The Roles and Expression of Cationic Host Defence Peptides in Normal and Compromised Pregnancies

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BSc (Hons) PG Dip

Masters by Research

October 2014

A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University, for the award of Masters by Research

Contents

Acknowledgements

I'd firstly like to kindly thank my supervisors, Drs. Peter Barlow, Mick Rae and Nick Wheelhouse for their outstanding support, patience and guidance. I'd also like to thank the University for awarding the Strategic Funding Award to cover tuition fees and consumables in the lab, and the Moredun Research Institute for the use of their facilities and the support whilst there. Finally I'd also like to thank Mr Maxime Jacques who carried out some of this work as part of his placement project in the lab, Dr David Longbottom for their support and Dr Douglas Fraser-Pitt for provision of primers.

Abstract

Cationic Host Defense Peptides (CHDP), also known as antimicrobial peptides, are key components of the host innate immune response that have a wide range of direct microbicidal activities against a range of bacterial and viral pathogens, as well as a variety of immunomodulatory effects. CHDP are primarily expressed in tissues exposed to the external environment, and by cells of the innate immune system such as epithelial cells, macrophages, neutrophils and monocytes.

During pregnancy it is critical that the steroid milieu and the sterility of the reproductive tract are maintained for a successful pregnancy. In this study a mixture of ovine *in vivo* and *in vitro* steroid manipulations studies and an *in vitro* model of ovine placental infection are used. Using a fetal ovine animal model of dexamethasone and testosterone excess as models of compromised steroid milieu, we assessed the mRNA expression of the sheep cathelicidin SMAP-29 (Sheep Myeloid Antimicrobial Peptide-29) and sheep β-defensin 1 & 2 (sBD-1 and sBD-2) in the placenta, fetal thymus and fetal lung. These *in vivo* observations were followed up by *in vitro* steroid manipulation studies using the ovine trophoblast cell line AH-1 and the human lung epithelial cell line A549. *Chlamydia abortus* and *Waddlia chondrophilia* are both pathogens known to cause abortion in both livestock and humans. These pathogens were incubated with CHDP to determine their efficacy against such pathogens using an *in vitro* model of placental infection.

In vivo steroid manipulations resulted in significantly increased SMAP-29 levels in the fetal thymus of female sheep that were administered testosterone *in utero*. *In vitro* experiments found that the levels of SMAP-29, sBD1 and sBD2 were significantly altered by dexamethasone and only SMAP-29 was significantly altered by testosterone in the trophoblast cell line, AH-1.

The pre-incubation of *C.abortus* and *W.chondrophilia* with CHDP appears to aid organism growth, which is a novel observation for CHDP contributing to a potential role in pathogenicity. The proinflammatory profile of *W.chondrophilia* infection in the AH-1

cell line shows that the organism initiates an aggressive inflammatory response indicated by IL-8, IL-1β and TNFα expression. *Waddlia* infection also stimulates expression of sBD1 and sBD2 in the AH-1 cell line but, interestingly, not SMAP-29.

These data show how the steroid milieu and sterility, in the context of infection, of the reproductive tract can regulate the expression of CHDP, which could ultimately have an impact on the success of a pregnancy.

Chapter 1 – Introduction

1.1 General Introduction & Overview

Cationic Host Defence Peptides (CHDP), also known as antimicrobial peptides, have a wide range of direct microbicidal activities against a range of bacterial and viral pathogens (Zasloff, 2002; Ramanathan et al., 2002; Brown & Hancock, 2006). One of the major CHDP families are cathelicidins, which are primarily expressed in tissues exposed to the external environment, and by cells of the innate immune system such as epithelial cells, macrophages, neutrophils and monocytes (Bowdish, Davidson & Hancock, 2005). A CHDP with some of the best-characterised immunomodulatory activities is the sole human cathelicidin, LL-37, a pluripotent peptide with functions that include immunomodulation, angiogenesis, chemotaxis, LPS binding and neutralisation, and the modulation of cell death pathways (Barlow et al., 2011; Bowdish, Davidson & Hancock, 2005). The other major family of CHDP are the defensins, which are divided into three structural groups; α, β, and θ (Ganz, 2003). The molecules of interest in this study are the β-defensins as they have a significant role in defence against infection. β-defensins have similar antimicrobial properties to cathelicidins but fewer immunomodulatory properties (Semple & Dorin, 2012). CHDP are known to be present on mucosal surfaces, in the reproductive tract, and within the amnion during pregnancy (Frew & Stock, 2011; Horne et al., 2008).

Steroids are frequently used as therapeutics in a wide range of pathophysiological conditions and are also thought to underlie the progress of some developmental diseases (Barnes, 1998; Tegethoff et al., 2009; Walker, 2007). Glucocorticoids (GC's) are administered therapeutically in pregnancies at risk of preterm delivery in order to increase surfactant protein production in the lungs to prepare the fetus for the extra-uterine environment and as potent anti-inflammatory agents (Reynolds & Seckl, 2012; Lee et al., 2006; Newnham et al., 1999). Male sex steroids (androgens) are required for masculinisation of the fetus and program the development of the primary and secondary

male sex organs (Scott et al., 2009). *In utero* exposure to excess androgens is thought to "program" the development of polycystic ovary syndrome (PCOS) in females (Padmanabhan & Veiga-Lopez, 2013; Rae et al., 2013). Another potent secosteroid, which is crucial during the development of a healthy skeletal system, is Vitamin D_3 (VitD) (Lagishetty et al., 2011; Luk et al., 2012). There is an increasing prevalence for the role of VitD in relation to efficacy of the host innate immune response (Lagishetty et al., 2011). This is primarily thought to be due to the ability of VitD to stimulate production of cathelicidin due to a Vitamin D response element upstream of the cathelicidin antimicrobial peptide gene (*CAMP*) (Gombart et al., 2005). The wide-ranging effects of steroids, either as part of normal development, a disease process, or as a therapeutic, have resulted in a significant amount of research into the effects of steroids, pre- and post-natally, on the metabolic and reproductive systems. The effect of steroids on the developing immune system, specifically in the context of antimicrobial effector molecules such as CHDP remains undetermined.

The clinically relevant livestock pathogen *Chlamydia abortus* is a pathogen of ruminant and porcine pregnancy primarily (Wheelhouse et al., 2008; Kerr et al., 2005). *C.abortus* does have the ability to infect humans but can only infect women during pregnancy. The organism is an abortifacient agent in human pregnancy (Pospischil et al., 2002). *C.abortus* is an obligate intracellular pathogen and infects its host and lays dormant within host cells until the animal is pregnant, once pregnancy is detected through some unknown mechanism the bacteria become pathologic and cause destruction of the trophoblast of the placenta (Sammin et al., 2006; Sammin et al., 2009). It has been hypothesised that the bacterium lays dormant within the lymphatic tissue (namely the tonsils) before being released into the blood and lymph to further infection (Papp et al., 1993). Additional to this, the emerging abortifacient pathogen, *Waddlia chondrophilia* is a chlamydia-like organism that has been isolated from bovine abortion tissues (Rurangirwa et al., 1999). Less is known about *Waddlia*'s route of infection and pathogenesis, although it has been shown that *Waddlia* is less discriminate about which

species it will inhabit meaning it has higher potential to cause human miscarriage (Baud et al., 2007; Baud et al., 2014).

This project aims to discover if either pharmacological or pathological changes during pregnancy such as manipulated steroid status of the fetus or chlamydial infection will alter or modulate CHDP expression in tissues of the fetus (lung and thymus) and the placenta. Using these tissues, the presence of CHDP will initially be confirmed and subsequent analysis will determine if there are any changes in CHDP expression from treatment with steroids. In addition, infection with the clinically significant reproductive pathogens *Chlamydia abortus* and *Waddlia chondrophilia* will also be examined to assess the role of CHDP in the pathophysiological effects of the organism. CHDP are known to play key antimicrobial and immunomodulatory roles for both the mother and fetus during pregnancy, and perturbation of peptide expression or activity could have adverse effects in the successful completion of a pregnancy.

1.2 Cationic Host Defence Peptides

CHDP are antimicrobial effector peptides of the innate immune system. These molecules are highly conserved not only in mammals but also across animals and plants, where they have wide roles in antimicrobial defence and immunomodulation (Zasloff, 2002). In humans, there are two main families of CHDP, cathelicidins and defensins. CHDP have multiple actions within the innate immune system ranging from inflammatory cell recruitment to modulation of cell death (Bowdish et al., 2006).

1.2.1 Cathelicidins

Cathelicidins are characterised by a highly conserved cathelin region that does not possess any measureable antimicrobial action. In humans the only known cathelicidin is hCAP18/LL-37, hCAP18 being the inactive pro-protein and LL-37 the active antimicrobial cleavage product (Ramanathan et al., 2002; Dürr et al., 2006). The pro-protein is predominantly stored in specific granules of the neutrophils, but is also expressed by other cells and tissues such as epithelial cells, various immune cells (NK cells, B cells,

monocytes), keratinocytes, and at sites of chronic inflammation (psoriasis and atherosclerotic plaques) (Bals et al., 1998; Vandamme et al., 2012; Sørensen et al., 1997). Following release from inflammatory cell granules, this pro-protein is cleaved by extracellular proteinase-3 to release the mature variable active LL-37 peptide (Sørensen et al., 1997). In ovine species, the comparable analogue of LL-37 is sheep myeloid antimicrobial peptide–29 (SMAP-29) (Mahoney et al., 1995). Notably, sheep possess several other cathelicidins such as the ovine bactenecins (OaBac) OaBac-5, 6, 7.5 and 11, which are similar peptides of differing molecular weights (Huttner et al., 1998).

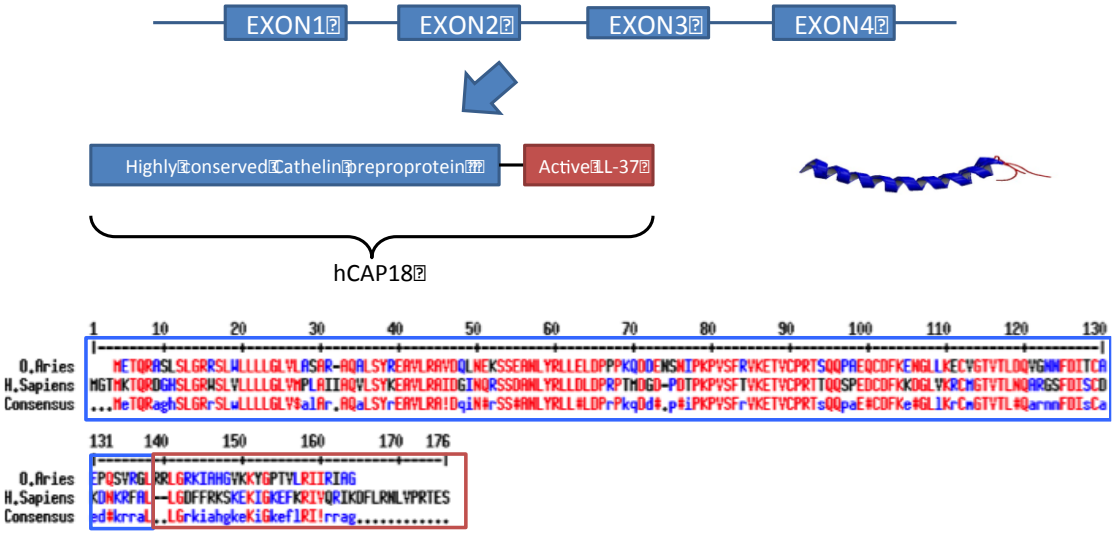


Figure 1. Diagram showing; exon arrangement of *CAMP* gene, basic pro-protein structure (hCAP18/LL-37) and 3D structure of LL-37 and a protein sequence alignment of human cathelicidin protein sequence alongside the ovine cathelicidin protein sequence with a consensus alignment below (conserved cathelin preprotein highlighted by the blue box, functional cleavage product ,LL-37 for humans, SMAP-29 for sheep, highlighted by red box).

The *CAMP* gene encodes hCAP18/LL-37, which comprises of 4 exons and 3 introns. Exon 1-3 contains the highly conserved cathelin region and the signal peptide and exon 4 contains the cleavage site and active peptide (Fig 1). LL-37 is named as such as the peptide is cleaved from the hCAP18 proprotein at the double leucine residue (LL) and is 37 amino acid residues in length (Yang et al., 2001). LL-37 has alpha helical structure with a net positive charge, and these characteristics are thought to give LL-37 its potent antimicrobial effects by facilitating rapid interactions with negatively charged cell walls of bacteria (Sørensen et al., 1997; Pazgier et al., 2013). LL-37 has well characterised

interactions with formyl peptide receptor-like 1 (FPRL1) and $P2X_7$ receptors but more putative cellular interactions have being proposed such as the intracellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) receptor (De Yang et al., 2000; Mookherjee et al., 2009; Nagaoka et al., 2006).

In addition to its antimicrobial potential, LL-37 has a large repertoire of immunomodulatory action within the innate immune system including; lipopolysaccharide (LPS) binding and sequestration, chemotactic recruitment of innate and adaptive immune cells, angiogenic properties during would healing and tissue remodelling, dendritic cell maturation and differentiation (Bowdish et al., 2006; Bowdish, Davidson, Scott, et al., 2005). Barlow *et al* has shown that during infection with *Pseudomonas aeruginosa*, LL-37 promotes apoptosis in damaged cells via mitochondrial depolarisation, thereby potentially reducing inflammation at the site of infection through the onset of programmed cell death (Barlow et al., 2010). Barlow and colleagues have also established that LL-37 is effective against influenza virus through as yet undetermined mechanisms although it is thought that this occurs through direct interaction with the virus itself (Barlow et al., 2011). Although the immunomodulatory actions of cathelicidins have not been fully established in sheep, it is thought that due to the highly conserved nature of cathelicidins in other species, SMAP-29 possesses similar multifunctional properties as LL-37. CHDPs are required in the reproductive tract to maintain a sterile environment for the zygote to implant and develop. LL-37 has been identified from fetal skin swabs and also within the vernix and amniotic fluid (Frew & Stock, 2011). As such, any perturbation of CHDP in the reproductive tract may lead to failed implantation or infection of the neonate.

1.2.1.1 Cathelicidin response to Vitamin D_3

In primates, including humans, it has been shown that Vitamin D_3 has an ability to stimulate production of cathelicidin through a Vitamin D response element (VDRE) upstream of the *CAMP* gene (Fig. 2).

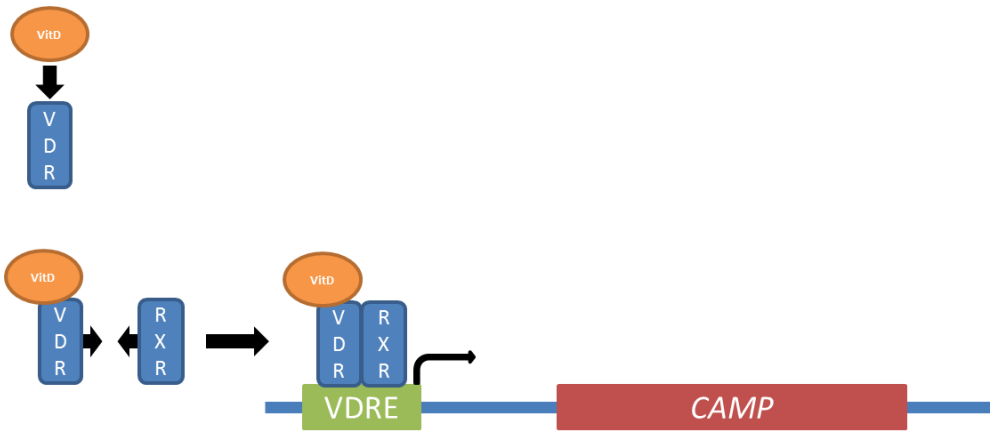


Figure 2. Diagram illustrating Vitamin D_3 regulation of *CAMP* gene. Vitamin D_3 will bind with the Vitamin D receptor. VitD/VDR complex will then dimerise and form a heterodimer with Retinoid-X receptor (RXR) which can then bind to the VDRE and induce expression.

