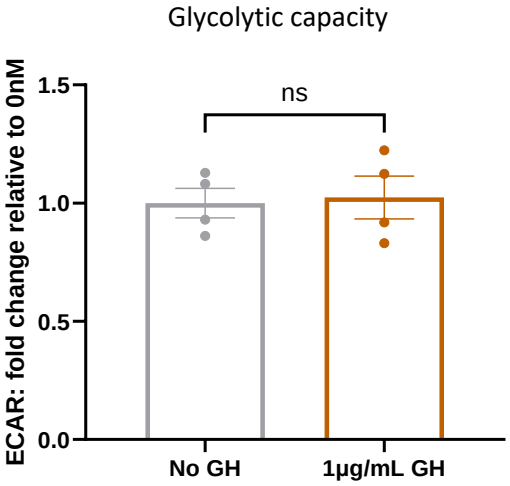


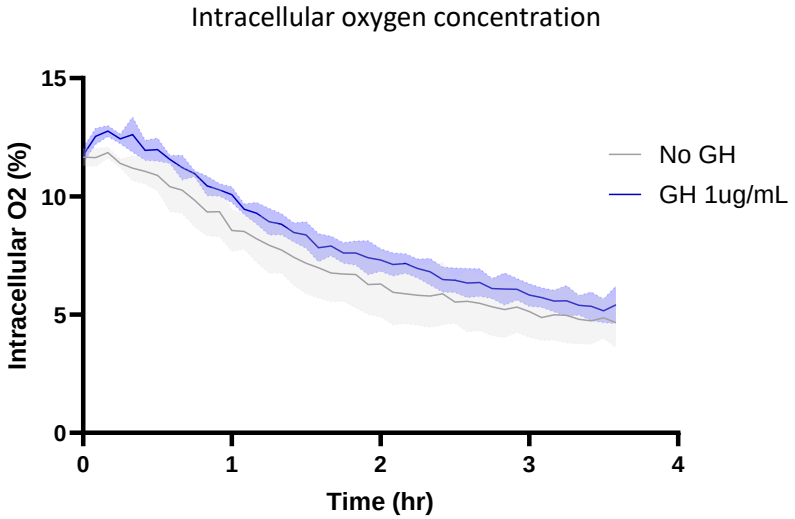
Figure 53. Effect of GH on glycolytic capacity in lactogenic HC11 cells. (A) pH Xtra lifetime in lactogenic HC11 cells treated with (orange) or without (grey) GH 1µg/mL, shown as mean ± SEM for 4 replicates, after addition of oligomycin. (B) Glycolytic capacity, calculated as ECAR following oligomycin addition minus ECAR following 2-deoxyglycose addition, between 0 and 70 minutes, plotted as mean ± SEM. Data analysed using unpaired t-test; ns, non-significant.

5.3.3.2. Effects of growth hormone on oxidative phosphorylation

Milk production is energy-consuming, yet the hormonal drivers underlying mammary bioenergetics remain poorly understood. GH promotes mitochondrial function and oxidative capacity in myocytes, macrophages and oocytes in both human and non-human settings (492-495), although the signalling pathways producing these changes are uncertain. GH infusion in humans leads to increased ATP production, increased expression of mitochondrial proteins, and mitochondrial oxidative capacity in muscle (495). This makes GH a candidate mediator of the mitochondrial changes reported at the onset of lactation (92, 93). We therefore assessed the effects of GH on oxygen consumption, which is an indicator of mitochondrial oxidative phosphorylation, in HC11 mammary cells with a lactogenic phenotype (section 3.8).

As shown in figure 54, 24 hours of treatment with 1µg/mL GH does not alter OCR at baseline, indicating that GH does not promote basal oxygen consumption. This suggests that GH is not directly stimulating mitochondria, nor indirectly increasing mitochondrial activity by activating ATP-consuming metabolic processes in cells.

(A)



(B)

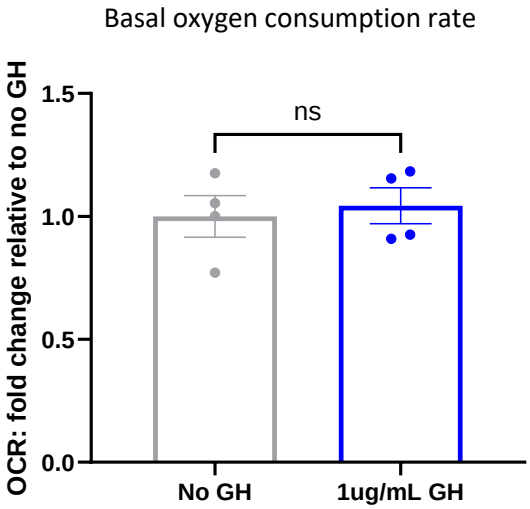
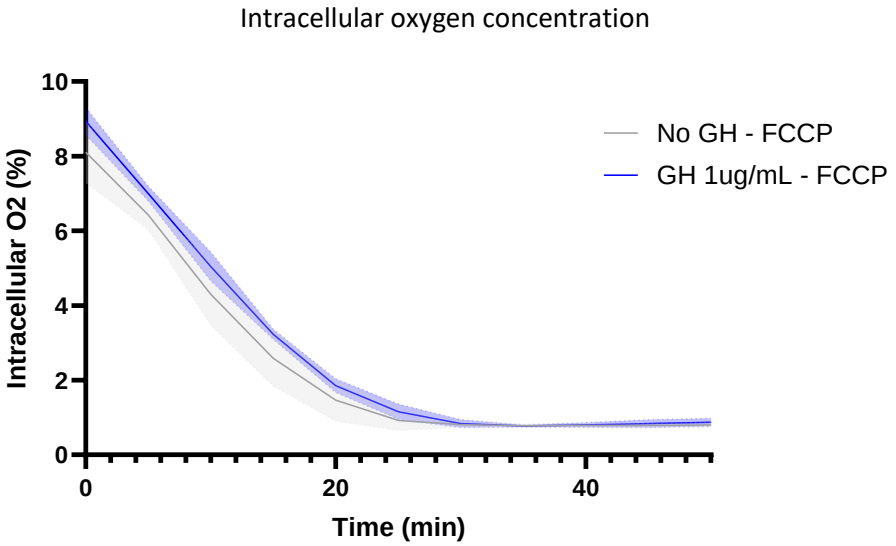


Figure 54. Effect of GH on basal respiration in lactogenic HC11 cells. (A) Calculated intracellular oxygen (%) in lactogenic HC11 cells treated with (blue) or without (grey) 1µg/mL GH, shown as mean ± SEM for 4 replicates. (B) Basal oxygen consumption rate (OCR) determined over recording period, plotted as mean ± SEM. Data analysed using unpaired t-test; ns, non-significant.

I next assessed whether GH influences maximal oxygen consumption, which was induced by the addition of FCCP, a mitochondrial uncoupling agent (section 3.8). Figure 55 demonstrates that GH also does not alter maximal oxygen consumption in HC11 cells with a

lactogenic phenotype. Therefore, GH treatment does not alter basal or maximal oxidative phosphorylation, at the concentration and duration assessed, in HC11 mammary cells.

(A)



(B)

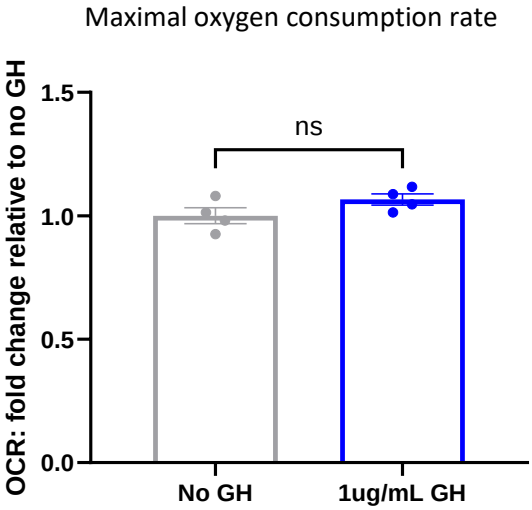


Figure 55. Effect of GH on maximal oxygen consumption in lactogenic HC11 cells. (A) Calculated intracellular oxygen (%) in lactogenic HC11 cells treated with (blue) or without (grey) GH 1µg/mL, shown as mean ± SEM for 4 replicates, after addition of FCCP. (B) Maximal oxygen consumption rate (OCR) determined between 0 and 20 minutes, plotted as mean ± SEM. Data analysed using unpaired t-test; ns, non-significant.

5.3.4. Transcriptomic assessment of growth hormone in mammary epithelial cells

5.3.4.1. Quality assessment of transcriptomic data

To further evaluate the cellular mechanisms by which GH might act on mammary epithelial cells, transcriptomic analysis of RNA obtained from GH-treated HC11 cells was undertaken (section 3.11). HC11 cells that had gone lactogenic differentiation were treated with or without 1 μg/mL rhGH and extracted at the following time points: 5 minutes, 30 minutes, 2 hours and 8 hours (n=5 per condition).

Extracted RNA was sent to Azenta Life Sciences for RNASeq analysis. Data was returned in the form of raw counts, which reflects the number of transcripts obtained from a single gene. Raw counts were normalised to counts per million (CPM), and a comparison of log_2-transformed CPM was used to ensure comparability between samples. As shown in figure 56, the median counts across all conditions are similar (log_2-transformed median count 5.14).

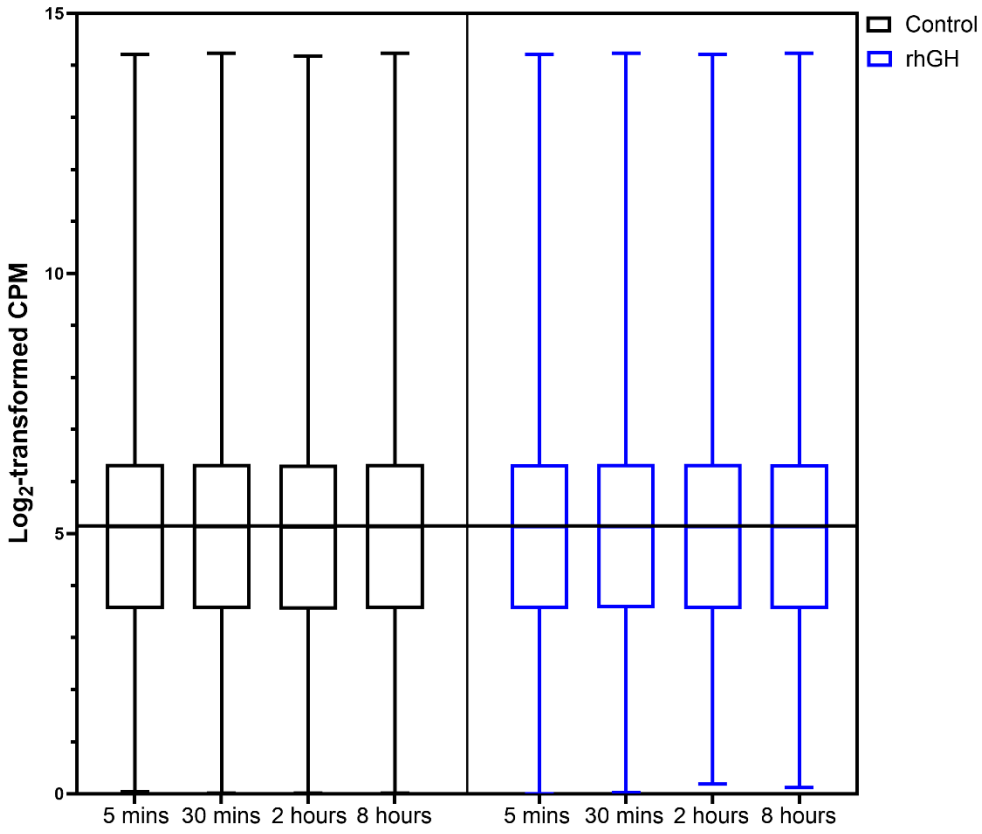


Figure 56. Counts in GH-treated and control HC11 cells. Log_2 transformed counts for control (left-hand side, white) and rhGH-treated (right-hand side, yellow) HC11 cells. Median, 25th and 75th centiles, and range plotted.

In addition, the RNA integrity number (RIN) provides an estimation of the integrity of RNA obtained. This ranges from 1-10, with 10 representing samples with the best integrity, while a RIN of >8 is considered acceptable for use. All RIN values were greater than 9.5 (figure 57).

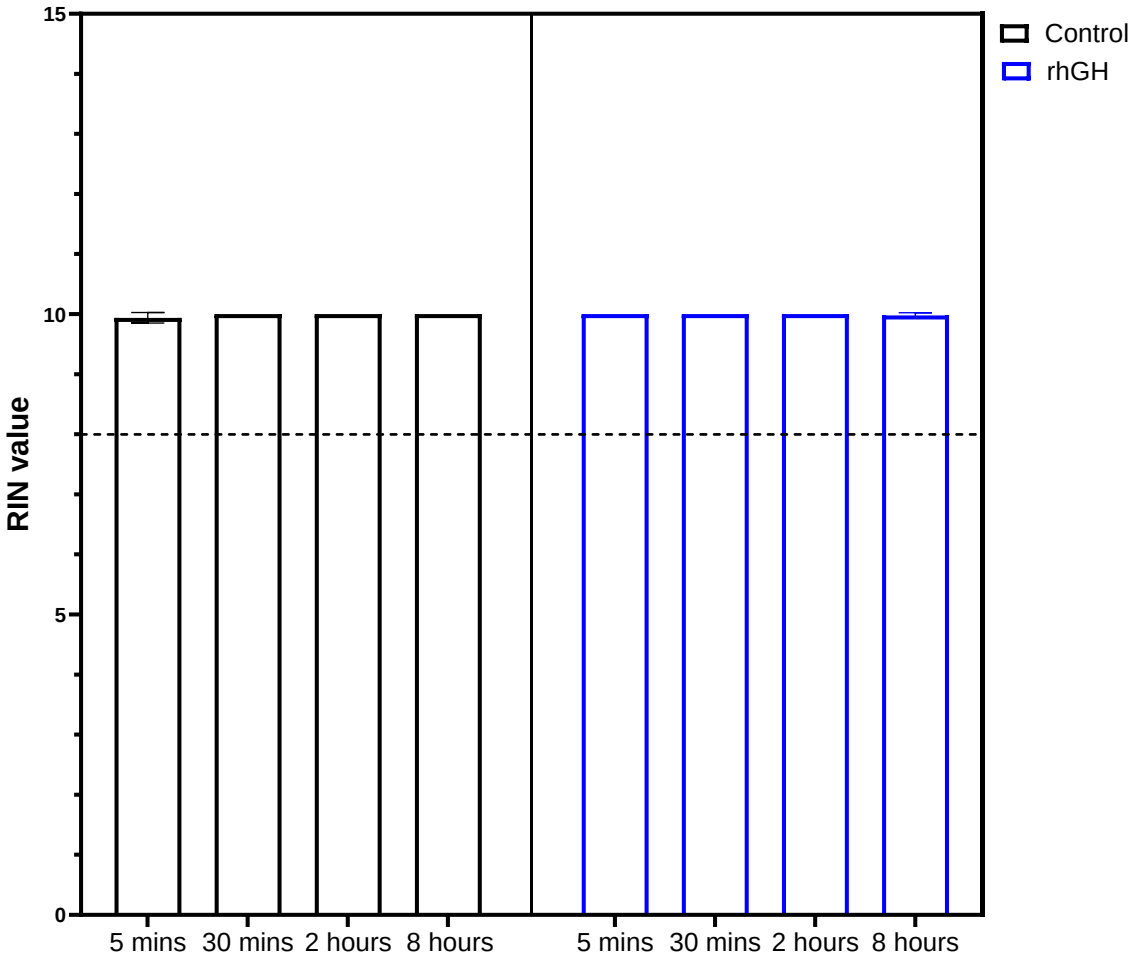


Figure 57. RIN values in GH-treated and control HC11 cells. RIN values for control (left-hand side, black) and rhGH-treated (right-hand side, blue) HC11 cells. Data shown as mean ± SD. The dashed horizontal line marks a RIN value of 8, above which RNA is generally considered of good quality.

In order to provide an initial evaluation of similarity between samples from the same group, as well as to compare different conditions, cluster analysis was undertaken with a PCA plot (figure 58). This demonstrates that samples from the same condition broadly cluster together, apart from one control sample at 5 minutes, one rhGH sample at 2 hours, and one rhGH and one control sample at 8 hours. There is separation of control and rhGH groups at 5 minutes, 30 minutes and 8 hours.

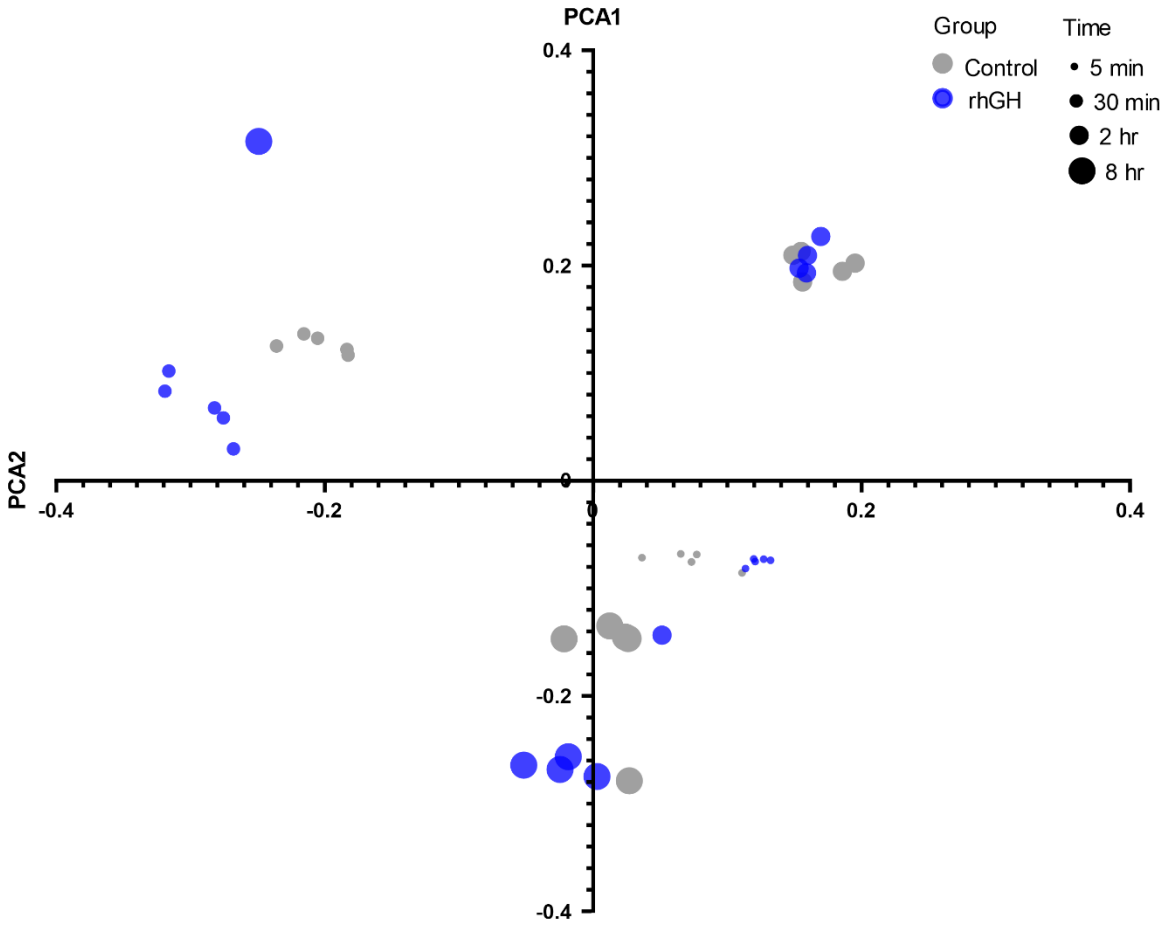


Figure 58. Cluster analysis of GH-treated and control HC11 cells. PCA plot of rhGH-treated (blue circles) and control (grey circles) HC11 cells, according to time point of extraction (5 minutes, 30 minutes, 2 hours, 8 hours). Each circle represents an individual sample.

The number of DEGs upregulated and downregulated at each extraction time point is summarised in figure 59. This demonstrates a time-dependent increase in the number of DEGs, with n=0 DEGs at 5 minutes, n=5 DEGs at 30 minutes, n=45 DEGs at 2 hours and n=60 DEGs at 8 hours. The majority of DEGs are upregulated genes in response to GH treatment.

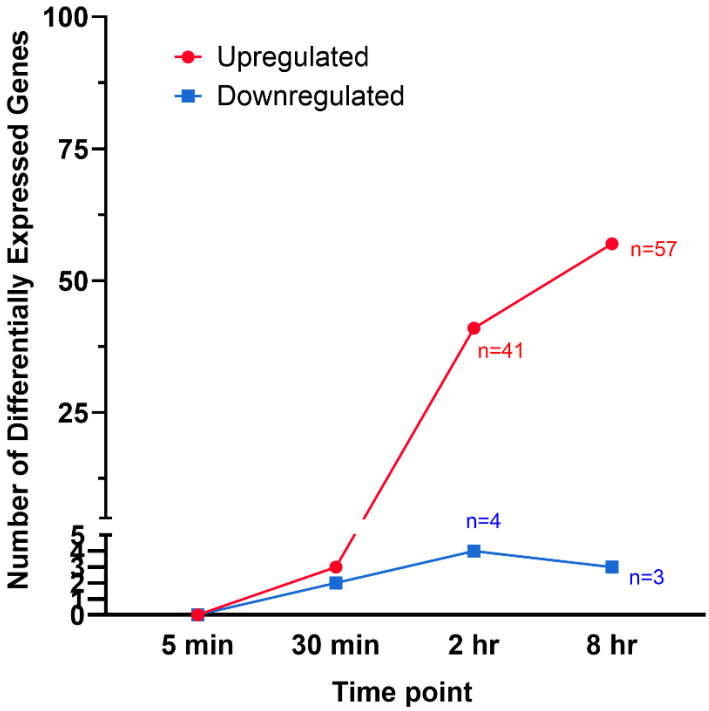


Figure 59. Summary of number of DEGs at each extraction time point. Numbers of significantly downregulated (blue) and upregulated (red) genes in rhGH-treated compared to control cells.

To visualise which biological pathways are affected by GH, gene set enrichment was performed using databases including Hallmark, MSigDB, KEGG and GO. For gene set enrichment analysis (GSEA), gene sets with a false discovery rate (FDR) <0.05 were selected. This identified n=2 gene sets upregulated at 2 hours and n=7 gene sets upregulated at 8 hours. No gene sets were significantly downregulated.

5.3.4.2. Metabolic genes and processes altered by GH

The gene sets significantly changed by GH at 8 hours are plotted in figure 60.

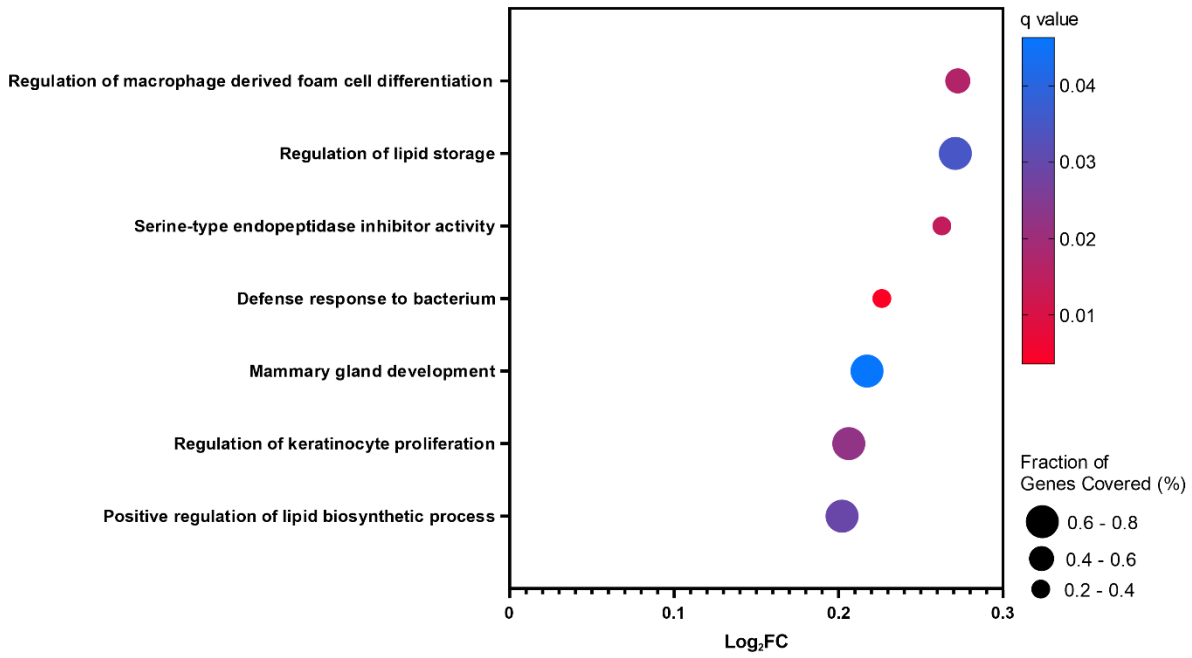


Figure 60. Differentially expressed gene sets at 8 hours. Gene sets arranged according to the log_2-fold change. The colour of each circle represents the q value, while its size corresponds to the fraction of total genes covered by that gene set.

