Stillbirth Prevention by Combating Placental Rejection

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Contents

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List of Abbreviations

AF	Alexa-Fluor
ALP	Alkaline phosphatase
AMR	Antibody mediated rejection
APC	Antigen presenting cell
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CHI	Chronic histiocytic intervillositis
CI	Chronic intervillositis
CIUE	Chronic intervillositis of unknown etiology
CK7	Cytokeratin 7
CO_2	Carbon dioxide
CR	Complement receptor
cRF	Calculated reaction frequency
CTB	Cytotrophoblast
CTL	Cytotoxic lymphocytes
CTLpf	Cytotoxic T lymphocyte precursor frequency
CXCL	Chemokine (C-X-C) ligand
DAB	Diaminobenzidine
DAMP	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DCDA	Dichorionic-diamniotic
DNA	Deoxyribonucleic acid
DSAs	Donor-specific antibodies
EDTA	Ethylenediaminetetraacetic acid
EVT	Extravillous trophoblast
FCXM	Flow cytometry crossmatch
FFPE	Formalin-fixed paraffin embedded
FGR	Fetal growth restriction
FIZZ1	Resistin-like α
FNAIT	Fetal and neonatal alloimmune thrombocytopaenia
Foxp3	Forkhead box P3
FSA	Fetal-specific anti-HLA antibodies
GLUT1	Glucose transporter isoform-1
H&E	Haematoxylin and eosin
HBC	Hofbauer cell
hCG	Human chorionic gonadotropin

HLA	Human leukocyte antigen
IBC	Individualised birthweight centile
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IMS	Industrial methylated spirit
iNOS	Inducible nitric oxide synthase
IVIG	Intravenous immunoglobulin
IVS	Intervillous space
KIRs	Killer immunoglobulin-like receptors
LFA-1	Leukocyte function-associated antigen-1
LMWH	Low-molecular-weight heparin
LPS	Lipopolysaccharide
MAC	Membrane attack complex
mAb	Monoclonal antibody
MBL	Mannose-binding lectin
MCI	Massive chronic intervillositis
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPFD/MFI	Massive perivillous fibrin deposition/Maternal floor infarction
Ms	Mouse
MΦ	Macrophage
NBF	Neutral buffered formalin
NHS	National Health Service
NK	Natural killer
NRES	National Research Ethics Service
O_2	Oxygen
OCT	Optimal cutting temperature compound
pAb	Polyclonal antibody
PAMP	Pathogen-associated molecular patterns
PAPP-A	Pregnancy associated plasma protein-A
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-L1	Programmed death ligand 1
PE	R-phycoerythrin
PMN	Polymorphonuclear leukocytes
Rb	Rabbit
RM	Recurrent miscarriage
ROI	Regions of interest
RPL	Recurrent pregnancy loss
SAPE	R-phycoerythrin-conjugated streptavidin

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SGA	Small for gestational age
SSO	Sequence specific oligonucleotide
STB	Syncytiotrophoblast
TBS	Tris buffered saline
TGF-β	Transforming growth factor beta
Th	T helper cell
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TOP	Termination of pregnancy
TOPFA	Termination of pregnancy for fetal anomaly
Treg	Regulatory T cell
UK	United Kingdom
uNK	Uterine natural killer cell
VEGF	Vascular endothelial growth factor
VUE	Villitis of unknown etiology
VXM	Virtual crossmatching
Ym1	Chitinase 3-like 3
β_2-GPI	beta$_2$-glycoprotein I

Scientific Abstract

As a semi-allogeneic organ, the human placenta must be tolerated by the maternal immune system throughout pregnancy in order to maintain the survival and development of the fetus. Any failure in this process is suspected to underpin several pregnancy complications including miscarriage, stillbirth and perinatal death. Chronic histiocytic intervillositis (CHI) is one such example of a severe inflammatory placental lesion, characterised by maternal macrophage infiltration of the intervillous space. Presently, CHI can only be diagnosed by histopathological examination (usually performed) after poor pregnancy outcome has already occurred, and there is no standardised protocol for its prevention or treatment. CHI's cause remains largely unknown, though its incidence in women with autoimmune disease, high recurrence rate and similarities shared with rejected allografts has given rise to the theory that it is a disorder of maternal anti-fetal rejection. This study aimed to investigate this hypothesis by characterising the placental inflammatory profile, evaluating the efficacy of immunomodulatory medication in reducing CHI severity, and applying crossmatching techniques commonly used in the prediction of antibody-mediated allograft rejection to affected women.