This VDRE has been translocated upstream of the cathelicidin gene within a short interspersed nuclear element (SINE) (Gombart et al., 2005). The response to VitD has been shown in macrophages and trophoblasts (Gombart et al., 2005; Liu et al., 2009). Both active 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) and the less potent precursor 25-hydroxyvitamin D_3 (25OHD) can stimulate the expression of cathelicidin through the vitamin D receptor in these tissues. Active Vitamin D_3, $1,25(OH)_2D_3$ is generated locally by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) from 25OHD classically in the kidney. CYP27B1 is also expressed in the trophoblast of the placenta as Vitamin D_3 metabolites are required for successful pregnancy.

1.2.2 Defensins

Defensins are a diverse family of CHDP, comprising of three structural families, α-, β- and θ-defensins. Most animal species have either α- or β-defensins or both, although θ-defensins are only found in old world monkeys and higher primates. While the gene sequences for these peptides are present in the human genome, they are non-functional (Semple & Dorin, 2012; Ganz, 2003). The functional defensin families α and β are characterised by their β-sheet structure and are classified as such by the number of residues between their six cysteines and the pairing patterns of the disulphide bonds

between the cysteine residues. In sheep only β-defensins have thus far been identified and are of focus in this study (Ganz, 2003; Semple & Dorin, 2012; Bowdish et al., 2006).

In similarity with cathelicidins, β-defensins are also positively charged, although there are thirty-three different β-defensins in humans that are very similar in their core structure, but with slight variances in amino acid sequence between the cysteine residues. The genes which encode the β-defensins are all denoted as DEFB(number) i.e. *DEFB1* encodes β-defensin 1, and β-defensin genes are found in two clusters upon chromosome 8 and 6 respectively (Pazgier et al., 2006). Defensins are constitutively expressed in the early stages of neutrophil development in the bone marrow reservoir of progenitor myeloid cells and stored in azurophilic granules until required as constitutive expression tails off during neutrophil maturation (Semple & Dorin, 2012). The azurophilic granules are destined to fuse with phago-lysosomes indicating that β-defensins are used by neutrophils predominantly to aid in the phagocytic destruction of bacteria and other pathogens. β-defensins produced via pathogen recognition in epithelial tissues are expressed and released to aid in host defence and not as part of phagocytic attack. β-defensins have also been attributed with immunomodulatory functions similar to cathelicidins including potent antimicrobial and antiviral effects. In addition, the immunomodulatory capacity of defensins has been shown to include the recruitment of adaptive immune cells such as CD4+ Memory T-cells and immature dendritic cells via interaction with the CCR6 receptor (Bowdish et al., 2006; Ganz, 2003; Semple & Dorin, 2012; Pazgier et al., 2006).

1.3 The Steroid Environment during Pregnancy

Pregnancy and parturition are two processes that are intricately linked to the steroid state of the mother in order for a healthy pregnancy and birth of the child. Recent studies have associated changes in the maternal/fetal steroid environment to predisposition of ‘first world diseases’ such as type 2 diabetes, cardiovascular disease and obesity through a phenomenon called fetal programming (Lee et al., 2006). Fetal programming, is a

phenomenon whereby the maternal environment can affect the developing fetus during pregnancy (Fowden & Forhead, 2004; Harris & Seckl, 2011; Roseboom et al., 2001). As several aspects of pregnancy are dependent on tightly regulated specific steroid profiles, any changes in steroid concentrations may potentially predispose to altered health in the offspring

Therapeutic administration of exogenous synthetic GC can be used as a prophylactic treatment in pregnancies where the mother is at risk of premature delivery. Steroid treatment can encourage lung development and surfactant production, and ready the fetus for the *ex utero* environment in the event of an early delivery (Reynolds & Seckl, 2012). In these instances GCs, such as dexamethasone (Dex) or betamethasone, are given post- or ante-partum to promote pulmonary maturation and surfactant protein production in the neonate (Tegethoff et al., 2009).

Interestingly, excess male steroids during pregnancy are thought to be a cause for PCOS (Veiga-Lopez et al., 2011). This hypothesis came about from psychosexual studies carried out in the 70's that discovered by injecting pregnant rhesus macaque monkeys with testosterone had effects on the metabolic system of female offspring showing a PCOS as well as effecting the behaviour of the monkeys. This research was later revisited by Abbot *et al* and has since become the gold-standard model of investigating PCOS in terms of generation of animal models (Abbott et al., 1998).

1.3.1 Dexamethasone

Dex is a synthetic GC and is used in a clinical setting as a potent, long-lasting, stable surrogate of the corticosteroid cortisol. Unlike cortisol, Dex only interacts with the GC receptor, whereas the more promiscuous cortisol can also interact with the mineralocorticoid receptor. Dex is used primarily for the replacement of GCs in conditions where GC production has been lost or diminished (e.g. Addisons disease), although it is also used as a promoter of fetal lung surfactant protein production in pregnancies at risk of premature delivery (Tegethoff et al., 2009). Dex is approximately

thirty fold more potent than the native cortisol and this is attributed to the fact that Dex cannot be metabolised by the cortisol inactivation enzyme 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). In a physiological context, 11βHSD2 is used as an active barrier in the placenta preventing fetal exposure to maternal GCs (Kapoor et al., 2008).

Given that Dex cannot be metabolised by 11βHSD2, it is an ideal candidate to be used to model fetal exposure to excess GCs. In experimental models of maternal stress, Dex is used to simulate an increase in cortisol secretion from the maternal adrenals, which is thought to cross the placental barrier and affect the developing fetus. These studies so far have concluded that fetal exposure to GCs can result in rodent and ovine hypertension, altered HPA axis, impaired glucose homeostasis and obesity (reviewed in Fowden & Forhead, 2004). It should be noted that the steroid manipulation in these studies described here occurs at early/mid gestation and not completely translatable to effect that an antenatal treatment regime would have as they are only administered in the last few weeks of pregnancy or diagnosis of a condition where the neonate is at risk of delivering early. Large cohort studies on the risks of using Dex/beta-methasone as a antenatal intervention have been carried out to assess the long-term side effects of these treatments (Tegethoff et al., 2009). The immediate benefits are clear to be seen with a reduction in infant mortality and morbidity but from animal studies the consequences of excess GCs on development and in later life are well known (Harris & Seckl, 2011). The large scale cohort studies to date haven't concluded whether the long-term effects are adequately balanced from a risk/reward standpoint but these studies have certainly identified areas of further study which will help answer if *in utero* GCs are beneficial or not (Tegethoff et al., 2009; Reynolds & Seckl, 2012).

1.3.2 Testosterone

Testosterone is the male sex hormone that is responsible for development of male sexual organs and production of spermatozoa in the testis. It is produced primarily in the testis, but also in the ovary and adrenal, by a suite of steroidogenic enzymes in which 17β-

hydroxysteroid dehydrogenase (17βHSD) is the final step. Testosterone can be metabolised by P_{450} aromatase to oestradiol. Aromatase is present in the placenta to prevent fetal exposure to maternal androgens as the timing of sexual characteristics development is controlled by fetal production of androgens (Padmanabhan & Veiga-Lopez, 2013; Padmanabhan & Veiga-Lopez, 2011; Veiga-Lopez et al., 2011; Abbott et al., 1998). Male sexual development is a delicately timed process where deviations thereof can cause disorders of sexual development such as, testis dysgenesis syndrome (cryptorchidism, hypospadias and increased incidence of male germ cell tumors) (Scott et al., 2007; Welsh et al., 2008; Sharpe, 2006). Testosterone propionate (TP) is the injectable formula of testosterone and has negligible differences in activity compared to testosterone. Testosterone is primarily used as a treatment in gender reassignment patients and men suffering from hypogonadism (small or underdeveloped testes) where lowered serum testosterone levels require supplementation (Scott et al., 2009).

1.4 Chlamydial pathogens

1.4.1 *Chlamydia abortus*

Chlamydia abortus is an obligate intracellular pathogen that primarily infects ruminants and pigs and causes enzootic abortion of ewes (EAE), which has a large economic impact on the livestock industry (Kerr et al., 2005; Wheelhouse et al., 2008; Wheelhouse et al., 2009). A *Chlamydia* infection has a biphasic development cycle where the microbe inhabits two states that facilitate intra and extracellular existence of this pathogen. The elementary body (EB) state enables *Chlamydia* spp to survive in the extracellular environment. In this phase there is no metabolic activity and in this state the microbe is infective and able to interact with the mucosal membranes and invade host cells using as yet undetermined mechanisms. The EB is taken into an intracytoplasmic inclusion that is prevented from binding to phagolysosomes and at this point the *Chlamydia* switches to its other state, the reticular body (RB). The RB is metabolically active and during this phase the *Chlamydia* reproduces using binary fission within the inclusion.

Before the inclusion becomes large enough to lyse the cell the *Chlamydia* switches back to the infective EB state to enable further infection (Longbottom & Coulter, 2003). In *C.abortus* infection this process wipes out specifically the trophoblast within the placenta, which eventually prevents the transfer of nutrients and O_2 to the fetus (Sammin et al., 2009; Sammin et al., 2006). In the context of this project, infection with *Chlamydia* spp provides an appropriate model of infected pregnancy where CHDP modulation and activity can be assessed.

To date most major studies using CHPD against *chlamydia* spp have been directed towards the human pathogens *C.trachomatis* and *C.pneumoniae* (Donati & Leo, 2005). Both cathelicidins and defensins have been tested against these *Chlamydia* spp. and have been found to be effective as antimicrobial agents against these species. One study tested the efficacy of different cathelicidins from different host species against clinical strains of *C.trachomatis* and *C.pneumoniae*. Additional to these test groups there was also an 'animal *chlamydia* strain' group which were assessed as a group for susceptibility but the group comprised of a mixture of *C.psittaci* (parakeet), *C.felis* (cats), *C.abortus* (livestock, sheep, cows and pigs) and *C.pecorum* (koala and livestock). Cathelicidins were shown to have an antimicrobial effect on these species of *chlamydia* but as they were assessed as a group we cannot infer too much on specific reactions from specific species. One interesting finding from the paper was that the sheep cathelicidin SMAP-29 had differing efficacies being more potent against the human species as compared to the animal strains (Donati & Leo, 2005). This therefore lends support to using cathelicidins from other animals as therapies against human pathogens. Evolutionarily human pathogens have co-evolved with human cathelicidins and thusly have developed some form of tolerance or resistance but as can be seen in Donati & Leo (2005), cathelicidins from another host, ovine cathelicidins for example, are shown to be effective at lower concentrations indicating a higher potency and therefore potential therapeutic benefit.

1.4.2 *Waddlia chondrophilia*

Waddlia chondrophilia is an emerging intracellular pathogen of the order chlamydiales (Rurangirwa et al., 1999). Like other members of this order *Waddlia* has a biphasic reproductive cycle, multiplying via binary fission in the inclusion body of infected cells in the metabolically active reticulate stage. Then whilst in the metabolically inactive EB stage, is infective (Goy et al., 2008; de Barsy & Greub, 2013). *Waddlia* was originally isolated from an aborted bovine fetus in 1986 but has since been identified in other bovine abortions as well as being implicated in human birth complications and spontaneous abortions/miscarriages (Baud et al., 2014; Goy et al., 2008). Recent studies have shown that *Waddlia* is capable of infecting several cell lines of varying species as well as primary human macrophages (Kebbi-Beghdadi et al., 2011 a,b). To date there has been no attempts to test the efficacy of CHDP against this emerging zoonotic pathogen.

1.5 Study purpose and outline

The sterility and the steroid milieu of the reproductive tract are both of key importance to ensure a successful pregnancy and birth of the newborn. An aim of this study is to assess the impact of an altered steroid environment (excess androgens or GCs) during pregnancy on the expression of CHDP in the functional subunit of the placenta, the trophoblast. In addition to this, fetal lung and thymus (sites of regular CHDP production) will be assessed to determine if altered steroid concentrations *in utero* will program any lasting effects in the CHDP expression of the offspring.

Sterility of the reproductive tract during pregnancy is paramount for a successful pregnancy. Some reproductive pathogens, such as the *C.abortus*, and *W.chondrophilia* have specifically evolved in livestock species (sheep, pigs and goats) to only become pathogenic during pregnancy. The immunomodulatory roles of CHDP, together with their antimicrobial and LPS neutralising properties are if significant interest, and designing novel antimicrobial agents based on naturally occurring peptide is now a popular

strategy. A number of new therapeutic agents based upon naturally occurring antimicrobial peptides are currently undergoing clinical trails (Baltzer & Brown, 2011; Padhi et al., 2014; Yeung et al., 2011). Here, CHDP (human, ovine and porcine) was tested to assess whether it could be used a viable novel antimicrobial agent against this economically relevant pathogen.

1.5.1 Tissues analysed

1.5.1.1 Fetal Lung

The lung is a well-known and well characterized site of CHDP expression due to its constant exposure to the external environment (Tecle et al., 2010; Seaborn et al., 2010; Collie et al., 2013; Scott et al., 2002). The lungs develop throughout pregnancy but aren't fully matured and able to facilitate oxygen exchange until the latter stages of pregnancy (Grenache & Gronowski, 2006). Lung development is a tightly regulated and sequential process beginning around week 3 of embryological development and not fully completing until postnatal life (Seaborn et al., 2010; Grenache & Gronowski, 2006; Schaller-Bals et al., 2002). Surfactant protein, which is vital for gas exchange within the alveoli, begins production around mid-late gestation in preparation for the extrauterine environment. In human neonates the expression of certain CHDP are developmentally regulated throughout pregnancy showing that they are key to a healthy epithelial environment for when the lungs are exposed to the extrauterine environment for the first time (Starner et al., 2005).

1.5.1.2 Fetal Thymus

The thymus is the organ where immature T-cells migrate to, from the lymphoid progenitor cells in the bone marrow, to undergo maturation (Blackburn & Manley, 2004). The thymus can be spilt into two distinct zones, the outer cortical area (thymic cortex) and the inner medullary zone (thymic medulla) that are demarcated by a cortico-medullary junction. Immature T-cells enter the thymus and migrate to the outer cortical region to undergo the first steps of maturation (thymopoiesis), β-selection and lineage

commitment (Blackburn & Manley, 2004). Thymocytes are now either CD4 or CD8 positive with a functional T-cell receptor (TCR) they move to the cortico-medullary junction where they encounter plasmacytoid dendritic cells, which present 'self' antigens to test the T-cells for autoreactivity (Lee et al., 2010; Blackburn & Manley, 2004). T-cells that recognise 'self' antigens are negatively selected and programmed into apoptosis, T-cells that don't react with self-antigens are transported to the periphery and matured further into specific lineages of T-cell (e.g. T-helper, T-reg and T-suppressor) (Lee et al., 2010; Blackburn & Manley, 2004). There is a body of literature showing LL-37 has a role in constitutively expressing interferon-α stimulated genes which confer a resistance to viral infection within the medulla of the thymus (where T-cells that have undergone positive-selection are found) (Colantonio et al., 2011; Lee et al., 2010).

1.5.1.3 Placenta (Placentome/Cotyledon)

The ovine placenta is different to the human placenta in a number of aspects. There is not one single placental unit like a human placenta, the ovine placenta consists of ~90 discreet individual placentomes interconnected by the intercotyledonary membrane (Sammin et al., 2006). The ovine system consists of an intercotyledonary membrane with individual cotyledons interspersed throughout the membrane (Entrican, 2002; Entrican et al., 2010). When these cotyledons (which are the fetal side of the placentome) interface with the maternal caruncles (cup-like features) of the endometrium the fetal and maternal villi interdigitate to form a functional placentome (Sammin et al., 2009). Each of these placentome structures has a common blood supply and coalesces to form the umbilical cord that supports the fetal lamb. The ovine placenta is synepthelichorial, which means that the fetal chorion and maternal uterine epithelium come into direct contact with one another and transfer nutrients directly between each other. Whereas the human placenta is haemochorial, where the trophoblasts of the placenta are bathed in maternal blood and transfer nutrient to the fetus from the maternal circulation directly (Sammin et al., 2009; Sammin et al., 2006; Entrican et al., 2010). It has been shown in

human trophoblasts and choriocarcinoma cell lines (BeWo) that LL-37 can be expressed in response to VitD in these tissues in humans (Liu et al., 2009; Gombart et al., 2005).