We focused on the metabolic changes induced by GH. Of the significantly changed pathways, 2 out of 7 relate to cellular lipid handing (figure 60). Upregulated genes relevant to this process include that encoding lipoprotein lipase (3.8-fold increase), which mediates hydrolysis of triglycerides in plasma lipoproteins, enabling uptake of fatty acids into cells (500). Furthermore, there is upregulated expression of fatty acid desaturase 1 and 2 (2.6-fold and 2.1-fold, respectively), which convert saturated to unsaturated fatty acids (501). There is also increased expression of lipin 1 (3.2-fold), an enzyme that catalyses the penultimate step in triglyceride formation (502). We also observed increased expression of *CSN1S1* and *CSN2* (5.6-fold and 3.3-fold, respectively), which encode the α-casein S1 and β-casein milk proteins, with *CSN1S1* being the most upregulated gene in response to rhGH treatment. A heatmap of these changes is shown in figure S1(A) (appendix 4).

5.4. Discussion

My clinical findings demonstrate that serum GH concentrations increase in women both in the early post-partum period and during breastfeeding. I also confirmed that GH signals through the STAT5 pathway in cultured mammary cells, and promotes transcription of genes important for lipid uptake and synthesis, as well as for milk protein synthesis, in lactogenically differentiated HC11 cells. By contrast, GH does not signal through the Akt pathway in HC11 cells nor alter cell metabolism. These findings suggest a predominantly synthetic role for GH in mammary cells during lactation.

5.4.1. Novel post-breast feed increases in growth hormone suggest a role in lactation

Evidence for a role for GH in milk production comes from observational studies in non-pregnant women and animals with GH excess. Acromegaly, caused by GH-secreting pituitary tumours, has long been known to induce milk release in non-pregnant women (374). Transgenic female mice that overexpress human GH produce milk and can successfully raise foster litters to weaning without having been pregnant (375). Moreover, there is data from interventional studies to support the role of GH. Injection of GH releasing factor into cows stimulates milk production (376). In addition, GH injection into healthy women or women of pre-term infants increases milk volume (377, 378). It is, however, notable that studies of GH in breastfeeding women are small in size and not all studies confirm a role for GH in milk production (379).

There is a paucity of existing data assessing GH concentrations in women in the early post-partum period. Existing data has demonstrated that pituitary GH production is suppressed

in late pregnancy, increases after delivery, but is not maximal until the late post-partum period (424). This is in keeping with our data, which shows GH suppression in late pregnancy followed by increased concentrations on days 1-4 post-partum. Furthermore, our study describes novel increases in serum GH concentrations during a breastfeeding episode (figure 32), which suggests that GH may have a role in lactation during the early post-partum period.

Prolactin is necessary for lactation in humans and other species, and its concentration is approximately an order of magnitude higher in the early post-partum period compared to non-pregnant, non-lactating women (161, 351). However, concentrations of prolactin do not increase substantially during breastfeeding on days 1-4 post-partum (figure 29). By contrast, GH concentrations on days 1-4 post-partum are similar to those in non-pregnant, non-lactating women (503). However, GH concentrations increased during breastfeeding in some women. These findings suggest that a consistently high prolactin concentration is required for lactation, while GH changes during breastfeeding may have a role in augmenting milk production.

5.4.2. Lactating human mammary cells express the growth hormone receptor

Evaluating hormone receptor expression in human mammary epithelial cells at lactation onset is limited by the inability to obtain the cells of interest, namely *in situ* luminal mammary epithelial cells. Mammary cells are shed into milk but their phenotype is likely to change during this process (504). Furthermore, milk is composed of a number of different cell types, many of which are non-epithelial (385). This limits the use of cells in milk as a

means of understanding gene expression in human mammary epithelial cells. RNA in milk fat globules, however, is considered to be representative of gene expression in lactating mammary epithelial cells and is well validated (77). This is because milk fat globules excreted from epithelial cells also contain cytoplasm abundant in mammary cell RNA. Analysis of gene expression in milk fat globule RNA confirms that mammary *GHR* is expressed in the early post-partum period, and is more abundant than the prolactin receptor (figure 43). GH is also capable of activating the prolactin receptor (423) and this may be another mechanism by which GH influences mammary epithelial cells.

5.4.3. Evaluation of GH-induced milk component synthesis in cultured mammary cells

Growth hormone signals via the Jak2-STAT5 pathway (479), which is the major pathway by which prolactin promotes milk protein synthesis (505). We therefore hypothesised that GH induces expression of β-casein, a major milk protein expressed in mammary epithelial cells.

In order to consider how GH might signal and act in lactating mammary epithelial cells, an appropriate cellular model of lactation is required. Our aim is to better understand human lactation biology, yet primary human mammary epithelial cells (hMECs) obtained from benign, non-pregnant and non-lactating tissue are difficult to differentiate *in vitro* (388). While human mammary epithelial cell lines exist, these are challenging to differentiate into cells with a lactogenic phenotype and have lost many of the features of primary cells (387, 388). We therefore used the HC11 mouse mammary cell line. This cell line is well-established to acquire lactogenic features, such as β-casein expression, with appropriate hormone treatment (395). Their phenotype can be studied at various stages relevant to the

mammary gland such as proliferation and differentiation to a milk protein expressing phenotype.

We utilised these cells to confirm that GH promotes STAT5 phosphorylation, with the response being greatest in β-casein-expressing cells (figure 44). This suggests that differentiation to a lactogenic phenotype increases sensitivity to GH.

Through a transcriptomic assessment of GH-treated β-casein-expressing HC11 cells, we have shown that GH increases the expression of genes mediating lipid uptake and synthesis. Thus, GH increases expression of lipoprotein lipase, which hydrolyses plasma triglycerides and allows uptake of fatty acids into mammary epithelial cells (500). Furthermore, there is upregulated expression of fatty acid desaturases, which convert saturated fatty acids to unsaturated fatty acids (501). This is important for milk synthesis, as approximately half of the fatty acids in milk are unsaturated (506). There is also increased expression of the gene encoding lipin 1 (3.2-fold), an enzyme that catalyses the penultimate step in triglyceride formation (502). These findings are in keeping with known GH-mediated increases in expression of fatty acid desaturases and lipin 1 in non-pregnant, non-lactating rodent adipose tissue and liver, while effects on lipoprotein lipase are tissue-dependent (507-510). Such mammary lipogenic effects of GH may augment those of prolactin, which increases lipid synthesis in the rat mammary gland (511). In addition to these changes in lipid biosynthesis, GH increases expression of α-casein S1 and β-casein. Caseins are the most abundant proteins in milk, and represent an important source of calories, amino acids, calcium and phosphate in milk (512).

5.4.4. Growth hormone does not alter metabolism in HC11 cells

Growth hormone is a metabolic hormone with direct cellular effects through the GHR, as well as exerting indirect effects through IGF-1. Direct effects of GH in non-mammary tissues and cells are predominantly mediated by STAT5 (497, 498, 513). However, conflicting glycolytic effects of GH have been demonstrated in different settings. Thus, GH inhibits glycolysis in mouse tissues (497, 498), in keeping with anabolic roles for GH. By contrast, GH is reported to promote aerobic glycolysis in the immortalised 293T cells (513). Furthermore, a study involving breastfeeding women showed that daily rhGH injections increase expression of mammary genes involved in glycolysis and the TCA cycle, and also increased cell proliferation genes (379). These findings highlight a potential role for GH in mammary cell metabolism. However, our *in vitro* data demonstrates that treatment with GH for 24 hours has no effect on either basal glycolysis or glycolytic capacity in HC11 cells with a lactogenic phenotype (figures 52, 53). This is in keeping with our finding that GH does not induce phosphorylation of the Akt metabolic signalling protein, while the GH-mediated STAT5 activation observed is not associated with altered glycolytic flux in this setting.

Our data in lactogenic HC11 cells shows no effect of GH on either basal or maximal oxygen consumption (figures 54, 55). Therefore, GH does not promote mitochondrial activity, and this likely relates to the lack of PI3K-Akt pathway activation, as Akt is well established to promote mitochondrial energy metabolism (514). Overall, these findings suggest no role for GH in modulation of the two key mechanisms for cellular energy generation, glycolysis and mitochondrial oxidative phosphorylation.

5.4.5. Limitations

The post-breast feed increases described for serum GH in the early post-partum period represent a novel finding and suggest that GH secretion may be stimulated by breastfeeding. However, it is notable that these post-breast feed increases did not occur in all women, but were observed in around 50% of study participants on days 1-4 post-partum (figure 32). There are several potential reasons for different GH responses between women. Firstly, the time course of GH increases during breastfeeding has not been determined, and in some women may not occur within the 45 minute blood sampling duration utilised in the INSIGHT study (section 3.1). Secondly, other factors not measured in this study may be important for determining GH concentrations. For example, a fall in blood glucose is known to increase in pituitary GH release (515) while dietary intake is also important, with protein meals increasing GH and high-fat meals blunting GH responses (470, 471, 516). Ongoing work should therefore measure blood glucose concentrations before and after feeds, something that is now being undertaken prospectively as part of the INSIGHT study, and should consider the use of meal diaries. Finally, the rate of decline of the placental GH isoform after birth is uncertain and may vary between women. The Siemens Immulite 2000 assay used in this study does not provide information on isoform specificity, similar to other commercial assays (448). However, it seems unlikely that placental GH is detected by this assay, given the absence of GH in the third trimester of pregnancy (figure 32). Therefore, mass spectrometry to assess GH isoforms in the post-partum period may provide additional information about the observed GH variation between women.

We assessed *GHR* expression in human mammary epithelial cells at lactation onset using RNA obtained from milk fat globules and demonstrated no significant change in *GHR*

between days 2-5 post-partum. However, marked variability between women is observed and the sample sizes, particularly on days 2 and 5, are small. Measuring protein expression of the GHR in mammary epithelial cells, obtained by flow sorting of milk-derived cells, is a more accurate representation of GHR expression. In addition, direct assessment of STAT5 signalling following GH stimulation of milk-derived cells may provide further insights into hormone sensitivity, although this would require maintenance of milk-derived cells. This may be feasible, given that 90% of cells in milk are viable and culture of milk-derived cells has been reported (517), but requires validation of appropriate protocols that reliably select for mammary epithelial cells.

While HC11 cells represent a cellular model capable of displaying lactogenic features (395), they are derived from mouse mammary epithelial cells and it is uncertain if the findings we have observed represent the mammary effects of GH in humans. There are significant developmental and functional inter-species differences in the mammary epithelium during lactation (518-521), reflecting distinct demands. Metabolism in the mammary gland varies between species and is reflected in the differing composition of milk, such that rodent milk is relatively protein- and lipid-rich, while human milk has more lactose and water (518). Furthermore, rodents have large litters and require an earlier onset of copious milk production, and this is accompanied by differences in hormonal control of milk production (522). Notably, pituitary GH concentrations in rodents do not rise in the post-partum period as we have observed for humans (523).

Primary human mammary cells and human mammary cell lines are challenging to use for these *in vitro* studies as they difficult to differentiate into cells with a lactogenic phenotype in two-dimensional culture (387, 388). However, development of human mammary

organoids may provide a future means to evaluate cells with a lactogenic phenotype. Mouse mammary organoids capable of displaying lactogenic features have been developed (524), and there is increasing interest in the development of relevant human mammary organoids (525). Thus, future studies of hormone action in the cell culture setting should consider evaluation and use of human models of lactation.

It is important to note that neither the human studies performed to date, nor our cellular studies, replicate the normal physiological fluctuations that GH exhibits in women (526) or the fluctuations we have seen in the serum of breastfeeding women (figure 32). Furthermore, rhGH injections used in previous studies are likely to lead to suppression of endogenous pituitary GH production (527). Therefore, a model to better understand the effects of GH on the mammary epithelium could be a mammary- and lactation-specific knockout of the GHR in an animal model, while accepting that mammary GH effects may differ between species.

Finally, we have only assessed treatment with a single concentration of GH, using a single treatment duration. Other cellular studies assessing the effect of GH have used a similar concentration and treatment duration (493, 499, 528-530). However, whether additional GH concentrations or treatment durations may be more likely to induce glycolytic or mitochondrial effects and should be considered in future experimental work. Alternatively, GH may induce mammary transcription of IGF-1, which may in turn have metabolic effects (531, 532), and this should be assessed in future studies.

6. STUDY 3: INFLUENCE OF INSULIN ON MAMMARY CELL METABOLISM

6.1. Background

Insulin is an essential metabolic hormone, and a candidate mediator of the mammary changes required for milk synthesis, as highlighted in insulin receptor knockout mouse model studies (section 1.5.1) and by women with insulin-dependent diabetes who are at risk of delayed lactogenesis (361).

Insulin is produced by pancreatic β-cells, and has roles in glucose, lipid and protein metabolism (533). Insulin signals through the insulin receptor (INSR), a member of the tyrosine kinase receptor family (534). Thus, ligand binding to the α-subunits receptor induces a conformational change and activation of the kinase activity in the β-subunits. The resulting transphosphorylation of the β-subunits permits recruitment and phosphorylation of insulin receptor substrate (IRS) proteins, of which IRS1 and IRS2 mediate the effects of insulin in most cell types (535). Phosphorylated IRS proteins are able to act as scaffolds for downstream effector proteins (536). PI3K is an important mediator of the actions of insulin and is recruited to the insulin receptor-IRS complex (537). PI3K catalyses the formation of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate at the plasma membrane, enabling recruitment of phosphoinositide-dependent kinase (PDK1/2) and Akt, and leading to phosphorylation and activation of the latter (538). Insulin is capable of activating other intracellular pathways, including the Ras-Raf-MEK-MAPK pathway, which mediates mitogenic functions of insulin (539). The insulin signalling pathway is outlined in figure 61.

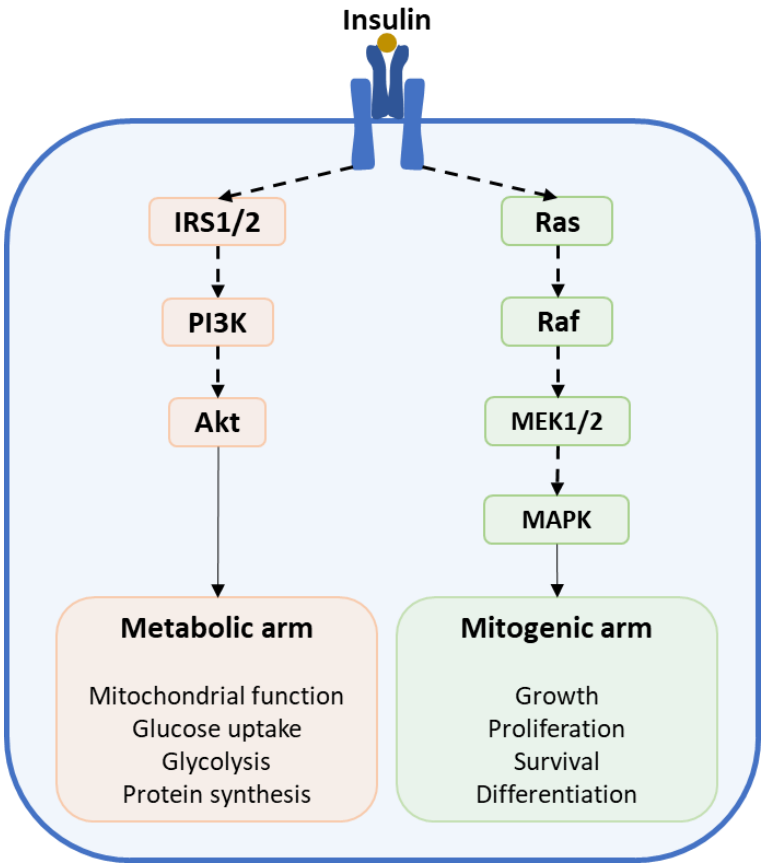


Figure 61. Schematic of cellular insulin signalling. Insulin binds and activates the insulin receptor, which signals through two well-established pathways. The PI3K-Akt pathway is responsible for most of the metabolic actions of insulin, including promoting mitochondrial function, glucose uptake, glycolysis and protein synthesis. The MAPK pathway mediates mitogenic effects, leading to increased growth, proliferation and cell survival.

Akt is considered to mediate the metabolic effects of insulin (540). With respect to mitochondrial function, Akt has been shown to promote mitochondrial biogenesis in 293T cells and rat hepatoma cells through activation of the transcription factors NRF1 and cAMP response element binding protein (CREB) (490, 491). Furthermore, Akt is reported to promote mitochondrial fusion and the formation of mitochondrial networks in mice through interaction with mitochondrial fission and fusion proteins such as dynamin-related protein 1 (DRP1) and mitofusin 2 (MFN2) (541-543). In addition, the insulin-Akt pathway is reported to

promote mitochondrial respiration in mouse and human embryonic stem cells and mouse myocytes (544-546). Insulin, through Akt, therefore acts to promote mitochondrial activity.

Akt also has well-established effects on increasing glucose uptake and glucose metabolism within cells. Akt promotes trafficking of GLUT1 and GLUT4 to the plasma membrane through inhibition of thioredoxin-interacting protein (TXNIP) in multiple cell types and mouse tissues, including skeletal muscle and adipose tissue (486). Furthermore, Akt phosphorylates and inhibits TBC1 domain family member 4 (TBC1D4) in a mouse adipose cell line, which promotes GLUT4 trafficking to the cell surfaces (547). Alongside increased glucose uptake, Akt promotes the expression and activity of glycolytic enzymes. Thus, Akt increases the activity of hexokinase 2 (HK2) and phosphofructokinase 1 (PFK1) in rodent and bovine cardiomyocytes, and rodent fibroblasts (548-550). In human HEK293T and HeLa cell lines, Akt also promotes increased expression of glycolytic enzymes through increased activity of transcription factors including HIF1α and MYC (551-554). Moreover, Akt inhibits the activity of the FOXO transcription factors in mice adipose cell lines, which in turn inhibit the transcription of glycolytic genes (555). Therefore, insulin-mediated Akt activation promotes glucose uptake and use in a range of non-mammary cells and tissues. These non-mammary actions of the insulin-PI3K-Akt pathway also represent candidate mechanisms of action for insulin in the mammary gland during lactation, and may highlight a role for this hormone at lactation onset.

6.2. Aims

The aims of this chapter are to:

1. Evaluate how mammary insulin sensitivity (receptor expression) changes during lactogenic differentiation in cultured mammary cells.

2. Determine whether insulin signals through the PI3K-Akt signalling pathway in cultured mammary cells.

3. Characterise the effects of insulin in a lactogenic mammary cell model, specifically on:

    i. Cell metabolism (glycolysis and mitochondrial activity).

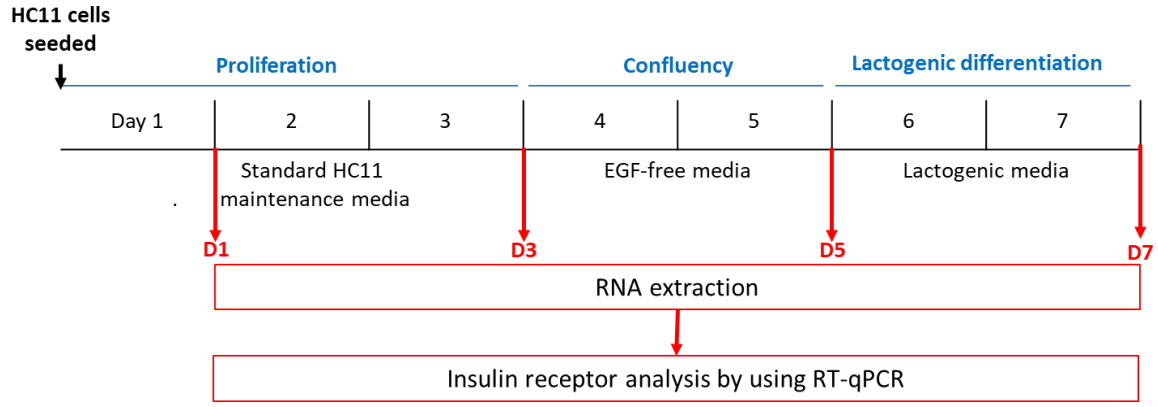
    ii. Production of intermediates required for milk synthesis.

6.3. Results

6.3.1. Changes in mammary cell insulin sensitivity during lactogenic differentiation

Insulin receptor expression was assessed in HC11 cells. RNA from HC11 cells was extracted to assess *INSR* expression at four time points: during proliferation (D1), on reaching confluency (D3), after an EGF-free period (D5) and after hormone treatment to induce a lactogenic phenotype (D7) (figure 62(A)). As shown in figure 62(B), *INSR* expression showed an increasing trend through these stages and is greatest in cells with a lactogenic phenotype (D7).

(A)



(B)

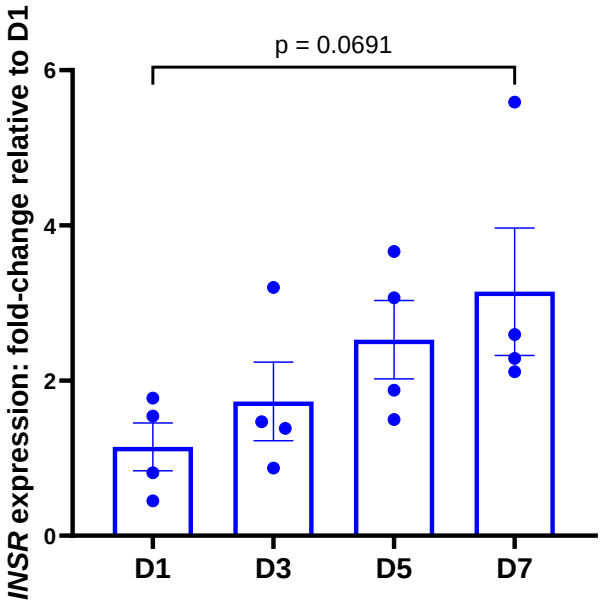


Figure 62. Insulin receptor expression in HC11 cells. (A) The extraction time points are shown. HC11 cells are extracted while proliferative (D1), when reaching confluency (D3), after the EGF-free period (D5), and after hormone treatment to induce lactogenic differentiation (D7). (B) Insulin receptor expression at each extraction time point, plotted as mean ± SEM for n=4 replicates per time point, and analysed by one-way ANOVA.

6.3.2. Insulin signalling in a mammary cell model

6.3.2.1. Insulin-induced Akt phosphorylation in HC11 cells

Insulin mediates many of its effects through the PI3K-Akt pathway (section 6.1). To evaluate insulin-mediated Akt activation, HC11 cells that have undergone lactogenic differentiation were stimulated with insulin at concentrations between 0 and 100 nM (figure 63). Insulin produced a dose-dependent increase in Akt phosphorylation, with a 6.8-fold greater Akt phosphorylation when using 100 nM compared to control cells. Given the greatest Akt response was induced by 100 nM insulin, this concentration was used for further cellular studies.

(A)

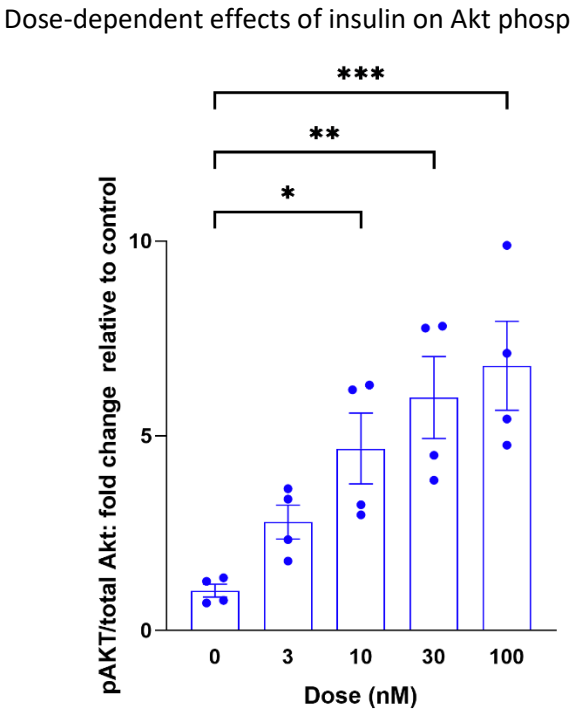
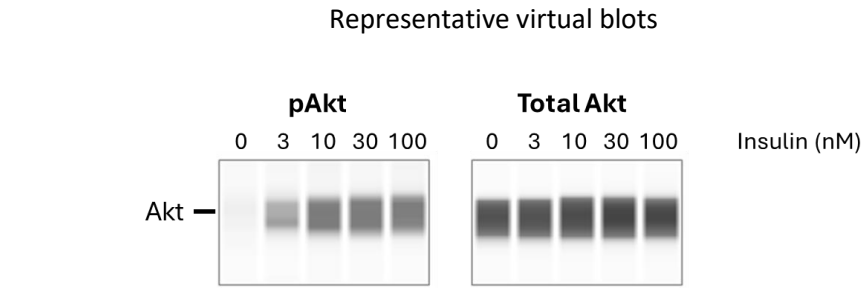


Figure 63 (continued overleaf).

