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The role of *Chlamydia trachomatis* infection in adverse pregnancy outcomes

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Thesis submitted to the University of Edinburgh for the Degree of Doctor of Philosophy

April 2017

Declaration

This thesis has been composed by the author. The studies presented in this thesis were the unaided work of the author, except where acknowledgement is made. The work has not been submitted for any other degree or professional qualification. All included publications are the author's own work, except where indicated throughout the thesis.

Sevasti Giakoumelou

April 2017

Acknowledgements

I would like to thank my supervisors Professors Andrew Horne, Sarah Howie and Gary Entrican for giving me the opportunity to work on this project and advising me throughout. I'm extremely grateful especially to Andrew and Sarah for always being there for me over the past four years, supporting me and encouraging me, for their patients and guidance both with experimental work as well as writing this thesis and our papers.

I am also grateful to former and current members of the Horne lab group. Dr Jeremy Brown for offering advice and technical support during the first years of my PhD. Jean Wade for teaching me various methods as well as supporting my experimental work and helping me day to day in the lab. Dr Vicky Young for helpful advice not only experimental but also on PhD life in general. Dr Furquan Ahmad for assisting with sample processing.

The CRH is a great place for a PhD student. I am extremely grateful to everyone who has helped me and answered my questions during the past four years. Professor Hilary Critchley not only for donating ESC used in this study but also for always making me feel like a member of the Critchley lab group. Dr Alison Murray for calming me down during many moments of panic, Dr Moira Nicol for all her support. Dr Pamela Brown and Linda Fergusson for being PCR gurus and helping me through some very important experiments, Forbes Howie for being my ELISA advisor. Professor Philippa Saunders, Dr Douglas Gibson and Frances Collins for always being supportive whenever I needed help. Mike Millar, Sheila Mcpherson, Debbie Mauchline.

I was fortunate to be involved in patient recruitment during my PhD, an experience that taught me a lot. I would have never been able to do it without the help of Ann Doust, who guided me and advised me throughout the process for several months and was actively involved in recruiting. I would like to thank the nurses at Pregnancy support centre and especially Nic Watson for all her help and advice. I would also like to thank Ana Calarrao and Hayley Moir of Edinburgh Tissue biobank for all their help and being actively involved in recruitement.

I am very grateful to Dr Nick Wheelhouse for helping me generate the *Ct* stock used for the in-vitro and in-vivo studies at Moredun Institute. I am also grateful to Professor Paddy Horner for valuable advice on statistical analysis. I would like to thank Professor Myra McClure and Dr Gill Wilson for carrying out the Pgp3 assay of study samples.

I am very grateful to all my friends that made my time in Edinburgh so much more fun and helped me push through and reach the finish line sane. I don't think I have to mention you by name, you know who you are! Particularly I would like to thank Elena, Elpida and Diane for proofreading my thesis. And I would also like to thank my officemates of the past three years, for being partners in too much coffee drinking.

I am extremely grateful to Diane, Mariana, Dawn,, Yanni, Annalucia, Elpida and Callum for helping me finish the corrected thesis.

I am also extremely grateful to Dr Yannis Papastathopoulos for reviewing the statistics of Chapters 3 and 4.

Last, but most definitely not least, I would like to thank my family for everything. Without them, I wouldn't have been here today.

Publication arising from this work

Sevi Giakoumelou, Nick Wheelhouse, Jeremy Brown, Jean Wade, Ioannis Simitsidellis, Douglas Gibson, Philippa TK Saunders, Paddy Horner, Gary Entrican, Sarah EM Howie and Andrew W Horne

Chlamydia trachomatis infection of human endometrial stromal cells induces defective decidualisation and chemokine release.

Scientific Reports- accepted for publication

Presentation of this work

Defective decidualisation: a possible mechanism of *Chlamydia trachomatis* induced miscarriage

Presented at Society for Reproduction and Fertility- July 2015. Won Best PhD student Oral presentation award.

Declaration of work

All of the work in the present thesis has been carried out by Sevasti Giakoumelou with the following exceptions:

Chapter 3: I was helped by Dr Nick Wheelhouse Dr Sean Wattegedera and Prof. Gary Entrican at Moredun Institute during the production of *Ct* stocks for in-vivo and in-vitro experiments.

Chapter 4: *Ct* PCR swab test was carried out by Mike Shepherd at the Microbiology lab of the Royal Infirmary of Edinburgh.

Chapter 5: The Pgp3 assay of study samples was carried out by Dr Gill Wilson at Imperial College London.

Patient recruitment: I personally identified 130 eligible control patients from the Edinburgh Tissue Biobank and recruited 10 patients from the Obstetric Triage Department at the Royal infirmary. Furthermore, I personally recruited 58 women at the Pregnancy Support Centre of the Royal Infirmary of Edinburgh. Ann Doust, Nicola Watson, Priscilla Fernandez, Lisa Starrs, Jean Wade and Hayley Muir have recruited the remaining patients to date.

Thesis Abstract

Chlamydia trachomatis (*Ct*), the most common sexually transmitted bacterium, has been associated with adverse pregnancy outcomes including controversial data on miscarriage, intrauterine growth restriction and low birth weight, however the causative mechanisms are unknown. A successful pregnancy requires normal endometrial stromal cell (ESC) decidualisation and trophoblast invasion, processes that involve chemokine action and lead to successful implantation. My objectives were to determine whether *Ct* infection impacts upon ESC decidualisation and chemokine secretion on human primary ESC in-vitro, to investigate the role of *Ct* infection in pregnancy in-vivo using a murine model of pregnancy and to investigate the role of *Ct* in miscarriage in a statistically powered case control study.

A novel finding is that *Ct* can infect and proliferate in ESC, resulting in suboptimal decidualisation as measured by decidualisation marker prolactin's reduced mRNA and protein levels in infected ESC. Furthermore, the altered secreted chemokine profile of decidualised ESC suggests an attenuated innate immune response from infected ESC. Focusing on chemokines C-X-C motif chemokine 12 (CXCL12) and CXCL16, important for trophoblast invasion, decreased mRNA and protein concentrations were detected in infected decidualised cells.

From the in-vivo mouse model of past *Ct* infection in pregnancy, it was demonstrated that *Ct* infection did neither affect the fertility of the mice, pregnancy or resorption numbers in C3H mice nor alter embryonic and placental weight on e12 embryos. However, *Ct* infection caused reduction of embryo and placenta weight on e14 embryos.

Finally, preliminary data from the case control study indicate that past *Ct* infection is not associated with miscarriage. Our in house PGP3 ELISA that detects past *Ct* infection was more sensitive than a commercially available MOMP ELISA.

My data suggests that *Ct* infection affects pregnancy during the implantation stage by impairing decidualisation and altering chemokine secretion predisposing for adverse pregnancy outcomes that include growth restriction during later gestation.

Lay abstract

Chlamydia trachomatis (Ct) is the most common sexually transmitted bacterial disease worldwide. Controversial research data indicates that it may be a cause of pregnancy complications including miscarriage, growth restriction of the embryo during pregnancy and decreased birth weight. It is not yet known how *Ct* infection can cause this, however it is known that a successful pregnancy is determined during the first days, when the embryo attaches itself to a receptive womb, a process called implantation. A womb is not receptive to an embryo throughout the menstrual cycle, it has to prepare during every cycle for a possible implantation, a process called decidualisation. Decidualisation happens in a specific part of the womb that consists of cells called endometrial stromal cells (ESC) and following decidualisation the functional role of ESC is to allow and assist the implantation of an embryo. In combination with generation of new blood vessels and invasion of the embryonic trophoblast (the cells that will form the placenta) in the womb, these processes are essential for a successful pregnancy. Signalling molecules of the immune system called chemokines play an important role in regulating and coordinating all the above processes. My assumption was that *Ct* infection interferes with decidualisation resulting in pregnancy complications.

To investigate the effect of infection in decidualisation, I showed for the first time that *Ct* can infect the ESC of the womb. The infected ESC seem to not be able to undergo a decidualisation-like process they were subjected on as efficiently as non infected ESC. This was measured by reduced levels of molecules known to be essential for decidualisation and implantation, including chemokines that guide foetal trophoblast cells during implantation.

Furthermore, to clarify whether maternal *Ct* infection that has been cleared (past *Ct* infection) can affect pregnancy outcome and embryo growth I used a mouse model of *Ct* infection. The mice were infected with *Ct* and a month later were impregnated. The results from the *Ct* infected mice were compared to pregnancy outcomes of mice that had not been infected. My experiments showed that past infection did not affect the pregnancy rate of the mice and did

not result change the number of miscarried pups when the pregnancy outcome was examined on day 12 (e12) of mouse pregnancy. Also, there was no change in the weight of the pups and the placentas on e12, however pups and placentas from e14 of mouse pregnancy weighed less compared to their e14 counterparts from mice that were not infected with *Ct.*

Finally, I examined women that miscarried compared to women with healthy pregnancies to see if they had *Ct* infection in the past to verify whether this increases the miscarriage incidence in women. Even though the study is not yet complete, preliminary data suggest that past *Ct* infection is not likely to increase miscarriage incidence. The same samples were tested using a commercially available ELISA that proved to be less accurate at detecting past infection in patients.

To summarise, the present study suggests that changes caused by *Ct* infection in the womb can potentially disrupt delicate and strictly regulated processes during early pregnancy that could lead to pregnancy complications such as restricted embryonic growth. Since *Ct* is treatable but difficult to diagnose, screening for *Ct* infection in high-risk populations could decrease these incidents.

Table of Contents

List of Figures

List of Tables

Abbreviations

CHX	Cycloheximide
Ct	*Chlamydia trachomatis*
CXCL12/SDF1	C-X-C motif chemokine/stromal derived factor 1
CXCL16	C-X-C motif chemokine 16
CXCL8/IL8	C-X-C motif chemokine/ interleukin 8
CXCR1/2/4/6	C-X-C motif chemokine receptor 1/2/4/6
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
EB	Elementary body
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ERB	Estrogen receptor B
ESC	Human primary endometrial stromal cells
FOXO	Forkhead box
HB-EGF	Heparin binding epidermal growth factor
HLA	Human leukocyte antigen
HOX	Homeobox
IFNγ	Interferone γ
IFV	In-vitro fertilization
IFU	Inclusion forming unit
IGFBP1	Insulin-like growth factor-binding protein 1
IUGR	Intrauterine growth restriction
IVC	Individually ventilated cage
KO	Knockout

LH	Luteinising hormone
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MOMP	Major outer membrane protein
NK	Natural killer cells
PAMP	pathogen-associated molecular pattern
PGRA&B	Progesterone receptor A&B
PID	Pelvic inflammatory disease
PRL	Prolactin
PRLR	Prolactin receptor
PRR	Pattern recognition receptor
RB	Reticulate body
SGK1	Serum and glucocorticoid-regulated kinase 1
SPG buffer	Succinic acid, sodium phosphate monobasic monohydrate and glycine buffer
STD	Sexually transmitted disease
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNFα	Tumour necrosis factor α
uDC	Uterine dendritic cell
uNK	Uterine natural killer cell
PCR	Polymerase chain reaction
VEGF	Vascular endothelial growth factor

1. Chapter 1: Literature review

1.1 *Chlamydia trachomatis (Ct)*

Chlamydia trachomatis (Ct) is the most common sexually transmitted pathogen worldwide (Howie *et al.*, 2011).

1.1.1 Taxonomy

Ct's taxonomical lineage is: *Chlamydiae/Verrucomicrobia group; Chlamydiae; Chlamydiia; Chlamydiales; Chlamydiaceae; Chlamydia/Chlamydophila group; Chlamydia*. *Ct* serovars (bacterial strains that have different antigenic properties as determined by a microimmunofluorescence test) A, B, Ba and C infect mainly the conjunctiva and are associated with trachoma (Monroy and Villalba-magdaleno, 2012). Serovars D, Da, E, F, G, Ga, H, I, J and K are predominantly isolated from the urogenital tract and are associated with sexually transmitted diseases (STDs), inclusion conjunctivitis or neonatal pneumonitis in infants born to infected mothers (Clarke, 2011; Klint *et al.*, 2007; Monroy and Villalba-magdaleno, 2012). Serovars L1, L2, L2a and L3 can be found in the inguinal lymph nodes and are associated with lymphogranuloma venereum (Clarke, 2011; Klint *et al.*, 2007; Monroy and Villalba-magdaleno, 2012).

1.1.2 Prevalence, symptoms, diagnosis, treatment, persistence and vaccine

Ct prevalence is high, estimated at 97 million new cases in 2005 worldwide (World Health Organisation (WHO), 2011). The most recent data from Public Health England for 2015 show that the most commonly diagnosed sexually transmitted infection was *Ct* as 200,288 infections were diagnosed, a small decrease from 206,774 diagnoses made during 2014 (*Health Protection Report*, 2016). More specifically, 1.5 million young people aged 15 to 24 years old were tested, the target population for the National *Chlamydia* Screening Programme (*Health Protection Report*, 2016).

Though untreated *Ct* infection is often asymptomatic in women, it can result in mucopurulent cervicitis (Brunham *et al.*, 1984), acute urethral syndrome (Stamm *et al.*, 1980) and pelvic inflammatory disease (PID, Paavonen and Lehtinen, 1996). Reinfection with *Ct* can result in a strong adaptive immune response and the increased inflammation may cause further damage to the reproductive tract, as it is presumed to be the case in chronic PID (Hillis *et al.*, 1997; Price *et al.*, 2013).

Diagnosis is carried out by PCR on swab samples. Treatment includes administration of antibiotics, such as tetracyclines, azithromycin, or erythromycin (Brocklehurst and Rooney, 2000; MedlinePlus, 2014).

Following infection, *Ct* can under specific conditions convert into a dormant form that remains undetected by the immune system of the host. This form does not multiply although continues to survive in the cell for 6 months up to 5 years (Meijer *et al.*, 2005; Morré *et al.*, 2002). In-vitro data have demonstrated that exposure to penicillin and interferon-γ (IFN-γ) can result in "persistent" *Ct* infection (Beatty *et al.*, 1993; Lambden *et al.*, 2006). Persistence also depends on the serovar causing the infection, as it has been shown that serovar E has lower clearance rate compared to other serovars (Meijer *et al.*, 2005; Morré *et al.*, 2002).

There is no vaccine available for *Ct* yet, despite many attempts at developing one (Cambridge *et al.*, 2013; Peterson *et al.*, 1999; Schautteet, Stuyven, *et al.*, 2011; Singh *et al.*, 2006; Stary *et al.*, 2015). These attempts often resulted in partial to non prophylactic protection from *Ct* reinfection. However, even when protection against reinfection has been demonstrated by a study (Brunham and Rey-Ladino, 2005), there is significant serovar to serovar variation between characteristic surface proteins such as major outer membrane complex (MOMP) that hinders the development of a universal vaccine of the host against any future infection. Furthermore, persistent *Ct* infection that results in sophisticated mechanisms of avoiding detection from the host immune system is an additional challenge that needs to be addressed (Brunham and Rey-Ladino, 2005). Recent vaccines tend to shift towards prevention of disease

rather than protection from reinfection, however an effective vaccine has not yet been developed (reviewed in Brunham and Rey-Ladino, 2005; Schautteet, De Clercq, *et al.*, 2011).

1.1.3 Life cycle of *Ct*

Ct has two developmental stages, the infectious but metabolically inactive elementary body (EB) and the metabolically active intracellular reticulate body (RB). EBs measure from 200 to 400 nm in diameter, are susceptible to penicillin and are resistant to mechanical shock. In contrast, RBs are between 500 and 1500 nm in diameter, are not infective or affected by penicillin however they are susceptible to mechanical shock (Monroy and Villalba-magdaleno, 2012). *Ct* is known to infect epithelial cells of mucosal tissues (Hossain, 1989; Monroy and Villalba-magdaleno, 2012; Rödel, Grosse, Yu, Wolf, Otto, Liebler-Tenorio, Forsbach-Birk, Straube, *et al.*, 2011). Briefly, as seen in Figure 1.1, a *Ct* EB enters a host epithelial cell, transitions to RB that multiplies by binary fission and approximately 24-48 hours later the RBs transform to EBs, the host cell is lysed and the EBs are released to infect nearby healthy cells.

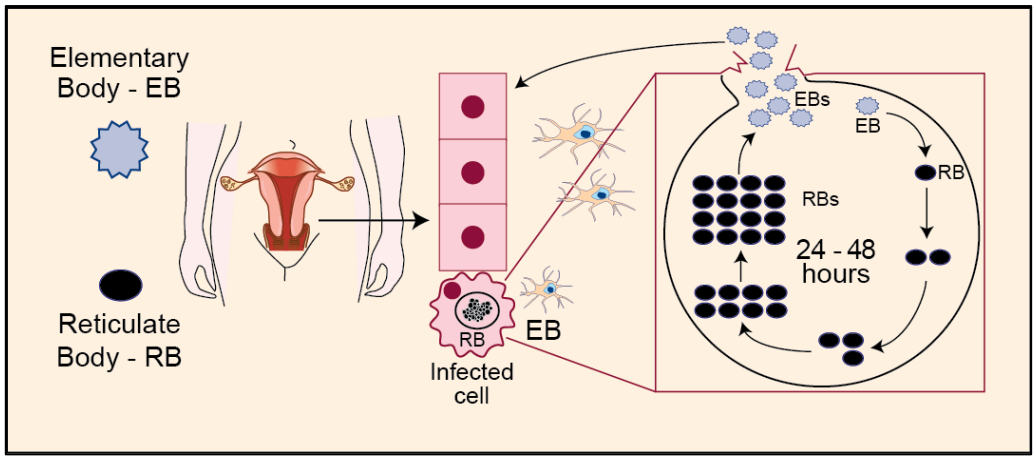

Figure 1.1. Life cycle of *Ct* in the female genital tract. The *Ct* infectious elementary body (EB) enters a host epithelial cell of the female genital tract and transforms to the metabolically active reticulate body (RB). RBs multiply by binary fission utilising host cell nutrients within a protective membrane called inclusion and approximately 24-48 hours later the RBs transform to EBs. The host cell is lysed and the released EBs are able to infect nearby healthy cells.

1.1.3.1 Molecular mechanisms of *Ct* infection

The infectious process begins with attachment of an EB to an eukaryotic epithelial cell (Dautry-Varsat *et al.*, 2005). *Ct* serovars utilise slightly different mechanisms of entry to host cells (Dautry-Varsat *et al.*, 2005). Serovars such as L1-L3 utilize a surface heparan-sulfate-like ligand that binds to a specific host cell receptor that facilitates bacterial entry into the cell (Chen and Stephens, 1997; Jian Ping Zhang and Stephens, 1992). Serovar E attachment to the HEC-1B epithelial cell line was not affected by heparin or heparan sulphate, indicating it enters host cells via another mechanism (Davis and Wyrick, 1997). It has been suggested that serovar E uses lipid microdomains in the host cell plasma membrane that are known as detergent insoluble glycolipid-rich domains or lipid "rafts" (Stuart *et al.*, 2003), however a more recent study reported that serovar E is not using this mechanism (Gabel *et al.*, 2004). Although the paper by Gabel *et al.* does not show data from several results, both studies appear to have conducted very similar experiments including same concentration of drugs used to inhibit *Ct* infection of HeLa cells. Another study has implicated the protein disulfide isomerase, a component of the estrogen receptor complex, to attachment of serovar E to endometrial epithelial cells (Davis *et al.*, 2002). The precise entry process of serovar E EBs remains therefore unclear to date.

Following internalization, a vacuole called inclusion forms from *Ct*-originating vesicles in the infected cell. *Ct,* as an obligate intracellular bacterium, requires host nutrients to proliferate whilst it modifies the cell defence mechanisms to avoid destruction. All such interactions are facilitated by the inclusion membrane. It was originally thought that *Ct* depends completely on the host cell's energy molecules ATP, GTP, CTP and UTP to acquire energy, however it was later revealed that *Ct* can synthesize CTP (Tipples and McClarty, 1993). Immediately after cell entry, *Ct* protein synthesis produces inclusion proteins (Li *et al.*, 2008; Scidmore-Carlson *et al.*, 1999). Sphingomyelin and cholesterol are both examples of materials required by the growing RBs and are transported from the Golgi apparatus, a process that has been shown to involve *Ct* encoded proteins such as the *Ct* inclusion membrane protein (IncA) and is

promoted by microtubule trafficking (Carabeo *et al.*, 2003; Hackstadt *et al.*, 1995, 1999; Richards *et al.*, 2013).

As seen in Figure 1.2, it is believed that the *Ct* developmental cycle is regulated at a transcriptional level and is divided in three stages (Nicholson *et al.*, 2003). Very little is known regarding the transformation of EBs to RBs that occurs within the first 2-6 hours following internalisation. During early differentiation of stage I, genes that are expressed are involved in transcription or nutrient transport, genes inhibiting fusion of the *Ct* inclusion with the host cell lysosomal pathway and inclusion membrane proteins (AbdelRahman and Belland, 2005; Belland *et al.*, 2003). Stage II, 18 hours post infection, is when RB proliferation has reached a plateau (Nicholson *et al.*, 2003; Stephens *et al.*, 1998). Genes upregulated during this stage include "cell envelope biogenesis components (18%), energy metabolism (11%), protein folding (3%), and DNA replication, modification, repair, and recombination (4%)" and some genes that are in common with stage III (Nicholson *et al.*, 2003). During stage III of the developmental cycle, RBs transition to EBs, therefore proteins specific for EBs are expressed among other genes (Nicholson *et al.*, 2003). It is known that the *Ct* cell envelope becomes rigid and septated at the time of transition from RB to EB (Peterson and de la Maza, 1988).

Finally, at the end of a *Ct* life cycle, RBs transform back into EBs and EBs are released by host cell lysis or extrusion (packaged EB release that leaves host cell intact, Hybiske and Stephens, 2007).

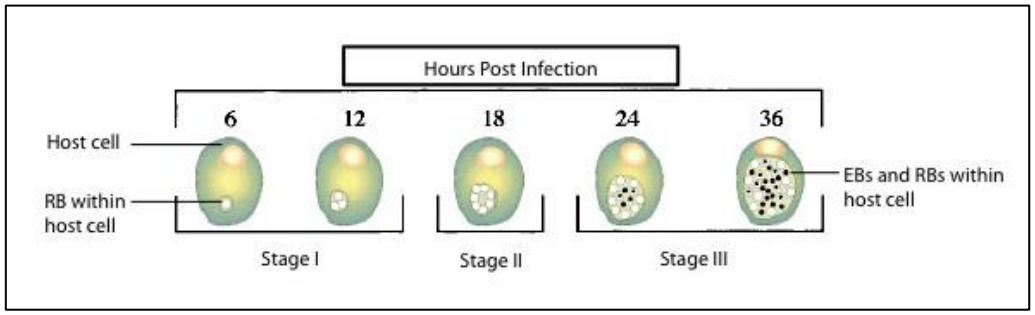

Figure 1.2. The three stages of *Ct* developmental cycle within the cell. Early differentiation during the first 12 hours that is characterised by transformation of an EB to RB and transcription of early stage genes that are involved in *Ct* growth and protection from host cell defence mechanisms. During stage II, up to 18 hours post infection, the RBs proliferate and divide. Finally, 24 hours post infection the first RBs begin to transform to EBs that will infect nearby cells when the cell is lysed 48 hours post infection. (Image adapted from open access article Nicholson *et al.*, 2003).

1.1.3.2 Host immune response to *Ct* infection

The host immune response to *Ct* has two stages: an early response caused by the innate immune system and a more delayed response of the adaptive immune system. The innate immune system acts both as a first barrier against pathogens and as an activator of the adaptive immune system (Iwasaki and Medzhitov, 2010).

The innate immune system is the first line of defence against pathogens. It includes physical barriers such as the epithelial cell barrier, the complement system (a number of proteins found in the blood that enhance the ability of phagocytes and antibodies to eliminate pathogens) as well as specialised cells including mast cells, macrophages, neutrophils, dendritic cells (DC), basophils, eosinophils and natural killer (NK) cells circulating throughout the whole body.

Host–pathogen interactions are induced via host recognition of conserved molecular structures of the pathogen that are known as pathogen-associated molecular patterns (PAMPs). PAMPS are sensed by the host's pattern recognition receptors (PRRs). Several classes of PRRs recognize distinct microbial components and directly activate immune cells (reviewed in Uematsu and Akira, 2008). PRRs are expressed on innate immune cells such as DCs, neutrophils, NK cells and macrophages. These receptors include among

others the highly conserved among species family of transmembrane Toll-like receptors (TLRs), the Scavenger receptors and the NOD-like receptors (Dempsey *et al.*, 2003; Iwasaki and Medzhitov, 2010; Kumar *et al.*, 2011; Meylan *et al.*, 2006).

Ct is a Gram-ve bacterium that has an inner and outer membrane similar to Gram-ve bacteria and a lipopolysaccharide (LPS) but does not have a peptidoglycan layer (Monroy and Villalba-magdaleno, 2012). However, it does not contain peptidoglycan (Hatch, 1996). As demonstrated in Figure 1.3, *Ct* is known to contain unique PAMPs including MOMP (Caldwell *et al.*, 1981) and rough LPS (Heine *et al.*, 2003). *Ct* LPS has been reported to be recognised by both TLR2 (Erridge *et al.*, 2004) and TLR4 (Heine *et al.*, 2003). Another known antigen of *Ct* that induces a strong immunological response is *Chlamydia* heat shock protein 60 (Hsp60) that is located on the inclusion (Raulston *et al.*, 1998) and is recognised by TLR4 (Sasu *et al.*, 2001).

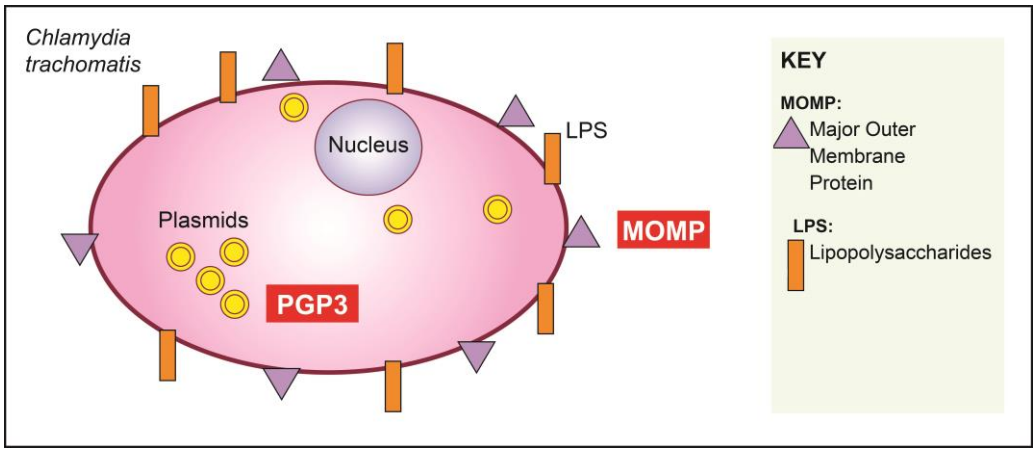

Figure 1.3. Notable antigens of *Ct* include Lipopolysaccharides (LPS), Major outer membrane protein (MOMP) and secreted plasmid protein PGP3. LPS is targeted by pattern recognition receptors (PRRs) of the innate immune system whereas MOMP and PGP are recognised by antibodies and memory cells of the adaptive immune system. These antigens are also commonly used to detect presence of *Ct* in diagnostic and laboratory techniques.

If a PAMP is successfully recognised, signalling pathways that activate host immune responses mediated by various cytokines and chemokines are mobilised, including immune cell recruitment (Dempsey *et al.*, 2003; Iwasaki and Medzhitov, 2010; Kumar *et al.*, 2011). These processes aim to eradicate the pathogens from the host organism. One such chemokine that is known to be

strongly induced by *Ct* infection in epithelial cells is C-X-C motif chemokine 8 or interleukin 8 (CXCL8 or IL8, Buckner *et al.*, 2013; Das *et al.*, 2010; Natividad *et al.*, 2009). CXCL8 attracts neutrophils to the cite of infection (Buckner *et al.*, 2013; Das *et al.*, 2010; Natividad *et al.*, 2009). As seen in Figure 1.4, other innate immune cells recruited in response to *Ct* infection include macrophages and DCs. DCs secrete Interleukin 12 (IL-12) that acts as an attractant to NK cells (Bas *et al.*, 2008; Hook *et al.*, 2004; Lu and Zhong, 1999).

The NACHT, LRR, and pyrin domain-containing (NALP) protein family are a subtype of the NOD-like receptor family. NALP proteins have an important role in activation of proinflammatory caspases through formation of a complex called "inflammasome". NALP2/NALP3 inflammasomes contain the proteins NALP2, NALP3, CARDINAL, the adaptor protein ASC and caspase 1. The NALP1 inflammasome consists of NALP1, ASC, caspase 1 and caspase 5. Both inflammasomes control the activation of proinflammatory cytokines IL-1β and IL-18 (van de Veerdonk *et al.*, 2011).

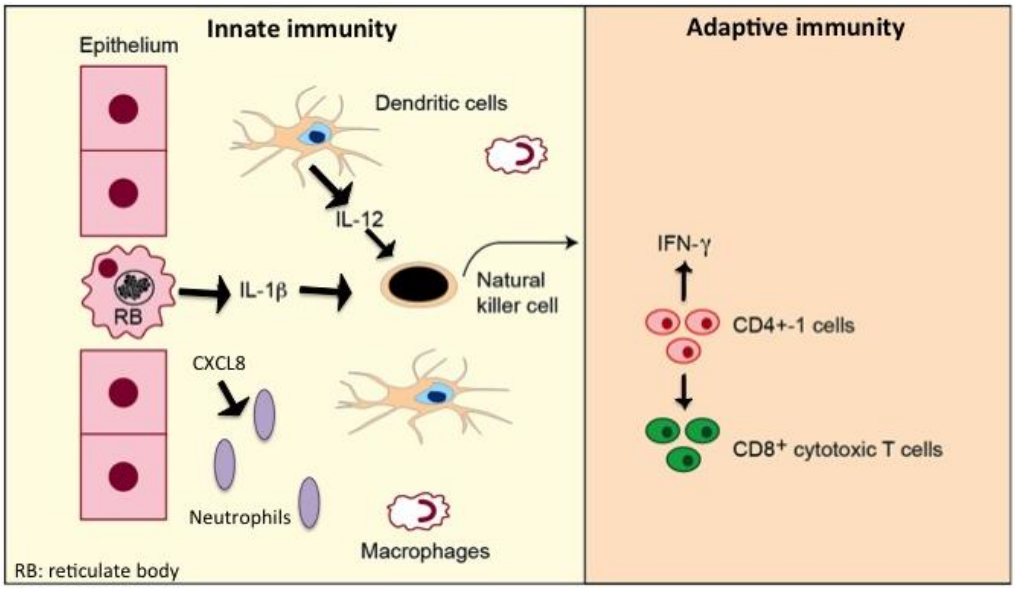

Figure 1.4. Host immune response to *Ct* infection is facilitated by both the innate and the adaptive immune system.

Following the innate immune response, defensive mechanisms of the adaptive immune system are activated by antigen-presenting cells including macrophages and DCs.

The adaptive immune system has two arms, humoral (antibody mediated) and cellular (cell mediated). T-cells and B-cells are the main effector and memory cells of adaptive immunity. In the thymus, naive T-cells that encounter self-antigens are deleted (central tolerance). Mature T-cells that exit the thymus can also become tolerant to antigens at other anatomical sites (peripheral tolerance). T-cells are divided into distinct populations characterised by specific cluster of differentiation (CD) markers expressed on each population: $CD4^+$ T-cells mediate the innate immune response by secreting various cytokines and this group includes include T-helper 1(Th1), T-helper 2 (Th2), T-regulatory cells (Treg) and other subtypes. Cytotoxic $CD8^+$ T-cells are the cells that have the potential to that "kill" infected cells, cancer cells and generally damaged cells. T-cell activation is a highly complex process (reviewed in Smith-Garvin *et al.*, 2009). Briefly, to activate a cytotoxic or helper T-cell to proliferate and differentiate into an effector cell, an antigen-presenting cell provides a signal by an antigen bound to a MHC protein on the surface of the presenting cell and a second signal provided by co-stimulatory proteins. This process is known as antigen presentation. Following activation, T-cells are believed to migrate from the thymus to other areas in the body. B-cells, following activation via T-cells or APCs produce antibodies and interact with various T-cell subtypes in a complex network that mediates the innate immune response (Dempsey *et al.*, 2003).

At the site of infection there is a strong inflammatory reaction mediated mainly by activated $CD4^+$ T-cells with a Th1 phenotype aiming to clear the *Ct* infection (Hafner *et al.*, 2008). Despite the fact that this dual response of innate and adaptive immune systems is usually effective in clearing the pathogens, in several cases *Ct* evades eradication resulting in a persistent infection. *Ct* uses a plethora of mechanisms to evade the host immune response. It has been established that *Ct* can infect macrophages and this results in cytotoxic effect

of the macrophages on the T-cells via tumour necrosis alpha factor (TNFα) secretion (Jendro *et al.*, 2000, 2004). However, one of the most known mechanisms is IFN-γ mediated persistence (Perry *et al.*, 1997). Th1 cells produce IFN-γ, which is known to inhibit *Ct* reproduction (Perry *et al.*, 1997). The resolution of infection depends on the concentration of IFN-γ. It is known that high levels of IFN-γ are associated with the clearance of infection whereas in low levels the bacteria stop replicating however they survive within the cells resulting in a persistent infection (Beatty *et al.*, 1993).

1.2 *Ct* and adverse pregnancy outcomes

As *Ct* infects the female genital tract, its role in adverse pregnancy outcome has been a subject of research interest during the past decades. Studies indicate a strong association with ectopic pregnancy supported by mechanistic data (Bakken *et al.*, 2007; Shaw *et al.*, 2011). However, the data on miscarriage (Baud *et al.*, 2011; Wilkowska-Trojniel *et al.*, 2009; Nigro *et al.*, 2011), low birth weight (Berman *et al.* 1987; Johnson *et al.*, 2011; Blas *et al.*, 2007), intrauterine growth restriction (Investigators of the John Hopkins Study of Cervicitis and Adverse Pregnancy, 1989; Vedmedovska *et al.*, 2010) and preterm labour (Hollegaard *et al.*, 2007; Blaset *et al.*, 2007; Silveira *et al.*, 2009; Johnson *et al.*, 2011) are the subject of on-going debate.

Controversial data exist due to the many confounders that can affect pregnancy outcomes in humans as well as issues with study design, including underpowered sample size, difficulty in recruitment, variable diagnostic test sensitivity that can differ significantly from study to study. Further research into the potential association between *Ct* and these adverse pregnancy outcomes is essential.

1.2.1 *Ct* and miscarriage

1.2.1.1 Miscarriage definition and statistics

Miscarriage is the spontaneous loss of pregnancy during the first 24 weeks of gestation (Figure 1.5), with the majority noted during the first 12 weeks. For the majority of women, a miscarriage is an isolated event and is followed by a successful pregnancy ("spontaneous miscarriage", termed "miscarriage" from this point onwards). A smaller number (0.5-1%) of women may experience three or more successive miscarriages, a condition known as "recurrent miscarriage" (Bulletti *et al.*, 1996). "Early miscarriage" is defined as pregnancy loss during the first trimester of pregnancy (less than 12 weeks of gestation) and occurs in up to one in five pregnancies. A "late miscarriage" during the second trimester (12-24 weeks of gestation) is less common, occurring in 1-2% of pregnancies (Hay, 2004). Foetal death from the 25th week of gestation onwards is defined as stillbirth (Figure 1.5).

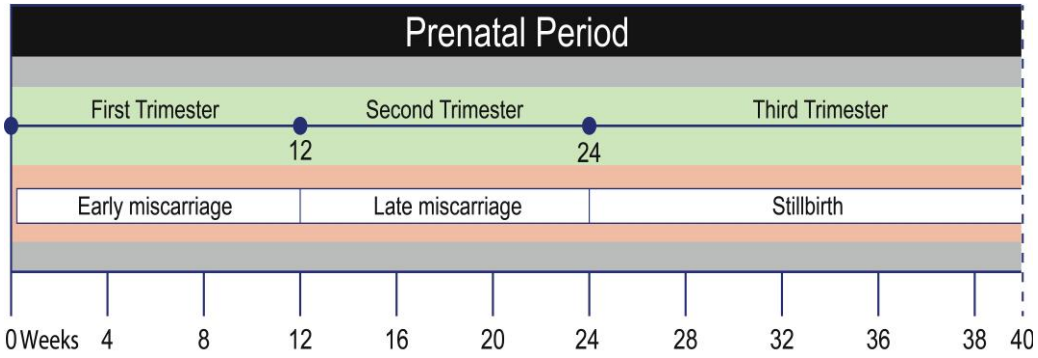

Figure 1.5. Adverse pregnancy outcomes throughout gestation. (Image from Giakoumelou *et al.*, 2015, reproduced with publisher's permission).

Although miscarriage is considered the most common adverse pregnancy outcome, worldwide figures are not available. It is generally accepted that miscarriage occurs in 20% of all pregnancies (Everett, 1997; Hure *et al.*, 2012). Measurements from a recent Australian prospective cohort study including 14,247 women aged 18-23 years, indicate a miscarriage rate between 11.3 to 86.5 per 100 live births amongst different groups. Miscarriage occurred in 25% of the women in the study when the women were 31-36 years old (Hure *et al.*, 2012). Contributing to the inaccuracy of miscarriage statistics is the fact that many miscarriages remain unreported and are managed at home.

Miscarriages are associated with physical and psychological morbidity. An early miscarriage is managed expectantly, medically or surgically often with no complications, however 2-3% of patients may develop an infection (Trinder *et al.*, 2006). Bleeding due to miscarriage can lead to haemodynamic shock and maternal death (Everett, 1997). The emotional response of a woman to miscarriage can include depression and anxiety (Blackmore *et al.*, 2011). It was reported that 25% of women one month after miscarriage display symptoms of post-traumatic stress disorder (PTSD) and depression. Four months after the occurrence, PTSD symptoms were reduced to 7% of the population, however depression rates remained similar, an indication as to how seriously an adverse pregnancy outcome can affect the psychological health of a woman (Engelhard *et al.*, 2001).

1.2.1.2 Aetiology of miscarriage

Approximately 50% of early miscarriages are attributed to chromosomal aberrations such as a structural alteration or abnormal chromosomal numbers of the foetus (Eiben *et al.*, 1990; Suzumori and Sugiura-Ogasawara, 2010). Several other factors have been associated with increased risk of miscarriage. The age of both parents has a significant role as the risk of an adverse pregnancy outcome is increased if the parents are 35 years old or older and it is 50% higher if the mother is 42 years of age (Fretts *et al.*, 1995; de la Rochebrochard and Thonneau, 2002; Maconochie *et al.*, 2007; Nybo Andersen *et al.*, 2000; Slama *et al.*, 2005). In addition, factors such as ethnic origin, psychological state of the mother, very low or very high pre-pregnancy BMI, feelings of stress, use of non-steroidal anti-inflammatory drugs, smoking and alcohol consumption have also been associated with significantly higher rates of miscarriage (Coste *et al.*, 1991; Lashen *et al.*, 2004; Maconochie *et al.*, 2007; Nielsen *et al.*, 2001; Sopori, 2002). Moreover, it has been reported that women whose first pregnancy resulted in miscarriage are at a higher risk of the second pregnancy resulting in miscarriage compared to women who had a live birth (Kashanian *et al.*, 2006).

1.2.1.3 The role of infection in miscarriage

Maternal infection causes 15% of early miscarriages and 66% of late miscarriages (Baud *et al.*, 2008; Srinivas *et al.*, 2006). It has been reported that 78% of 101 tissue samples from miscarriage were infected with bacteria (chorioamnionitis), whereas all the control samples from medically-induced abortions were uninfected in the same study (Allanson *et al.*, 2010). Studies published in the last five years suggest that infections such as bacterial vaginosis, malaria, cytomegalovirus, dengue fever, brucellosis and HIV may result in miscarriage (reviewed in Giakoumelou *et al.*, 2015, Appendix 2). On the contrary, there is no evidence to support that *Coxiella burnetii*, adeno-associated virus, Bocavirus, Hepatitis C and M*ycoplasma genitalium* are associated with miscarriage. More importantly though, there are contradicting data regarding the effects of *Ct*, *Toxoplasma gondii*, Human papillomavirus, human herpes virus 1&2, Polyomavirus BK, Hepatitis B and B19V infection. The study quality and results reported in each individual study have been assessed in the review included in Appendix 2 (Table 1.1,from Giakoumelou *et al.*, 2015).

Table 1.1. Pathogens and their association with miscarriage (from Giakoumelou *et al.*, 2015, reproduced with publisher's permission).

	Bacteria	Viruses	Protozoa
Associated with miscarriage	• Bacterial vaginosis (including *Mycoplasma hominis and Ureaplasma urealyticum*) • Brucellosis • Syphilis	• Cytomegalovirus • Dengue fever (*Flavivirus*) • HIV • Rubella	• Malaria (*Plasmodium*)
Little or no evidence for association with miscarriage	• *Coxiella burnetii* • *Mycoplasma genitalium*	• Adeno-associated virus • Bocavirus • Hepatitis C	• None
Conflicting evidence for association with miscarriage	• *Chlamydia trachomatis*	• Human papillomavirus • Herpes simplex virus 1 and 2 • Parvovirus B19 • Polyomavirus BK • Hepatitis B	• *Toxoplasma gondii*

HIV, human immunodeficiency virus.

1.2.1.4 The role of *Ct* infection in miscarriage

Several studies have been published regarding the association of *Ct* with miscarriage and they will be discussed in more detail in Chapter 5. Briefly, active infection has been shown to be associated with miscarriage in two studies (Vigil *et al.*, 2002, Arsovic *et al.*, 2014), however a number of studies

reported no association between active infection and miscarriage (Baud *et al.*, 2011; Grönroos *et al.*, 1983; Munday *et al.*, 1984; Sozio and Ness, 1998). On the contrary, studies have been published in support of the association of past *Ct* infection with miscarriage, with *Ct* prevalence ranging between 11%-69% in miscarriages compared to 2-7% in healthy pregnant controls (Arsovic *et al.*, 2014; Baud *et al.*, 2011; Licciardi and Grifo, 1992; Wilkowska-Trojniel *et al.*, 2009), a fact disputed only by one study (Paukku *et al.*, 1999).

1.2.2 The role of *Ct* infection in low birth weight, premature labour and intrauterine growth restriction

1.2.2.1 Definitions, prevalence statistics and aetiology

Low birth weight is defined by the World Health Organisation (WHO) as birth weight below 2,500g and is a significant cause of neonatal mortality and childhood morbidity (Kramer, 1987; McCormick, 1985). The most recent prevalence data from the US show that 8.0% of babies born in 2014 were born with low birth weight, a percentage that remains unchanged since 2013 (National Vital Statistics Reports, 2015). In England and Wales in 2015, 7% of babies had low birth weight, a percentage that remains unchanged since 2011 (Low birth weight of term babies, 2015). Low birth weight infants have been shown to have decreased motor development and have moderate-to-severe deficits in academic achievement, attention problems and internalising behavioural problems that continually hinder these individuals compared to their peers up to early adulthood (Aarnoudse-Moens *et al.*, 2009; de Kieviet *et al.*, 2009). Low birth weight can be attributed to either premature delivery or intrauterine growth restriction commencing as early as the first trimester (Kramer, 1987; Smith *et al.*, 1998).

Labour can be termed “preterm” or premature if the onset of labour is prior to 37 weeks of gestation (CDC - Centre for Disease Control and Prevention, 2013). Cases are classiffied as “extremely preterm” when gestational age is less than 28 weeks, “very preterm” when gestational age is between 28 and 32 weeks and as “moderate to late preterm” from 32 to less 37 gestational weeks. The most recent prevalence data from the US show that 11.4% of babies born

in 2013 were born prematurely (CDC - Centre for Disease Control and Prevention, 2013). Premature labour has been associated with intrauterine growth restriction (Ananth *et al.*, 2001).

Intrauterine growth restriction (IUGR) is most commonly defined as embryonic weight decreased by 10% compared to the expected weight at any gestational age and it occurs in approximately 10% of pregnancies (American College of and Gynecologists, 2013). IUGR is a significant cause of morbidity and mortality among premature neonates (Garite *et al.*, 2004). The aetiology of IUGR is attributed to maternal, placental and foetal causes (reviewed in Suhag, A & Berghella, 2013). Known maternal risk factors for IUGR include parity, maternal age, a history of preterm delivery, smoking, short stature, low weight, low weight gain (Horta *et al.*, 1997; Wen *et al.*, 1990) and many more. IUGR caused by placental insufficiency occurs in up to 3% of all pregnancies (Suhag, A & Berghella, 2013). The most common causes include abnormal implantation such as placenta previa, reduced uterine blood flow and reduced placental nutrient transport (Ananth *et al.*, 2001; Battaglia, 2003; Laurini *et al.*, 1994). Similar results identifying these parameters have been previously demonstrated by IUGR animal models such as sheep and rat (Block *et al.*, 1990; Gilbert and Leturque, 1982). Furthermore, disruptions of the innate immune system in the fetomaternal interface including the lack of dendritic cell activation in the maternal decidua have also been demonstrated in IUGR pregnancies (Cappelletti *et al.*, 2013). Common foetal factors known to result in IUGR are often genetic such as chromosomal abnormalities (Snijders *et al.*, 1993). Additionally, preeclampsia is a serious condition that can occur in pregnancy after 20 weeks and is associated with premature labour and IUGR (Srinivas *et al.*, 2009). Finally, it has been suggested that maternal infection with some pathogenic factors might result in IUGR, however the data is sparse and further studies are required identify potential risk factors (reviewed in Vedmedovska *et al.*, 2015).

1.2.2.2 The role of *Ct* infection in low birth weight, premature labour and intrauterine growth restriction

A prospective study of 801 women within 22 to 30 weeks of gestation examined the role of several pathogens including *Ct* in IUGR and preterm delivery (Investigators of the John Hopkins Study of Cervicitis and Adverse Pregnancy, 1989). Active *Ct* infection was significantly associated both with intrauterine growth retardation (odds ratio = 2.4, 90% Confidence Interval (CI) 1.32-4.18) and preterm delivery (odds ratio = 1.6, 90% CI 1.01-2.50) after adjustment for confounding factors (Investigators of the John Hopkins Study of Cervicitis and Adverse Pregnancy, 1989). Conversely, in a 1994 cohort study of 13,914 women, active *Ct* infection was not associated with IUGR (Germain *et al.*, 1994). The above studies both used cell culture as a detection method for *Ct* infection. New diagnostic tests with higher sensitivity than cell culture have been developed such as PCR detection (Centers for Disease Control and Prevention., 2014) and a new study should be carried out to investigate the association of *Ct* infection and IUGR utilising modern detection methods.

A strong association of *Ct* infection with premature birth is supported by data from several case control, cohort, retrospective or prospective studies (Andrews *et al.*, 2000; Baud *et al.*, 2015; Folger, 2013; Hollegaard *et al.*, 2007; Martius *et al.*, 1988; Rours, de Krijger, *et al.*, 2011; Rours, Duijts, *et al.*, 2011). *Ct* was detected in 25% of IUGR placentae and was shown to induce placental inflammation (Rours, de Krijger, *et al.*, 2011).

There is little evidence regarding the role of *Ct* infection with low birth weight. Among women with diagnosed *Ct* infection, recent *Ct* infection was identified as a risk factor for low birth weight compared to persistent *Ct* infection (Berman *et al.*, 1987). A study associated maternal *Ct* infection with bronchopulmonary dysplasia in very low birth weight babies (Da Silva *et al.*, 1997), however another study reported zero prevalence of *Ct* infection in low birth weight babies (Garland and Bowman, 1996).

1.3 The role of the maternal environment in successful pregnancy

A receptive uterus and successful implantation are crucial factors for normal pregnancy development.

1.3.1 Endometrial decidualisation and embryo implantation

1.3.1.1 Anatomy of human endometrium

The human endometrium is the innermost layer of the human uterus (Figure 1.6). It undergoes cyclic remodelling in response to sex steroid hormones during the menstrual cycle. The endometrium is comprised of luminal epithelial cells and beneath them lies a stromal compartment that contains endometrial fibroblasts/stromal cells (ESC), blood vessels created by endothelial cells, secretory epithelial cells forming glands and several types of immune cells including the uterine natural killer cells (uNK), DCs and macrophages (Aplin *et al.*, 2008a).