Chapter 2 – CHDP and Steroids during Pregnancy

2.1 Methods

2.1.1 Tissues

All tissues used were collected from ovine models of steroid manipulation during pregnancy which were conducted under a Home Office Project Licence as regulated by the Animals (Scientific Procedures) Act, 1986. Briefly, 20mg of testosterone propionate (TP) or 100 µg dexamethasone (Dex) dissolved in vegetable oil or oil vehicle alone were the treatments. Injections were carried out using an ultrasound-guided needle into the rump of the fetus under maternal anaesthesia at days 62 and 82 of a 147 day of pregnancy. Fetuses from both singleton and multiple pregnancies where injected directly with either TP, Dex or vegetable oil. Female Scottish Greyface fetuses were injected with TP, male Scottish Greyface fetuses were injected with Dex and both male and female fetuses injected with vegetable oil as a vehicle control. Fetal lung and thymus were gathered from fetuses sacrificed on day 90 of gestation and from the same fetus a cotyledon/placentome structure from the placenta was also collected. These tissues were snap frozen and stored at -80^{0}C for mRNA expression analysis.

2.1.2 *In Vitro* Steroid Treatments

The ovine trophoblast cell line, AH-1 and human lung epithelial cell line A549 were used to model the response of the placenta and lungs to different steroid exposures *in vitro*. AH-1 cells were kindly provided by Drs Nicholas Wheelhouse and David Longbottom from Moredun Research Institute, Edinburgh, UK, and were grown and maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal bovine serum (PAA Laboratories Ltd, Yeovil, Somerset, UK) at 37^{0}C with 5% CO_2. A549 cells were obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK) and were grown and maintained in Dulbeccoo's modified eagles medium with high glucose (4500mg/L) and GlutaMAX (L-alanyl-Glutamine, 862mg/L) (DMEM High glucose with

GlutaMAX, PAA Laboratories Ltd, Yeovil, Somerset, UK) supplemented with penicillin/streptomycin (50U/mL) and 10% fetal bovine serum (Sigma, UK). Treatment medias containing TP, Dex and VitD were created in serum free media (IMDM for AH-1 and DMEM for A549). All steroids were dissolved in the absolute alcohol at a stock concentration of 1mM and stored at -80^{0}C. In all steroid treatments an ethanol (EtOH) vehicle control was run (1µl/mL) to ensure EtOH wasn't having an independent effect. A 1:10 serial dilution from 1µM to 1nM (1µM, 100nM, 10nM and 1nM) was run for both Dex and TP. AH-1 cells were exposed to VitD at 100nM, 10nM and 1nM to accommodate LPS stimulation (500ng/mL, lipopolysaccharide from *Escherichia coli* O111:B4, Sigma, L2630-10mg). AH-1 cells were serum starved for 24h and steroid treatments were carried out in respective serum-free medias for 24hrs and were maintained in a heated, humidified incubator at 37^{o}C with 5% CO_2.

2.1.3 RNA Extraction, DNase treatment and cDNA Synthesis

For all RNA extractions RLT buffer (with 2-mercaptoenthanol, 1% v/v) from Qiagen RNeasy mini kits (Qiagen, Crawley, UK) were used for lysis of both *in vitro* and *ex vivo* samples. For frozen tissues, 30mg tissue was weighed and lysed in RLT buffer using a magnetic bead and the Qiagen TissueLyser (Qiagen, Crawley, UK). *In vitro* samples were lysed *in situ* and collected into sterile DNase/RNase free eppendorfs and stored at -80^{0}C until ready for extraction. Once samples were lysed, RNA was extracted from the lysates using the RNeasy mini kit system. All samples were analysed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) to ascertain RNA quantity. Representative samples from each treatment group were assayed for RNA quality using the Agilent Bioanalyser (Agilent, UK). Genomic DNA (gDNA) was removed from the extracted RNA using PrimerDesign Precision DNase Kits (PrimerDesign, Southampton, UK). DNase treatments were carried out on all samples as per manufacturer's protocol. Complimentary DNA (cDNA) was synthesised from the extracted RNA using Precision nanoScript reverse transcription kits as per the manufacturers protocol (PrimerDesign, Southampton, UK) and stored at -20^{0}C until

ready for downstream uses. Negative controls with no reverse transcriptase (no RT) in the reaction were ran for every treatment.

2.1.4 Quantitative Real Time – Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Applied Biosystems StepOne Real-Time PCR machine with SYBR green detection and a 96-well format setup. qRT-PCR primers for the ovine genes; SMAP-29, sBD1, and sBD2, were designed and optimised by PrimerDesign Ltd (PrimerDesign Ltd, Southampton, UK). Primers for androgen receptor (AR), glucocorticoid receptor (GR), 11β-hydroxysteroid dehydrogenase type 1 and 2 (11βHSD1/2) and interleukin-8 (IL-8) were obtained from MGW Eurofins with primer sequences optimised and previously published (Rae et al., 2013; Fach et al., 2007). Human primers for LL-37 were obtained from a previous publication (Li et al., 2013), hBD2 and GAPDH (sequences in Fig 2.2) were kindly provided by Dr Douglas Fraser-Pitt (Edinburgh Napier University). Melt curve analysis was carried out with every GOI (available in Appendix) to determine specificity of the primers alongside a no-template H_2O control. Housekeeper genes were identified using a GeNorm Kit to establish the most stable housekeeping genes (PrimerDesign GeNorm Ovine 12 reference gene kit, PrimerDesign, Southampton, UK). All samples were analysed using ΔΔCt method, $2^{-(\Delta\Delta Ct)}$ with quenching against a mixed reference sample. Each 10µl reaction consisted of 1µl (40ng) cDNA template, 0.5µl (300nM) gene specific primers, 5µl SYBR Green (Precision 2X qMasterMix with SYBR Green, PrimerDesign, Southampton, UK) and the reaction was brought to 10µl with DNase/RNase free H_2O. No RT and no template H_2O controls were performed on each plate as negative controls.

Gene Name	Forward Primer 5'-3'	Reverse Primer 5'-3'
SMAP-29	TAGACCCGCCTCCCAAGC	CCATTCTCCTTGAAGTCACACT
sBD1	GGCTGTCTAATGTTCATCTTACC	TCTCCGCATCCTCTCTCTC
sBD2	CTCCATCACCTGCTCCTC	GCTTAGACTATCTGTTACTCCAT
IL-8	AAGCTGGCTGTTGCTCTC	GGCATCGAAGTTCTGTACTC
GR	AAGTCATTGAACCCGAGGTG	ATGCCATGAGGAACATCCAT
AR	GCCCATCTTTCTGAATGTCG	CAAACACCATAAGCCCCATC
11βHSD1	AGCATTGTGGTCGTCGTCTCCT	CCTTGGTCGCCTCATATTCC
11βHSD2	TAAGGCGAGATTAGGTAGGTTG	ACCCTTCAAATCACAGCACTG

Table 2.1. Primer sequences for Ovine genes.

Gene Name	Forward Primer 5'-3'	Reverse Primer 5'-3'
GAPDH	CCAGCCGAGCCACATCGCTC	ATGAGCCCCAGCCTTCTCCAT
LL-37	TGGGCCTGGTGATGCCT	CGATGTTCCTTCGACAGGAAGC
hBD2	ATCAGCCATGAGGGTCTTGT	GAGACCACAGGTGCCAATTT

Table 2.2. Primer sequences for Human genes.

2.1.5 Statistical analysis

All qRT-PCR data were analysed using ΔΔCt method, $2^{-(\Delta\Delta Ct)}$ with quenching against a mixed reference sample. For all *in vivo* experiments in placenta, fetal lung, and fetal thymus were analysed by Students un-paired t-test. *In vitro* analysis of Dex, TP and VitD was carried out using ANOVA and comparisons between groups were carried out using Fisher least significant difference (LSD) test. Results were considered significant if P values were ≤ 0.05. All statistical analyses were ran using GraphPad Prism v6.0 (GraphPad Software Inc., San Diego, CA).

2.2 Results

2.2.1 qRT-PCR results from *in utero* dexamethasone exposures

Fetal lung and thymus and the placenta were assessed by qPCR to determine whether exposure to excess GCs during pregnancy will program changes in the CHDP production in these tissues.

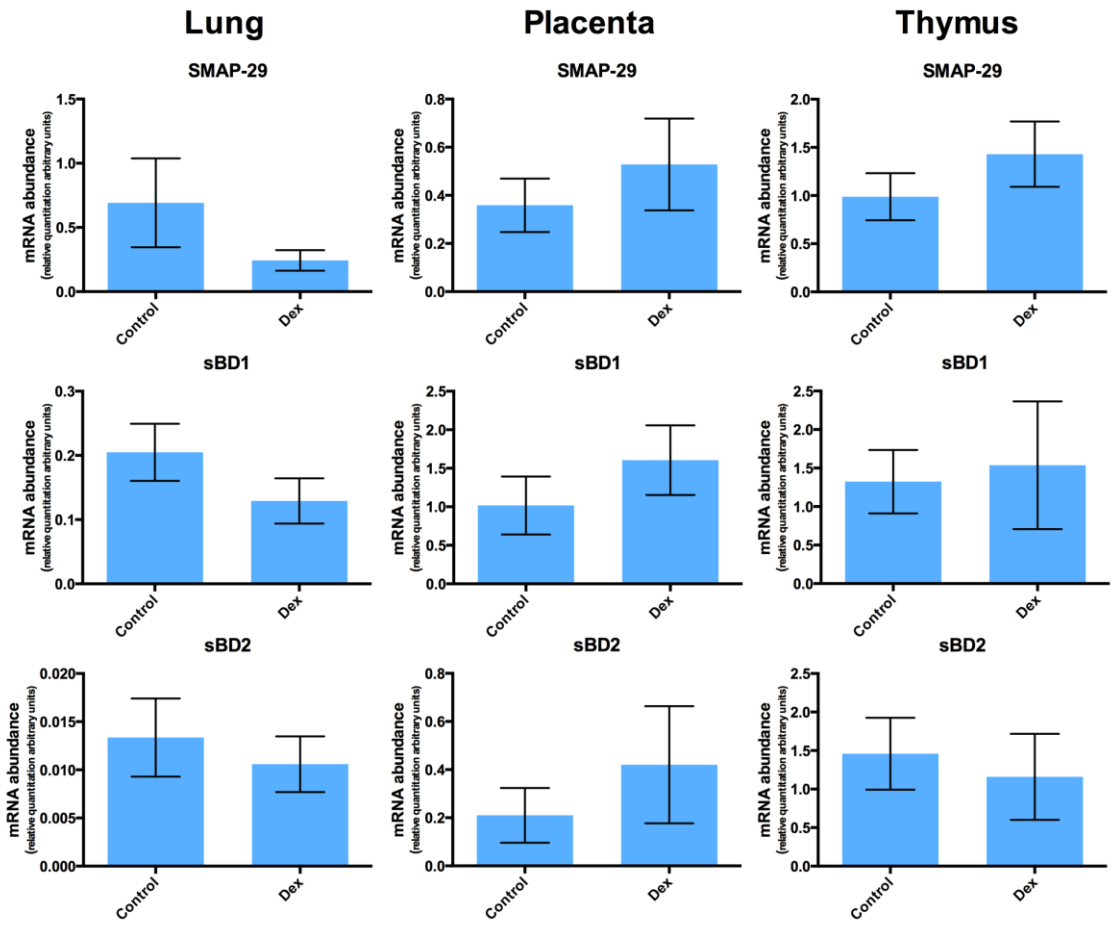


Figure 2.1. qRT-PCR results from fetal ovine lung, thymus and placenta of SMAP-29, sBD1 and sBD2 expression, after exposure in utero. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (lung: RPS2 and RPL19, placenta: RPS2 and YWHAZ, and thymus: G3PD and RPS2). Data were analysed by Students un-paired t-test. No statistical significance was found P> 0.05 in all instances. n=6 for each treatment group, control animals injected with vehicle (vegetable oil) and treatment animals injected with 20mg Dex. Error bars represent SEM.

The expression of CHDP in the fetal lung, thymus and placenta is not significantly regulated by *in utero* exposure to Dex (P>0.05) (Fig 2.1, lung - SMAP29 P=0.235, sBD1

P=0.211 and sBD2 P=0.591, thymus - SMAP29 P=0.315, sBD1 P=0.509 and sBD2 0.689, placenta SMAP29 P=0.460, sBD1 P=0.341 and sBD2 P=0.452).

2.2.2 qRT-PCR results from *in utero* testosterone propionate exposure

Testosterone is known to have effects on the developing metabolic system during pregnancy (Rae et al., 2013). With this known developmental effect of TP, the impact of excess upon the development of the innate immune system is still unknown. TP excess had no significant effects on CHDP expression in the fetal lung or the placenta with P>0.05 in all instances (SMAP29 P=0.836 and 0.688 respectively, sBD1 P=0.374 and 0.631 respectively and sBD2 P=0.399 and 0.236 respectively) (Fig 2.2 A & B). TP significantly increased SMAP29 expression in the fetal thymus (P=0.023), but the defensins are not altered by TP in the thymus (sBD1 P=0.673 and sBD2 P=0.558) (Fig 2.2 C).

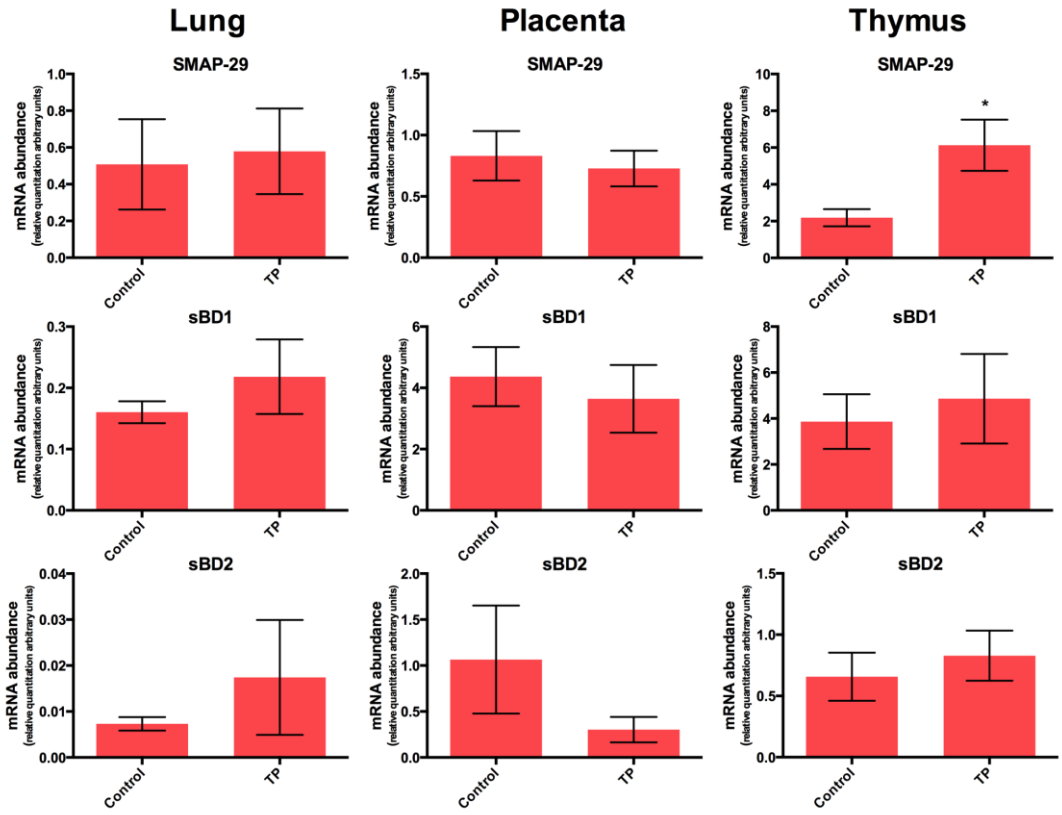


Figure 2.2. qRT-PCR results from fetal ovine lung, placenta and thymus of SMAP29, sBD1 and sBD2 expression after Testosterone propionate (TP) exposure in utero. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (lung: RPS2 and RPL19, placenta: ATPSynth and RPS26, and thymus: RPS2 and 18S). Data were analysed by Students un-paired t-test. SMAP-29 in thymus is significant P≤0.05 (*). No other statistical significance was found P> 0.05 in all other instances. n=6 for each treatment group, control animals injected with vehicle (vegetable oil), treatment animals injected with 20mg TP. Error bars represent SEM.