(B)



(C)

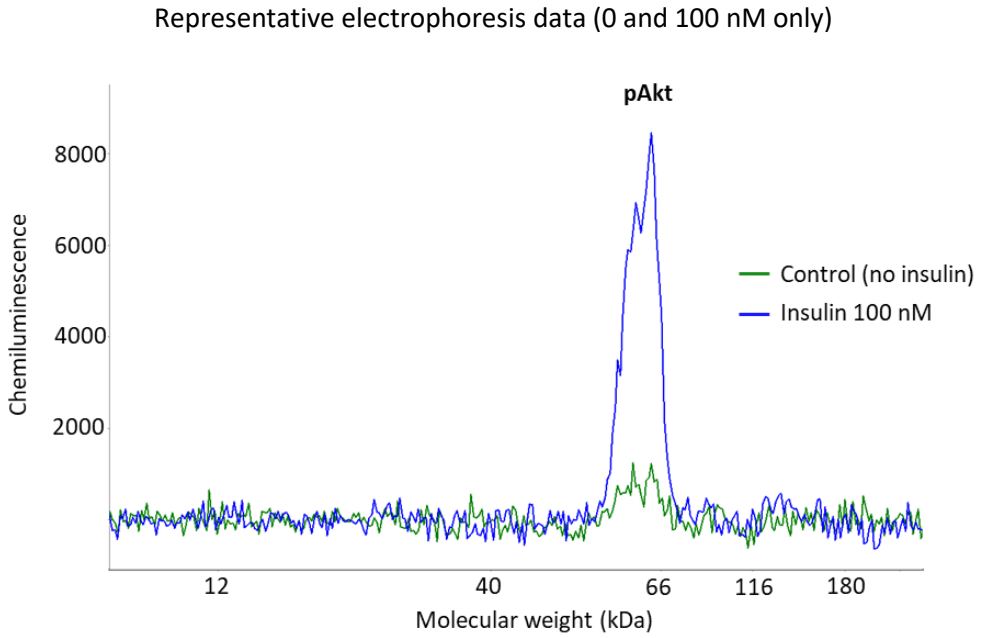
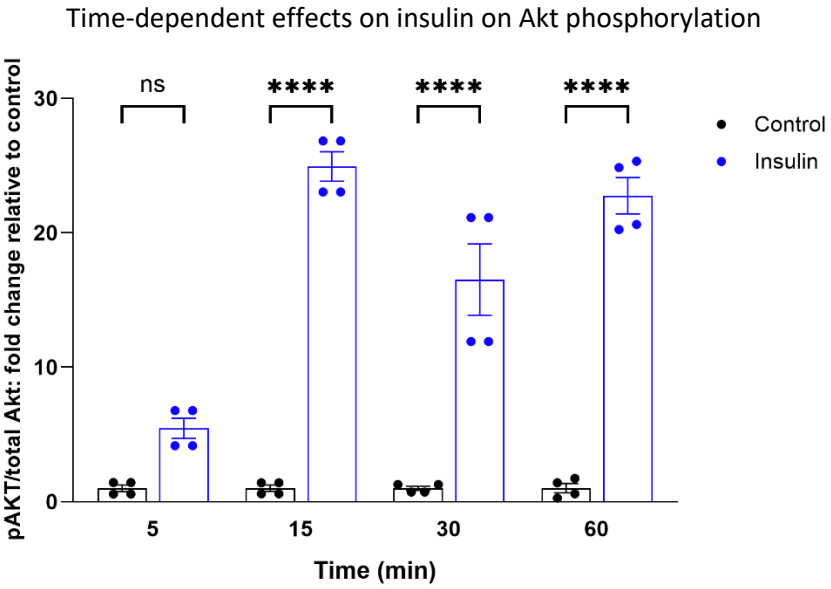


Figure 63. Dose-dependent effects of insulin on Akt phosphorylation in lactogenic HC11 cells. (A) Dose-dependent effects of insulin on Akt phosphorylation, assessed following 30 minutes insulin stimulation using concentrations ranging from 0-100 nM. Plotted as mean ± SEM for n=4 replicates. All phosphorylated Akt (pAkt) values are normalised to total Akt. (B) Representative virtual blots for pAkt and total Akt (n=1 replicate per condition shown). (C) Representative capillary electrophoresis data showing pAKt peak at 0 and 100 nM insulin (n=1 replicate per condition shown). The molecular weight of Akt is approximately 60 kDa. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To establish the time-course of insulin-mediated Akt activation, we assessed Akt phosphorylation in HC11 cells at 5, 15, 30 and 60 minutes after stimulation with 0 nM (control) or 100 nM insulin (figure 64). Akt phosphorylation begins to increase at 5 minutes,

peaks at 15 minutes (25-fold higher than control) and is sustained until 60 minutes after stimulation.

(A)



(B)

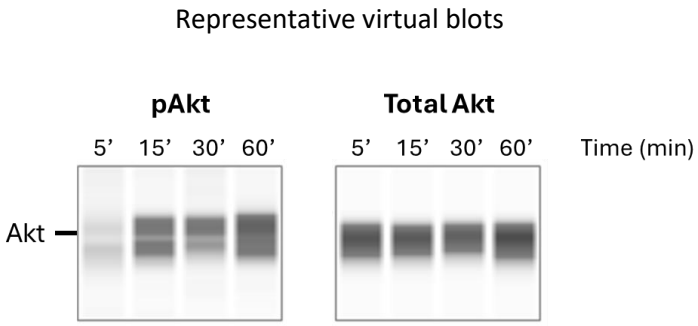


Figure 64 (continued overlead).

(C)

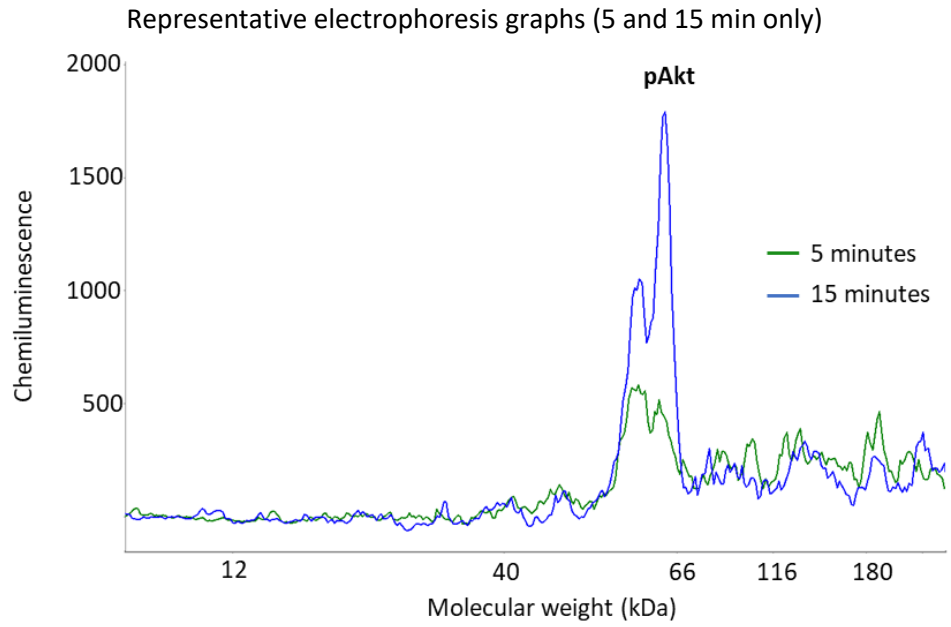


Figure 64. Time-dependent effects of insulin on Akt phosphorylation in lactogenic HC11 cells. (A) Time-dependent effects of insulin on Akt phosphorylation, assessed with 100 nM insulin stimulation between 5-60 minutes. Plotted as mean ± SEM for n=4 replicates All phosphorylated Akt (pAkt) values are normalised to total Akt. (B) Representative virtual blots for pAkt and total Akt (n=1 replicate per condition shown). (C) Representative capillary electrophoresis data showing pAKt peak at 5 and 15 minutes (n=1 replicate per condition shown). The molecular weight of Akt is approximately 60 kDa. ns, non-significant; **** $p<0.0001$.

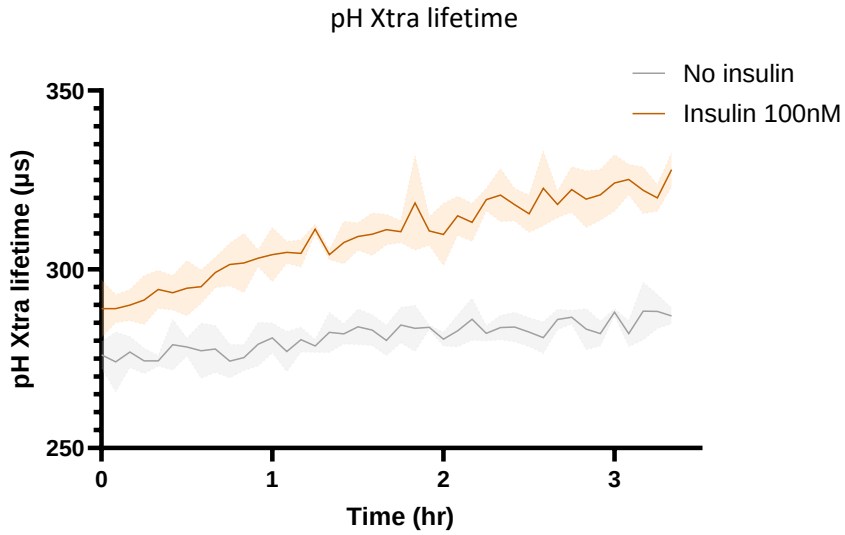
6.3.3. Metabolic effects of insulin on mammary epithelial cells

6.3.3.1. Insulin alters basal glycolysis in HC11 cells

To determine whether insulin promotes mammary glycolysis, HC11 cells were treated with 0 nM or 100 nM insulin for 24 hours before monitoring of media pH using the pH Xtra assay

(section 3.9). Figure 65 demonstrates that insulin significantly increases basal glycolytic rate in HC11 cells, from 0 µs/min to 0.15 µs/min.

(A)



(B)

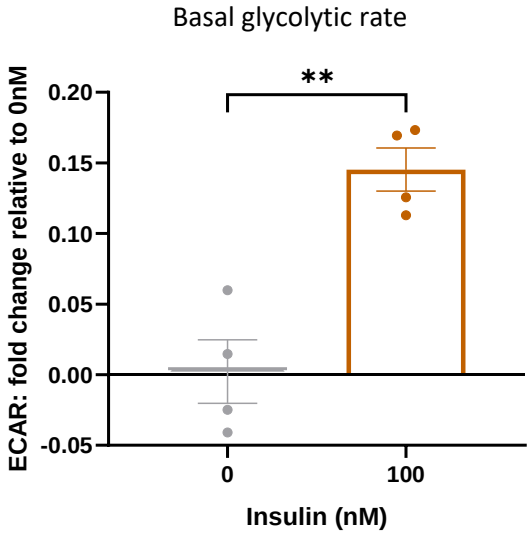
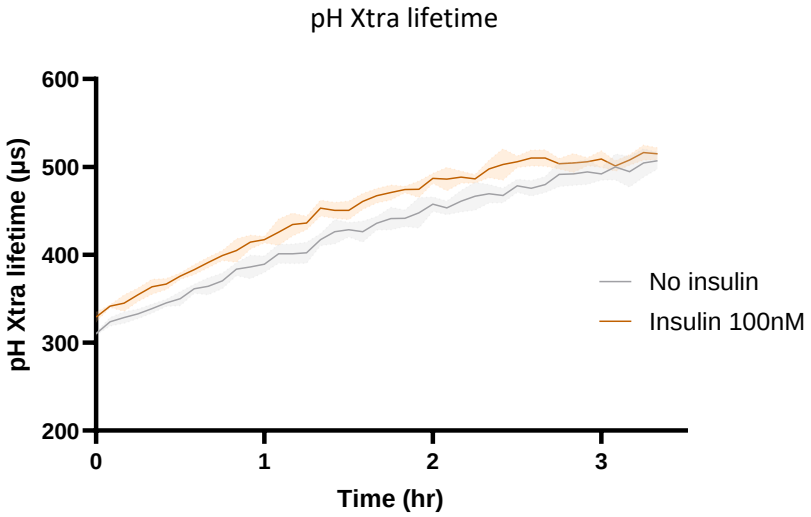


Figure 65. Effect of insulin on basal glycolytic rate in lactogenic HC11 cells. (A) pH Xtra lifetime in lactogenic HC11 cells treated with (orange) or without (grey) insulin 100 nM, shown as mean ± SEM for 4 replicates. (B) Extracellular acidification rate (ECAR) determined over recording period, plotted as mean ± SEM. Data analysed using unpaired t-test; ** $p<0.01$.

The effect of insulin on glycolytic capacity is plotted in figure 66. In contrast to basal glycolysis, insulin has no effect on glycolytic capacity in HC11 cells.

(A)



(B)

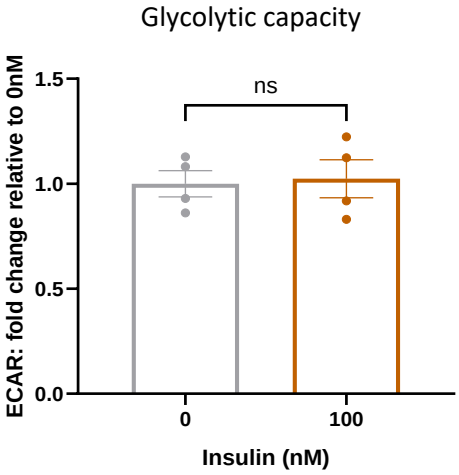
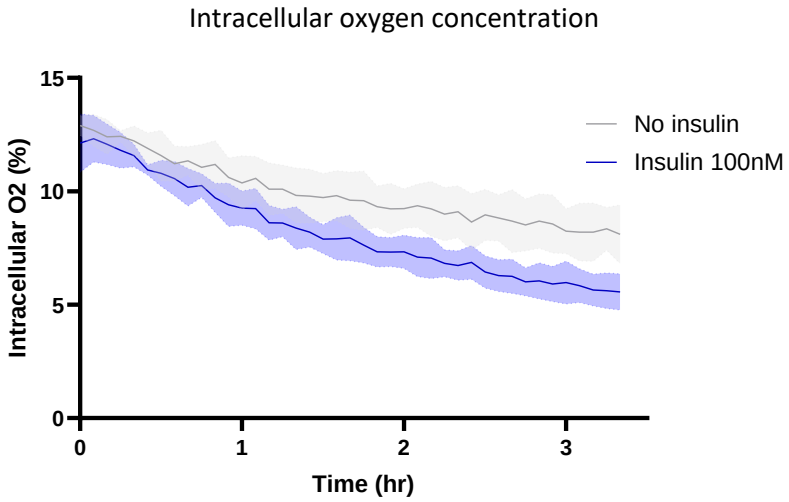


Figure 66. Effect of insulin on glycolytic capacity in lactogenic HC11 cells. (A) pH Xtra lifetime in lactogenic HC11 cells treated with (orange) or without (grey) insulin 100 nM, shown as mean ± SEM for 4 replicates, after addition of oligomycin. (B) Glycolytic capacity, calculated as ECAR following oligomycin addition minus ECAR following 2-deoxyglycose addition, between 0 and 70 minutes, plotted as mean ± SEM. Data analysed using unpaired t-test; ns, non-significant.

6.3.3.2. Insulin increases mitochondrial activity

We next sought to assess the effect of insulin on basal oxygen consumption (section 3.8). Treatment of differentiated HC11 cells with 100nM insulin for 24 hours led to a 1.7-fold increase in basal oxygen consumption , as compared to control cells (figure 67).

(A)



(B)

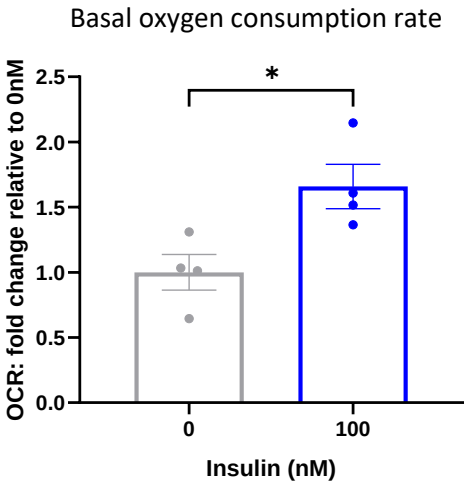
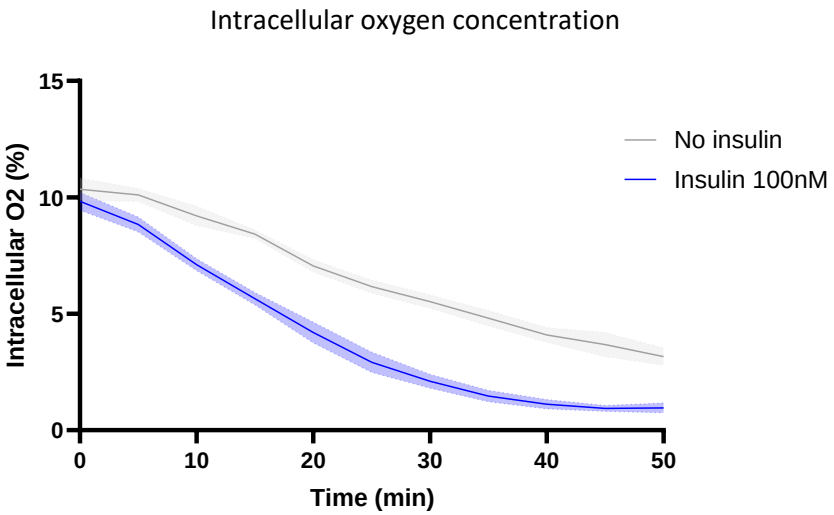


Figure 67. Effect of insulin on basal oxygen consumption in lactogenic HC11 cells. (A) Calculated intracellular oxygen (%) in lactogenic HC11 cells treated with (blue) or without (grey) insulin 100 nM, shown as mean ± SEM for 4 replicates. (B) Basal oxygen consumption rate (OCR) determined over recording period, plotted as mean ± SEM. Data analysed using unpaired t-test; * $p<0.05$.

To evaluate the effect of insulin on maximal oxygen consumption, HC11 cells with a lactogenic phenotype were treated with 0nM or 100nM insulin for 24 hours before addition of FCCP (section 3.8). Figure 68 demonstrates that insulin treatment increases maximal oxygen consumption 2.4-fold compared to control cells.

(A)



(B)

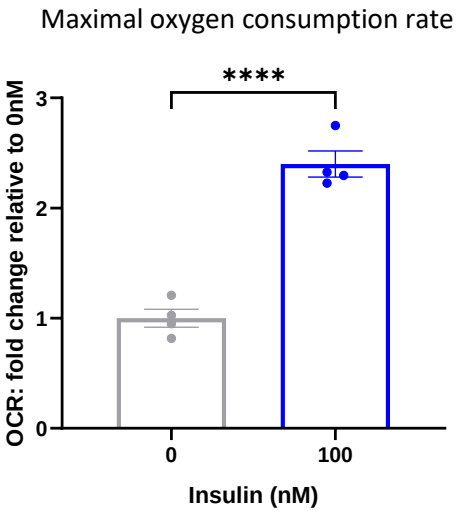


Figure 68. Effect of insulin on maximal respiration in lactogenic HC11 cells. (A) Calculated intracellular oxygen (%) in lactogenic HC11 cells treated with (blue) or without (grey) insulin 100 nM, shown as mean ± SEM for 4 replicates, after addition of FCCP. (B) Maximal oxygen consumption rate (OCR) determined between 10 and 30 minutes, plotted as mean ± SEM. Data analysed using unpaired t-test; **** $p<0.0001$.

6.3.4. Transcriptomic assessment of insulin in mammary epithelial cells

6.3.4.1. Quality assessment of transcriptomic data

To evaluate the cellular mechanisms underlying the bioenergetic changes described, transcriptomic analysis of RNA obtained from insulin-treated HC11 cells was undertaken (Section 3.11). HC11 cells that had gone lactogenic differentiation were treated with or without 100 nM insulin and extracted at the following time points: 5 minutes, 30 minutes, 2 hours and 8 hours (n=5 per condition). These times were selected to capture the fact that insulin can induce transcriptional changes in cultured cells within minutes, and with effects extending >4 hours (556).

Extracted RNA was sent to Azenta Life Sciences for RNASeq analysis. Data was returned in the form of raw counts, which reflects the number of transcripts obtained from a single gene. Raw counts were normalised to counts per million (CPM), and a comparison of log_2-transformed CPM was used to ensure comparability between samples. As shown in figure 69, the median counts across all conditions are similar (log_2-transformed median count 5.19).

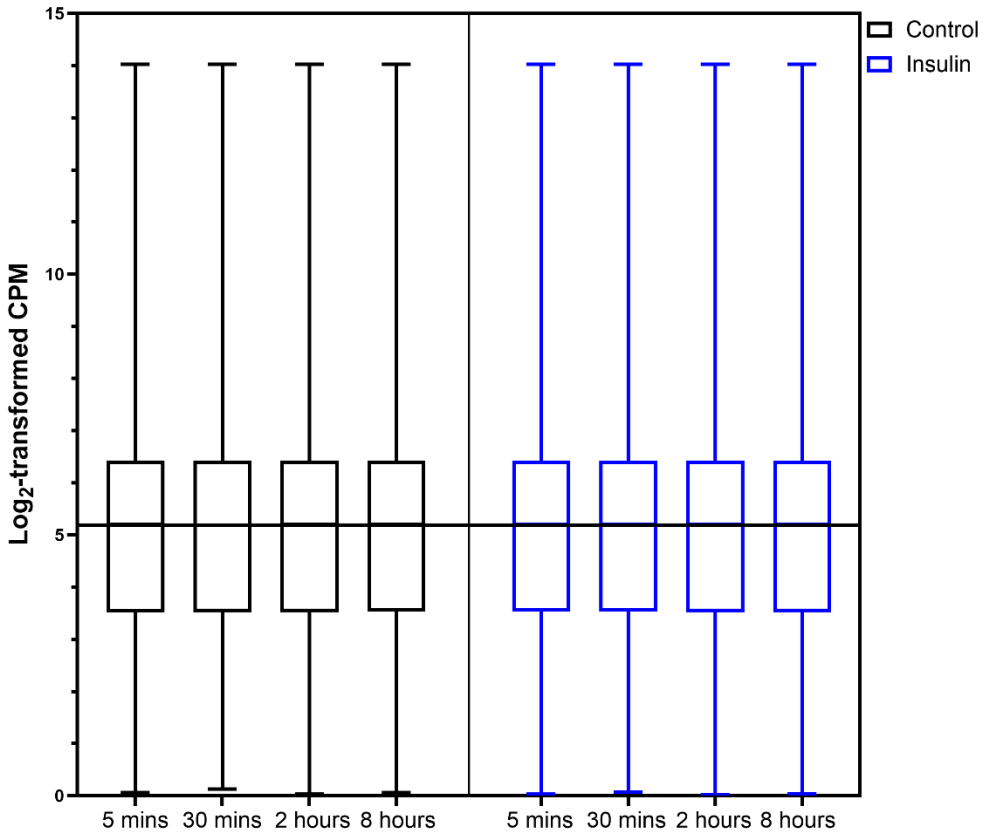


Figure 69. Counts in insulin-treated and control HC11 cells. Log_2 transformed counts for control (left-hand side, white) and insulin-treated (right-hand side, yellow) HC11 cells. Median, 25th and 75th centiles, and range plotted.

In addition, the RNA integrity number (RIN) provides an estimation of the integrity of RNA obtained. This ranges from 1-10, with 10 representing samples with the best integrity, while a RIN of >8 is considered acceptable for use. All RIN values were greater than 8, with most being 9-10 (figure 70).