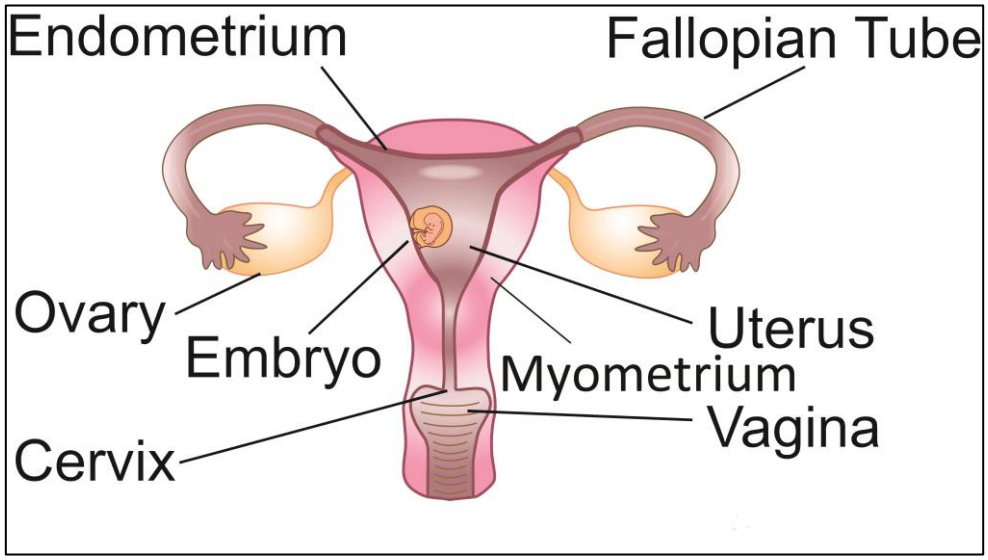

Figure 1.6. Anatomy of the human uterus.

The endometrium is composed of two layers; the basalis or basal layer and the functionalis or functional layer. The basal layer is located over the myometrium and does not alter significantly during the menstrual cycle. The

functional layer contains the stromal compartment and the luminal epithelium that are affected cyclically by the steroid hormones during the menstrual cycle (Aplin *et al.*, 2008a).

1.3.1.2 Endometrial decidualisation

A 28-day menstrual cycle has three distinct phases:

- Proliferative phase (days 5–14 of a 28-day menstrual cycle)

During the proliferative phase, the functional layer including the stromal compartment and the luminal epithelium regenerate, recovering from the previous menstrual phase. The main hormone of this phase is estrogen whose actions are mediated via various receptors. The endometrial thickness is increased from 1mm to 3-4 mm by ovulation (Figure 1.7, Aplin *et al.*, 2008a).

- Secretory phase: Decidualisation (days 14–28 of a 28-day menstrual cycle)

Around day 14 of a 28-day menstrual cycle, under the influence of anterior pituitary gland hormones luteinising hormone (LH) and follicle-stimulating hormone (FSH), ovulation occurs. During the secretory phase, the dominant hormone in the endometrium is progesterone that is secreted by the corpus luteum that in the ovary from a follicle following oocyte ovulation (Aplin *et al.*, 2008a; Graham and Clarke, 1997). In early secretory phase, the endometrial glands proliferate and, at a later stage, angiogenesis occurs whilst the stromal compartment undergoes decidualisation increasing in thickness.

Endometrial decidualisation is the process whereby, under the influence of estrogen and progesterone (P4), the stromal compartment of the endometrium proliferates and differentiates during the mid secretory phase of the menstrual cycle (Figure 1.7, Aplin *et al.*, 2008a). This process ensures that the endometrium transiently becomes receptive to a blastocyst during a brief period called the “window of implantation” at days 18-22 of a 28-day menstrual cycle (Gellersen and Brosens, 2014a). The ESC undergo structural changes as they become rounder and bigger as well as functional changes

displayed by secretion of decidualisation-specific markers such as prolactin (PRL, Gellersen and Brosens, 2014). Primary cells isolated from human endometrium have been used in in-vitro studies as a model for investigating the molecular events that occur during decidualisation, discussed further in section 1.3.6.

The molecular pathways of decidualisation are analysed in section 1.3.1.3.

- Menstrual phase (days 1–5 of a 28-day menstrual cycle)

If there is no blastocyst implantation, progesterone secretion ceases and as a result of progesterone withdrawal the functional layer of the endometrium is shed during the menstrual phase as the cells undergo apoptosis (Figure 1.7, Aplin *et al.*, 2008a).

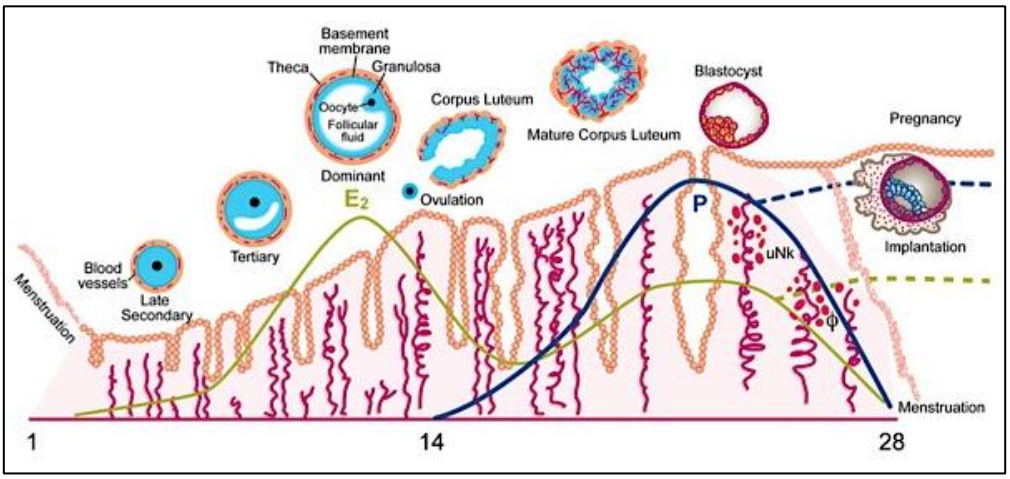

Figure 1.7. The menstrual cycle is governed by ovarian estrogen and progesterone secretion. It has three phases; proliferative, secretory and menstrual phase. During the proliferative phase, the functional layer of the endometrium regenerates under the influence of estrogen. When secreted estrogen levels drop, due to an increase in progesterone the stromal compartment commences decidualisation during the secretory phase. The stromal cells become bigger and rounder and increased angiogenesis is observed as the endometrium prepares of a possible blastocyst implantation (termed 'decidualisation'). In the event of no implantation, progesterone levels drop and the functional layer is shed during the menstrual phase. (Image drawn by T Pinner and obtained from Prof Philippa Saunders).

1.3.1.3 The fetomaternal interface during implantation

In humans, the embryo enters the uterine cavity at 72–96 hours after fertilization. Hatching of the embryo (escape from the zona pellucida) occurs

by day 5 (about 110–120 hours after ovulation). The embryo is prepared to implant, however whether this occurs depends on maternal receptivity and whether implantation occurs during the window of implantation (Gellersen *et al.*, 2010).

A successful pregnancy depends upon maternal receptivity during the implantation window. The importance of conception timing and ovulation has been of interest for several years. In a prospective study of 221 women attempting to get pregnant, it was shown that 84% of implantations occurred 8-10 days post ovulation and late implantation was associated with early pregnancy loss (Wilcox *et al.*, 1999). Implantation is a process that involves maternal and embryonic participation and interaction, however as the above study highlights, a healthy embryo is unlikely to survive in a non receptive uterus (Lessey, 2000).

Placenta formation begins as the embryonic trophoblast cells come into contact maternal epithelium (blastocyst apposition) and differentiate further into syncytiotrophoblast cells that invade the epithelial layer during blastocyst adhesion (Figure 1.8A). Syncytiotrophoblasts, supported by the decidualised stroma (Godbole *et al.*, 2011), penetrate the endometrium and surround the embryo, whilst it embeds itself in the stromal compartment at the final stage of implantation (blastocyst invasion/embedment). A second trophoblast layer, the cytotrophoblast, is an inner layer without contact with the maternal cells. At days 10-12 of gestation, the embryo is completely embedded in the endometrium, the epithelium has grown over it and the implantation process is complete (Figure 1.8B). The maternal decidua is not “passive” during the invasion process as it was widely believed for many decades; on the contrary it was recently shown that the ESC acquire invasive capabilities in response to trophoblast-derived signals and ESC migration is essential to facilitate implantation (Gellersen *et al.*, 2010; Grewal *et al.*, 2008).

Trophectoderm in the blastocyst gives rise to the trophoblast lineages of the placenta, whereas the placental stroma and vasculature rise from the extraembryonic mesoderm. 20-22 days post conception, vasculogenesis

commences as the first placental villi appear. The foetal heart begins beating around day 22 of gestation, the embryonic circulatory system is formed around day 32 and by day 45 blood begins to circulate in the placenta (Gellersen *et al.*, 2010; Godbole *et al.*, 2011; Grewal *et al.*, 2008).

Cytotrophoblast invasion of the uterine wall begins after implantation until week 18 of gestation. As a result, cytotrophoblast cells infiltrate the maternal decidua and spiral arteries, reaching as far as the uterine myometrium during the second semester. The invasion of the maternal spiral arteries is essential for the transformation of the vessels during pregnancy, that includes the loss of vascular smooth muscle that results in reduced blood flow typical of the fetomaternal interface during pregnancy (Gellersen *et al.*, 2010; Godbole *et al.*, 2011; Grewal *et al.*, 2008).

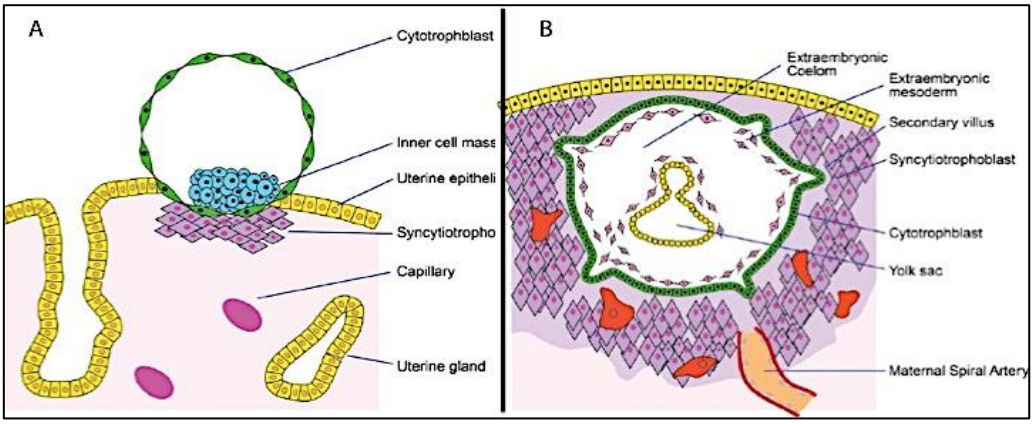

Figure 1.8. Blastocyst implantation in receptive human uterus during the window of implantation. (A) The trophoblast comes into contact with the epithelium and differentiates further into syncytiotrophoblast that invades the epithelial layer. (B) Syncytiotrophoblasts, supported by the decidualised stroma penetrate the endometrium and surround the embryo, whilst it embeds itself in the decidual stroma. The implantation process is complete by day 12 of gestation, rendering the embryo completely embedded in the endometrium (from Giakoumelou *et al.*, 2015, reproduced with publisher's permission).

The process of implantation is highly complex and, although many key pathways involved have been identified, new discoveries are still made (reviewed in Salamonsen *et al.*, 2015). P4 is an important regulator of endometrial receptivity during the menstrual cycle and can also negatively regulate trophoblast invasion via regulation of the matrix metalloproteinases (MMPs, reviewed in Halasz and Szekeres-Bartho, 2013). MMPs mediate

extracellular matrix degradation and are involved in several normal and pathological processes, including implantation (reviewed in Nardo *et al.*, 2003). The blastocyst also utilises adhesion proteins, called integrins, during the implantation window (Merviel *et al.*, 2001). In addition, the blastocyst has been shown to regulate apoptosis of the maternal epithelial cells in-vitro via the Fas ligand pathway (Galán *et al.*, 2000). Several studies confirm heparin-binding epidermal growth factor (HB-EGF) is required for trophoblast cell survival, in particular during hypoxic conditions (Armant *et al.*, 2006; Imudia *et al.*, 2008). The action of HB-EGF is known to impact on decidual ESC polyploidy and was shown to be mediated by cyclin D3 (Tan *et al.*, 2004). Increased HB-EGF levels were reported in maternal decidual cells and placental stromal cells from miscarriages compared to normal pregnancies (Ozbilgin *et al.*, 2015). Finally, deregulation of serum and glucocorticoid-inducible kinase 1 (SGK1) were shown to interfere with implantation and predispose to pregnancy complications such as RPL (Salker *et al.*, 2011).

1.3.2 Molecular pathways of endometrial decidualisation

Several molecular cues, pathways and cell types are involved in stromal cell transformation to decidualised ESC. Decidualisation in humans will be discussed first, followed by decidualisation in mice.

1.3.2.1 Endocrine signalling

As mentioned in paragraph 1.3.1 estrogen and P4, the ovarian steroid hormones, predominantly control uterine physiology (Gellersen and Brosens, 2014a). The postovulatory rise in circulating progesterone, produced by luteinizing granulosa cells, dictates the onset of decidualisation of estrogen-primed endometrium (Aplin *et al.*, 2008b).

1.3.2.2 Autocrine and paracrine signalling

The endometrial microenvironment is crucial to decidualisation; autocrine signals from ESC and paracrine cues from neighbouring cell populations such as epithelial cells, immune cells and endometrial vasculature cells all contribute to successful decidualisation (Gellersen and Brosens, 2014a).

1.3.2.2.1 Prolactin (PRL)

PRL is a peptide hormone and its levels are correlated with the progression of ESC decidualisation (Bole-Feysot *et al.*, 1998), hence its extensive use as a decidualisation marker in in-vitro studies (Telgmann and Gellersen, 1998). PRL expression is regulated by P4 (Brosens *et al.*, 1999). The role of PRL in decidualisation and early pregnancy, though crucial, is not completely known yet. It is has been reported that PRL has an active role in epithelial cell differentiation, as it has been shown to promote epithelial cell growth via crosstalk with the epidermal cell factor. It is known to be implicated in immune cell regulation, to induce angiogenesis and promote trophoblast cell growth and invasion (Corbacho *et al.*, 2002; Montgomery, 2001; Reuwer *et al.*, 2012; Stefanoska *et al.*, 2013). PRL also plays an important role in implantation and early pregnancy as it has been demonstrated that prolactin receptor (PRLR) knock-out mice have implantation failure (Lucas *et al.*, 1998). PRLR has also been shown to increase cell proliferation, implantation, angiogenesis, trophoblast cell growth and immune regulation during early pregnancy (Jabbour and Critchley, 2001). More recently it was shown that this effect attributed to ovarian not uterine PRLR (Reese *et al.*, 2000).

1.3.2.2.2 Growth factors

Decidualisation induces the expression of soluble and transmembrane HB-EGF, which has many roles in pregnancy. It facilitates embryo development, mediates implantation and is thought to have a function in endometrial receptivity and maturation (as reviewed in Iwamoto and Mekada, 2000; Raab and Klagsbrun, 1997).

Regarding in-vitro decidualisation, HB-EGF and its receptors EGFR and ERBB4/HER4 are increased in ESC after cAMP treatment and its expression is regulated by TNFα and TGFβ, which suggests that it inhibits stromal cell apoptosis whilst a decidualisation signal is present. Inhibition of HB-EGF results in reduction of PRL and IGFBP1 (Chobotova *et al.*, 2005).

Members of the transforming growth factor β (TGFβ) superfamily, including activins, inhibins and follistatin, play a major role in the paracrine/ autocrine

regulation of the decidual process (as reviewed in Jones *et al.*, 2002). TGFβ is a multifunctional modulator that is involved in processes ranging from apoptosis promotion to cell differentiation both in health and disease in various cell types and tissues (as reviewed in Wajant *et al.*, 2003). It has three isoforms, TGFβ1-3. TGFβ1 is induced by progesterone by endometrial epithelial cells in-vitro and acts on stromal cells to commence PRL secretion (Kim, 2006). Another autocrine modulator, a TGFβ family member, is LEFTY2, an important inhibitor of the NODAL signalling pathway (Derynck and Zhang, 2003; Saijoh *et al.*, 2000). LEFTY2 is increased during decidualisation and is activated by pro-protein convertase 5/6 (PC5/6, Okada *et al.*, 2005; Tang *et al.*, 2005). PC5/6 also activates vascular endothelial growth factor (VEGF, Siegfried *et al.*, 2003). VEGF, a known proangiogenic modulator (as reviewed in Ferrara *et al.*, 2003) is known to be activated by cAMP (Popovici *et al.*, 1999) and was shown to be increased in an in-vitro decidualisation model (Matsui *et al.*, 2004). Endothelial growth factor (EGF) is another angiogenic factor that was recently shown to be increased during the secretory phase together with VEGF (Möller *et al.*, 2001), however its role in decidualisation has not been further investigated.

Furthermore, activin and inhibin pathways as well as notch and lipid signalling, such as endocannabinoids and phosphatidic acid are also involved in decidualisation (as reviewed in Gellersen and Brosens, 2014).

1.3.2.3 Regulation of endometrial decidualisation

Progesterone and cAMP signalling pathways are crucial for successful decidualisation and both are required for the process to be completed (Brosens *et al.*, 1999).

P4 acts by binding and activating progesterone receptor (PGR). Two main PGR isoforms act in the endometrium, PGRA and B, which are both regulated by estrogen (Kastner *et al.*, 1990). PGRA and PGRB regulate different genes and pathways and PGRA even acts as a suppressor of PGRB (Gao *et al.*, 2000; Kastner *et al.*, 1990; Lockwood *et al.*, 2000). Progestin-dependent activation of the MAPK pathway through c-Src is mediated by PGRB (Migliaccio *et al.*,

1998). PGRB together with the estrogen receptor B also induce MAPK and AKT activation in response to progestin signalling (Vallejo *et al.*, 2005).

PGRA is the main PGR isoform in the endometrial stromal compartment (Mote *et al.*, 1999). It was also shown to be a stronger activator of insulin-like growth factor-binding protein 1 (IGFBP1) and decidual PRL promoters than PGRB (Christian, Pohnke, *et al.*, 2002; Gao *et al.*, 2000).

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger produced upon binding of extracellular ligands to Gs protein coupled receptors. Subsequent activation of membrane-bound adenylyl cyclase leads to generation of cAMP from adenosine triphosphate (ATP). A major target for cAMP is protein kinase A (PKA), a holoenzyme composed of two regulatory and two catalytic subunits. Binding of two cAMP molecules to each regulatory subunit leads to dissociation of the catalytic subunits, which in turn catalyse phosphorylation of various cytoplasmic target proteins (as reviewed in Skalhegg and Tasken, 2000). Catalytic subunits also phosphorylate and activate nuclear targets like cAMP response element (CRE) binding protein (CREB) or the related CRE modulator (CREM) (as reviewed in Lamas *et al.*, 1996; Mayr and Montminy, 2001) that act as activators of a plethora of genes.

Together, cAMP and progesterone are known to significantly induce expression of decidualisation markers IGFBP1 and PRL in-vitro decidualised ESC, thus providing a reliable in-vitro model of decidualisation (Brosens *et al.*, 1996, 1999). Many other genes are induced or suppressed during decidualisation and some of them have been identified in a microarray analysis of human decidualisation (Brar *et al.*, 2001).

Crucial to the decidualisation process, downstream of PI3K/AKT pathway are the Forkhead box (FOXO) subfamily of Forkhead transcription factors that are involved in cell cycle arrest, senescence, and apoptosis as well as cellular differentiation (as reviewed in Accili and Arden, 2004; Downward, 2004; Greer and Brunet, 2005). Phosphorylation in response to SGK1 or AKT results in the cytoplasmic retention of FOXO3A resulting increased proliferation

(Brunet *et al.*, 2001). FOXO1 is significantly upregulated in the human endometrium during decidualisation in-vivo, an effect also seen in-vitro (Christian, Zhang, *et al.*, 2002; Labied *et al.*, 2006). FOXO1 is required to activate IGFBP1 and PRL in ESC (Gao *et al.*, 1999; Kim *et al.*, 2005).

IGFBP1 and PRL are also regulated by HOX transcription factors, proteins that bind DNA through a conserved sequence, the homeobox. HOXA10 and HOXA11 both can induce these two classic decidualisation markers (Godbole and Modi, 2010; Lynch *et al.*, 2009).

A plethora of other signalling pathways, molecules and transcription factors are involved in decidualisation, an indication of how tightly regulated this process is in humans (reviewed in Gellersen and Brosens, 2014b).

1.3.3 Decidualisation and early pregnancy in mice

1.3.3.1 Anatomy of the mouse uterus and estrous cycle

The mouse (*Mus musculus)* reproductive tract is bilateral as it consists of two ovaries, a bicornuate uterus (two oviducts, two uterine horns), one cervix and one vagina (Figure 1.9). All these organs respond to hormonal changes due to the mouse estrous cycle and are very dynamic as they undergo many morphological changes during estrous cycles and gestation (Bertolin and Murphy, 2014).

The mouse uterus consists of the perimetrium, myometrium, endometrium, lumen, endometrial epithelium, stromal compartment that contains several immune cells and the uterine glands (Figure 1.9E). The mesometrium (the part that is closer to the abdominal cavity and contains uterine vessels and nerves) when decidualised contributes to the formation of placenta. The antimesometrial region (opposite the mesometrium) decidua supports the embryo during early pregnancy (reviewed in Groothuis *et al.*, 2007).

In female mice, similarly to women, gonadotropin-releasing hormone (GnRH) released by the hypothalamus causes secretion of LH and FSH from the gonadotrophs of the anterior pituitary, leading to ovulation (Bertolin and

Murphy, 2014). Ovarian follicles secrete estrogen, the action of which is followed by progesterone to render the endometrium receptive to implantation (Groothuis *et al.*, 2007).

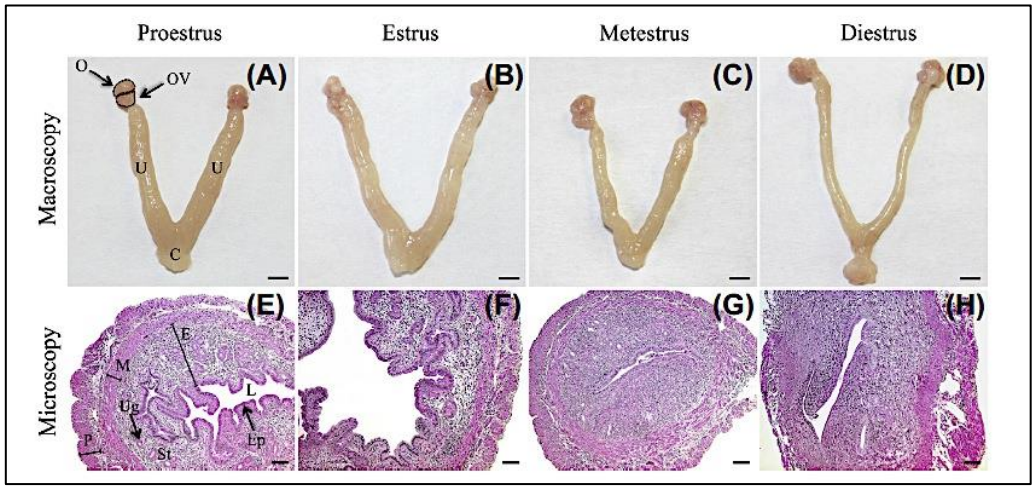

Figure 1.9. Mouse uterus during the four phases of the estrous cycle. (A–D) Female internal reproductive tract, (O - ovary, OV - oviduct, U – uterus, C – cervix). Scale bars represent 2 mm. (E–H) Bright field microscopy images of hematoxylin–eosin stained sections of the mouse uterus (P – perimetrium, M – myometrium, E – endometrium, L – lumen, Ep - endometrial epithelium, St - stromal compartment, Ug - uterine glands) Scale bars represent 0.1 mm. Image adapted from Bertolin and Murphy, 2014.

The equivalent of the menstrual cycle in rodents is called the estrous cycle, lasts approximately 4-5 days and has many similarities to the menstrual cycle (reviewed in Groothuis *et al.*, 2007).

The estrous cycle in mice consists of four stages:

- Proestrus (day 1)

  This stage corresponds to the proliferative phase of the menstrual cycle, characterised by follicle and uterine growth. During this stage, a *Prl* surge occurs following the LH and FSH surge (Figure 1.9A,E, reviewed in Bertolin and Murphy, 2014; Groothuis *et al.*, 2007).

- Estrous (day 2)

This stage is similar to the early secretory phase of the menstrual cycle; following ovulation there is a shift from estrogen to progesterone secreted by the corpus luteum as the main hormone influencing the murine endometrium. During this phase the female is receptive "in heat". Furthermore, observed *Prl* concentrations peak during this phase (Figure 1.9B,F, reviewed in Bertolin and Murphy, 2014; Groothuis *et al.*, 2007).

- Metestrous (day 3)

Corresponds to late secretory phase of humans. Progesterone secretion inhibits LH and thus prevents further ovulation. Towards the end of metestrous of an unmated mouse, the corpus luteum ceases progesterone secretion as it becomes non functional (Figure 1.9C,G, reviewed in Bertolin and Murphy, 2014; Groothuis *et al.*, 2007).

- Diestrous (day 4)

Similar to late secretory/ menstruation phase. Regression of the corpus luteum, followed by reabsorption of the endometrium in the uterus instead of menstrual shedding. Finally, estrogen levels start to increase preparing for the next proestrous phase. (Figure 1.9D,H, reviewed in Bertolin and Murphy, 2014; Groothuis *et al.*, 2007).

1.3.3.2 Decidualisation, implantation and early pregnancy in mice

Another major similarity between humans and mice is that implantation is critical to a successful pregnancy and occurs only during an "implantation window" during which the endometrium is receptive to the embryos (Favaro *et al.*, 2014; Yoshinaga, 1988).

Trophoblast differentiation in the mouse begins with the embryo polarisation at the 8-cell stage (reviewed in Sutherland, 2003). The outer polarised cells form the epithelial trophectoderm layer of the trophoblast that at the time of implantation transform to the invasive population called trophoblast giant cells.

These cells mediate embryo invasion in the uterus and vasculogenesis, as seen in Figure 1.10. This transformation includes morphological changes such as cytoskeleton modifications and is a complex process that involves factors such as *Oct3/4* and *Cdx2* (Carney *et al.*, 1993; Niwa *et al.*, 2005; Parast *et al.*, 2001; Ralston and Rossant, 2008; Strumpf *et al.*, 2005). Once the giant trophoblast cells adhere to the uterine epithelium, the epithelial cells undergo apoptosis and the giant trophoblast cells contact first the epithelial membrane and then the decidualised stromal cells as they invade the uterus (Favaro *et al.*, 2014; Parr *et al.*, 1987; Sutherland, 2003). Maternal receptivity is mediated mainly by estrogen and P4 action in the endometrium through a highly complex and tightly regulated process leading to decidualisation (reviewed in Ozturk and Demir, 2010). The blastocyst growth stage also known to influence maternal receptivity (Paria, Huet-Hudson, *et al.*, 1993).

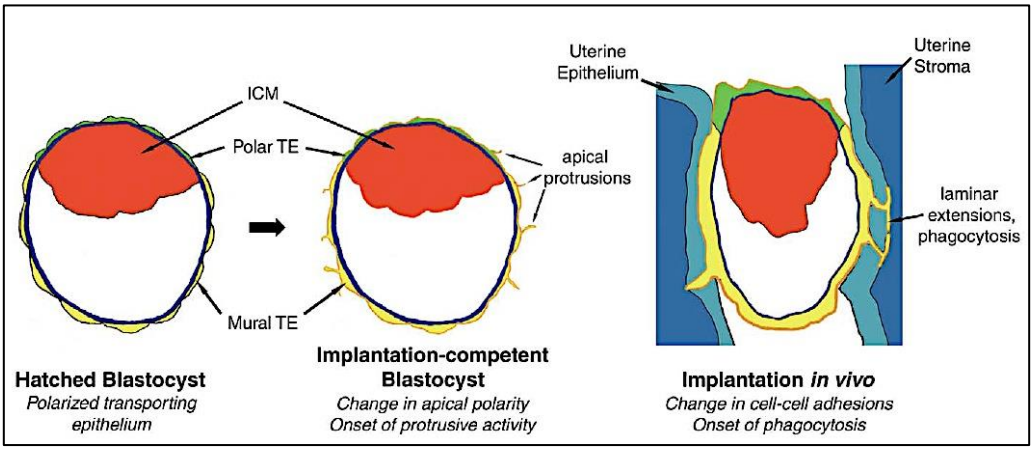

Figure 1.10. Transition of the trophectoderm to an invasive phenotype. The hatched blastocyst has a polarized, transporting, epithelial trophectoderm layer (TE), which exhibits no motility. The trophectoderm can be divided into two regions based on proximity to the inner cell mass (ICM): the polar TE (in green) and the mural TE (in yellow). As the blastocyst becomes competent to implant, the TE cells alter their polarity and begin to extend apical protrusions. During implantation in-vivo, these protrusions aid in displacement and phagocytosis of the apoptotic uterine epithelial cells, and give rise to the laminar extensions that ultimately form the blood sinuses of the yolk sac placenta. Figure from open access article (Sutherland, 2003).

Hb-egf is the first marker of embryo implantation in the mouse. It is expressed in the luminal epithelium prior to implantation at the site of blastocyst apposition on day 4 of gestation (Das *et al.*, 1994). *Hb-egf* was also shown to activate the epidermal growth factor receptor *(Egfr,* Das *et al.*, 1994) that is

known to be expressed by the blastocyst trophectoderm and its expression is regulated by maternal steroid hormones (Paria, Das, *et al.*, 1993). *Hb-egf* was later shown to be also expressed by the mouse uterus at the site of implantation, therefore suggesting involvement in foeto-maternal cross-talk during implantation (Hamatani *et al.*, 2004). Furthermore, *Hb-egf*$^{-/-}$ mice display reduced litter size as a result of defective implantation.

The prolactin homologue in the mouse (*Prl)* has multiple roles during decidualisation and early gestation. It is also responsible for the mammary gland development during early pregnancy (reviewed in Mizoguchi *et al.*, 1997).

1.3.4 The role of the immune system in endometrial decidualisation and implantation

1.3.4.1 Innate immune system in early pregnancy

Evidence has emerged in the past few years supporting multiple roles of immune system regulators in decidualisation, angiogenesis and implantation during early pregnancy (Bowen *et al.*, 2002; Nagamatsu and Schust, 2010). Whilst the immune tolerance of the semi-allogeneic foetus is maintained, several components of the immune system fulfil their designated roles in preparation for implantation as well as during gestation (Chaouat *et al.*, 2004; Entrican, 2002).

NK cells, macrophages and DCs have all been detected in the foeto-maternal interface and there is strong evidence of the critical role of the maternal innate immune system in early pregnancy (Gardner and Moffett, 2003; Guleria and Pollard, 2000; Moffett-King, 2002).

Approximately 40% of cells in the decidua are uNK ($CD56^{bright}CD16^{-}$ NK cells) that are in immediate contact with invading extravillous trophoblasts (Bulmer *et al.*, 1991; Hanna *et al.*, 2006) CXCL12 expression by invasive trophoblasts induces the specific migration of $CD16^{-}$ human NK cells, human placental cytotrophoblasts attract monocytes and $CD56^{bright}$ NK cells via the actions of MIP1a (Wu *et al.*, 2005). Hanna *et al* demonstrated in-vitro and in-

vivo that decidual NK cells regulate trophoblast invasion by production of the interleukin-8 (CXCL8/IL8) and interferon-inducible protein–10 chemokines (Hanna *et al.*, 2006)

Macrophages in the placental bed have been shown to induce apoptosis of extravillous trophoblast via tumour necrosis factor α (TNFα) secretion, that resulted in limited extravillous trophoblast invasion of spiral arterial segments in-vitro (Reister *et al.*, 2001).

1.3.4.2 Adaptive immunity in early pregnancy

Cytotoxic immune cells, including T-cells and B cells are not entirely absent during gestation. Gestation is not immunologically privileged; rather the maternal immune system is accepting a semi-allogeneic foetus. Several aspects of the processes governing maternal acceptance have been investigated and it is now known that multiple mechanisms are involved, making this process highly complex and tightly regulated (Chaouat, 2007; Entrican, 2002).

The traditional view of maternal acceptance was a shift in the ratio of Th1/Th2 cells in the uterus towards the Th2 during gestation, suggesting that Th1 population decrease is essential for the maternal acceptance of the semi-allogeneic foetus (Chaouat, 2007). Women with recurrent miscarriage displayed non-pregnancy Th1/Th2 ratio, indicating the shift to Th2 did not occur post conception (Reinhard *et al.*, 1998). However, research emerging during the past few decades has revealed several other mechanisms and cell types involved in maternal acceptance.

Decidualised ESC have been shown to actively ensure that the foeto-maternal interface is populated by $CD56^{bright}$ NK cells and macrophages but not $CD56^{dim}$ NK cells, T-cells, B cells or uterine DCs (uDCs, Silasi and Mor, 2012; Volchek *et al.*, 2010). Normally, DCs initiate T-cell responses to antigens they present in lymph nodes. However, uDCs have been shown in mice studies to be "entrapped" in the decidua and thus unable to present antigens in lymph nodes (Collins *et al.*, 2009; Tagliani and Erlebacher, 2011). Another mechanism via which T-cell recruitment in the uterus is minimised is epigenetic silencing of

chemokines that attract T-cells (Nancy *et al.*, 2012). Inflammatory cytokines such as IFN-γ and TNFα are not usually expressed in the placenta and have been associated with miscarriage in mouse models (Entrican, 2002).

Treg cells control immune homeostasis and immunosuppression (Saito *et al.*, 2010). Studies have demonstrated that unexplained infertility, miscarriage and preeclampsia are often associated with deficit in Treg cell number and function while normal pregnancy selectively stimulates the accumulation of maternal forkhead-box-$P3^+$ ($FoxP3^+$) $CD4^+$ Treg cells with foetal specificity (reviewed in La Rocca *et al.*, 2014; Roncarolo and Battaglia, 2007). Interleukin-10 (IL-10), a chemokine that mediates immunosuppression, is known to be highly expressed during early pregnancy until before the onset of labour, assisting in maternal acceptance and cross-talk between the placenta and decidua (reviewed in Thaxton and Sharma, 2010). Finally, the newly discovered immune effector cells, T-helper 17 cells (Th17) have also been associated with maternal acceptance and their role is still under investigation (Saito *et al.*, 2010).

1.3.4.3 Chemokines of particular importance in early pregnancy

Chemokines are a family of small secreted chemotactic cytokines. The chemokine superfamily consists of a large number of ligands and receptors with an important role in the immune system (Soares, 2004). According to the motif displayed by the first two cysteines at the amino end, they have been classified into four families: CXC, CC, XC and CX3C, named due to their ability to induce chemotaxis not only on immune cells but also on various other cell types (Luster, 1998; Rossi and Zlotnik, 2000).

1.3.4.3.1 Stromal derived factor 1 (SDF-1/CXCL12)

Stromal derived factor 1 (SDF-1/CXCL12) is a proliferative and chemotactic factor for cells as well as endothelial cells and is also involved in haematopoiesis, germ cell development, cardiogenesis and neurogenesis together with its receptor CXCR4 (Nagasawa, 2014).

Estrogen induces expression of CXCL12 in ESC (Tsutsumi *et al.*, 2011) and promotes the survival of secretory phase ESC via CXCL12/CXCR4 up-regulation-mediated autophagy inhibition (Mei *et al.*, 2015). However, CXCL12 levels were not found to be different throughout the menstrual cycle, in contrast to CXCR4 levels which were higher in proliferative phase (Laird *et al.*, 2011). Laird *et al* also reported strong immunostainning of CXCL12 and CXCR4 in the glandular epithelium and no staining of CXCL12 in stromal cells, whereas CXCR4 seemed present in low levels (Laird *et al.*, 2011). CXCL12 mRNA was detected in stromal and in decidual endothelial cells and its levels in stromal cells were not altered due to progesterone or estrogen treatment in another study (Carlino *et al.*, 2008). Another study reported few patient samples staining positive for CXCL12 during secretory phase and researchers were unable to detect any CXCL12 in cell supernatants in-vitro in response to 17β-estradiol or progesterone treatment (Kitaya *et al.*, 2004).

The CXCL12/CXCR4 axis is involved in the maintenance of Th2 bias at the maternal/foetal interface in early human pregnancy (Piao *et al.*, 2012). More importantly, CXCL12 controls over-invasion of trophoblasts via upregulating CD82 expression in DCs at maternal-foetal interface of human early pregnancy in a paracrine manner (Li *et al.*, 2011) and promotes the cross-talk between trophoblasts and decidual stromal cells in human first-trimester pregnancy (Zhou *et al.*, 2008). This is potentially mediated by recruitment of $CD56^{bright}CD16^{-}$ NK cells into decidua due to CXCL12 secretion by trophoblast cells (Carlino *et al.*, 2008; Hanna *et al.*, 2003; Wu *et al.*, 2005). CsA was shown to increase trophoblast invasiveness through strengthening the cross-talk of trophoblasts and decidual stromal cells mediated by CXCL12 and CD82 in early pregnancy (Meng *et al.*, 2012). Finally, human cytomegalovirus infection was shown to result in trophoblast invasion failure due to CXCL12 inhibition (Warner *et al.*, 2012).

In mice, $Cxcr4^{+}$ DCs were shown to promote angiogenesis during embryo implantation (Barrientos *et al.*, 2013). DCs are known to be crucial for mouse implantation as depletion of uterine DCs causes implantation impairment that

resulted in embryonic resorptions (Plaks *et al.*, 2008). *Cxcl12* has also been shown to enhance exogenous $CD4^+CD25^+$ T-cell migration in the uterus and thus prevent embryo loss in non-obese diabetic mice (Lin *et al.*, 2009).

1.3.4.3.2 CXCL16/ SRPSOX

CXCL16/ SRPSOX is a recently characterised chemokine (Matloubian *et al.*, 2000) that together with its receptor CXCR6 (Bonzo/STRL33/TYMSTR) has been associated with Th1 cells (Kim *et al.*, 2001), DCs and $CD4^+$ T-cells (Tabata *et al.*, 2005) as well as NK cells (Unutmaz *et al.*, 2000). CXCR6 was found to be critical for activation, homeostasis and recruitment of natural killer T (NKT) (Germanov *et al.*, 2008; Kim *et al.*, 2002). CXCL16 mRNA is activated by IFN-γ and TNFα (Abel *et al.*, 2004). Soluble CXCL16 (chemokine domain) induces chemotaxis of activated T-cells and bone marrow plasma cells via its receptor CXCR6, but the transmembrane molecule functions as a scavenger receptor for OxLDL, phosphatidylserine, dextran sulphate and bacteria and can uptake OxLDL and phagocytose bacteria as well as adhere to CXCR6-expressing cells (Abel *et al.*, 2004; Shimaoka *et al.*, 2004; Shimaoka *et al.*, 2004 (1)). The CXCL16/CXCR6 pathway has been associated also with cancer (Darash-Yahana *et al.*, 2009; Deng *et al.*, 2010), angiogenesis (Isozaki *et al.*, 2013) and juvenile idiopathic arthritis (Martini *et al.*, 2008).

Human trophoblast- recruited T-cells and monocytes into decidua by secretion of chemokine CXCL16 and interaction with CXCR6 in the first-trimester pregnancy (Huang *et al.*, 2008). Furthermore, CXCL16 has been reported to induce proliferation and invasion of first-trimester human trophoblast cells in an autocrine manner (Huang, Zhu, *et al.*, 2006).

In mice, the role of *Cxcl16* and its receptor *Cxcr6* in pregnancy remains unknown.

1.3.4.3.3 Interleukin 8 (IL8/CXCL8)

CXCL8 is a proinflammatory chemokine that has many identified roles. It acts as a chemoattractant and activator for neutrophils (Das *et al.*, 2010; Himmel *et al.*, 2011; Loos *et al.*, 2009) and T-cells (Larsen *et al.*, 1989). It is

proinflammatory (as reviewed in Huang *et al.*, 2006), proangiogenic (Heidemann *et al.*, 2003) and has also been associated with cancer and response to drugs (as reviewed in Gales *et al.*, 2013). It acts via surface receptors CXCR1 and CXCR2.

CXCL8 is expressed in perivascular cells and glandular epithelium and increases in late secretory phase endometrium and decidua during early pregnancy (Arici *et al.*, 1998; Caballero-Campo *et al.*, 2002; Critchley *et al.*, 1994; Milne *et al.*, 1999). This effect seems to be induced via lysophosphatidic acid (LPA) signalling in ESC through the LPA receptor and Nuclear Factor-κB-Dependent Pathway (NF-κB, Chen *et al.*, 2008). CXCL8 has furthermore been reported to increase proliferation of ESC (Arici *et al.*, 1998). CXCL8 expression in endometrial epithelial and stromal cells is induced by IL-1α and TNFα and its mRNA is slightly increased due to progesterone, even though there is no significant change in protein levels (Arici *et al.*, 1996). Stable protein levels throughout the menstrual cycle were also reported by another study that further demonstrated inhibition of CXCL8 secretion by P4 in endometrial explants (Kelly *et al.*, 1994).

Finally, CXCL8 secretion by uNK cells is reported to increase the invasiveness of extravillous trophoblast cells treated with conditioned supernatants in an in-vitro model (De Oliveira *et al.*, 2010). Decidual CXCL8 was shown to enhance trophoblast invasion by increasing MMP2 and MMP9 and integrins α5 and β1 (Jovanović *et al.*, 2010). The human blastocyst does not secrete measurable amounts of CXCL8, CCL2 and CCL5, however it does upregulate mRNA and protein levels of CXCL8 within endometrial epithelial cells whilst inhibiting protein secretion (Caballero-Campo *et al.*, 2002).

1.3.5 Abnormal decidualisation, implantation and placentation in adverse pregnancy outcomes

As presented above, extensive research indicates that decidualisation and implantation are inextricably associated and normal completion of both processes is essential for the establishment of a successful pregnancy.

1.3.5.1 Abnormal early gestation and miscarriage

The maternal endometrium plays a very important role to pregnancy outcome. It has recently been demonstrated that maternal decidualised ESC actively select blastocysts that are of good quality and this ability is not displayed by non decidualised stromal cells (Salker *et al.*, 2010; Teklenburg, Salker, Molokhia, *et al.*, 2010). Furthermore, both undifferentiated and decidualised endometrium has been shown to be altered compared to normal pregnancies in women with spontaneous miscarriage (Plaisier *et al.*, 2009). Additionally, impaired decidualisation, measured by a reduction in the decidualisation marker PRL in the endometrium, has been associated with recurrent miscarriage (Salker *et al.*, 2010) and in rodent models decidual cell prolactin production is essential for successful pregnancy (Bao *et al.*, 2007).

The timing of implantation is crucial for pregnancy outcome (Mahendru *et al.*, 2012). Among the pregnancies examined in this study, from 102 that implanted on day 9 after ovulation, 13% ended in early miscarriage. This proportion was increased to 26% with implantation on day 10, 52% on day 11, and 82% when implantation occurred after day 11 (Mahendru *et al.*, 2012).

1.3.5.2 Premature labour, low birth weight, IUGR and abnormal early gestation

IUGR has also been linked to abnormal implantation and placentation. Delayed implantation was also associated with decreased foetal size in the first trimester measured by smaller crown-rump length of the foetus (Mahendru *et al.*, 2012). It has been shown that smaller crown-rump length during the first trimester correlates with lower birth weight and subsequently IUGR (Salomon *et al.*, 2011). Furthermore, abnormal implantation that leads to defective placentation, such as in the case of placenta praevia, has been linked to low birth weight due to premature delivery and IUGR (Ananth *et al.*, 2001). The role of decidualisation to development of IUGR, low birth weight and premature labour has not yet been elucidated.

1.3.6 Studying decidualisation and early pregnancy: in-vitro and in-vivo models

In-vitro models have been used in research for many decades. They are generally faster and easier to use than in-vivo models. Furthermore, they allow for experimentation on primary cells and small tissue samples, thus enabling scientists to investigate if certain mechanisms and effects could exist in a living subject (Wu and Swartz, 2014). Importantly, they can replace certain in-vivo studies and preliminary data from in-vitro studies can inform on subsequent in-vivo experiments, thus reducing the number of animals required for research. As a result, they have a significant ethical advantage over in-vivo studies (Balls and M., 2002). However, as in-vitro models consist of the study of either individual cells or few cell types in co-culture (primary cells, cell lines or combination), they do not represent the complex microenvironment of a tissue or organ (James, Carter, *et al.*, 2012). Moreover, in several cases primary cells in single cell culture display different behaviour and transcriptional profiles due to the lack of interaction with other cell types (James, Carter, *et al.*, 2012). The advantages of immortalised cell lines are the ease of obtaining a culture stock, ease of handling and culture manipulation and generally faster cell proliferation compared to primary cells. However, they can significantly differ from their primary cell counterparts (Kaur and Dufour, 2012). Despite these limitations, in-vitro experiments are used to test whether a hypothesis is plausible prior to progressing to more complex in-vitro experiments or studies in humans such as clinical trials.

Several in-vitro models have been used to study decidualisation of primary ESC (reviewed in Gellersen and Brosens, 2014). ESC can be decidualised in-vitro when treated with medroxyprogesterone acetate (MPA) combined with enhancer cAMP for 4 to 10 days (Brosens *et al.*, 1999). When using MPA alone culture of ESC can last between 10-20 days (Tseng *et al.*, 1992). On the contrary, using cAMP alone was shown to induce decidualisation in a cell line derived from immortalised ESC within three days (Samalecos *et al.*, 2009). Estrogen followed by P4 can induce decidualisation of ESC in-vitro, however

it is a long protocol as it lasts 10-14 days (Hess *et al.*, 2007; Popovici *et al.*, 2000). Treatment of primary ESC using P4 and cAMP results in decidualised phenotype from day 4 of treatment (Gibson *et al.*, 2016). All of the above protocols induce mRNA and protein expression of classic decidualisation markers such as PRL and IGFBP1.

Mice have a number of advantages as research model organisms. The mouse genome is similar to the human genome (99%) and mouse biology has been well characterised due to years of extensive research (Peters *et al.*, 2007). Mice have short gestation period (less than 20 days), produce plenty of offspring and their reproductive biology is similar enough to humans to allow extrapolation of conclusions of animal studies to humans, often followed by validation of identified mechanisms (Lim and Wang, 2010). Mouse models have been developed utilising genetic manipulations such as gene deletion (Woods *et al.*, 1996). In addition, as a result of their small size and short life span, the financial cost of murine studies is often significantly less compared to larger organisms, such as sheep. However, important factors taken into consideration when using mouse models are that mice have several differences with humans: an estrous cycle, decidualisation occurs after fertilisation only, they usually produce multiple offspring per gestation, they have bicornuate uteruses, gestation lasts 20 days whereas the human gestation is much longer and some human genes may not have a mouse homolog or the mouse gene may have a different function to the human (Arck and Hecher, 2013).

Extensive gestational studies are conducted in other species, such as sheep and cows, due to similarities to human gestation and the industrial use of these animals. Several observational studies have been carried out in ruminants (Wu *et al.*, 2006). A comparison of human and sheep gestation and advantages of using sheep as an animal model is reviewed in (Barry and Anthony, 2008).

Due to the limitations in studying reproductive outcomes with relation to *Chlamydia* infection in humans, several mouse models have been established and used to investigate whether infection has a causative role. These include *Chlamydia psittaci (C. psittaci,* Buzoni-Gatel and Rodolakis, 1983), *C.abortus*

*(*de Oca *et al.*, 2000), *C.muridarum (*Habbeddine *et al.*, 2013) and various serovars of *Ct* (de la Maza *et al.*, 1994; Pal *et al.*, 1999, 2001). Detailed analysis of the above studies can be found in Chapter 4, section 1.