Additional genes were analysed in placentomes from the TP and Dex treated animals (Fig 2.3). HSD11B1 and HSD11B2 are responsible for the interconversion between cortisol and cortisone, were analysed in addition to the androgen and GC receptor (AR and GR). There were no significant differences upon statistical analysis (P>0.05).

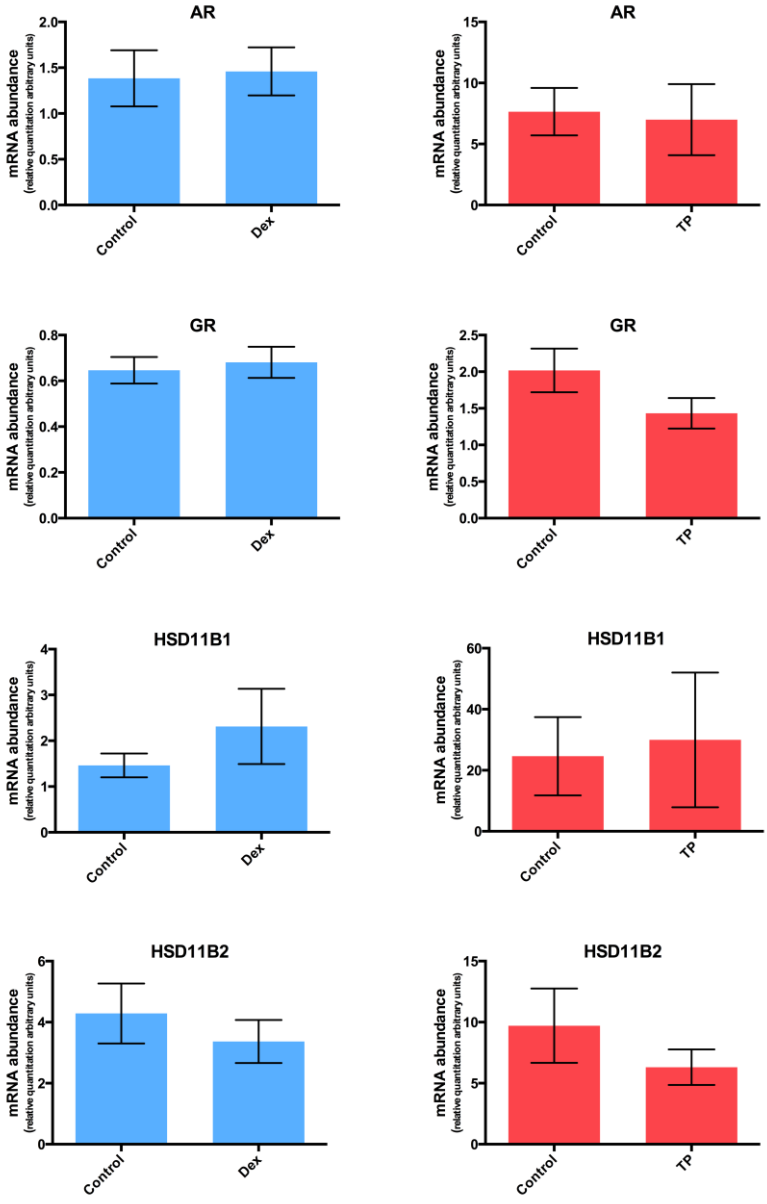


Figure 2.3. qRT-PCR results from ovine placenta of AR, GR, HSD11B1 and HSD11B2 expression after Dex (blue bars) and Testosterone propionate (TP, red bars) exposure in utero. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (TP treatments ATPSynth and RPS26, Dex treatments RPS2 and YWHAZ). Data were analysed by Students un-paired t-test. No statistical significance was found P> 0.05 in all other instances. n=6 for each treatment group, control animals injected with vehicle (vegetable oil) treatment animals injected with 20mg TP or Dex. Error bars represent SEM.

2.2.3 AH-1 qPCR results after 24h steroid exposure

Using AH-1 cells as a model for ovine placenta, steroid exposures were carried out to determine whether steroids (Dex and TP) had any short-term effects (24h post-exposure). In all CHDP genes examined, there is a significant increase in expression attributable to treatment with 1nM Dex (SMAP29 P=0.0007, sBD1 P=0.04 and sBD2 P=0.03) (Fig 2.4 A, B and C). As the concentration of Dex increases from 1nM to 1µM

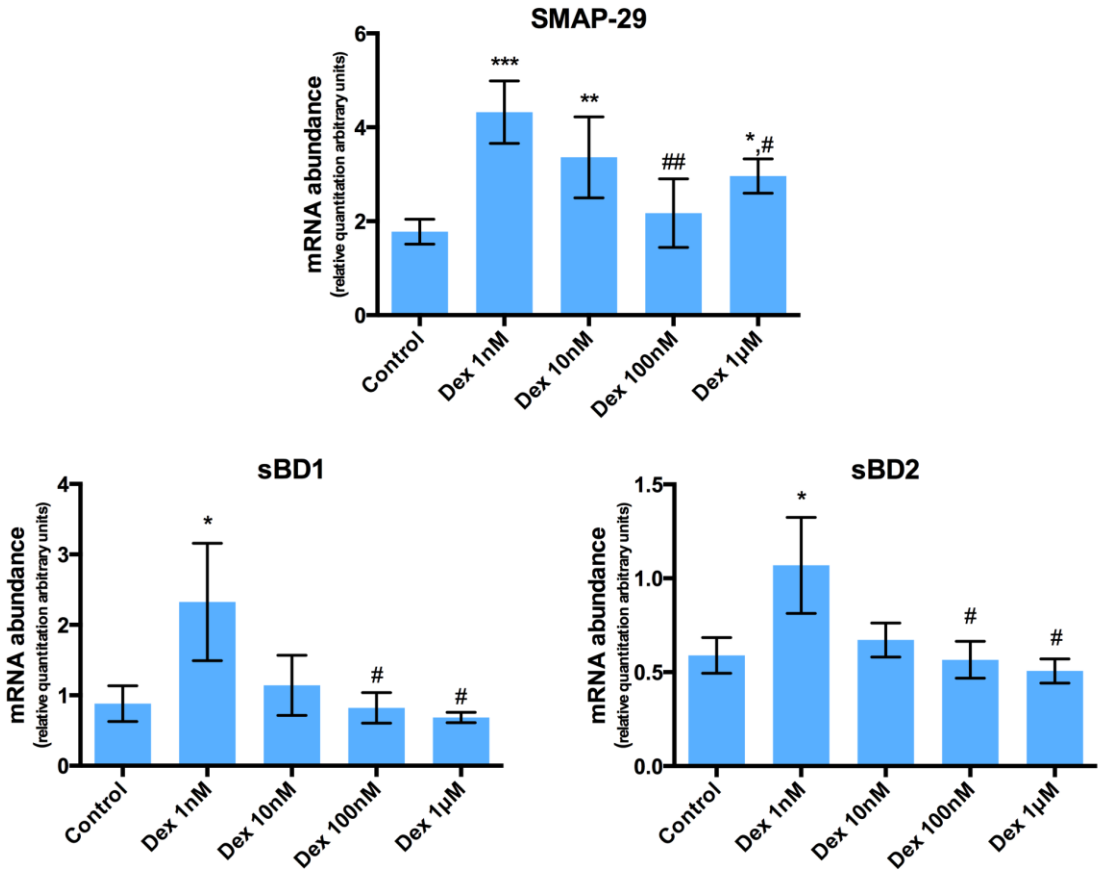


Figure 2.4. AH-1 cell line *in vitro* SMAP-29, sBD1 and sBD2 expression after 24h Dex exposure. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (bActin and GAPDH). Statistically-significant differences to control are denoted by * (*** P<0.001, * P<0.05) and statistically-significant differences to 1nM Dex are denoted by # (## P<0.01, # P<0.05). Error bars represent SEM. Data were analysed by ANOVA. Comparisons between treatments were made using Fisher's LSD. n=3 for both all treatments and control.

CHDP expression decreases back to control. SMAP29 has a notably different profile where at 1µM the expression increases to that observed at 10nM Dex. This is not

significantly greater than 100nM so does not fit a classical non-monotonic response (Fig 2.4 A).

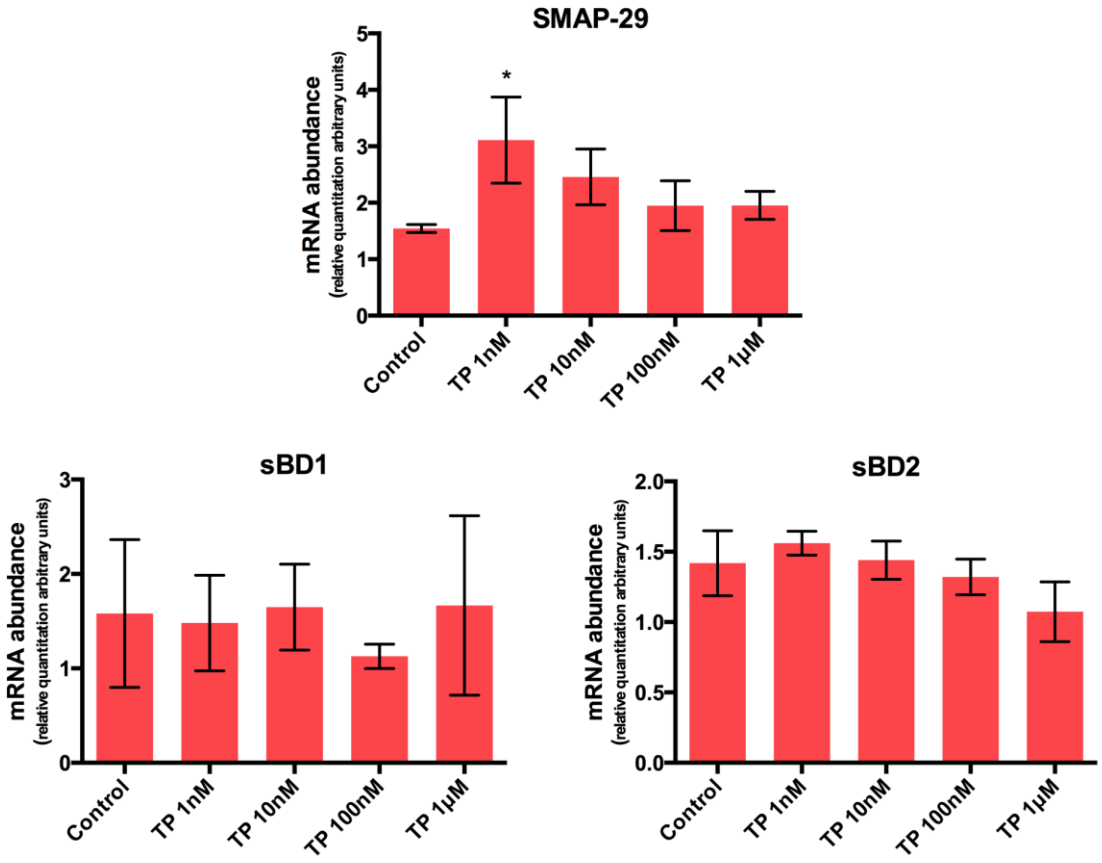


Figure 2.5. AH-1 cell line *in vitro* SMAP-29, sBD1 and sBD2 expression after 24h testosterone propionate exposure. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (bActin and GAPDH). Statistically-significant differences to control are denoted by * P<0.05. Error bars represent SEM. Data were analysed by ANOVA. Comparisons between treatments were made using Fisher's LSD. n=3 for both all treatments and control.

AH-1 cells were exposed to testosterone propionate to elicit whether trophoblasts will express CHDP in response to androgens. SMAP-29's expression significantly increased at 1nM TP (P=0.038). As the concentration of TP increased from 1nM to 1mM there was a return to control expression (Fig 2.5 A). Defensins don't respond significantly to TP exposure (P>0.05) (Fig 2.5 B & C).

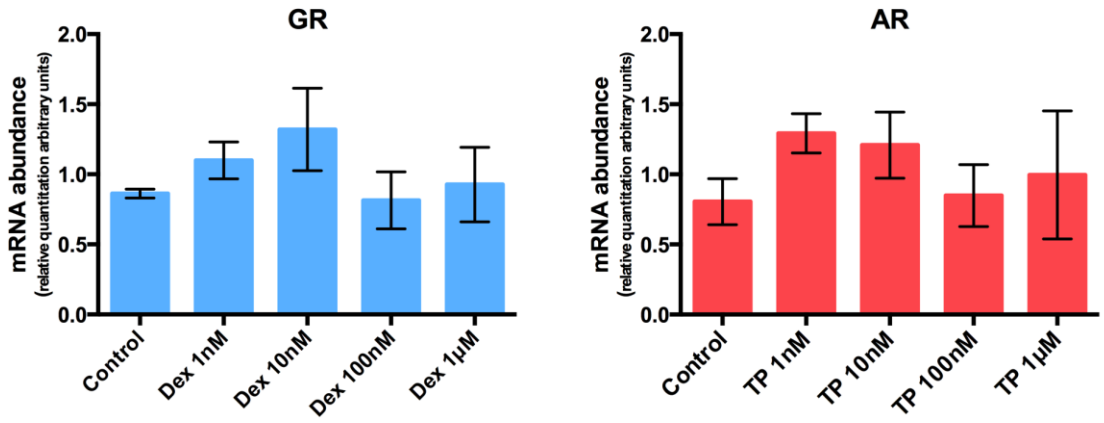


Figure 2.6. AH-1 cell line *in vitro* AR and GR expression after 24h Dex or testosterone propionate exposure. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (bActin and GAPDH). Error bars represent SEM. Data were analysed by ANOVA. Comparisons between treatments were made using Fisher's LSD. n=3 for both all treatments and control.

To elucidate whether the effects found in the AH-1 cell line in response to the steroid treatments were mediated via androgen receptor or glucocorticoid receptor (AR or GR) regulation the expression of both receptors were assessed. No significant differences (P>0. 05) attributable to steroid treatment were observed (Fig 2.6).

In humans there is a Vitamin D response element upstream of the *CAMP* gene, which stimulates production of LL-37. AH-1 cells (ovine) were assessed for their response in terms of SMAP-29 mRNA expression after VitD exposures. There was no significant change in expression of SMAP-29, sBD1 or sBD2 across the series of VitD concentrations that were tested (Fig 2.7 A-C, P>0.05). To ensure that the TLR signalling was still operational within the AH-1 cell line IL-8 messenger RNA abundance was measured. After LPS stimulation IL-8 message is 300x more than control (P<0.0001) and there was no significant difference in IL-8 message with 100nM VitD (P>0.05) (Fig 2.7 D).