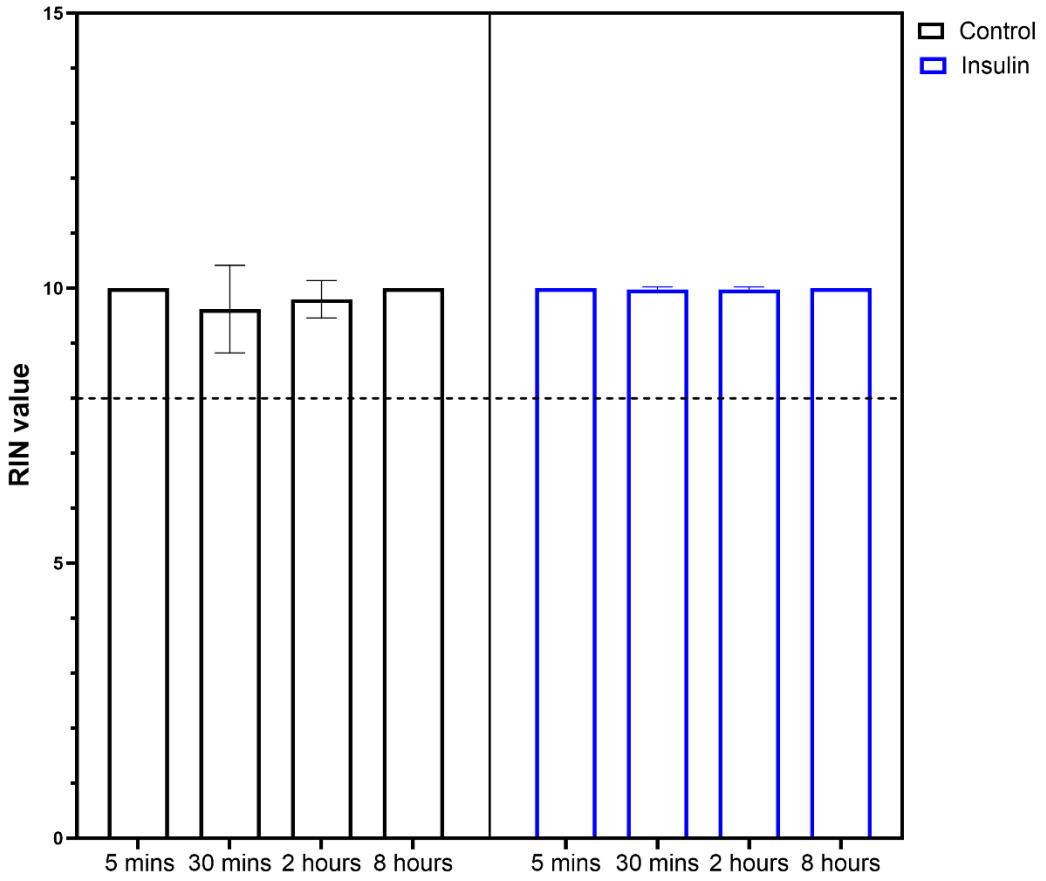


Figure 70. RIN values in insulin-treated and control HC11 cells. RIN values for control (left-hand side, black) and insulin-treated (right-hand side, blue) HC11 cells. Data shown as mean ± SD. The dashed horizontal line marks a RIN value of 8, above which RNA is generally considered of good quality.

In order to provide an initial evaluation of similarity between samples from the same group, as well as to compare different conditions, cluster analysis was undertaken with a PCA plot (figure 71). This demonstrates that samples from the same condition cluster together closely. It also highlights that the differences between insulin-treated and control HC11 cells are small at 5 and 30 minutes, with significant overlap between data points, but increase at 2 and 8 hours.

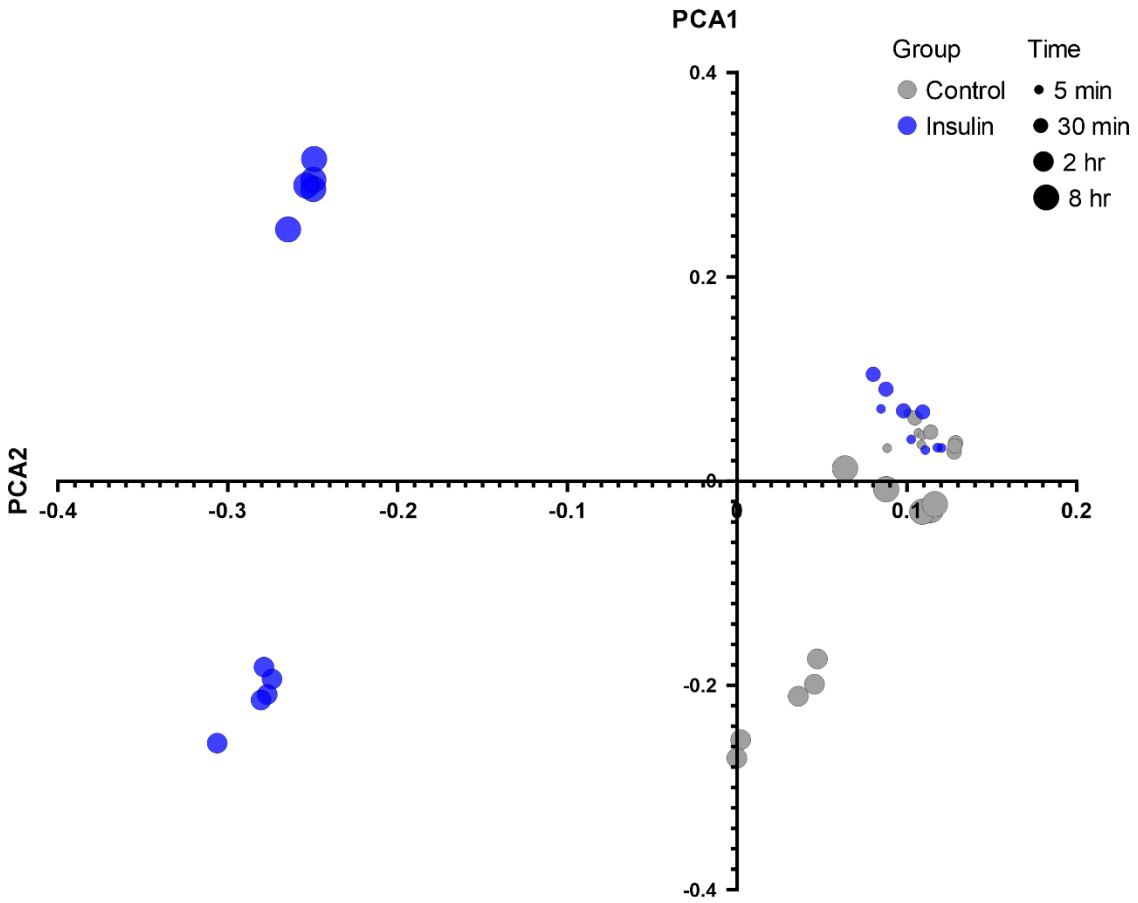


Figure 71. Cluster analysis of insulin-treated and control HC11 cells. PCA plot of insulin-treated (blue circles) and control (grey circles) HC11 cells, according to time point of extraction (5 minutes, 30 minutes, 2 hours, 8 hours). Each circle represents an individual sample.

In keeping with the PCA plots, volcano plots of differentially expressed genes (DEGs) highlight the increase in DEGs with increasing time of stimulation (figure 72). Thus, there are no DEGs when comparing insulin-treated and control cells at 5 minutes. However, by 8 hours, there are a total of 710 DEGs, with 322 of these upregulated in insulin-treated cells and 388 downregulated.

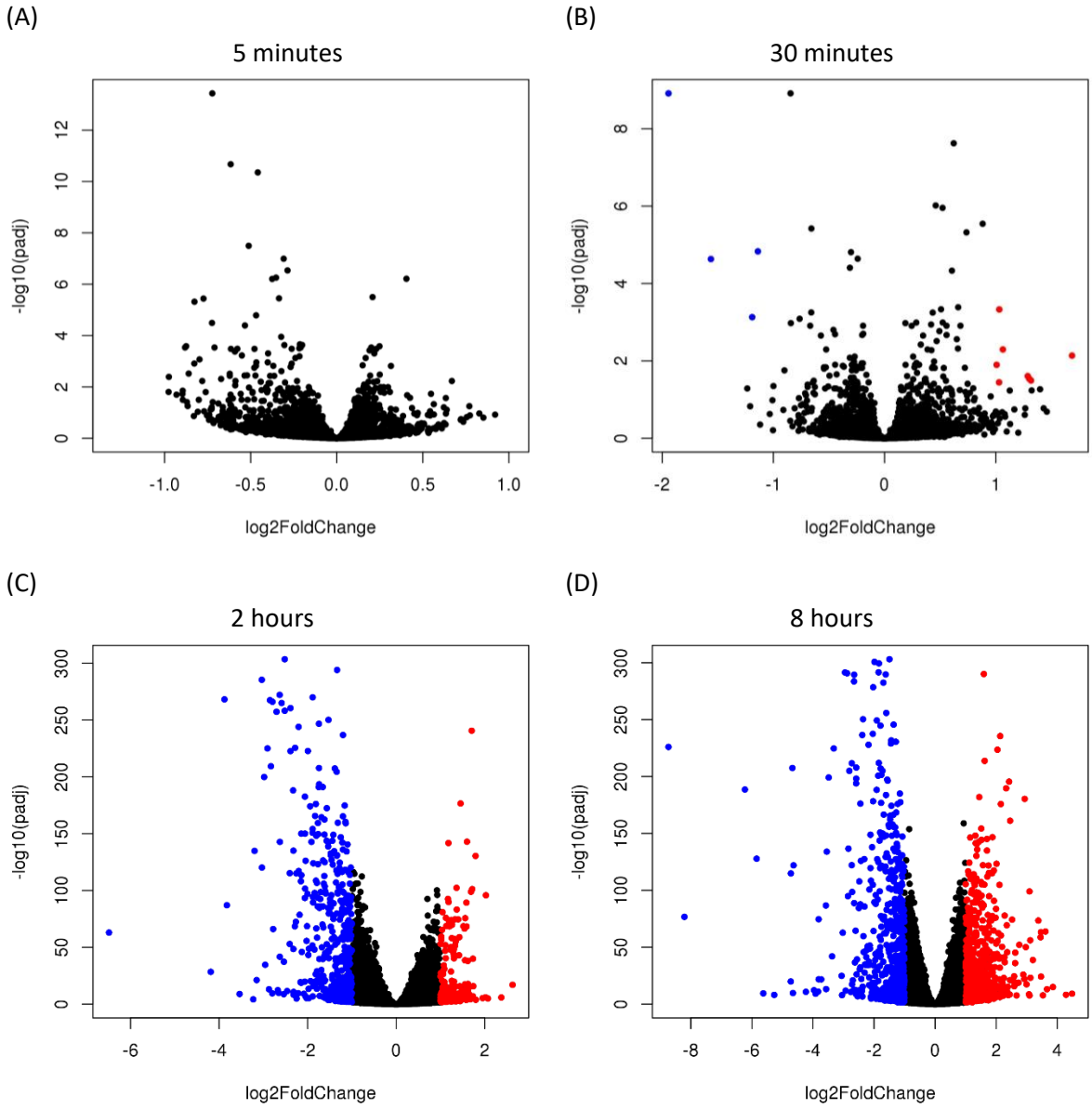


Figure 72. Volcano plots of differentially expressed genes (DEGs). Volcano plots showing significantly downregulated (blue) and upregulated (red) genes in insulin-treated compared to control HC11 cells at 5 minutes (A), 30 minutes (B), 2 hours (C) and 8 hours (D).

The number of DEGs upregulated and downregulated at each extraction time point is summarised in figure 73. This confirms the time-dependent increase in the number of DEGs.

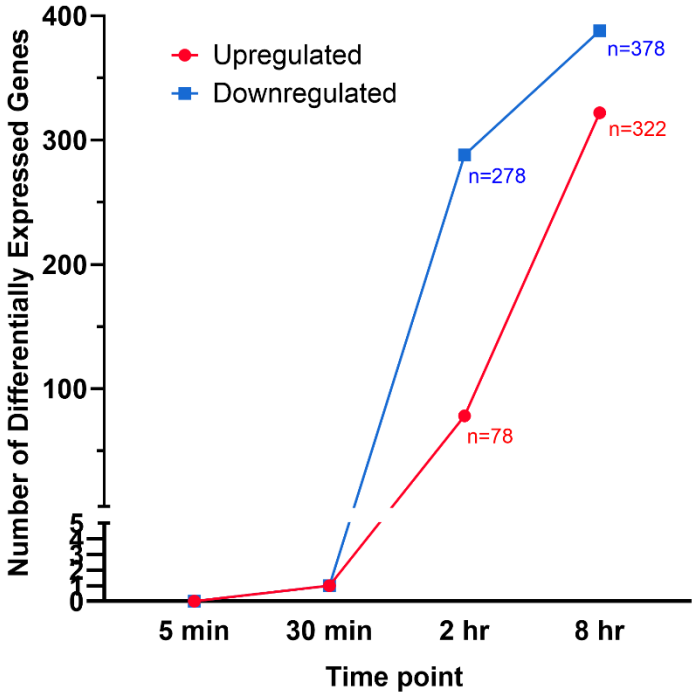


Figure 73. Summary of number of DEGs at each extraction time point. Numbers of significantly downregulated (blue) and upregulated (red) genes in insulin-treated compared to control cells.

In order to visualise which biological pathways are affected by insulin, the gene set enrichment was performed using databases including Hallmark, MSigDB, KEGG and GO. The parameters used for gene set enrichment analysis (GSEA) were a $\log_2$-fold change of >0.5 (equating to a fold change of >1.4) and a FDR of <0.05. As plotted in figure 74, there are no differentially expressed gene sets at 5 or 30 minutes. At 2 hours, 9 gene sets are differentially regulated (5 upregulated, 4 downregulated) and this increases to 22 gene sets at 8 hours (19 upregulated, 3 downregulated).

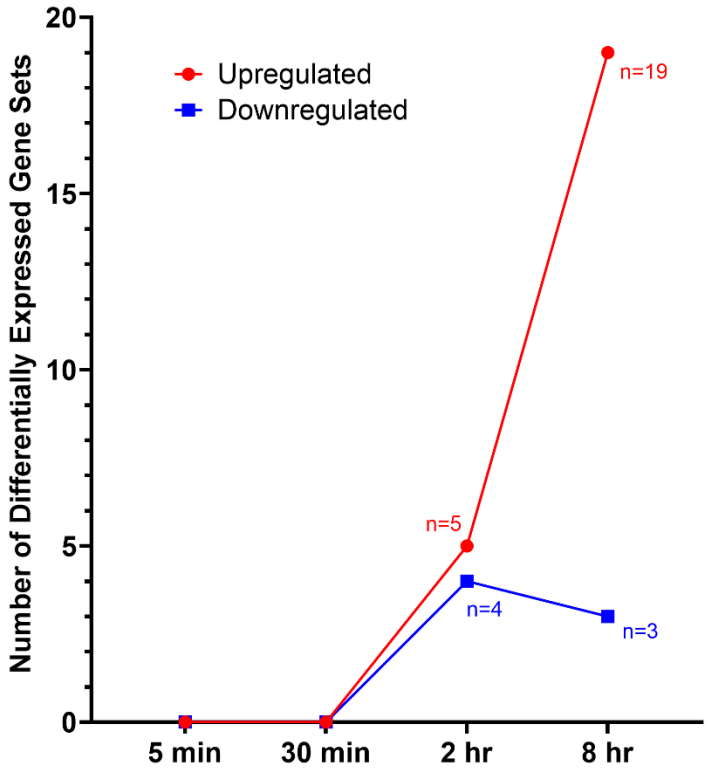


Figure 74. Summary of number of differentially expressed gene sets at each extraction time point. Numbers of significantly downregulated (blue) and upregulated (red) genes in insulin-treated compared to control cells.

6.3.4.2. Metabolic pathways altered by insulin

The ten most significantly changed gene sets after 8 hours of stimulation were selected and plotted (figure 75). The largest change induced by insulin was upregulation of genes involved in glycolysis, while genes involved pentose phosphate pathway were also increased. Six of the gene sets upregulated were related to mitochondrial oxidative phosphorylation and ATP synthesis. Forkhead box O (FOXO)-mediated transcription of genes related to cell death and oxidative stress was downregulated by insulin treatment.

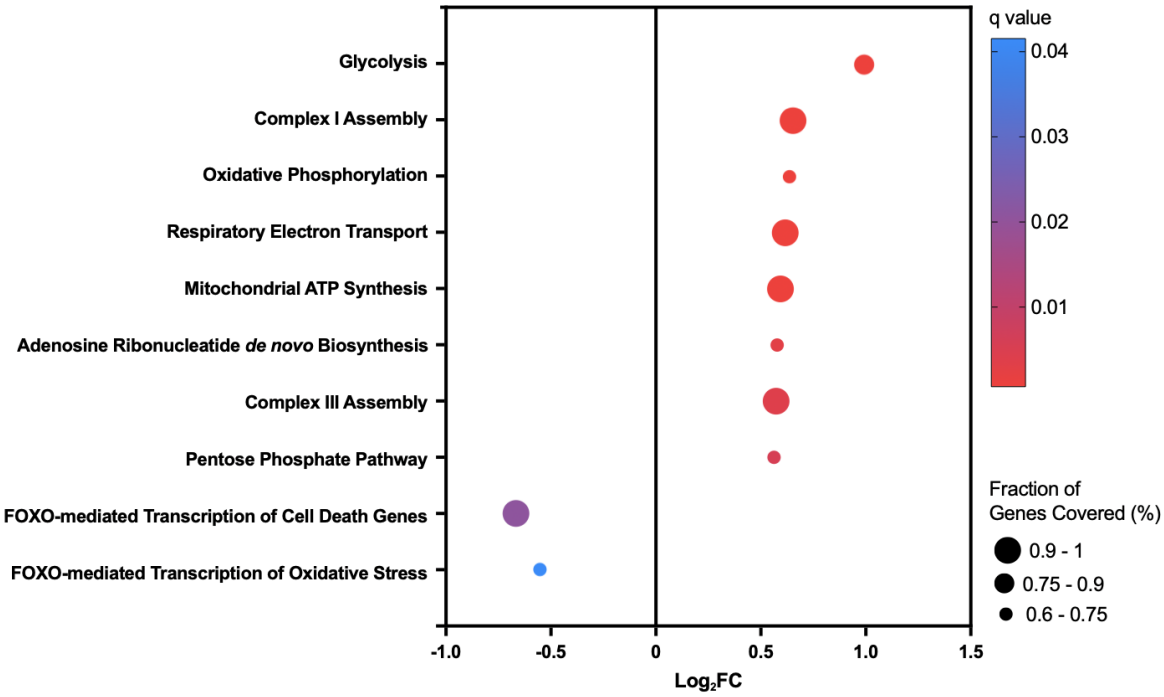


Figure 75. Top 10 most significant differentially expressed gene sets. Gene sets arranged according to the log_2-fold change. The colour of each circle represents the q value, while its size corresponds to the fraction of total genes covered by that gene set.

6.3.4.3. Effects of insulin on glycolytic gene expression

Glycolysis encompasses a series of enzymatic reactions that metabolise glucose to pyruvate, which can then be converted to acetyl coenzyme A (acetyl-CoA), for use in the tricarboxylic acid (TCA) cycle, or to lactic acid (557). The glycolytic pathway and associated genes is outlined in figure 76, with the expression of genes significantly changed in response to insulin highlighted.

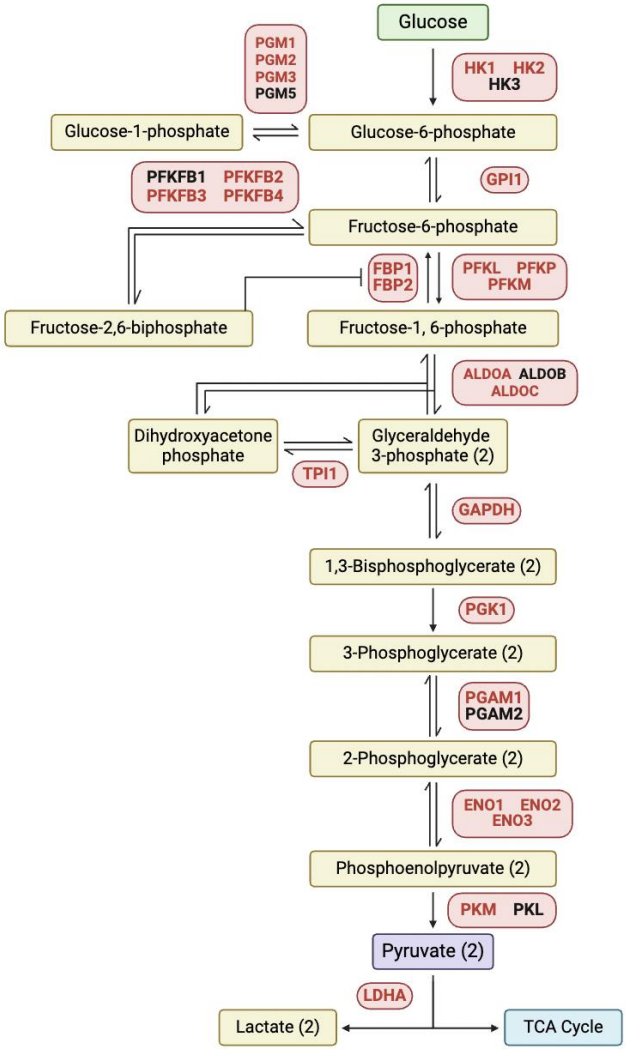


Figure 76. Genes encoding proteins in the glycolytic pathway. The glycolytic pathway is outlined, beginning with glucose (green box) and including the intermediates shown in yellow boxes. The enzymes catalysing each reaction are in red boxes. Significantly upregulated genes are in red text, while unchanged genes are in black. Abbreviations: HK, hexokinase; GPI, glucose-6-phosphate isomerase; PGM, phosphoglucomutase; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; FBP, fructose-1,6-bisphosphatase; PFK, phosphofructokinase; ALDO, aldolase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; LDHA, lactate dehydrogenase A.

A heatmap of significantly changed genes at each time point is shown in figure 77. There is upregulation of 25 out of 31 genes encoding glycolytic enzymes, starting at 2 hours and being most pronounced at 8 hours, while there are no changes at 5 and 30 minutes. The most proximal steps in glycolysis, catalysed by hexokinase (HK) and glucose-6-phosphate isomerase (GPI) have only small increases in expression (1.2-fold and 1.3-fold for *HK1* and

HK2, respectively, and 1.3-fold for *GPI*). By contrast, enzymes lower down in the glycolytic pathway show greater insulin-induced changes in expression, with the gene for lactate dehydrogenase A (LDHA) demonstrating the greatest increase (4.0-fold). In addition, the *SLC2A1* gene, which encodes the main mammary glucose transporter GLUT1, shows a 2.6-fold increase in expression (not shown).

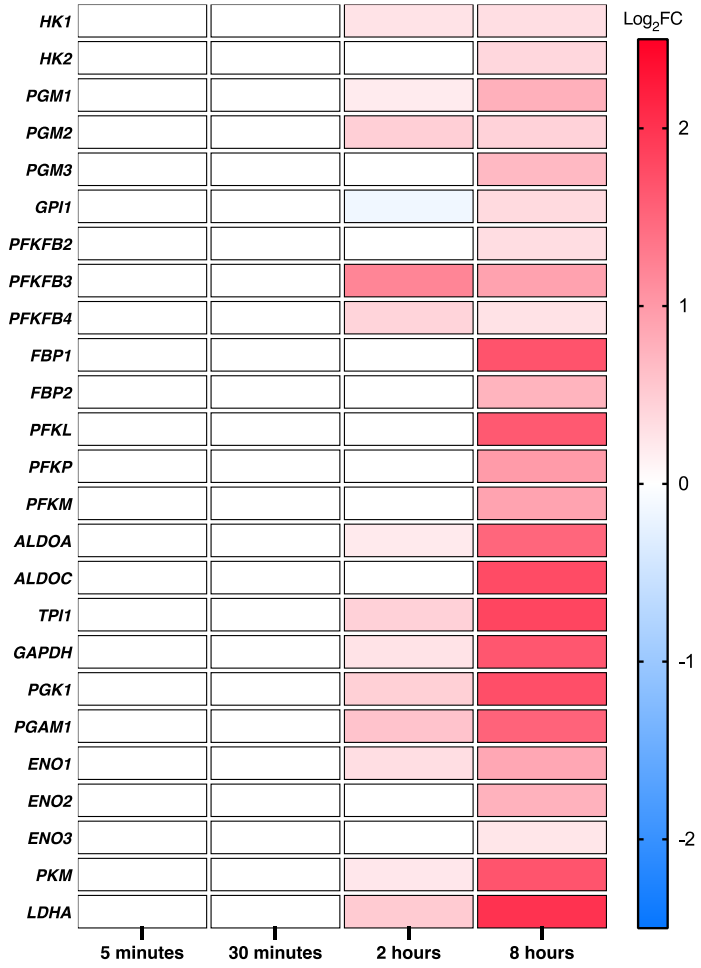


Figure 77. Heatmap of effects of insulin on glycolytic genes. A heatmap showing the fold change in expression of each DEG displayed as log_2-fold change, at each extraction time point. Where an enzyme has multiple isoforms, the genes for each of these isoforms are displayed. Upregulation in response to insulin treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading.