When the focus of research is human health and disease, in-vitro and in-vivo studies can inform on potential mechanisms involved as well as the potential efficacy of a treatment, however further investigation on humans in the form of a study is required to confirm each hypothesis. When designing a study, statistical power is calculated based on previous studies that inform on prevalence or predictions of the extend of the effect under investigation if this is a novel investigation (Concato *et al.*, 2000). Following strict ethical rules, prospective and retrospective large-scale studies can be used to investigate prevalence of a disease and whether it is associated with a certain outcome. These allow for direct extrapolation of results regarding patient health and can impact upon health policies (Song and Chung, 2010). Limitations of these studies include adequate sample size and inclusion bias that can lead to conclusions that are not representative for the whole population. Due to the complexity of humans biology, multiple factors can result in the same outcome (Saeidnia *et al.*, 2015). Suboptimal study design that does not take into account all confounding factors can be a serious limitation for many studies (Saeidnia *et al.*, 2015). However, carefully designed studies that investigate issues regarding human health are one of the pillars of current research.

1.4 Hypothesis and aims of PhD

The hypothesis of the work presented in this thesis is that *Ct* infection of the maternal endometrial stromal compartment has a detrimental effect on decidualisation leading to adverse pregnancy outcomes including miscarriage and IUGR. To address this hypothesis, the aims of my PhD were:

1. To determine whether *Ct* infection impacts upon decidualisation and chemokine secretion in a human model of endometrial stromal cell decidualisation

2. To investigate the role of *Ct* infection on adverse pregnancy outcome in a murine model of pregnancy

3. To determine the association of *Ct* in miscarriage in a case-control study

2. Chapter 2: Materials and Methods

Unless otherwise stated all chemicals and reagents were purchased from Sigma (Sigma- Aldrich Company Ltd, Dorset, UK).

2.1. *Chlamydia trachomatis* (*Ct*) stock production

2.1.1. Production of *Ct* stocks for in vitro and in-vivo experiments

Ct serovar E was used for both in-vitro and in-vivo infection studies. Stocks were produced at the Moredun Research Institute, with the generous help and guidance of Dr Nick Wheelhouse, Dr Sean Wattegedera and Prof. Gary Entrican.

2.1.2. *Ct* elementary bodies (EBs) growth and purification

Ct serotype E stock was produced in HEp2 cells using well established protocols. Sub-confluent flasks of HEp2 cells were inoculated with *Ct*. Infected cells were cultured for 48-72 hours in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Paisley, UK) supplemented with 2% heat inactivated foetal calf serum (FCS) and 1 µg/ml cycloheximide (Sigma, Cat. No C4859, PAA laboratories Ltd, Yeovil, Somerset, UK) until high numbers of mature inclusions were observed by optical microscopy. The cell monolayers were disrupted with glass beads and the medium containing cell debris was briefly sonicated (Vibracell, Sonics & Materials, Connecticut, USA) and centrifuged at 50 x g for 5 minutes at 4°C to remove intact cells. The supernatant was removed and centrifuged at 12,000 x g using a J-LITE JLA-16.250 rotor (Beckman Coulter Ltd. High Wycombe, UK). The pellet was resuspended by sonication in Tris/KCl and 25ml of inoculum was layered onto 10ml 40% Gastrografin® (Bayer plc, Berkshire, UK) and centrifuged at 20,000 rpm at 4°C for 45 minutes using a SW 32Ti rotor on Optima L-90K ultracentrifuge (Beckman Coulter Ltd). The pellet was re-suspended into 1ml Tris/KCl and layered onto discontinuous gradient (54%, 44%, 34% Gastrografin®) and centrifuged at 20, 000 rpm as above at 4°C for two hours.

The interface between the 44% and 54% layers, containing the EBs was carefully removed. The EBs were washed by resuspension in 10ml Tris KCl and centrifuged at 20, 000 rpm as above at 4°C for a further 45 minutes to remove residual traces of Gastrografin®. The final pellet was resuspended by sonication in 10ml of *Chlamydia* Transport Medium (218mM Sucrose, 3.76 mM KH_2P0_4, 7.1 mM K_2HPO_4, 4.9 mM L-glutamic acid, 10% FCS, 0.05 mg/ml Gentamycin, 0.1mg/ml Streptomycin, 150U/ml Nystatin) and the aliquots stored at -80^{O}C.

2.1.3. UV inactivation of Ct elementary bodies

To UV inactivate *Ct* EB's, 500 µl of inoculum was exposed to 2J UV-C. The successful inactivation of *Ct* was confirmed by culturing HeLa cells, known to be infected by *Ct* (Rödel, Grosse, Yu, Wolf, Otto, Liebler-Tenorio, Forsbach-Birk, and Straube, 2011), with UV inactivated bacteria for 96 hours without the development of any inclusions.

2.1.4. Titration of *Ct* stock

To determine the titre of the *Ct* stock, HEp2 cells were plated at 10^5 per well in 8 well glass chamber slides, infected with serial tenfold dilutions of *Ct* inoculum (Multiplicity of infection [MOI] 1 – 10^{-8}) and cultured for 48 hours. The cells were fixed in ice-cold acetone for 5 minutes, left to air dry and stored at -20°C. After thawing, the slides were rehydrated in phosphate-buffered saline (PBS) and incubated with the primary antibody (mouse monoclonal anti-*C. abortus* LPS, Santa Cruz Biotechnology, Cat. No. 13/4) for 1 hour at room temperature. Following PBS washes, the slides were incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Sigma) for 60 minutes at room temperature in a light-tight humidity chamber. Slides were mounted using ProLong® Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain (lifeTechonologies, Cat. No. P36930). The total number of inclusions per well was counted at serial dilutions 10^{-5}-10^{-7} and the titre of stock was calculated. The titre is a measurement of EBs that can form inclusions and are called inclusion-forming units (IFU).

2.2. RNA extraction, quantification and quality assessment

To assess changes in expression levels of genes of interest, total ribonucleic acid (RNA) extraction was performed using RNeasy® Mini (Qiagen, Crawley, UK, Cat. No. 74104) and RNeasy Micro (Qiagen, Cat. No. 74004) kits for RNA extraction from tissue and cells respectively.

2.2.1. RNA extraction from cultured cells

Cultured cells were grown as monolayers in six-or 12-well plates, as described in Chapter 3, section 2. Per well of each plate, 350 μl of RLT lysis buffer containing 0.01% β-mercaptoethanol were added following PBS washes to remove cell media. The cell lysates were immediately stored at -80°C until extraction. Frozen lysates were defrosted, mixed at 1:1 ratio with 70% ethanol and the sample was added to an RNeasy MinElute® Spin Column. Following a series of wash steps with wash buffers provided in the kit and on-column DNaseI digestion to remove contaminating DNA (Qiagen, Qiagen RNAse-Free DNase kit, Cat. No.79254), 80% ethanol was added to the samples and the column membranes were dried using centrifugation. During the final step, the samples were eluted in 15 μl RNase-free H_2O.

2.2.2. Tissue RNA extraction

To extract RNA from mouse tissue (model described in Chapter 4) a phenol/chloroform protocol provided by the RNeasy® Mini Kit was used. Tissue samples weighing 5-10 μg were transferred in sterilized 2ml tubes containing a steel bead and 1 ml TRI-Reagent® (Cat. No.T924, Sigma). Samples were lysed for three minutes at 25 Hz twice using a tissue lyser. Lysates were transferred to 1.5 ml PhaseLock Heavy Gel™ tubes and 200 μl of chloroform was added. Following mixing of the two phases in each tube by shaking and room temperature incubation for five minutes, samples were centrifuged at 14,000 g at 4°C. The aqueous top layer was poured into new tubes and 1:1 volume 70% ethanol was added. The mixture was transferred to RNeasy mini spin columns and the manufacturer's protocol was followed.

Briefly, after a series of wash steps and on-column DNaseI digestion, samples were eluted in 30 µl RNase-free H_2O.

2.2.3. Quantification and quality assessment of RNA samples

Quantification of all RNA samples was done using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilminton, DE, USA). All extracted samples were stored at -80°C.

To assess the efficacy of the RNA extraction protocol and the quality of the extracted RNA, the Agilent RNA 6000 Nano Kitsystem was used (Agilent, CA, USA, Cat. No. 5067-1511). The manufacturer's protocol was followed - briefly, the gel-dye mix was prepared and added to the chip provided from the specified well. The RNA marker was loaded in all wells, followed by the ladder and the samples. The chip was then vortexed and run on the Agilent 2100 Bioanalyzer.

2.3. Quantitative-Real Time PCR

Quantitative-Real Time- polymerase chain reaction (qPCR) is widely used to investigate changes in transcription levels of genes of interest, the expression level compared to genes that are known to be stably expressed across all experimental conditions, called 'housekeeper' genes (relative expression). Furthermore, qPCR can be used to quantify levels of a target in samples utilising a standard curve of samples with known levels (absolute quantification).

2.3.1. Preparation of complementary DNA (cDNA) using SuperScript® VILO™ Synthesis Kit

First-strand complementary DNA (cDNA) synthesis was carried out using a SuperScript® VILO™ Synthesis Kit (InVitrogen, Paisley, UK, Cat. No. 11754050) according to the manufacturer's protocol, as summarised in Table 2.1.

Table 2.1. Reaction mix for cDNA synthesis using SuperScript VILO Synthesis Kit for a 20 µl reaction.

Stock	Final concentration	Volume per 20 µl
5x VILO Reaction mix	1 x	4 µl
10x Superscript Enzyme Mix	0.125 x	0.25 µl
RNA (100 ng/µl)	100 ng	1 µl
Nuclease Free H2O		14.75 µl

Samples were incubated in a thermo-cycler (MJ Research PTC-200 Thermo Cycler, BC-MJPC200) under the following conditions:

- 25°C for 10 minutes
- 42°C for 60 minutes
- 85°C at 5 minutes

The samples were stored at –20°C until use.

2.3.2. Housekeeping gene assay

The choice of housekeeping genes is an essential first step to ensure the results of each qPCR experiment are accurate and reproducible. To determine how many and which genes were appropriate, a housekeeping gene assay was performed for both the in-vitro human cell studies and the in-vivo mouse model (Primerdesign, Southampton, UK, Cat. No. ge-SY-12). Candidate housekeeping genes included in the geNorm housekeeping assay kit were 18S, RPL13A, EIF4A2, YWHAZ, ACTB, GAPDH, CANX, UBC, SDHA, CYC1 and ATP5B. The optimal amount of genes and the best housekeeping genes were selected by analysing the data using the qbase+ program.

This qPCR assay was based on the TaqMan probe method, whereby the gene specific probes are bind to single stranded DNA and are separated from the attached fluorophore only if the specific gene sequence is amplified by the DNA polymerase. The fluorophore is then cleaved and omits light when excited by the qPCR cycler.

Samples from all treatments derived from four different patients and five different mice were used. The assay was carried out in 384-well clear optical pates (Applied Biosystems, Cat. No.4309849) plates at 10 µl/ well final reaction volume, as described in Table 2.2.

Table 2.2. Sample preparation for geNorm Housekeeping gene qPCR assays (TaqMan method)

Stock	Final concentration	Volume per 10 µl
2X Taqman universal master mix	1 X	5 µl
Primer pair	20 µM	0.5 µl
Nuclease free H_2O		3.5 µl
cDNA	5 ng	1 µl

The experiments were carried out using ABI7900 thermal cyclers (instruments that employs precise temperature control and rapid temperature changes to conduct PCR) under standard conditions.

The results were analysed using qbase+ (Biogazelle), which contains the geNorm program that: "calculates the gene expression stability measure M for a reference gene as the average pairwise variation V for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability"(geNorm manual). The principles on which this analysis is based are widely accepted (Vandesompele *et al.*, 2002).

2.3.3. Quantitative-Real Time-PCR using SYBR green

SYBR green is a cyanide dye which only binds to double stranded DNA, the result of amplification of the double-stranded DNA due to gene-specific primers (Zipper *et al.*, 2004). The resulting complex, when excited with blue light, omits green light that is detected by the qPCR thermal cycler machine.

All reactions were carried out in 384-well plates at a final reaction volume of 10µl per sample, as seen in Table 2.3. The samples were mixed by repeat pipetting and centrifuged in a mini plate spinner (Applied Biosystems,

Warrington, UK) at 1000 g for 20 seconds to remove any bubbles. A standard curve of cDNA made from standardised placenta RNA (Ambion, Cat. No AM7950) was used on every plate to ensure comparability between all plates.

Table 2.3. qPCR sample using SYBR green

Stock	Final concentration	Volume per 10 µl
2x Brilliant III SYBR Mix	1 X	5 µl
Primer pair	10 uM	0.5 µl
Diluted Reference Dye		0.15 µl
Nuclease Free H2O		3.35 µl
cDNA	2.5 ng	1 µl

Plates were assayed using the cycle conditions described in Table 2.4.

Table 2.4 qPCR cycling protocol on ABI 7900 using Brilliant III ultra-fast SYBR green master mix

Cycles	Temperature	Time
1 cycle	95°C	180 seconds
40 cycles	95°C	5 seconds
	60°C	15 seconds
1 cycle	95°C	15 seconds
	60°C	15 seconds
	95°C	15 seconds

All primers were predesigned KiCqStart® SYBR® Green Primers (Cat. No. KSPQ12012). Furthermore, all primers were validated using a standard curve of placental RNA with known concentration (Ambion, Cat. No AM7950). The placental RNA sample was used as positive control sample across all plates to further assess the variability of the assay among plates. Dissociation curves were used for every primer, as shown by a single peak, to confirm specific products. Quantification was performed using the $2^{-\Delta\Delta Ct}$ method.

3. Chapter 3: *Chlamydia trachomatis* infection of human endometrial stromal cells induces defective decidualisation and chemokine release.

3.1. Introduction and aims

The development of a successful pregnancy depends upon maternal receptivity. This is largely established during decidualisation, and the normally decidualised endometrium plays a crucial role in successful pregnancy. It has recently been demonstrated that decidualised ESC actively select blastocysts based on quality and this ability is not exhibited by non decidualised stromal cells (Salker *et al.*, 2010; Teklenburg *et al.*, 2010). Decidualised ESC in the presence of arresting embryos in an in-vitro co-culture displayed suppression of important factors for implantation such as HB-EGF, an effect not seen in when embryos were cultured with non decidualised ESC (Teklenburg *et al.*, 2010).

In women with spontaneous miscarriage, decidualised endometrium is altered compared to normal pregnancies as it displayed decreased vascularisation evident by fewer blood vessels presented and accompanied by decreased expression of pro-angiogenic factors such as VEGF-A (Plaisier *et al.*, 2009). Novel findings highlight that endometrial stem cells may also impact in the establishment of a receptive endometrium. Specifically, ESC from patients with recurrent miscarriage have been recently discovered to be in a premature status of senescence that results in impaired decidualisation response, a fact attributed to the absence of stem cells in the uteri of women with recurrent miscarriage (Lucas *et al.*, 2016). Furthermore, impaired decidualisation, measured by a reduction in the decidualisation marker PRL in the endometrium, has also been associated with recurrent miscarriage (Salker *et al.*, 2010) and in rodent models decidual PRL production has been shown to be critical for successful pregnancy (Bao *et al.*, 2007). Moreover, in endometrial biopsies of women with miscarriage, use of electron microscopy revealed a reduction in intracellular communication gaps compared to healthy women

(Kara *et al.*, 2007). However, it is not specified whether the samples were from recurrent or spontaneous miscarriage cases (Kara *et al.*, 2007).

The maternal immune system impacts significantly upon decidualisation, implantation and pregnancy maintenance, with immune cells and chemokines being involved in processes such as the menstrual cycle, trophoblast invasion and spiral artery remodelling among many others (Critchley *et al.*, 2001; Lash *et al.*, 2010; Smith *et al.*, 2009). Secretion of appropriate chemokine signals by decidual cells contributes to the recruitment of predominantly anti-inflammatory leukocyte subpopulations necessary for pregnancy maintenance (He *et al.*, 2012), and prevent recruitment of potentially damaging Th1-cells in response to inflammation (Nancy *et al.*, 2012). A Th1 response was associated with recurrent miscarriage in a small study (Jenkins *et al.*, 2000). Furthermore, chemokines are also involved in trophoblast invasion and angiogenesis during early pregnancy (Du *et al.*, 2014). As discussed in Chapter 1, important chemokines shown to induce trophoblast invasion include CXCL12, CXCL16 and CXCL8 among others.

The maternal immune response to miscarriage associated infections can have detrimental effects on pregnancy maintenance, a characteristic example of which is seen when malaria pathogens are detected in the placenta (reviewed in Giakoumelou *et al.*, 2015).

Ct is the most common sexually transmitted bacterial disease and its potential association with adverse pregnancy outcomes such as miscarriage is discussed in Chapter 1. However, even studies that suggest an association between *Ct* and miscarriage (Baud *et al.*, 2011; Licciardi *et al.*, 1992; Vigil *et al.*, 2002) have not yet identified the mechanism(s) responsible.

It is well established that *Ct* infects endometrial epithelial cells (Buchholz and Stephens, 2006; Rasmussen *et al.*, 1997; Rödel, Grosse, Yu, Wolf, Otto, Liebler-Tenorio, Forsbach-Birk, and Straube, 2011), but the effect of *Ct* infection on ESC function and decidualisation is yet undetermined and may have a role in the association of *Ct* infection with adverse pregnancy outcomes.

Ct is known to cause endometritis, namely inflammation of the endometrium that can be asymptomatic, in non-pregnant women (Tait *et al.*, 1997). Data from animal studies indicate that in mice, *C. abortus* induces the murine equivalent of miscarriage without foetal harm, likely due to decidual damage (Buendía *et al.*, 1998). In cattle *C. psittaci* associated chronic endometritis is a recognised cause of infertility (strain now known as *C.pecorum*, (Wittenbrink *et al.*, 1993). To my knowledge, no study to date has identified why *Ct* can cause endometritis in women or how infection of the stromal compartment of the endometrium might alter the function of human ESC.

The hypothesis of the work presented in this Chapter was that *Ct* can infect ESC and impact on decidualisation and immune response.

To address this hypothesis I aimed:

1. to determine whether *Ct* can infect human ESC in-vitro

2. to examine the effect of *Ct* infection on decidualisation and chemokine secretion in an in-vitro model of human ESC decidualisation.

3.2. Materials and methods

Details of *Ct* elementary bodies (EBs) growth, purification and detection by immunohistochemistry, UV inactivation of *Ct* elementary bodies, titration of *Ct* stock, RNA extraction, quantification and quality assessment and Real time PCR have been described in Chapter 2. All cell lines were *Mycoplasma* free.

3.2.1. Patients and Samples

3.2.1.1. Primary human endometrial stromal cells (ESC)

The ESC used in this study were isolated from endometrial tissue samples from women undergoing hysterectomy for benign gynaecological conditions collected after informed written consent (kindly supplied by Professor Hilary Critchley; LREC10/S1402/59). Samples from women in the proliferative phase of the menstrual cycle (determined by the last menstrual period and measurement of serum estradiol and progesterone levels) were selected for the experiments described in this Chapter. The tissue underwent dissociation using enzymatic digestion with 1 mg/ml of collagenase type IV for two hours at 37°C, (Sigma C5138), followed by mechanical break down using a 70 μm and subsequently 40μm filters (Falcon, Corning, Cat. No 352350 and 352340 respectively), allowing the dissociated stromal cells to pass through the filter pores, while the partly digested epithelial-containing glands were discarded. Single cells were plated in RPMI 1640 medium (Sigma, Cat. No. R0883) supplemented with 10% heat inactivated foetal calf serum (HIFCS, Gibco, Cat. No 10082147), 1% L-glutamine (Sigma, Cat. No G-7513) and 1% penicillin/ streptomycin (Sigma, Cat. No. P4333), prepared under sterile conditions. The medium was maintained for a maximum of seven days after which fresh medium was prepared. The cells were monitored on a daily basis, cultured until confluent, harvested and stored in medium containing 0.5% FCS and 15% Dimethyl sulfoxide (DMSO, Sigma, Cat. No. 472301) in liquid nitrogen until required.

3.2.2. Cell culture

3.2.2.1. ESC

Proliferative phase ESC isolated as detailed in section 3.2.1.1 were thawed and re-suspended in RPMI 1640 medium supplemented as described above. The medium, containing DMSO, was changed following cell attachment to the flasks and culture was maintained until sufficient cell numbers were reached for up to five passages. Prior to any treatments, cells were transferred to phenol red-free RPMI 1640 (Sigma, Cat. No R7509) containing 10% charcoal stripped foetal calf serum (CSFCS, prepared in house from FCS), 1% L-glutamine and 0.5% gentamycin (Sigma, Cat. No. G1272) for 48 hours prior to use. The antibiotic gentamycin was used instead of penicillin/ streptomycin, because it does not inhibit *Ct* growth (Bowie *et al.*, 1978). Cells were incubated at 37°C in 5% CO_2. Treatments were performed in duplicate per experiment and cells from 2-7 different patients were used per experiment, as detailed in individual sections of this chapter.

3.2.2.2. Immortalised trophoblast cell line (SW71)

Swan-71 (SW71) is a first trimester human trophoblast cell line that has been telomerase immortalised (Straszewski-Chavez *et al.*, 2009). Cell stock aliquots were stored in liquid nitrogen. After being thawed, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, LifeTechnologies, Cat. No. 11966-025) with 10% HIFCS, 1% L-glutamine and 0.5% gentamycin.

3.2.2.3. HeLa cell line

The HeLa cell line was a kind gift from Professor Sarah EM Howie, Centre for Inflammation Research, University of Edinburgh. HeLa cells were cultured in RPMI 1640 media containing phenol, supplemented with 10% HIFCS, 1% L-glutamine and 0.5% gentamycin.

3.2.3. In-vitro decidualisation

To assess the effects of infection on the decidualisation of primary ESC treatment with P4 and cAMP for 6 days (Gibson *et al.*, 2016) was selected from the published models of in-vitro decidualisation (discussed in 1.3.6) as an

optimal protocol length for ESC decidualisation and survival, as it was to be followed by a 48 hour *Ct* infection.

Cells were trypsinised, centrifuged for 3.5 minutes at 115 g and resuspended in phenol red-free RPMI 1640 with 10% CSFCS. They were then plated at $2x10^5$ cells per well in a six-well plate or 10^5 cells per well in a 12-well plate for a minimum of 24 hours. Before the decidualisation protocol commenced, cells were serum starved in 2% CSFCS medium for 24 hours, supplemented as previously described. The cells were treated with 10^{-6} M P4 (Sigma, Cat. No. P0130) and cAMP (Sigma, Cat. No. A9501) at a final concentration of 0.1 mg/ml. The medium was changed every 48 hours over a six-day period (Table 3.1).

To assess the decidualisation of ESC, levels of the secreted classic decidualisation marker PRL were measured using ELISA according to the manufacturer's protocol (R&D, Cat. No. DY682). Consistent with PRL secretion, morphological changes of the cells (cells became bigger, rounder and multinuclear) were observed as the protocol was progressing.

3.2.4. In-vitro *Ct* infection of decidualised ESC

ESC, decidualised as described in 3.3, were infected with multiplicity of infection (MOI) 0.001, 0.01, 0.1, 1, 2 or 3 on day 6 of the decidualisation protocol to determine optimal *Ct* concentration for ESC infection as described in Table 3.1. For example, infection with MOI 1 was conducted by adding 10^5 *Ct* EBs on 10^5 decidualised ESC in 2% CSFCS medium for 48 hours. Following infection, the cells were fixed in ice cold methanol for 20 minutes, air dried and stored at -20°C until further use.

Table 3.1. Decidualisation protocol for ESC. Treatments of progesterone (P4) and cyclic-AMP (cAMP) were administered in the cell media on days 1, 3 and 5. On day 6 the cells were infected with *Ct* or vehicle control and 48 hours later samples were collected.

	Day 1	**Day 3**	**Day 5**	**Day 6**	**Day 8**
ESC	P4/cAMP treatment	P4/cAMP treatment	P4/cAMP treatment	*Ct*/mock infection	Sample collection

3.2.4.1. Counting *Ct* inclusions and cell numbers

To visualise ESC infection, ESC fixed on plates were stained for 10 minutes using Giemsa stain (Sigma, Cat. No. 48900) diluted at 0.04 % (w/v) in pH6 buffer followed by a wash with distilled water. When air-dried, pictures were immediately taken using an Axiovert 200 Zeiss microscope to analyse the infection status of cells.

When performing cell and *Ct* inclusion counts, 15 images were taken for each well at random locations of the well by the author and were subsequently analysed using ImageJ. The numbers of both inclusions (infected) and uninfected cells were counted in all 15 images and the mean was used in calculating the different cell and inclusion numbers as a response to *Ct* infection, treatment with UV-*Ct* or uninfected media control.

3.2.4.2. Detection of *Ct* inclusions using Immunofluorescence

To stain the *Ct* inclusions, ESC and HeLa cells were grown on round glass coverslips (FisherScientific, Cat. No015) that were sterilised in 70% ethanol and then transferred in six-well plates. ESC and HeLa cells were grown at 10^4 cells/coverslip on glass slides coated with Matrigel (Corning, Cat. No 354248). Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumour rich in proteins such as laminin (a major component), collagen IV, heparin sulphate proteoglycans, entactin/nidogen, and a number of growth factors that improves cell adhesion and survival.

An antibody for lipopolysaccharide (LPS), a protein found on the outer wall of Gram⁻ve bacteria (anti- *C.abortus* LPS, Santa Cruz Biotechnology, Cat. No. sc-101593) was tested. The second antibody tested was an anti-*Ct* MOMP antibody (ThermoFisher, Cat. No MA1-7334). Several concentrations and antigen retrieval conditions (1 μg/ml - 100 ng/ml incubated overnight at 4°C,

pH6 heat induced epitope retrieval, pH9 heat induced epitope retrieval, no retrieval) were tested and the anti-LPS antibody did not stain *Ct* inclusions in infected samples. However, the anti-MOMP antibody's optimal concentration was 1 μg/ml with overnight incubation at 4°C. To visualise the primary antibody staining, Tyramide amplification system (Perkin Elmer, Cat. No SAT701B001EA for FITC, NEL745B001KT for Cy5) was used according to the manufacturer's instructions. Cells were visualised using an LSM 710 confocal microscope.

3.2.4.3. DNA extraction and *Ct* plasmid copy assay analysis of *Ct* infected cells

To elucidate whether *Ct* was multiplying in ESC, I used a *Ct* plasmid copy assay that utilised a standard curve to estimate the plasmid copies of *Ct* in the samples. This is a qPCR based method that detects the *Ct* Cryptic Plasmid Helicase (DnaB) gene that is found in 7-9 copies per bacterium.

3.2.4.4. DNA extraction from ESC

To extract DNA from infected cells, the MagMAX™ Total Nucleic Acid Isolation Kit (Ambion, Cat. No. AM1840) was used. 235 μl of lysis/binding solution were added in bead tubes. Cells were washed with PBS three times and scraped off using a cell scraper in 175 μl of PBS. The mixture was transferred into bead tubes containing magnetic zirconia beads that bind nucleic acids and vortexed for 15 minutes. Tubes were then centrifuged at 8,000 g for 30 seconds to pellet the zirconia beads. 115 μl of each sample were moved to a processing plate and remaining samples were stored at -20°C. 65 μl of 100% isopropanol was added in each well and it was mixed for 1 minute. 20 μl of Bead Mix were subsequently added and mixed for 5 minutes at a plate shaker. Binding beads that were holding the DNA were captured using a magnetic plate and washed twice with 150 μl of wash solution followed by two washes with 150 μl wash solution 2. Beads were dried by shaking for 2 minutes and 30 μl 65°C elution buffer and mixed for three minutes. The beads were captured and supernatant containing purified DNA was stored at -20°C.

3.2.4.5. *Ct* plasmid copy assay

To assess whether *Ct* could proliferate in ESC the *Chlamydia trachomatis* Genesig Standard Kit (PrimerDesign, Cat. No Path-C.trachomatis-standard) was used. Using a standard curve of known concentration provided by the kit, I was able to do absolute quantification of *Ct* in DNA extracts from ESC cells. The qPCR protocol is described in 2.3.3 (TaqMan method) respectively.

3.2.5. Human Chemokine Array

To investigate changes in secreted chemokines from decidualised primary ESC in response to *Ct* infection, the Proteome Profiler™ Human Chemokine Array Kit (R&DSystems, Abingdon, UK, Cat. No. ARY017) was used with the help of Mrs June Stroud, MRC Centre for Inflammation Research. This proteomic assay is a membrane based antibody sandwich immunoassay that detects 33 different chemokines, shown in Table 3.2.

Table 3.2. Chemokines detected by the Proteome Profiler™ Human Chemokine Array Kit

CCL1/I-309	CCL21/6Ckine	CXCL8/IL-8
CCL2/MCP-1	CCL22/MDC	CXCL9/MIG
CCL3/CCL4 (MIP-1 alpha/MIP-1 beta)	CCL26/Eotaxin-3	CXCL10/IP-10
CCL5/RANTES	CCL28	CXCL11/I-TAC
CCL7/MCP-3	Chemerin	CXCL12/SDF-1
CCL14/HCC-1/HCC-3	CX3CL1/Fractalkine	CXCL16
CCL15/MIP-1 delta/LKN-1	CXCL1/GRO alpha	CXCL17/VCC-1
CCL17/TARC	CXCL4/PF4	IL-16
CCL18/PARC	CXCL5/ENA-78	Midkine
CCL19/MIP-3 beta	CXCL7/NAP-2	XCL1/Lymphotactin
CCL20/MIP-3 alpha		

Samples used were pooled supernatants from three in-vitro *Ct* infected ESC and the manufacturer's protocol was followed without deviation. Briefly, the membranes were blocked for one hour on a rocking platform. After several washes, sample/detection antibody mix was added on the membranes, overnight at 4°C. The next day the membranes were washed and Streptavidin-HRP was added for 30 minutes, after which they were developed in X-ray film for 1, 3, 5 and 7 minutes. Each chemokine was represented in film (if present)

by a circle. Varying diameters corresponded to various concentrations of chemokines. Using ImageJ and Photoshop, the pixel density of each chemokine was estimated across all samples.

3.2.6. Enzyme-linked Immunosorbent Assay

3.2.6.1. Protocol of sandwich ELISA

Sandwich ELISA was used to detect IGFBP-1 (R&DSystems, Cat. No.DY871), PRL (R&DSystems, Cat. No.DY682) and CXCL8 (R&DSystems, Cat. No. DY208). All ELISAs were used as per manufacturer's instructions. . For more information regarding the reagents involved in this ELISA, see Table 1.3.

Clear flat-bottomed 96-well plates (Sigma, Cat. No. CLS3590) were coated with 100 µl per well of capture antibody diluted to working concentration in PBS. Plates were incubated overnight at room temperature. After incubation, the solution was aspirated, all wells were washed three times with wash buffer and the plate was blotted on a paper towel, a procedure that was repeated after every protocol step. Plates were blocked using blocking buffer for one hour during which a two-fold serial dilution standard curve and appropriate sample dilutions in reagent diluent were prepared. Standard curve dilutions were commencing from 2000 ng/µl. The wash step was repeated and 100 µl of sample or standard were added to each plate, along with appropriate negative controls, for two hours at room temperature on a plate rocker. Plates were again washed and detection antibody was added for two hours at 100 µl per well on a plate rocker. The antibody was then removed, and the wells were washed and incubated with Streptavidin-HRP solution for 12 minutes on a plate rocker. A final wash step was then performed and substrate, 3,3',5,5'-Tetramethylbenzidine (TMB) was added in the wells under dark on a plate rocker. TMB is colourless but is converted to light blue by streptavidin, if it is present in each well. This was converted to yellow with the addition of 50 µl 2M sulphuric acid, which also stopped the reaction. Optical density was measured immediately after, using a microplate reader at appropriate wavelengths, as specified by each manufacturer (Table 3.3).

Table 3.3. Concentrations of capture antibody, detection antibody, streptavidin-HRP and recipe of wash buffer, blocking buffer used in IGFBP-1, PRL and CXCL8 ELISAs. *Antibody concentration differed between ELISA kit batches.

Reagent	IGFBP-1 ELISA	PRL ELISA	CXCL8/IL8 ELISA
Wash buffer	0.05% Tween® 20 in PBS	0.05% Tween® 20 in PBS	0.05% Tween® 20 in PBS
Blocking buffer	5% Tween® 20 in PBS	1% bovine serum albumin (BSA) in PBS	1% bovine serum albumin in PBS
Capture antibody*	as specified on bottle	as specified on bottle	as specified on bottle
Detection antibody*	as specified on bottle	as specified on bottle	as specified on bottle
Reagent diluent	5% Tween® 20 in PBS	0.1% BSA and 0.05% Tween® 20 in PBS	0.1% BSA and 0.05% Tween® 20 in PBS
Streptavidin	as specified on bottle	as specified on bottle	as specified on bottle
Substrate solution	1:1 mixture of colour reagent A and B	1:1 mixture of colour reagent A and B	1:1 mixture of colour reagent A and B
Stop solution	2N H_2SO_4	2N H_2SO_4	2N H_2SO_4

Sample concentrations were calculated using MasterPlex® QT (Hitachi) software, courtesy of Dr Forbes Howie.

3.2.7. Magnetic Luminex® Screening Assay

The Luminex® Screening Assay (R&D Systems, Cat.No. LXSAHM) was used to detect CXCL12 and CXCL16 as per manufacturer's instructions. Supernatants from *Ct* infected, UV-*Ct* treated and uninfected ESC originating from three patients were pooled and the assay membranes were incubated in these supernatants and following washes, Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents were added. The signal produced is in proportion to the amount of cytokine bound on the capture antibodies located on each membrane. Using an X-ray developer, the signal was visualised. To compare the quantity of each chemokine across the membranes, each chemokine was expressed as a % proportion of the average of three reference dots that are present on each membrane.

3.2.8. Trophoblast cell migration assays

To analyse the migration potential of first trimester trophoblast cell line SW71 in response to chemokines CXCL12, CXCL16, their combination and conditioned media from infected ESC and controls, a migration assay was designed.

The minimal concentrations of CXCL12 and CXCL16 recombinant proteins (R&D systems, Cat. No. 350-NS/CF and 976-CX-025 respectively) were determined using the following scratch assay. Scratch assays are widely used to assess cell migration in in-vitro culture systems (Liang *et al.*, 2007). SW71 cells were plated at 10^5 in 12-well plates and left to rest for four hours. Subsequently, the media was changed to media containing 5 ng, 25 ng, 50 ng and 100 ng of each chemokine and their combination per ml. Using a 1 ml pipette tip, the well was scratched in the middle and pictures were taken using an Axiovert 200 Zeiss microscope at this point termed 0 hours. 30 hours later, the distance covered between cells on 30 points randomly chosen was counted using surface measure in ImageJ.

Due to large variations observed in the scratch assay, a spheroid assay was developed to assess SW71 migration in response to chemokines and ESC conditioned media. Briefly, 10^5 SW71 cells were plated in round bottom non-adhesive 96-well plates overnight. The following morning, the cell spheroids formed were transferred in 48-well plates (2 spheroids per well), that were containing 1ml of conditioned media, with and without chemokines as seen in Table 3.4. 30 hours later, the assay was stopped, the spheroids were photographed using an Axiovert 200. The migration are was defined as the one cell layer surface originating from an attached spheroid, excluding the area covered by the spheroid and was calculated using ImageJ. Each treatment was completed in duplicate, for a total of three experiments.

Table 3.4. Conditions of trophoblast migration assay experiment.

Conditioned ESC Media-	DMEM	Total volume	Chemokines added
Decidualised *Ct* infected 300 µl	200 µl	500 µl	+/- 50 ng/ml CXCL12 +/- 100 ng/ml CXCL16
Decidualised UV-*Ct* treated 300 µl	200 µl	500 µl	+/- 50 ng/ml CXCL12 +/- 100 ng/ml CXCL16
Decidualised uninfected 300 µl	200 µl	500 µl	+/- 50 ng/ml CXCL12 +/- 100 ng/ml CXCL16
Non decidualised uninfected – 300 µl	200 µl	500 µl	none
RPMI control – 300 µl	200 µl	500 µl	none
DMEM control – 300 µl	200 µl	500 µl	none

3.2.9. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6. Repeated measures non parametric ANOVA- Friedman's test was used for all sample sets. By using a non parametric test it was assumed that the samples are not following a Gaussian distribution, as the sample size was not suitable for a normality test. Friedman's test was selected as using a repeated-measures test (RM one way ANOVA) controls for experimental variability attributed to patient-to-patient response variations. Some factors not controlled for in the experiment will affect all the measurements from one subject equally, so they will not affect the difference between the measurements in that subject. By analyzing only the differences, therefore, a matched test controls for some of the sources of scatter. Furthermore, to compare specific groups when Friedman's test showed a significant difference, Dunn's method for non parametric multiple comparisons was used, as it allows for comparison of two particular groups by determining each rank from the entire data set (all groups), not just the two groups being compared. All graphs are representing mean with standard deviation (SD) of the data.

Dr Ioannis Papastathopoulos, Chancellor's Fellow at the University of Edinburgh's School of Mathematics has reviewed the statistical analysis and approved it for analysis of the data presented in this chapter.

3.3. Results

3.3.1. *Ct* stock viability and titration

Two *Ct* stocks used for in-vitro experiments were produced as described in 2.1. Titration of stock was carried out using anti-LPS immunohistochemistry on 8-well chamber slides containing HEP2 cells infected with multiplicities of infection (MOI) of *Ct* (10^{-1} to 10^{-7}), as described in 2.1. Following thorough counting of all inclusions in dilutions 10^{-5} and 10^{-6}, the titre of the first stock was estimated at 2.8 x 10^{8} bacteria per ml of stock and the second stock 2 x 10^{6} bacteria/ml. A portion of this stock was UV-inactivated (UV-*Ct*, see paragraph 2.1). Aliquots were transported from Moredun Institute to the QMRI according to safety regulations and were used for all experiments described in this chapter unless otherwise specified.

CHX was used for *Ct* stock production as explained in 2.1. As it was shown not to be required for successful infection in-vitro and it is highly toxic for cells (as seen in Figure 3.1), all experiments in the present thesis were carried out without CHX.

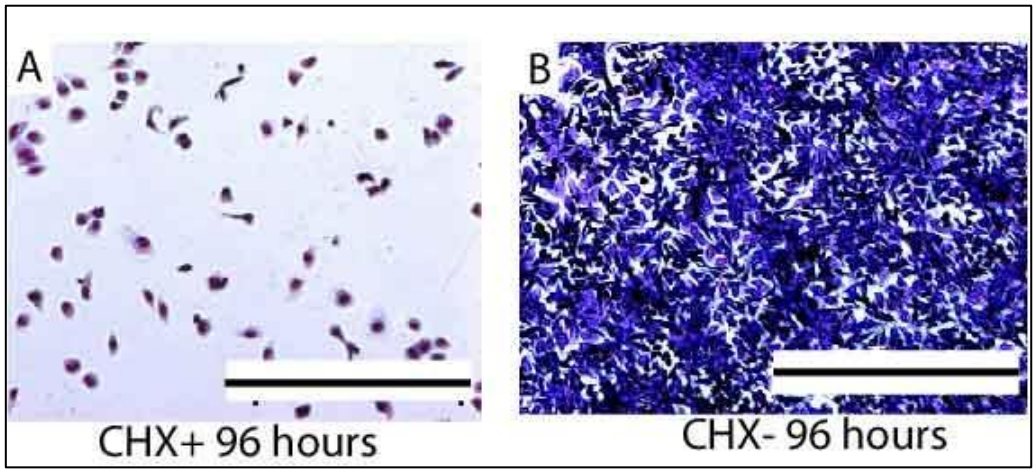

Figure 3.1. Cycloheximide (CHX) has a highly toxic effect on HeLa cells. (A) Significantly reduced numbers of uninfected HeLa cells were observed when CHX was included in their media. (B) Uninfected HeLa cells without CHX in their media. Both pictures were taken 96 hours after plating**.** Scale bars equal 200 μm.

To confirm the viability of *Ct* stock after transfer and the successful UV-inactivation of the *Ct* stock, HeLa cells were infected at MOI 1 for 96 hours and treated with UV-*Ct* at the same concentration.

In cultures infected with viable *Ct,* visible inclusions were seen in Giemsa stained cells from 48 hours, with an exemplar image seen in Figure 3.2A and highlighted in Figure 3.2B.

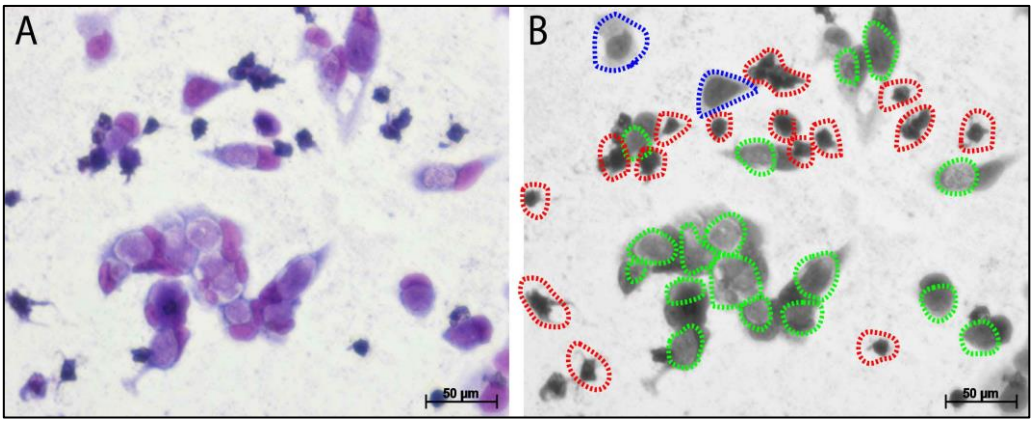

Figure 3.2. *Ct* inclusions in HeLa cells infected with MOI 1. Cells were stained with Giemsa 48 hours post infection. (A) Staining revealed large *Ct* inclusions within infected HeLa cells, coloured purple darker than the cytoplasm with a grainy appearance. Several dead cells were also visible, as absorbing Giemsa stained them dark blue. (B) In the sketch corresponding to panel A, *Ct* inclusions are highlighted in green, dead cells in red and uninfected HeLa cells in blue. Scale bars equal 50 µm.

The number of cells containing inclusions increased over time. By 96 hours post infection, very few live HeLa cells remained in the wells and some inclusions were visible (

Figure 3.3A). In contrast, UV-*Ct* treated HeLa and uninfected controls proliferated as expected in a 96-hour cell culture (

Figure 3.3B,

Figure 3.3C). Moreover, no inclusions were seen in cells cultured with UV-*Ct* at the same MOI as *Ct* over the same time course, indicating no infectious EBs remained after UV inactivation of the *Ct* stock.

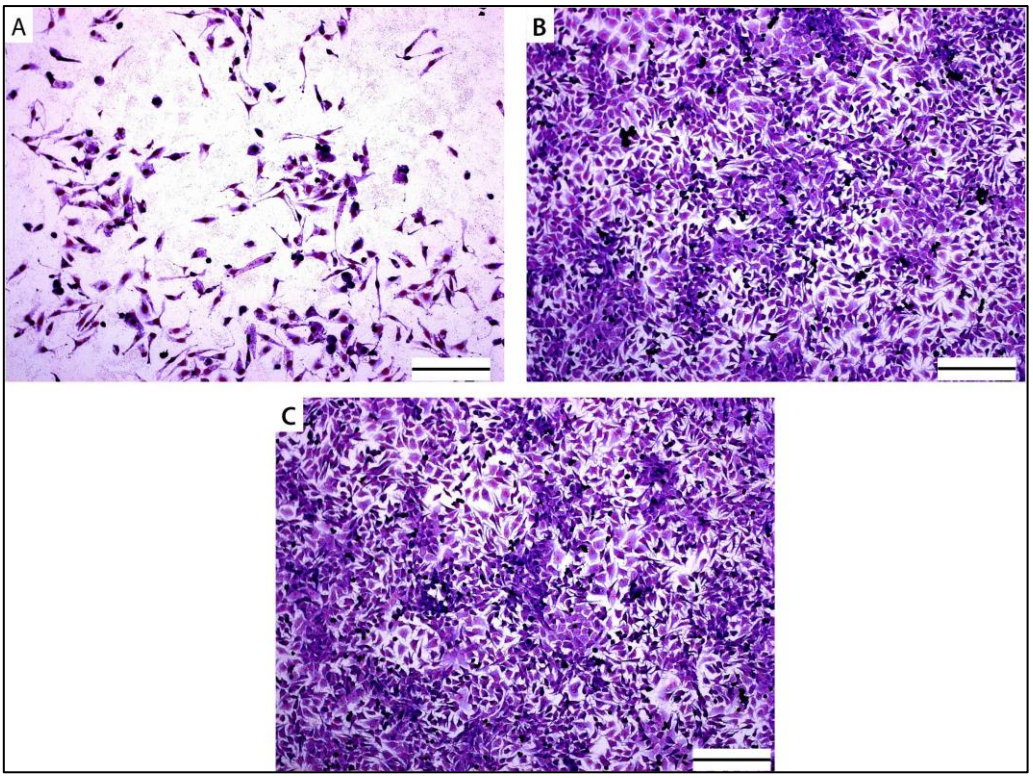

Figure 3.3. *Ct* infection of HeLa cells causes reduction in cell numbers and cell death. HeLa cells were infected with MOI 1 of *Ct*, then cells were fixed and stained with Giemsa 96 hours post infection. (A) *Ct* inclusions (stained light purple) were visible in some cells. Uninfected live HeLa cells were scarce and there was a clear reduction in cell numbers, as well as many dead cells and cell debris (stained blue). (B) UV-*Ct* did not infect HeLa cells and their appearance resembled that of uninfected HeLa cells. (C) Uninfected HeLa cells cultured for 96 hours proliferated and as a result high cell density was observed. Scale bars equal 200 μm.

3.3.2. *Ct* can directly infect human ESC

Primary ESC were infected in 12-well plates with *Ct* serovar E at MOI 0.01, 0.1, 1, 2 and 3. No visible inclusions were present in cells at MOI 0.01 and MOI 0.1 48 hours post infection and very few inclusions were seen at MOI 1, whereas ESC infected with MOI 2 and 3 showed similar numbers of inclusions following infection. The non infected non decidualised and decidualised ESC displayed no signs of infection however uninfected decidualised ESC were visibly enlarged and rounder as expected (**Figure 3.4**A, **Figure 3.4**B) whereas visible *Ct* inclusions were seen in cells infected at MOI 2, as well as dead cells and cell debris (**Figure 3.4**C). Decidualised ESC treated with UV-*Ct* were morphologically similar to uninfected decidualised ESC and displayed no signs of infection (**Figure 3.4**.D). No copies of *Ct* cryptic plasmid were detected in uninfected cells, as assessed using qPCR. UV-*Ct* treated ESC (which still contained bacterial DNA) had 2.5 $x10^4$ – 7.5 $x10^4$ plasmid copies per well. *Ct* infected wells contained 1.2 $x10^6$ – 3.3 $x10^6$ plasmid copies indicating that significant replication had occurred (**Figure 3.4**.E). Although the number of decidualised uninfected ESC was decreased compared to non-decidualised ESC, UV-*Ct* treated and *Ct* infected ESC samples contained similar numbers of cells compared to decidualised uninfected ESC (**Figure 3.4**.F). In infected wells, between 0.05% - 20% of ESC contained *Ct* inclusions (**Figure 3.5**). These conditions were used for all subsequent experiments.

To visualise *Ct* more accurately, two anti-*Ct* antibodies were tested under various antigen retrieval conditions and primary antibody concentration as described in 3.4.2. Using the Tyramide amplification system for immunofluorescence, *Ct* inclusions were clearly stained in HeLa cells but ESC cells were autofluorescing when stained with Cy3 and FITC, thus imaging and detecting *Ct* inclusions was difficult. When using Cy5, detection of inclusions was possible in ESC. However, ESC were not surviving on glass coverslips longer than 72 hours, regardless of coverslip coating with Matrigel. Decidualised ESC had poor survival if trypsinised and replated. *Ct* infected

decidualised ESC proved to be particularly fragile and could not be transferred onto glass slides to perform immunofluorescence. Finally, as ESC were grown in 12-well plates, the cost of immunofluorescence was quite high and the available visualisation methods did not produce good quality images. Therefore, Giemsa stain was selected as the optimal staining method for visualising *Ct* infection in ESC.