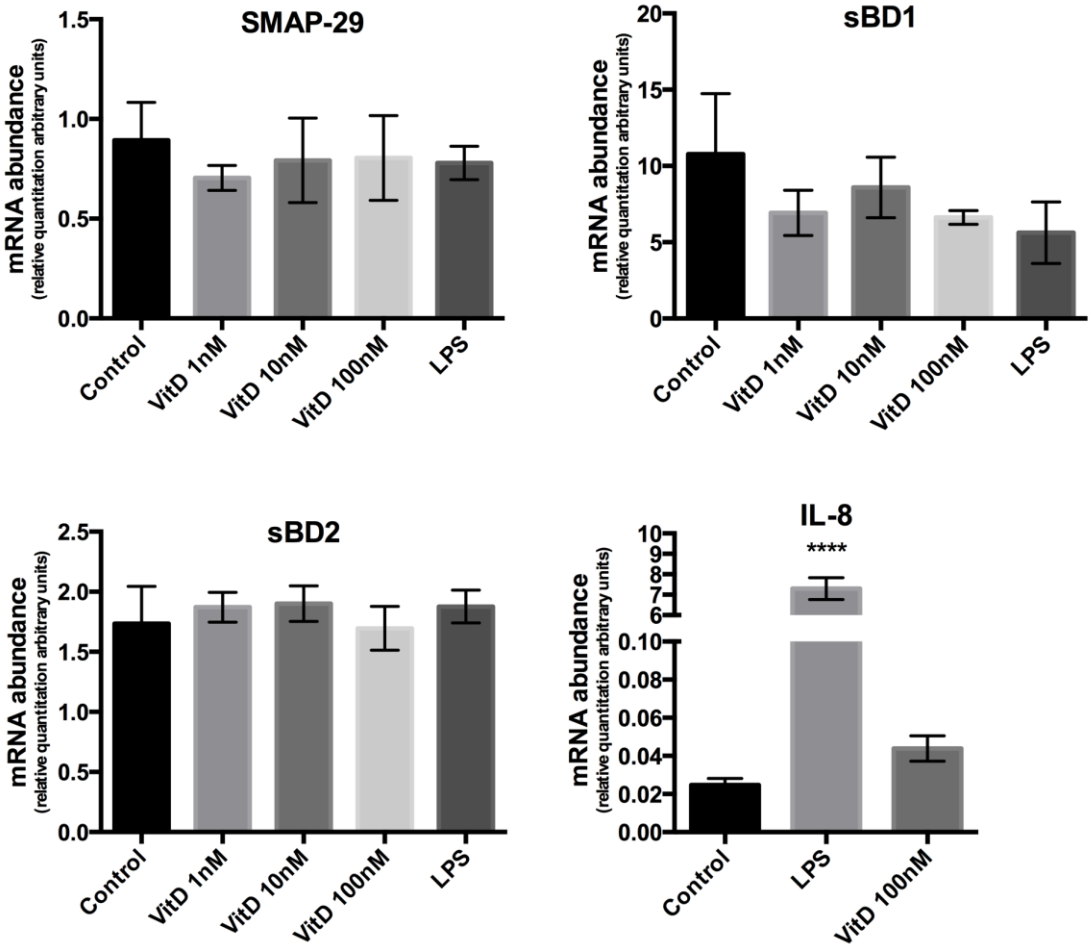


Figure 2.7. AH-1 cell line *in vitro* SMAP-29, sBD1, sBD2 and IL-8 expression after 24h vitamin D_3 (calcitriol) exposure. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (bActin and GAPDH). Statistically-significant differences to control are denoted by **** P<0.0001. Error bars represent SEM. Data were analysed by ANOVA. Comparisons between treatments were made using Fisher's LSD. n=3 for both all treatments and control.

2.3 Discussion

In vivo study results demonstrate that administration of Dex during pregnancy has no significant effects upon fetal lung, thymus and placenta in terms of SMAP-29, sBD1 and sBD2 expression (Fig 2.1). TP administration similarly has no effect in the fetal lung and the placenta on SMAP-29, sBD1 and sBD2 expression (Fig 2.2A and B); however there was a significant increase in expression in SMAP29 in response to *in utero* direct fetal administration of TP in the thymus (Fig 2.2C). The relevance that this has in terms of the development of the thymus and thymopoiesis (T-cell maturation), given the importance of these systems throughout life, requires further investigation.

The thymus in humans and rodents constitutively expresses LL-37/mCRAMP (mouse cathelicidin-related antimicrobial peptide) during fetal and postnatal life (Colantonio et al., 2011). This expression is part of a mechanism responsible for constitutive expression of interferon stimulated genes (ISG's) in the thymus to increase resistance to viral infection. LL-37's role in this mechanism is to complex with DNA or RNA from apoptotic T-cells undergoing negative selection and to stimulate IFN-α expression though TLR7/9 signaling which will then cause downstream expression of ISG's via IFN-α receptor (IFNαR) (Lande et al., 2007; Ganguly et al., 2009). This constitutive expression of IFN-α is localised to the medulla of the thymus where mature T-cells, pDCs, and, macrophages reside. This localised expression is thought to confer a host defence advantage against pathogens such as CCR-5 tropic HIV that targets mature thymocytes as compared to CXCR-4 tropic strains (Colantonio et al., 2011).

Classically, androgens are known to be suppressive of thymopoiesis via AR signalling in the thymic epithelial cells, as upon withdrawal or suppression of androgens, there is an enlargement of the thymus and increase in T-cell production (Olsen et al., 2001). This increase in SMAP-29 could serve two functions in response to androgens; first to help co-ordinate the suppression of thymopoiesis seen in response to androgens (Olsen et al., 2001; Olsen & Kovacs, 2001). Secondly, with the known ability of cathelicidins to

mediate the inflammatory response (Barlow et al., 2006; Li et al., 2009; Nagaoka et al., 2006), the increase in expression of SMAP-29 could control the inflammatory state of the thymus in a similar manner to how LL-37 acts as an anti-inflammatory mediator during secondary necrosis of apoptotic neutrophils (Li et al., 2009). Thus , this would ensure that regulated induction of apoptosis, due to negative selection during T-cell maturation, carries on without a large inflammatory response.

A final theory, which is difficult to link to the effects of androgens on the female thymus in the context of SMAP-29, is that the expression of SMAP-29 in response to androgens could allude to a mechanism that confers the sexual dimorphism found in the development of autoimmune diseases (Hince et al., 2008; Gubbels Bupp et al., 2008; Gui et al., 2011). It has been shown, in a mouse model of systemic lupus erythematosus, that there is a male specific delay in the onset of symptoms; although after castration the males mimic the progression of the females (Gubbels Bupp et al., 2008; Gui et al., 2011). There is no direct evidence linking SMAP-29 or any other cathelicidin to the stringency of negative selection during T-cell maturation, but with the discovery here that there is a sex difference in expression due to androgens, this is a result that warrants further investigation.

The key organ in successful mammalian pregnancy is the placenta, which is responsible for the transport of nutrients and O_2 to the developing fetus (Igwebuike, 2006; Sammin et al., 2009; Burton, 2009). The function of this organ is intricately regulated by the maternal and fetal steroid milieu. The placenta also acts as an immunological barrier to the fetus We have shown here using the ovine trophoblast cell line AH-1 to model the placenta *in vitro*, that contradictory to the *in vivo* results, there is a short-term effect (24h post exposure) on the placental expression of CHDP after exposure to excess GC's. In these experiments, the levels of CHDP mRNA expression at 1nM Dex are double that of control. As the concentration of Dex increases towards 1μM the expression of sDB1 and sBD2 returns to control levels showing an inverse dose response. SMAP-29 also has this apparent inverse dose response reducing towards control levels at 10nM and

100nM, but unlike the defensins at 1μM the mRNA abundance of SMAP-29 significantly increases above control but not to levels (Fig. 2.4). These data suggests that there could be some type of non-monotonic dose relationship with SMAP-29 and Dex but a wider range of concentrations would have to be tested to elicit whether this response exists or not. Inverse dose responses are common in the field of endocrine disrupting chemicals (reviewed in; Lagarde et al., 2015).

In an attempt to explain this response, the expression of GR mRNA was measured to assess whether the reduced CHDP responses at the higher Dex concentrations were receptor mediated. The data indicates that there is no significant change in the expression of GR mRNA after Dex treatment (Fig. 2.6) but this does not rule out GR-mediated actions such as receptor ubiquitination and subsequent degradation (Wallace & Cidlowski, 2001).

TP has a similar effect on SMAP29 expression only in AH-1 cells, while defensin expression remains unchanged in response to androgens (Fig. 2.5). Again, to determine whether this inverse dose response is receptor mediated, AR receptor mRNA abundance was assayed and similarly did not show any change in expression in response to TP.

The stimulation of CHDP production in response to Dex in the placenta is interesting as Dex is a potent anti-inflammatory drug used to ameliorate inflammation in autoimmune conditions and severe inflammatory disorders and here it is paradoxically being shown to stimulate production of a proinflammatory peptide.

Similarly with the increase in SMAP-29 expression in response to TP is also interesting as TP is generally a suppressant on the immune system (Olsen et al., 2001; Sakiani et al., 2013). These findings require further investigation as they could have an impact in neonatal medicine (Dex) and also our current understanding of fetal programming of innate immunity (Dex or TP), which currently is very scarce. Whether this stimulation of CHDP are a direct action of steroid signaling remains to be seen as these increases in

expression are only found *in vitro* after 24h exposure but *in vivo* there are no prolonged effects programmed in the placenta.

In humans and higher primates there has been a translocation of a short interspersed nuclear element (SINE) containing a VDRE upstream of the *CAMP* gene (Gombart et al., 2005). This translocated response element has allowed humans and primates to increase expression of cathelicidin in response to Vitamin D_3. Here we show that sheep do not have this VDRE upstream of their cathelicidin, as they do not increase expression of SMAP-29 in response to VitD (Fig 2.7 A). Interestingly we also show a possible tolerance in the AH-1 cell line within the LPS stimulation pathway (Toll-like receptor 4) as when challenged with LPS, SMAP-29, sBD1 and sBD2 expression is not altered. However, IL-8 message after LPS stimulation is drastically increased compared to control and VitD stimulation (Fig 2.7 D). Further experiments challenging AH-1 with peptidoglycan or poly I:C would assess the involvement of TLR4 ,or if other TLR pathways or NOD-like receptors are altered (Lappas, 2013). However, the lack of VitD stimulation is not unexpected as Gombert *et al*, shown that the VitD response is not present in the rat, mouse and dog but is present in the higher primates. This highlights that this translocation event must have happened in primate and human evolution and has not occurred earlier in evolutionary terms (Gombart et al., 2005).

Chapter 3 – Expression and Activity of CHDP and Chlamydial Pathogens

3.1 Methods

3.1.1 *Chlamydia abortus* and *Waddlia chondrophilia* CHDP exposure experiments

Chlamydia abortus (*C.abortus*) strain S26/3 and *Waddlia chondrophilia* strain ATCC VR-1470 were exposed to LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN-LVPRTES), scrambled LL-37 (sLL-37, RSLEGTDRFPFVRLKNSRKLEFKDI-KGIKREQFVKIL), SMAP-29 (RGLRRLGRKIAHGVKKYGPTVLRIIRIAG) and PG-1 (RGGRLCYCRRRFCVCVGR), the porcine cathelicidin, to assess the ability of the antimicrobial peptides to kill or inhibit the infectivity of the organisms. Both organisms are obligate intracellular and were grown in McCoy cells (murine fibroblast cell line regularly used to propagate *Chlamydia spp*.) in RMPI 1640 supplemented with 5% fetal calf serum (PAA Laboratories, Peterborough, UK). Frozen stocks of *C.abortus* and *W.chondrophilia* that had been previously propagated and stored were used in our experiments. Placental infection was modelled by seeding AH-1 cells in 48-well plates (Corning) and grown to 80% confluency overnight in IMDM growth medium (w/ 5% FCS). Organisms were then incubated with CHDP in serum-free media at 100µg/ml, 50µg/ml, 25µg/ml, 10µg/ml and serum-free media as a control (0µg/ml). For LL-37, a scrambled peptide, sLL-37, was available and was used as a negative control (equivalent of 100µg/ml) as well as a serum free media control. Approx. 400000 organisms, equal to MOI 1 (multiplicity of infection, MOI 1 = 1 intracellular organism per cell) were pre-incubated with the CHDP enriched mediums for 1h at 4^{o}C. Exposure mediums were diluted 1 in 10 in 2% FCS IMDM media, then transferred to AH-1 cells and incubated for 1h at 37^{o}C to allow infection of cells and then replaced with normal growth medium and

grown for a further 48h. Infected cells were then lysed *in vitro* with AL buffer from DNeasy kit (Qiagen, Crawley, UK) and lysates collected and stored at -20°C for DNA extraction.

3.1.2 DNA Extraction

DNA was extracted from AH-1 cells infected with *C.abortus* and *W.chondrophilia* using DNeasy Blood and Tissue kit (Qiagen, Crawley, UK) as per manufacturer's instructions with no amendment to protocol. Extracted DNA was stored at -20°C for PCR analysis.

3.1.3 RNA Extraction, DNase treatment and cDNA Synthesis

All RNA extraction, DNase treatments and cDNA synthesis was carried out as per protocol explained in Chapter 2 section 3 (2.3).

3.1.4 Quantitative Real Time – Polymerase Chain Reaction (qRT-PCR)

3.1.4.1 Measurement of CHDP and inflammatory response to *W.chondrophilia*

qRT-PCR was performed using Applied Biosystems StepOne Reat-Time PCR machine with SYBR green detection and a 96-well format setup. PCR conditions were as follows; 10 mins at 95°C, then 40 cycles of 15 secs at 95°C, and 1min at 60°C, with a melt curve analysis included in each run. Primers for IL-1α, IL-1β, IL-8 and TNFα were obtained from MGW Eurofins with primer sequences optimised and previously published (Fach et al., 2007; Smeed et al., 2007) and SMAP-29, sBD1 and sBD2 primers from PrimerDesign Ltd (PrimerDesign Ltd, UK). Housekeeper genes were identified using a GeNorm Kit to establish the most stable housekeeping genes (PrimerDesign GeNorm Ovine 12 reference gene kit, PrimerDesign, Southampton, UK). All samples were analysed using ΔΔCt method, $2^{-(\Delta\Delta Ct)}$ with quenching against a mixed reference sample. Each 10µl reaction consisted of 1µl (40ng) cDNA template, 0.5µl (300nM) gene specific primers, 5µl SYBR Green (Precision 2X MasterMix, PrimerDesign) and the reaction was brought to 10µl with DNase/RNase free H_2O. No RT and no template H_2O controls were run on each plate as negative controls.

3.1.4.2 qRT-PCR determination of bacterial growth

Chlamydiales 16S gene copy number was used to quantify the number of organisms and indirectly bacterial growth in the CHDP exposure experiments. Taqman pan-chlamydiales 16S gene PCRs were carried out in 20µl reactions consisting of, 10µl Quanta PCR MasterMix (Quanta Toughmix Low ROX PCR MasterMix) 0.2µl forward primer (100nM), 0.2µl reverse primer (100nM) and 0.2µl probe (100nM) (FAM/BlackHole Quencher) 1µl of DNA template and 8.4µl of DNase/RNase free H_2O. Using Lienard's method (Lienard et al., 2011), a standard curve using a plasmid with Chlamydiales 16S gene from *Parachlamydia acanthamoeba* inserted, at predetermined concentrations was run on each plate to determine copy number. This was used as a marker of organism growth.

Gene Name	Forward Primer 5'-3'	Reverse Primer 5'-3'
IL-1α	TTGGTGCACATGGCAAGTG	GCACAGTCAAGGCTATTTTTCC
IL-1β	ATGGGTGTTCTGCATGAG	AAGGCCACAGGAATCTTG
IL-8	AAGCTGGCTGTTGCTCTC	GGCATCGAAGTTCTGTACTC
TNFα	CTCATCTACTCGCAGGTCCTC	ACTGCAATGCGGCTGATGG

Table 3.1: qPCR primers for genes of interest

Primer Name	Primer sequence 5'-3'
panCh16F2	CCGCCAACACTGGGACT
panCh16R2	GGAGTTAGCCGGTGCTTCTTTAC
pan16S probe	FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1

Table 3.2: Taqman qPCR primers for pan-chlamydiales 16S gene

3.1.5 Statistical analysis

All CHDP exposures were run in duplicate so statistical analysis was not performed. All qRT-PCR results from 24hr Waddlia infections were analysed using ΔΔCt method, 2^{-}

$^{(\Delta\Delta Ct)}$ with quenching against a mixed reference sample. Statistical analyses were carried out using ANOVA and comparisons between groups were carried out using Fishers LSD test. Results were considered significant if $P \leq 0.05$. Statistical analyses were run using GraphPad Prism v6.0 (GraphPad Software Inc., San Diego, CA).

3.2 Results

3.2.1 *Chlamydia abortus* and *Waddlia chondrophilia* growth after incubation with CHDP

To determine the efficacy of cathelicidins against *C.abortus* and *W.chondrophilia*, LL-37, SMAP-29 and PG-1 were tested against these reproductive pathogens.