6.3.4.4. Effects of insulin on pentose phosphate pathway gene expression

The pentose phosphate pathway represents a parallel metabolic pathway to glycolysis, incorporating some glycolytic intermediates. It generates NADPH, pentose sugars, and ribose-5-phosphate, a precursor for nucleotide synthesis (558). The pentose phosphate pathway and significantly changed genes are shown in figure 78.

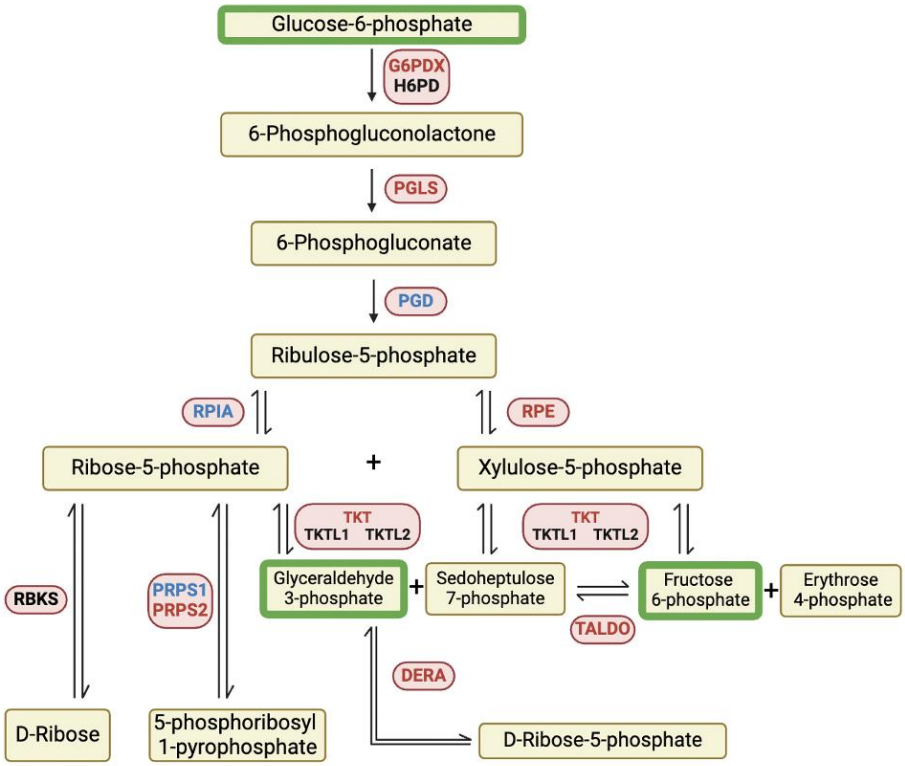


Figure 78. Genes encoding proteins in the pentose phosphate pathway. The pentose phosphate pathway is outlined on the left. Intermediates are shown in yellow boxes, while those intermediates coming from the glycolytic pathway are outlined in green. The genes representing pentose phosphate pathway enzymes are in red boxes. Significantly upregulated genes are in red text, downregulated genes are in blue, while unchanged genes are in black. Abbreviations: G6PDX, glucose-6-phosphate dehydrogenase; H6PD, hexose-6-phosphate dehydrogenase; PGLS, phosphogluconolactonase; PGD, phosphogluconate dehydrogenase; RPE, ribulose-5-phosphate 3-epimerase; RPIA, ribose 5-phosphate isomerase; TKT, transketolase; TALDO1, transaldolase 1; RBKS, ribokinase; PRPS, phosphoribosyl pyrophosphate synthetase; DERA, deoxyribose-phosphate aldolase; PGM, phosphoglucomutase.

A heatmap showing the significant effects of insulin on genes comprising the pentose phosphate pathway at each stimulation time point is shown in figure 79. While 7 out of 14 genes are upregulated by insulin, 4 out of 14 are unchanged (*H6PD*, *RBKS, TKTL1, TKTL2*) and 3 out of 14 are downregulated (*PGD*, *RPIA*, *PRPS1*).

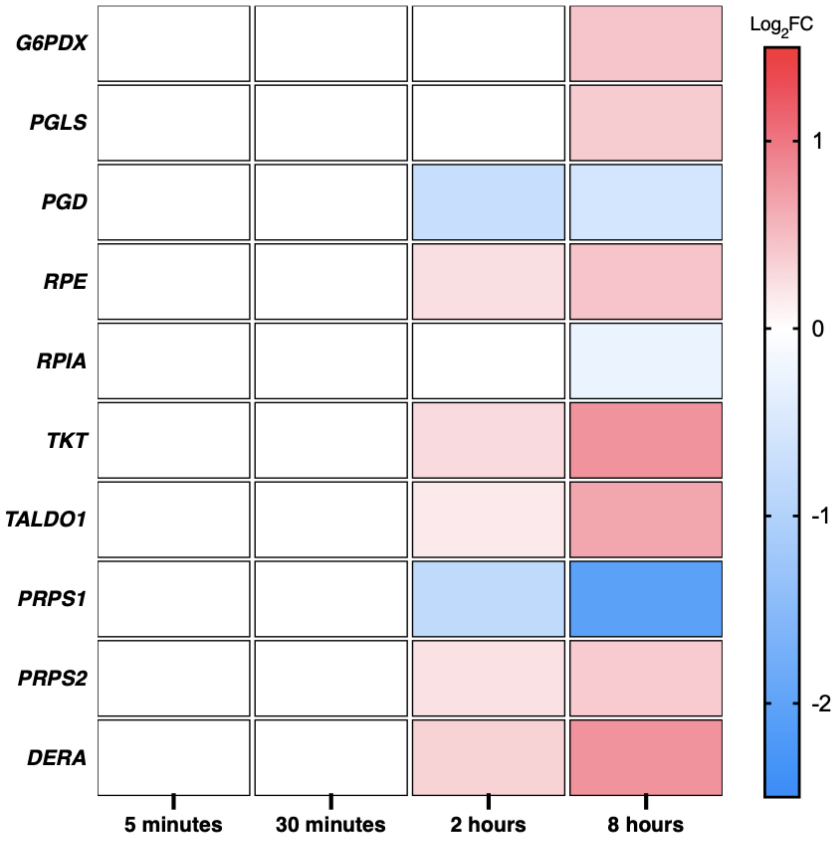


Figure 79. Heatmap of effects of insulin on pentose phosphate pathway genes. A heatmap showing the fold change in expression of DEGs, displayed as log_2-fold change, at each extraction time point. Where an enzyme has multiple isoforms, the genes for each of these isoforms are displayed. Upregulation in response to insulin treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading.

6.3.4.5. Effects of insulin on mitochondrial ETC gene expression

The mitochondrial electron transport chain (ETC) is responsible for oxidative phosphorylation (559). The ETC is composed of complexes I-V, and each complex involved in is composed of multiple subunits (560). Reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) generated from glycolysis and the TCA cycle donate electrons to complex I and complex II, respectively. The subsequent transfer of electrons through complexes I-IV results in the pumping of protons from the mitochondrial matrix into the mitochondrial intermembrane space. These protons then re-enter the mitochondrial matrix through a pore located within complex V (ATP synthase), which drives this complex to produce ATP from ADP. This is summarised in figure 80.

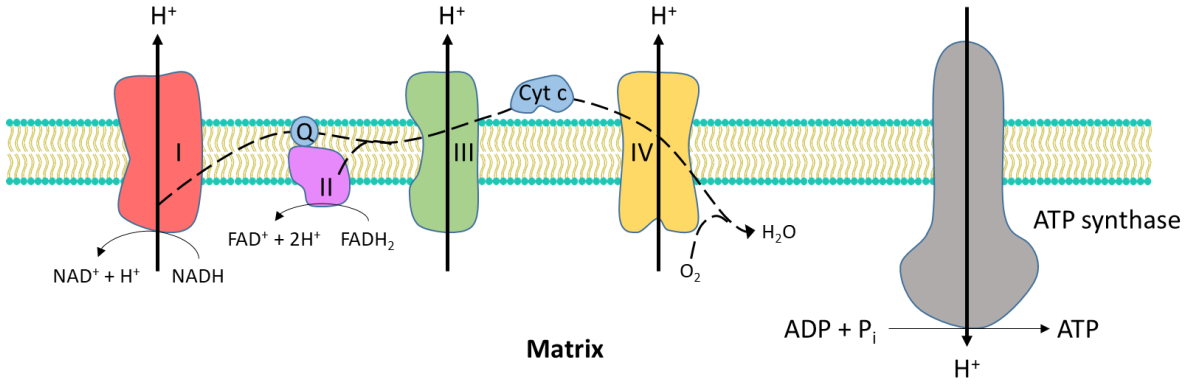


Figure 80. The mitochondrial electron transport chain. Donation of electrons by NADH and $FADH_2$ to complexes I and II, respectively, and subsequent electron transport through complexes I-IV drives proton pumping into the intermembrane space and formation of a proton gradient. This gradient is used by complex V (ATP synthase) to produce ATP from ADP and phosphate (P_i).

Each of the five complexes consist of 'core' proteins, which have catalytic activity, and a number of additional proteins involved in assembly, regulation and stability of complexes

(561). In total, insulin upregulated expression of 127/160 genes associated with the ETC in HC11 cells. The effect on each ETC complex is described below.

The subunits comprising complex I are shown in figure 81.

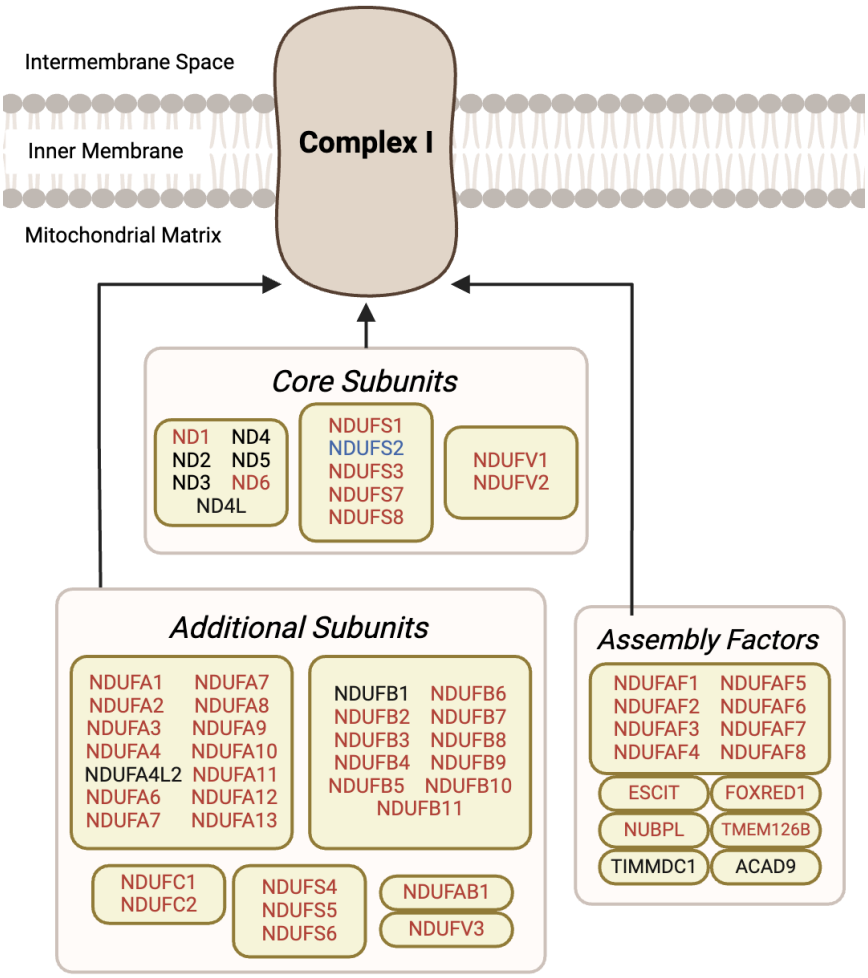


Figure 81. Genes encoding proteins in mitochondrial complex I. The subunits forming and required for assembly of complex I, are subdivided according to their role. Significantly upregulated genes are in red text, downregulated genes are in blue, while unchanged genes are in black. Abbreviations: ND, NADH dehydrogenase; NDUF, NADH:ubiquinone oxidoreductase; NDUFAF, NADH:ubiquinone oxidoreductase complex assembly factor; FOXRED, FAD-dependent oxidoreductase domain-containing protein; ESCIT, evolutionarily conserved signalling intermediate in Toll pathway; NUBPL, nucleotide-binding protein-like; TMEM, transmembrane protein; TIMMDC, translocase of inner mitochondrial membrane domain containing; ACAD9, acyl-CoA dehydrogenase.

GSEA of complex I demonstrates that insulin upregulates core subunits, assembly subunits, and other assembly factors (figure 82). This begins at 2 hours and is greatest at 8 hours, with almost universal upregulation of subunit expression at this time point. Thus, 50 out of 60 genes are upregulated at 8 hours, with a the most increased gene (*NDUFB7*) showing a 2-fold increase in expression. *NDUFS2* is downregulated in response to insulin, while 9 out of 60 genes are unchanged in response to insulin treatment.

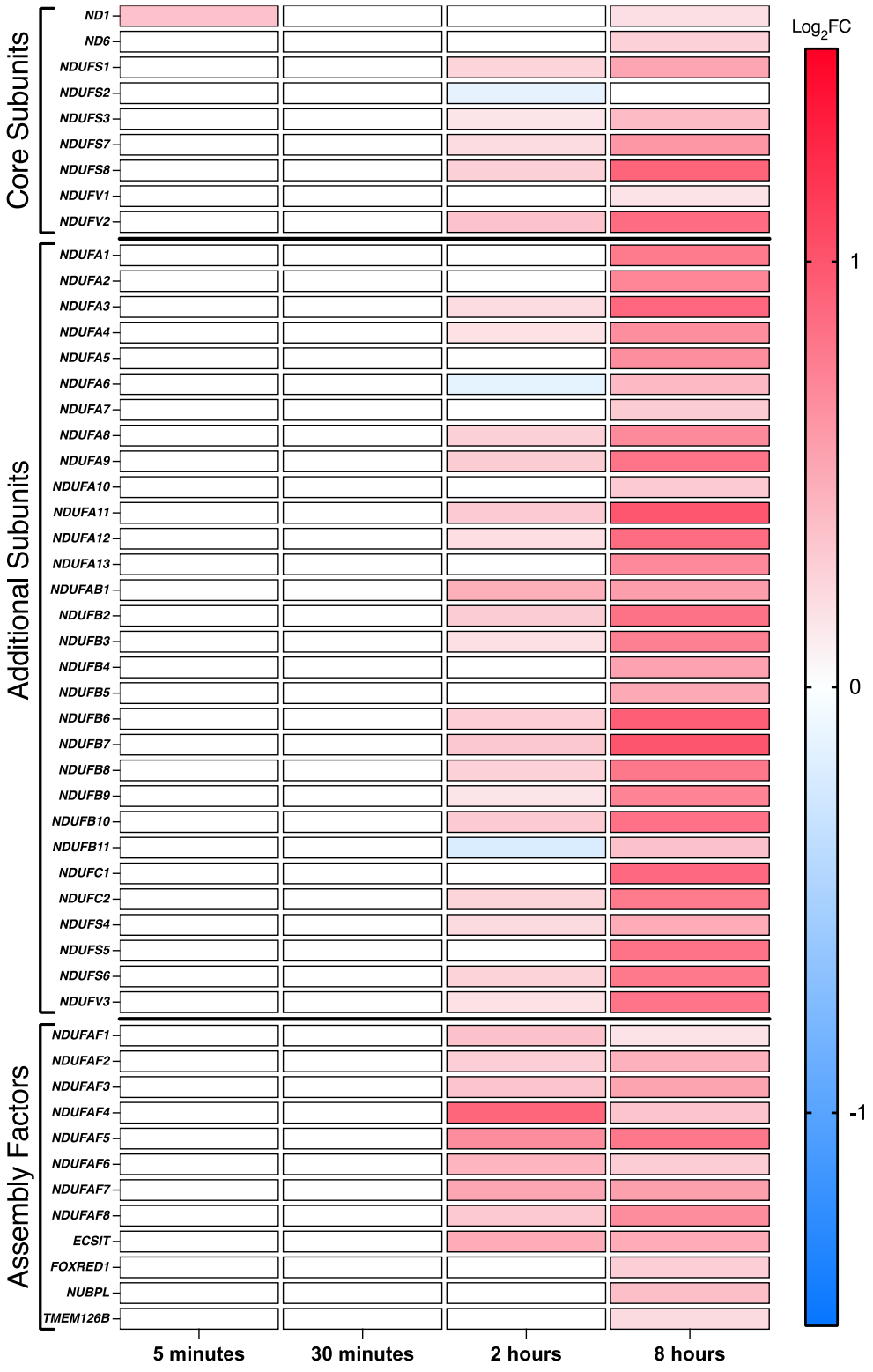


Figure 82. Heatmap of effects of insulin on mitochondrial complex I subunit gene expression . The heatmap shows fold change in expression of each subunit gene, displayed as log_2-fold change, at each extraction time point. Upregulation in response to insulin treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading.

Complex II is composed of four subunits and requires fewer subunits for its assembly compared to complex I (figure 83). Coenzyme Q_{10} (CoQ_{10}) is a lipophilic molecule that resides in the inner mitochondrial membrane and is critical for electron transfer from complexes I and II to complex III (562). Similar to complex I, the majority of complex II subunits (16 out of 21) demonstrate upregulation in response to insulin treatment, particularly at 8 hours (figure 83).

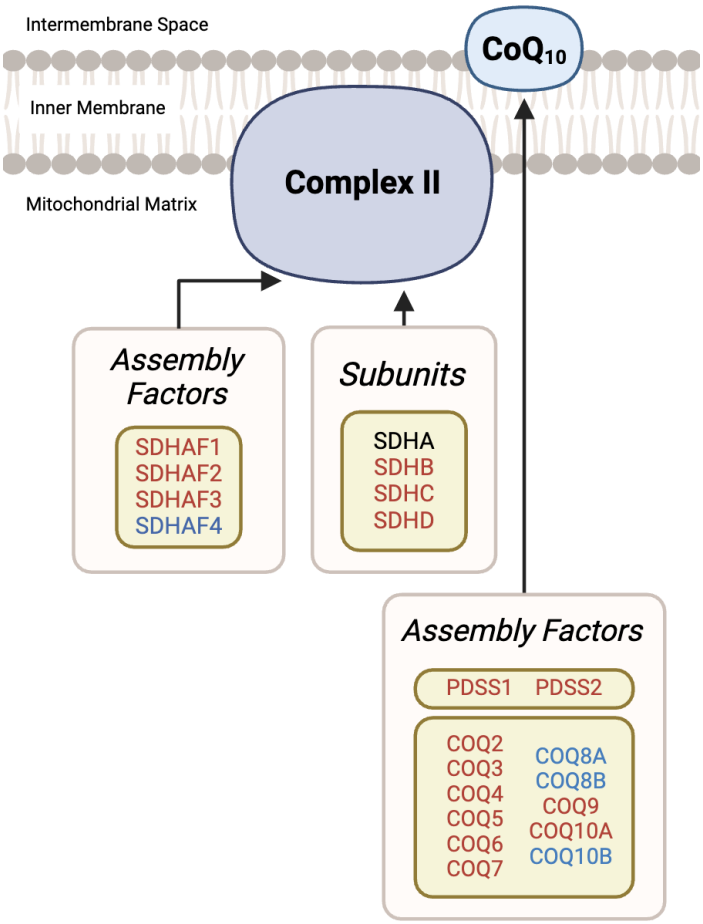


Figure 83. Genes encoding mitochondrial complex II subunits and factors involved in CoQ_{10} synthesis. The subunits forming and required for assembly of complex II and CoQ_{10}, subdivided according to their role. Significantly upregulated genes are in red text, downregulated genes are in blue, while unchanged genes are in black. Abbreviations: SDH, succinate dehydrogenase; SDHAF, succinate dehydrogenase assembly factor; PDSS, decaprenyl diphosphate synthase; COQ, coenzyme Q.

Genes upregulated in response to insulin include 3 out of 4 of the core complex subunits (*SDHB*, *SDHC*, *SDHD*). Genes related to formation of CoQ_{10} show variable responses in response to insulin. Thus, expression of *COQ8A, COQ8B* and *COQ10B* decreases in response to insulin, while the other 8 out of 11 factors increase in response to insulin (figure 84).

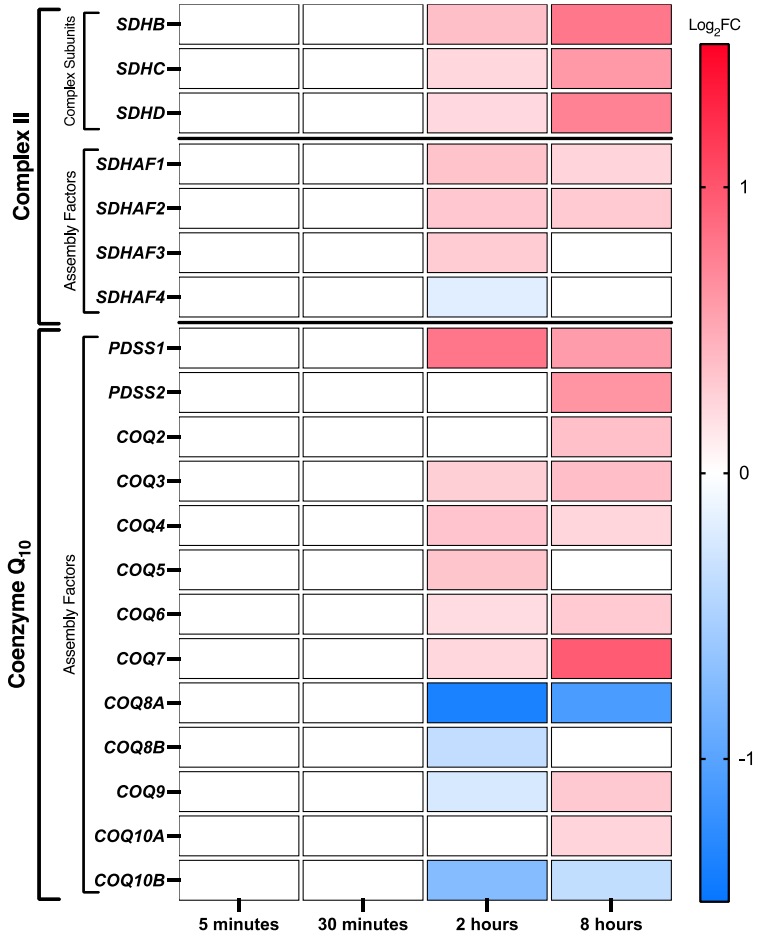


Figure 84. Heatmap of effects of insulin on the expression of genes encoding mitochondrial complex II and factors involved in CoQ_{10} synthesis. The heatmap shows the fold change in expression of each gene displayed as log_2-fold change, at each extraction time point. Upregulation in response to insulin treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading.

Complex III transfers electrons received from CoQ_{10} to cytochrome c which, in turn, carries electrons to complex IV. Complex III is composed of a number of subunits, while cytochrome c is derived from a single nuclear encoded gene (figure 85) (563, 564). Insulin treatment of HC11 cells upregulates 15 out of 17 of these genes.

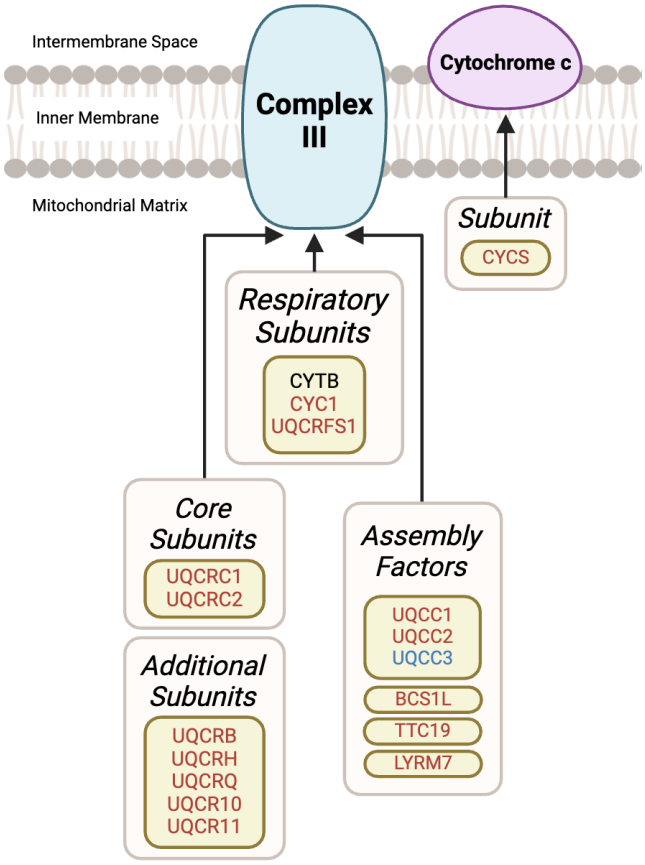


Figure 85. Genes encoding mitochondrial complex III subunits and cytochrome c. The subunits forming and required for assembly of complex III and cytochrome c, subdivided according to their role. Significantly upregulated genes are in red text, downregulated genes are in blue, while unchanged genes are in black. Abbreviations: CYTB, cytochrome B; CYC1, cytochrome C1; UQCRFS, ubiquinol-cytochrome c reductase Rieske iron sulphur polypeptide; UQCEC, ubiquinol-cytochrome c reductase core protein; UQCRB, ubiquinol-cytochrome c reductase binding protein; UQCRH, ubiquinol-cytochrome c reductase hinge protein; UQCR, ubiquinol-cytochrome c reductase subunit; UQCC, ubiquinol-cytochrome c reductase assembly factor; BCS1L, ubiquinol-cytochrome c reductase chaperone; TTC19, tetratricopeptide repeat domain-containing protein; LYRM7, LYR motif-containing protein 7; CYCS, cytochrome c.