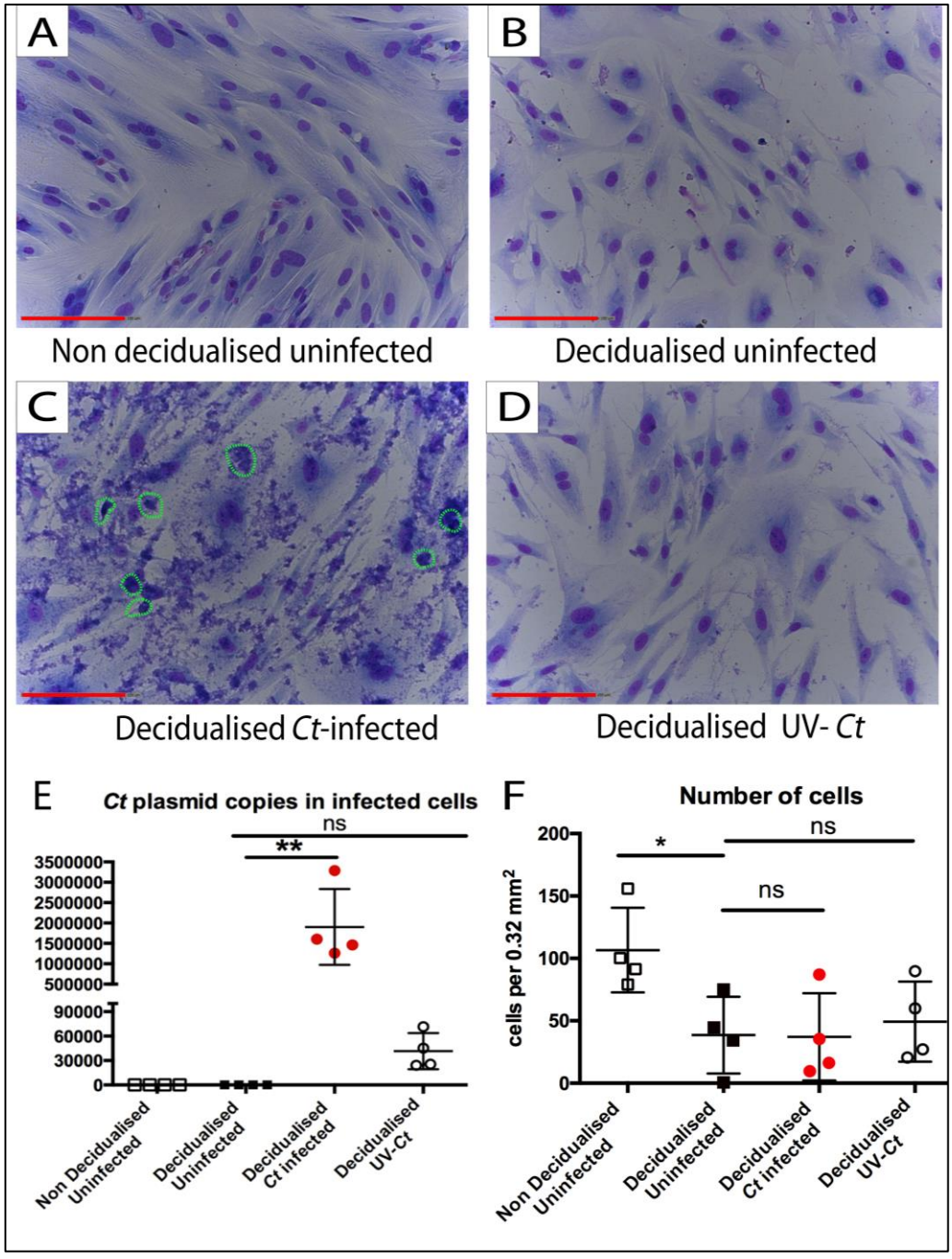

Figure 3.4. *Ct* can infect decidualised ESC. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-*Ct* treated ESC were used as controls. 48 hours post infection DNA was collected for qPCR and cells were stained with Giemsa. Cell counts were conducted in 15 fields of view per well measuring 0.32 mm^2 each. (A) Non decidualised uninfected ESC were elongated and thin. (B) Decidualised uninfected ECS became rounder and larger compared to non decidualised ESC. (C) *Ct* infected decidualised ESC displayed signs of infection and contained inclusions that were stained purple by Giemsa stain. The wells also contained dead cells and cell debris. (D) UV-*Ct* treated decidualised ESC did not contain inclusions and resembled uninfected decidualised ESC in appearance. (E) 25.000 – 75.000 *Ct* plasmid DNA copies were detected in UV-*Ct* treated ESC. *Ct* infected ESC had a significantly higher number of 1.200.000 – 3.300.000 plasmid copies per sample, indicating proliferation of *Ct* only in infected cells (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p=0.0094, n=4). (F) Cell counts on Giemsa stained ESC indicated that decidualised uninfected cells were significantly fewer compared to non-decidualised controls (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p= 0.0185, n=4). In contrast, UV-*Ct* treated cells and *Ct* infected cell numbers were similar to uninfected decidualised ESC. Scale bars equal 200 µm. Graphs show the mean and standard deviation.

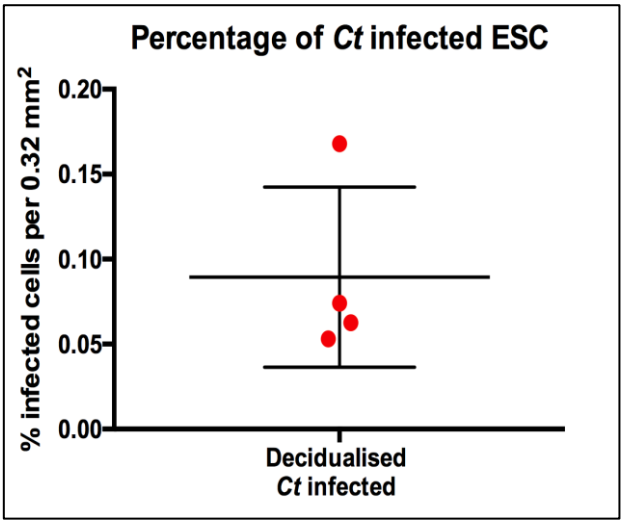

Figure 3.5. *Ct* infects 0.05% - 18% of decidualised ESC 48 hours post infection. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol, then were fixed 48 hours post infection and stained with Giemsa stain. Cell counts were conducted in 15 fields of view per well measuring 0.32 mm^2 each. Graph shows the mean and standard deviation.

3.3.3. *Ct* infection of human ESC results in defective decidualisation

3.3.3.1. *Ct* infection did not affect decidualisation marker IGFBP1

For decidualisation marker IGFBP1, both mRNA and protein were significantly upregulated in decidualised uninfected ESC compared to non decidualised controls (Figure 3.6A&B). However, no differences in IGFBP1 levels were observed as a result of UV-inactivated *Ct* treatment or *Ct* infection (Figure 3.6A&B).

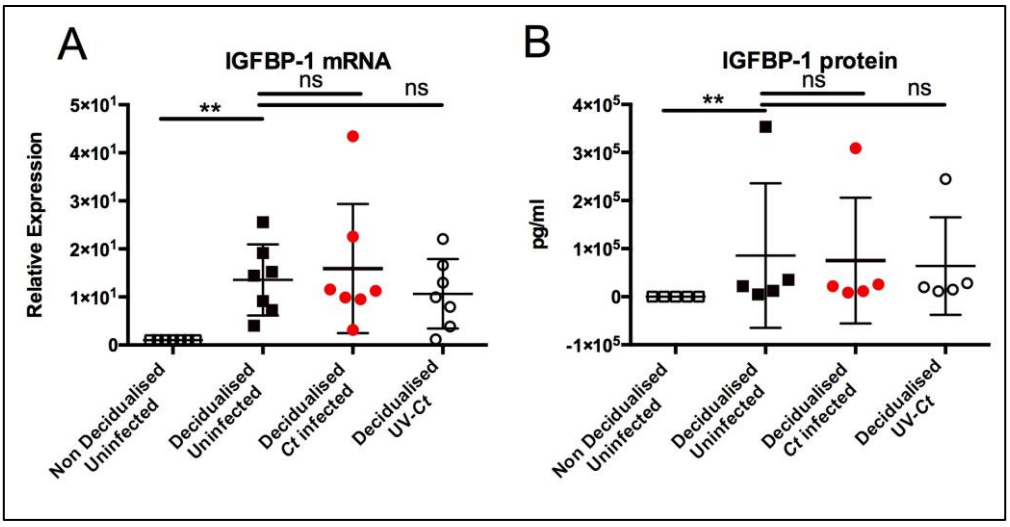

Figure 3.6. *Ct* infection does not affect mRNA and protein levels of decidualisation marker IGFBP1. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-*Ct* treated ESC were used as controls. 48 hours post infection mRNA and supernatants were collected for qPCR and ELISA tests respectively. (A) IGFBP1 mRNA levels were elevated in response to decidualisation signals as expected, however no difference was observed between decidualised *Ct* infected and UV-*Ct* treated or uninfected decidualised ESC (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p=0.0057, n=7). (B) Secreted IGFBP1 protein concentration was not altered by *Ct* infection, however it was higher in decidualised ESC compared to non decidualised cells (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p=0.044, n= 5). Graphs show the mean and standard deviation.

3.3.3.2. *Ct* infection affected decidualisation marker PRL

Infection with *Ct* reduced the mRNA expression of the classic decidualisation marker PRL at mRNA but not protein levels in decidualised *Ct* infected ESC (Figure 3.7A&B). Decidualised uninfected ESC had increased levels of both PRL mRNA and protein compared to non decidualised ESC (Figure 3.7A&B).

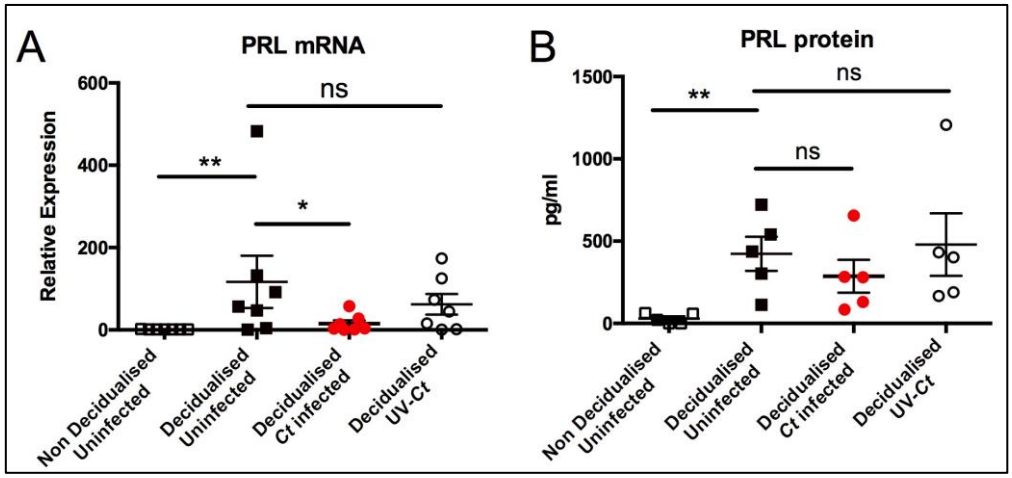

Figure 3.7. *Ct* infection of ESC reduces mRNA but not protein levels of decidualisation marker PRL. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-*Ct* treated ESC were used as controls. 48 hours post infection mRNA and supernatants were collected for qPCR and ELISA tests respectively. (A) PRL mRNA was upregulated in response to decidualisation stimulus, whereas it was downregulated in infected decidualised cells compared to uninfected controls indicating the adverse effect of *Ct* infection on decidualisation (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p=0.0009 and 0.0151 respectively, n=7). (B) PRL protein levels were increased in decidualised non infected cells compared to non decidualised controls as expected (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p=0.0044, n=5). However, PRL levels were not significantly different between *Ct* infected ESC compared to decidualised uninfected controls, (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p>0.05, n=5). Graphs show the mean and standard deviation.

3.3.4. *Ct* infection does not affect SGK1, VEGF, FOXO3A, HB-EGF but reduces EGF expression

To further investigate the effect of *Ct* on other genes that have been identified as important to decidualisation, implantation or association with miscarriage (discussed in Chapter 1), the mRNA levels of SGK1, VEGF, FOXO3A, EGF and HB-EGF were investigated by qPCR. However, with the exception of reduced EGF mRNA levels in *Ct* infected decidualised ESC compared to

uninfected non decidualised controls, no change was observed in response to *Ct* infection in any of the above genes (Figure 3.8).

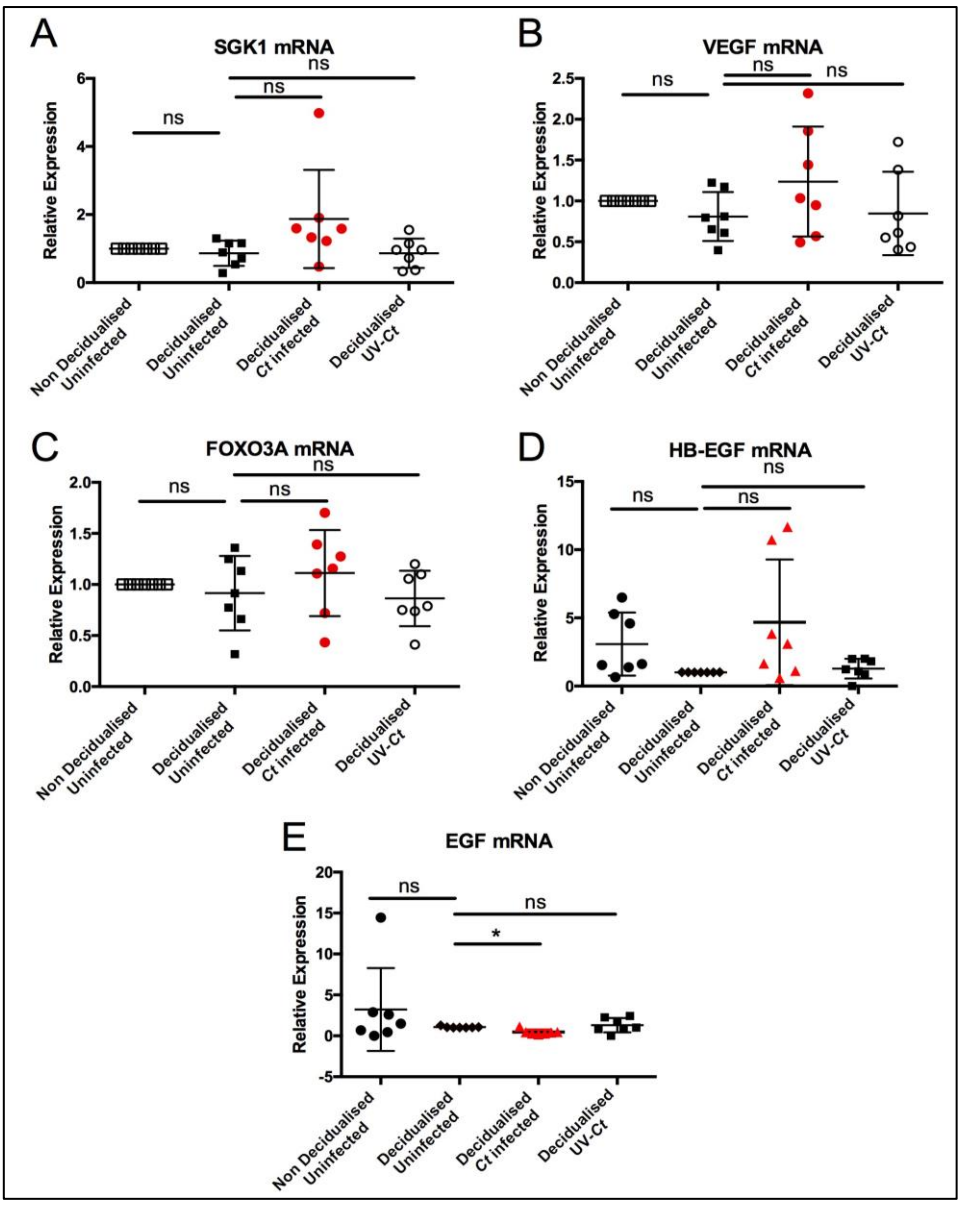

Figure 3.8. *Ct* infection has no effect on SGK1, VEGF, FOXO3A, HB-EGF but reduces the levels of EGF mRNA. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-*Ct* treated ESC were used as controls. 48 hours post infection mRNA was collected test the effect of *Ct* infection on VEGF, EGF, SGK1, FOXO3A and HB-EGF expression levels using qPCR. (A) SGK1 mRNA levels were not altered by *Ct* infection (RM one-way ANOVA-Friedman's test, $p>0.05$, n=7). (B) VEGF mRNA levels did not differ among all groups (RM one-way ANOVA-Friedman's test, $p>0.05$, n=7). (C) FOXO3A mRNA expression levels remained unchanged (RM one-way ANOVA-Friedman's test, $p>0.05$, n=7). (D) HB-EGF mRNA levels did not differ among the four groups (RM one-way ANOVA-Friedman's test, $p>0.05$, n=7). (E) EGF mRNA levels were reduced in *Ct* infected ESC (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, $p=0.0389$, n=7). Graphs show the mean and standard deviation.

3.3.5. *Ct* infected human ESC have an altered chemokine profile

To assess the immune response of ESC to *Ct* infection, CXCL8 mRNA and protein levels were examined and a significant increase of this chemokine was observed in response to active *Ct* infection, however the levels of CXCL8 were not affected by decidualisation or exposure to UV-inactivated *Ct* (Figure 3.9).

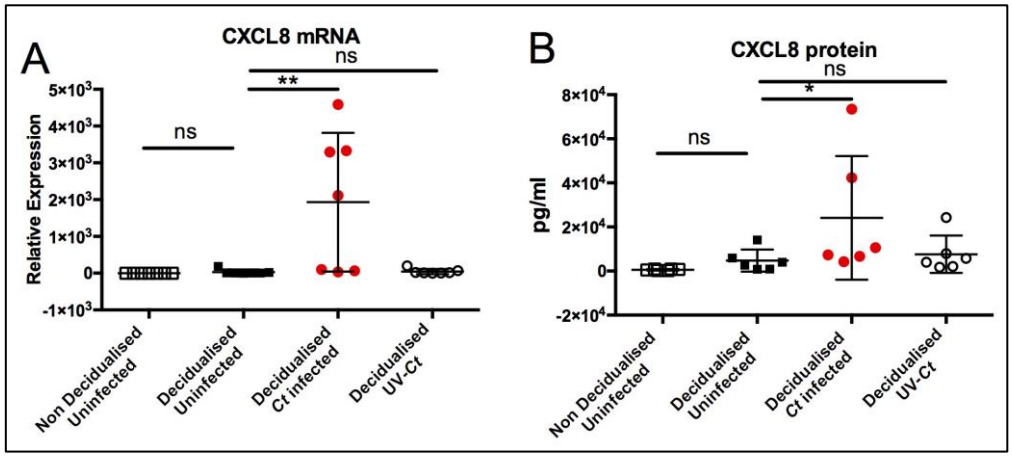

Figure 3.9. CXCL8 expression is induced by *Ct* infection. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-*Ct* treated ESC were used as controls. 48 hours post infection mRNA and supernatants were collected for qPCR and ELISA tests respectively. (A) CXCL8 mRNA was upregulated in *Ct* infected decidualised ESC (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p= 0.001, n=7). CXCL8 was not upregulated as a response to decidualisation or UV-*Ct* treatments alone. (B) CXCL8 protein secretion was increased in infected ESC but not in controls (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p= 0.011, n=6). CXCL8 protein levels in decidualised ESC supernatants were not higher compared to non decidualised cells. All graphs show the mean and standard deviation.

To investigate the effect of *Ct* infection on chemokine secretion by decidualised ESC, the levels of 31 secreted chemokines were examined by proteomic array in supernatants from decidualised uninfected and infected ESC. A pattern of differential expression between the cell types was observed (Figure 3.10).

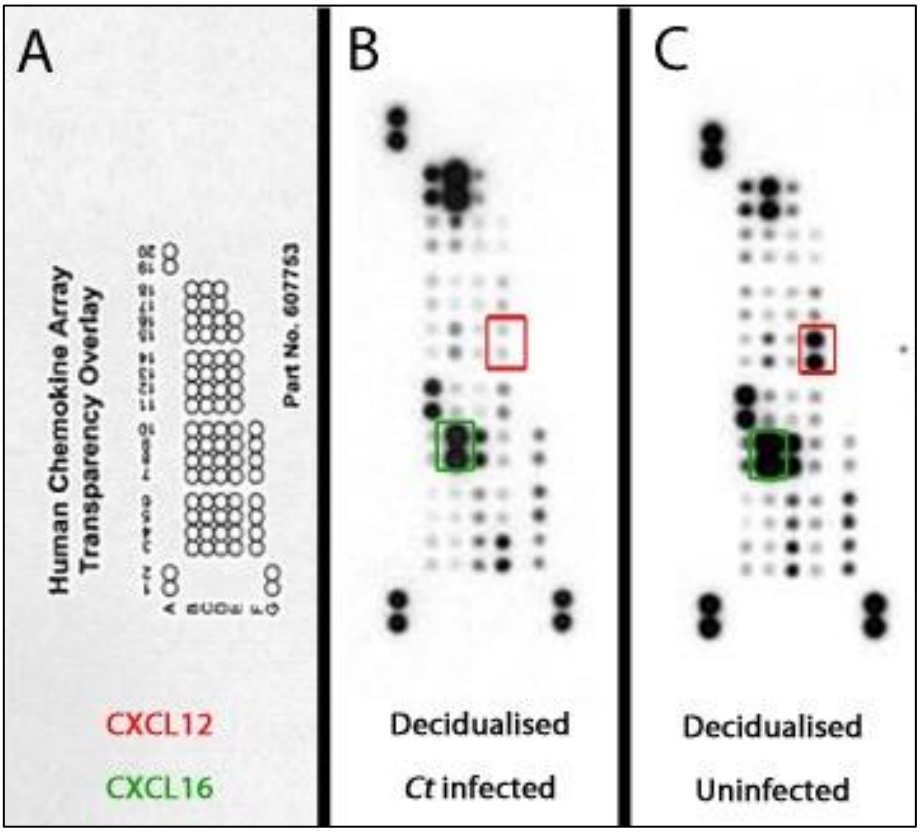

Figure 3.10. Chemokine proteomic array of 31 chemokines on pooled cell supernatants indicated altered chemokine secretion from decidualised *Ct* infected compared to uninfected decidualised ESC. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC were used as controls. 48 hours post infection supernatants were collected. The secretion levels of 31 chemokines in pooled supernatants from three patient samples were analysed on blot membranes. Each chemokine was represented by two dots. (A) Layout of each membrane with cytokines CXCL12 and CXCL16 highlighted in red and green respectively (B) *Ct* infected decidualised ESC secreted chemokine profile, demonstrating ESC secreted all 31 chemokines. (C) Uninfected decidualised ESC secreted chemokine profile is altered compared to *Ct* infected ESC. (n=1)

Table 3.2 shows that four chemokines were upregulated and 15 downregulated in *Ct* infected decidualised compared to non infected ESC (threshold 5%). Of the chemokines known to be involved with early pregnancy, trophoblast invasion and/or genital infection CXCL12 and CXCL16 were both strongly downregulated in infected decidualised ESC, as were chemokines CCL7, CCL12 and Midkine whilst CXCL7, CXCL1 and XCL1 were strongly upregulated.

Table 3.5. Chemokine proteomic array showed altered chemokine secretion due to *Ct* infection in decidualised ESC. Results were calculated as % of the average of positive controls present in each membrane. Significant differences higher than 5% are highlighted in grey. Table contains information about the role of each chemokine in decidualisation, early pregnancy and association with miscarriage and *Ct* infection.

Protein	**% of positive control Decidualised**		**Δ % of positive control**	**Role in decidualisation/early pregnancy/miscarriage/association with *Ct* in humans**
	***Ct* infected**	**Uninfected**		
6Ckine/CCL 21/ Exodus-2	14.90	19.43	-4.53	mRNA detected in the endometrium throughout the menstrual cycle and increased in early pregnancy (GCID:GC09M034709). No information on miscarriage and association with *Ct* infection.
CCL28/ MEC	6.42	7.78	-1.36	Detected by immunohistochemistry in decidualised ESC and first trimester decidua, was shown to induce apoptosis in ESC and its receptors were increased in ESC from miscarriage (Sun *et al.*, 2013). No information on association with *Ct* infection.
CXCL16/ SRPSOX	24.59	53.31	-28.72	Human trophoblasts recruited T lymphocytes and monocytes into decidua by secretion of CXCL16 in early pregnancy (Huang *et al.*, 2008). CXCL16 also induces invasion and proliferation of first-trimester human trophoblast cells (Huang, Zhu, *et al.*, 2006). No information on decidualisation, miscarriage or association with *Ct* infection.
Chemerin /TIG-2/ RARRES2	91.66	99.69	-8.03	Chemerin serum concentration was significantly higher in pregnant women in the 3rd trimester than in non-pregnant and pregnant women in the 1st trimester, whilst no differences were observed during the menstrual cycle (Garces *et al.*, 2013).No information on miscarriage or association with *Ct* infection.
ENA-78/ CXCL5	10.67	14.48	-3.82	Elevated ENA-78 levels were associated with increased risk of miscarriage (Whitcomb *et al.*, 2007). mRNA levels were increased in trachoma patients (Burton *et al.*, 2011). No information on decidualisation.
Eotaxin-3/ CCL2	16.52	23.75	-7.23	IL-33 enhances proliferation and invasiveness of ESC by up-regulation of CCL2 and its receptor CCR2(Hu *et al.*, 2014). No association between maternal circulation levels during the first trimester of CCL2 and miscarriage(Hannan *et al.*, 2014). No information on association with *Ct* infection.

Protein	% of positive control Decidualised		Δ % of posi-tive control	Role in decidualisation/early pregnancy/miscarriage/association with *Ct* in humans
	Ct infected	Uninfected		
Fractalkine/ CX3CL1/ Neurotactin	28.68	30.31	-1.63	Is expressed in maternal decidua and promotes human trophoblast invasion (Hannan *et al.*, 2006). No association between maternal circulation levels during the first trimester of CCL2 and miscarriage(Hannan *et al.*, 2014). No information on association with *Ct* infection.
GROα/ CXCL1	94.17	66.23	27.94	Expressed in early pregnancy decidua (GCID:GC04P073869). Upregulated in women with antiphospholipid antibodies inducing a placental inflammatory response via the TLR-4/MyD88 pathway, which in turn compromises trophoblast survival(Mulla *et al.*, 2009). Associated with asymptomatic *Ct* infection in men (Hakimi *et al.*, 2014).
HCC-1/ CCL14/ HCC-3	10.22	15.23	-5.01	Promotes human trophoblast invasion (Hannan *et al.*, 2006). No information on miscarriage or association with *Ct* infection.
I309/ CCL1/ TCA3	10.82	18.54	-7.72	No information on decidualisation, early pregnancy, miscarriage or association with *Ct* infection.
IL-8/CXCL8	98.02	100.15	-2.13	Promotes trophoblast invasion (Jovanović *et al.*, 2010). Upregulated in women with antiphospholipid antibodies inducing a placental inflammatory response, which in turn compromises trophoblast survival(Mulla *et al.*, 2009). No association of serum levels with miscarriage (Whitcomb et al., 2007). Induced by *Ct* infection (Natividad *et al.*, 2009).
IP-10/ CXCL10	36.13	44.75	-8.62	Increased in decidua during the implantation window (Sela *et al.*, 2013). Also reported to be increased in women with *Ct* associated infertility (Gupta *et al.*, 2009). No information on miscarriage.
I-TAC/ CXCL11	8.70	13.58	-4.88	Increased levels in euthyroid women with autoimmune thyroiditis with recurrent miscarriage and compared to diabetics with successful pregnancy (Aktas *et al.*, 2014). Secreted by progesterone primed primary endocervical epithelial cells infected with *Ct* (Wan *et al.*, 2014). No information on decidualisation or early pregnancy.

Protein	% of positive control Decidualised		Δ % of posi-tive control	Role in decidualisation/early pregnancy/miscarriage/association with *Ct* in humans
	Ct infected	Uninfected		
Lymphotactin/ SCM-1α	46.07	23.09	22.98	Upregulated during implantation window (Lobo *et al.*, 2004). No information on miscarriage or association with *Ct* infection.
MCP-1/ CCL2/ MCAF	101.83	99.08	2.75	Localised in glandular epithelium and endothelial cells, not secreted by blastocyst during early implantation window (GCID: GC17P034255). Upregulated in women with antiphospholipid antibodies inducing a placental inflammatory response which in turn compromises trophoblast survival (Mulla *et al.*, 2009). Elevated mRNA due to *Ct* infection (Molestina *et al.*, 2000).
IL-16/ LCF	20.71	36.59	-15.88	Expressed in decidua during the implantation window (GCID:GC15P081159). Induced in response to *Ct* infection in cell lines (Marti *et al.*, 2014). No information on miscarriage.
MCP-3/ CCL7	38.34	61.95	-23.61	Detected in plasma of women during 1st trimester of pregnancy but not altered in miscarriage cases (Hannan *et al.*, 2014). Does not induce trophoblast migration (Hannan *et al.*, 2006).
MDC/ CCL22/ STCP1	31.95	46.88	-14.93	Detected on maternal stromal cells and increased in placentae from miscarriage cases (Freier *et al.*, 2015). No information on association with *Ct* infection.
MDK/MK/ Midkine	76.92	97.66	-20.75	No information on decidualisation, early pregnancy, miscarriage or association with *Ct* infection.
MIG/ CXCL9	10.27	17.56	-7.29	Associated with asymptomatic *Ct* infection in men (Hakimi *et al.*, 2014).No information on decidualisation or early pregnancy.
MIP-1α/β / CCL3/ CCL4	10.44	18.14	-7.70	Secreted by ESC in response to hCG (Srivastava *et al.*, 2013). Promotes trophoblast migration (Hannan *et al.*, 2006). No association of serum levels with miscarriage (Whitcomb *et al.*, 2007). Levels were not affected by *Ct* infection (Maxion and Kelly, 2002).

Protein	% of positive control Decidualised		Δ % of posi-tive control	Role in decidualisation/early pregnancy/miscarriage/association with *Ct* in humans
	Ct infected	Uninfected		
MIP-1δ/ CCL15/ Leukotactin 1/ HCC-2	10.74	16.56	-5.82	No information on decidualisation, early pregnancy, miscarriage or association with *Ct* infection.
MIP-3α/ CCL20/ LARC/ Exodus-1	14.12	14.36	-0.24	Was shown to be increased due to *Ct* infection (Porcella *et al.*, 2015). No information on decidualisation, early pregnancy or miscarriage.
MIP-3β/ CCL19/Exodus-3	36.15	37.03	-0.88	No information on decidualisation, early pregnancy, miscarriage or association with *Ct* infection.
NAP-2/ CXCL7/ CTAP III	78.93	18.09	60.84	No information on decidualisation, early pregnancy, miscarriage or association with *Ct* infection.
PARC/ CCL18/ MIP-4/ AMAC-1	15.85	25.42	-9.57	Increased in trachoma patients in response to Il-17 (Burton *et al.*, 2011). No information on decidualisation, early pregnancy or miscarriage.
PF4/ CXCL4	19.27	31.85	-12.57	No information on decidualisation, early pregnancy, miscarriage or association with *Ct* infection.
RANTES / CCL5/ SISd	34.42	25.36	9.07	Localised in endometrial stromal and endothelial cells during the implantation window and is not produced by the trophoblast (GCID:GC17M035871). No association of serum levels with miscarriage (Whitcomb *et al.*, 2007).

Protein	**% of positive control Decidualised**		**Δ % of posi-tive control**	**Role in decidualisation/early pregnancy/miscarriage/association with *Ct* in humans**
	***Ct* infected**	**Uninfected**		
SDF-1/ CXCL12/ PBSF	15.12	94.86	-79.75	Associated with trophoblast invasion and implantation in primary stromal cells (Hanna *et al.*, 2003; Zhou *et al.*, 2008). There is also a study showing inhibition of CXCL12 due to cytomegalovirus infection that resulted in impaired migration and invasion of human extravillous cytotrophoblasts (Warner *et al.*, 2012). Levels not altered in semen of men with asymptomatic *Ct* infection (Hakimi *et al.*, 2014). No information on association with miscarriage.
TARC/ CCL17	17.75	30.25	-12.50	Induces trophoblast migration (Li *et al.*, 2014). No information on decidualisation, miscarriage or association with *Ct* infection.
VCC-1/ CXCL17/ DMC	6.77	10.00	-3.23	No information on decidualisation, early pregnancy, miscarriage or association with *Ct* infection.

To validate the array results, qPCR and ELISA were used to measure changes in mRNA and protein levels of chemokines CXCL12 and CXCL16 as a result of infection with *Ct*. As seen in Figure 3.11A and Figure 3.11C, there were no significant differences in mRNA encoding CXCL12 and CXCL16. In contrast, infected ESC secreted significantly less CXCL12 and CXCL16 (Figure 3.11B, Figure 3.11D). CXR4 (the receptor for CXCL12) mRNA was significantly decreased in infected decidualised ESC (Figure 3.11E). However, CXCR6 (the receptor for CXCL16) mRNA was not affected by *Ct* infection (Figure 3.11F).

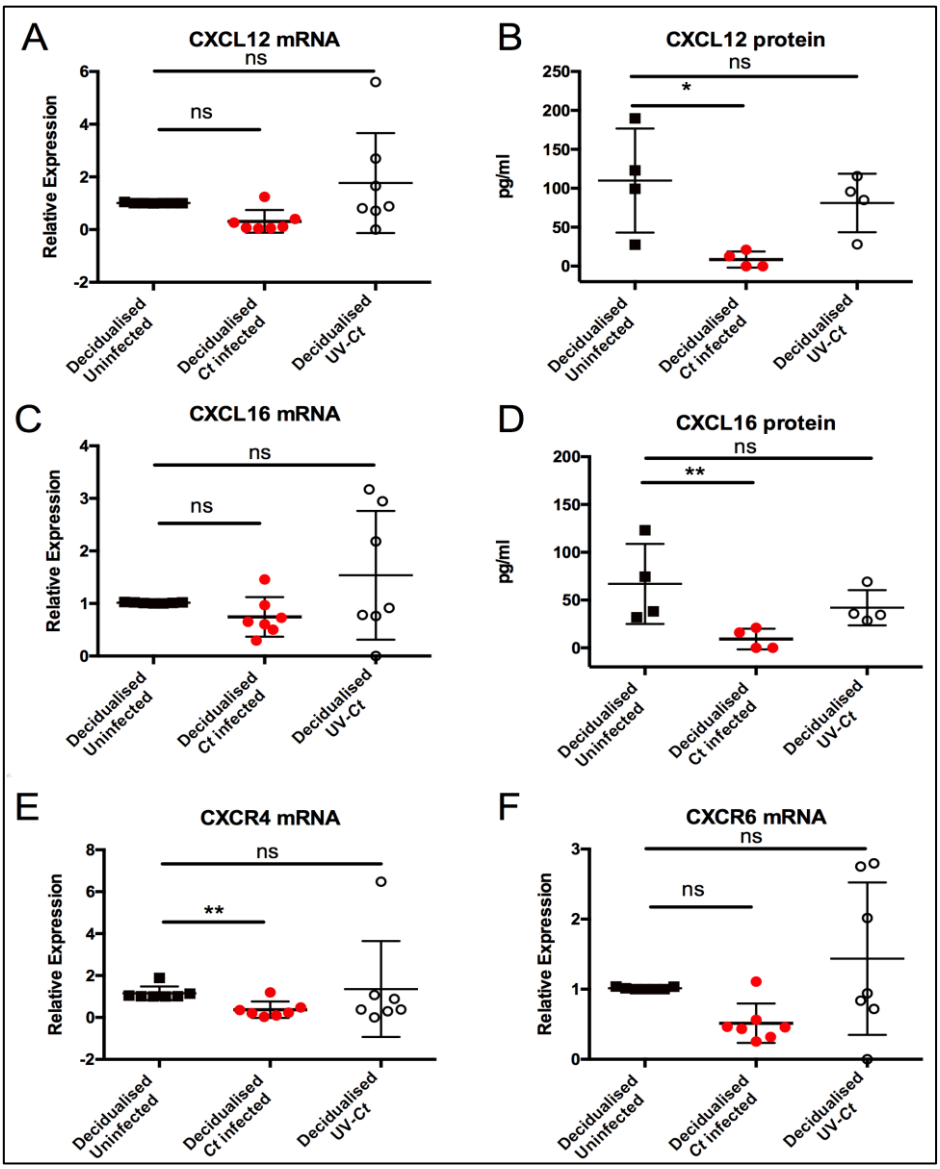

Figure 3.11. *Ct* infection alters innate immune response of decidualised ESC as measured by attenuated mRNA and protein levels of trophoblast invasion-associated chemokines CXCL12 and CXCL16. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-*Ct* treated ESC were used as controls. 48 hours post infection mRNA and supernatants were collected for qPCR and ELISA tests respectively. (A) CXCL12 mRNA was not altered in response to infection (RM one-way ANOVA-Friedman's test, $p>0.05$ n=7). (B) Reduction of secreted CXCL12 in *Ct* infected cell supernatants (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, $p= 0.0267$, n=4). (C) mRNA levels of CXCL16 were not altered (RM one-way ANOVA-Friedman's test, $p>0.05$, n=7). (D) Decreased CXCL16 protein levels were measured in *Ct* infected ESC supernatants (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, $p= 0.0078$, n=4). (E) CXR4 mRNA, receptor of CXCL12, was increased only in infected cells (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, $p=0.0267$, n=7). (F) CXCR6, receptor of CXCL16 was not affected by *Ct* infection in mRNA level CXR4 mRNA, receptor of CXCL12, was increased only in infected cells (RM one-way ANOVA-Friedman's test, $p>0.05$, n=7). Graphs show the mean and standard deviation.

3.3.6. Supernatants from *Ct* infected cells do not affect SW71 trophoblast cell line migration

Since CXCL12 and CXCL16 are known to affect trophoblast migration (as discussed in Chapter 1), I hypothesised that decreased secretion of these two chemokines might affect trophoblast migration thus potentially impairing implantation.

To test this hypothesis, SW71 first trimester trophoblast cells were treated with conditioned media from *Ct* infected ESC with and without recombinant CXCL12 and CXCL16. The optimal concentration of CXCL12 and CXCL16 that can induce SW71 migration was calculated from a scratch assay was completed in triplicate as described in paragraph 3.2.8.

SW71 spheroids were plated in 48-well plates and treated as seen in Table 3.4. There were no other significant differences in migration among all treatment groups (Figure 3.12A). Supernatants from primary decidualised ESC alone was not sufficient to induce migration of SW71 cells compared to media from non decidualised ESC (Figure 3.12B). Similarly, addition of recombinant CXCL12 and CXCL16 did not affect trophoblast migration in cells treated with various conditioned media (Figure 3.12C, Figure 3.12D). However, when both CXCL12 and CXCL16 were both added in trophoblast spheroids treated with *Ct* infected conditioned media, cells expanded further away from the main spheroid body (Figure 3.12E). An example can be seen in Figure 3.12F.

Further validation and troubleshooting of this experiment was not possible due to time restriction.

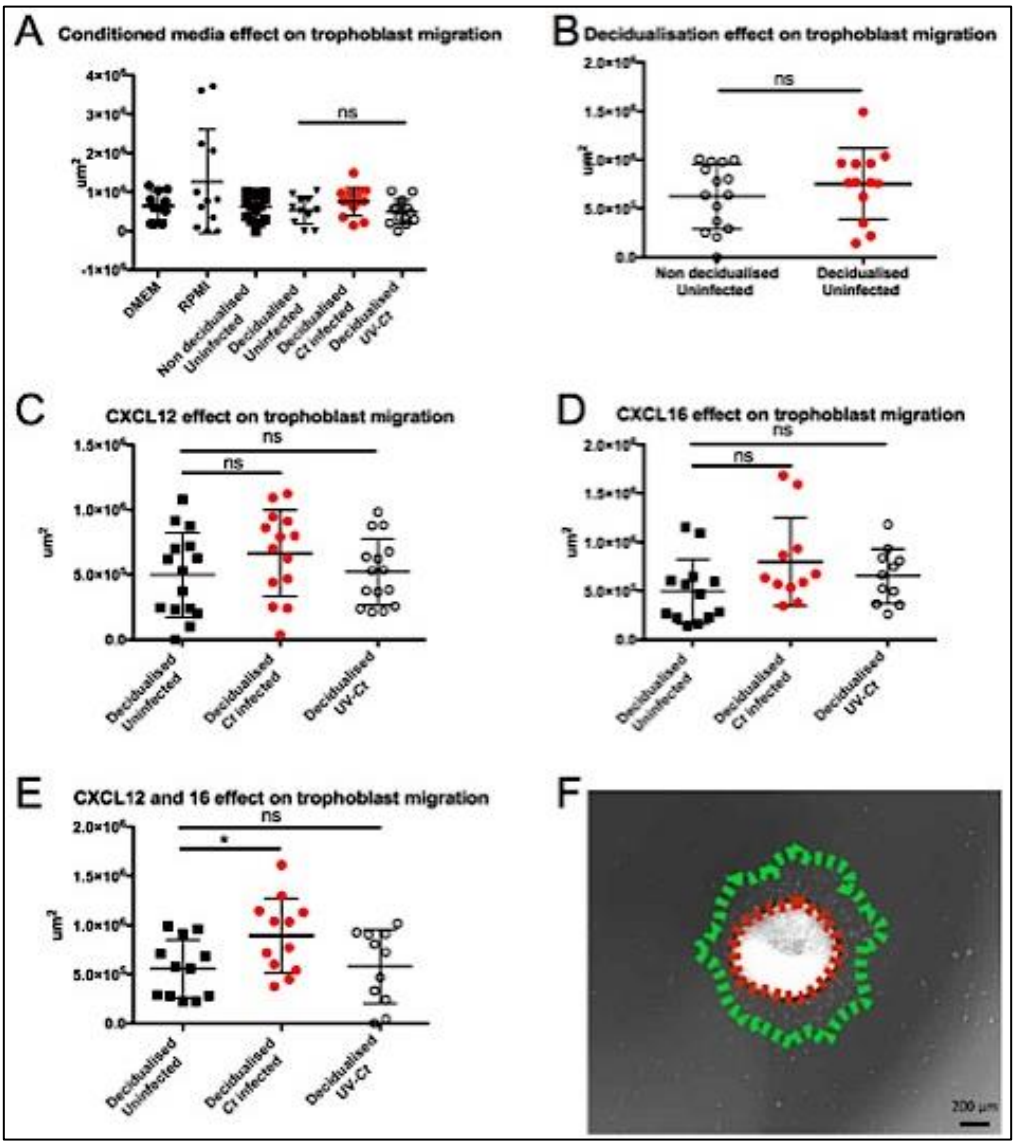

Figure 3.12. Supernatants from *Ct* infected ESC do not affect migration of trophoblast cell line SW71. ESC were infected with *Ct* MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-*Ct* treated ESC were used as controls. Supernatants were collected from ESC 48 hours post infection and added to SW71 spheroids. Recombinant CXCL12 and CXCL16 proteins were added either individually or combined in conjunction with ESC cell supernatants. 30 hours later the area between the main spheroid body (marked in red) and the outer ring of cells (marked in green) was measured to calculate migration. (A) SW71 migration distance was not different among decidualised uninfected and *Ct* infected ESC supernatants (RM one-way ANOVA-Friedman's, $p>0.05$, n=3). (B) Supernatants from decidualised ESC did not induce increased migration (RM one-way ANOVA-Friedman's test, $p>0.05$, n=3). (C) Addition of CXCL12 to SW71 media did not induce migration (RM one-way ANOVA-Friedman's test, $p>0.05$, n=3). (D) Addition of CXCL16 to SW71 media did not induce migration (RM one-way ANOVA-Friedman's test, $p>0.05$, n=3). (E) Trophoblast cells treated with *Ct* infection supernatant supplemented with CXCL12 and CXCL16 migrated more compared to controls (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, $p=0.047$, n=3). (F) Representative image of expansion of trophoblast spheroids in response to *Ct* infected conditioned media combined with CXCL12 and CXCL16, indicated by the green line. Scale bar equals 200µm. Graphs show the mean and standard deviation.

3.4. Discussion

The data presented in this chapter demonstrate that *Ct* can infect ESC and infection could impair decidualisation as it decreases the mRNA levels of decidualisation marker PRL and alters the secretion of chemokines CXCL12 and CXCL16.

It was previously believed that *Ct* can only infect epithelial cells (Buchholz and Stephens, 2006; Rasmussen *et al.*, 1997; Rödel, Grosse, Yu, Wolf, Otto, Liebler-Tenorio, Forsbach-Birk, and Straube, 2011). I demonstrated for the first time that *Ct* can also infect and proliferate in ESC, the functional compartment of the endometrium that is responsible for decidualisation. It is therefore possible that ascending genital *Ct* infection might be a more complicated process than previously thought. *Ct* could breach the epithelial barrier of the endometrium and infect other cell types such as stromal cells, endothelial cells or glandular epithelial cells causing extended inflammation (endometrial structure reviewed in Chapter 1). *Ct* is known to cause endometritis, namely inflammation of the endometrium that is often asymptomatic, in non-pregnant women (Tait *et al.*, 1997). Some studies have suggested an association of chronic endometritis with miscarriage and implantation failure (Cicinelli *et al.*, 2014; McQueen *et al.*, 2014). Data from animal studies indicate that in mice, *C. abortus* induces the murine equivalent of miscarriage likely due to decidual damage (Buendía *et al.*, 1998). The effect of a *Ct* infected endometrial stromal compartment on gestation-related processes, such as decidualisation, might be a possible mechanism of *Ct* infection induced pregnancy complications.

Using a novel in-vitro model of active *Ct* infection on ESC cells, I demonstrated that infection can impact ESC decidualisation by affecting expression of widely used decidualisation marker PRL. PRL is a key molecule of decidualisation, also thought to be involved in epithelial cell differentiation (Haines *et al.*, 2009). Furthermore, PRL has been associated with angiogenesis, trophoblast cell growth and immune regulation during early pregnancy

(reviewed in Jabbour and Critchley, 2001). The PRL receptor (PRLR) has also been shown to increase cell proliferation (Olazabal *et al.*, 2000). Given the crucial role of PRL during decidualisation and early gestation, it is possible that reduced PRL levels due to *Ct* infection might have a detrimental effect on a potential pregnancy. It is known that PRL mRNA and protein was expressed in some patients suffering from recurrent miscarriage and infertility but not in others (Garzia *et al.*, 2004). Reduction of endometrial PRL has been linked recently to recurrent miscarriage (Salker *et al.*, 2010). Finally, in rodent models decidual cell prolactin production has been shown to be critical for successful pregnancy (Bao *et al.*, 2007). Although the exact mechanism remains unknown, reduction of PRL due to *Ct* infection might be a potential mechanism of *Ct* induced miscarriage.

On the contrary, *Ct* infection did not affect the expression of IGFBP1 mRNA or protein. This could be attributed to the fact that infection occurred after ESC were decidualised and IGFBP1 is the first marker to be induced in high levels (Gellersen and Brosens, 2014b). It is therefore possible that at that stage of decidualisation, the impact of *Ct* infection on IGFPB1 was negligible. However, PRL is induced at a later stage and although IGFBP1 and PRL are similarly regulated by various transcriptional factors, there is one study to date that has reported slightly differential regulation (Buzzio *et al.*, 2006). Buzzio *et al.* reported that FOXO1A and FOXO3A transcriptional factor silencing was sufficient to downregulate IGFBP1 but PRL levels were not affected, although overexpression of FOXO1 activated both molecules as expected (Buzzio *et al.*, 2006). However, my data demonstrated that FOXO3A mRNA was not affected by *Ct* infection in decidualised ESC (Figure 3.9C). This mechanism is therefore not likely to be responsible for the different effect of *Ct* infection on IGFPB1 and PRL observed in this model. Another possibility is that the ratio between FOXO1 and FOXO3A can impact upon decidualisation markers expression (Buzzio *et al.*, 2006); examination of FOXO1 mRNA levels in further experiments is required to clarify this possibility. Alternatively, there may be yet more unknown mechanisms involved in induction of

decidualisation marker secretion, mechanisms that result in differential regulation of IGFBP1 and PRL in the presence of *Ct* infection.