Cases of CHI were identified retrospectively from medical records and clinical data on treatment and pregnancy outcomes collected. In participants who were treated with one or both of prednisolone and hydroxychloroquine in their subsequent pregnancy following diagnosis, CHI severity was reduced in 86.7% of cases compared to 33.3% of those treated without (p=0.02). A reduction in placental lesion severity was also associated with a 62.3% increase in livebirth rate (p=0.003). These data provide preliminary evidence to support the use of immunomodulatory medication in the treatment of CHI.

Unbiased computerised quantification of immune cells in untreated (index) cases of CHI showed a 28-fold median increase in intervillous $CD68^+$ macrophages compared to healthy controls (p<0.0001), though some cases did not display elevated infiltration. Increased fibrin deposition was also present in certain placentas. In subsequent pregnancies following diagnosis, the number of macrophages resembled those of healthy controls and fibrin deposition was significantly reduced (p=0.04). However, $CD3^+$ T cells were increased above controls (p<0.0001). In index cases of CHI, intervillous macrophages were positive for M2 marker CD163, and did not display M1 markers (CD80 and CD86) suggesting they may be resolving inflammation.

In both index cases of CHI and subsequent pregnancies, immunohistochemical staining for intervillous C4d deposition did not differ compared to controls. Screening of maternal plasma in subsequent pregnancies revealed no significant differences in antibodies toward fetal HLA or placental antigens compared to that from healthy pregnancies. Antibody-mediated rejection in these cases could therefore not be confirmed, though immunomodulatory treatment was a likely confounding factor.

This study provides evidence that CHI is an inflammatory placental condition, which without specialist treatment has severe effects on fetal development and survival. Though CHI shares features with allograft rejection, further research in larger cohorts is required to investigate any possible antibody-mediated component to its pathophysiology independent of treatment effect.

Lay Abstract

The placenta is the organ which connects a mother and her baby during pregnancy, transferring oxygen and nutrients and removing waste to allow the baby to grow and survive. As the placenta has half its mother's DNA and half its father's, the mother's immune system has developed ways to prevent the placenta being seen as not part of the mother and rejected. In a disease called chronic histiocytic intervillositis (CHI), this process appears to fail, and the mother's immune cells (macrophages) build up in the placenta, similar to rejection of an organ transplant. In pregnancies with CHI, there is a high chance that the baby will fail to grow, or die before or shortly after birth. Although it is a rare condition, CHI returns in 25-100% of future pregnancies, and there is no proven way to treat affected pregnancies because its cause is not fully understood. This study aimed to find out whether treatments to reduce the mother's immune response (immunomodulatory medications) were effective at reducing the severity of CHI in pregnancies after diagnosis. Untreated pregnancies were also studied to identify which immune cells are involved in CHI and whether antibodies produced by the mother towards the baby are increased.

In women who were treated with at least one medication to reduce inflammation, the severity of CHI was reduced in 54% more pregnancies compared to women who did not use this type of medication. In pregnancies where CHI was reduced, there was a 62% higher chance that the baby would survive past birth. This data provides evidence to support treating women with CHI using immunomodulatory medication to improve the chance they will have a healthy baby in future pregnancies.

Computer software was used to analyse placentas from pregnancies in women with a history of CHI and compare them to healthy pregnancies. As expected, macrophages were increased in untreated cases of CHI, and some cases had increased levels of blood clots. In treated pregnancies, placentas had numbers of macrophages similar to healthy pregnancies, and clotting was reduced. In untreated pregnancies, macrophages appeared to be trying to prevent inflammation.

Laboratory tests routinely used to show evidence of organ rejection were carried out in both women with previous pregnancies affected by CHI and healthy women – to find antibodies that reacted with the placenta. In women who had a history of CHI, there was no evidence of increased antibodies toward the placenta or baby, meaning rejection could not be confirmed.