As shown in figure 86, insulin upregulates genes for all subunits associated with complex III apart from two (*CTYB* and *UQCC3*). In addition, insulin upregulates expression of the gene encoding cytochrome c. These effects begin at 2 hours of insulin treatment and peak at 8 hours.

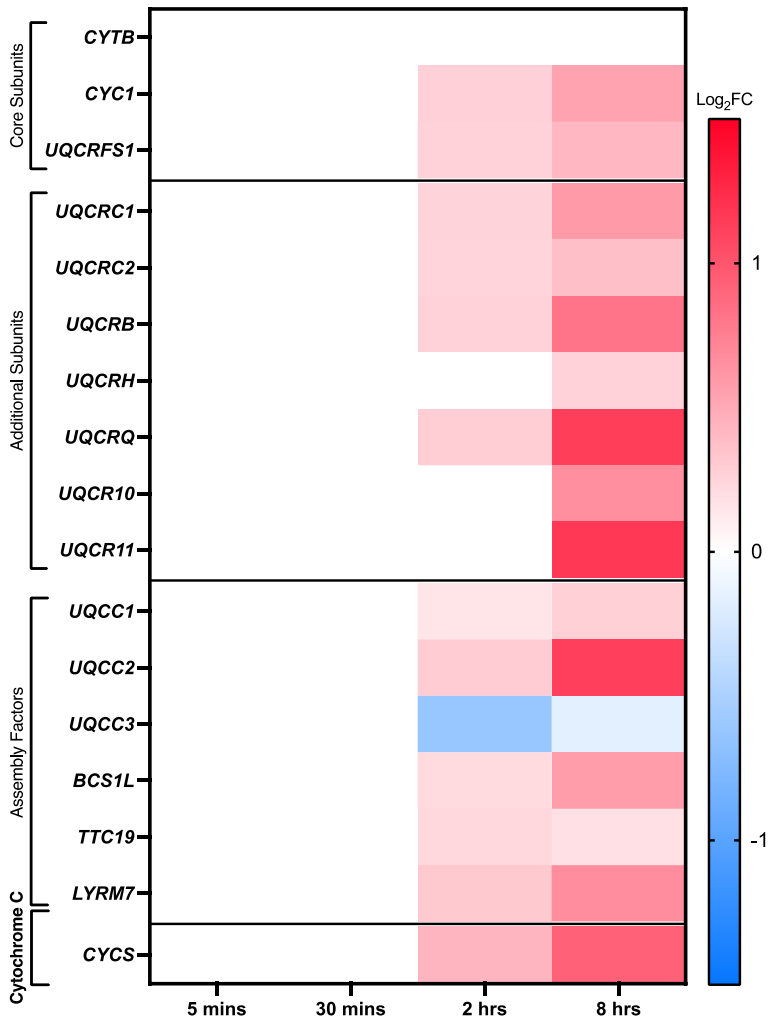


Figure 86. Heatmap of effects of insulin on the expression of genes encoding mitochondrial complex III subunits and cytochrome c. The heatmap shows the fold change in expression of each protein, displayed as log_2-fold change, at each extraction time point. Upregulation in response to insulin treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading.

Complex IV is the terminal electron acceptor, receiving electrons from cytochrome c and reducing oxygen to water (565). The core subunits and assembly factors comprising complex IV are shown in figure 87.

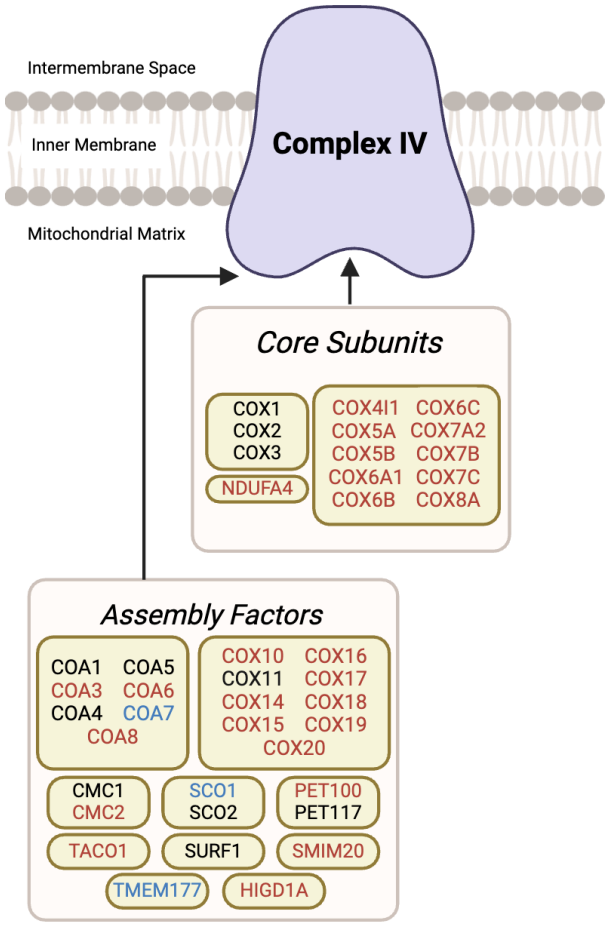


Figure 87. Genes encoding mitochondrial complex IV subunits. The subunits forming and required for assembly of complex IV, subdivided according to their role. Significantly upregulated genes are in red text, downregulated genes are in blue, while unchanged genes are in black. Abbreviations: COX, cyclo-oxygenase; NDUF, NADH-ubiquinone oxidoreductase; COA, cytochrome c oxidase assembly factor; TACO1, translational activator of cytochrome c oxidase 1; CMC, C-X9-C motif-containing protein; SURF1, surfeit locus protein 1; SMIM20, small integral membrane protein 20; TMEM177, transmembrane protein 177; SCO1, synthesis of cytochrome c oxidase 1; PET100, cytochrome c oxidase chaperone; HIGD1A, hypoxia inducible gene 1 (HIG1) domain family member 1A.

The majority of genes encoding complex IV proteins are altered after 8 hours of insulin treatment, while only a minority change at 2 hours and none at 5 or 30 minutes (figure 88). At 8 hours, most (27 out of 41) genes show increased expression, but some decrease (*COA7*, *TMEM177*, *SCO1*) and others are unchanged (*COX1*, *COX2*, *COX3*, *COA1, COA4*, *COA5*, *COX11*, *CMC1*, *SURF1*).

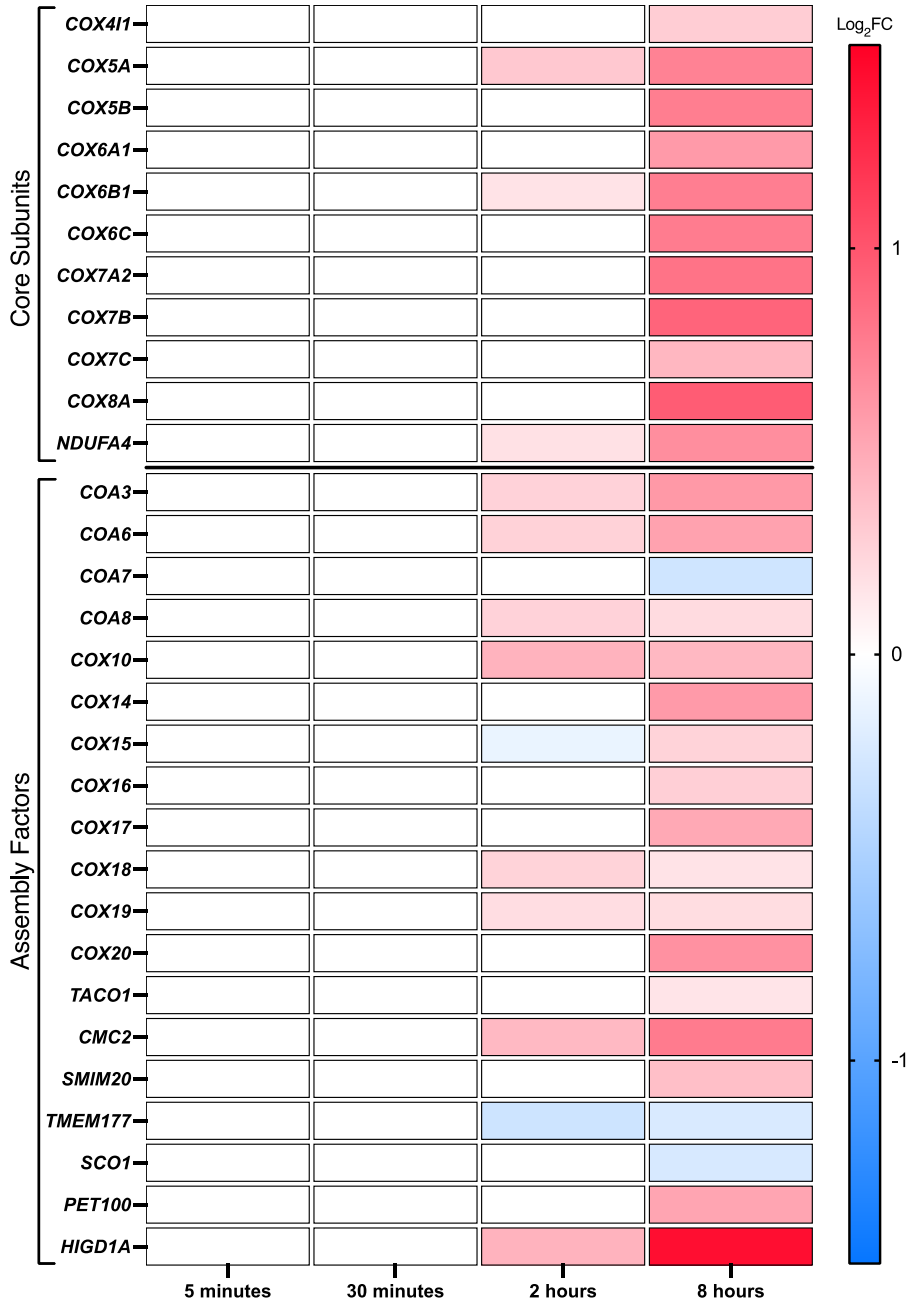


Figure 88. Heatmap of effects of insulin on the expression of genes encoding mitochondrial complex IV. The heatmap shows the fold change in expression of each protein, displayed as log_2-fold change, at each extraction time point. Upregulation in response to insulin treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading.

ATP synthase (complex V) is responsible for generating ATP. The proton gradient generated by complexes I-IV flows through the F_0 component of ATP synthase, driving rotary motion of the F_0-F_1 complex (566). This results in conversion of ADP and phosphate to ATP. The subunits that make up ATP synthase are shown in figure 89.

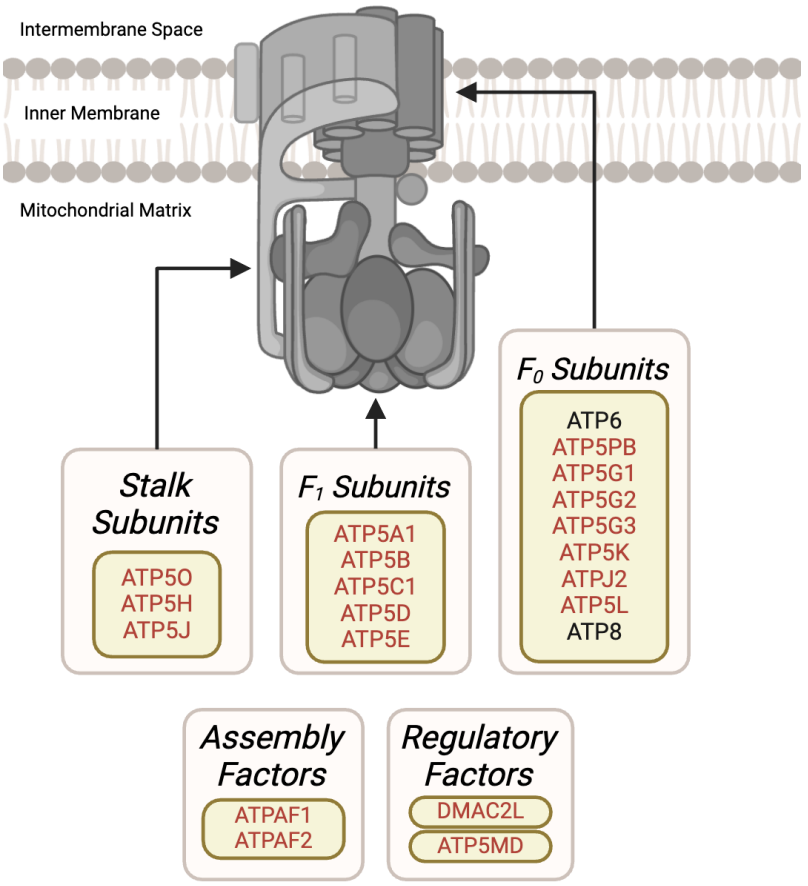


Figure 89. Genes encoding mitochondrial ATP synthase subunits and assembly and regulatory factors. The subunits forming and required for assembly of ATP synthase, subdivided according to their role. Significantly upregulated genes are in red text, while unchanged genes are in black. Abbreviations: ATP, ATP synthase subunit; ATPAF, ATP synthase mitochondrial F_1 complex assembly factor; DMAC2L, distal membrane arm assembly component 2 like.

In response to insulin, no changes in expression of ATP synthase genes are seen at 5 and 30 minutes (figure 90). Some insulin-induced increases in expression are apparent at 2 hours,

while 19/21 are upregulated by insulin at 8 hours. The only unchanged genes are *ATP6* and *ATP8*.

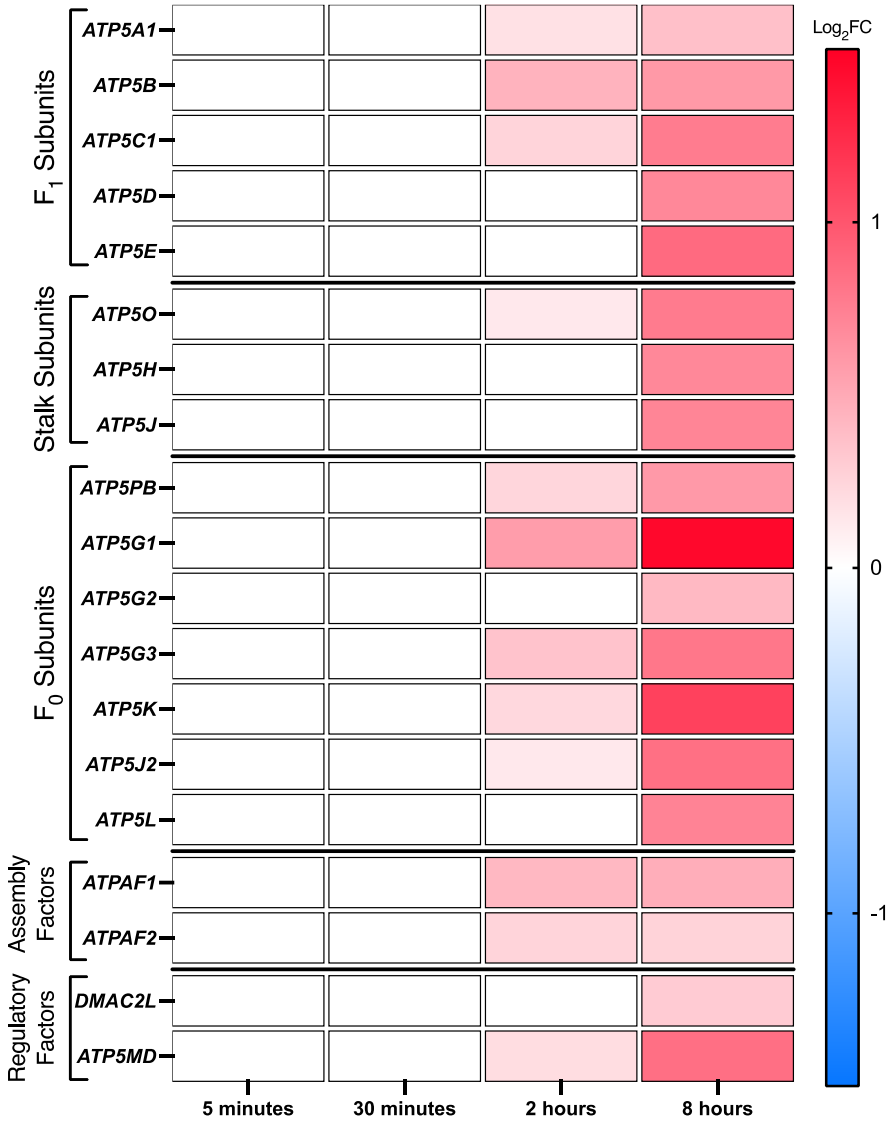


Figure 90. Heatmap of effects of insulin on the expression of genes encoding mitochondrial ATP synthase subunits and assembly and regulatory factors.. The heatmap shows the fold change in expression of each protein, displayed as log_2-fold change, at each extraction time point. Upregulation in response to insulin treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading.

We found no significant increase in expression of genes encoding proteins considered important for mitochondrial biogenesis, namely peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α), nuclear respiratory factor 1 (NRF1), nuclear respiratory factor 2 (NRF2) and transcription factor A mitochondrial (TFAM) (data not shown) (567). Furthermore, we found no changes in expression of the core regulators of mitochondrial fusion and fission, optic atrophy protein 1 (OPA1), mitofusin 1 (MFN1), mitofusin 2, and dynamin-related protein 1 (DRP1) (data not shown) (568).

6.3.4.6. Synthetic effects of insulin

Milk production is associated with upregulation of lipid, protein and lactose synthetic processes (108). To evaluate whether insulin might have any synthetic role in HC11 cells, we evaluated expression of genes related to lactose synthesis, lipid synthesis and milk proteins (appendix 4). The catalytic subunit of lactose synthase (*B4GALT1*) was not increased following insulin treatment, while the regulatory subunit (*LALBA*) was not expressed. Similarly, several genes related to fatty acid synthesis (*ACLY, ACACA, ACACB, FASN*) were decreased. Finally, we observed no increase in expression of key milk proteins, namely casein proteins (*CSN3, with CSN1S1, CSN1S2, CSN2* not expressed in insulin-treated cells).

6.4. Discussion

These cellular studies highlight the importance of insulin in regulating mammary epithelial bioenergetics at lactation onset. My work has shown that insulin signals through the PI3K-

Akt pathway in HC11 cells. The major effect of insulin is to increase basal and maximal mitochondrial oxygen consumption (1.7-fold and 2.4-fold respectively). Transcriptomic analysis of HC11 cells stimulated with insulin demonstrates that the underlying mechanism for this involves global upregulation of mitochondrial electron transport chain components. Insulin also upregulates basal glycolysis in HC11 cells and induces transcription of enzymes related to glycolysis and to the pentose phosphate pathway.

6.4.1. Mammary insulin sensitivity increases during lactogenic differentiation

Rodent and human studies have shown that mammary insulin sensitivity increases during lactation (569). Importantly, increased insulin responsiveness in the mammary gland, rather than that of conventional insulin-responsive tissues such as adipose tissue, underlies the increased systemic insulin sensitivity during lactation (569). My clinical studies have demonstrated an increase in systemic insulin sensitivity, reflected by a fall in serum insulin concentrations, on days 1-4 post-partum (figure 37).

In keeping with this, I have shown increased insulin receptor (*INSR*) expression in HC11 cells following differentiation to a lactogenic phenotype, consistent with increased mammary cell insulin sensitivity. This is supported by previous work on differentiated HC11 cells, whereby *INSR* isoform B *(INSRB)* is upregulated during differentiation and considered functionally important for the terminally differentiated mammary epithelial cells (380). However, there remains a poor understanding of the mechanisms by which insulin acts on the mammary epithelium and we have therefore undertaken studies to investigate this further.

6.4.2. Insulin has a predominantly metabolic role in lactogenic mammary cells

6.4.2.1. Insulin signals through the PI3K-Akt pathway in mammary epithelial cells

Akt, particularly Akt isoform 1 (Akt1), has been shown to be essential for milk production in mice. Thus, Akt1 null mice are unable to produce sufficient quantities of milk due to dysregulation of metabolic pathways that support milk production (570). Mammary metabolic defects in these mice are related to glucose and lipid metabolism, while cell proliferation, differentiation, and apoptosis are unaffected. In keeping with this, overexpression of Akt in the CIT3 mammary epithelial cell line leads to upregulation of glucose transport and lipid metabolism (571).

A major activator of the PI3K-Akt pathway is insulin, and this pathway is essential in mediating the cellular metabolic effects of insulin (539, 540). The importance of insulin for lactation in regulating metabolic changes required for milk production is highlighted by early animal studies (572-577). Furthermore, insulin has been shown to activate Akt in the lactating mouse mammary gland (578). We have therefore focused on insulin-mediated activation of the PI3K-Akt pathway, rather than the Ras-Raf-MEK-MAPK pathway which mediates mitogenic functions of insulin (539).

We sought to evaluate whether insulin signals through this pathway in HC11 cells. We have demonstrated a dose-dependent Akt phosphorylation in response to insulin treatment, with the greatest effect induced by 100 nM insulin (figure 63). Furthermore, we have shown that insulin produces a rapid induction of Akt phosphorylation, starting within 5 minutes, peaking at 15 minutes, and persisting until 60 minutes post-stimulation (figure 64). This pattern of Akt activation is typical of insulin-responsive cells, and in keeping with the effects of insulin

in other cell types (579-583). Thus, insulin is capable of producing a rapid and robust activation of Akt in the HC11 mammary cell model.

6.4.2.2. Insulin does not have a major synthetic role in differentiated HC11 cells

Mice with a mammary-specific insulin receptor knockout show reductions in expression of genes related to milk proteins, lipid synthetic enzymes and lactose synthesis (45). These mice also display impaired mammary development. Furthermore, treatment of HC11s with insulin has previously been reported to increase expression of β-casein and WAP (380). Notably, this protocol involved treatment with or without insulin during the two-day differentiation period, meaning that cells were not fully differentiated when treatment was commenced.

Our work has demonstrated that insulin does not promote synthetic processes. However, it is important to note that our studies have involved treatment of cells that have undergone lactogenic differentiation, unlike previous studies. This implies that insulin may be important for lactogenic differentiation but has little role in synthesis of milk components in mammary epithelial cells that are already differentiated.

6.4.2.3. Insulin upregulates glycolytic and pentose phosphate pathways

Early studies in mice showed upregulation of glycolysis at lactation onset (584-586). However, a more recent transcriptomic study has demonstrated that the early steps in glycolysis are inhibited in order to divert glucose to lactose synthesis (108). Similarly,

analysis of human milk fat globule RNA during the shift from colostrum to transitional milk is associated with inhibition of glycolysis (77). Thus, there remains uncertainty about the direction of changes in the glycolytic pathway at lactation onset and how these changes may be regulated. Understanding this is of importance in mammary epithelial cells because cellular glycolysis is important for generating metabolic intermediates for synthetic processes, producing pyruvate for entry into the TCA cycle, and also synthesising ATP under conditions of oxygen deprivation (587).

Insulin, through the PI3K-Akt pathway, is an important regulator of cellular glycolysis (548-554). Our work is the first to assess the effect of insulin treatment on the entire glycolytic and pentose phosphate pathway in cultured mammary cells that express milk proteins. We have observed an increase in basal glycolysis, but not glycolytic capacity, in response to treatment with 100 nM insulin (figures 65 and 66). In addition, GSEA of transcriptomic data generated from insulin-treated HC11 cells identified glycolysis as the most changed pathway with insulin treatment leading to the upregulation of the majority of glycolytic enzymes (25 out of 31) (figures 75, 76 and 77). We also observed increased expression of the gene encoding the major mammary glucose transporter, GLUT1, which may enable increased glucose uptake.