Ct infection had no effect on decidualisation and miscarriage-associated gene SGK1 and it's downstream target FOXO3A, indicating that this is not a pathway that is likely to be affected by *Ct* infection of ESC. Furthermore, mRNA levels of proangiogenic factor VEGF and HB-EGF were similarly not altered. HB-EGF has been shown to mediate decidualisation and ESC survival (Chobotova *et al.*, 2005); it therefore appears that *Ct* infection does not affect this decidualisation pathway. On the contrary, EGF mRNA was reduced in infected ESC compared to decidualised controls. A study reported that increasing concentration of EGF resulted in inhibition of cAMP induced in-vitro decidualisation of ESC (Sakamoto *et al.*, 2000). Since EGF mRNA was reduced by *Ct* infection, the impaired decidualisation observed in infected ESC is not likely to be mediated via EGF. Reduction of EGF could be the result of a mechanism attempting to compensate for the reduced PRL mRNA levels, however this requires further investigation.

It would be very interesting to induce decidualisation on ESC infected with a lower MOI, allowing to experiment to be stopped at various time points after the onset of decidualisation, to investigate whether active infection would impair the process by decreasing PRL and/or IGFBP1 secretion. Unfortunately, it was not possible to conduct these experiments due to time restriction, however future studies could further explore the mechanism via which *Ct* infection impacts on decidualisation.

Ct infection of ESC induces a strong innate immune response from the host, indicated by high levels of transcript and secreted CXCL8 in decidualised ESC. Several studies have already shown this in epithelial cells and cell lines such as HeLa cells, identifying NOD1 pattern recognition receptor as responsible for CXCL8 induction (Buchholz and Stephens, 2006, 2007, 2008; Cheng *et al.*, 2008). Importantly, CXCL8 recruits proinflammatory M1 macrophages (Xuan *et al.*, 2015), thus tipping the M1/M2 balance in the fetomaternal interface

from M2 to M1, a fact recently shown to be associated with miscarriage (Brown *et al.*, 2014; Guenther *et al.*, 2012). Innate immune response of maternal decidua to active *Ct* infection during gestation could potentially have serious implications on pregnancy outcome.

Furthermore, data presented in this study demonstrate that *Ct* infection changes the chemokine secretion profile in decidualised ESC. Interestingly, a reduction in the levels of CXCL12 and CXCL16 was observed. The reduction in protein expression in the culture supernatants cannot be attributed to increased receptor-ligand interactions as the receptor for neither chemokine showed increased expression. This observation may have relevance to the role of *Ct* in miscarriage because both CXCL12 and CXCL16 are known to promote trophoblast migration (Huang, Zhu, *et al.*, 2006; Isozaki *et al.*, 2013). Furthermore, reduced CXCL12 levels were shown to be the cause of decreased trophoblast migration as a result of human cytomegalovirus infection (Warner *et al.*, 2012). Importantly, in a pituitary adenoma cell line it was shown that CXCL12/CXCR4 pathway activation resulted in increased PRL secretion, suggesting a potential link between reduced PRL and CXCL12 levels (Barbieri *et al.*, 2007). It is possible that *Ct* infection could subsequently lead to defective trophoblast invasion due to lack of essential attractant signalling molecules secreted from maternal decidua, such as CXCL12 and CXCL16.

To test this hypothesis, a trophoblast invasion assay was conducted using spheroid trophoblast SW71 cells treated with conditioned media from infected ESC and controls. Expansion of trophoblast SW71 cells from the central spheroid body was not hindered by conditioned media from *Ct* infected ESC or UV-*Ct* treated ESC. This could be due to the fact that ESC supernatants were mixed with trophoblast DMEM media and this might have reduced the impact on SW71 spheroids. However, when lack of both CXCL12 and CXL16 was compensated by supplementing recombinant chemokines in *Ct* infection media, trophoblast cells surprisingly migrated over a wider area compared to controls. This observation could be attributed to many factors. The chemokine array conducted in this study indicated highly secreted RANTES/CCL5 (regulated

on activation, normal T-cell expressed and secreted) and MCP1/ CCL2 (monocyte chemotactic protein 1), chemokines that are also known to induce trophoblast migration (as seen in Table 3.5). A study showed that trophoblast cells migrated into media containing CXCL8, RANTES and MCP1 (James, Cartwright, *et al.*, 2012). CXCL8 alone has been reported to induce trophoblast migration (Jovanović *et al.*, 2010). It is thus possible that high levels of CXCL8, RANTES and MCP1 found in *Ct* infection conditioned media, when combined with additional CXCL12 and CXCL16 result in increased trophoblast migration. The complicated interactions between various cytokines and their pleiotropic action pose a particular challenge when trying to investigate the effect of infection on trophoblast migration. An interesting future experiment would be investigating the effect of a co-culture of *Ct* infected decidualised ESC and SW71 cells. If *Ct* infection hindered SW71 cell migration, the experiment could be repeated using primary first trimester trophoblast cells and analysis could result in identification of more factors involved in this process.

Developing a novel in-vitro model of infection of decidualised ESC proved particularly challenging due to the difficulties of ESC cell culture. Each experiment lasted approximately two months as ESC are growing at a slow rate in cell culture and are quite sensitive. Several repetitions were required to establish the model, ensuring both successful decidualisation via secretion of decidualisation markers and successful infection with the presence of inclusions and CXCL8 secretion. A limitation of the present study was the quantification of *Ct* inclusions was conducted using observation of inclusion staining by Giemsa. Due to significant difficulties of immunohistochemical staining of *Ct* inclusions, the fixed cells could be fluorescently stained for *Ct* and results visualised to facilitate counting. For a more user-independent quantification, the automated snapshot function of an appropriate imaging program could be utilised to confirm unbiased selection of areas. The experiment could be further supported by a second individual independently counting infected cells, which unfortunately was not possible at this time.

In summary, my data suggest that *Ct* infection leads to defective endometrial stromal cell decidualisation and results in an altered immune response that could impact on trophoblast migration and immune cell recruitment. Future work to clarify the role of *Ct* infection on trophoblast invasion via CXCL12 and CXCL16 dysregulation and immune cell recruitment in the endometrium due to altered chemokine profile could further our understanding of the mechanism of *Ct*-induced miscarriage.

4. Chapter 4: The effect of *Ct* infection in a murine model of pregnancy

4.1. Introduction and aims

Studies indicate that during gestation presence of acute infection leads to premature labour (de la Maza *et al.*, 1994; Pal *et al.*, 1999). In a study by Pal *et al.* (Pal *et al.*, 1999), *Ct* mouse pneumonitis (MoPn) biovar was used to infect BALB/c mice intravaginally at concentrations ranging between 10^1 and 10^7 IFU during gestation in a model of acute infection. It was shown that high concentrations of MoPn EBs (10^6 or 10^7) led to premature pup delivery (Pal *et al.*, 1999). Mice infected with lower concentrations mostly delivered at term and no differences were observed in pup weight, however weight measurements were not taken in prematurely delivered pups as they were cannibalized by their mothers upon delivery (Pal *et al.*, 1999). This study had good methodology, thus the data presented appears to be reliable. Using the same MoPn biovar in an earlier study (de la Maza *et al.*, 1994), it was shown that 6 weeks post intravaginal inoculation with $3x10^7$ bacteria, C3H/HeN, C57BL/6N and BALB/cAnN mice were less fertile compared to controls as indicated by a reduction in the number of pregnant mice and total pup number per mouse (de la Maza *et al.*, 1994). However, the methodology of this study is not described in detail and no statistical power calculation is given (de la Maza *et al.*, 1994). Another study (Pal *et al.*, 2001) demonstrated that susceptibility of BALB/c, C3H, and C57BL/6 mice ranging from 5 to 14 weeks of age to intravaginal infection with various concentrations (10^4 - 10^7) of *Ct* pneumonitis serovar was shown to be age-dependant. Younger mice had higher number of IFU recovered per mouse, vaginal shedding lasted longer and following mating higher rates of infertility and a decrease in the number of embryos were observed in the infected young mice (Pal *et al.*, 2001). The methodology of this study is however not described in detail.

Additionally, vaginal infection with *Ct* serovars prior to gestation in mice can result in infertility (Igietseme *et al.*, 2013). C57Bl/6 mice that were infected

twice with 10^5 IFU of *Ct* serovar L2 were infertile, an effect reversed by local caspase activity inhibition in a more clearly designed study by Igietseme *et al* (Igietseme *et al.*, 2013).

On the contrary, in a different study (Tuffrey *et al.*, 1988) when TO mice were infected with $1x10^7$ IFU of *Ct* serovar E during gestation, the number of fetuses was not altered and the bacteria failed to cross the foetal membranes. Placental colonisation was detected especially when the infection was intraperitoneal versus intravaginal or intravenous infection, however this outcome was not observed if the mice were infected prior to day 5 of gestation (Tuffrey *et al.*, 1988). This study is an old study and further details on the mouse strain used could not be obtained, a serious limitation of this study. Furthermore, it seems low numbers of mice were used as well as both intraperitoneal and intravaginal inoculation of *Ct*, however the experiments are not clearly described and that needs to be taken into consideration when interpreting the data.

Despite their limitations, the majority of the above studies indicate that vaginal infection with *Ct* serovars prior to gestation in mice can result in infertility and during gestation presence of acute infection leads to premature labour. However, no study to date has examined the effect the effect of *Ct* serovar E past infection on mouse implantation, pregnancy and resorption, and embryonic growth. In addition, the mechanism(s) by which *Ct* could result in these adverse pregnancy outcomes remains unknown.

The hypothesis of this chapter was that past *Ct* infection with serovar E in C3H mice can affect pregnancy outcomes.

To address this hypothesis I aimed:

1. To investigate the effect of past *Ct* serovar E infection in C3H mice on pregnancy outcomes including implantation rates, pregnancy resorption numbers, and embryonic growth
2. To investigate the effect of past *Ct* serovar E infection in C3H mice on genes of importance to mouse gestation and embryonic growth

4.2. Materials and methods

All procedures were undertaken according to UK Home Office regulations after ethical review, under project license 60/3544 held by Professor Andrew Horne.

4.2.1. Murine pregnancy *Ct* infection model

C3H mice were selected as several studies have indicated they are susceptible to infection with *Ct* (Carmichael *et al.*, 2013; Darville *et al.*, 1997; de la Maza *et al.*, 1994; Peterson *et al.*, 1999). Assuming the null hypothesis that prior *Ct* infection has no effect on pregnancy rate, a 95% infection rate with *Ct* and a viable pregnancy rate of 95%, this study required 80 female (40 infected and 40 sham infected) and therefore 12 male mice to achieve significant results. Due to time restrictions, from the first set of experiments (termination of pregnancy at e12 of gestation) 60 out of 80 mice in total were used in the experiment. Only preliminary experiments were conducted for the second set (termination of pregnancy at e14) due to time restrictions and 10 infected and 10 sham infected animals were sacrificed.

Mice were kept in a 12-hour light/dark cycle with humidity maintained at 55% and temperature between 20°C and 25°C. Food and water were available as required. Mice from C3H strain at seven weeks of age were used for all experiments and mice were housed in individually ventilated cage (IVC) conditions at a maximum of five mice per box. All experiments were performed in a Class II hood according to health and safety regulations of the BRR animal facility.

4.2.2. Preparation of *Ct* stock for intravaginal inoculation

Ct stock at a titre of $3x10^6$ /ml was used and each mouse was inoculated vaginally with 10^5 EBs. Prior to inoculation, the bacterial stock was spun at 5,000g for 5 minutes and subsequently re-suspended in Succinic acid, sodium phosphate monobasic monohydrate and glycine buffer (SPG) buffer at pH7 (for protocol see Table 4.1) at a final volume of 30 µl per mouse. Control C3H mice

were inoculated vaginally with 30 µl of SPG buffer that did not contain any *Ct* (sham infection). Prior to vaginal inoculation, the mice were anaesthetised using 4% isofluorane for 30 seconds.

Table 4.1. SPG buffer recipe.

Component	Concentration	Company and Cat. No
Sucrose	250 mM	Sigma, Cat. No S0389
Sodium Phosphate	10 mM	Sigma, Cat. No 342483
L-glutamate	5 mM	Sigma, Cat. No G1251
Sterile water	volume as required	Baxter, Cat. No BHL2F7113CS

4.2.3. Mouse model protocol

The following protocol was used:

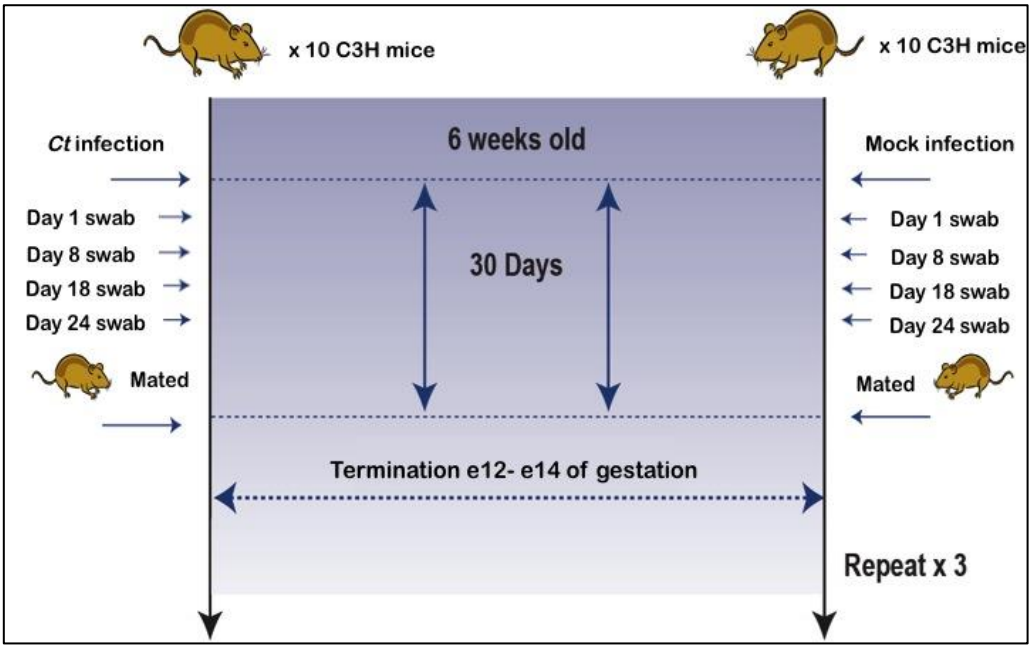

Figure 4.1. In-vivo murine model of past *Ct* infection.
Day 0: Control mice received intravaginal injection of 30µl SPG buffer. Case mice received 30µl of SPG buffer containing 10^5 *Ct* each.
Day 1: All mice were vaginally swabbed to test for *Ct* presence.
Day 8: All mice were vaginally swabbed to test for *Ct* presence.
Day 18: All mice were vaginally swabbed to test for *Ct* presence.
Day 24: All mice were vaginally swabbed to test for *Ct* presence.
Day 30: All mice were mated and checked for plugs daily until pregnancy was established. Mice that remained unplugged over three consecutive cycles (3x4-day-cycles) were removed from the male mice and culled when each experiment was finished.
e12-e14 of gestation: pregnant mice were culled at appropriate time points as required for each experiment.

4.2.4. Timed mouse mating

C3H mice were mated 30 days post infection. For timed mating, each male was housed in an individual cage and was allocated a maximum of two females. Vaginal plug checks of breeding females were undertaken each morning before 10 am to allow accurate calculation of their gestation stage. The day of plug discovery was designated e1 of gestation.

4.2.5. Testing for presence of *Ct* infection

To ensure that the mice were infected, vaginal FLOQswabs (COPAN floc technologies, Cat. No 518CS01) were collected and subsequently tested for evidence of *Ct* infection at the Microbiology lab of the Royal Infirmary of Edinburgh by Mike Shepherd using Abbott RealTime *Ct*/NG Amplification Reagent Kit (Abbot, Cat. No 08L07-091). Mice were immobilized by hand and swabbed vaginally without anaesthesia.

4.2.6. Tissue collection and processing

Mice were killed by a schedule 1 method.

The uterus was removed from each pregnant mouse post-mortem. The number of live pups and non viable pups (resorption sites) was recorded. Each embryo and placenta was morphologically observed to identify potential malformations. Embryonic and placental weights were recorded immediately after dissection using a scale by one observer. All measurements were recorded on paper and subsequently imported in Excel.

Embryonic and placental weight data were available for 12/19 non infected mice, 15/16 *Ct* exposed mice and 7/9 *Ct* infected mice at e12 as they were not initially included in the measured outcomes of this study and therefore not recorded.

Tissue samples from each uterus, placenta and embryo were collected in RNAlater (ThermoFisher Scientific, Cat.No AM7021) to study mRNA levels of genes of interest. RNA samples were stored at -80^{o}C following a 24-hour incubation period in RNAlater.

4.2.7. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6. Normality was assessed using Kolmogorov Smirnoff tests. Where data was not normally distributed, the Kruskal-Wallis non-parametric test was used to analyse the data. This was followed by Dunn's multiple comparison's test as it allows for comparison of two particular groups by determining each rank from the entire data set (all groups), not just the two groups being compared. If the data was normally distributed, a parametric ordinary one-way ANOVA (Brown-Forsythe test) was used to determine if there were any significant differences. This was followed by the Bonferroni's post-hoc test for comparison among groups if any significant differences were indicated by the ANOVA test. All graphs show mean and standard deviation of the data.

Dr Ioannis Papastathopoulos, Chancellor's Fellow at the University of Edinburgh's school of mathematics, reviewed the statistical analysis and approved it for analysis of the data presented in this chapter.

4.3. Results

4.3.1. Natural history of *Ct* infection in the exposed cohort of mice

As seen in Table 4.2, none of the control mice tested positive for *Ct* one day post infection but all mice inoculated with *Ct* tested positive. Seven days later, 20 of 40 case mice remained positive for *Ct* and these mice were considered '*Ct* infected'. The remaining case mice were considered '*Ct*-exposed' (n=20). All mice tested negative for *Ct* 18 and 24 days post-infection, indicating that infected mice cleared the infection in maximum three weeks time.

Table 4.2. Vaginal swab test for *Ct* infection results from C3H mouse experiments.

	Number of mice			
	Sham infected		***Ct* infected**	
	Negative swab test	**Positive swab test**	**Negative swab test**	**Positive swab test**
Swab day 1	40	0	0	40
Swab day 8	40	0	20	20
Swab day 18	40	0	40	0
Swab day 24	40	0	40	0

4.3.2. Pregnancy outcomes of *Ct* infection in the exposed cohort of mice

Mice were killed at e12 and e14 of gestation as calculated based on vaginal plug detection. An example of a pregnant mouse uterus at e12 can be seen in Figure 4.2A. Resorptions, namely pups that are no longer viable and are subsequently absorbed by the mouse uterus (Passey *et al.*, 1999), were classified as "early" when at the time of dissection it was not possible to distinguish the embryonic tissue from the placenta (Figure 4.2B) and as "late" if the distinction was clear (Figure 4.2C). From here onwards, with the term "resorption" both early and late resorptions are included and normally developed pups are named "NDev". The experiment was conducted on 60 out of 80 mice for e12 and 20 out of 80 mice for e14 due to time restrictions.

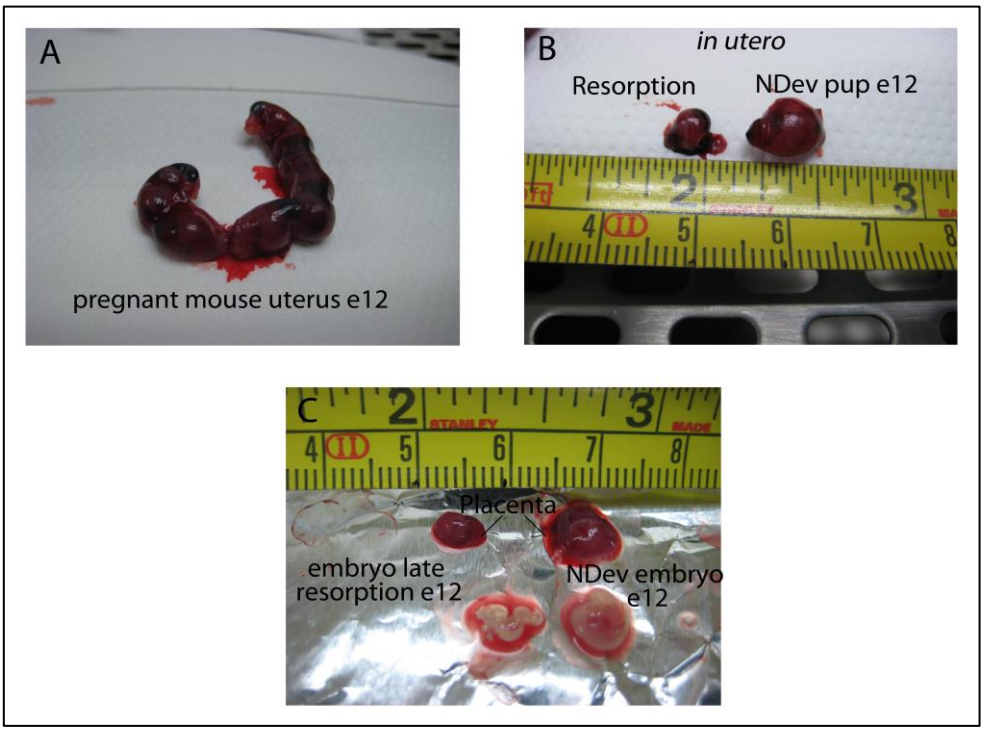

Figure 4.2. C3H mouse uterus, resorption and embryos at e12 of gestation. (A) Uterus with normally developed (NDev) pups and pups that were no longer viable, namely resorptions. (B) *In utero* resorption and live pup. (C) Dissected resorption placenta and embryo (left) and live embryonic and placental tissue (right). Ruler in cm.

As some mice were not successfully mated, in a total of five experiments, the outcomes were 23 out of 40 control "non infected" mice. 18 negative cases or "*Ct*-exposed" and 12 out of a total of 40 positive cases termed "*Ct* infected" mice (Table 4.3). Embryonic and placental weight data were available for 12/19 non infected mice, 15/15 *Ct* exposed mice and 7/9 *Ct* infected mice at e12 as they were not initially included in the outcomes of this study and therefore not recorded.

Table 4.3. Number of C3H mice per non infected, *Ct* exposed and *Ct* infected group, classified by gestation age at day of termination.

number of mice / day of gestation	Non-pregnant mice	Mice with missed plug	Non infected mice	*Ct* exposed mice	*Ct* infected mice
e12	9	8	19	15	9
e14	4	6	4	3	3

4.3.3. Past *Ct* infection does not affect implantation rate, or resorption or pup numbers

The total number of implantation sites was calculated per mouse (live pups plus resorption sites), similar levels of total implantation sites per mouse were demonstrated (Figure 4.3A).

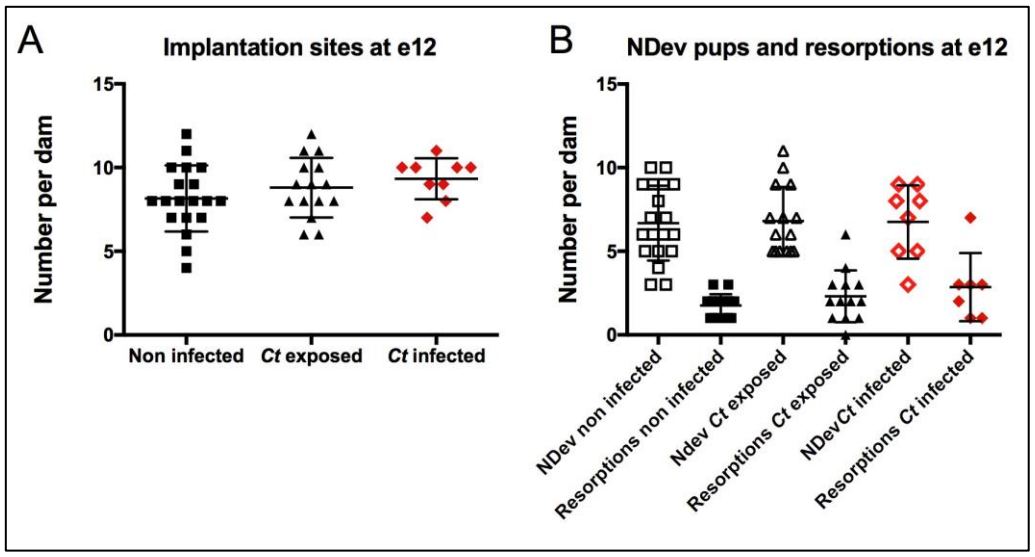

Figure 4.3. Past *Ct* infection of C3H mice does not impact on implantation and pregnancy outcome at e12 of gestation. C3H mice were intravaginally inoculated with *Ct* or sham infected and mated 30 days post infection. They were classified as *Ct* infected if they presented with infection 8 days post inoculation, *Ct* exposed if they cleared the infection by day 8 and controls (non infected mice). (A) *Ct* infection did not alter total embryo implantation site numbers per mouse, therefore not affecting the implantation rate of the mice (ordinary one-way ANOVA, $p>0.05$, n=9-19 mice per group). (B) Past *Ct* infection and exposure to *Ct* did not affect pup or resorption numbers (ordinary one-way ANOVA, $p>0.05$, n=9-19 mice per group). Graphs show mean and standard deviation.

Furthermore, the number of normally developed pups and resorptions did not differ among non infected, *Ct* exposed and *Ct* infected mice (Figure 4.3B).

4.3.4. Past *Ct* infection does not affect placental or embryo weight at e12

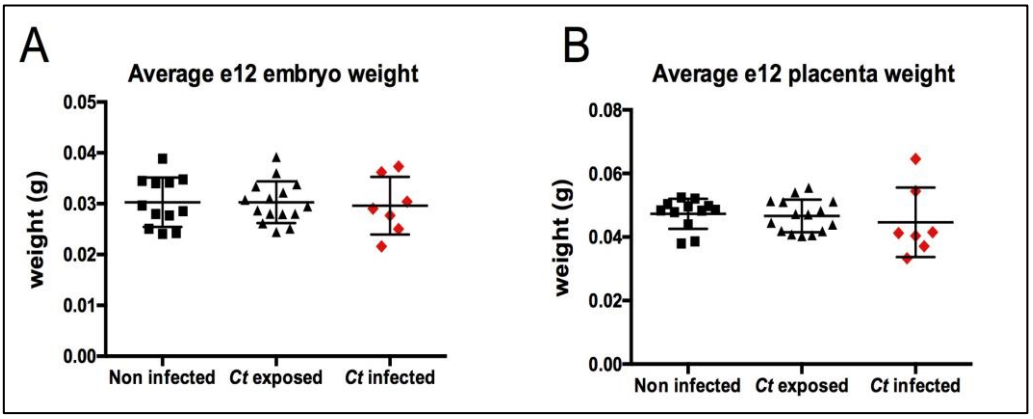

Figure 4.4. Past *Ct* infection does not affect the average embryonic and placental weight of e12 pups. C3H mice were intravaginally inoculated with *Ct* or sham infected and mated 30 days post infection. They were classified as *Ct* infected if they presented with infection 8 days post inoculation, *Ct* exposed if they cleared the infection by day 8 and controls (non infected mice). (A) No difference was observed in the average e12 embryo weight among all groups (ordinary one-way ANOVA, $p>0.05$, n=7-15 mice per group). (B) The average e12 placental weight was not affected by *Ct* infection or exposure to *Ct* (ordinary one-way ANOVA, $p>0.05$, n=7-15 mice per group). Graphs show mean and standard deviation.

The average weight of embryos and placenta per mouse did not differ among the three groups (Figure 4.4). When examining each pup as an individual independent unit, data shows the weight of each pup and placenta was not altered due to exposure to *Ct* or presence of *Ct* infection on e12 (Figure 4.5A, Figure 4.5B). The embryo to placenta ratio per mouse was also not different among the three groups (Figure 4.5C).

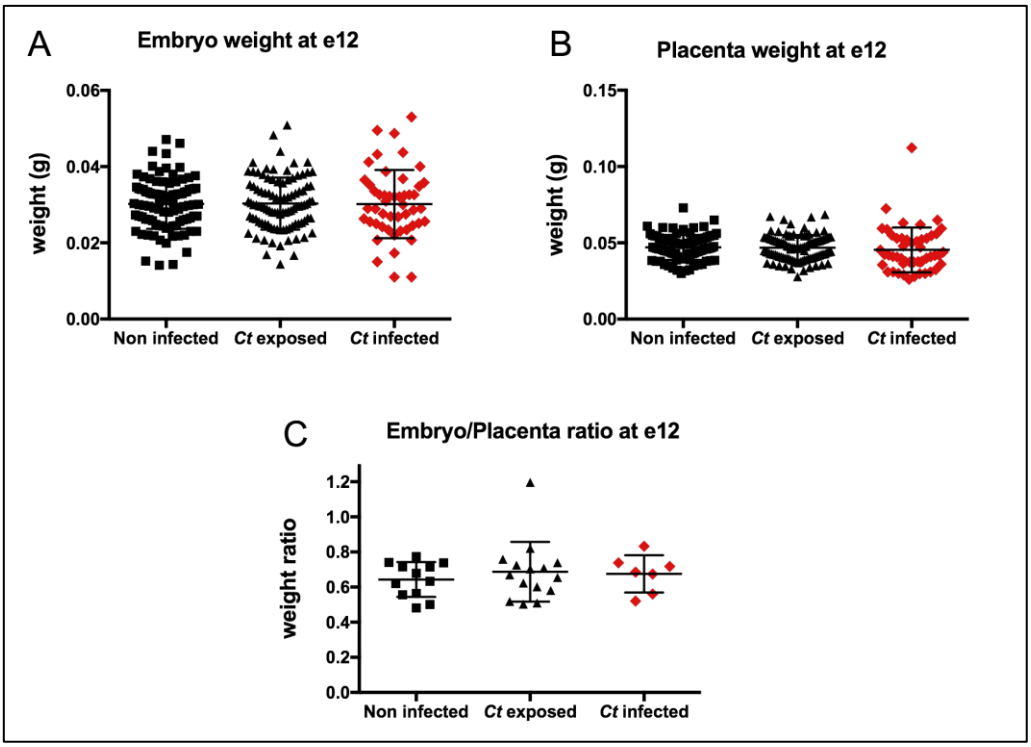

Figure 4.5. Past *Ct* infection of C3H mice does not affect embryonic and placental weight at e12 of gestation. C3H mice were intravaginally inoculated with *Ct* or sham infected and mated 30 days post infection. They were classified as *Ct* infected if they presented with infection 8 days post inoculation, *Ct* exposed if they cleared the infection by day 8 and controls (non infected mice). (A) No change in embryonic weight was observed (ordinary one-way ANOVA, $p>0.05$, n=49-98 pups**)**. (B) Placental weight did not differ among all groups (Kruskal-Wallis test, $p>0.05$, n=49-98 pups**)**. (C) Embryo to placenta ratio was not altered due to exposure to *Ct* or *Ct* infection (ordinary one-way ANOVA, $p>0.05$, n=7-15 mice**).** Graphs show mean and standard deviation.

4.3.5. Past *Ct* infection does not affect implantation rate, pup or resorption numbers at e14

At e14 no differences were observed on the number of normally developed pups and resorptions or total implantation sites among non infected, *Ct* exposed and *Ct* infected mice (Figure 4.6A, Figure 4.6B).

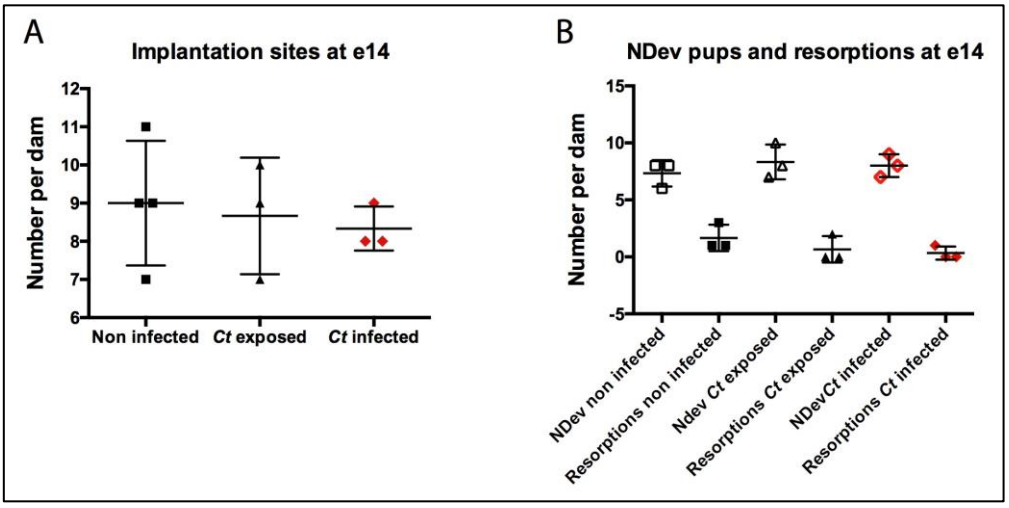

Figure 4.6. Past *Ct* infection does not impact on C3H mice implantation and pregnancy outcome at e14 of gestation. C3H mice were intravaginally inoculated with *Ct* or sham infected and mated 30 days post infection. They were classified as *Ct* infected if they presented with infection 8 days post inoculation, *Ct* exposed if they cleared the infection by day 8 and controls (non infected mice). (A) The number of implantation sites did not differ among all groups (Kruskal-Wallis test, $p>0.05$, n=3-4 mice). (B) Similar numbers of NDev pups and resorption sites per mouse were observed at e14 (Kruskal-Wallis test, $p>0.05$, n=3-4 mice). Graphs show mean and standard deviation.

4.3.6. Past *Ct* infection reduces embryo and placenta weight at e14

When examining the average embryo and placenta weight per dam, no differences were observed among *Ct* infected, *Ct* exposed and non infected dams at e14 as seen in Figure 3.7.

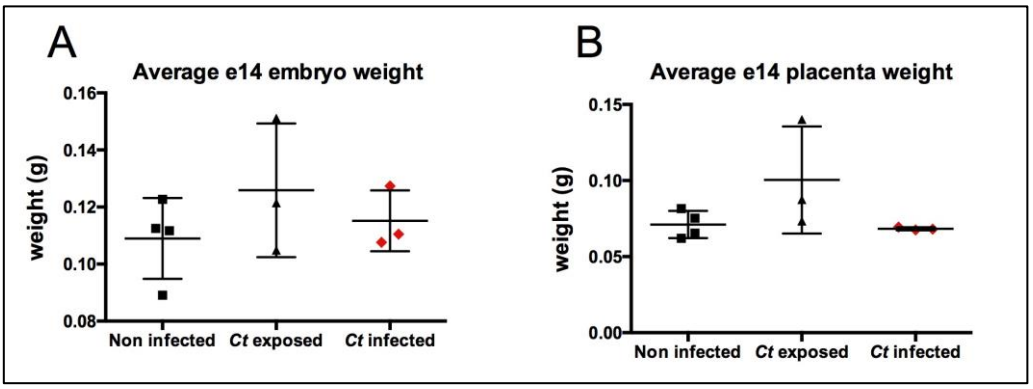

Figure 4.7. Past *Ct* infection does not affect neither the average embryonic nor the average placental weight of e14 C3H mice pups. C3H mice were intravaginally inoculated with *Ct* or sham infected and mated 30 days post infection. They were classified as *Ct* infected if they presented with infection 8 days post inoculation, *Ct* exposed if they cleared the infection by day 8 and controls (non infected mice). (A) No change in average embryonic weight was observed among all groups (Kruskal-Wallis test, $p>0.05$, n=3-4 mice). (B) The average placental weight did not differ among all groups. (Kruskal-Wallis test, $p>0.05$, n=3-4 mice). Graphs show mean and standard deviation.

The weight of e14 embryos from *Ct* infected mothers was reduced compared to non infected pups (mean of 0.1138g compared to a mean of 0.1219g, Figure 4.8A). Similarly, placentae from *Ct* infected mice weighed less than those from non infected controls (a mean of 0.06828g compared to a mean of 0.07598g, Figure 4.8B). On the contrary, the embryo/placenta ratio of individual mice did not differ among groups (Figure 4.8C).

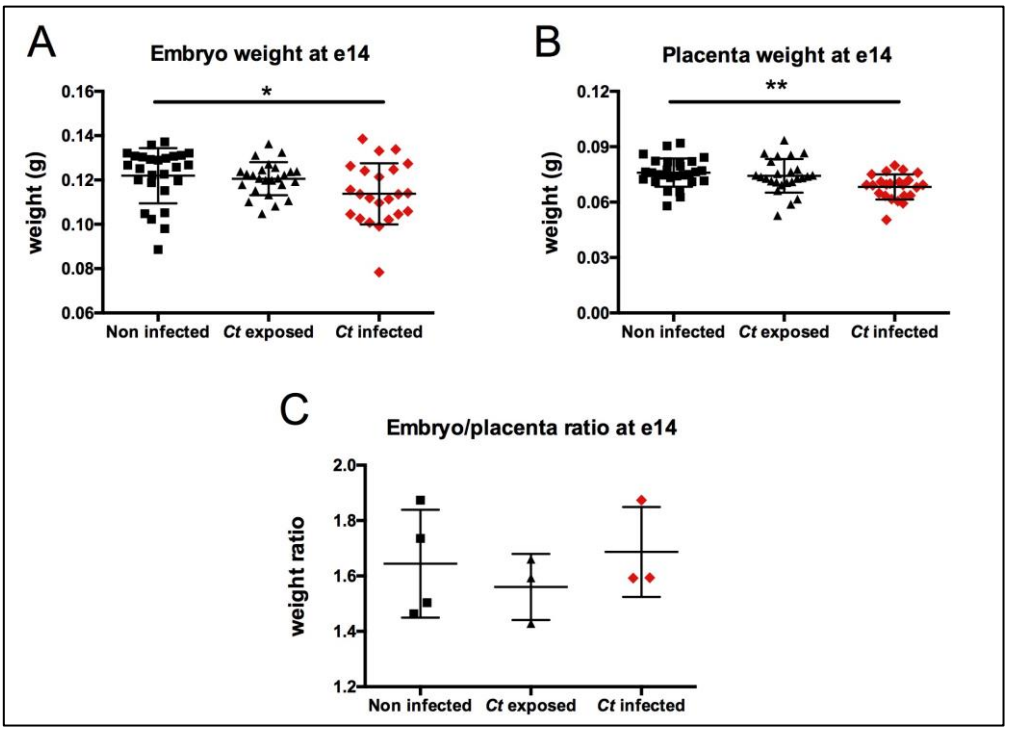

Figure 4.8. *Ct* past infection of C3H mice results in reduced embryonic and placental weight of e14 C3H pups. C3H mice were intravaginally inoculated with *Ct* or sham infected and mated 30 days post infection. They were classified as *Ct* infected if they presented with infection 8 days post inoculation, *Ct* exposed if they cleared the infection by day 8 and controls (non infected mice). (A) Reduction in embryonic weight of e14 pups was measured in *Ct* infected embryos compared to non infected control mice (Kruskal-Wallis test with Dunn's multiple comparison's test, p=0.034, n=23-26 pups). (B) Similarly, placentae of pups from *Ct* infected mice weighed less than non infected controls (ordinary one-way ANOVA with Bonferroni's multiple comparison's test, p=0.0019, n=23-26 pups). (C) No difference was observed in the embryo/placenta ratio per mouse among all groups (Kruskal-Wallis test, p>0.05, n=3-4 mice). Graphs show mean and standard deviation.

4.3.7. *Ct* infection induces *Prlr*, *Cxcl16* and *Egf* expression in e12 mouse uterus surrounding resorption sites but does not alter *Cxcl12* or their respective receptor expression levels

To investigate potential changes in my genes of interest for this study, the levels of on *Prl*, *Cxcl12*, *Cxcl16*, *Egf* and their receptors were examined (Figure 4.9). The mRNA levels of *Prlr*, *Cxcl16* and *Egf* were elevated in uterine tissue in proximity to mouse resorption implantation sites in *Ct* infected mice. In contrast, *Prl*, *Cxcl12*, *Cxcr4* and *Cxcr6* were not affected by past *Ct* infection.

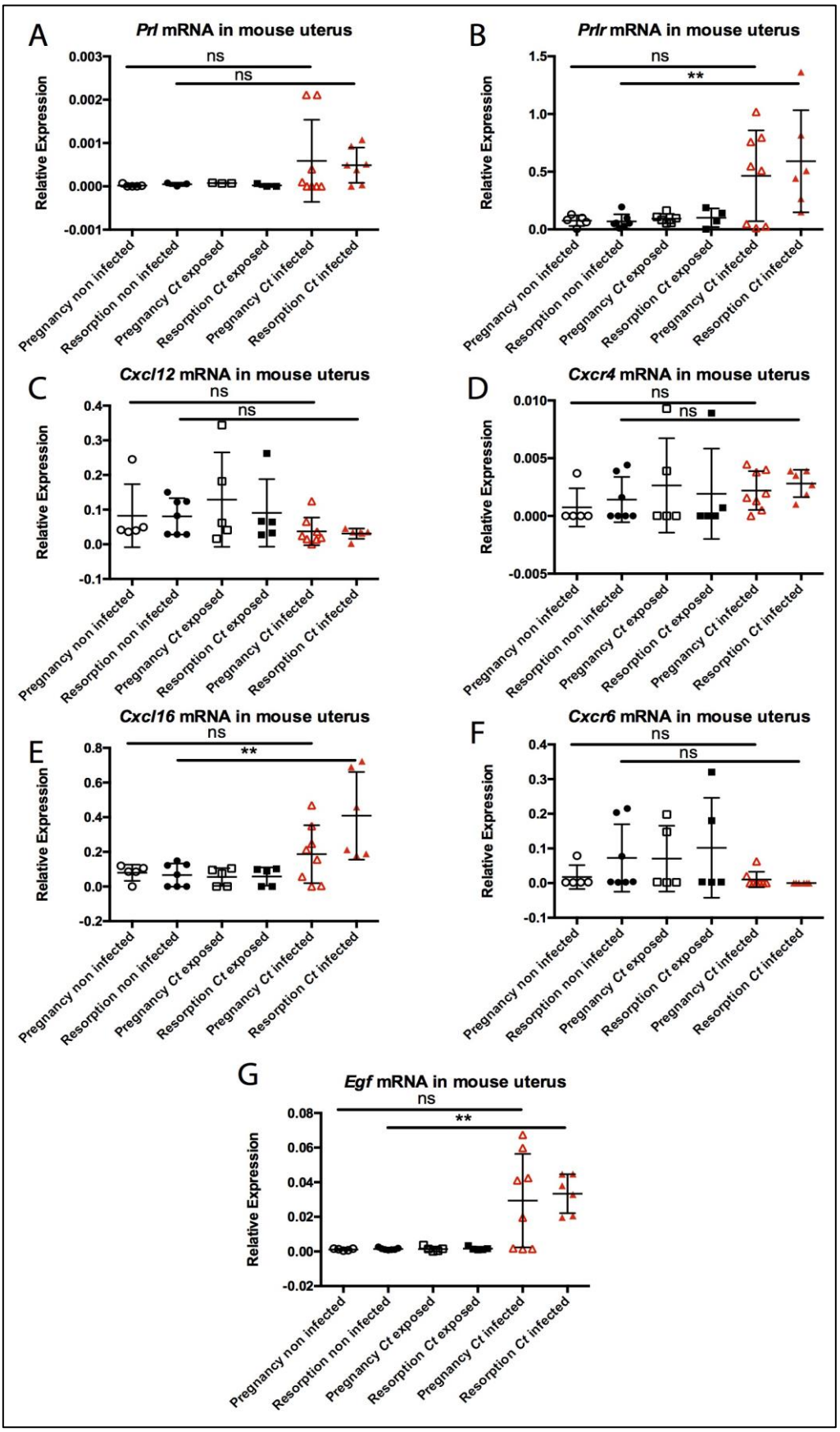
A
Prl mRNA in mouse uterus
Relative Expression
0.003
0.002
0.001
0.000
-0.001
ns
ns
Pregnancy non infected
Resorption non infected
Pregnancy Ct exposed
Resorption Ct exposed
Pregnancy Ct infected
Resorption Ct infected
B
Prlr mRNA in mouse uterus
Relative Expression
1.5
1.0
0.5
0.0
ns
**
Pregnancy non infected
Resorption non infected
Pregnancy Ct exposed
Resorption Ct exposed
Pregnancy Ct infected
Resorption Ct infected
C
Cxcl12 mRNA in mouse uterus
Relative Expression
0.4
0.3
0.2
0.1
0.0
-0.1
ns
ns
Pregnancy non infected
Resorption non infected
Pregnancy Ct exposed
Resorption Ct exposed
Pregnancy Ct infected
Resorption Ct infected
D
Cxcr4 mRNA in mouse uterus
Relative Expression
0.010
0.005
0.000
-0.005
ns
ns
Pregnancy non infected
Resorption non infected
Pregnancy Ct exposed
Resorption Ct exposed
Pregnancy Ct infected
Resorption Ct infected
E
Cxcl16 mRNA in mouse uterus
Relative Expression
0.8
0.6
0.4
0.2
0.0
-0.2
ns
**
Pregnancy non infected
Resorption non infected
Pregnancy Ct exposed
Resorption Ct exposed
Pregnancy Ct infected
Resorption Ct infected
F
Cxcr6 mRNA in mouse uterus
Relative Expression
0.4
0.3
0.2
0.1
0.0
-0.1
ns
ns
Pregnancy non infected
Resorption non infected
Pregnancy Ct exposed
Resorption Ct exposed
Pregnancy Ct infected
Resorption Ct infected
G
Egf mRNA in mouse uterus
Relative Expression
0.08
0.06
0.04
0.02
0.00
-0.02
ns
**
Pregnancy non infected
Resorption non infected
Pregnancy Ct exposed
Resorption Ct exposed
Pregnancy Ct infected
Resorption Ct infected

Figure 4.9. Past *Ct* infection on does not affect the mRNA levels of *Prl* and *Cxcl16* but alters the expression levels of *Prlr, Cxcl12, Cxcr4, Cxcr6* and *Egf* in the uterus at e12 of C3H mouse gestation. C3H mice were intravaginally inoculated with *Ct* or sham infected and mated 30 days post infection. They were classified as *Ct* infected if they presented with infection 8 days post inoculation, *Ct* exposed if they cleared the infection by day 8 and controls (non infected mice). (A) The mRNA of *Prl* was not altered among all groups (Kruskal-Wallis test, $p>0.05$, n=5-7 mice). (B) *Prlr* mRNA was increased in resorption sites of *Ct* infected C3H mice compared to resorptions of non infected mice (Kruskal-Wallis test with Dunn's multiple comparisons test, $p=0.0052$, n=5-7 mice). (C) *Cxcl12* expression levels were not altered among all groups (Kruskal-Wallis test, $p>0.05$, n=5-7 mice). (D) mRNA levels of *Cxcr4*, receptor of *Cxcl12* chemokine, remained unchanged among all groups (Kruskal-Wallis test, $p>0.05$, n=5-7 mice). (E) *Cxcl16* mRNA was increased in resorption sites of *Ct* infected mice (Kruskal-Wallis test with Dunn's multiple comparisons test, $p=0.0061$, n=5-7 mice). (F) In contrast, mRNA for *Cxcl16* receptor *Cxcr6* remained unchanged (Kruskal-Wallis test, $p>0.05$, n=5-7 mice). (G) *Egf* messenger RNA was also upregulated in *Ct* infected mice resorptions (Kruskal-Wallis test with Dunn's multiple comparisons test, $p=0.0049$, n=5-7 mice). Graphs show mean and standard deviation.

To summarise, pregnancy outcomes including implantation rate and pregnancy or resorption numbers remained unchanged in *Ct* exposed and *Ct* infected mice compared to non infected controls at e12 and e14 of gestation. Embryonic and placental weight of *Ct* infected mice was similar to controls on e12 however a reduction was observed on e14 of gestation. *Prl, Cxcl12, Cxcr4* and *Cxcr6* mRNA levels were not affected by past *Ct* infection, whereas *Prlr*, *Cxcl16* and *Egf* where elevated in uterine tissue in proximity to mouse resorption implantation sites in *Ct* infected mice compared to tissue near normally developed pups from non infected mice.