This study supports CHI as a disease where the mother's immune cells build up in the placenta, which when treated without immunomodulatory medication can result in the baby failing to grow or survive. Antibodies towards the baby or placenta could not be identified in pregnant women after a diagnosis of CHI, although medication may have affected this result. Future studies with larger numbers of women are needed to identify why immune cells are attracted towards the placenta in CHI, and to find ways to predict which women are at risk before they have an affected pregnancy.

Declaration

Data produced from quantification of $CD3^+$ cells in Chapter 4 was submitted by Tihesia Riley to The University of Manchester for the degree of Master of Research in Reproduction and Pregnancy, 2021. The remainder of work referred to in this thesis has not been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Undertaking my PhD has been one of the most challenging, yet exciting periods of my life. Throughout the last three years, I have developed profound confidence in myself and furthered an already intense passion for science, and for that I have many people to thank.

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I owe a huge thank you to my family for unconditionally supporting my academic goals and reading (or attempting to read) my papers time after time even after getting lost on the first page. To my friends, I feel lucky that you all stuck around and took interest in my work despite my tendency to get carried away talking about how cool the placenta is. Special thanks to the PubMed group for being the best support network and lunchtime companions.

Finally, thank you Lee, for being there to share my problems with along the way and ensuring we always had the biggest celebration no matter how small the success. I can't decide if it's me or you who is most excited for me to finish this and gain my title as Dr, but I think the pride and confidence you have in me has played a major part in me making it this far.

The Author

Chloe A Brady graduated from the University of Manchester in July 2018 with a First Class Honours integrated Master's degree in Neuroscience before starting her PhD at Tommy's Maternal and Fetal Health Research Centre in September 2018.

Alternative Format

This thesis was written in the journal format after a review of the literature (included in Chapter 1) and results Chapter 3 were published in the journals *American Journal of Reproductive Immunology* and *Frontiers in Medicine*, respectively. Subsequent data formed separate Chapters 4 and 5 which have been written in preparation for submission to the journals *Archives of Pathology and Laboratory Medicine* and the *American Journal of Reproductive Immunology.*

Chapter 1: Introduction

Chapter 1: Introduction

The Immunological Paradox of Pregnancy

In the United Kingdom, one in every four pregnancies ends in loss.[1] Stillbirth is defined in the UK as the death of a baby after 24 weeks' gestation with 1 in 225 pregnancies affected annually, constituting one of the highest rates in Europe.[2] Despite their frequency, the majority of pregnancy losses remain unexplained, posing a distressing problem for expectant parents and clinicians alike. In those with a known cause, around half are attributed to placental pathologies.[3] A subset of these, especially recurrent cases, are suspected to be immunological in nature,[4,5] and there is a growing body of evidence associating inflammation to other common obstetric conditions including preeclampsia and preterm birth.[6]

Pregnancy as a unique immunological state allowing survival and growth of the genetically foreign fetus first garnered the interest of biologists in the early 20th century. In 1924, geneticist C. C. Little first suggested the existence of 'protective mechanisms' in pregnancy which prevented recognition of the embryo by the mother.[7] However, it was not until the 1950s that the immunological problem of pregnancy became a research focus, when transplantation biologist Sir Peter Medawar asked:

'…how does the pregnant mother contrive to nourish within itself, for many weeks or months, a foetus that is an antigenically foreign body?'.[8]

Medawar also acknowledged that there exists an equal requirement during pregnancy to conserve the maternal immune response against pathogens to protect survival of both mother and fetus. Since then, this 'paradox' of pregnancy has prompted investigation into the specific mechanisms allowing fetal cells expressing paternal antigens to survive, as well as how this may be deficient in certain pregnancy complications. Adaptation of the immune system during pregnancy is evident in pregnant women's greater susceptibility to infection,[9] exacerbation or relief of symptoms of autoimmune disorders[10] and increased risk of allograft dysfunction or rejection.[11] Likewise, the immunological microenvironment *in utero* exerts significant effects on the health of the developing fetus, and is closely linked to fetal growth restriction (FGR) and death.[12-14] Inflammation during intrauterine life also has implications for long term health, predisposing offspring to neurological conditions and an increased likelihood of adult disease.[15,16] A greater understanding of immunological adaptation during pregnancy and where it may be dysfunctional therefore holds significance for research into a range of disorders, including but not limited to those occurring during pregnancy.