These changes may help to support lactation. At lactation onset, there is substantial diversion of glucose towards lactose synthesis (588). Furthermore, glycolytic intermediates are diverted towards other synthetic processes. For example, dihydroxyacetone phosphate can be metabolised to glycerol-3-phosphate, which in turn is converted to produce glycerol necessary for triglyceride synthesis (589). In addition, there may be diversion of substrates to the pentose phosphate pathway. This pathway operates in parallel to the glycolytic

pathway and is necessary for production of nicotinamide adenine dinucleotide phosphate (NADPH), required for maintaining redox balance and for use in anabolic reactions, as well as ribulose-5-phosphate, which is a precursor for nucleotide synthesis (590). Previous analysis of mouse mammary glands at lactation onset has demonstrated upregulation of genes encoding proteins which form this pathway (108). Here, we have demonstrated that insulin increases expression of 7 out of 14 components of the pentose phosphate pathway in HC11 cells (figures 78 and 79). Importantly, there is upregulation of glucose-6-phosphate dehydrogenase (G6PD), the rate limiting enzyme in the pentose phosphate pathway (591). Finally, increased pyruvate produced by glycolysis can enter the TCA cycle, supporting the increased mitochondrial activity in insulin-stimulated HC11 cells (592).

However, my study showed that the effects of insulin on glycolysis are mild, and glycolytic capacity is unchanged. This could relate to the experimental conditions used. The pH Xtra assay is run under atmospheric conditions, meaning that atmospheric oxygen concentrations (approximately 20%) are higher than those which normal breast tissue might experience *in vivo* (median 8.7%) (593). This could cause HC11 cells to preferentially use mitochondrial oxidative phosphorylation, and therefore reduce their glycolytic activity, which may mask any effects of insulin on glycolysis.

It is also important to note that the majority of glycolytic reactions are bidirectional. For example, fructose-6-phosphate is converted to fructose-1,6-bisphosphate through the actions of phosphofructokinase (PFK), while the reverse reaction is catalysed by fructose-1,6-bisphosphatase (FBP). Isoforms of both enzymes are upregulated by insulin in HC11 cells, and it is not possible to determine the overall effect of this by looking at gene expression alone.

The effects of insulin on glycolysis and the pentose pathway are complex and require further evaluation. Use of a metabolomic approach, coupled to ^{13}C metabolic flux analysis, would allow a detailed assessment of metabolite flux (594).

6.4.2.4. Insulin has a major role in mammary mitochondrial function

Milk production is highly energy-consuming and requires significant mammary mitochondrial changes to support this (94, 95, 430). In keeping with this, oxygen consumption is reported to increase in HC11 cells as they differentiate to a lactogenic phenotype (592). However, the underlying drivers for the mammary mitochondrial changes are not known. The insulin-Akt pathway is known to promote mitochondrial respiration in non-mammary cells (544-546). For example, insulin increases mitochondrial biogenesis and network formation (490, 491, 541-543), and is reported to upregulate the expression of genes encoding mitochondrial electron transport chain proteins in skeletal muscle cells (595-597). We therefore assessed the effects of insulin on mammary mitochondrial function and possible mechanisms underlying this.

Insulin-treated HC11 cells with a lactogenic phenotype showed an increase in basal and maximal oxygen consumption compared to control cells, confirming a role for insulin in increasing mitochondrial activity (basal respiration) as well as capacity of cells to respond to increased bioenergetic demand (maximal respiration) (figures 67 and 68).

We next sought to evaluate the mechanisms underlying this by undertaking transcriptomic assessment of insulin-treated lactogenic HC11 cells. We focused on the effects of insulin on the mitochondrial ETC. The ETC consists of complexes I-IV, which generate a proton

gradient, and complex V (ATP synthase), which uses the proton gradient to produce ATP (559, 560). We assessed expression of genes related to these complexes and to CoQ_{10} and cytochrome c, which are important for electron transfer between complexes. We found that the majority of genes encoding proteins forming core, assembly and regulatory proteins are upregulated in differentiated HC11 cells in response to insulin treatment (figures 81-90).

Complex I of the ETC oxidises NADH generated by the TCA cycle, and passes two electrons to CoQ_{10}. Complex I is the main point of entry of electrons into ETC and is considered the rate-limiting step in mitochondrial respiration (598). Complex I consists of 'core' subunits which form the functional complex, and a number of proteins which are required for assembly of the complex (599). In a mouse model of diabetes, insulin signalling counteracts FOXO-mediated inhibition of complex I subunit gene expression in muscle (597). In keeping with this, we have shown that insulin increases the expression of the majority of genes encoding complex I proteins in HC11 cells. Given this complex is rate-limiting, this may account for the increase in basal oxygen consumption that we observed.

Complex II (succinate dehydrogenase) has roles in both the ETC and TCA cycle, oxidising succinate to fumarate and transferring electrons from succinate to CoQ_{10} (600). While complex I is rate-limiting and therefore the principal determinant of basal respiration, complex II appears to be the main source of reserve respiratory capacity, and therefore important in determining maximal respiration (601). Insulin treatment of differentiated HC11 cells results in increased expression of core subunits and assembly factors of complex II, which is likely to account for the increase in maximal oxygen consumption that we have observed.

CoQ_{10} (ubiquinone) is a lipophilic molecule located in the inner mitochondrial membrane, which is essential for transport of electrons from complexes I and II to complex III (602). Biosynthesis of CoQ_{10} involves a series of enzymatic steps catalysed by COQ1, COQ2, COQ3, COQ5, COQ6 and COQ7, while COQ4, COQ8, COQ9 and COQ10 are regulatory factors (603). This work is the first to show that insulin treatment results in increased expression of most of these factors, with the exception of COQ8A, COQ8B and COQ10B, which are decreased. The overall effect of these changes on ubiquinone requires further investigation. However, given COQ8 activity is not considered rate limiting, and COQ10A and COQ10B have identical functions (604), the overall effect of insulin is likely to be an increase in CoQ_{10}.

Electron transfer from CoQ_{10} is to complex III, which subsequently transfers electrons to cytochrome c (560). Insulin treatment of HC11 cells increases expression of core and additional subunits of complex III except for *CYTB* (unchanged), and all assembly factors except for *UQCC3* (1.1-fold decrease). UQCC3 is involved in assembly of mitochondrial supercomplexes including complex III (605), which are higher-order arrangements comprising several ETC complexes, and may increase efficiency of mitochondrial electron transfer (606). The effect of decreased *UQCC3* expression is unclear. These effects of insulin on complex III proteins have not been previously described.

Complex IV (cytochrome c oxidase) catalyses the final transfer of electrons from cytochrome c to oxygen, forming water in the process (565). This complex is considered to have a low reserve capacity and is therefore represents a major site of regulation of mitochondrial respiration (607, 608). Insulin has been previously reported increase expression of genes encoding complex IV proteins in muscle (595). In HC11 cells, most genes show increased expression, but some decrease and others are unchanged. Of the genes that are

downregulated, the effect of reduced expression of the assembly factors COA7, TMEM177 and SCO1 is uncertain and requires further investigation.

ATP synthesis is performed by complex V (ATP synthase), a rotary protein complex that uses the proton gradient established by complexes I-IV to convert ADP and phosphate to ATP (566). Withdrawal of insulin in individuals with type 1 diabetes has been reported to decrease expression of ATP synthase components and mitochondrial ATP production rate in muscle (609). We have shown that insulin treatment of HC11 increases expression of nearly all genes encoding proteins that form ATP synthase, an effect which is likely to support mitochondrial capacity to generate ATP.

This study is the first to comprehensively assess the effect of insulin on the mitochondrial ETC in mammary cells and provides insights into how insulin might support the production of energy required for milk synthesis. Our cellular findings indicate that insulin may play a key role in promoting mammary mitochondrial biogenesis with increased synthesis of mitochondrial ETC components required for ATP production. The effects of insulin on the ETC in HC11 cells are aligned with previous work in non-mammary cells. Thus, insulin increases expression of ETC complex subunits in dorsal root ganglia neuronal cells, while insulin resistance in skeletal muscle cells leads to reduced expression of subunits in complexes I, III and IV (610, 611). These effects are also in keeping with the observed reduction in ETC subunit expression and ETC activity observed in liver and skeletal muscle of people with insulin resistance (612-615).

The mechanism underlying insulin-mediated increases in ETC subunit expression requires further investigation. PGC1α is considered the central regulator of mitochondrial gene expression, and in turn promotes expression of the nuclear transcription factors NRF1 and

NRF2, as well as the mitochondrial transcription factor TFAM (616). Transcriptomic assessment of insulin-treated HC11 cells demonstrates no changes in expression of the genes encoding these proteins. This suggests an alternative mechanism is important for the actions of insulin. One possible mechanism is the Akt-mediated phosphorylation and inhibition of FOXO proteins (555). The FOXO transcription factors inhibit expression of ETC complexes, as well as their reducing activity through induction of the haem-catabolising HMOX1 protein (617-619). Delineating the pathways by which insulin produces transcriptional changes should be addressed in future studies.

The effects of insulin in HC11 cells are summarised in figure 91.

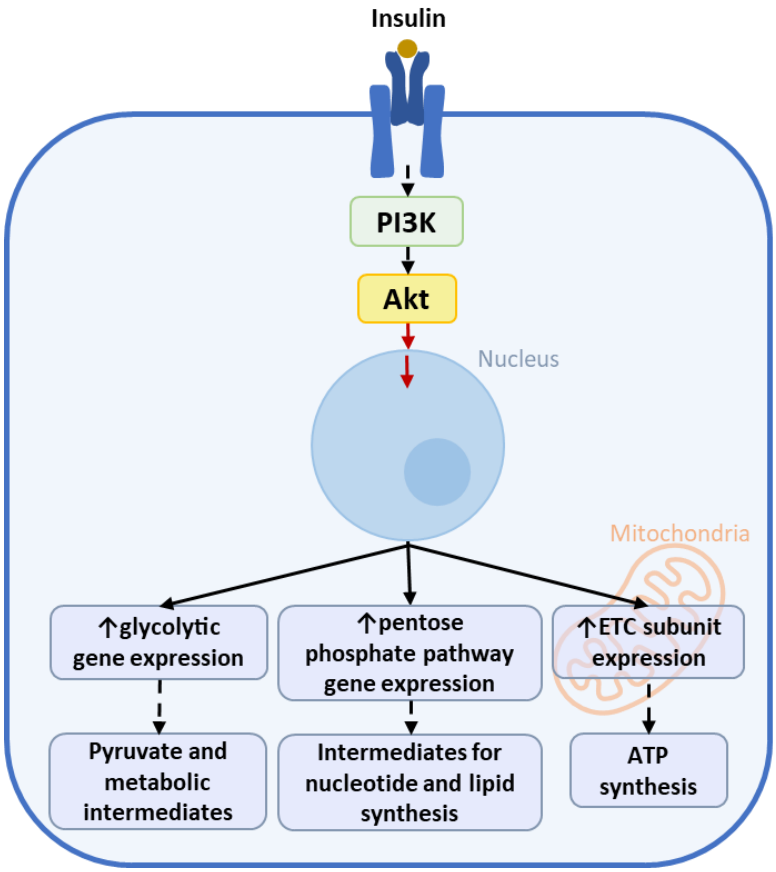


Figure 91. Summary of changes produced by insulin in HC11 cells. Key cellular metabolic pathways altered by insulin in HC11 cells with a lactogenic phenotype.

6.4.3. Potential implications of insulin resistance on lactation

The global prevalence of obesity has increased considerably over the last four decades, from 4.6% in 1980 to 14.0% in 2019 (620). Obesity is associated with numerous adverse health outcomes, including non-alcoholic fatty liver disease (NAFLD), cardiovascular disease and malignancy (621-623). Importantly, obesity causes insulin resistance and predisposes to the development of type 2 diabetes (624). It is therefore unsurprising that the global prevalence of type 2 diabetes is also increasing and is expected to affect 0.7% of individuals by 2030, with an even higher prevalence than this in Western Europe (625, 626).

Overweight and obese women (BMI 25-29.9 and BMI ≥30, respectively) are at risk of delayed lactation onset and early cessation of breastfeeding (381, 382). We have demonstrated that women with an increased BMI have systemic insulin resistance (figure 39). Given that insulin responsiveness of the mammary gland is the major determinant of systemic insulin sensitivity during lactation (569), it is likely that women with an elevated BMI have mammary insulin resistance. However, this requires further investigation and could be done through comparative analysis of *INSR* expression in milk fat globule RNA obtained from women with normal and high BMIs when sufficient sample numbers from each group are available.

Our cellular data provides insights into how insulin resistance may impair lactation. In differentiated HC11 cells, insulin appears to have a major role in promoting mitochondrial oxidative phosphorylation and is therefore likely to be important for generating the large amounts of energy required to support lactation. Insulin resistance is therefore likely to impair mitochondrial function and impair generation of ATP required for key metabolic processes. This is in keeping with the mitochondrial dysfunction observed in skeletal muscle, adipose tissue and liver in states of insulin resistance (627). Insulin also appears to have a role in regulating glycolysis and the pentose phosphate pathway, and therefore availability of intermediates required for milk synthesis. Insulin resistance may therefore limit availability of key substrates required for milk production.

Understanding the impact of insulin resistance in lactation requires further work. Collection of sufficient numbers of milk samples from breastfeeding women in the early post-partum period will allow comparison between women with normal and high BMIs. Thus, milk fat globule RNA can be used for transcriptomic assessment of gene expression, with a particular

focus on genes related to mitochondrial function and milk synthesis. In parallel, metabolomic assessment of whole milk can help to determine if milk composition is affected by insulin resistance. Use of a mouse model of insulin resistance may provide adjunctive information, enabling transcriptomic, proteomic and/or metabolomic assessment of mammary glands, and provide further mechanistic insights. This work is necessary considering the increasing global burden of obesity and the need to develop therapeutic strategies to counteract the impact of insulin resistance on lactation.

6.4.4. Limitations

While *INSR* expression in HC11 cells provides some insights, evaluation of mammary insulin sensitivity in the human mammary setting is important. This would also provide insights into differences between women with a normal and high BMI. Such evaluation would require n=10 women with normal BMI and n=10 women with high BMI on each post-partum day, based on a mean difference of 1.25 *INSR* normalised expression units (628), a pooled SD of 0.72, an alpha error probability of 0.05, a power of 0.95 and an allocation ratio of 1:1. This work is important for understanding how mammary insulin sensitivity increases at lactation onset, and how this may differ in women with a high BMI. Moreover, this will allow confirmation that the increase in systemic insulin sensitivity that we have observed is due to increased mammary insulin sensitivity.

Understanding the metabolic effects of insulin by using the HC11 cellular model of lactation is also associated with limitations. Lactogenic differentiation of HC11 cells is defined by expression of β-casein, but HC11 cells do not differentiate to the same extent as mammary

epithelial cells *in vivo* and do not produce milk (396). Furthermore, there is no established protocol for evaluation of insulin resistance in a cellular model of lactation. While use of cellular inhibitors of insulin signalling, such as PI3K and Akt inhibitors (629), can help in understanding insulin resistance at a cellular level, this non-physiological. A complete understanding of the physiological effects of insulin, as well as the adverse effects of insulin resistance, therefore requires further work using animal models and samples collected from breastfeeding women.

Assessment of mitochondrial and glycolytic activity through use of OCR and ECAR, respectively, is associated with a number of challenges (630). The pH Xtra assay evaluates pH change in the media but does not account for buffering capacity. The respiratory buffer we have used is designed to have a low buffering capacity but there will be some reduction of the pH change observed. Furthermore, production of CO_2 and atmospheric gas exchange can confound pH changes. The MitoXpress Intra assay looks at total oxygen consumption but is unable to separate this into oxygen consumption coupled to ATP production versus that uncoupled from ATP production. Thus, further metabolic studies should be considered in future work. One option for these is the Seahorse system (Agilent Technologies, USA) which is specifically designed to take measurements from media in proximity to cells, and measure the relative contributions of coupled and uncoupled respiration to oxygen consumption.

Finally, the existing transcriptomic work on HC11 cells suffers from the broader limitations of assessing gene expression, specifically that it does not necessarily directly correlate with protein activity or metabolite flux (631). Proteomic and/or metabolomic assessment of insulin-treated HC11 cells will therefore provide additional information. This is particularly

relevant for assessment of glycolysis and the pentose phosphate pathway, where the widespread transcriptomic changes do not correlate with absence of changes observed using end-point assessment of basal glycolysis and glycolytic capacity with media pH. Use of metabolite flux analysis would therefore help in understanding the effect of the transcriptomic changes observed on glycolytic intermediates necessary for milk synthesis. In a similar manner, mitochondrial function could be assessed more directly through measurement of ATP production.

7. CONCLUSION

7.1. Improved understanding of human lactation biology

7.1.1. The INSIGHT study: investigating secretory activation in humans

Breastfeeding confers important benefits for both mother and infant, and a normal onset of secretory activation is an important determinant of breastfeeding success (2-10, 13-17, 53). However, the systemic hormone concentrations required for secretory activation, as well as the mammary epithelial cell changes during this critical period, have not previously been systematically evaluated in humans.

The INSIGHT study represents the first study to evaluate both hormonal and mammary epithelial changes during the first few post-partum days and is a significant step in understanding secretory activation in women. The INSIGHT study has been designed to collect antenatal and post-natal clinical information, blood samples and milk samples from women. Blood samples collected have been used to evaluate hormonal changes during post-partum days 1-4, and across a breastfeeding episode, providing significant insights into the physiology of human lactation. Furthermore, these samples can be used for untargeted proteomic and metabolomic analyses, in order to better understand the systemic adaptations required to support lactation.

Milk samples collected from breastfeeding women can be used to understand mammary epithelial biology at lactation onset. Changes in cell phenotype during shedding and the presence of non-immune cells limit the utility of whole cells in milk, but the transcriptome of milk fat globule RNA is considered to be representative of mammary epithelial cells (77, 386). Thus, changes in mammary function can be assessed in the immediate post-partum

phase through analysis of RNA in milk fat. The milk collected can also be used for proteomic and metabolomic analysis, enabling a better understanding of milk composition and mammary biology on days 1-4 post-partum.

Combined collection of clinical data, blood and milk on days 1-4 post-partum is particularly important to the success of this study. The wealth of information generated is not only important in understanding the biology of normal lactation but can also be used to assess for relationships between lactation hormones and mammary epithelial function, as well as to evaluate the physiological basis underlying clinical risk factors for impaired lactation.

7.1.2. Novel hormone changes described during secretory activation

The INSIGHT study has identified several novel hormone changes at lactation onset in women, summarised in figure 92. Prolactin is well-established as an essential hormone for lactation, yet the concentrations of prolactin required for secretory activation have not previously been defined. We have shown that prolactin concentrations in maternal serum increase approximately 20% between the end of pregnancy and day 1 post-partum, and this may be important in stimulating milk synthesis after childbirth. Moreover, we have identified higher post-partum prolactin concentrations in multiparous women as compared to primiparous women. This may underlie the greater lactation success observed in multiparous women (25). How prolactin acts to promote lactation can be assessed in future work comparing the impact of parity and prolactin concentrations on mammary epithelial transcription and milk composition.

The INSIGHT study has also demonstrated novel changes in GH in the post-partum period. In addition, there is reduced insulin sensitivity in women with a high BMI, a known risk factor for delayed lactation onset and impaired lactation (460, 461). These findings have suggested a role for GH and insulin at lactation onset.

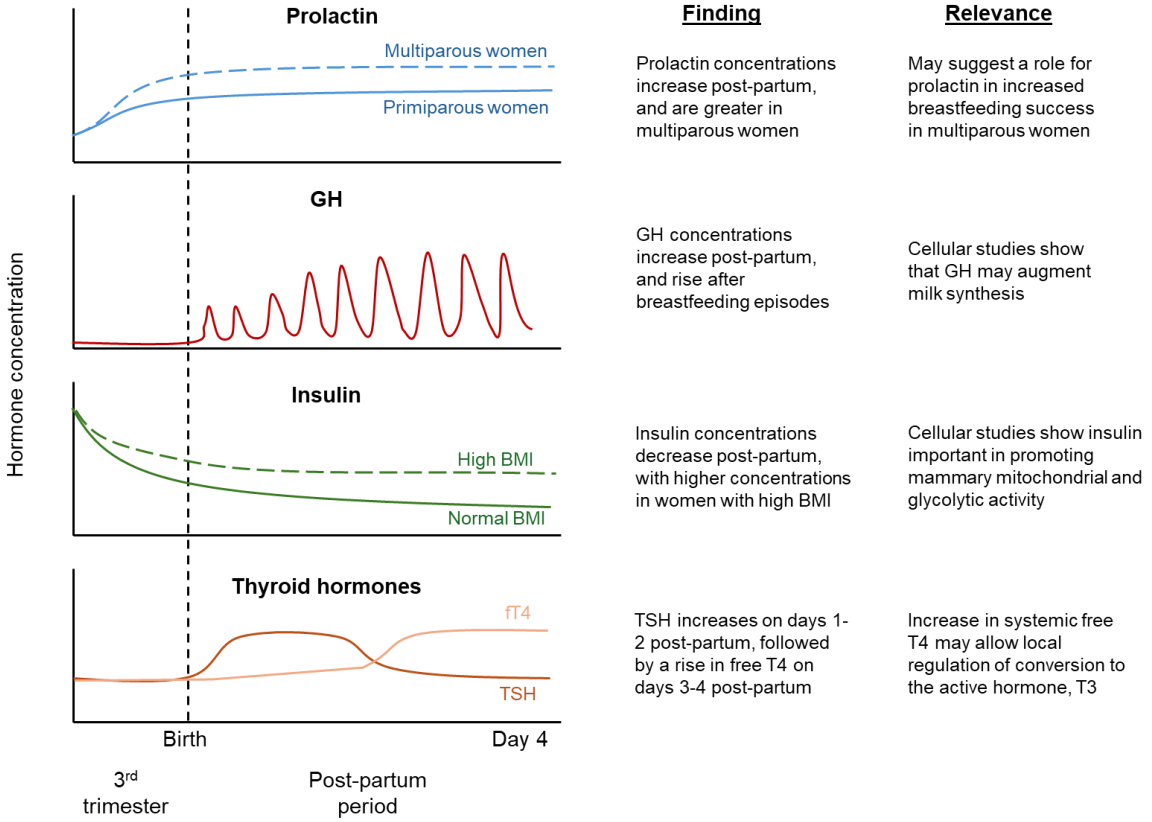


Figure 92. Summary of hormone changes in breastfeeding women at lactation onset. Schematic graphs of hormone changes on days 1-4 post-partum changes shown for prolactin, GH, insulin and thyroid hormones (TSH and free T4), alongside a summary of findings and potential relevance.

7.2. Growth hormone promotes milk synthesis during lactation

We have demonstrated that GH concentrations increase in the post-partum period and across a breastfeeding episode, supporting a role for GH in lactation. We also investigated

GH function using the HC11 mammary cell model and confirmed that GH, like prolactin, signals through STAT5. GH increases expression of milk proteins and proteins associated with lipid uptake and synthesis. Thus, GH may augment the synthetic effects of prolactin in the early post-partum period. These findings are consistent with the increased milk production in women injected with recombinant GH (377, 378). Furthermore, this work highlights the importance of the STAT5 pathway in milk production and shows potential redundancy between GH and prolactin in activation of this pathway.

7.3. Insulin promotes mammary metabolism during lactation

Insulin sensitivity falls post-partum, and we have demonstrated relative insulin resistance in women with a high BMI. Given that insulin resistance is a known risk factor for delayed and insufficient lactation, we sought to evaluate the role of insulin in lactation. Our data reveals that insulin signals through the PI3K-Akt pathway and promotes both mitochondrial respiration and glycolysis. Through transcriptomic assess of lactogenic HC11 cells, we have shown that insulin induces upregulation of mitochondrial ETC components and glycolytic enzymes and is therefore important in supporting the high mammary energetic requirements at lactation onset. This work has advanced our understanding of how insulin may support milk synthesis during lactation, as well as elucidating mammary processes that may be downregulated in states of insulin resistance.

7.4. Future work

7.4.1. Hormone reference intervals during secretory activation

Data from the INSIGHT study will be used to produce hormone reference intervals for each sampling time point, once a minimum of 120 measurements are made per time point (632). The first benefit of these reference intervals is the ability to identify endocrine abnormalities in women who suffer from delayed lactation. Investigation and correction of such abnormalities may improve lactation outcomes in these women. A second advantage is the improved diagnosis of endocrine disorders through use of time-specific reference intervals for the early post-partum period. Current diagnosis of these conditions in the post-partum period relies on reference intervals developed for non-pregnant, non-lactating women. Our data demonstrates changes in several hormones on post-partum days 1-4, suggesting reference intervals specific to this period are warranted.

To understand which hormonal axes are implicated in delayed onset of lactation, hormone data needs to be collected from affected mothers. Furthermore, recruitment of increased numbers of participants with conditions that affect lactation, such as diabetes or obesity, will help in understanding how these disorders affect lactation.