4.4. Discussion

This study demonstrates that *Ct* infection, cleared prior to pregnancy, reduces embryonic and placental weight at e14 but has no effect on implantation or pup resorption rates in a murine model of pregnancy. Furthermore, changes in gestation related genes *Prlr, Cxcl16* and *Egf* indicate possible mechanisms that might be responsible for *Ct* induced adverse pregnancy outcomes in mice.

There are few models of ascending *Ct* infection in the mouse female reproductive tract (Gondek *et al.*, 2012; Pal *et al.*, 1994; Swenson *et al.*, 1983) and even fewer models of ascending infection leading to adverse pregnancy outcomes (Igietseme *et al.*, 2013; de la Maza *et al.*, 1994; Pal *et al.*, 1999, 2001; Tuffrey *et al.*, 1988). In my model using *Ct* serovar E, vaginal inoculation with 10^5 IFU of *Ct* was sufficient to induce ascending and subsequent uterine infection in C3H mice that was cleared 18 days post infection in all mice. This could be a useful model to study processes and pathways related to early pregnancy that are affected by serovar E, the human sexually transmitted *Ct* serovar.

The number of total implantation sites per mouse was not altered following exposure to *Ct* indicating no effect of past *Ct* infection on implantation. Similarly, no differences were observed in the number of live embryos or resorptions at both e12 and e14 gestational days. Although the experiments were not completed due to time restrictions, it is likely that past *Ct* infection does not alter implantation rates or fertility in C3H mice. However, infertility has been induced by the MoPn serovar (in high IFU doses, such as 10^6 and 10^7, Carmichael *et al.*, 2013). Serovar E has been shown to be less potent pathogenic factor in mice compared to other *Ct* serovars such as D and F in C3H mice (Carmichael *et al.*, 2013). Although my results indicate that it is not likely that past infection with serovar E results in infertility, future experiments utilizing higher IFU for serovar E infection and comparison with MoPn serovar infection should be carried out to further investigate this hypothesis.

The average embryonic and placental weight per mouse was not affected by *Ct* infection or exposure to *Ct* at e12 of gestation. Similarly, in preliminary experiments at e14 of gestation, no difference was observed when the average pup and placental weight was examined per dam. However, *Ct* infection was shown to decrease placenta and embryonic weight at e14 but not e12 if each embryo was considered an individual value. Some published studies have analysed gestational parameters by regarding each embryo and placenta as an individual value (Constância *et al.*, 2002; Mu *et al.*, 2008), whereas others either analysed the mean weight per dam or analysed the data both ways (Cosselman *et al.*, 2012; Hubbard *et al.*, 2013). Both ways of analysing the data are valid, however they highlight the need of further experiments to investigate the potential link between past *Ct* infection and IUGR.

Although these were preliminary experiments, this is the first murine model to demonstrate that past maternal infection with *Ct* infection could potentially lead to IUGR. IUGR has been induced in other mouse models with a maternal low-protein diet and hypercholesterolemia (Bhasin *et al.*, 2009). In the absence of maternal malnutrition, insufficient vascularisation as a result of abnormal vessel development in $Vegf^{-/-}$ mice caused IUGR (Carmeliet *et al.*, 1996). Nitric oxide synthase knockout ($eNOS^{-/-}$) mice display suboptimal uterine artery function and placental nutrient transport is impaired leading to IUGR (Kusinski *et al.*, 2012). Insulin-growth factor II (*IgfII)* depletion has been shown to induce reduction of placenta weight that also results in IUGR (Constancia *et al.*, 2002). It would be interesting to determine whether *Ct* infection leads to IUGR through any of these pathways in future experiments utilising the mouse model of infection and gestation developed in this study.

It was surprising that *Prlr* and *Egf* transcripts were upregulated in uterine tissue of mouse resorption in *Ct* infected mice. There are no data available regarding pathogenic roles of excess *Prlr* or *Egf.* It is known however that $Prl^{-/-}$ and $Prlr^{-/-}$ mouse models display serious reproductive failure phenotypes including impaired fertilisation, implantation failure and abnormal mammary gland development (Bao *et al.*, 2007; Horseman *et al.*, 1997; Lucas *et al.*, 1998;

Ormandy *et al.*, 1997). Furthermore, reduced *Prl* and *Egf* protein levels have been associated to placental induced IUGR by several studies in mice (Ain *et al.*, 2005; Cellini *et al.*, 2004; Kamei *et al.*, 1999; Rab *et al.*, 2013). Taking into consideration that elevated mRNA levels do not necessarily result in increased protein levels, it is possible that the mRNA levels of *Prlr* are increased in an attempt to compensate for reduced protein levels due to the past *Ct* infection. This suggests a potential effect of *Ct* in post-translational regulation of these critical genes and requires further investigation.

Finally, the role of *Cxcl16* during mouse gestation is completely unknown and the presence of mRNA on gestational day e12 is a novel finding. As *Cxcl16* is affected by *Ct* infection in a pattern similar to *Prlr* and *Egf*, it is likely that it is also involved in mouse gestation similarly to its role in human gestation, including recruitment of immune cells and interaction with trophoblast cells or maternal decidua. It would be very interesting to explore the role of *Cxcl16* utilising a knock-out mouse model, with and without *Ct* infection during gestation.

A limitation of the current study is that weight data were not available for all dams at e12 as this was not an outcome initially included in the study aims. Future studies should be planned in great detail in advance and include embryonic and placental weight measurements. Furthermore, due to time restrictions, the experiments presented in this study are preliminary and should be repeated until a sufficient sample number is reached, as established in the statistical power calculation of this study.

In future studies of past *Ct* infection utilising the current C3H model of gestation, it would be very interesting to investigate whether the growth restriction is continued till labour and whether the labour onset is premature or not. Moreover, another study could be designed to explore whether the effect of antibiotic treatment of *Ct* infection could prevent any of the above complications in a future gestation.

This study demonstrated that past *Ct* infection not only reduced embryonic and placental growth on e14 pups but also attenuated the expression levels of gestation related genes *Prlr, Cxcl16* and *Egf*. These findings require further investigation initially using the murine model and further evaluation in humans, as they may highlight a previously unknown effect of past *Ct* infection to gestation and provide a link between infection, IUGR and potentially other adverse pregnancy outcomes such as premature labour and reduced birth weight.

5. Chapter 5: Interim study analysis: association between *Ct* infection and miscarriage

5.1. Introduction and aims

Active *Ct* infection is defined as 'current' infection and is detected by PCR of a cervical swab (Centers for Disease Control and Prevention., 2014) although historically this was done by the less sensitive method of tissue culture (Centers for Disease Control and Prevention., 2014; Sozio and Ness, 1998). Persistent *Ct* infection is defined as infection that is still active for a minimum of 6 months up to 5 years after initial diagnosis (Meijer *et al.*, 2005; Morré *et al.*, 2002). Past *Ct* infection, which has been either treated using antibiotics or spontaneously cleared, can be diagnosed by proxy via detection of anti-*Ct* IgG antibodies in serum (Baud *et al.*, 2011). Several ELISAs based on the detection of antibodies against *Ct* antigen MOMP are commercially available at the moment such as the MEDAC ELISA plus, the Savyon Sero *Ct*-IgG ELISA and the Anilabsystems IgG EIA (Wills *et al.*, 2009b). A newly developed ELISA targeting antibodies against the Pgp3 protein of the *Ct* plasmid was shown to be the most sensitive assay when detecting past *Ct* infection (Horner *et al.*, 2013; Wills *et al.*, 2009c).

Active infection was shown to be associated with miscarriage in a study of 66 women with spontaneous miscarriage (Vigil *et al.*, 2002). In this study (Vigil *et al.*, 2002), 21% (14 of 66) were *Ct* infected compared with 8.9% (23 of 59) of women with full-term pregnancies ($p<0.05$). The percentage increased to 68.8% (22 of 32) when both parents were infected.

Conversely, other studies have reported no association between active *Ct* infection and miscarriage (Baud *et al.*, 2011; Grönroos *et al.*, 1983; Munday *et al.*, 1984; Sozio and Ness, 1998). In a study of 145 women with miscarriage and 261 controls (Baud *et al.*, 2011), *Ct* was detected in 4% (5 of 145) of placentae from women with miscarriage and 0.7% (18 of 261) of control women with healthy pregnancies, however no difference was observed in the

prevalence of active *Ct* infection between women with miscarriage and controls (p=0.026). Baud *et al.* (Baud *et al.*, 2011) more recently conducted a statistically powered study, with defined patient recruitment criteria for both cases and controls and examined for the presence of *Ct* infection in women and placentae using sensitive methods (ELISA and PCR), therefore this study is of high quality. In a separate study (Sozio and Ness, 1998), the proportion of women with *Ct* infection was 3.8% (2 of 52) among spontaneous miscarriage cases and 8.5% (5 of 59) controls under 22 weeks of gestation, therefore the researchers reported no increased risk of miscarriage due to *Ct*. In another prospective study (Munday *et al.*, 1984), 241 pregnant women, 76 women miscarried and *Ct* was detected in the cervix of one woman in the study using cell culture followed by Giemsa stain, leading the authors to believe there was no association between *Ct* and miscarriage. In a study by Grönroos *et al.* (Grönroos *et al.*, 1983), detection of IgG and IgA *Ct* antibodies by radioimmunoassay in sera and cervical secretions of 189 women with threatened miscarriage, showed no difference in *Ct* prevalence among women that subsequently delivered a healthy baby compared to women that miscarried. Further details of these studies are shown in Table 5.1.

Several studies have been published in support of the association of past *Ct* infection with miscarriage, with *Ct* prevalence ranging between 11%-69% in miscarriages compared to 2-7% in healthy pregnant controls (Arsovic *et al.*, 2014; Baud *et al.*, 2011; Licciardi and Grifo, 1992; Wilkowska-Trojniel *et al.*, 2009). In one study (Arsovic *et al.*, 2014), 10 of 54 women with miscarriage were shown to have persistent *Ct* infection. Arsovic *et al.* (Arsovic *et al.*, 2014), suggest an association between persistent *Ct* infection and miscarriage, however a limitation of this study is these cases were compared only against patients with tubal infertility and not uninfected healthy pregnant women (Arsovic *et al.*, 2014). In another study (Baud *et al.*, 2011), 145 women with miscarriage and 261 controls, a positive association of past *Ct* infection with miscarriage was observed. The prevalence of *Ct* antibodies was 15.2% (2 of 145) in women with miscarriage compared to 7.3% (19 in 261) in controls

(p=0.018, Baud *et al.*, 2011). Furthermore, in an observational study of 4,920 women with genital tract infections (Kortekangas-Savolainen *et al.*, 2012), Kortekangas-Savolainen *et al.*, have suggested that late complications can occur in *Ct* infected pregnant women. Moreover, Wilkowska-Trojniel *et al.* (Wilkowska-Trojniel *et al.*, 2009) observed the prevalence of *Ct* antibodies in 11.28% (9 of 76) patients with one spontaneous miscarriage compared to 2.2% (1 of 46) of 2nd and 3rd pregnancy trimester controls (p=0.029). In an older study of 195 miscarriage cases (Feist *et al.*, 1999), 4.1% (8 of 195) patients were seropositive for *Ct* and none of the placenta sections examined were infected with the bacterium. This study examined no controls (Feist *et al.*, 1999). Finally, 69.0% (20 of 29) women with miscarriage were found to be positive for *Ct* antibodies compared to 23.7% controls (9 of 38, p<0.001) or 25.6% of women who did not become pregnant (20 of 78, p<0.001) after IVF (Licciardi *et al.*, 1992). In contrast, a number of older studies have disputed the association of past *Ct* infection and miscarriage (Coste *et al.*, 1991; Feist *et al.*, 1999). In a case control study (Coste *et al.*, 1991) comprising of 279 miscarriage and 279 control cases, previous miscarriage, ethnic origin, maternal age above 30 at time of conception but not *Ct* infection were highlighted as significant risk factors to miscarriage. Additionally, a different study (Feist *et al.*, 1999) examined 195 placental samples from miscarriage cases where examined using anti-*Ct* LPS antibody and were all negative. In the same study, serum samples from 187 patients were tested for detection of anti-*Ct* antibodies by microimmunofluorescence. Serological findings indicated that only 8 patients had had past *Ct* infection (Feist *et al.*, 1999). Finally, Paukku *et al.* (Paukku *et al.*, 1999) examined 72 recurrent miscarriage patients and 132 controls using serum *Ct* IgG and IgA ELISA to detect *Ct* infection and reported no association between *Ct* and miscarriage. This paper appears to have contradictory statements, as the microimmunofluorescence data the authors also presented in the paper demonstrated a higher prevalence of *Ct* infection antibodies in the control group. This was also openly criticised also in a letter to the same journal (Witkin, 2000). Further details of these studies and their limitations are shown in Table 5.1.

To summarise, the evidence supporting an association of *Ct* with miscarriage is inconsistent. This can be attributed to poor sensitivity of older detection assays and the inability of assays that detect current infection to detect past infection. Moreover, many of the above studies have been poorly designed, with some having unequal group sizes or missing a control patient population.

I therefore set out to investigate the association of lifetime exposure to genital *Ct* infection with miscarriage using a novel ELISA that detects antibodies to the Pgp3 protein of *Ct* plasmids with more sensitivity than widely used commercial assays (Wills *et al.*, 2009a). The Pgp3 antigen is unique to *Ct*, eliminating cross-reactivity with antibodies to other *Chlamydia* species such as *C.pneumoniae*, a major weakness of previous serological tests (Wills *et al.*, 2009a). A well-powered case-control study to investigate whether past *Ct* infection is a risk factor for miscarriage (for further details see sections 5.2.1).

The hypothesis of this study was that past *Ct* infection is associated with increased risk of miscarriage.

To address this hypothesis I aimed:

1. To compare the detection of past *Ct* infection
2. To investigate whether lifetime exposure to *Ct* is associated with increased risk of miscarriage
3. To investigate the prevalence of active *Ct* infection in women presenting with miscarriage.

Table 5.1. Analysis of studies investigating the role of past and active *Ct* infection and miscarriage.

Study	Pregnancy complication	Case population	Control population	Type of infection	Assay used	Result	Study quality
Grönroos *et al.*, 1983	Spontaneous miscarriage (not further defined)	189 consecutive patients with threatened miscarriage	None	Active infection	Active infection: cell culture from cervical swab and IgA radioimmunoassay on cervical and serum samples	No association between active infection and spontaneous miscarriage	Less reliable due to lack of control population and less sensitive detection methods
Munday *et al.*, 1984	1st and 2nd trimester miscarriage	76 women with spontaneous miscarriage under 28 weeks of gestation	149 women whose pregnancies ended in live-births	Active infection	Active infection: cell culture detection using GIEMSA stain from cervical swab test	No association between active infection and miscarriage	Less reliable due to less sensitive detection methods
Sozio and Ness, 1998	1st and 2nd trimester miscarriage	60 women between the ages of 14 and 40 with miscarriage up to 22 weeks of gestation	60 women chosen randomly with uncomplicated pregnancies continuing past 22 weeks of gestation	Active infection	DNA testing on urine samples using the Abbott LCR Probe system	No association between active infection and miscarriage	Limitation is the sample size of both groups
Vigil *et al.*, 2002	1st trimester miscarriage	66 women with history of one or more 1st trimester spontaneous miscarriages	259 women with children and no history of spontaneous miscarriage recruited during annual gynaecological examination	Active infection	Immunofluorescence on cervical smears using MicroTrak kit	Active infection significantly associated with miscarriage	More reliable study although the much smaller size of the case population is a limiting factor
Licciardi *et al.*, 1992	Spontaneous miscarriage (not further defined)	29 women undergoing IVF treatment	116 women undergoing IVF treatment	Active and past infection	Active: cell culture, DNA probe Past: serum IgG detection using	Past infection significantly associated with spontaneous miscarriage	Prospective study with case group much smaller compared to control group – significant limitation

Study	Pregnancy complication	Case population	Control population	Type of infection	Assay used	Result	Study quality
Wilkowska-Trojniel *et al.*, 2009	1st trimester miscarriage: spontaneous and recurrent (>2 miscarriages)	120 women aged 19-44 years with miscarriage history	46 women, in the 2nd and 3rd trimester with normal pregnancy, no history of miscarriage or urogenital disorders	Active and past infection	Active: PCR of endocervical swabs Past: serum IgG and IgA detection using MEDAC p-ELISA	Past infection associated with spontaneous and recurrent miscarriage	Serious limitation is the smaller sample size of the control group therefore not as reliable
Baud *et al.*, 2011	Miscarriage (not further defined)	125 women consulting for an acute miscarriage.	261 women attending the labour ward with no past miscarriage, stillbirth, preterm labour.	Active and past infection Foetal infection	Active: PCR of endocervical swabs, Past: serum IgG and IgA using Ridascreen ELISA Foetal: PCR on placentae from miscarried foetuses	Past infection associated with miscarriage, *Ct* detection foetal placentae	More reliable study using sensitive detection methods despite the smaller size of the case population
Arsovic *et al.*, 2014	Spontaneous miscarriage (not further defined)	54 women with spontaneous miscarriage	33 women with tubal factor infertility	Past and active infection	Serum samples: ELISA on IgG and IgA *Ct* antibodies using MEDAC p-ELISA	Association only of persistent active infection with miscarriage	Serious limitation is the small sample size of both groups therefore not as reliable
Paukku *et al.*, 1999	Recurrent miscarriage	70 selected women from RPL clinic	40 normal parous women, 94 asymptomatic sexually active women	Past infection	Serum samples: ELISA on IgG and IgA *Ct* antibodies microimmunofluorescence	No association between past infection and miscarriage	Serious limitations: small sample size of both groups, contradictory statements
Coste *et al.*, 1991	Miscarriage (expulsion of a fetus weighing <500g)	279 women between 15 and 44 years with miscarriage	279 women 15 - 44 years, giving birth in the same hospitals on date close to a case	Not specified	Microimmunofluorescence method on unspecified *Ct* antibodies on serum samples	No association with miscarriage	Limitation is the detection method sensitivity
Feist *et al.*, 1999	Spontaneous miscarriage (not further defined)	195 women between 19 and 44 years, miscarriage-gestational ages of 3-37 weeks	None	Not specified	Microimmunofluorescence method on unspecified *Ct* antibodies on serum. Immunohistochemistry, histological examination of placental samples	No association with miscarriage	Less reliable due to lack of control population, detection method sensitivity

5.2. Materials and Methods

5.2.1. Statistical power of study

This is a large study of women with miscarriage with sufficient power to estimate the *Ct* population attributable risk in miscarriage (300 women in the miscarriage group and 300 women in the control group). Past *Ct* infection seroprevalence of 20% in the control group and 40% in women with miscarriage is anticipated based on literature review and pilot work (Baud *et al.*, 2010; Lyytikäinen *et al.*, 2008). The study has over 99% power to demonstrate an association between *Ct* exposure and miscarriage. It will have 86% power to detect a dose response effect of *Ct* titre, if 35% of the estimated 60 pregnant *Ct* positive controls have a high titre compared with 60% of the expected 120 miscarriage *Ct* positive population. With 300 women who have miscarried and 300 women with normal pregnancies, this will allow estimation of exposure within the miscarriage group (95% CI) 34.5%- 45.8% and 15.7%-25.1% within the control group respectively.

5.2.2. Study participants

All women gave written informed consent to the study, which was approved by the Scotland Ethics committee (REC reference number: 12/SS/0098).

Women who attended the Pregnancy Support Centre of the Royal Infirmary of Edinburgh with a miscarriage that had been confirmed with an ultrasound scan were recruited. These women were aged between 16 and 45 years old with no history of HIV. The miscarriages were all within the first 12 weeks of pregnancy.

Details of age, ethnicity, patient reported number of previous episodes of *Ct*, height and weight, medical history, current medication and current *Ct* infection status (cervical swab tested by Abbott RealTime *CCOt*/NG Amplification Reagent Kit, Abbot, Cat. No 08L07-091, and/or results obtained from the clinic notes if taken as part of routine clinical care) were collected for each patient. Additionally, birth weight (grams) of the baby was recorded for control patients.

Patients in the control group were recruited from the Edinburgh Tissue Biobank and the Obstetric Triage Department at the Royal infirmary. The Edinburgh Tissue Biobank database folders were checked for eligible patients. Inclusion criteria included were between 15 and 45 years old, had no history of miscarriage or stillbirth

in previous pregnancies, no history of HIV, and were in their third trimester of their current pregnancy when approached. If information regarding history of *Ct* infection was recorded in the Biobank database, it was recorded. Details of age, ethnicity, patient reported number of previous episodes of *Ct*, height and weight, medical history, current medication were also recorded. However, routine *Ct* PCR swab test was not conducted by the Biobank at the time of recruitment. Serum samples were retrieved from the Biobank and analysed. The same inclusion criteria were used for women approached at the Obstetric Triage Department at the Royal infirmary, however a *Ct* PCR swab test was also conducted during recruitment in addition to serum sample collection.

5.2.3. *Ct* antibody detection in serum samples

5.2.3.1. Pgp3 indirect ELISA assay

Samples were analysed for evidence of past *Ct* infection by Dr Gillian Wills as published in (Wills *et al.*, 2009a) in Professor Myra McClure's laboratory (Jefferiss Trust Laboratories, Wright-Fleming Institute, Imperial College London). This assay was selected as it is the most sensitive and specific assay compared to the most widely used anti-MOMP ELISAs (Wills *et al.*, 2009c).

Briefly, immunosorb 96-well microtitration plates were coated with Pgp3 protein for 1 h at 37°C (20 ng per well in 100 mM sodium carbonate buffer, pH 9.6). The bound protein was washed with PBST-B (PBS, pH 7.2, containing 0.05% Tween 20), blocked with 200 μl of 1% Hammersten casein in PBST-B for 2 h at 37°C, and washed three times. All sera were then assayed in duplicate at a 1:100 dilution in the blocking buffer. After 1 h at 37°C, the bound protein and antibody were washed three times, and 100 μl of an HRP-labelled goat anti-human antibody (Fc fragment) diluted 1:8,000 was added. After 1 h at 37°C, unbound antibody was removed by six washes with PBST-B; then 100 μl TMB solution was added, and the mixture was incubated for 10 min at 25°C. The reaction was stopped with 50 μl 2 M H2SO4, and absorbance was read at 450 nm. Readings were corrected for background by subtracting the average absorbance of two (blank) wells with no serum.

5.2.4. RIDASCREEN® *Chlamydia* IgG/IgM assay

The second *Ct* detection assay used was the RIDASCREEN® *Chlamydia* IgG/IgM kit, used in the study by in the study by Baud and colleagues (Baud *et al.*, 2011), R-biopharm, Cat. no: KGM3101).

The kit was used according to the manufacturer's protocol. Briefly, 100 µl sample buffer was added into each of the microwells followed by 5 µl sample or control. The plate was mixed on a plate mixer and incubated for 45 minutes at 37 °C in an incubator. The wells were emptied into a waste container containing hypochlorite solution for disinfection. Then the plate was blotted onto absorbent paper in order to remove the residual moisture and washed 5 times using 300 µl diluted wash buffer each time. Subsequently, 100 µl of the streptavidin conjugated- IgG was added and incubated at 37 °C for 30 minutes. Following 5 washes, 100 µl of substrate were added to each well and incubated at room temperature for 20 minutes. The reaction was stopped by adding 50 µl stop reagent and the absorbance was measured at 450 nm (reference wavelength 620 nm).

5.2.5. Statistical analysis

Statistical analysis was carried out by the Chi square test (used to test for a relationship between two groups of categorical values), Mann-Whitney test (used to test for a relationship between two groups of continuous values) and Fisher's exact test (is used to analyse contingency tables with two nominal variables). The GraphPad Prism 6 software was used with the guidance of my study collaborator Dr Paddy Horner (Consultant Senior Lecturer in the School of Social and Community Medicine at the University of Bristol and expert in statistical analysis and modelling) (Horner *et al.*, 2013; Wills *et al.*, 2009c).

5.3. Results

5.3.1. Demographic characteristics of study participants

During January 2013–November 2015, a total of 199 women with miscarriage (‘miscarriage group’) were recruited to my study at the Pregnancy Support Centre of the Royal Infirmary of Edinburgh. 141 ‘control’ women were prospectively recruited from the Obstetric Triage Department at the Royal Infirmary of Edinburgh (‘control group’) and the Edinburgh Tissue Biobank.

I personally identified 130 eligible control patients from the Edinburgh Tissue Biobank and recruited 10 patients from the Obstetric Triage Department at the Royal infirmary. Furthermore, I personally recruited 58 women at the Pregnancy Support Centre of the Royal Infirmary of Edinburgh.

Due to difficulties with recruitment, I did not achieve the target set out in my sample size calculation and I therefore present an interim analysis. Recruitment is on-going by the Horne group and the target of 300 women in each group should be achieved by 2017.

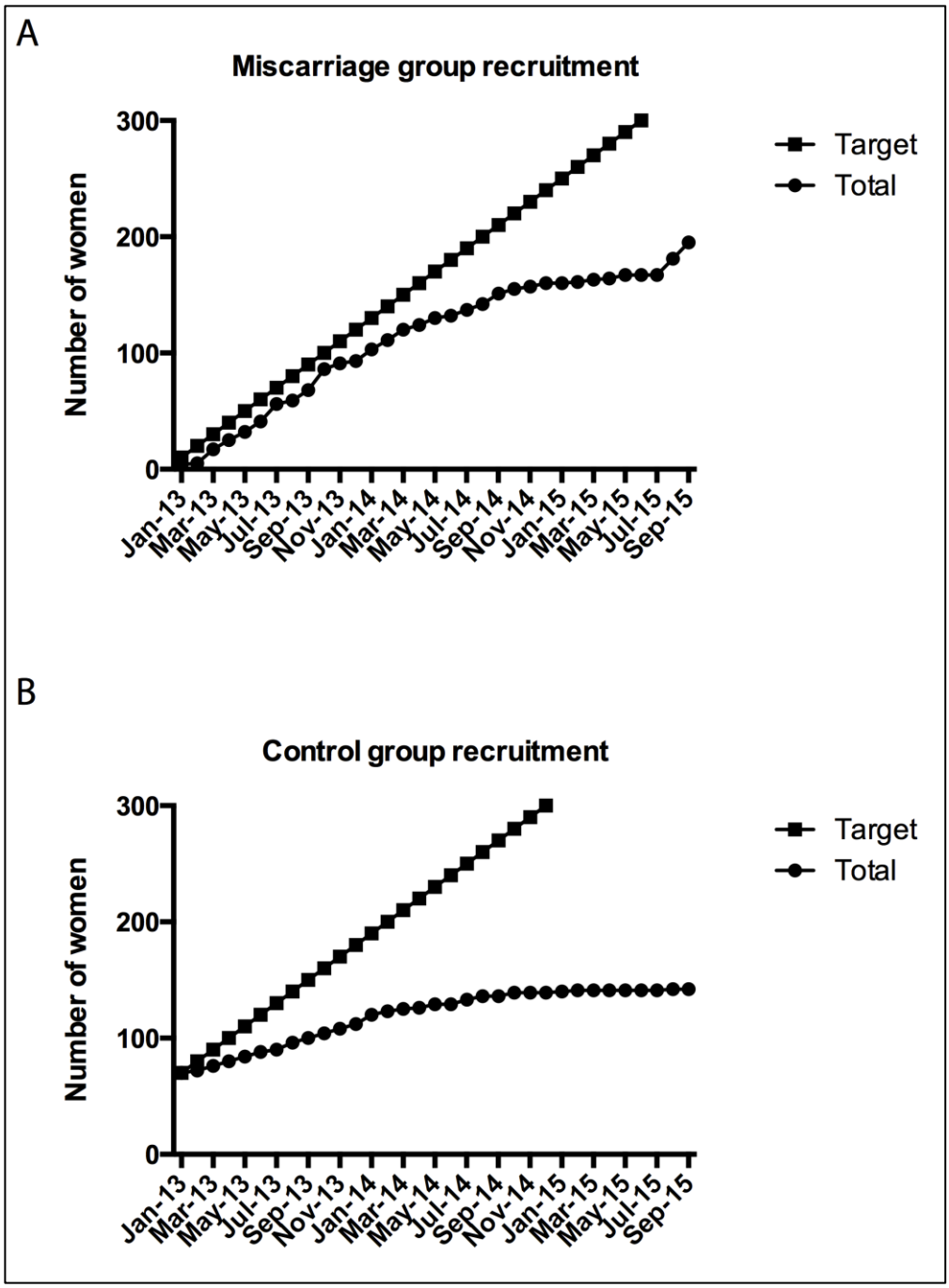

Figure 5.1. Case-control study recruitment was not completed till July 2015. (A) The target of 300 women in the control group was not reached by May 2016, a total of 199 women were recruited. (B) Similarly, 141 of 300 women of the control group were recruited.

As shown in Table 5.2, the groups did not significantly differ in age, as the average was 32.87 years of age for the control group and 36.62 for the miscarriage group. Similarly, no differences were observed in weight, height or body mass index (BMI)

between both groups. Ethnicity was a parameter that differed between groups, as more participants in the control group were of "unknown/other" ethnicity (9,3% compared to 0% whereas in the miscarriage group the majority of participants were Caucasian (95.5% compared to 87.9% in the control group).

Table 5.2. Study participants displayed no difference in demographics including age, weight, height, smoking status and body mass index (BMI), however a difference was observed in the ethnicity between the control and miscarriage group.

Parameter	Statistic	Control group (n=141)	Miscarriage group (n=199)	p value
Age (years)	Mean± Std. dev.	32.87 ± 5.419	32.62 ± 5.816	0.9506*
Weight (kg)	Mean ± Std. dev.	70.84 ± 13.76	70.18 ± 17.42	0.2131*
Height (cm)	Mean ± Std. dev.	164.8 ± 5.785	165.2 ± 6.619	0.4059*
Body mass index (BMI)	Mean ± Std. dev.	26.22 ± 5.374	25.72 ± 6.024	0.0991*
Smoking status	Smoker (%)	13 (9.6%)	21 (10.7%)	0.6720**
	Ex smoker (%)	27 (19.9%)	46 (23.4%)	
	Never smoked (%)	96 (70.6)	130 (66%)	
Ethnicity	Caucasian (%)	123 (87.9%)	190 (95.5%)	< 0.0001**
	Black (%)	3 (2.1%)	3 (1.5%)	
	Asian (%)	1 (0.7%)	6 (4%)	
	Other/Unknown (%)	13 (9.3%)	0 (0%)	
*Mann-Whitney test, **Chi square test				

Detailed statistical parameters can be found in Appendix 3.

5.3.2. Pgp3 ELISA detected more patients exposed to *Ct* than RIDASCREEN Chlamydia IgG ELISA

Using the RIDASCREEN ELISA to detect past infection, 12 women with miscarriage and ten women from the control group were found to be seropositive for past *Ct* infection. However, using the Pgp3 ELISA 36 women with miscarriage and 31

women from the control group were positive for the Pgp3 antigen, indicating past *Ct* infection (Figure 5.2).

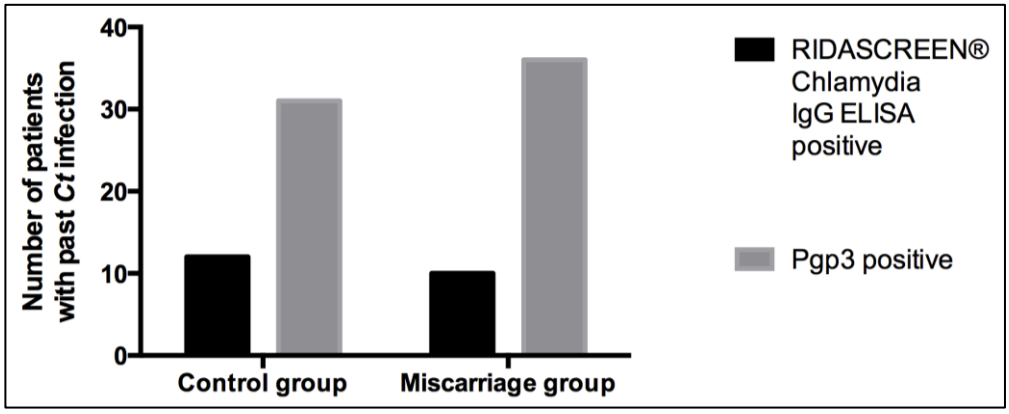

Figure 5.2. The Pgp3 ELISA detected more women that had been exposed to past *Ct* infection compared to the RIDASCREEN ELISA. Serum samples were collected from 36 versus 12 patients in the miscarriage group and 31 compared to 10 in the control group. Past *Ct* infection was detected using an in-house ELISA assay detecting antibodies against *Ct* cryptic plasmid protein Pgp3 and a commercially available ELISA detecting anti-*Ct* LPS antibodies.

5.3.3. No association with miscarriage and past *Ct* infection (based on interim analysis)

Past *Ct* infection was detected by both the Pgp3 and the commercially available anti-LPS ELISA. 31 women from the control group and 36 women from the miscarriage group were seropositive for Pgp3, whereas 96 and 124 respectively were seronegative. Using the commercially available IgG LPS ELISA on the same samples, 12 women from the control group where positive with 113 negatives and 10 women from the miscarriage group were positive whilst 113 were negative (Table 5.3).

Table 5.3. Interim analysis of 141 serum samples from women with normal pregnancies and 199 women with miscarriage demonstrates no association between past *Ct* infection and miscarriage. Past *Ct* infection was detected in serum samples using an in-house ELISA assay detecting antibodies against *Ct* cryptic plasmid protein Pgp3 and a commercially available ELISA detecting anti-*Ct* LPS antibodies. In the control group, 31 participants tested positive and 96 tested negatively for past *Ct* infection, whereas in the miscarriage group 36 were seropositive and 124 were seronegative as detected by the Pgp3 assay.

Parameter	Outcome	Control group	Miscarriage group	p value

		(n=141)	(n=199)	
Pgp3 result	Positive	31	36	0.7790**
	Negative	96	124	
	Not yet tested	25	40	
LPS RIDASCREEN Elisa result	Positive	12	10	0.8240**
	Negative	113	113	
	Not yet tested	0	0	
**Chi square test				

History of past *Ct* infection was reported by 4 women of the control group and 17 women with miscarriage, with 17 women from the control group and 182 women from the miscarriage group reporting no previous *Ct* diagnosis. Information on history of *Ct* infection was not available for 120 women from the control group retrieved from the Biobank.

Taking into account the self-reported history of past infection and the past *Ct* infection seroprevalence data from the Pgp3 assay, it is evident that several women had been exposed to *Ct* in the past without ever been diagnosed. More specifically, out of 31 seropositive women in the control group, only 4 reported past *Ct* diagnosis, whereas in the miscarriage group more women had received a diagnosis as 17 out of 36 seropositive patients reported history of *Ct* infection (p=0.0034).

5.3.4. Active *Ct* infection was not detected in any of the study participants

None of the 14 women from the control group and 177 women from the miscarriage group was infected with *Ct* at the time of enrolment. The remaining 129 women of the

control group were not tested for active infection as the serum samples had been collected previously by Edinburgh tissue Biobank (Table 5.4).

Table 5.4. Active *Ct* infection was not detected in any of the study participants. Participants were tested for active *Ct* infection at the time of enrolment in the study by PCR (Abbott RealTime *Ct*/NG Amplification Reagent Kit) on a cervical swab sample the participants collected themselves. This was not possible for control samples retrieved from the Edinburgh tissue Biobank, thus only 14 patients were tested and found seronegative. In the miscarriage group, none of the 177 women tested were diagnosed with active *Ct* infection.

Parameter	Outcome	Control group	Miscarriage group
Current *Ct* infection	Positive	0	0
	Negative	14	177

5.4. Discussion

To summarise, interim analysis of the samples from this study to date indicates that past *Ct* infection is not associated with miscarriage. If this holds true for the completed dataset, this is a very important public health message.

The Pgp3 assay is more sensitive than other commercially available ELISAs (Wills *et al.*, 2009a). As I have demonstrated, the Pgp3 ELISA detected more cases of past *Ct* infection compared to the RIDASCREEN *Chlamydia* IgG assay used by Baud and colleagues in the most recent study showing an association between *Ct* and miscarriage (Baud *et al.*, 2011), however a study on samples with known history of *Ct* infection is required to establish the difference in sensitivity of these two ELISAs.

With a total of 340 participants from both the miscarriage and control groups, the present study has a sample size larger than most studies published on the association between past and active *Ct* infection and miscarriage (Arsovic *et al.*, 2014; Coste *et al.*, 1991; Feist *et al.*, 1999; Grönroos *et al.*, 1983; Munday *et al.*, 1984; Paukku *et al.*, 1999; Sozio and Ness, 1998; Wilkowska-Trojniel *et al.*, 2009). The interim analysis suggests no association between past *Ct* infection and miscarriage, a result that, if confirmed upon study completion, contradicts several published studies (Arsovic *et al.*, 2014; Baud *et al.*, 2011; Licciardi and Grifo, 1992; Wilkowska-Trojniel *et al.*, 2009), whilst adding to those arguing against past *Ct* infection being a risk factor for miscarriage (Coste *et al.*, 1991; Feist *et al.*, 1999). It is likely that upon completion of the study the lack of association will be proved given the statistical power of the present study, thus clarifying that lifetime exposure to *Ct* is not a risk factor for miscarriage.

An important factor that must be taken into consideration is that this study has 99% power to detect the association between *Ct* and miscarriage provided the prevalence of past *Ct* infection is 20% in the control group and 40% in the miscarriage group. Preliminary data indicate that the difference in *Ct* prevalence is not as high as 20% between the two groups. Although the study is still on-going, it is possible that a far larger scale study may be required to have sufficient power to establish an association between *Ct* and miscarriage.

Maternal weight and obesity, defined as body mass index (BMI) >=28, as well as smoking status were not associated with miscarriage. Obesity has been identified as a risk factor for miscarriage (Boots and Stephenson, 2011). The results reported in the present interim analysis could be attributed to similar weight, height and BMI distribution between the two groups, leading to no association between BMI and miscarriage. Furthermore, published systematic review of 98 studies has demonstrated that active smoking during pregnancy, including passive smoking, increased the risk of miscarriage (Pineles *et al.*, 2014). However, similarly to BMI, smoking status was not associated with miscarriage in my analysis and a similar pattern of smokers, ex smokers and non smokers was observed among both groups.

An important outcome from the interim analysis was that no active *Ct* infection was detected in any of the women in the miscarriage group so it is not going to be possible to come to any conclusion on active infection from this study. A past study on pregnant women in England has reported prevalence ranging from 0.08% to 2.1% in adult women 25-44 years old (Price *et al.*, 2014). Similar prevalence statistics have been reported across Europe, ranging between 1.7% and 17% (Rours, Duijts, *et al.*, 2011; Wilson *et al.*, 2002). Crucial to prevalence of the disease is country of origin as prevalence rates are higher in developing countries (27% in Saudi Arabia (Ashshi *et al.*, 2015), 23% (Patel *et al.*, 2010)and 29.8% in India (Yasodhara *et al.*, 2001), and 10.9-12.5% in Argentina (Golijow *et al.*, 2005)). Furthermore, younger women are at higher risk of *Ct* infection and detection rates in pregnant adolescents have been reported as high as 37% (Hardy *et al.*, 1984). In my sample set, zero prevalence reflects on *Ct* testing policies in Scotland that have been applied to young women 17-24 years old and have resulted in 21% reduction of *Ct* cases (Looker *et al.*, 2015) as well as the fact that the average age of both groups was 32 years old, suggesting a population of women at a lower risk of *Ct* infection (Adams *et al.*, 2004; Price *et al.*, 2014). It is also possible that women who wish to become pregnant are more conscious of their health and ask to get tested if they think they might be at risk. It is therefore likely that potential infections might have been treated at the time of recruitment. As *Ct* prevalence rates are higher in developing countries, risk of adverse pregnancy outcomes due to past *Ct* infections might be more relevant in developing countries, highlighting the importance of the conduction of2 a statistically powered study in these areas.

The recruiting process for this study proved particularly challenging. Abiding by the schedule of each patient visit can be time consuming, as appointments can be delayed. This could result in missing patients if appointments occur in parallel in different departments. Furthermore, many patients did not consent to discussing the study or refused to participate after discussion. Another important factor was the lack of eligible patient samples from the Edinburgh Tissue Biobank for inclusion in my study. Another limitation of the Edinburgh Tissue Biobank samples was the lack of past *Ct* infection medical history information and current infection tested at the time of recruitment, as this was not a process normally undertaken by the Biobank. This resulted in a need for additional recruitment of patients attending their elective C-section appointments at the Triage for the control group of this study.

Recruitment to this study should be completed by 2017 and detailed statistical analysis will be followed by publication of the findings that will contribute to a better understanding of *Ct* infection and risk of miscarriage.

6. Chapter 6: General discussion

In the present thesis three questions were addressed:

1. Does *Ct* infection impact upon decidualisation and chemokine secretion?
2. Does past *Ct* infection cause adverse pregnancy outcomes in a murine pregnancy model?
3. Is past *Ct* infection associated with miscarriage in humans?

The hypothesis was that *Ct* can infect ESC that undergo decidualisation. *Ct* infection could result in suboptimal decidualisation and attenuated chemokine secretion, which could negatively impact on a potential implantation. Furthermore, I hypothesised that there is a causal relationship between past *Ct* infection and adverse pregnancy outcomes including increased number of pup resorptions and IUGR in a murine model of pregnancy. Finally, drawing parallels between the mice and humans, I postulated that past *Ct* infection could be a risk factor for miscarriage.

6.1. The role of the endometrium in *Ct* infection and adverse pregnancy outcomes

The fetomaternal microenvironment during decidualisation and early pregnancy is a result of complex interactions between various cell types of the mother and the fetus. Consequently, any disruption of this delicate balance may lead to adverse pregnancy outcomes. As demonstrated in Chapter 3, *Ct* can infect not only the endometrial luminal epithelium but also the stromal cells. Furthermore, it can disrupt decidualisation as measured by reduced levels of decidualisation marker PRL, without affecting the high mRNA and protein levels of the other important decidualisation marker, IGFBP1.

PRL is a key molecule for gestation and decidualised, with a well characterised role, and reduced PRL secretion has been linked to recurrent miscarriage (Salker *et al.*, 2010). In contrast, the role of PRL in IUGR remains unclear. It has been shown that mRNA and protein levels of decidualisation marker IGFBP-1 and leptin were elevated in IUGR placentae compared to placentae from normal birth weight babies, whereas PRL levels were found to be similar between the two groups (Struwe *et al.*, 2010).Interestingly, the same group detected elevated levels of IGFBP-1, leptin and PRL levels in the periphery and intermediate zone of IUGR placentae compared to

normal birth weight control placentae, demonstrating that sampling area is crucial for accurate quantification of proteins of interest (Tzschoppe *et al.*, 2010).

As demonstrated in Chapter 4, within a controlled experimental environment where the only variable is *Ct* infection of dams a month prior to mating, it is evident that past *Ct* infection did not cause increased resorption and did not evidently affect pregnancy up to e12 of gestation. However, preliminary experimental data collected by examination of individual pups at e14 have shown that *Ct* infection causes an apparent reduction in fetal size, indicating IUGR. *Prl* is expressed during the first half of mouse gestation and it is downregulated from e10 onwards (Barkley *et al.*, 1978; Mednick *et al.*, 1980), similar to the pattern observed during human gestation (Rigg *et al.*, 1977). It was therefore not unexpected that the mRNA levels of *Prl* (Figure 4.9) were barely detected in the whole uteri examined in my mouse model. This does not necessarily mean that it has no role in contributing to IUGR caused by *Ct* infection in a mouse model of gestation. Future experiments are needed to investigate changes in *Prl* protein and mRNA levels during the early mouse gestation, namely at the stage corresponding to early decidualisation of the in-vitro experiments.

Another largely unexplored field is the role of the immune system in IUGR. More specifically, there is no information on the role of CXCL12 and CXCL16 in IUGR development. A recent study has reported raised levels of interleukin-6, CXCL8 and interleukin-18 in IUGR new born babies compared to healthy babies, indicating that inflammation might be associated with IUGR (Krajewski *et al.*, 2014). Moreover, increased umbilical artery CCL16 levels were associated with IUGR and preterm preeclampsia (Mäkikallio *et al.*, 2012). I showed that CXCL12 and CXCL16 were reduced in-vitro in decidualised ESC infected with *Ct* (Figure 3.11) whereas CXCL8 was elevated (Figure 3.9). As the role of maternal decidua is critical during early gestation, this could be a potential mechanism via which *Ct* infection could result in IUGR. A study utilising a proteomic array on babies and mothers with IUGR compared to healthy babies and mothers could identify more cytokines involved in IUGR pathogenesis and thus contribute to clarifying factors responsible for this adverse pregnancy outcome.

6.2. The role of past *Ct* infection in IUGR

The role of active *Ct* infection in IUGR has not been firmly established, although most studies indicate that it can have a causative effect (Germain *et al.*, 1994; Investigators of the John Hopkins Study of Cervicitis and Adverse Pregnancy, 1989; Vedmedovska *et al.*, 2010). The complexity of mechanisms associated with adverse pregnancy outcomes has been furthermore demonstrated by the several risk factors associated with IUGR such as smoking, high BMI and drug use among others (reviewed in Suhag, A & Berghella, 2013). Therefore, a future study should account for all the above factors in order to elucidate whether *Ct* is a risk factor for IUGR.

In this study, mRNA levels of *Prl* and *Egf* were upregulated in uterine tissue of mouse resorption in *Ct* infected mice indicating that past *Ct* infection might affect gestation related pathways. . Contrary to expectations none of the gene changes reported in a 2010 microarray analysis of mice uteri in an IUGR model induced by the human pathogen *Campylobacter rectus* infection (Bobetsis *et al.*, 2010) were observed here, which highlights the possibility that different pathogens could impact upon different pathways whilst resulting to similar outcome. In a study of IUGR, induced by dexamethasone in rats, in addition to the placental prolactin family genes, the *insulin-like growth factor-II* and the *Akt* signalling pathway were dysregulated (Ain *et al.*, 2005). Due to attenuated expression of *Akt/ protein kinase B*, increased apoptosis was observed from placentae of IUGR mothers, partially explaining the reduced placental size observed (Ain *et al.*, 2005). Insulin growth factor 1 (IGF1) mutation was shown to be responsible for growth retardation of a 15-year old boy (Woods *et al.*, 1996). The role of IGF1 in IUGR is further supported by another reported case of IUGR in a female patient (Wallborn *et al.*, 2010). It is therefore important for future experiments with the in-vivo model to further investigate these genes as potential targets.

An interesting question raised is the mechanism via which past *Ct* infection results in permanent uterine damage that could affect future pregnancies. It is has been well established that *Ct* can regulate the transcription of host cell genes to promote its multiplication and protect itself from the host organism's immune system (Nicholson *et al.*, 2003). An emerging new field is examining the effect of infections on the epigenetic regulation of genes, called "patho-epigenetics" (Minárovits, 2009). The field of epigenetics examines the effect of DNA methylation, mainly at gene

promoters and enhancers that results in gene silencing, post-translational modifications of histone proteins, and RNA-based silencing on the phenotype of an individual (Riddihough and Zahn, 2010). Epigenetic alterations have been proven to be involved in disease development, including cancer and schizophrenia (Kanwal and Gupta, 2012; Van Vliet *et al.*, 2007). Emerging data indicates that some viral and bacterial infections can attenuate epigenetic modifications resulting in abnormal phenotype that could lead to disease development (reviewed in Bierne *et al.*, 2012; Minárovits, 2009). *Helicobacter pylori* infection of gastric mucosal cells was shown to increase methylation of several genes such as p16, resulting in increased risk of gastric cancer (Maekita *et al.*, 2006). Infection with intracellular *Listeria monocytogenes* but not non-invasive *Listeria innocua* altered the epigenetic modifications of CXCL8 promoter, resulting in increased CXCL8 by HUVEC cells, however it did not affect IFN-γ (Opitz *et al.*, 2006; Schmeck *et al.*, 2005). No research to date is available on whether *Ct* infection can alter the epigenome of infected cells and whether this alterations could be lingering after the infection has been cleared, thus impairing normal cell function. This is an interesting new parameter of the potential long term health effects of bacterial infections that should be further investigated in the future.