The Immune System

To recognise how the immune system can be vital for healthy pregnancy whilst holding the potential to contribute towards poor neonatal health or fetal death, it is first necessary to understand its role outside of pregnancy.

Throughout life, there is continuous exposure to infectious agents in the environment. The majority of these are unable to penetrate tissue and cells due to exterior barriers and defences, including the skin, stomach acid, tears, mucus and excretion via urination. In addition, there are commensal bacteria, e.g. those in the gut and vagina, which compete with invading pathogens.[17] For

pathogens which are able to infect the body, cells and signalling molecules of the immune system have evolved to recognise infection and orchestrate a response to effectively protect the health and survival of the individual. Due to the wide variety of pathogens which may be encountered in the external environment, there exists a diverse population of cells each with differing roles and functions, which form the innate and adaptive arms of the immune system (Figure 1).

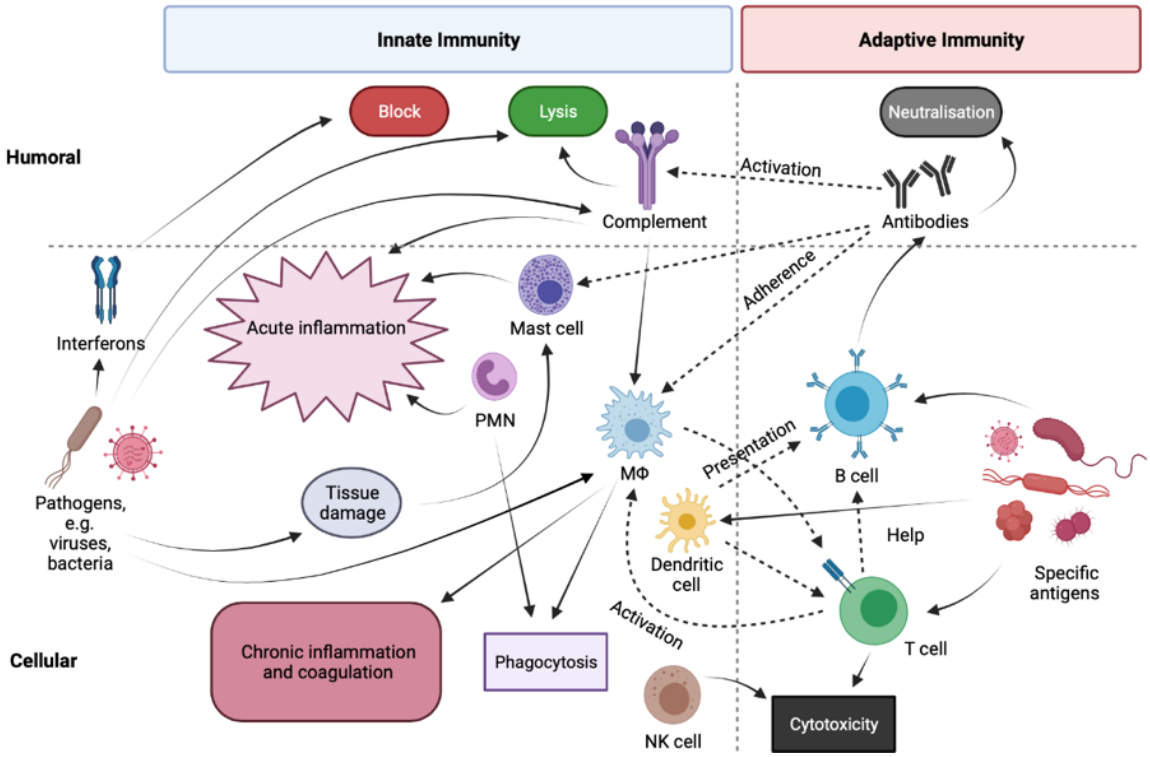

Figure 1. Components of the innate and adaptive immune systems and their interactions. MΦ – macrophage; NK – natural killer; PMN – polymorphonuclear leukocyte. Adapted from Playfair and Chain, Immunology at a Glance.[18] Created with BioRender.com.