These studies will facilitate an improved understanding of the basis of normal and impaired lactation. The data generated will also form a potential basis for identification of some women who may have delayed onset of lactation before milk comes in, where these are caused by altered lactation hormones. They may also identify endocrine abnormalities that may be targeted therapeutically in women with delayed or insufficient lactation.

7.4.2. Development of lactation biomarkers

While measurement of hormones may help to identify women with endocrine disorders of lactation, hormone measurements are unlikely to be sufficient in identifying women at risk of delayed lactation from other causes. It is therefore important to identify biomarkers linked to secretory activation. Previous work has evaluated electrolyte, protein, lactose and citrate concentrations in milk as potential markers of secretory activation, but none of these markers have demonstrated utility in prospectively predicting normal lactation onset (633, 634).

The INSIGHT study offers scope to assess for prospective markers of delayed secretory activation, through proteomic and metabolomic evaluation of blood and milk samples. Use of biomarkers will allow targeted breastfeeding support to be provided to women at risk of delayed lactation. Furthermore, discovery of proteomic or metabolomic signatures of specific lactation disorders may allow targeted management of these disorders.

7.4.3. New therapies for low milk supply

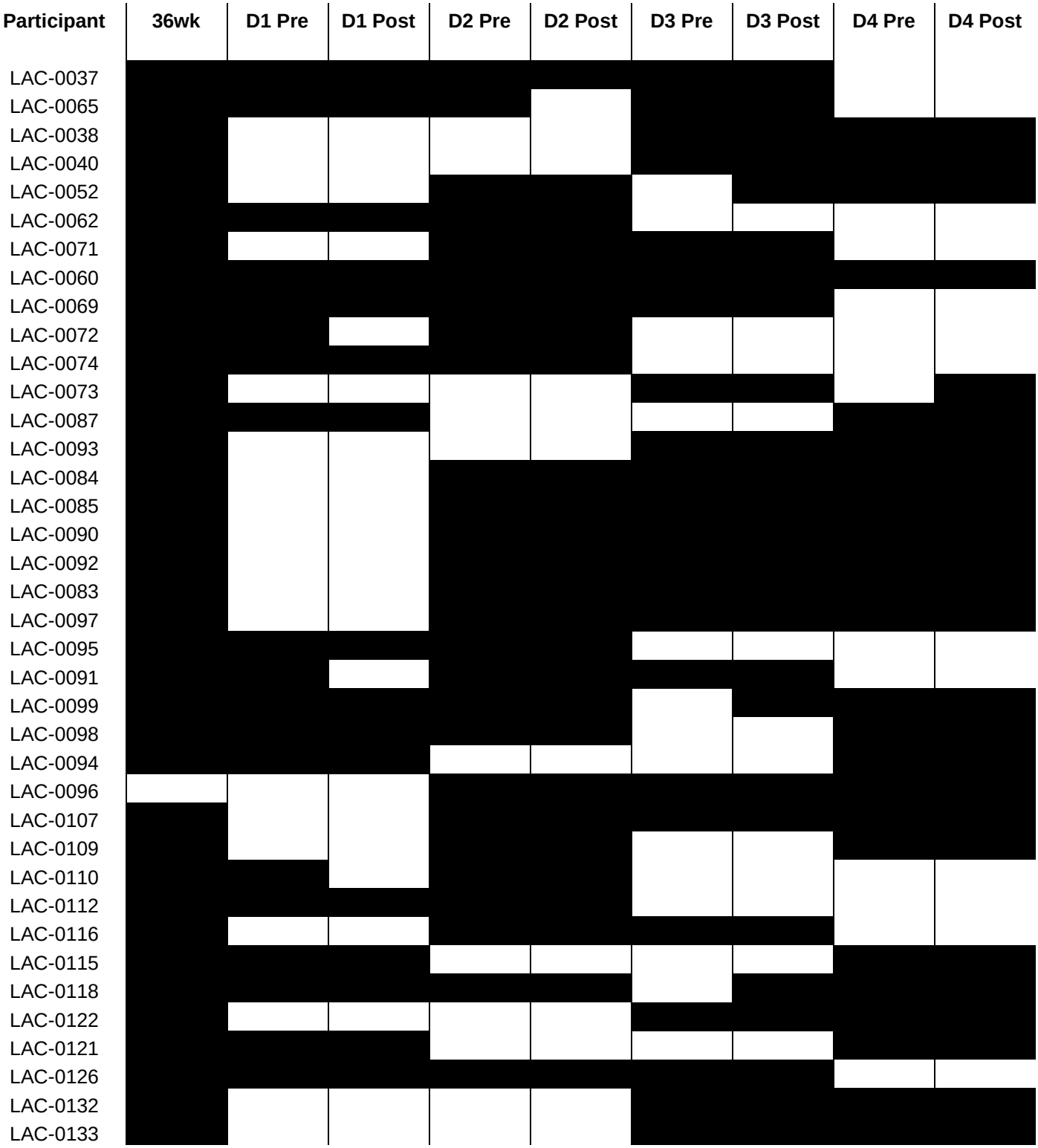
There is little research done in normal and abnormal human lactation, and currently no universally accepted systematic approach to evaluating and managing women who experience impaired lactation. Improved understanding of normal systemic and mammary changes at lactation onset is an essential first step in identifying pathways that may be dysregulated in disorders of lactation. Furthermore, understanding the key biological pathways offers increased scope to identify therapeutic targets in women with delayed or insufficient lactation. No pharmacological therapies currently exist for women suffering

from delayed or absent secretory activation (635). The clinical and cellular data reported in this thesis represent an advance in our understanding of human lactation biology, and a necessary foundation for the identification and management of lactation disorders.

8. APPENDICES

8.1. Appendix 1: Samples collected from each participant

Table S1. Blood samples collected for each participant, by sampling time point. A filled box indicates sample collection. Samples used for serum hormone analysis.

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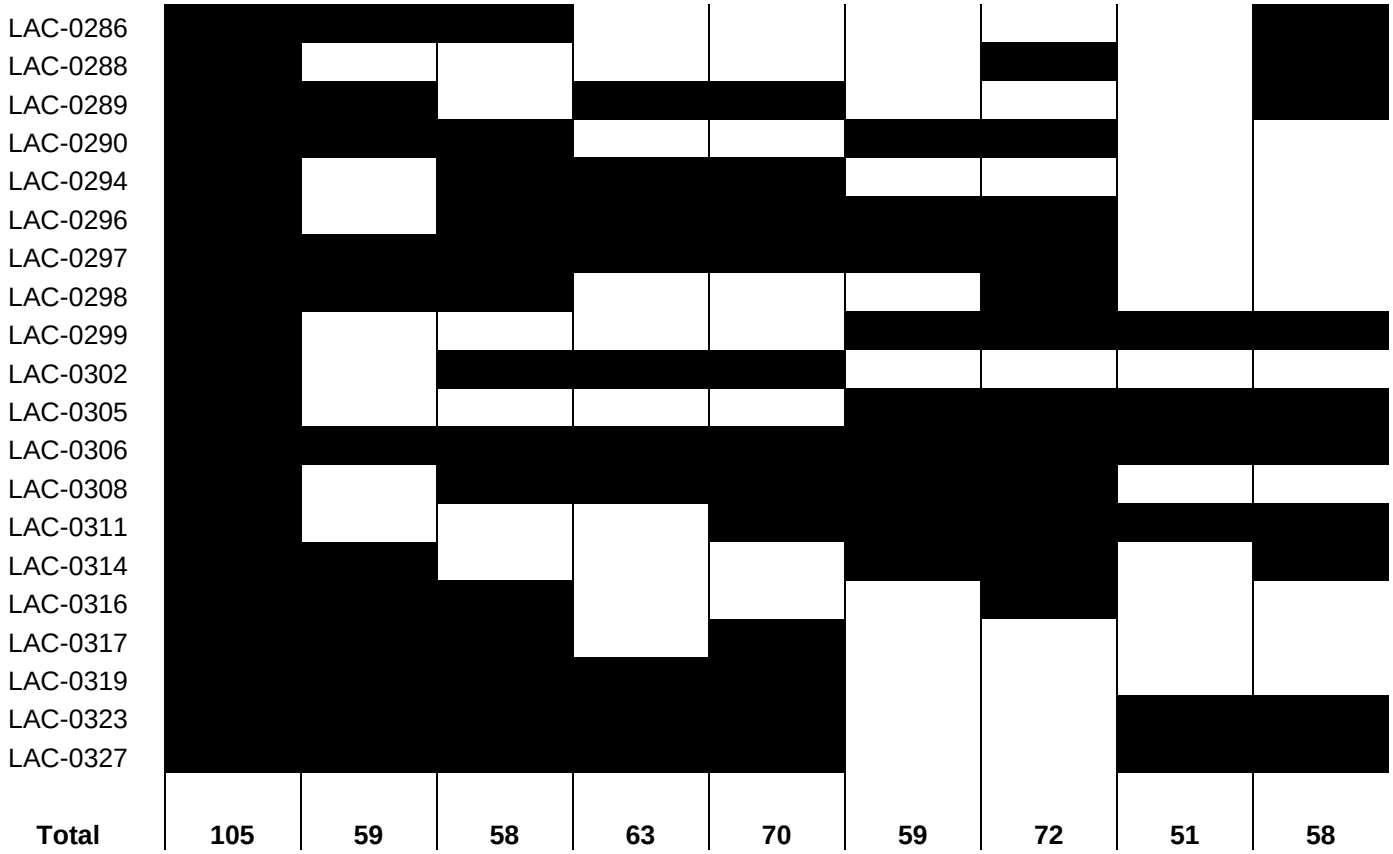
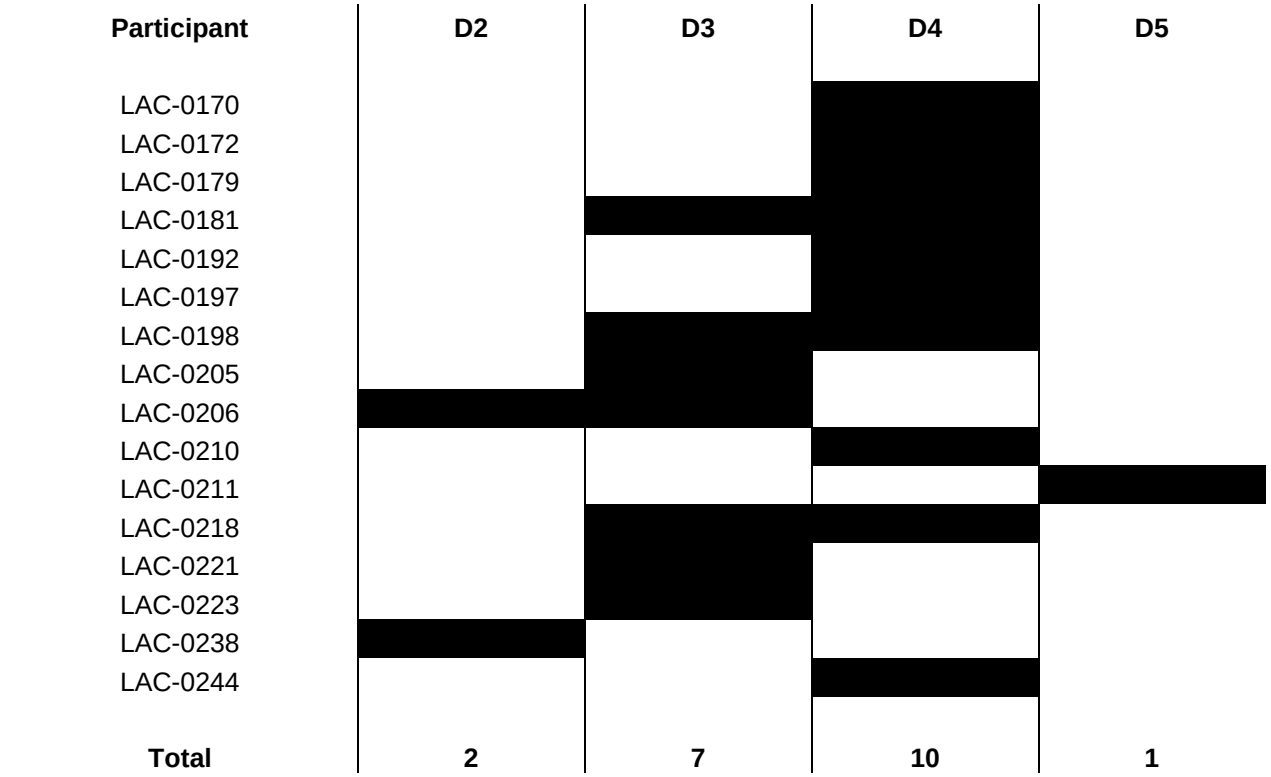


Table S2. Milk samples collected for each participant, by sampling time point. A filled box indicates sample collection. Samples used for assessment of *GHR* and *PRLR* expression by qRT-PCR.



8.2. Appendix 2: Primers used

Table S3. Primers used for qRT-PCR.

Gene	Species	Forward primer sequence	Reverse primer sequence
Actb	Mouse	GATGTATGAAGGCTTTGGTC	TGTGCACTTTTATTGGTCTC
B2m	Mouse	CGAACATACTGAACTGCTAC	AGCCAGGATATAGAAAGACC
Csn2	Mouse	GTATTTCCAGTGAGGAATCTG	ATTGCAAGAGATGGTTTGAG
GAPDH	Human	TCGGAGTCAACGGATTTG	CAACAATATCCACTTTACCAGAG
FAM190B	Human	GGAGTGTGACAATATGAACC	TCTGCTCAAGTCACTTTTTC
PGK1	Human	CTCAACAACATGGAGATTGG	CTTTGGACATTAGGTCTTTGAC
PRDX1	Human	CTGCCAAGTGATTGGTGCTTCTG	AATGGTGCGCTTCGGGTCTGAT
SYMPK	Human	ATGAGGACAAAGACTTGGAG	GACCAGATTAGCCACATTATC
RPL13A	Human	GTCTGAAGCCTACAAGAAAG	TGTCAATTTTCTTCTCCACG
RPLP0	Human	CGGTTTCTGATTGGCTAC	ACGATGTCACTTCCACG
YWHAZ	Human	AACTTGACATTGTGGACATC	AAAACTATTTGTGGGACAGC
PRLR	Human	CAAGTCAAGAGAGAGAACAG	GATGTTGTTATCCATGACCC
GHR	Human	CTCCTCAAGGAAGGAAAATTAG	GTGGAATTCGGGTTTATAGC
INSR	Human	GATCCAATCTCAGTGTCTAAC	CCTTTGAGGCAATAATCCAG
INSRA	Human	TTCGTCCCCAGGCCATCT	ATGCGATAGCCCGTGAAG
INSRB	Human	ACCTCTTCAGGCACTGGT	TCACATTCCCAACATCGC
THRA	Human	ACAAGATCGAGAAGAGTCAG	ACTTCTCTCTCCTTCATCAG
THRB	Human	GGACTAGCTCAATATTCCATC	GTACCCTTTACATTTCTTCTCC

8.3. Appendix 3: Serum hormone means, ranges and standard deviations

Table S4. Hormone concentrations at 36 weeks' gestation and post-partum days 1 and 2. Shown as mean ± SD (range).

Hormone	**Sampling time point**				
	36 weeks	**Day 1 (pre-feed)**	**Day 1 (post-feed)**	**Day 2 (pre-feed)**	**Day 2 (post-feed)**
Prolactin (mU/L)	4128±1614 (1513-9005)	5369±2047 (1804-12288)	6040±2065 (2058-13844)	5425±1808 (2572-10620)	5648±1915 (2600-11308)
GH (mU/L)	0.3±0.4 (0.0-3.6)	0.8±1.5 (0.0-8.2)	0.9±1.9 (0.0-12.3)	0.5±1.0 (0.0-6.4)	0.9±1.2 (0.0-4.6)
IGF-1 (nmol/L)	39.0±13.8 (13.4-82.7)	27.1±9.7 (12.1-55.3)	26.6±10.5 (11.1-64.8)	18.0±7.1 (6.2-44.0)	17.9±7.2 (6.0-43.2)
Progesterone (nmol/L)	487.8±156.4 (148.0-1127.0)	30.0±16.6 (9.0-92.0)	34.4±14.9 (11.0-70.0)	12.3±5.4 (4.0-26.0)	14.7±5.6 (5.0-29.0)
Oestradiol (pmol/L)	84554±105716 (23202-854700)	665±356 (149-1487)	681±325 (159-1290)	344±225 (48-1024)	315±203 (86-802)
Insulin (pmol/L)	216±352 (23-3303)	134±105 (13-425)	94±89 (13-420)	118±92 (13-434)	83±75 (9-345)
Leptin (ng/mL)	22.9±20.2 (4.9-94.0)	16.1±20.9 (3.7-82.2)	N/A	12.7±20.9 (1.0-52.7)	N/A
Adiponectin (µg/mL)	8.7±3.4 (3.6-15.3)	8.4±2.7 (4.9-13.7)	N/A	8.6±3.1 (5.2-14.1)	N/A
TSH (mU/L)	1.5±0.7 (0.2-4.5)	2.8±1.7 (0.4-8.5)	2.8±2.0 (0.4-10.7)	2.5±1.6 (0.7-10.6)	2.5±1.6 (0.7-10.4)
Free T4 (pmol/L)	10.6±1.1 (8.5-14.8)	10.8±1.3 (8.2-13.9)	10.6±1.2 (8.7-14.0)	11.1±1.1 (9.0-14.5)	11.2±1.3 (8.3-14.1)
Free T3 (pmol/L)	4.1±0.6 (2.8-5.3)	3.8±0.7 (2.7-6.5)	3.8±0.8 (1.8-6.2)	4.1±0.6 (2.7-5.4)	4.1±0.6 (2.7-5.4)

Table S5. Hormone concentrations on post-partum days 3 and 4. Shown as mean ± SD (range).

Hormone	**Sampling time point**			
	Day 3 (pre-feed)	**Day 3 (post-feed)**	**Day 4 (pre-feed)**	**Day 4 (post-feed)**
Prolactin (mU/L)	5001±1762 (1666-9259)	5695±2168 (2094-12354)	4802±1815 (1979-9647)	5537±1982 (2106-11398)
GH (mU/L)	1.0±1.6 (0.0-6.8)	1.1±1.3 (0.0-5.4)	0.7±1.6 (0.0-10.5)	1.2±2.3 (0.0-11.8)
IGF-1 (nmol/L)	14.1±5.7 (5.9-32.3)	13.8±5.3 (5.9-32.0)	12.4±4.3 (6.4-23.5)	12.5±4.1 (6.4-23.9)
Progesterone (nmol/L)	5.9±2.6 (2.0-18.0)	5.6±3.2 (2.0-17.0)	3.5±1.8 (0.0-9.0)	3.5±2.1 (1.0-10.0)
Oestradiol (pmol/L)	182±100 (70-498)	172±119 (69-549)	148±119 (0-696)	144±84 (69-383)
Insulin (pmol/L)	92±81 (0-317)	69±64 (0-250)	97±82 (13-363)	69±57 (0-236)
Leptin (ng/mL)	11.6±13.2 (1.9-53.8)	N/A	8.7±7.8 (1.2-24.7)	N/A
Adiponectin (µg/mL)	7.3±2.3 (3.8-11.9)	N/A	7.4±2.1 (3.9-11.4)	N/A
TSH (mU/L)	2.0±1.5 (0.5-11.7)	2.0±1.5 (0.4-12.2)	2.1±2.4 (0.6-16.5)	2.0±2.2 (0.7-15.7)
Free T4 (pmol/L)	11.7±1.2 (8.9-15.4)	11.7±1.3 (8.2-15.9)	12.0±1.3 (9.1-14.5)	12.1±1.3 (9.1-14.9)
Free T3 (pmol/L)	4.3±0.7 (1.8-6.2)	4.3±0.6 (2.0-5.6)	4.3±0.6 (2.7-5.7)	4.3±0.6 (2.5-5.7)

8.4. Appendix 4: Changes in synthetic genes in response to GH and insulin

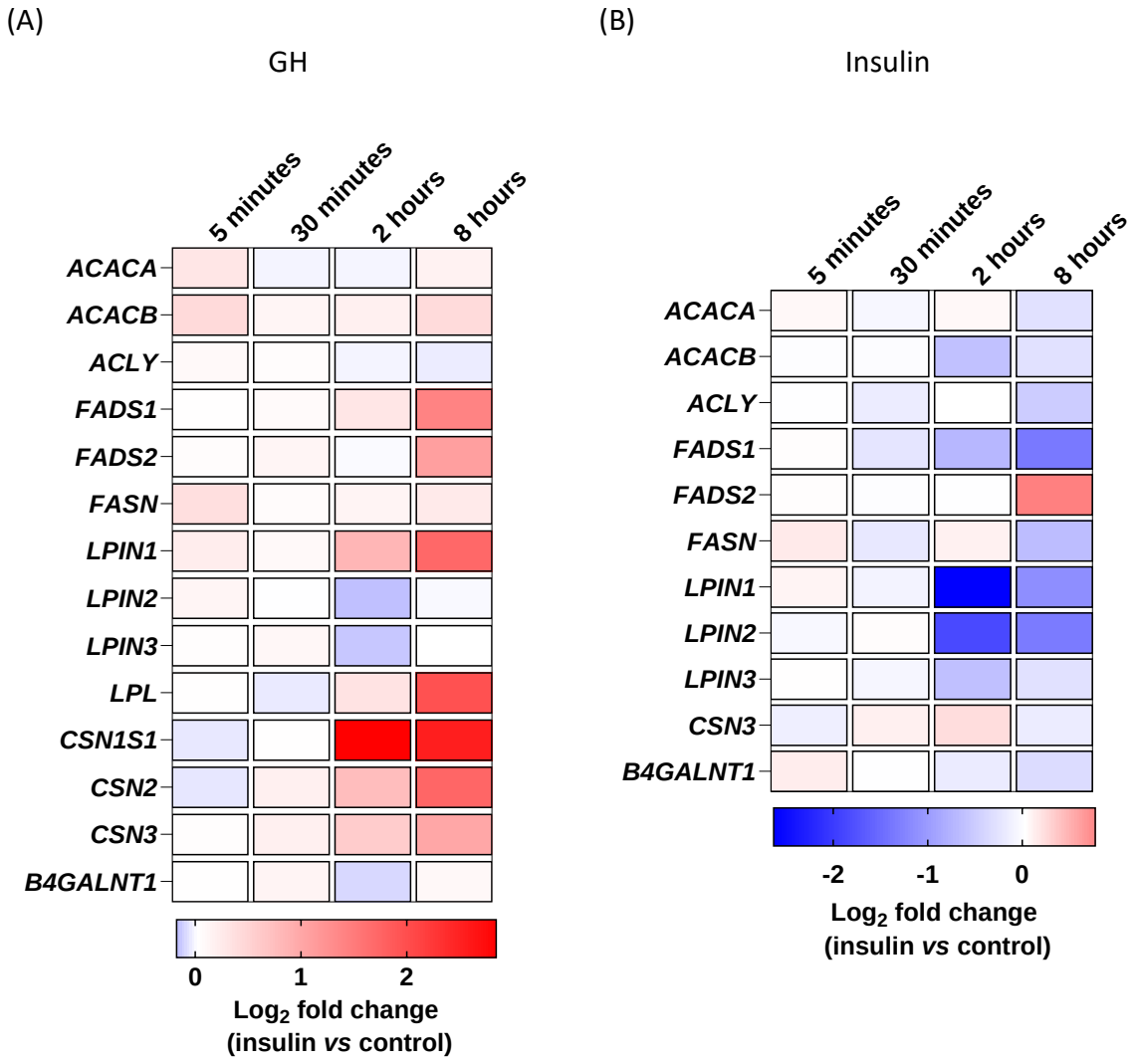


Figure S1. Heatmap of effects of GH and insulin on the expression of genes related to fatty acid, protein and lactose synthesis. The heatmap shows the fold change in expression of each protein, displayed as log_2-fold change, at each extraction time point for (A) GH and (B) insulin. Upregulation in response to hormone treatment is represented by red shading and downregulation by blue shading, with a darker colour indicating a greater fold-change. Unchanged gene expression is marked by white shading. Note that some genes were not expressed insulin-treated HC11 cells (*LPL, CSN1S1, CSN2*).

8.5. Appendix 5: Publications to date

Rostom H, Meng X, Price H, Fry A, Elajnaf T, Humphrey R, Guha N, James T, Kennedy S, Hannan FM. Protocol for an observational study investigating hormones triggering the onset of sustained lactation: the INSIGHT study. BMJ Open. 2022;12(8):e062478.

Cranston T, Boon H, Olesen MK, Ryan FJ, Shears D, London R, **Rostom H**, Elajnaf T, Thakker RV, Hannan FM. Spectrum of germline AIRE mutations causing APS-1 and familial hypoparathyroidism. Eur J Endocrinol. 2022; 187(1):111-122

PDF copies of these publications are submitted as additional files.

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