Pan-chlamydiales 16S gene primers were used to detect 16S rRNA gene copy number, which was used as an indirect measure of organism growth for both *C.abortus* and *W.chondrophilia*. There is a noticeable difference in copy numbers between both species, this is due to the fact that *W.chondrophilia* enters its exponential phase around 30-36hrs whereas, *C.abortus* does not enter exponential phase until after 48hrs. When incubated with human cathelicidin, LL-37, *C.abortus* copy number increased as the concentration of the peptide was increased from 0µg/ml to 25µg/ml. At 50µg/ml of LL-37 the *C.abortus* copy number drops to that of 10µg/ml (~80 copies) but as the concentration of peptide increases from 50µg/ml to 100µg/ml the copy number begins to rise again. Scrambled LL-37 (sLL-37, 100µg/ml) peptide was used as a negative control and interestingly shows that it encourages *C.abortus* growth as well as 100µg/ml LL-37 (Fig 3.1).

When *W.chondrophilia* is incubated with the control, 0µg/ml LL-37, there were approx. 3800000 copies of the 16S gene. Treatment with LL-37 at 10µg/ml and 25µg/ml results in a slight increase in the 16S gene to ~5000000 copies which indicates organism growth. At 50µg/ml there is a similar number of copies of the 16S gene to control (~4000000), although this effect is lost at 100µg/ml where the copy number increases to ~4800000 copies. Scrambled LL-37 is shown to have the greatest effect against *W.chondrophilia* reducing the 16S copy number to ~1800000 copies (52% reduction).

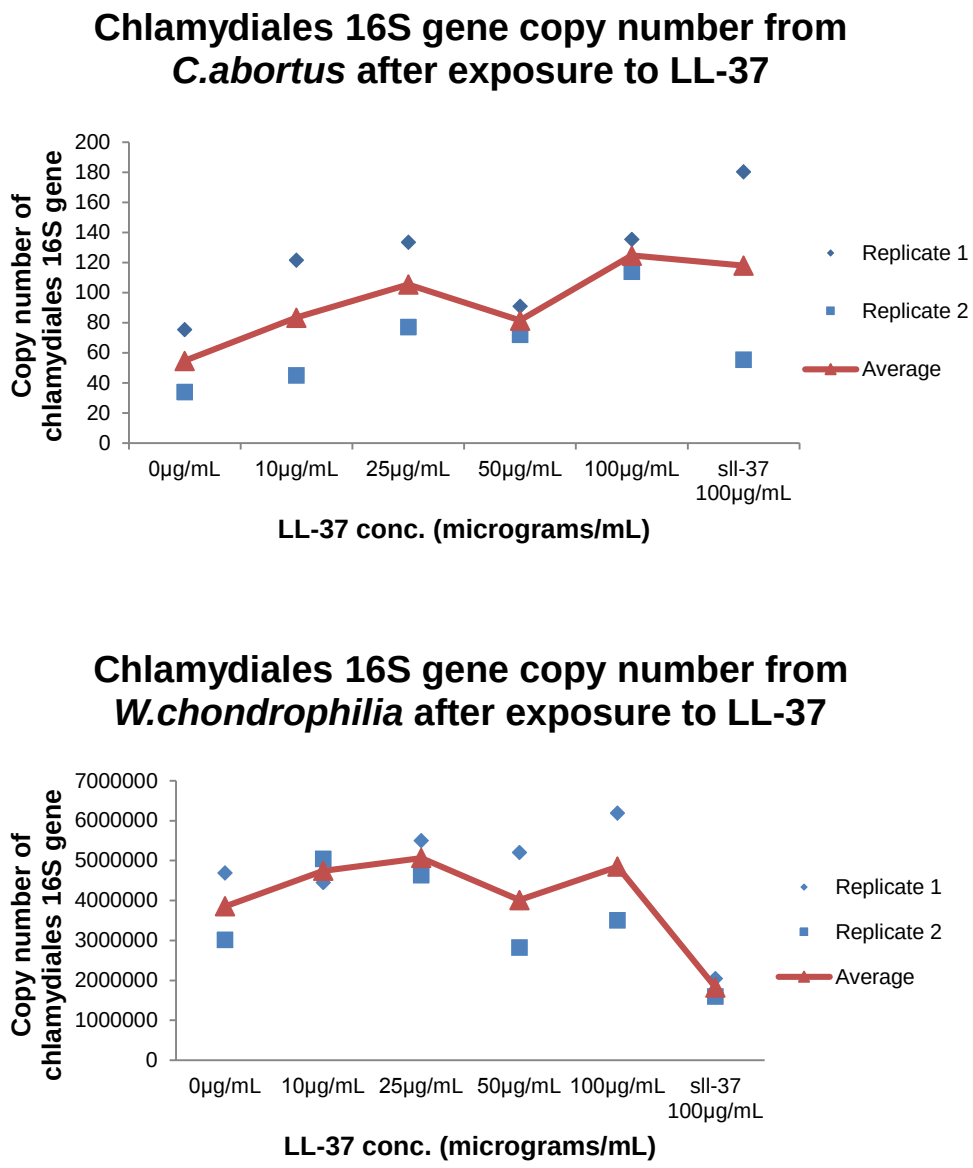


Figure 3.1: 16S copy number of *Chlamydia abortus* and *Waddlia chondrophilia* after exposure to LL-37 derived by qRT-PCR. Copy number calculated against standard curve. Data displayed as individual replicates and average, n=2.

Upon incubation with the ovine cathelicidin SMAP-29 at 10µg/ml the number of *C.abortus* 16S gene copies (~75 copies) is half that of control levels, 0µg/ml SMAP29 (~145 copies). Increasing the level of SMAP-29 to 25µg/ml where the copy number increases to ~130 copies voids this effect. As the concentration of SMAP-29 is increased to 50µg/ml and 1000µg/ml, *C.abortus* appears to grow, as the copy number increases to ~210 and ~280 copies respectively (Fig 3.2).

SMAP-29 had a similar effect on *W.chondrophilia* as it does with *C.abortus* reducing the copy number at 10µg/ml (~210000 copies at control down to ~120000 copies at 10µg/ml). Additionally to this SMAP-29 at, 25µg/ml and 50µg/ml both seem to have an antimicrobial effect reducing the 16S copy number by ~100000 copies. At 100µg/ml SMAP-29 the copy number returns to control levels of ~210000 copies.

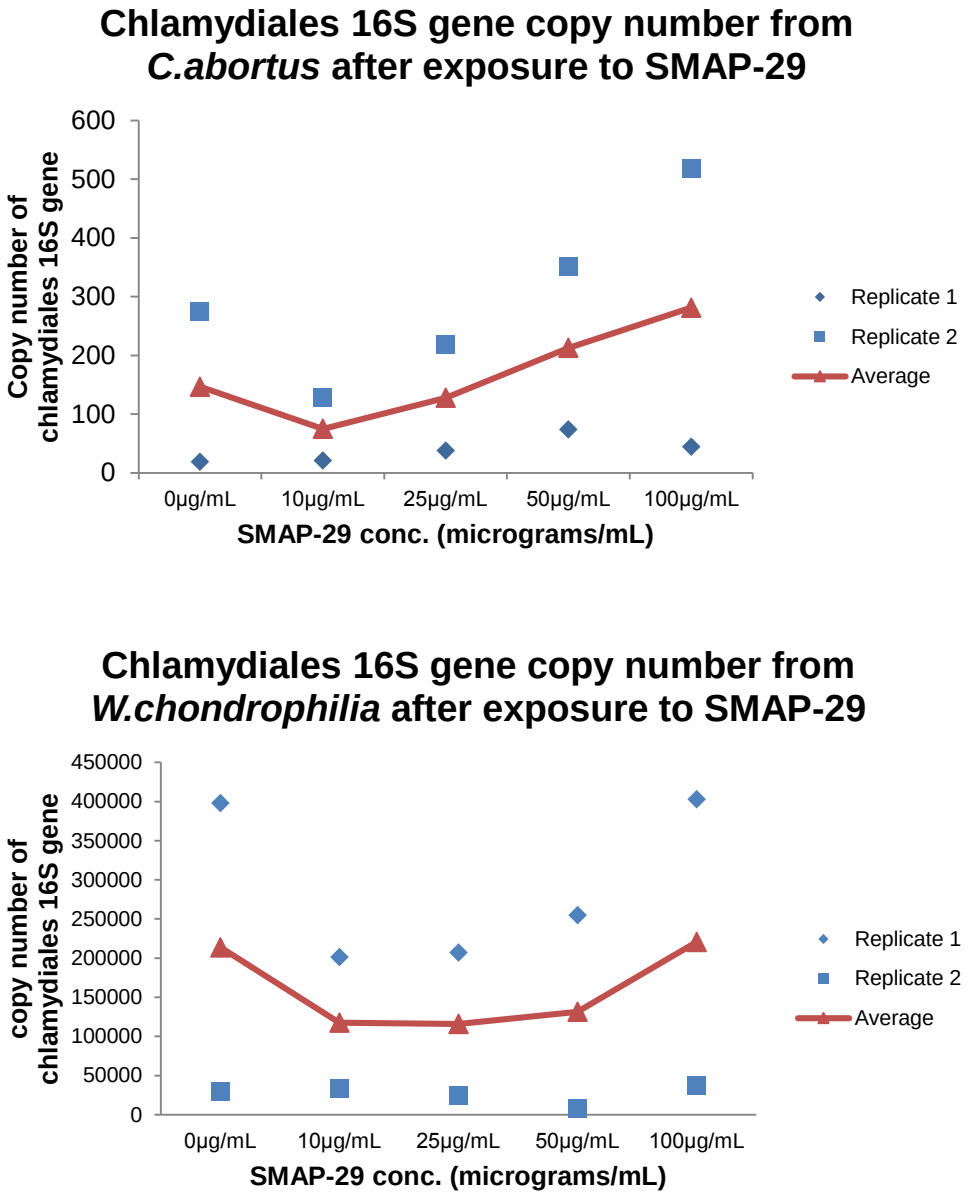


Figure 3.2: 16S copy number of *Chlamydia abortus* and *Waddlia chondrophilia* after exposure to SMAP-29 derived by qPCR. Copy number calculated against standard curve. Data displayed as individual replicates and average, n=2.

To test whether there is a conserved activity of cathelicidins across different species the porcine protegrin PG-1 was also investigated. This is of particular importance as porcine

livestock can suffer from Chlamydial infection, albeit a different *Chlamydia* strain (spp. suis). PG-1 also has less cytotoxic effects against mammalian cells so would be an ideal candidate as a blueprint for a novel therapeutic. At control levels (0µg/ml) there was a very low *C.abortus* 16S copy number present (~10 copies). Upon incubation with PG-1 at 10µg/ml this increased to over 200 copies. Further addition of PG-1 (25µg/ml) reduced the number of 16S copies to ~150. As the dose of PG-1 is increased to 50µg/ml and 100µg/ml, the copy number of *C.abortus* increases to ~250 and ~320 respectively (Fig 3.3). PG-1 seemed to promote *W.chondrophilia* growth as the copy number increased from control at ~16000 copies to ~23000 copies at 10µg/ml, and increases again to ~27000 copies at 25µg/ml where this effect seems to plateau at 50µg/ml. There is only a slight increase at 100µg/ml (~29000) relative to the previous increases.

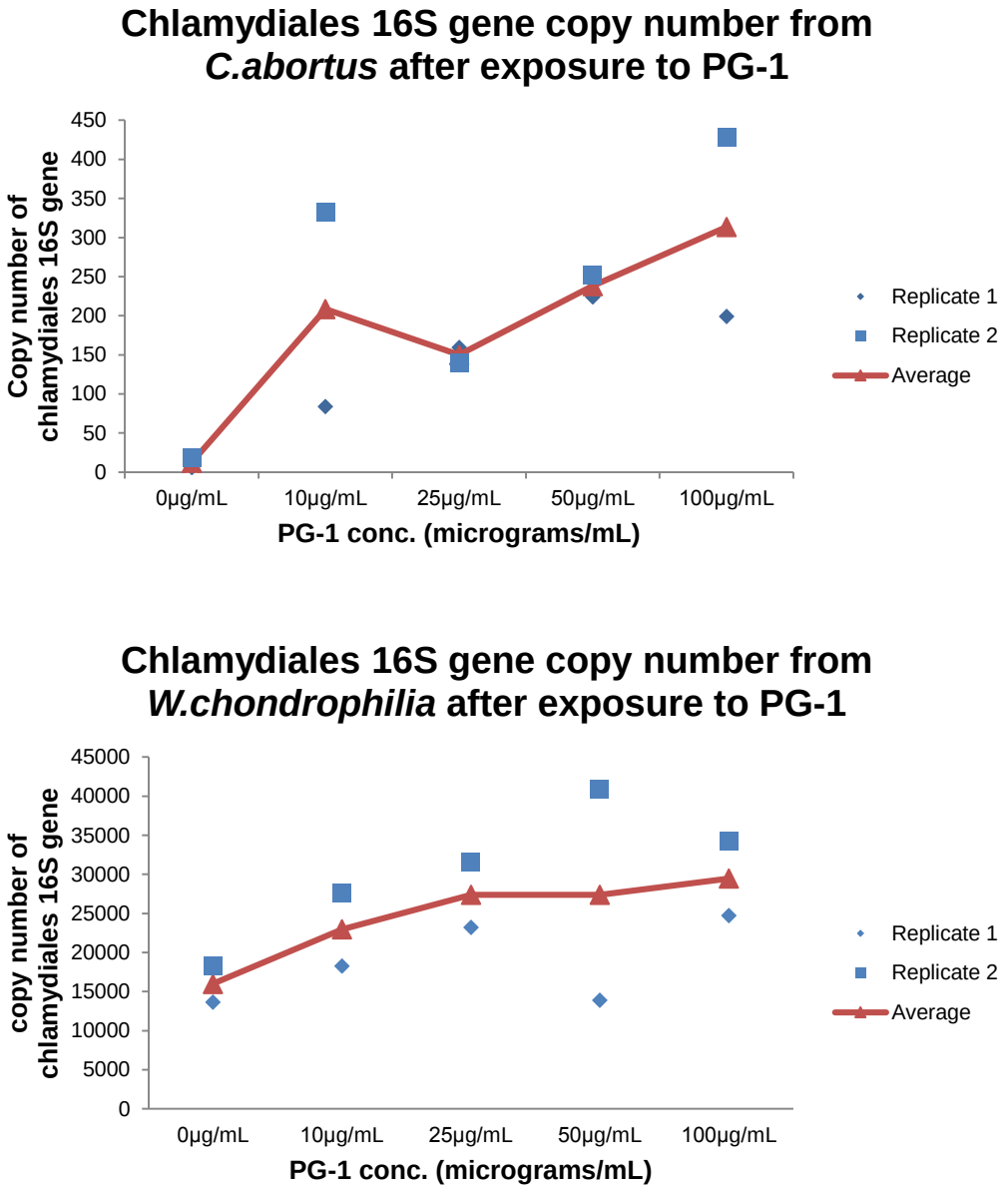


Figure 3.3: 16S copy number of *Chlamydia abortus* and *Waddlia chondrophilia* after exposure to PG-1 derived by qPCR. Copy number calculated against standard curve. Data displayed as individual replicates and average, n=2.

3.2.2 CHDP and cytokine expression after *W.chondrophilia* exposure

24h post infection with *Waddlia*, AH-1 cells were lysed and the expression of CHDP and cytokines measured by qPCR. *Waddlia* is shown here to stimulate the expression of the ovine β-defensins in this model of placental infection at an MOI 10 (Fig. 3.4B & C). This also shows that defensins are upregulated in response to intracellular pathogens but not via extracellular microbe associated molecular peptides (MAMPs) as UV killed *Waddlia*

(equivalent to MOI 10) does not elicit any response from the defensins. *Waddlia* does not stimulate any effect from SMAP-29 (Fig 3.4A).