Innate Immunity

The innate immune system acts as first-line defence, immediately recognising a wide range of pathogens in a non-specific manner via molecules known as pathogen-associated molecular patterns (PAMPs). Innate cells arise from the myeloid lineage and include polymorphonuclear leukocytes (PMNs) (e.g. neutrophils, eosinophils, basophils and mast cells), monocytes and macrophages, natural killer (NK) cells and dendritic cells (Figure 1). In the cellular response, microorganisms can be phagocytosed by PMNs and macrophages, or lysed via lysozyme - an enzyme released from macrophages and granules within neutrophils.[19] Viral particles result in cellular production of interferons (IFN), which enable other cells within the body to become resistant.[17] The innate response also has a humoral component, wherein foreign cells are labelled with complement, a series of proteins which act as a signal, enabling immune detection and lysis. Importantly, inflammation can also occur in the absence of microorganisms, in a process termed ‘sterile inflammation’. Sterile inflammation is triggered by the release of intracellular contents known as damage-associated molecular patterns (DAMPs) from damaged and necrotic cells, and can be a result of injury, hypoxia or chemically induced.[20]

A particularly vital component of innate immunity are antigen presenting cells (APCs), including dendritic cells and macrophages.[21] APCs take up antigens from pathogens and display them to T and B cells, stimulating their activation and connecting the innate and adaptive systems.[17]

The Complement System

The complement system is comprised of over 30 serum proteins, originally named after their ability to 'complement' the action of antibodies.[22] Complement functions via a cascade of proteolytic reactions leading to inflammation, lysis of pathogenic cells, labelling of foreign antigens (opsonisation), and immune cell priming (Figure 2).[22] There are three pathways by which the cascade is activated; the classical, lectin and alternative pathways. The classical pathway is activated via binding of component C1q to antibody-antigen complexes, and the lectin pathway via binding of mannose-binding lectin (MBL) to carbohydrates on bacterial cells. Finally, in the alternative pathway, C3 circulating within the blood is prone to spontaneous hydrolysis by water, activating the cascade in the event it encounters and binds foreign bodies.[17] All three pathways converge at the point of C3 and C5 convertase formation, which result in the production of anaphylatoxins, the membrane attack complex (MAC) and opsonins. Anaphylatoxins produced include C3a, C4a and C5a which contribute toward inflammation via leukocyte recruitment and binding to numerous immune cells possessing their receptors, namely those of the myeloid lineage including macrophages, PMNs and dendritic cells.[22] The MAC is formed by a series of enzymatic reactions beginning with C5 convertase binding to C3b, and non-enzymatic assembly of several complement proteins including C5b-9.[17] Upon formation of the MAC, pores are made within the cell membrane of target cells, resulting in their lysis. Complement product C3b acts as an opsonin, labelling pathogens and increasing their susceptibility to phagocytosis.[17] The presence of circulating C3b also creates an amplification loop to ensure inflammation is sustained long enough to clear invading pathogens.

Cells of the body must employ mechanisms to prevent their own destruction via the complement pathway, especially that of the alternative pathway which is constitutively active. These include expression of membrane-bound complement regulatory proteins e.g. CD59, regulation of convertases via decay-accelerating proteins, cleavage of essential amino acid residues and the requirement of cofactors at various stages of the pathway.[22]