6.3. Investigating the role of past and current *Ct* infection in adverse pregnancy outcomes

Upon completion of sample collection for the case control study, analysis of the results will elucidate the role of past *Ct* infection and miscarriage. Secondary outcomes of the study will include prevalence of current *Ct* infection in women of reproductive age in Scotland and association of factors such as maternal BMI and smoking status with miscarriage. Interim analysis of the data suggests no association of past *Ct* infection with miscarriage, an outcome that coincides with the findings from the in-vivo mouse model.

The role of maternal infections in the development of IUGR has not been elucidated. It is postulated that infections including *C.pneumoniae* and Cytomegalovirus are an important risk factor to development of preeclampsia, a condition that includes complications such as premature labour and IUGR (von Dadelszen and Magee, 2002). The association between IUGR and past *Ct* that was indicated by data from the in-

vivo model presented in Chapter 4 could potentially be addressed by examining the birth weight and placental weight of embryos born from control women enrolled in the study. However, as this study has not been statistically powered to answer this question, a new appropriately designed case control study should be carried out. It is vital to take into consideration in the design of such a study important risk factors for IUGR development, such as maternal malnutrition and socioeconomic status (reviewed in Suhag, A & Berghella, 2013).

Past and active *Ct* infection has been linked to female infertility, more specifically tubal factor infertility (Brunham *et al.*, 1985; Mania-Pramanik *et al.*, 2012; Patton *et al.*, 1994). If the lack of association between past *Ct* infection and miscarriage is confirmed when the present study analysis is complete, this will indicate that although some women may have fertility problems as a result of exposure to *Ct* infection, women can be reassured that if they do have a normal uterine pregnancy there is no evidence of an adverse outcome.

6.4. Future studies

Sample collection for the case control study should be completed by the end of 2017. Statistical analysis of the results will be followed by publication of the findings.

Future experiments utilising the in-vivo *Ct* infection model, in addition to further elucidation the role of past *Ct* infection in IUGR as suggested in Chapter 4, could focus on exploring the mechanism via which this effect is taking place. Molecules such as leptin, interleukin-6, CXCL8 and interleukin-18 that have been highlighted by other studies could be targets of interest. However, another future step could be a large-scale proteomic or mRNA array that could potentially reveal pathways affected by *Ct* infection that have never previously been identified. Sample sets could include pregnant mice with active infection and past infection, with and without antibiotic treatment at early and later gestational time points. This could lead not only to important discoveries regarding the role of past and active *Ct* infection in gestation, but also reveal potential implications to the general health of the mother.

The role of many PRR receptors such as the NOD family of receptors in gestation is a new field of scientific interest (Abrahams, 2011). NOD1 and NOD2 are expressed in first trimester trophoblast cells and their activation may induce a proinflammatory

response (Costello *et al.*, 2007). Another member of the NOD family, NALP3 was also shown to be expressed in trophoblast cells (Mulla *et al.*, 2013). Inflammasome-dependent caspase-1 activation (via NALP3 and ASC) was shown to induce *Ct* intracellular growth (Abdul-Sater *et al.*, 2009). This could potentially have detrimental effect in establishing *Ct* infection in the decidua and placenta and is a direction that future experiments both in-vitro and in-vivo could explore.

Moreover, a study in epigenetic changes such as changes in DNA methylation and histone modification in infected ESC could be a clue as to how does past *Ct* infection have long lasting effects. Similarly, this could be explored in uterine samples from the mouse model.

As active *Ct* infection in pregnancy is recognised to be a risk factor for adverse pregnancy outcomes, an optimised in-vitro model of trophoblast invasion could be used to ascertain whether active *Ct* infection can impact on trophoblast cell migration. This could be conducted in parallel with studies in mice that could focus on active maternal infection and onset of gestation and placentation.

Finally, the efficacy of antibiotic treatment in preventing adverse consequences of *Ct* infection could be explored both in-vivo and in-vitro. An interesting set of experiments could focus on key targets highlighted by the above studies and whether antibiotic treatment can reverse the attenuated expression/ regulation of genes of interest. If this hypothesis were to be proven true, the importance of diagnosis and treatment of *Ct* infection would be particularly highlighted.

6.5. Conclusions of PhD

In summary, the data presented in this thesis suggests that *Ct* has the capability to infect the stromal compartment of the endometrium and infection could result in suboptimal decidualisation as reduced mRNA and protein levels of the decidualisation marker PRL were observed in *Ct* infected ESC in-vitro. Furthermore, infection altered chemokine secretion of decidualised ESC in-vitro, thus attenuating levels of chemokines essential for normal implantation. The role of past *Ct* infection in causing miscarriage was investigated in a mouse model, however the number of resorptions did not differ between control and *Ct* infected mice. Importantly, the

embryonic and placental weight of pups of *Ct* infected dams was reduced compared to controls, a consequence that could lead to IUGR later during gestation.

Though it may be the case that already exposed women may be at a higher risk of pregnancy complications, quick diagnosis and effective treatment could result in minimising the risk for a future pregnancy. The current study indicates that it is preferable to maintain vigilance and prophylactic policies as a precaution whilst future research efforts should be focused on elucidating key issues that remain unknown regarding the role of *Ct* infection in pregnancy.

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7. Appendix 1

The following paper based on Chapter 3 has been accepted for publication in Scientific Reports.

Chlamydia trachomatis infection of human endometrial stromal cells induces defective decidualisation and chemokine release.

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Miscarriage affects ~20% of pregnancies and maternal infections account for ~15% of early miscarriages. *Chlamydia trachomatis* (*Ct*) has been associated with miscarriage but the underlying mechanisms are unknown. Successful implantation requires endometrial stromal cell (ESC) decidualisation. Maintenance of pregnancy requires angiogenesis, establishment of the correct cellular milieu and trophoblast invasion, all of which involve the action of chemokines. Our objective was to determine whether *Ct* infection impacts upon ESC decidualisation and chemokine secretion. Human primary ESC were decidualised in-vitro, infected with *Ct* serovar E, and changes in expression of genes of interest were measured using RT-PCR, proteomic array and ELISA. We demonstrate for the first time that *Ct* can infect and proliferate in ESC. Expression of the decidualisation marker prolactin was decreased in *Ct*-infected ESC at both mRNA and protein levels. *Ct* infection altered the chemokine profile of decidualised ESC as shown by proteomic array. Chemokines CXCL12 and CXCL16, important for trophoblast invasion, were analysed further and expression was reduced in infected decidualised cells at mRNA and protein levels. Our data indicate that *Ct* infection of ESC impairs decidualisation and alters chemokine release. These findings at least partially explain how *Ct* infection could result in adverse pregnancy outcomes.

Word count: 198/200

Introduction

A miscarriage is defined as the spontaneous loss of a pregnancy during the first 24 weeks of gestation and occurs in approximately 20% of clinically recognised pregnancies[1]. Miscarriages are associated with considerable physical and psychological morbidity. Bleeding due to miscarriage can lead to haemodynamic shock and death and the emotional response to miscarriage can include depression and anxiety[1]. Approximately, 50% of early miscarriages are attributed to fetal chromosomal abnormalities, however, the underlying cause in other cases is often undefined. A number of infections have also been linked to miscarriage and infections are thought to account for 15% of early and 66% of late miscarriages (reviewed in[1]). Several studies have been published regarding the association of pelvic *Chlamydia trachomatis* (*Ct)* infection with miscarriage, with *Ct* prevalence ranging between 11%-69% in miscarriages compared to 2-7% in healthy pregnant controls[2–6]. The mechanisms underlying this association between *Ct* infection and miscarriage are unknown, though a recent study suggests *Ct* may interfere in essential early pregnancy inflammatory processes[7].

The development of a successful pregnancy depends upon maternal receptivity during the implantation window. This is largely established during decidualisation, the process whereby the stromal cells of the endometrium undergo structural and morphological changes to prepare for possible embryo implantation. Secretion of appropriate chemokine signals by decidual cells contributes to the recruitment of predominantly anti-inflammatory leukocyte subpopulations necessary for pregnancy maintenance[8], and prevents recruitment of potentially damaging T lymphocytes[9]. The maternal immune response to miscarriage associated infections can have detrimental effects on pregnancy maintenance, a characteristic example of which is seen when malaria pathogens are detected in the placenta (reviewed in[1]). Furthermore, chemokines not only recruit and impact on immune cells but are also involved in trophoblast invasion and angiogenesis during early pregnancy[10].

Both undifferentiated and decidualised endometrium has been shown to be altered compared to normal pregnancies[11] in women with spontaneous miscarriage. Impaired decidualisation, measured by a reduction in the decidualisation marker prolactin (PRL) in the endometrium, has been associated with recurrent miscarriage[12] and in rodent models decidual cell prolactin production has been shown to be critical for successful pregnancy[13]. Infection can markedly change the chemokine profile to recruit pro-inflammatory cell subsets.

It is well established that *Ct* infects endometrial epithelial cells[14–16], however the effect of *Ct* infection on endometrial stromal cell function and decidualisation is yet undetermined and may have a role in the association of *Ct* infection with miscarriage. *Ct* is known to cause endometritis, namely inflammation of the endometrium that is often asymptomatic, in non-pregnant women[17]. Data from animal studies indicate that in mice, *C. abortus* induces the murine equivalent of miscarriage without fetal harm, likely due to decidual damage[18]. In cattle *C. psittaci* associated chronic endometritis is a recognized cause of infertility (strain now known as *Chlamydia pecorum*)[19]. To our knowledge, no study to date has identified why *Ct* can cause endometritis in women or how infection of the stromal compartment of the endometrium might alter the function of human endometrial stromal cells. We therefore aimed to determine whether *Ct* can infect human endometrial stromal cells (ESC) and examine the effect of *Ct* infection on decidualisation and chemokine secretion in an in-vitro model.

Results

Ct can directly infect human endometrial stromal cells (ESC)

Primary ESC (n=4) were infected in 12 well plates with *Ct* serovar E at a multiplicity of infection (MOI) of 0.01, 0.1, 1, 2 and 3. No visible inclusions were present in cells at MOI 0.01 and MOI 0.1 48 hours post infection and few inclusions were seen at MOI 1 (data not shown). The non-infected ESC and ESC treated with UV-inactivated *Ct* at MOI 2, displayed no signs of infection (Figure 1A, B, C) whereas visible *Ct* inclusions were seen in cells infected at MOI 2 (Figure 1, D, E). No copies of *Ct* cryptic plasmid were detected in uninfected cells, as assessed using qPCR. ESC exposed to UV-inactivated *Ct* (which still contained bacterial DNA) had $2.5 \times 10^4 – 7.5 \times 10^4$ plasmid copies per well. *Ct* infected wells contained $1.2 \times 10^6 – 3.3 \times 10^6$ plasmid copies indicating that significant replication had occurred (Figure 1F). Although the number of decidualised uninfected ESC was decreased compared to non-decidualised ESC, UV-treated and *Ct* infected ESC samples contained similar numbers of cells compared to decidualised uninfected ESC (Figure 1G). In infected wells, between 0.05% - 20% of ESC contained chlamydial inclusions (Supplementary Figure 1). These conditions were used for all subsequent experiments. Cells infected at MOI 3 appeared similar to those infected at MOI 2 (equivalent to 4×10^5 organisms per well, supplementary Figure 2).

Ct infection of human ESC results in defective decidualisation

Infection with *Ct*, but not treatment with UV-inactivated *Ct*, reduced the expression of the classic decidualisation marker prolactin (PRL) at both mRNA (Figure 2A) but not protein levels (Figure 2B) in decidualised *Ct* infected ESC. Similarly, exposure of decidualised ESC to either 200 or 100 μg/ml lipopolysaccharide (LPS) derived from either, *Escherichia coli* (*E. coli*) or *Salmonella enterica* serotype Minnesota (*S.* Minnesota) also failed to inhibit the of prolactin secretion (Supplementary figure 2). Decidualised uninfected ESC had increased levels of both PRL mRNA and protein compared to non decidualised ESC.

Ct-infected human ESC have an altered chemokine profile

To investigate the effect of *Ct* infection on chemokine secretion by decidualised ESC, the levels of 31 secreted chemokines were examined by proteomic array in supernatants from decidualised uninfected and infected ESC. All of the chemokines on the array were detected and a pattern of preferential expression between infected and uninfected cells was seen (Figure 3). Supplementary table 1 shows that four chemokines were upregulated and 17 downregulated in *Ct* infected decidualised compared to non-infected ESC (cutoff 5%). Of the chemokines known to be involved with early pregnancy, trophoblast invasion and/or genital infection CXCL12 and CXCL16 were both strongly downregulated in infected decidualised ESC, as were chemokines CCL7, CCL12 and Midkine whilst CXCL7, CXCL1 and XCL1 were strongly upregulated (see Supplementary table 1 for further details). To validate the array results, qPCR and ELISA were used to measure changes in mRNA and protein levels of chemokines CXCL12 and CXCL16 as a result of infection with *Ct*. As seen in Figures 4A and C, there were no significant differences in mRNA encoding CXCL12 and CXCL16. In contrast, infected ESC secreted significantly less CXCL12 and CXCL16 (Figure 4B, D). CXR4 (the receptor for CXCL12) mRNA was significantly decreased in infected decidualised ESC (Figure 4E). In contrast, CXCR6 (the receptor for CXCL16) mRNA was not affected by *Ct* infection (Figure 4F).

Discussion

Herein, we demonstrate for the first time that *Ct* can infect and proliferate in human endometrial stromal cells (ESC) and that active infection impairs decidualisation and alters the secretion of chemokines.

To our knowledge, all previous studies have only explored the effect of *Ct* on epithelial cells of the female reproductive tract[20–22], however as we have demonstrated here *Ct* can also infect and affect the function of ESC. In light of this novel finding, we propose that ascending genital *Ct* infection might be a more complicated process than previously thought, as *Ct* could breach the epithelial barrier of the endometrium and infect other cell types such as stromal cells, endothelial cells or glandular epithelial cells (endometrial structure reviewed in[1]) causing extended inflammation. Infection with *Ct* has been reported to cause endometritis and some studies have suggested an association of chronic endometritis with miscarriage and implantation failure[23,24]. The effect of a *Ct* infected endometrial stromal compartment on gestation-related processes, such as decidualisation, might at least partially explain infection related adverse pregnancy outcomes.

Using an in-vitro model, we demonstrated that *Ct* infection attenuates ESC decidualisation. Furthermore, in the current model active *Ct* infection, but not exposure to either UV-inactivated organisms or to purified *E. coli* or *S.* Minnesota LPS, caused a reduction in mRNA but not protein levels of the widely used phenotypic decidualisation marker, PRL. PRL protein levels may not be altered due to the infection occurring after ESC decidualisation however a reduction of mRNA potentially indicates impaired decidualisation capability of *Ct* infected ESC. PRL is a key factor in the process of decidualisation and is thought to also be involved in epithelial cell differentiation, implantation, angiogenesis, trophoblast cell growth and immune regulation during early pregnancy[25]. We believe this observation is important because a reduction of endometrial PRL has been linked recently to recurrent miscarriage[12].

Furthermore, we show that *Ct* infection changes the chemokine secretion profile in decidualised ESC. Interestingly, we observed a reduction in the levels of CXCL12 and CXCL16. The reduction in protein expression in the culture supernatants cannot be attributed to increased receptor-ligand interactions as the receptor for neither chemokine showed increased expression. We believe that this may have relevance to the role of *Ct* in miscarriage because these cytokines are known to promote trophoblast migration[26–28]. It is possible that that infection could therefore lead to defective trophoblast invasion due to lack of essential

attractant signalling molecules secreted from maternal decidua, such as CXCL12 and CXCL16.

Our findings are also important because decidualised endometrium from spontaneous and recurrent miscarriages have been reported to have different immune cell profiles compared to viable pregnancies, including increased levels of uterine natural killer cells (uNK) and macrophages[29,30]. Dysregulated chemokines due to *Ct* infection, as indicated in our array, could therefore also impact on the population of immune cells at the feto-maternal interface by altering immune cell recruitment.

In summary, our data suggest a novel mechanism through which infection leads to defective endometrial stromal cell decidualisation, resulting in an altered immune response that could impact upon trophoblast migration and immune cell recruitment. Future work to clarify the potential role of *Ct* and other bacterial infections upon trophoblast invasion via CXCL12 and CXCL16 dysregulation and immune cell recruitment in the endometrium due to altered chemokine profile could further our understanding of this potential mechanism of infection associated miscarriage.

Materials and methods

Subjects

Ethical approval for this study was obtained from the Lothian Research Ethics Committee (LREC10/S1402/59). Informed written consent obtained from all patients and all of the methods were carried out in accordance with the approved guidelines. Human endometrial stromal cells (ESC) were collected after informed written consent from women undergoing hysterectomy for benign gynaecological conditions. Only samples from women in the proliferative phase of the menstrual cycle (cycle staging determined by the last menstrual period and measurement of serum estradiol and progesterone levels) were selected for further experiments. The tissue was dissociated using enzymatic digestion with 1mg/ml of collagenase type IV for two hours at 37$^{\circ C}$, (Sigma C5138), followed by mechanical breakdown using a 70μm and subsequently 40μm filter (Falcon, Corning, Cat. No 352350 and 352340 respectively). Single cells were plated in RPMI 1640 media (Sigma, Cat. No. R0883) supplemented with 10% heat inactivated foetal calf serum (HIFCS, Gibco, Cat. No 10082139), 1% L-glutamine (Sigma, Cat. No G-7513) and 1% Penicillin/ Streptomycin (Sigma, Cat. No. P4333).

Primary human endometrial stromal cell (ESC) culture

Prior to any treatments, cells were transferred to phenol red-free RPMI 1640 (Sigma, Cat. No R7509) containing 10% charcoal stripped fetal calf serum (CSFCS, prepared in house from FCS), 1% L-glutamine and 0.5% gentamycin (Sigma, Cat. No. G1272) for 48 hours prior to use. The antibiotic gentamycin was used instead of the standard penicillin/ streptomycin regimen, because it does not inhibit *Ct* growth[31]. Cells were maintained at 37°C and 5% CO2. Treatments were completed in duplicate per experiment and were repeated on 4 (for initial chlamydial growth experiments) or 6 (prolactin/ chemokine expression) times using cells derived from different patients on each occasion.

In-vitro decidualisation

To assess the effects of chlamydial infection on the decidualisation of primary endometrial stromal cells, an in-vitro decidualisation method previously described was used[32]. Cells were trypsinised, spun for 3.5 minutes at 800rpm and resuspended phenol red-free RPMI 1640 with 10% CSFCS. They were then plated at 10^5 cells per well of a 12-well plate for a minimum of

24 hours. Before the decidualisation protocol commenced, cells were serum starved in 2% CSFCS medium for 24 hours, supplemented as previously described. The cells were treated with 10-6M progesterone (Sigma, Cat. No. P0130) and 8-Bromo-adenosine 3'-5'-cyclic monophosphate (cAMP, Sigma, Cat. No. A9501) at a final concentration of 0.1 mg/ml. The medium was changed every 48 hours over a six-day period (supplementary table 2).

Ct elementary bodies (EBs) growth and purification

Chlamydia trachomatis (Ct) serotype E stock was produced in HEp2 cells using well established protocols. Sub-confluent flasks of HEp2's were inoculated with Ct. Infected cells were cultured for 48-72 hours in Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Paisley, UK) supplemented with 2% heat inactivated fetal calf serum (FBS) and 1 µg/ml cycloheximide (Sigma, Cat. No C4859) (PAA laboratories Ltd, Yeovil, Somerset, UK), until high numbers of mature inclusions were observed by optical microscopy. The cell monolayers were disrupted with glass beads and the medium containing cell debris was briefly sonicated (Vibracell, Sonics & Materials, Connecticut, USA) and centrifuged at 50 x g for 5 minutes at 4°C to remove intact cells. The supernatant was removed and centrifuged at 12,000 x g using a J-LITE JLA-16.250 rotor (Beckman Coulter Ltd. High Wycombe, UK). The pellet was resuspended by sonication in Tris/KCL and 25ml of inoculum were layered onto 10ml 40% Gastrografin® (Bayer plc, Berkshire, UK) and centrifuged at 20,000 rpm at 4°C for 45 minutes SW 32Ti rotor on Optima L-90K ultracentrifuge (Beckman Coulter Ltd). The pellet was re-suspended into 1ml Tris/KCL and layered onto discontinuous gradient (54%, 44%, 34% Gastrografin®) and centrifuged at 20, 000 rpm at 4°C for two hours. The interface between the 44% and 54% layers, containing the EBs was carefully removed. The EBs were washed by resuspension in 10ml Tris KCL and centrifugation at 20, 000 rpm at 4°C for a further 45 minutes, to remove residual traces of Gastrografin®. The final pellet was resuspended by sonication in 10 ml of Chlamydia Transport Medium (218mM Sucrose, 3.76mM KH2P04, 7.1mM K2HP04, 4.9mM L-glutamic acid, 10% FBS, 0.05 mg/ml Gentamycin, 0.1mg/ml Streptomycin, 150U/ml Nystatin). and the aliquots stored at -80OC.

UV inactivation of Ct elementary bodies

To UV inactivate *Ct* EB's, 500 µl of inoculum was exposed to 2J UV-C. The successful inactivation of *Ct* was confirmed by cell culture of HeLa cells with UV inactivated bacteria for 96 hours without the presence of any inclusions.

Titration of Ct stock

To determine the titre of the *Ct* stock, HEp2 cells were plated at 10^5 per well of 8 well glass chamber slides, infected with serial tenfold dilutions of *Ct* inoculum (Multiplicity of infection [MOI] $1 – 10^{-8}$) and cultured for 48 hours. The cells were fixed in ice-cold acetone for 5 minutes, left to air dry and stored at -20°C. After thawing, the slides were rehydrated in PBS and incubated with the primary antibody (*C. abortus* LPS, Santa Cruz Biotechnology, Cat. No. 13/4) for 1 hour at room temperature. Following PBS washes, the slides were incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Sigma) for 60 minutes at room temperature in a light-tight humidity chamber. Slides were mounted using ProLong® Gold Antifade Mountant with DAPI (lifeTechonologies, Cat. No. P36930). The total number of inclusions per well was counted at serial dilutions 10^{-5}-10^{-7} and the titre of stock was calculated.

_Ct_ infection of decidualised ESC

ESC were seeded at 12-well plates and decidualised as described above. On day 6 of the protocol, ESC were infected with *Ct* MOI 0.01, 0.1, 1, 2 and 3 or UV-*Ct* at the same MOI for 48 hours prior to sample collection. To determine whether decidualisation of ESC was affected by exposure to lipopolysaccharide (LPS) from other bacterial species, decidualised ESC isolated from a further five subjects were exposed to LPS from either *E. coli* or *S.* Minnesota (both Invivogen) (each at 200µg/ml and 100µg/ml) for 48 hours prior to sample collection.

RNA extraction, quantification and quality assessment

To assess changes in expression levels of genes of interest, total RNA extraction was performed Using the RNeasy® Micro kit (Qiagen, Crawley, UK, Cat. No. 74004) following the manufacturer's protocol.

Real time PCR

All reactions were carried out in 384 well plates at a final reaction volume of 10µl per sample, as seen in supplementary table 3. The samples were mixed by repeat pipetting and centrifuged in a mini plate spinner (Applied Biosystems, Warrington, UK) at 1000g for 20 seconds to remove any bubbles. A standard curve of cDNA made from standardised placenta RNA (Ambion, Cat.No AM7950) was used on every plate to ensure comparability among all plates. All primers were predesigned KiCqStart® SYBR® Green Primers (Cat. No. KSPQ12012).

PCR conditions were an initial step of 3 min at 95°C, followed by 40 cycles of 5s at 95°C, 15s at 60°C and a final disassociation step consisting of 15s at 95°C, 15s at 60°C and finally 15s at 95°C.

DNA extraction from ESC

To extract DNA from infected cells, the MagMAX™ Total Nucleic Acid Isolation Kit (Ambion, Cat. No. AM1840) was used according to the manufacturers' protocol.

Ct plasmid copy assay

To assess whether *Ct* could proliferate in ESC the '*Chlamydia trachomatis* Genesig Standard Kit' (PrimerDesign, Cat. No Path-C.trachomatis-standard) was used. A standard curve of known concentration provided by the kit was used to absolutely quantify *Ct* plasmid copy numbers in DNA extracts from ESC cells using qPCR.

Human Chemokine Array

Proteome Profiler™ Human Chemokine Array Kit (R&DSystems, Abingdon, UK, Cat. No. ARY017) was used. Samples were pooled supernatants from ESC cultures and the manufacturer's protocol was followed without deviation. The data are expressed as percentage of positive control of each membrane and pixel density was measured using ImageJ.

285

286 Enzyme-linked Immunoabsorbant Assay (ELISA)

287 The sandwich ELISA was used to detect PRL (R&DSystems, Cat. No.DY682) protein
288 concentrations as per manufacturer's instructions. Sample concentrations were calculated
289 using MasterPlex® QT (Hitachi) software.

290

291 **Magnetic Luminex®Screening Assay**

292 The Luminex®Screening Assay (R&D Systems,Cat.No. LXSAHM) was used to detect
293 CXCL12 and CXCL16 as per manufacturer's instructions.

294

295 **Statistical analysis**

296 Statistical analysis was carried out using GraphPad Prism 6. Friedman's non parametric test
297 was used for all sample sets. The "n" number corresponds to individual patients.

298 Repeated measures non parametric ANOVA- Friedman's test was used for all sample sets. By
299 using a non parametric test it was assumed that the samples are not following a Gaussian
300 distribution, as the sample size was not suitable for a normality test. Friedman's test was
301 selected as using a repeated-measures test (RM one way ANOVA) controls for experimental
302 variability attributed to patient-to-patient response variations. Some factors not controlled for
303 in the experiment will affect all the measurements from one subject equally, so they will not
304 affect the difference between the measurements in that subject. By analyzing only the
305 differences, therefore, a matched test controls for some of the sources of scatter. Furthermore,
306 to compare specific groups, Dunn's method for non parametric multiple comparisons was
307 used, as it allows for comparison of two particular groups by determining each rank from the
308 entire data set (all groups), not just the two groups being compared. However, the absolute
309 value of the difference between the mean rank of the groups being compared is entered into
310 the calculation of the z ratio. All graphs are representing mean with standard deviation (SD)
311 of the data.

312

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Funding

This work was funded by an MRC Centre Grant (MRC G1002033) and Tommy's.

Acknowledgments

We would like to thank Professor Hilary Critchley for kindly donating the primary endometrial stromal cells used for this study. We would also like to thank Dr Ioannis Papastathopoulos, Chancellor's Fellow at the University of Edinburgh's School of Mathematics for reviewing the statistical analysis of this study.

Author Contributions Statement

AH, GE, and SH conceived the project. SG, NW, JW, IS, DG, PS performed experimental work. SG, SH and AH analysed the data. SG, SH, JB, PH and GE and AH wrote the manuscript. SG, SH, NW and AH prepared the figures and the supplementary file. All authors reviewed the manuscript.

Additional Information

Accession codes N/A

Competing financial interests

The authors have no competing financial interests to declare.

Figure legends

Figure 1. Ct infects decidualised ESC. ESC were infected with Ct MOI 2 following an in-vitro decidualisation protocol. Non decidualised uninfected ESC, uninfected decidualised ESC and UV-Ct treated ESC were used as controls. 48 hours post infection DNA was collected for qPCR and cells were stained with Giemsa. Cell counts were conducted in 15 fields of view per well measuring 0.32 mm^2 each. (a) Non decidualised uninfected ESC were elongated and thin. (b) Decidualised uninfected ECS became rounder and larger compared to non decidualised ESC. (c) Ct infected decidualised ESC displayed signs of infection and contained inclusions that were stained purple by Giemsa stain (highlighted by green circles). (d) UV-Ct treated decidualised ESC did not contain chlamydial inclusions and resembled uninfected decidualised ESC in appearance. (e) 25.000 – 75.000 Ct plasmid DNA copies were detected in UV-Ct treated ESC. Ct infected ESC had a significantly higher number of 1.200.000 – 3.200.000 plasmid copies per sample, indicating proliferation of Ct only in infected cells (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p=0.0094, n=4). (f) Cell counts on Giemsa stained ESC indicated that decidualised uninfected cells were significantly fewer compared to non-decidualised controls (RM one-way ANOVA-Friedman's test with Dunn's multiple comparisons test, p= 0.0185, n=4). In contrast, UV-Ct treated cells and Ct infected cell numbers were similar to uninfected decidualised ESC. Scale bars equal 200µm. Graphs show the mean and standard deviation.

Figure 2. *Ct* serovar E infection of ESC results in suboptimal decidualisation of ESC. (a) PRL mRNA is upregulated in response to decidualisation stimulus, whereas it is downregulated in infected decidualised cells compared to uninfected controls (n=5, One way ANOVA Friedman's test with Dunn's multiple comparisons test, p=0.0009 and 0.0151 respectively). (b) PRL protein levels are increased in decidualised non infected cells compared

to non decidualised controls as expected (n=5, One way ANOVA Friedman's test with Dunn's multiple comparisons test, p=0.0044). Reduction of PRL is observed only in cells infected by *Ct* compared to decidualised controls, indicating the adverse effect of *Ct* infection on decidualisation (n=5, One way ANOVA Friedman's test with Dunn's multiple comparisons test, p=0.028). Each circle/square represents an individual patient.

Figure 3. Chemokine proteomic array of 31 chemokines on pooled cell supernatants from *Ct* infected and uninfected decidualised cells. Each chemokine is represented by a dot. (a) *Ct* infected decidualised ESC secreted chemokine profile, demonstrating ESC secrete all 31 chemokines. (b) Uninfected decidualised ESC secreted chemokine profile that is altered compared to *Ct* infected ESC. (n=3)

Figure 4. Innate immune response of decidualised ESC to *Ct* infection. The innate response was determined by measurement (at an mRNA and protein level) of trophoblast invasion-associated chemokines CXCL12 and CXCL16. (a-b). CXCL12 mRNA was not altered in response to infection, however there was a reduction in protein levels in cell supernatants (n=4, One way ANOVA Friedman's test with Dunn's multiple comparisons test, p= 0.0267). (c-d) Similarly, there was less secreted CXCL16 protein observed only in infected cells however no change in mRNA levels (n=4, One way ANOVA Friedman's test with Dunn's multiple comparisons test, p= 0.0078). (e) CXR4 mRNA, receptor of CXCL12, was increased only in infected

cells (n=7, One way ANOVA Friedman's test with Dunn's multiple comparisons test,, p=0.0267). f. CXCR6, receptor of CXCL16 does not seem to be affected by *Ct* infection in mRNA level. Each circle/square represents an individual patient.

8. Appendix 2

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Giakoumelou, S. *et al.* The role of infection in miscarriage. *Hum. Reprod. Update* (2015). (Epub ahead of print)

Human Reproduction Update Advance Access published September 19, 2015
Human Reproduction Update, Vol.0, No.0 pp. 1–18, 2015
doi:10.1093/humupd/dmv041

human reproduction update

The role of infection in miscarriage

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Submitted on May 5, 2015; resubmitted on August 27, 2015; accepted on September 1, 2015

TABLE OF CONTENTS

BACKGROUND: Miscarriage is the spontaneous loss of a pregnancy before 12 weeks (early miscarriage) or from 12 to 24 weeks (late miscarriage) of gestation. Miscarriage occurs in one in five pregnancies and can have considerable physiological and psychological implications for the patient. It is also associated with significant health care costs. There is evidence that potentially preventable infections may account for up to 15% of early miscarriages and up to 66% of late miscarriages. However, the provision of associated screening and management algorithms is inconsistent for newly pregnant women. Here, we review recent population-based studies on infections that have been shown to be associated with miscarriage.

METHODS: Our aim was to examine where the current scientific focus lies with regards to the role of infection in miscarriage. Papers dating from June 2009 with key words 'miscarriage' and 'infection' or 'infections' were identified in PubMed (292 and 327 papers, respectively, on 2 June 2014). Relevant human studies (meta-analyses, case–control studies, cohort studies or case series) were included. Single case reports were excluded. The studies were scored based on the Newcastle – Ottawa Quality Assessment Scale.

RESULTS: The association of systemic infections with malaria, brucellosis, cytomegalovirus and human immunodeficiency virus, dengue fever, influenza virus and of vaginal infection with bacterial vaginosis, with increased risk of miscarriage has been demonstrated. Q fever, adeno-associated virus, Bocavirus, Hepatitis C and *Mycoplasma genitalium* infections do not appear to affect pregnancy outcome. The effects of *Chlamydia trachomatis*, *Toxoplasma gondii*, human papillomavirus, herpes simplex virus, parvovirus B19, Hepatitis B and polyomavirus BK infections remain controversial, as some studies indicate increased miscarriage risk and others show no increased risk. The latest data on rubella and syphilis indicate increased antenatal screening worldwide and a decrease in the frequency of their reported associations with pregnancy failure. Though various pathogens have been associated with miscarriage, the mechanism(s) of infection-induced miscarriage are not yet fully elucidated.

CONCLUSIONS: Further research is required to clarify whether certain infections do increase miscarriage risk and whether screening of newly pregnant women for treatable infections would improve reproductive outcomes.

Key words: miscarriage / infection / female tract / pregnancy

Introduction

Miscarriage is one of the most common yet under-studied adverse pregnancy outcomes. In the majority of cases the effects of a miscarriage on women's health are not serious and may be unreported. However in the most serious cases symptoms can include pain, bleeding and risk of haemorrhage. Feelings of loss and grief are also common and the psychology and mental health of those affected can suffer (Engelhard et al., 2001).

For the purposes of this review 'miscarriage' is defined as the spontaneous loss of a pregnancy during the first 24 weeks of gestation (Fig. 1). For most women, a miscarriage is an individual event and will be followed by a successful pregnancy ('spontaneous miscarriage', termed 'miscarriage' from this point onwards). A small number (0.5–1%) of women wishing to have children may experience three or more successive miscarriages, a condition known as 'recurrent miscarriage' (Bullett et al., 1996). 'Early miscarriage' is defined as pregnancy loss during the first trimester of pregnancy (less than 12 weeks of gestation) and occurs in up to one in five pregnancies. 'Late miscarriage' occurs during the second trimester (12–24 weeks of gestation) and is less common, occurring in 1–2% of pregnancies (Hay, 2004). Fetal death from the 25th week of gestation onwards is defined as stillbirth, an outcome taken into consideration in some of the studies included here, however it is not the main focus of this review.

Although miscarriage is considered the most common adverse pregnancy outcome, worldwide figures are not available. In 2012–2013 there were 729 674 live births recorded in England and Wales (Office for National Statistics, 2012). Loss of one in five pregnancies suggests that this figure is accompanied by ~200 000 miscarriages. Statistics from England and Wales for 2012/13 report that 39 800 miscarriages resulted in a hospital stay (Office for National Statistics, 2012). In an Australian prospective cohort including 14 247 women aged 18–23 years, the rate of miscarriage varied from 11.3 to 86.5 per 100 live births amongst different groups; overall, miscarriage occurred in 25% of the women in the study when the women were 31–36 years old (Hure et al., 2012).

Aetiology of miscarriage

The causes of miscarriage are often unknown. However, in ~50% of early miscarriages the fetus exhibits chromosomal aberrations such as a structural alteration or abnormal chromosomal numbers (Eiben et al., 1990; Suzumori and Sugiura-Ogasawara, 2010). Several other factors have been associated with increased risk of miscarriage. The age of both parents has a significant role as the risk of an adverse pregnancy outcome is increased if the parents are 35 years old or older and it is 50% higher if the mother is 42 years of age (Fretts et al., 1995; Nybo Andersen et al., 2000; de la Rochebrochard and Thonneau, 2002; Slama et al., 2005; Maconochie et al., 2007). In addition, factors such as ethnic origin, psychological state of the mother, very low or very high pre-pregnancy BMI, feelings of stress, use of non-steroidal anti-inflammatory drugs, smoking and alcohol consumption have also been associated with significantly higher rates of miscarriage (Coste et al., 1991; Nielsen et al., 2001; Sapori, 2002; Lashen et al., 2004; Maconochie et al., 2007). Moreover, it has been reported that women whose first pregnancy resulted in miscarriage are at a higher risk of the second pregnancy resulting in miscarriage compared with women who had a live birth (Kazhanian et al., 2006). Finally, a number of infections have been linked to miscarriage (Benedetto et al., 2004) and to other adverse outcomes, such as stillbirth (Goldenberg and Thompson, 2003) and preterm delivery (Garland et al., 2002). Specifically, 15% of early miscarriages and 66% of late miscarriages have been attributed to infections (Srinivas et al., 2006; Baud et al., 2008). In a recent study, 78% of 101 tissue samples from miscarriage were infected with bacteria (chorioamnionitis), whereas all the control samples from medically induced abortions were uninfected (Allanson et al., 2010).

Methods

The aim of this review is to summarize present knowledge regarding the role of infection in miscarriage. In order to combine the most recent findings regarding infection and a potential association with miscarriage, we focused on studies published in the past 5 years. Our aim was to investigate current evidence regarding high-risk pathogens and scientific research trends. In PubMed, using the key words 'miscarriage' combined with 'infection' and 'infections', with 'human', 'English language' and '2009-present' filters, articles published in the past 5 years were identified. The search returned a total of 292 and 327 papers for 'miscarriage infection' and 'miscarriage infections' respectively (up to 02/06/2014). From these, single case reports and studies in animals were excluded. A total of 44 studies investigated the association of different pathogens with miscarriage and the findings are presented in this review. The studies were also scored by two individuals independently based on the Newcastle - Ottawa Quality Assessment Scale for case control studies. The score of random studies was further evaluated by two more individuals.

Results

Infections associated with miscarriage

An overview of all the studies analysed is presented in Supplementary Table S1, including pathogen(s) investigated, outcome of the study and

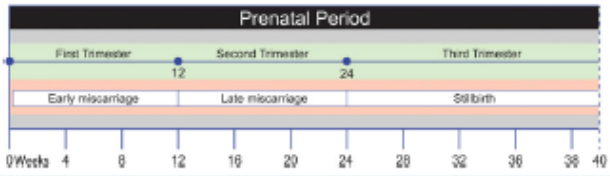

Figure 1 Adverse pregnancy outcomes across the three trimesters of pregnancy.

an estimation on the strength of each study, as described in Methods. Some of the most common caveats addressed in this review were variation in sample size and detection techniques, whether multivariate analysis was implemented or not and variation in study design.

Bacterial infections

Bacterial vaginosis

In healthy women, the normal genital tract flora consists for the most part of *Lactobacillus* species bacteria (Lamont *et al.*, 2011). Other potentially virulent organisms, such as *Gardnerella vaginalis*, group B streptococci, *Staphylococcus aureus*, *Ureaplasma urealyticum* (*U. urealyticum*) or *Mycoplasma hominis* (*M. hominis*) occasionally displace lactobacilli as the predominant organisms in the vagina, a condition known as bacterial vaginosis (BV) (Escherbach, 1993; Casari *et al.*, 2010). BV is present in 24–25% of women of reproductive age (Ralph *et al.*, 1999; Wilson *et al.*, 2002) and causes a rise in the vaginal pH from the normal value of 3.8–4.2 up to 7.0. It is usually asymptomatic but may result in a vaginal discharge, which can be grey in colour with a characteristic 'fishy' odour. BV is diagnosed using microscopic examination of vaginal swab samples for 'clue cells' and/or Nugent criteria and is commonly treated with antibiotics, such as metronidazole (Donders *et al.*, 2014). Change of sexual partner, a recent pregnancy, use of an intrauterine contraceptive device and antibiotic treatment have been identified as plausible causes of BV (Hay, 2004; Smart, 2004). BV has been associated with premature delivery (Hay *et al.*, 1994) and with miscarriage (Donders *et al.*, 2009; Rocchetti *et al.*, 2011; Tavo, 2013).

In a retrospective study from Albania, *U. urealyticum* and *M. hominis* were present in 54.3 and 30.4% of the patients (150 hospitalized women, presenting with infertility, who had had a miscarriage or medically induced abortion, Tavo, 2013). The prevalence of both pathogens was significantly higher among women with a history of miscarriage (*U. urealyticum*: $P = 0.04$ and *M. hominis*: $P = 0.02$) and women who reported more than one miscarriage ($P = 0.02$ for both pathogens). This study however has some weaknesses, as it is not clear whether the comparisons made were with non-infected women with a miscarriage history or non-infected women with no miscarriage history and the method by which prevalence of microbes was tested is not specified.

Data on the prevalence of group B streptococci and pregnancy outcome in 405 Brazilian women with gestational age between 35 and 37 weeks was published in 2011 (Rocchetti *et al.*, 2011). Overall, 25.4% of women were positive for *Streptococcus agalactiae* and infection was associated, among other factors, with a history of miscarriage (odds ratio (OR) 1.875; 95% confidence interval (CI) 1.038–3.387).

Association of BV and particularly *M. hominis* and *U. urealyticum* was reported from a study from Turkey (Bayraktar *et al.*, 2010). In total 50 pregnant women with BV symptoms were tested for *M. hominis* and *U. urealyticum* and observed until end of pregnancy. The pregnancy outcomes of 50 asymptomatic pregnant women were used as controls. Miscarriage was reported in 12 symptomatic women, in 8 of which *M. hominis* and/or *U. urealyticum* infection was confirmed. However, the definition of miscarriage used in this study was 'less' than 36 weeks. Furthermore, comparative analysis between the two groups was not carried out.

Ureaplasma urealyticum was also detected in 25% of 101 gestational tissue samples (chorion, amnion, umbilical cord) from miscarriage cases that were otherwise normal. Second most common pathogens were *M. hominis* and group B streptococci at 11.1%, whereas all controls were not infected (Allanson *et al.*, 2010).

In a further study using a cohort of 759 Belgian pregnant women following microbiological evaluation of vaginal flora, 8.4% of participants in the cohort presented with BV and were not treated (Donders *et al.*, 2009). BV was positively correlated with miscarriage, as 2% of positive women miscarried before 25 weeks gestation; with an OR of 6.6 (OR 6.6; 95% CI 2.1–20.9). An absence of lactobacilli was also associated with miscarriage (less than 25 weeks; OR 4.9; 95% CI 1.4–16.9, Donders *et al.*, 2009).

These studies indicate an association of BV with miscarriage. As BV is treatable, screening programmes for pregnant women can be used to prevent adverse pregnancy outcome. Current guidelines from the USA advise against screening asymptomatic pregnant women (U.S. Preventive Services, 2008). The same principle is applied in Canada (Yudin and Money, 2008) and the UK as of November 2014 (UK National Screening Committee, 2014). A recent Cochrane review, including 7847 women in 21 trials, found decreased risk of late miscarriage when antibiotic treatment was administered (relative risk (RR) 0.20; 95% CI 0.05–0.76; two trials, 1270 women, fixed-effect, $I^2 = 0\%$). As the authors highlight, further studies are required to establish the effect of screening programmes to prevent adverse pregnancy outcomes (Brocklehurst *et al.*, 2013).

Brucellosis

Bacteria of the genus *Brucella* can infect a variety of wild and domesticated mammals. Cattle and deer are susceptible to *Brucella abortus* (*B. abortus*) whereas *Brucella melitensis* affects goats and sheep, causing fever and abortion; a disease known as brucellosis (Asluri *et al.*, 2011; Moreno, 2014). Humans can contract infection via consumption of unpasteurised dairy products (Corbel, 1997). Infection is detected via bacterial isolation from blood samples or serology (CDC—Centre for Disease and Prevention, 2012a).

Kurdoglu and colleagues in Turkey (Kurdoglu *et al.*, 2010), conducted a case–control study examining the miscarriage rate of 342 pregnant women with brucellosis compared with 33 936 uninfected women of similar socioeconomic status treated in the same hospital. The researchers concluded that 24.14% of infected pregnant women miscarried versus 7.59% of the controls. This result however could be influenced by statistical power, as the cases are ~100 times smaller than the control group.

The seroprevalence of brucellosis among 445 miscarriage cases and 445 control pregnant Jordanian women with no history of miscarriage consecutively recruited, matched for age, socioeconomic status and area of residence, was not significantly different (Abo-shehada and Abu-Halaweh, 2011). In the paper the researchers state that a sample of 441 was adequate as the prevalence of brucellosis is 8% in high-risk patients in contact with livestock (Abo-Shehada *et al.*, 1996), though their reference for statistical power could not be reviewed. The overall prevalence was similar in both groups; 1% in controls and 1.8% in cases.

The evidence suggests brucellosis is still a risk factor for miscarriage in areas where the infection is endemic in farm animals. This is in accordance with older studies that have reported high miscarriage rates among women with brucellosis (Lulu *et al.*, 1988; Khan *et al.*, 2001).

Chlamydia trachomatis

Chlamydia trachomatis, an obligate intracellular bacterium, is the most common sexually transmitted bacterial disease worldwide (Howie *et al.*, 2011). The prevalence of the disease is high, estimated at 101 million new cases in 2005 worldwide (World Health Organization, 2011). Though in women it is often asymptomatic, untreated

C. trachomatis infection can result in mucopurulent cervicitis (Brunham et al., 1984), acute urethral syndrome (Stamm et al., 1980) and pelvic inflammatory disease (PID) (Paavonen and Lehtinen, 1996). *Chlamydia trachomatis* infection is a known risk factor for ectopic pregnancy and preterm birth (Martin et al., 1982; Hillis et al., 1997; Egger et al., 1998; Kovács et al., 1998; Bakken et al., 2007; Shaw et al., 2011). Diagnosis is carried out by PCR on vaginal swab samples and treatment includes the administration of antibiotics, such as tetracyclines, azithromycin or erythromycin (Brocklehurst and Rooney, 2000; MedlinePlus, 2014).

The most recent case–control study investigating a potential association of *C. trachomatis* and miscarriage was published in 2011 (Baud et al., 2011). Using an enzyme-linked immunosorbent assay (ELISA) to detect *C. trachomatis* antibodies in sera, as well as a standard vaginal swab for *C. trachomatis* detection by PCR, on 145 cases and 261 controls, a positive association with miscarriage was observed. Immunoglobulin (Ig)G antibodies against *C. trachomatis* were present in higher levels in the miscarriage group (15.2%) than in the controls (7.3%; $P = 0.018$). The same pattern was observed for IgA antibodies only after adjustment for age, origin, education and number of sexual partners. Furthermore, *C. trachomatis* was detected using PCR in the placentae from cases more often than those from controls (4.0 and 0.7% respectively, $P = 0.026$). Subsequently, an observational study from Finland on 4920 women with genital tract infections has suggested that late complications can occur in *C. trachomatis* infected pregnant women (Kortelainen-Savolainen et al., 2012). However there was no control group in this study and neither were the terms 'early' or 'late' pregnancy defined, therefore limiting extrapolation of the findings.

In a study from Serbia, 21.3% of 54 miscarriage cases were shown to have persistent *C. trachomatis* infection as determined by levels of sera IgA against *C. trachomatis* major outer membrane protein (Arsovic et al., 2014). The authors suggest an association between persistent *C. trachomatis* infection and miscarriage, however these cases were compared only against patients with tubal infertility and not uninfected pregnant women.

Chlamydia trachomatis has been studied extensively and a lot of data are available for this infection from over three decades of research. Contradicting studies have been published, resulting in conflicting evidence regarding the role of *C. trachomatis* in miscarriage (Feist et al., 1999; Witkowska-Trojniel et al., 2009). Taking into account the most recent findings and the increase in screening programmes worldwide, such as the screening offered to all pregnant women in the USA (CDC, 2014), public awareness of the possible risk of *C. trachomatis* infection to a future pregnancy might be advisable.

Mycoplasma genitalium

Mycoplasma genitalium is a sexually transmitted bacterium, known to cause urethritis, cervicitis and PID, but infection can also be asymptomatic (Taylor-Robinson and Jensen, 2011). It has been suggested that *M. genitalium* can enhance human immunodeficiency virus (HIV) infection and transmission (Napierala Mavedzenge and Weiss, 2009); diagnosis is via PCR on urine samples (CDC, 2012b) and treatments include azithromycin and doxycycline (Horner et al., 2014). The only published study of this infection, is a case–control study from the USA on 392 women with miscarriage before 22 weeks of gestation and 802 healthy pregnant controls, and used data from participants originally enrolled in another study. Overall, *M. genitalium* had a prevalence of 5.9% but no association with miscarriage was found (Short et al., 2010).