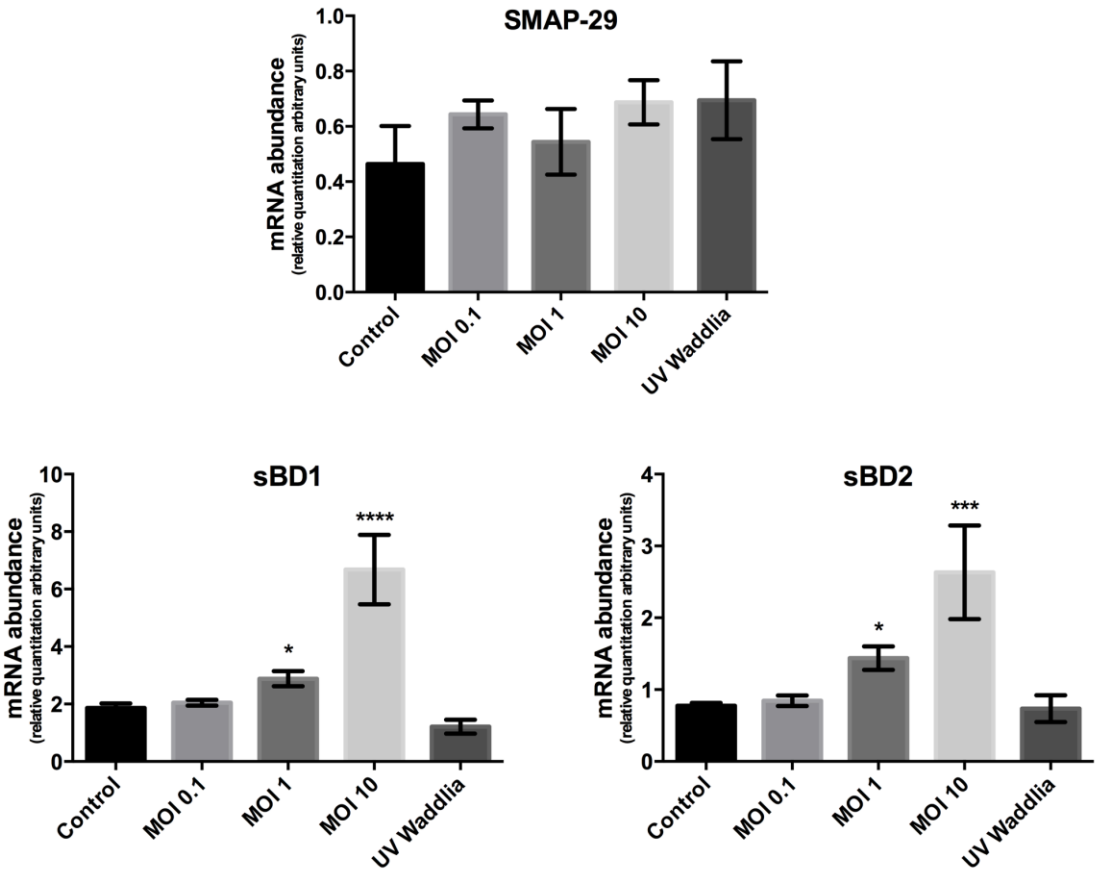


Figure 3.4. AH-1 cell line *in vitro* SMAP-29, sBD1 and sBD2 expression after 48h *Waddlia chondrophilia* infection. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (βActin and GAPDH). MOI stands for multiplicity of infection (i.e. MOI1 = 1 organism per cell). Statistically-significant differences to control are denoted by **** P<0.0001, *** P<0.001. Error bars represent SEM. Data were analysed by ANOVA. Comparisons between MOI's were made using Fisher's LSD. n=3 for all treatments and control.

An inflammatory response to infection with chlamydial species in the trophoblast is thought to contribute to the pathogenesis and pathology of chlamydial infection (Wheelhouse et al., 2009; Wheelhouse et al., 2014). Upon infection with *Waddlia* for 24h IL-8 message was significantly elevated at MOI 1 and MOI 10 showing that the *Waddlia* stimulates a potent proinflammatory reaction (Fig 3.5A). IL-1α message didn't change in response to *Waddlia* infection (Fig 3.5B). In response to exposure of *Waddlia* at MOI 10,

IL-1β was significantly increased (Fig 3.5C). TNFα was significantly increased at both MOI 1 and MOI 10 after 24h *Waddlia* infection (Fig 3.5D).

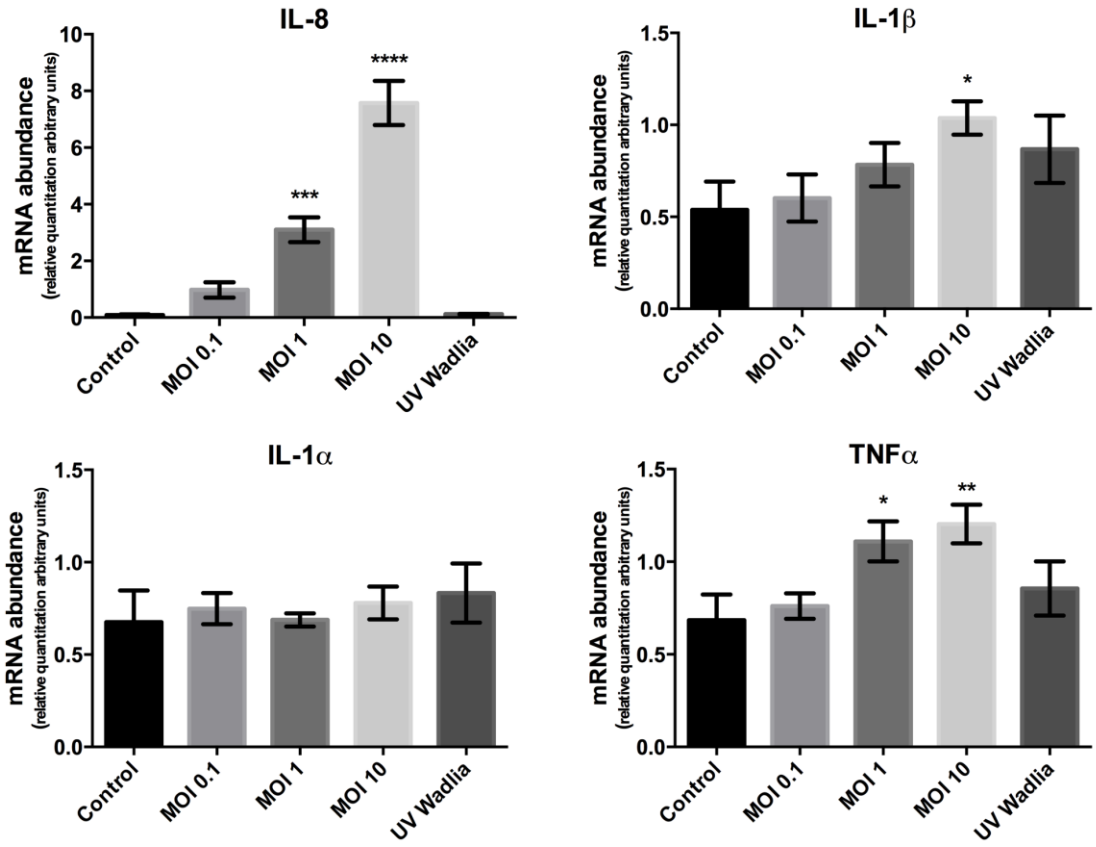


Figure 3.5. AH-1 cell line *in vitro* IL-8, IL-1α, IL-1β and TNFα expression after 48h *Waddlia chondrophilia* infection. Data represents mRNA abundance of GOI's in relatively quantified arbitrary units relative to geometric mean of 2 housekeeper genes identified by GeNorm analysis (βActin and GAPDH). MOI stands for multiplicity of infection (i.e. MOI1 = 1 organism per cell). Statistically-significant differences to control are denoted by ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. Error bars represent SEM. Data were analysed by ANOVA. Comparisons between MOI's were made using Fisher's LSD. These data are also published in (Wheelhouse et al., 2014). n=3 for all treatments and control.

Although MOI 0.1 wasn't significant in any of these experiments this is only a snapshot at 24h post infection, meaning there is not enough organisms to elicit the same response after 24h with MOI 0.1 as compared to MOI 1 or MOI 10. UV killed *Waddlia* also failed to elicit a response indicating that the cytokine responses observed must be due to intracellular abundance and subsequent detection of the organism.

3.3 Discussion

CHDP are an attractive novel class of antimicrobial therapeutics. Their resilience to microbial resistance and efficacy against a diverse range of targets including bacteria, fungi and viruses makes them very attractive as potential future therapies (Padhi et al., 2014; Tsai et al., 2014; Bucki et al., 2010; Barlow et al., 2014; Yeung et al., 2011). It has been shown previously that LL-37 can act against intracellular pathogens such as *Mycobacteria* spp. (Padhi et al., 2014; Sato et al., 2013).

With previous studies showing that these cathelicidins have the ability to act upon intracellular pathogens we have chosen three cathelicidins; human LL-37, ovine SMAP-29 and porcine PG-1, and tested their antimicrobial properties against the common intracellular pathogen *Chlamydia abortus* and a new emerging pathogen *Waddlia chondrophilia*. Both *of C.abortus* and *W.chondrophilia* are intracellular pathogens that can cause abortion during pregnancy in livestock and humans (Dilbeck-Robertson et al., 2003; Baud et al., 2007; Baud et al., 2008; Pospischil et al., 2002). Here, using 16S gene copy number as a proxy for microbial growth, we have shown that LL-37 and PG-1 are ineffective against *C.abortus* and *W.chondrophilia* as paradoxically they do not inhibit the growth of the organisms; they promote growth of the organisms. As the concentration of cathelicidin is increased to supraphysiological concentrations (>25-30μg/mL) (Nijnik & Hancock, 2009), the copy number of the 16S gene in both organisms increases in the presence of all CHDP tested (Fig. 3.1 and 3.3). SMAP-29 has a slightly different effect on both *C.abortus* and *W.chondrophilia*; showing that the 16S copy number is reduced at 10μg/mL, indicating a reduction in bacterial cell growth. Against *C.abortus* this effect of SMAP-29 is only found at 10μg/mL and the paradoxical increase in 16S gene copy number is found as the SMAP-29 concentration increases towards 100μg/mL. SMAP-29 seems to have a greater 'therapeutic window' and is able to maintain an bacteriostatic hold on 16S gene copy number at concentrations between 10μg/mL to 50μg/mL before bacterial growth resumes.

A classical antimicrobial function of CHDP has been the ability to interfere with the microbial cell wall and create perforations due to the net positive charge of the molecule and negative net charge of the cell wall of bacteria (Ramanathan et al., 2002). As indicated by our data, LL-37 and PG-1 in particular do not affect *C.abortus* and *W.chondrophilia* as we would expect them too by reducing bacterial growth. We have shown that the organisms exhibit greater growth when there is more cathelicidin present. One hypothesis that may explain this phenomenon is that at supra-physiological concentrations, LL-37 (Henzler-Wildman et al., 2003; Henzler-Wildman et al., 2004) and PG-1 (Heller et al., 2000) will disrupt the host cell membrane and this inadvertent property of these cathelicidins may make the host cells more permissive to infection. As the sLL-37 control has a similar number of copies of the 16S gene to 100µg/ml this must be due to some other mechanism that requires further investigation.

The approach we utilized here tested the effect of these cathelicidins against the microbes directly before they were able to establish an infection (Fig 3.6). By using this approach, we may have stressed the bacteria, which could explain our findings of an increase in bacterial growth as the concentration of LL-37 and PG-1 increase. *Chlamydia trachomatis* can secrete the serine protease chlamydial proteasome/protease-like activity factor (CPAF) which can cleave LL-37 and eliminate its antimicrobial actions so a similar mechanism cannot be ruled out here (Tang et al., 2015). In order to fully assess the efficacy of cathelicidins against *C.abortus* and *W.chondrophilia* infection further work is required. In the first instance, AH-1 cells could be pre-incubated with differing concentrations of cathelicidin to elucidate firstly whether there is an effect of the peptides on the cells themselves before the microbes are introduced, secondly if after introduction of the microbes, whether these cells are more or less permissive to infection (Fig. 3.6). We also did not test the ability of the cathelicidins to effect an established infection, which would be a more realistic scenario (Fig. 3.6).

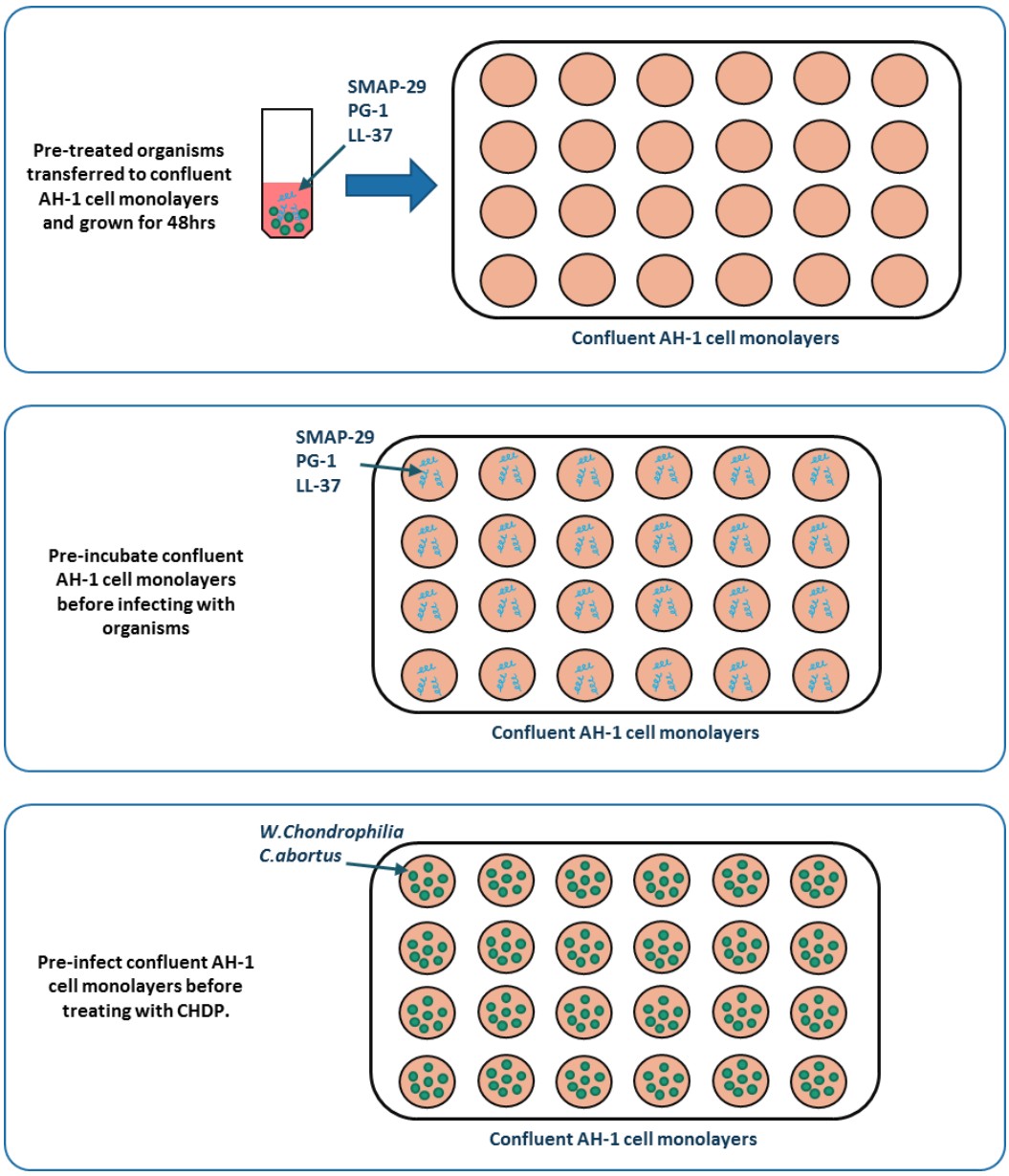


Figure 3.6. Diagram outlining CHDP pre-incubation experiment (first panel) and examples of possible permutations to fully understand the effect of CHDP on chlamydia-like organisms.