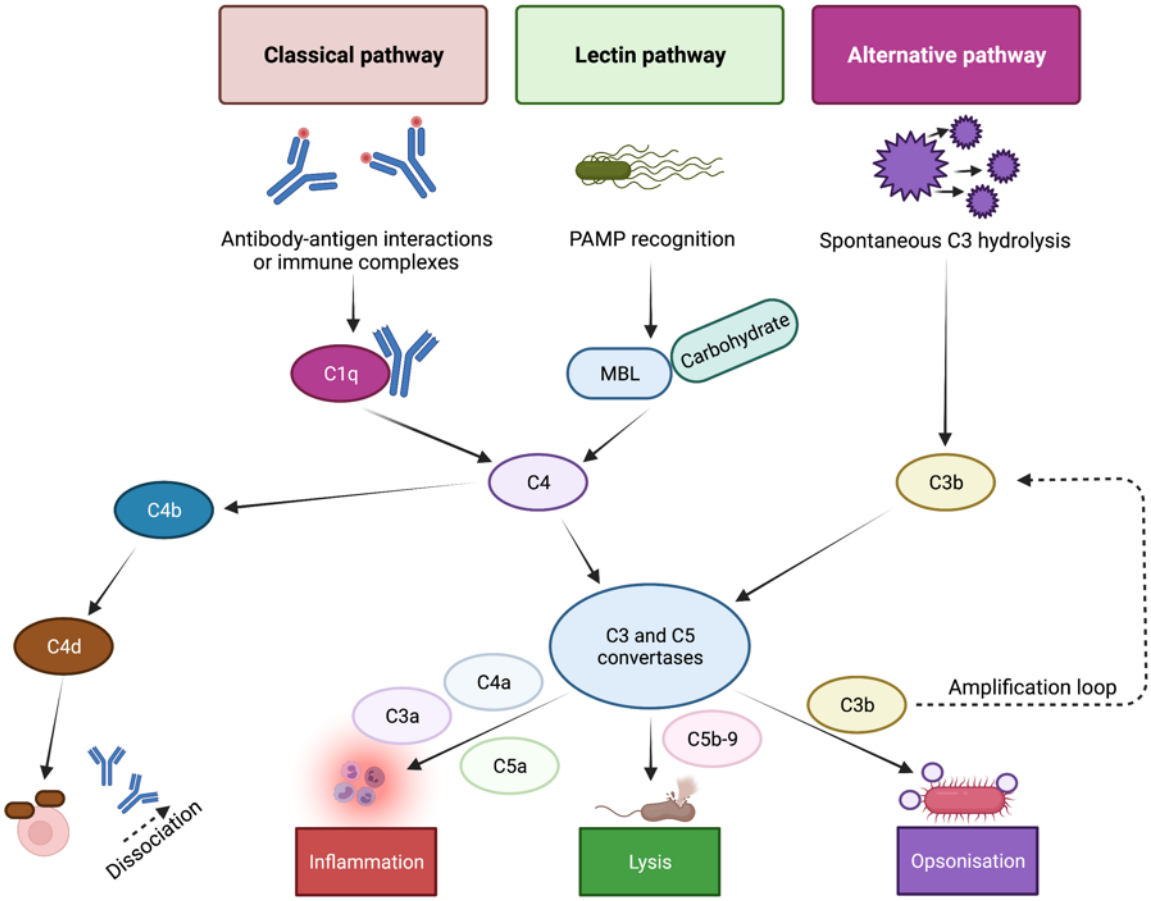

Figure 2. General overview of the complement system. Antibody binding, recognition of pathogens, and constitutive hydrolysis of complement within the blood can all activate the complement cascade. A series of proteolytic reactions occur which result in the production of C3 and C5 convertases, the point at which all three pathways converge. Several protein products are then produced which act as anaphylatoxins to stimulate inflammation, opsonins to label pathogens as foreign, or form the membrane attack complex to facilitate cell lysis. Note that C4d is a split product of both classical and lectin binding pathways, and forms a stable bond with cells long after antibodies have dissociated. MBL – mannose binding lectin; PAMP – pathogen-associated molecular pattern. Figure adapted from Dunkelberger and Song, and Cohen et al.[22,23] Created with BioRender.com.

Macrophages

Following stimulation, circulating monocytes infiltrate tissues and differentiate into macrophages. Macrophages play a pivotal role in the innate immune system, functioning not only as APCs but as key contributors to the promotion or resolution of inflammation and coagulation due to their plasticity.