Q fever

Q fever is a zoonotic infection, caused by the bacterium *Coxiella burnetii* (Maurin and Raoult, 1999). Infection is most commonly observed in humans who come into close proximity to livestock. *Coxiella burnetii* is usually transmitted via inhalation of infectious aerosols from animal fluids (Maurin and Raoult, 1999; van der Hoek et al., 2010). Infection is asymptomatic in half of all cases in adults but can present as an unspecific illness combined with pneumonia or hepatitis. Q fever is confirmed via PCR on blood samples (CDC, 2013). Recommended treatment in symptomatic adults and children is doxycycline administration. In pregnant women, Q fever infection has been associated with adverse pregnancy outcomes, as in a recent report from the United States Centres for Disease Control and Prevention (CDC) (Anderson et al., 2013). However, as the authors note, studies investigating serological evidence of infection and miscarriage have produced contradictory results. Screening of pregnant women is not currently recommended in the European Union (Munster et al., 2012).

Two Danish studies, one in 2012 and the second in 2013, concluded that *C. burnetii* is not linked to miscarriage (Nielsen et al., 2012, 2013). Both used randomized sera samples from the Danish National Birth Cohort. The first study was powered to detect whether infection could be associated with miscarriage. The presence of infection was investigated in a case group of 218 women with miscarriage (loss of pregnancy prior to 22 weeks of gestation) compared with 482 healthy pregnancies. The second study focused on pregnancy outcomes of 397 women exposed to cattle and sheep (high risk of exposure to *C. burnetii* infection) versus 459 women that had no contact with animals. *Coxiella burnetii* prevalence was 5% in cases and 6% in controls of the first study, whereas in the second study 19.5% of all women were positive, however 87% of these women had contact with livestock. Nielsen and colleagues (Nielsen et al., 2012) reported one positive miscarriage case (0.46%) and 3 (0.67%) seropositive among controls whereas in the second study two miscarriages were positive (Nielsen et al., 2013). These results suggest that, despite presence of *C. burnetii* infection especially in pregnant women in proximity with cattle and sheep, this bacterium does not seem to be widely associated with adverse pregnancy outcome, although individual cases have been reported.

Syphilis

Syphilis is a bacterial infection that can be transmitted sexually or via contact with the blood of an infected person. It is caused by *Treponema pallidum*, diagnosed using PCR, and is treated with antibiotics (Cohen et al., 2013). Stage one symptoms include a highly contagious sore that develops during stage two to a rash accompanied by sore throat. The third and final stage is tertiary syphilis, which is not contagious but is very harmful.

Casal and colleagues (Casal et al., 2012) assessed risk factors associated with syphilis and pregnancy outcomes in a Brazilian population. The cases consisted of women positive for syphilis, 169 with live births and 68 who had an adverse pregnancy outcome. This included miscarriage, stillbirth and neonatal death grouped together. The control group of women negative for syphilis included 219 women who had live births and 83 with adverse pregnancy outcome. Syphilis was significantly associated with history of miscarriage (OR 3.31; CI 2.20–4.99; $P < 0.0001$) after testing using a multiple regression model. Most of the pregnancies resulting in live births were not

completely asymptomatic when infection was present, resulting in outcomes such as prematurity, low birthweight and respiratory problems, among others. They also observed that maternal syphilis was associated with illegal drugs, alcohol, no counselling on syphilis, sexual activity initiation at 16 years of age or younger, two or more sexual partners during the preceding 1.5 years, life in a household with a low income and poorer sanitation; all factors that may also have a detrimental impact on reproductive outcome.

A study from China reported that, following a screening programme aiming to prevent mother-to-child syphilis transmission, the adverse pregnancy outcomes including miscarriage were reduced from 27.3% in 2003 to 8.2% in 2011 (Hong *et al.*, 2014).

The effect of syphilis on pregnancy has been a subject of interest for almost 100 years; general consensus is that syphilis can have a devastating effect on fetuses resulting in miscarriage, stillbirth and congenital transmission (Temmerman *et al.*, 1992; Oswal and Lyons, 2008). Syphilis screening programmes are in effect in the USA and EU (CDC, 2014; Janier *et al.*, 2014).

Viral infections

Herpes virus infections

The Herpes family of DNA viruses includes a number of pathogenic viruses of humans (Human Herpes Viruses/HHV) that can remain latent in the host and can reactivate (Whitley and Roizman, 2001). Two members of this family, HSV-1 (HHV1) and HSV-2 (HHV-2) establish latency in neuronal cells and on reactivation can cause herpes genitalis or labialis (Margolis *et al.*, 2007). Cytomegalovirus (CMV) (HHV-5) is also a very common virus, acquired by most people during childhood (Chisholm and Lopez, 2011). CMV infects mostly myeloid cells and is never eradicated from the body (Koch *et al.*, 2006). Herpes viruses can be diagnosed using PCR in sera samples (Singh *et al.*, 2005).

HSV-1 and HSV-2

HSV1 and/or HSV2 DNA were detected in 43.5% of 95 frozen trophoblastic tissue samples from Greek women with spontaneous pregnancy loss compared with 16.7% of women undergoing elective abortion ($n = 35$, $P = 0.03$, Fisher's exact test) (Kapranos and Kotronias, 2009). Using *in situ* hybridization HSV DNA was detected in the trophoblast of 18 out of 25 HSV positive cases. The authors concluded that HSV seems to have a role in early miscarriage, although they did not distinguish between the two types of HSV.

These data are supported by a more recent study from Korea (Kim, *et al.*, 2012b). The authors of this study tested sera of 500 pregnant women for HSV-2 and 85 (17%) were seropositive. Most of the women in both groups also tested positive for rubella, varicella zoster (HHV-3) and hepatitis B (HEPB), however the authors adjusted for this. Of HSV-2 seropositive women, 38.8% had a history of miscarriage compared with 29.6% of the control group ($P < 0.05$).

A possible association of HSV1 and HSV2 with miscarriage cannot be ascertained from these reports and further studies are required.

Human CMV/HHV-5

Hadar and colleagues studied a group of seropositive 59 women with peri-conceptional CMV infection, which occurred between 4 weeks prior to the last reported menstrual period and up to 3 weeks after the expected date of the period. Out of these women, four had miscarriages before undergoing amniocentesis to confirm intrauterine infection. The remaining patients either elected to terminate the pregnancy or gave birth to live infants. No conclusion could be drawn with regards to miscarriage association as no controls were included in this study (Hadar *et al.*, 2010).

Data from a Malaysian study (Saraswathy *et al.*, 2011) showed that anti-CMV IgG antibody was detected in 84% of healthy pregnant women as well as women with adverse pregnancy outcome, including 17 cases of miscarriage.

Despite the lack of recent studies supporting an association of CMV with miscarriage, *in vitro* studies have shown that CMV infection can result in placental dysfunctions (see below). However, further studies are required to elucidate the true role of CMV in adverse pregnancy outcomes.

Human papillomavirus

Human papillomaviruses (HPV) comprise a group of over 150 different types of small DNA viruses some of which cause common sexually transmitted infections (Cutts *et al.*, 2007). Sexually transmitted HPV infection has a prevalence rate of 11.7% in the general female population of reproductive age (Bruni *et al.*, 2010). According to CDC, sexually transmitted HPV prevalence nationwide in the USA among women 14–59 years old was 42.5% in 2003–2006, an estimation based on positive servicovaginal swab tests (Hariri *et al.*, 2011). Persistent infection with high-risk types of HPV (the most prevalent being HPV 16/18 worldwide) have been associated with cervical cancer, and others (HPV 6/11) with genital warts (Cutts *et al.*, 2007; Crosbie *et al.*, 2013). The vast majority of infections are asymptomatic and clear naturally without causing long-term disease and a vaccine is now available for types 6, 11, 16 and 18 (Cutts *et al.*, 2007). HPV infection cannot be diagnosed by blood tests, however PCR on cervical cell samples is used to determine specific viral genotypes following a positive Papanicolaou (PAP)-test (Molijn *et al.*, 2005).

The results of recent studies into the effects of HPV infection upon miscarriage are contradictory (Perino *et al.*, 2011; Skoczyński *et al.*, 2011; Yang *et al.*, 2013). A study in China on the effect of HPV on the pregnancy outcome of IVF treated patients found no difference in miscarriage rates between women with abnormal cervical cytology who had a positive high risk HPV test ($n = 56$) and those who tested negative for high-risk HPV ($n = 56$, Yang *et al.*, 2013). A second study from Poland tested for 33 HPV genotypes and also for specific HPV 16/18 DNA presence in placentae from miscarriages ($n = 51$) and from term deliveries from women who showed no signs of systemic infection (but were not tested, $n = 78$). They found HPV DNA in 17.7% of miscarriage cases and in 24.4% control placentae. A total of 11.8% of miscarriages and 12.8% of normal placentae were positive for HPV 16/18, but none of these differences reached statistical significance (Skoczyński *et al.*, 2011). Both of these studies suggest that HPV infection in women has no effect on pregnancy outcome, although no more than 150 women were examined in either study.

Conversely, results from a 2011 study significantly associated male partner HPV infection with miscarriage rate in 199 couples attending IVF clinics in Italy (66.7% in HPV infected couples versus 15% of controls with no HPV infection, $P < 0.01$, Perino *et al.*, 2011). The researchers also identified that all pregnancies in couples where both partners were infected resulted in miscarriage ($n = 9$).

These studies present contradictory data, however the first two examined infection in female partners whereas the second one

investigated male partners. Interestingly, an older study (Hermonat et al., 1997) reported HPV DNA presence in 15/25 early miscarriage samples compared with 3/15 first trimester elective abortion samples. Further well-designed, adequately powered studies are required to fully elucidate the role of HPV as a potential risk factor for miscarriage, whilst considering the role of an infected male partner as there are indications of a potential role in early miscarriage (Garolla et al., 2011).

Parvovirus infection

Parvoviruses belong to the Parvoviridae family and are very small single stranded DNA viruses that infect invertebrates and vertebrates (Cotmore et al., 2014). Of interest to studies of miscarriage are Adeno-associated virus (AAV), Parvovirus B19 (B19V) and Bocavirus (BC).

AAV

Antibodies against several serotypes of AAV show infection in various tissues, but it is asymptomatic (Gao et al., 2004). AAV needs the help of a helper virus, adenovirus, to replicate. Despite this, 80% of the human population is seropositive for AAV, as diagnosed by PCR (Gonçalves, 2005).

No association of AAV infection with serotypes 2, 3 and 5 with recurrent miscarriage (defined as two or more) was found in couples with subfertility (Schlehofer et al., 2012). A total of 146 semen samples as well as 134 endocervical samples from couples attending a fertility clinic were tested for the presence of AAV DNA and 14.9% of female and 19.9% of male samples were positive. No associations with other infectious pathogens, semen quality or subsequent fertility issues were indicated.

In another study (Pereira, 2010), the presence of AAV was examined in 81 patients, divided into three groups: 13 medically induced abortions, 29 miscarriages and 39 'undetermined' (including 66 decidua and 52 ovarian biopsies from the same patients). AAV DNA was detected in 23/81 (28.4%) of cases for at least one of the decidual or ovular fragments. Furthermore, 22/68 (32.3%) of spontaneous and 7.7% (1/13) of elective abortions (classified according to patient information) tested positive. The authors grouped cases with confirmed type of abortion and observed 28.6% (12/42) and 2.4% (1/42) AAV positive 'for spontaneous and medically induced abortion, respectively ($P < 0.05$)'. The classification of samples used as well as definition of the various groups compared in this study are unclear from the paper description, thus interpretation is challenging. The authors suggest a 'casual association' of AAV to miscarriage.

Despite the detection of AAV DNA in some miscarriage cases, there is inconclusive evidence for a role for this virus in miscarriage.

Parvovirus B19 (B19V)

Parvovirus B19 (B19V) is a small virus capable of causing different diseases in humans, such as 'Fifth disease' during childhood (Young and Brown, 2004). It is estimated that ~50% of young men and women have antibodies against B19V, determined via serology tests (Broliden et al., 2006). The remaining 50% of women are at risk of developing infection during pregnancy, which can lead to non-immune hydrops fetalis, a well-established cause of fetal death (Silingardi et al., 2009).

A recent study from Northern Ireland examined 3921 women of reproductive age and 33.5% of them were at risk of infection as they had no antibodies against B19V (Watt et al., 2013). Though fetal loss was reported in infected women with confirmed presence of the virus in miscarried fetuses, no increased association with miscarriage was observed. However, the authors reported 'inadequate follow-up' of pregnancies potentially associated with B19V infection.

In an earlier study of 72 pregnant women with B19V, it was noted that the risk of vertical transmission is higher if infection occurs by gestational week 20. Six out of eight cases of fetal loss observed were 'attributed to B19V infection' without further elucidation. No conclusions regarding the association were reached by the researchers (Bonvicini et al., 2011).

A higher percentage of IgM antibodies indicating recent infection was observed in women with adverse pregnancy outcomes (22.72%, $n = 88$) compared with 4.5% observed in 88 control healthy pregnant women (Brkic et al., 2011). Interestingly, anti B19V IgG antibodies were higher in controls than cases (70.5 and 53.4% respectively, $P = 0.046$). An important limitation of this study is that the adverse pregnancy outcome included miscarriage, non-immune hydrops fetalis and intrauterine fetal death, thus the association of miscarriage alone with B19V is not clear.

In a study from Nigeria, B19V prevalence among pregnant women was estimated at 40.7%, as 111 out of 273 patients in the study had detectable levels of either IgG or IgM antibodies, however these were not associated with a history of miscarriage (Emiasegen et al., 2011).

From the above, it is evident that a case–control study on women with miscarriage versus healthy pregnant controls, statistically powered to elucidate the role of B19V in miscarriage is required, as there are indications of high prevalence in pregnancy and fetal infection.

Bocavirus (BC)

The human BC is a newly discovered member of the parvovirus family detected in 93% of sera of children older than 3 years old (Kantola et al., 2010). In a study on 535 fetal biopsies (120 miscarriages, 169 intrauterine fetal deaths and 246 induced abortions), even though only 10% of women were seronegative, none of the fetuses tested positive and the authors concluded that BC could not have a possible role in miscarriage (Räsänen et al., 2010).

HIV

HIV is a retrovirus, and is most commonly transmitted via unprotected sexual intercourse or sharing of equipment for intravenous drug use. There are two types of HIV, HIV-1 and HIV-2, with the first being the most common (Gnann et al., 1987). The virus infects several cell types of the host immune system, such as CD4+ T lymphocytes (Miedema et al., 1988; Embretson et al., 1993), macrophages (Orenstein et al., 1997) and dendritic cells (Gringhuis et al., 2010). Worldwide, the World Health Organization (WHO) estimates that 34 million people are living with HIV, diagnosed by HIV viral load blood tests (PCR) (World Health Organisation, 2013a). Anti-retroviral treatment delays the onset of severe symptoms and protects the patient from opportunistic infections, which are the main cause of death among HIV-positive patients (Dybul et al., 2002).

A 2013 study from Nigeria examined 2381 pregnancies in 1702 women positive for HIV compared with 2381 pregnant non-infected women from the same hospitals. Following preterm delivery, miscarriage was significantly associated with HIV positivity (OR: 1.37; CI: 1.1–2.3). This association was retained after adjustment for several confounding variables such as age, parity, history of miscarriage and others. The infected women in this study were all receiving anti-retroviral treatment, however different regimes were used during the years in which the study was conducted. Limitations of this study include the number of controls

not being clearly stated and lack of testing for other sexually transmitted diseases (Ezechi et al., 2013).

In a study in Zambia 1229 HIV-positive pregnant women were followed up (Kim et al., 2012a, b). The ratio of miscarriages to live births was 3.1/100 and CD4 counts less than 350 cells/mm^2 were significantly associated with miscarriage. The women were recruited during both first and second trimesters and none of the women who miscarried had received anti-retroviral treatment. The study did not compare the cases with uninfected pregnant women.

In a study of 382 Ugandan/Zimbabwean HIV-infected pregnant women undergoing multiple anti-retroviral therapies (Gibb et al., 2012), miscarriage and medically induced abortions occurring prior to 22 weeks of gestation were assessed as one factor, not separate outcomes. Of note, fetal death after 22 weeks was classified as stillbirth. Therefore, no conclusions regarding miscarriage specifically can be drawn from this study. A study from India on 69 HIV-infected and 345 non-infected women demonstrated higher miscarriage/stillbirth risk amongst the infected group, however again there was no distinction between medically induced abortions and miscarriages (Datrik et al., 2011). In a retrospective analysis from Germany, 42% of HIV-positive women attending an outpatient clinic for preconception counselling became pregnant and only one miscarried (Gingelmaier et al., 2011).

HIV status was associated with miscarriage in a study of 1,218 pregnant women from Uganda (De Beaudrap et al., 2013). However, they defined stillbirth as the delivery of a non-living fetus ≥28 weeks gestation; and miscarriage as the delivery of a non-viable fetus either at <28 weeks gestation or weighing <500 g. Furthermore, as stillbirth and miscarriage were grouped together as one outcome, no definite conclusions regarding miscarriage can be drawn from this study.

To summarize, evidence suggests that HIV infection negatively affects pregnancy; however, anti-retroviral treatment can reduce the risk of adverse outcomes (Zolopa et al., 2009; Friedman et al., 2011). Furthermore, HIV has been associated with BV which could have a detrimental role on pregnancy outcome (Ledru et al., 1997). The presence of multiple diseases could further compromise a pregnancy. As most of the studies suggest, consultation and monitoring of HIV-positive women who wish to become pregnant is desirable. Women during their first antenatal visit are offered HIV tests in the UK, USA and EU (European Centre for Disease Prevention and Control, 2010; UK National Screening Committee, 2013; CDC, 2014).

Polyomavirus BK

Polyomavirus BK infects up to 90% of the general population via an unknown transmission route and is usually asymptomatic with the exception of immunocompromised individuals (Hirsch and Steiger, 2003). Antibodies against the virus can be detected using sera samples and PCR, urine cytology and viral immunostaining (Masutani, 2014).

Recent studies have investigated a potential role of BK virus infection on adverse pregnancy outcomes. A study on patients with unexplained villitis (infection of the placental villi associated with adverse pregnancy outcomes) detected no BK in placenta from miscarriages (Cajaiba et al., 2011). The authors state that 'For cases with diffuse villitis, the gestational age ranged from 31 to 41 weeks (average 37.2 weeks)'. It seems therefore more suitable to address these cases as stillbirths, not miscarriages. In another study from Italy, samples from five miscarried fetuses with chorioamnionitis and miscarriages due to chromosomal abnormalities (controls), BK was detected in fetal organs (Boldorini et al., 2010). Though this provides possible proof of vertical transmission of the virus, as it was detected in four out of five chromosomally abnormal controls and three out of five cases, the authors concluded that BK infection does not have a role in miscarriage. In accordance with the first study, the fetuses were between the 15th and the 28th week of gestation, so some of them were stillbirths according to our review's classification. Moreover, the fetuses were not matched for gestational age. In both studies, the numbers were small and no early miscarriages were tested. The question whether BK virus could be associated with miscarriage requires therefore further investigation.

Dengue fever

Dengue fever is a disease caused by four viruses of the single stranded RNA flaviviridae genus (DEN1-4), transmitted via mosquito bites usually in tropical and sub-tropical climates worldwide. WHO estimates 40–50 million new cases every year. Dengue is a flu-like illness with no vaccination and treatment currently available. Diagnosis is difficult as symptoms resemble other diseases, however usual approaches include DNA and antibody detection in serum samples using PCR and ELISA, respectively (CDC, 2012).

The role of dengue fever in miscarriage was examined in a prospective study from Malaysia on 115 women with miscarriage up to 22 weeks of gestation and 296 healthy pregnant controls. This study found significant association of recent dengue fever infection with miscarriage after adjusting for confounders such as maternal age, gestational age, parity and ethnicity (5.3% in cases versus 1.7% in controls, adjusted OR 4.2, 95% CI 1.2–14, $P = 0.023$, Tan et al., 2012).

In a case series report from Sri Lanka, two out of fifteen pregnant women experienced fetal death at 24 and 35 weeks of gestation, however the study provides no evidence of vertical transmission to the fetuses (Kariyawasam and Senanayake, 2010). In another case series report from French Guiana the authors reported two late miscarriages in 53 pregnant women with dengue fever. However, the infection could not be connected to the adverse pregnancy outcome (Basurko et al., 2009).

A systematic review on 30 studies concluded that it is unclear whether dengue fever is associated with adverse pregnancy outcomes (Poulliot et al., 2010). Based on recent evidence however, we can conclude that dengue fever seems to be a risk factor for miscarriage; therefore it is advisable to raise awareness regarding protective measures in high-risk areas and for people travelling to those areas.

HEPB and HEPC

The HEPB virus is a member of the Hepadnavirus family of small DNA viruses and the HEPC virus is a member of the flaviviridae genus of single stranded RNA viruses. Both viruses cause liver inflammation and disease and are both found in body fluids. HEPB is often resolved within a couple of months, however HEPC can develop into a chronic disease. Both diseases are diagnosed using blood serological tests (Gretch, 1997; Krajden et al., 2005).

In a case–control study from China, 75 couples that received assisted reproduction treatment were followed up, divided into a group with one partner diagnosed with chronic HEPB infection and a control group with both parents seronegative for HEPB (Ye et al., 2014). The early miscarriage rate (gestational week range not specified) was 44% in the case group compared with 9.1% in the control group ($P = 0.043$, Fisher's exact test). Highest miscarriage rates (60%) were observed when

mothers were seropositive and fathers seronegative ($P = 0.03$). Using PCR, HEPB DNA was detected in 6/62 'abandoned embryos' from the case group, whereas all embryos of the control group were negative. These results suggest a possible role of chronic HEPB infection in miscarriage.

Conversely, a cross-sectional study from Yemen examined the association of miscarriage with HEPB and HEPC infection in pregnant women, and found that 10.8% of women were positive for HEPB (95% CI: 8.0–14.0%) and 8.5% for HEPC (95% CI: 6.0–11.5%). No association of infection with miscarriage was apparent after multivariate analysis (Murad et al., 2013).

These studies raise questions regarding the role of persistent HEPB infection during pregnancy. At the moment, screening programmes of pregnant women for HEPB and HEPC during their first antenatal visit in the USA and UK aim to prevent adverse pregnancy outcome (UK National Screening Committee, 2013; CDC, 2014).

Rubella

Rubella is a mild childhood disease that, if acquired during the first 16 weeks of gestation, can result in miscarriage and serious fetal defects (Banatvala and Brown, 2004). A vaccine has been available for several years resulting in significant reduction in new cases according to the latest WHO progress report (Reef et al., 2011). Regardless of this progress, it is important to be aware that there remain a number of unvaccinated pregnant women in Europe and worldwide that do not have access to vaccination and who are still at risk of adverse pregnancy outcome due to rubella (Metcalf et al., 2011; Muscat et al., 2014).

Influenza virus

A study of the 1918 influenza pandemic concluded that it resulted in a decrease of live births due not only to high mortality but also to an increase of early miscarriages in pregnant women who were infected by the virus (Bloom-Feshbach et al., 2011). In a case series report regarding the H1N1 influenza A pandemic, six women were admitted to intensive care and had adverse pregnancy outcomes, however only one seriously ill patient had a spontaneous abortion as four cases occurred during the third trimester (Oluyomi-Obi et al., 2010).

Protozoan infections

Malaria

Malaria is caused by infection with protozoa of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*), is transmitted via mosquito bites and is endemic in more than 100 countries in Africa, Asia and South America (World Health Organisation, 2013b). In 2012 there were an estimated 207 million cases of malaria resulting in an approximately 627 000 deaths (90% of all malaria deaths occur in sub-Saharan Africa) (World Health Organisation, 2013b). Symptoms include fever, sweats, headache and diarrhoea and can be treated using different drugs depending on the symptoms and the specific pathogen causing the disease such as atovaquone plus proguanil or doxycycline (Kar and Kar, 2010). Malaria parasites are identified by microscopit examination of patients' blood samples. In 2007, 54.7 million pregnancies occurred in areas with endemic *P. falciparum* malaria and a further 70.5 million in areas with exceptionally low malaria transmission or with *P. vivax* only (Dellicour et al., 2010). Plasmodium can bind chondroitin sulphate A expressed on trophoblast and this is what causes local parasitaemia in the placenta (Agbor-Enoh et al., 2003). Maternal disease is most severe in primigravida women, and it reduces with each pregnancy as immunity builds up to those parasites that target the placenta (Fried et al., 1998).

Women with asymptomatic and symptomatic malaria (single episode before 14 weeks of gestation) are at a higher risk of miscarriage (adjusted OR 2.70, 95% CI 2.04–3.59 and 3.99, 95% CI 3.10–5.13, respectively). This study included 3527 women with miscarriage and 14 087 women that gave birth to live babies in Thailand. The risk ratios were not different for both *P. falciparum* and *P. vivax* (McGready et al., 2012).

De Beaudrap et al. (2013) also studied malaria during pregnancy in Uganda. In 1218 pregnant women no association of malaria with adverse pregnancy outcome was shown but an association with HIV status was demonstrated, as described above.

An association of malaria with adverse pregnancy outcomes, and more specifically miscarriage, is evident from the above studies. Prevention measures and screening of pregnant women at risk of malaria infection are advised.

Toxoplasmosis

Prevalence of Toxoplasmosis differs across the world, from 20–40% in the UK and USA (Food Standards Agency, 2012) to ~70% in tropical countries (Klaren and Kijlstra, 2002). In a recent study from London, 17.32% of 2610 samples tested were seropositive (Flatt and Shetty, 2013). Even though most patients are asymptomatic, immunocompromised individuals are susceptible to developing severe disease and women who become infected whilst pregnant can pass the infection vertically (Jones et al., 2001). The presence of *T. gondii* is confirmed by antibody detection.

Alvarado-Esquivel et al. (2014) showed that 6.7% of 326 women with a history of miscarriage had been exposed to *T. gondii*. This study however did not include a control group with no history of miscarriage. Miscarriage or stillbirth occurred in 28 out of 190 pregnant cases with toxoplasmosis presenting in England and Wales between 2008 and 2012, however these are data from the surveillance programme currently in place and not part of a study (Halsby et al., 2014). In a study on serum samples from 100 women who had miscarried, 86% of which were during the first trimester of pregnancy, 55% were seropositive for IgG against *T. gondii* (Vado-Solis et al., 2012), however, no comparison to uninfected pregnant women was made. A meta-analysis of several Mexican studies also indicates that infection rates are higher in women with miscarriage (Galvan-Ramirez et al., 2012). Despite this, as the authors highlight, only three out of the 132 studies included in their systematic review, were focused on women who had a miscarriage.

The likelihood of association of *T. gondii* infection resulting in miscarriage is highlighted by the present review of recent studies. Taking into account the significant worldwide prevalence of this protozoan infection, screening of pregnant women is recommended if it is established that this infection presents a significant risk for adverse pregnancy outcome.

How do infections lead to miscarriage?

Pregnancy is a complex process involving multiple cell types and regulated by several sophisticated mechanisms, which are still not fully

clarified despite years of research. To examine the negative impact of infections to pregnancy, we first need to understand how a normal, successful pregnancy is established.

Maternal-fetal interface: morphology, implantation process and the role of the immune system

The human endometrium is composed of several different cell types, including luminal and glandular epithelial cells, stroma with stromal fibroblastic cells, immune cells and blood vessels. During every menstrual cycle, in response to ovarian estrogen and progesterone via a process called 'decidualisation', the endometrial stromal compartment undergoes morphological and structural transformation to become receptive to implantation. Prior to implantation, the trophoblast differentiates into the growing blastocyst as it travels from the Fallopian tube to the uterus. The 'implantation window', during which the uterus is receptive to the embryo, is usually between 6 and 12 days after ovulation (Rashid *et al.*, 2011). The blastocyst attaches to the receptive endometrium utilizing adhesion proteins, called integrins, during the implantation window (Fig. 2A, Merviel *et al.*, 2001). Placenta formation begins as the trophoblast comes into contact with the epithelium and differentiates further into syncytiotrophoblast that invades the epithelial layer. Various other molecules, both from the maternal and fetal side, are involved in this process (reviewed in Dimitriadis *et al.*, 2005; Tranguch *et al.*, 2005; Achache and Revel, 2006; Chen *et al.*, 2009). Syncytiotrophoblasts, supported by the decidualised stroma (Godbole *et al.*, 2011), penetrate the endometrium and surround the embryo, whilst it embeds itself in the decidual stroma. A second trophoblast layer, the cytotrophoblast, is an inner layer without contact with the maternal cells. During the trophoblast invasion, cavities called lacunae develop, which, as they get filled with maternal blood, bring the maternal circulation into contact with the placental villi, thus marking the onset of placental circulation that includes exchange of nutrients and waste between the embryo and mother. At days 10–12 of gestation, the embryo is completely embedded in the endometrium, the epithelium has grown over it and the implantation process is complete (Fig. 2B). The three placental zones are now distinguishable: the early chorionic plate near the embryo, the intervillous space with the villous trees and the primitive basal plate in contact with the maternal endometrium (Pijnenborg *et al.*, 1980, 1981). Simultaneously, endovascular trophoblast cells stemming from the basal plate invade the walls of the spiral arteries, replacing the maternal muscular and endothelial cells with trophoblast cells, transforming the arteries into large diameter and low resistance blood vessels (Lyall, 2005).

The role of the immune system in a successful pregnancy is crucial (Fig. 3A). Whilst the immune tolerance of the semi-allogeneic fetus is maintained, several components of the immune system fulfil their designated roles in preparation for implantation as well as during gestation (Entrican, 2002; Chaouat *et al.*, 2004). Natural killer (NK) cells, macrophages and dendritic cells have all been detected in the feto–maternal interface (Guleria and Pollard, 2000; Moffett-King, 2002; Gardner and Moffett, 2003). Cytokines such as interleukin (IL-10), colony-stimulating factor (CSF-1) and transforming growth factor-β among others have been linked with the implantation process and are expressed in uterine cells (Altman *et al.*, 1990; Guleria and Pollard, 2000; Thaxton and Sharma, 2010). Implantation induces an inflammatory response because of invasion and damage of maternal tissue, with many cells undergoing apoptosis (Jerzak and Bischof, 2002; Joswig *et al.*, 2003). Conversely, inflammatory cytokines such as interferon-γ and tumour necrosis factor alpha (TNF-α) are not usually expressed in the placenta and have been associated with abortion in mouse models (Entrican, 2002).

Abnormal implantation, placentation or blood vessel transformation are thought to result in miscarriage (Michel *et al.*, 1990; Ball *et al.*, 2006). An active infection could interfere with the pregnancy by affecting any of the above-mentioned processes as well as disrupt the immune balance, whether it resulted in placental and fetal infection or not.

Examples of where we understand the mechanism of infection-induced miscarriage

For most of the pathogens where an association has been demonstrated, the exact mechanism that leads from infection to miscarriage is unknown. Bacteria, protozoa and viruses utilize different mechanisms to infect their host and each one seems to induce a unique cascade of events in the feto–maternal interface, most of which remains to be determined. Our knowledge is derived mostly from animal studies and data on human pregnancies are scarce.

Multiple mechanisms can be utilized by pathogens to cross the placental barrier. Plasmodium, as mentioned previously, enters the host via the maternal circulation and can infect and multiply in the trophoblast (Fig. 3B), even though its natural target cells are red blood cells (Agbor-Enoh *et al.*, 2003; Moreno-Pérez *et al.*, 2013). However, this mechanism of crossing the placental barrier is specific to malaria. *Listeria monocytogenes* uses two bacterial surface proteins called internalin A and B to invade the placenta, after passing from the intestinal barrier to the maternal circulation (Fig. 3B, Vázquez-Boland *et al.*, 2001; Lecuit *et al.*, 2004; Disson *et al.*, 2008). The presence of pathogenic organisms in the placenta induces a maternal immune response to infection that could result in miscarriage.

The susceptibility of placenta and fetus to several viruses has been investigated, as trophoblast cells have been identified as targets and viruses such as AAV and CMV have been detected in fetal tissue. CMV has been shown *in vitro* to replicate in trophoblast cells (Fig. 3B), in addition to epithelial, stromal cells and macrophages that are known target cells of the virus (Minton *et al.*, 1994; Fisher *et al.*, 2000; Sinzger *et al.*, 2008). In trophoblasts, CMV can induce an inflammatory response that increases apoptosis (Chou *et al.*, 2006). CMV has also been shown to activate TNF-α, again leading to cell death (Chan *et al.*, 2002). TNF-α is normally expressed in low levels by the placenta (Entrican, 2002); in the mouse CBA × DBA/2 model TNF-α was shown to increase fetal resorption via activation of NK cells, macrophages, and Th1-type cytokines (Clark *et al.*, 1998). Furthermore, decreased levels of implantation-associated matrix metalloproteinases 2 and 9 (MMP2 and MMP9) in the early pregnancy villi of women with CMV indicate compromised invasive capability, that could result in miscarriage (Tao *et al.*, 2011). These results suggest that CMV infection could lead to placental dysfunction as well as suggest possible routes of fetal infection resulting in miscarriage.

Bacterial infections initiate different responses from the immune system compared with viruses but gram-negative and gram-positive bacteria are both capable of activating the innate immune system

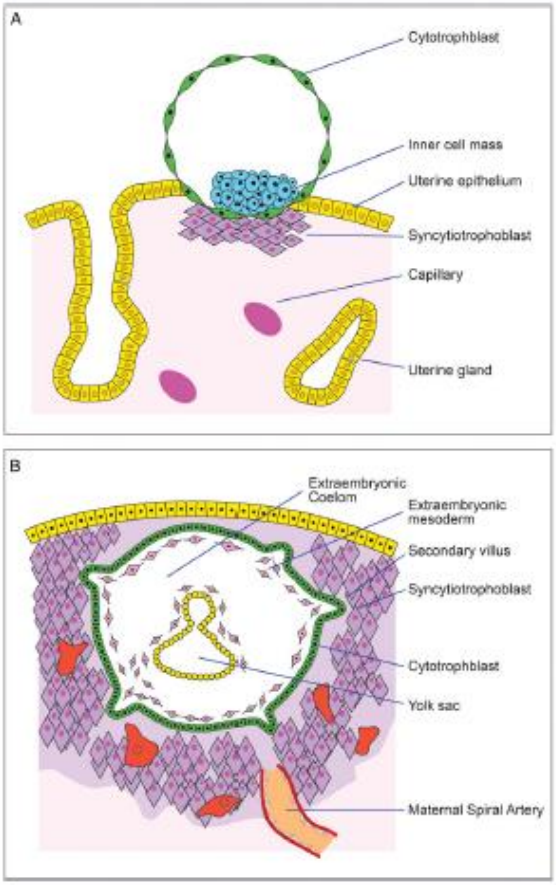

Figure 2 Implantation of blastocyst in the maternal endometrium. (**A**) During the implantation window (Day 6–12 post conception), the blastocyst adheres to the endometrium, and the placenta formation commences as the syncytiotrophoblast develops and invades the endometrium. (**B**) On Days 10–12 the implantation is completed as the embryo is encapsulated within the maternal tissue and the endometrial spiral arteries have been transformed into low resistance blood vessels, thus marking the onset of the placental blood flow.

(Takeuchi *et al.*, 1999; Yoshimura *et al.*, 1999). Most of our knowledge regarding bacterial infections and pregnancy comes from studies in mouse models. Nitric oxide and prostaglandins produced in the presence of bacterial lipopolysaccharides (LPS) were shown to be associated with embryonic resorption, as inhibition of this pathway reversed the effect in mice (Aisemberg *et al.*, 2010). Poor uterine receptivity and implantation failure due to exposure to bacterial LPS was also reported in another study in mice (Deb *et al.*, 2005).

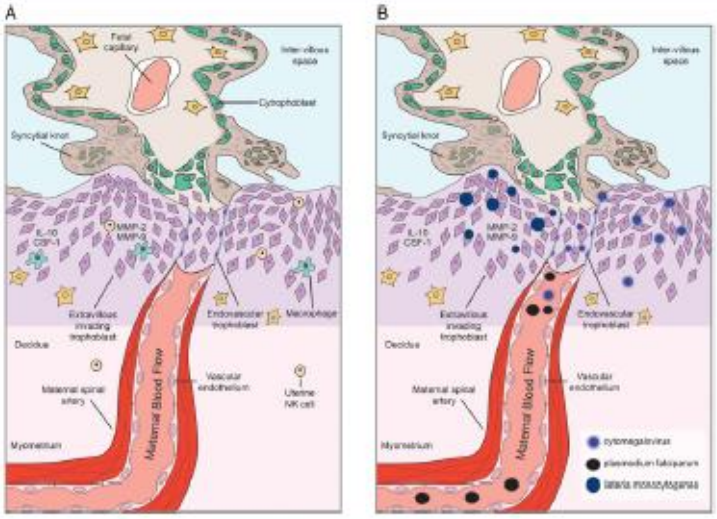

Figure 3 Healthy and infected feto–maternal interface. (**A**) During a healthy pregnancy, the interaction between maternal decidua, vasculature and immune cells (macrophages, uterine natural killer cells and dendritic cells) with fetal trophoblast and syncytial cells is the cornerstone of establishment and progression of pregnancy. Molecules such as interleukin (IL-10), colony stimulating factor (CSF-1) and transforming growth factor-β are essential for trophoblast invasion during the implantation process and are expressed by uterine cells. (**B**) Infections can disrupt the balance of feto–maternal interactions. *Plasmodium falciparum* can infect trophoblast cells entering via the maternal bloodstream. Cytomegalovirus and *Listeria monocytogenes* are examples of viral and bacterial infections known to interfere with trophoblast cells.

Table I Summary of pathogens and their association with miscarriage.

	Bacteria	Viruses	Protozoa
Associated with miscarriage	• Bacterial vaginosis (including *Mycoplasma hominis and Ureaplasma urealyticum*) • Brucellosis • Syphilis	• Cytomegalovirus • Dengue fever (Flavivirus) • HIV • Rubella	• Malaria (*Plasmodium*)
Little or no evidence for association with miscarriage	• *Coxiella burnetii* • *Mycoplasma genitalium*	• Adeno-associated virus • Bocavirus • Hepatitis C	• None
Conflicting evidence for association with miscarriage	• *Chlamydia trachomatis*	• Human papillomavirus • Herpes simplex virus 1 and 2 • Parvovirus B19 • Polyomavirus BK • Hepatitis B	• *Toxoplasma gondii*

HIV, human immunodeficiency virus.

Bacteria, viruses and protozoa utilize various mechanisms to infect fetal and maternal tissues (Fig. 3B), a few of which have been elucidated yet several remain unknown. These pathways are possibly implicated in miscarriage caused by infection. Further research is however required, as understanding the exact mechanisms behind infection-induced miscarriages could lead to effective treatment and thus prevention.

Table II Summary of the sites of detection of pathogens in the studies in the review.

Microbe	Site of detection			
	Fetus/placenta	Vagina/cervix	Serology/maternal blood/maternal urine	Paternal sample
Adeno- associated virus	Pereira (2010)	Schlehofer et al. (2012) Pereira (2010)		Schlehofer et al. (2012)
Bacterial vaginosis (including *M. hominis* and *U. urealyticum*)	Allanson et al. (2010)	Rocchetti et al. (2011) Reyniktar et al. (2010) Donders et al. (2009)		
Bocavirus	Räsänen et al. (2010)		Räsänen et al. (2010)	
Brucellosis			Kurdoglu et al. (2010) Abo-shehada and Abu-Halaweh (2011)	
Chlamydia trachomatis	Baud et al. (2011)		Baud et al. (2011) Kontiokangas-Savolainen et al. (2012) Arsovic et al. (2014)	
Coxiella burnetii			Nielsen et al. (2012, 2013)	
Cytomegalovirus			Hadar et al. (2010) Saraswathy et al. (2011)	
Dengue fever (Flavivirus)	Basurko et al. (2009)		Tan et al. (2012) Kariyawasam and Senanayake (2010) Basurko et al. (2009)	
Herpes simplex virus 1 and 2	Kapranos and Kotronias (2009)		Kim, et al. (2012a, b)	
Hepatitis B	Ye et al. (2014)		Ye et al. (2014) Murad et al. (2013)	Ye et al. (2014)
Hepatitis C			Murad et al. (2013)	
HIV			Eaichi et al. (2013) Kim et al. (2012a, b) Gibb et al. (2012) Darak et al. (2011) De Beaudrap et al. (2013)	
Human papillomavirus	Skoczyński et al. (2011)	Yang et al. (2013)		Perino et al. (2011)
Malaria (Plasmodium)	De Beaudrap et al. (2013)		McGready et al. (2012) De Beaudrap et al. (2013)	
Mycoplasma genitalium			Short et al. (2010)	
Parvovirus B19			Bitkic et al. (2011) Emiraegen et al. (2011)	
Polyomavirus BK	Cajaiba et al. (2011) Boldorini et al. (2010)			
Syphilis			Casal et al. (2012) Hong et al. (2014)	
Toxoplasma gondii			Alvarado-Esquivel et al. (2014) Habibi et al. (2014) Valdo-Solís et al. (2012)	

Conclusions

A plethora of bacterial, viral and protozoan infectious agents have been investigated to determine whether they are associated with an increased risk of miscarriage. The evidence presented in this review shows that infections such as BV, malaria, CMV, dengue fever, brucellosis and HIV may adversely affect pregnancy outcome. In contrast, there is no current evidence to suggest that *C. bumetii*, adeno-associated virus, Bocavirus, Hepatitis C and *M. genitalium* are associated with miscarriage. More importantly though, the lack of consensus regarding the effects of *C. trachomatis*, *T. gondii*, HPV, HSV1, HSV2, Polyomavirus BK, Hepatitis B and B19V infection reveals a gap in knowledge that future research should address, as these pathogens could potentially be harmful to early pregnancy development (Table I). This issue is of particular importance for public health practitioners as it could alter current policies of prevention of infection, diagnosis and treatment in pregnant women.

Even in diseases such as malaria and rubella, where a causative role is established, the underlying molecular cause of miscarriage is still unknown. The mechanism that has been proposed to explain how CMV infection could undermine a pregnancy could apply to other intracellular pathogens. *Chlamydia trachomatis* and *U. urealyticum* have been detected in placental cells, therefore they could cause a similar response (Joste *et al.*, 1994; Baud, *et al.*, 2011). It is well established that pregnancy is a balance between tolerance and rejection, as the maternal immune system is re-programmed to tolerate the allogeneic (paternal) fetal antigens (Thellin and Heinen, 2003). An active infection could destabilize this balance resulting in rejection, especially if it leads to a serious illness of the mother. Evidently, further research is required to understand the causes of pregnancy failure.

In severe maternal infection, such as with influenza, HIV, dengue fever and malaria, the maternal response may result in miscarriage instead of a direct placental infection effect. However, pathogens such as *Plasmodium* parasites and Dengue fever's *flavivirus* are known to be detected in fetal tissue and placenta, as are a plethora of other pathogens (Table II). This is of particular importance, as proof of vertical transmission that could interfere with an ongoing pregnancy is more likely to result in miscarriage than a maternal infection. Examination of fetal tissues from infected mothers is essential to clarify whether vertical transmission is possible for pathogens as this has not yet been elucidated and it is evident from recent studies that an association is likely: for example, brucellosis, *Mycoplasma genitalium* and *Coxiella burnetii* infections. A very significant issue is fetal specimen contamination in cases with presence in the vagina of common viruses, such as HPV and HSV, as this does not equate to causation of miscarriage.

One interesting outcome of our review was that studies regarding infections and pregnancy outcome were conducted worldwide. Despite this, it seems that most of the studies were from countries in the developing world where the prevalence of specific diseases is higher.

A commonly observed limitation of the studies presented in this review was that few studies tested for the presence of other pathogens except for the one of interest. Several pathogens are often associated with one another, such as HIV with BV and malaria (Ledru *et al.*, 1997; Taha *et al.*, 1998; De Beaudrap *et al.*, 2013).

Furthermore, the definition of terms, such as miscarriage and stillbirth, may differ from the ones generally accepted in some studies, as mentioned previously. For example, loss of pregnancy up to 36 weeks was considered miscarriage (Bayraktar *et al.*, 2010), or in other cases less than 22 gestational weeks (Nielsen *et al.*, 2012). Universal terminology guidelines are required to establish effective scientific communication.

The impact of immunization, if a vaccine is available such as in the case of HPV, could be detrimental in cases of infection-induced miscarriage. However, vaccine development for some of the pathogens of interest in this review is a complicated process and has been unsuccessful so far (Hafner *et al.*, 2008; Mouquet and Nussenzweig, 2013).

To our knowledge, there are no EU guidelines regarding screening for infectious diseases in pregnancy. Current screening guidelines in the UK include offering tests for HepB, HIV, rubella (testing for susceptibility) and syphilis to pregnant women (Public Health England, 2015). Systematic screening for infections such as BV or CMV is not currently recommended in the UK. Without accounting for cost, screening for pathogens highlighted as high risk for miscarriage in this review should be reconsidered as an option worldwide.

New policies including public education to raise awareness and screening programmes for appropriate pathogens associated with adverse pregnancy outcomes could result in a decrease in the number of miscarriages.

Supplementary data

Supplementary data are available at http://humupd.oxfordjournals.org/.

Acknowledgements

The authors would like to acknowledge Sharon Cameron's critical review of the manuscript and suggestions, Ronnie Grant for the electronic drawing of the figures in the present review, Dr Colin Duncan for his expert opinion on statistics and Miss Eleni Fitsiou for proofreading the manuscript.

Authors' roles

S.G. drafted the manuscript, substantially contributed to conception and design, analysis and interpretation of data. N.W. contributed to manuscript preparation and critically revised important intellectual content. K.C. critically revised important intellectual content. G.E. critically revised important intellectual content. S.E.M.H. substantially contributed to conception and design, and critically revised important intellectual content. A.W.H. substantially contributed to conception and design, and critically revised important intellectual content.

Funding

S.G. is funded by the MRC Centre for Reproductive Health and Tommy's Charity. N.W. is funded by the Biotechnology and Biological Sciences Research Council (BBSRC)/Zoetis Industrial Partnership award. G.E. is funded by the Scottish Government Rural and Environment Science and Analytical Services Division (RESAS). Funding to pay the Open Access publication charges for this article was provided by RCUK UK Open Access Fund.

Conflict of interest

The authors have no conflict of interests in relation to this work.

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9. Appendix 3

Detailed interim study statistical analysis:

Parameter	Statistic	Control group (n=141)	Miscarriage group (n=199)	p value
Age (years)	Range	18-47	16-46	0.9506 (Mann-Whitney test)
	Mean	32.87	32.62	
	Median	34	34	
	Std. deviation	5.419	5.816	
Weight (kg)	Range	49-121	36.8-146	0.2131 (Mann-Whitney test)
	Mean	70.84	70.18	
	Median	67	65.7	
	Std. deviation	13.76	17.42	
Height (cm)	Range	152-185	143-183	0.4059 (Mann-Whitney test)
	Mean	164.8	165.2	
	Median	164.5	165	
	Std. deviation	5.785	6.619	
BMI	Range	18-47.27	15-46	0.0991 (Mann-Whitney test)
	Mean	26.22	25.72	
	Median	24.66	24	
	Std. deviation	5.374	6.024	
Ethnicity	Caucasian (%)	123 (87.9%)	190 (95.5%)	< 0.0001 (Chi square test)
	Black (%)	3 (2.1%)	3 (1.5%)	
	Asian (%)	1 (0.7%)	6 (4%)	
	Other/Unknown (%)	13 (9.3%)	0 (0%)	
Smoking status	Smoker (%)	13 (9.6%)	21 (10.7%)	0.6720 (Chi square test)
	Ex smoker (%)	27 (19.9%)	46 (23.4%)	
	Never smoked (%)	96 (70.6)	130 (66%)	

PGP3 result	Positive	31	36	0.7790 (Chi square tests)
	Negative	96	124	
	Not yet tested	25	40	
LPS Elisa* result	Positive	12	10	0.8240 (Chi square test)
	Negative	113	113	
	Not yet tested	0	0	
Reported previous *Ct* infection	Yes	4	17	
	No	17	182	
	Unknown	120	0	
Current *Ct* infection	Positive	0	0	
	Negative	14	177	
	Unknown	126	15	
* RIDASCREEN® Chlamydia IgG, Cat no: KGM3101				