Waddlia chondrophilia is a member of the order Chlamydiales known to cause zoonotic abortion in humans and in cattle (Baud et al., 2014; Dilbeck et al., 1990). These data show that the AH-1 cell line has the capacity to detect the pathogen intracellularly and cause a pro-inflammatory response similar to that of *C.abortus* and other *Chlamydia* spp. (Wheelhouse et al., 2008). Waddlia must be more readily detected via the intracellular pattern recognition receptors such as NOD1 and 2 like other *chlamydia* spp. (Wheelhouse et al., 2009; Lappas, 2013; Buchholz & Stephens, 2008; Welter-Stahl et al., 2006; Wheelhouse et al., 2014; Moreira & Zamboni, 2012) as there is only a

noticeable response to live infection and not to the UV killed organisms. For the first time this data demonstrates a change in CHDP production during *Waddlia* infection. The increase in ovine β-defensins could be alluding to a mechanism were the defensins can be simulated by intracellular pathogen detection potentially via NOD1/2 (Moreira & Zamboni, 2012).

Final Conclusions and Further Work

Within this body of work the importance of the steroid milieu and consequences of chlamydial infection during pregnancy in the context of cationic host defense peptides and innate immunity have been outlined, but a significant body of work remains to be undertaken.

These data show that there is a potential mechanism for manipulating immunological tolerance within the thymus via excess exposure to androgens *in utero*. This mechanism could also be underpinning the sexual dimorphism found in autoimmunity but a whole suite of studies would be required to fully investigate this hypothesis.

Through the LPS exposures and the chlamydial infection studies in the AH-1 cell line, a better understanding of pathogen recognition and innate immune response has been started, Further work using inhibitors of TLR4/2 and NOD1/2 signal transduction is required to fully understand the mechanism by which *W.chondrophilia* is detected and reported to the rest of the immune system. Furthermore, to assess whether there is some form of dampening or modulation of the classical TLR4 pathway in the AH-1 cell line and subsequently the placenta as this could be a unique phenomenon, as macrophages exposed to LPS will produce LL-37 via TLR4 signaling (Bucki et al., 2010).

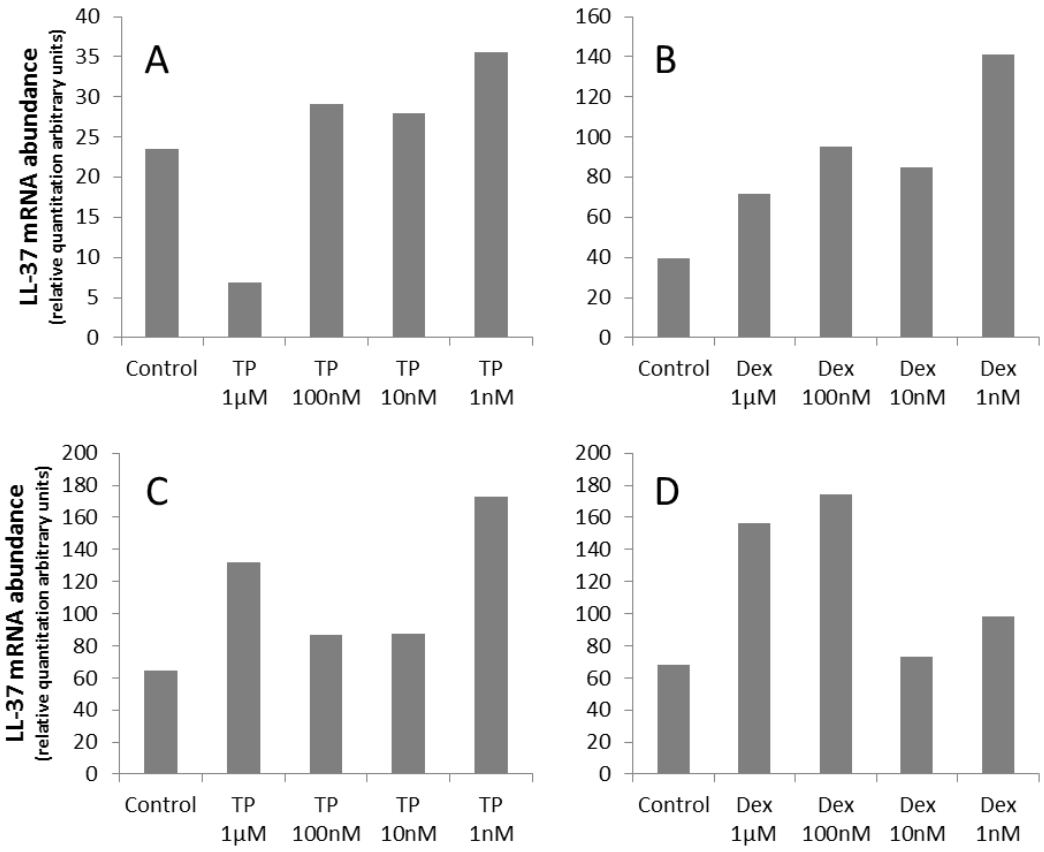
The expression of cathelicidin in the uterus remains underinvestigated, and it is unclear whether the ability of cathelicidins to modulate the adaptive immune system is contributory to the tolerance of heterogeneous fetus during implantation and subsequently the duration of pregnancy.

These data show how the steroid milieu and sterility, in the context of infection, of the reproductive tract can regulate the expression of CHDP, which could ultimately have an impact on the success of pregnancy.

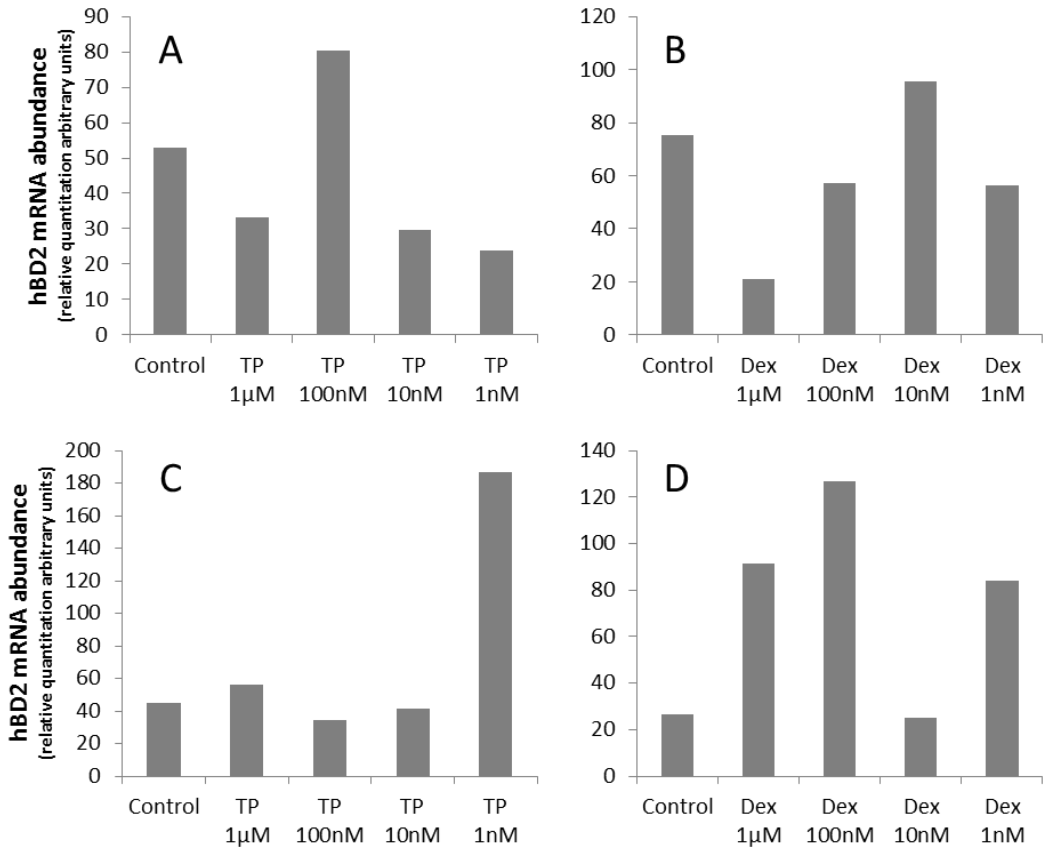
Appended data from Chapter 2

A549 qPCR results after 24h steroid exposure

Human lung epithelium is a key site of CHDP expression. To assess whether steroids impact on the expression of CHDP, the human alveolar epithelial cell line A549 was exposed to differing concentrations of Dex and TP for 24 and 48hrs.

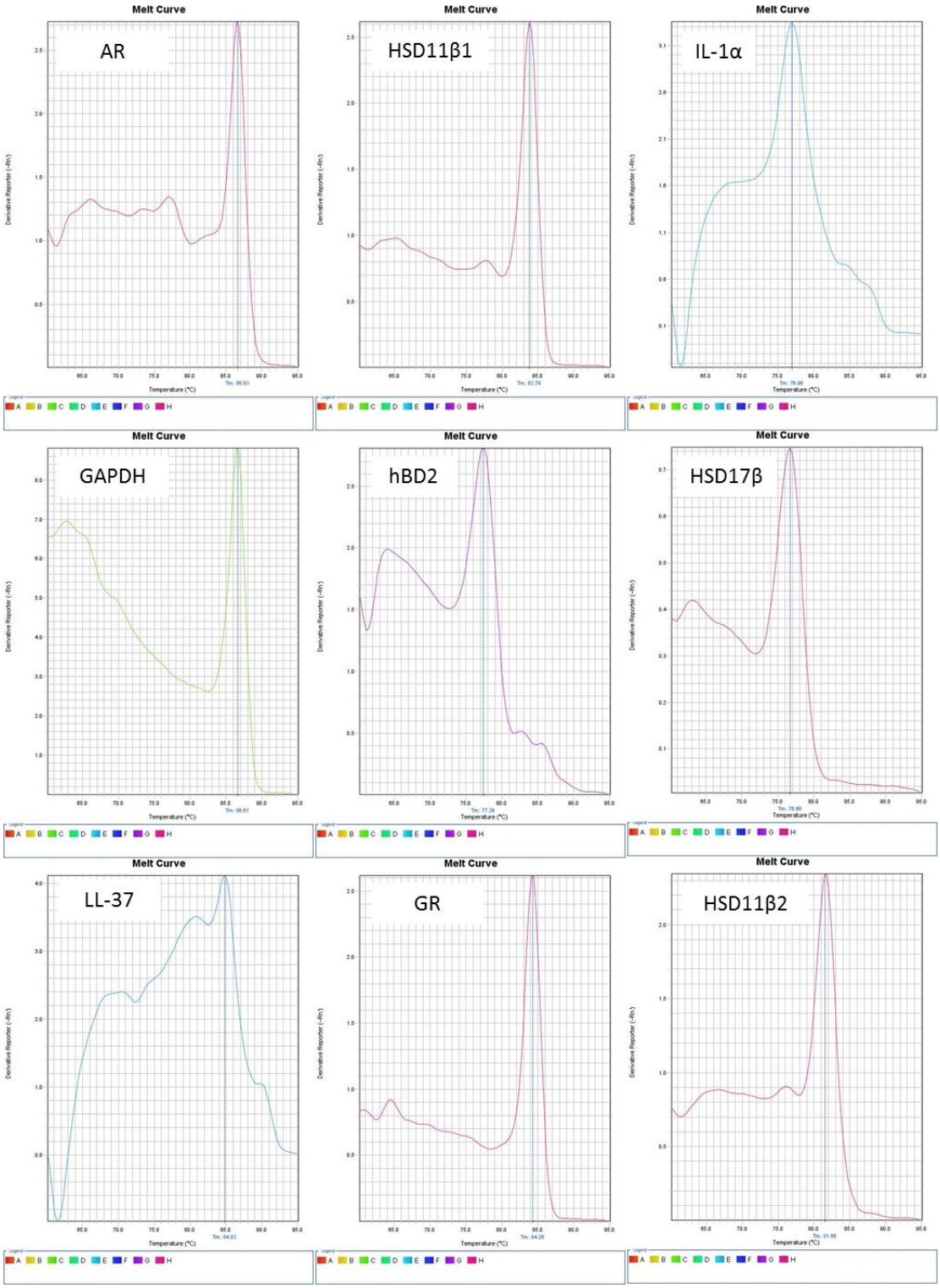


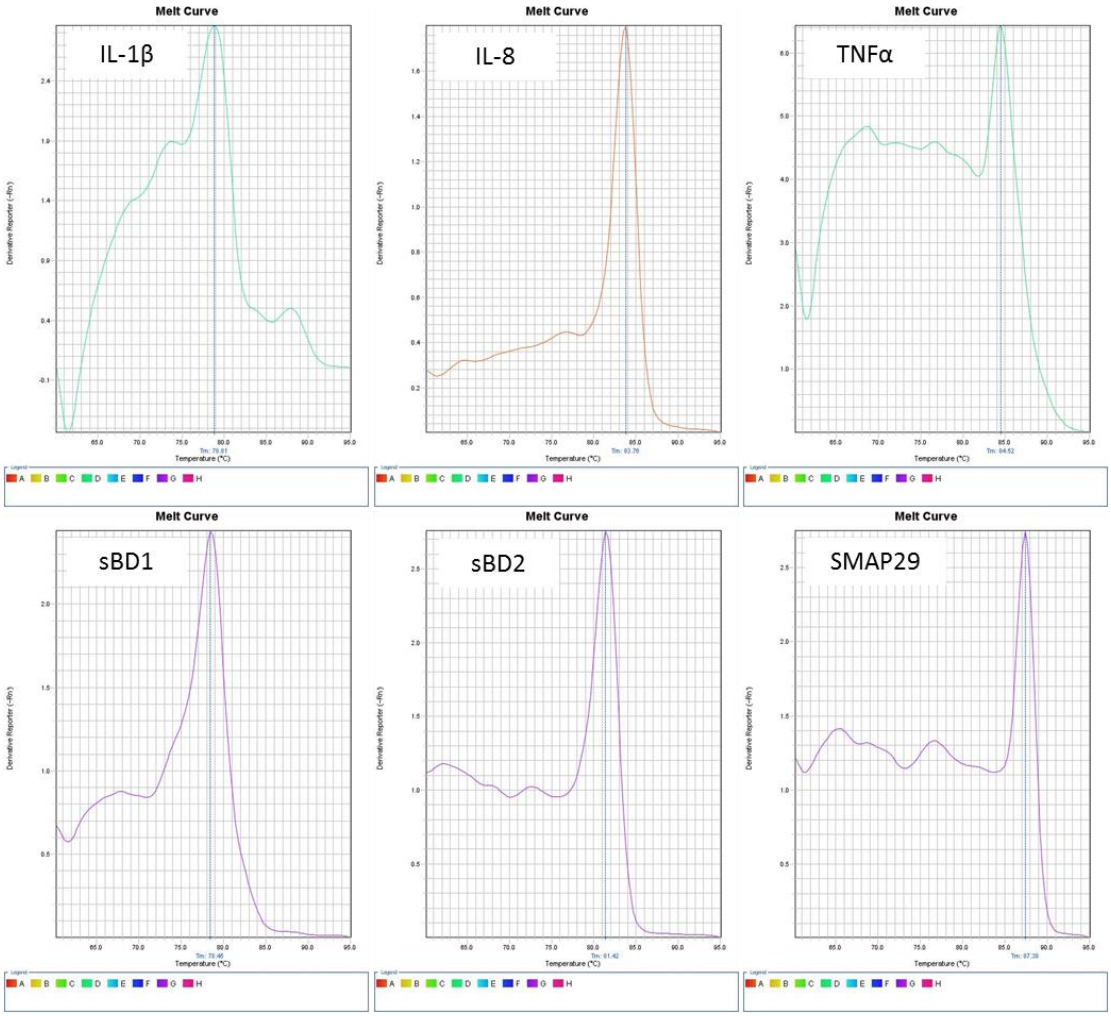
Appended Figure 1. A549 cell line *in vitro* LL-37 expression after exposure to Dex and testosterone propionate after 24h (A & B) and 48h (C & D). Data represents mRNA abundance of LL-37 in relatively quantified arbitrary units relative the housekeeping gene GAPDH. n=1 for all treatments and control.



Appended Figure 2. A549 cell line *in vitro* hBD2 expression after exposure to dexamethasone and testosterone propionate after 24h (A & B) and 48h (C & D). Data represents mRNA abundance of hBD2 in relatively quantified arbitrary units relative the housekeeping gene GAPDH. n=1 for all treatments and control.

Melt Curve analysis





Appended Figure 3. Melt curve analysis of GOIs and human GAPDH. Single peak identifies single PCR product.

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