Upon exposure to differing environmental stimuli, macrophages can be classically activated to adopt a pro-inflammatory M1 phenotype, or alternatively activated resulting in an anti-inflammatory M2 phenotype (Figure 3).[24] Polarisation of macrophages is considered a continuum as opposed to a fixed state, and balance between M1 and M2 functions is essential for tissue homeostasis and the appropriate control of inflammation.

When stimulated by lipopolysaccharide (LPS), tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), macrophages adopt an M1 phenotype characterised by expression of cell surface markers e.g. CD80, CD86, and production of pro-inflammatory cytokines and chemokines including interleukins (ILs)-1α and 1β (Figure 3). This in turn promotes other neighbouring macrophages to

adopt an M1 phenotype in a positive feedback loop, resulting in microbicidal and tumouricidal activity.[24]

On the other hand, M2 macrophages are stimulated by a selection of interleukins toward a phenotype whereby anti-inflammatory cytokines, chemokines and growth factors are released (Figure 3). M2 macrophages can be further divided into M2a, M2b, M2c and M2d subsets, all of which have specific roles in tissue growth and repair, phagocytosis and angiogenesis.[25]

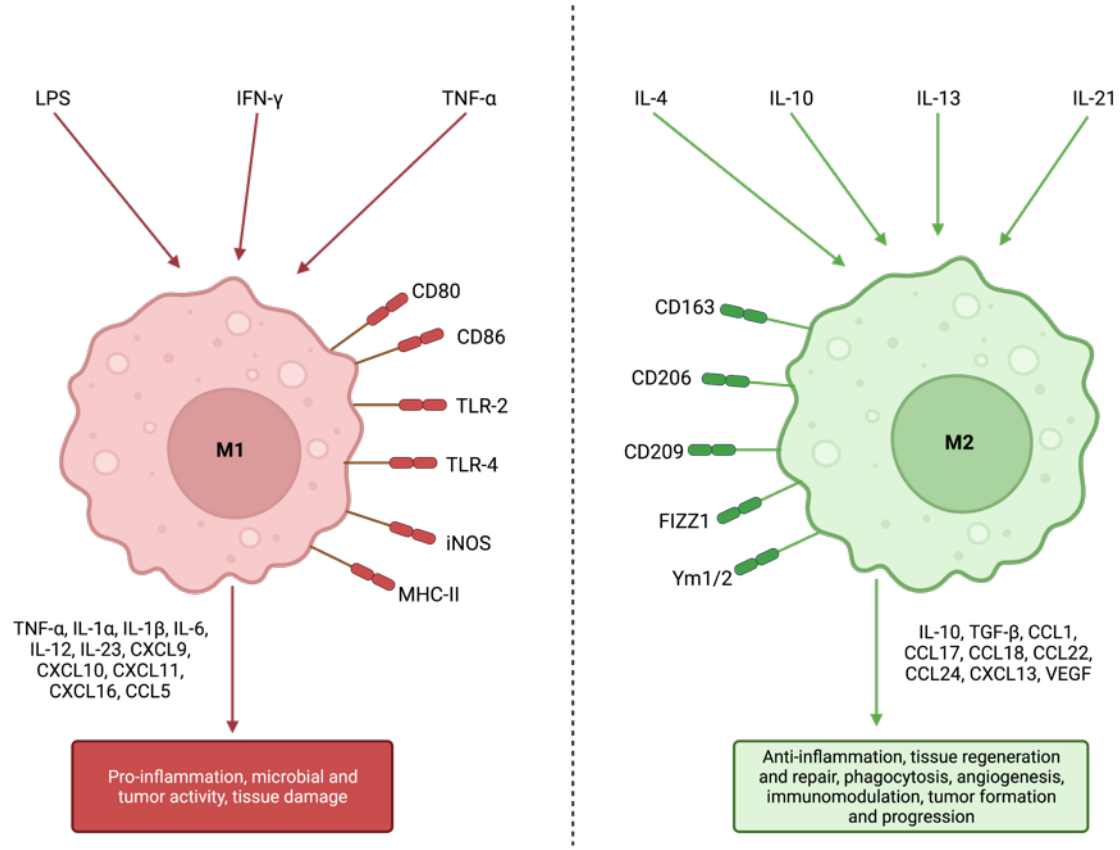

Figure 3 – M1 and M2-polarised macrophages: their stimuli, cell surface markers, secretion of cytokines and chemokines and ultimate functions. CCL - chemokine (C-C motif) ligand; CD – cluster of differentiation; CXCL - chemokine (C-X-C) ligand; FIZZ1 - resistin-like α; iNOS - inducible nitric oxide synthase; IFN-γ - interferon-gamma; IL - interleukin; LPS - lipopolysaccharides; MHC - major histocompatibility complex; TLR - Toll-like receptor; TNF-α - tumor necrosis factor alpha; TGF-β - transforming growth factor beta; VEGF - vascular endothelial growth factor; Ym1 - chitinase 3-like 3. Figure adapted from Yao et al.[24] Created with BioRender.com.

As key mediators of tissue repair, macrophages are tightly linked to the clotting cascade, a process initiated by inflammation and essential for wound healing. Pro-inflammatory M1 macrophages involved in the initial immune response stimulate recruitment of fibroblasts which synthesise components of the extracellular matrix to resolve tissue injury.[26,27] Following this phase, polarisation towards an M2 phenotype and secretion of growth factors including TGF-β and vascular endothelial growth factor (VEGF) suppresses excessive inflammation which may prevent sufficient healing. In addition, clearance of apoptotic fibroblast cells and debris by M2 macrophages limits the stimulation of a further immune response.[27] Therefore, macrophages regulate all stages of inflammation, and disturbances in their function can result in excessive fibrosis and collateral tissue damage evident in inflammatory disease.[27]

Adaptive Immunity

Unlike the innate immune system which responds non-specifically to insults, adaptive immunity involves cells which can recognise molecules, known as antigens, on the surface of both foreign cells and those belonging to the body (termed 'non-self' and 'self', respectively). As a result, the response initiated is specific to the antigen encountered, enabling the generation of 'memory' and long-term immunity in the event of re-exposure.[17] The cellular element of the adaptive immune response consists of T and B cells (Figure 1), named after the area of the body where they mature – the thymus and bone marrow, respectively.

B Cells

B cells are mainly responsible for the production of antibodies, termed immunoglobulins (Ig), which are Y-shaped proteins with a constant region and a variable binding region specific to the structure of a particular antigen.[28] Antibodies are expressed on the B cell surface or secreted, and exert their effect by neutralising antigens, stimulating leukocytes expressing Fc receptors, and activating the complement cascade. There are five antibody classes: IgA, IgD, IgE, IgG and IgM, each of which have differing properties. For example, IgG and IgM function best at activating the classical complement pathway, whilst IgD and IgE are largely involved in the activation of basophils and mast cells respectively.[29] Additionally, antibody isoforms have differing relevance in health and disease, with the majority of autoimmune disorders characterised by the production of IgG.[30] Following activation, B cells form long-lived memory B cells and plasma cells, which provide long-term immunity towards specific antigens.[17]

Alongside production of antibodies, B cells are also able to influence the activity of other cells within the immune system, and can themselves secrete cytokines including IFN-γ and TNF-α which influence downstream inflammation.[31] In addition, B cells can act as APCs in the activation and stimulation of T cells which constitute the other cellular component of adaptive immunity.

T Cells

T lymphocytes constitute several cells with differing functions and immunomodulatory capabilities and are classified into $CD8^+$ cytotoxic or $CD4^+$ helper T cells. $CD8^+$ cells differentiate into cytotoxic lymphocytes (CTLs), which can directly lyse infected or cancerous cells possessing their target antigen.[32] $CD4^+$ cells are further divided into several subsets including Th1 and Th2, Th17 and regulatory T cells (Tregs), each producing a specific range of cytokines which either have direct cytotoxic effects on foreign cells, activate B lymphocytes or modulate other immune responses.[32] The Th1 response is characterised by the production of IFN-γ, IL-2, and TNF-β, which facilitate the killing of intracellular parasites and promote cellular immunity.[33] On the other hand, cytokines of the Th2 response include IL-4, 5 and 13 stimulated by extracellular pathogens and allergens, and IL-10 which has a potent anti-inflammatory effect.[33] Macrophages have the capability to skew T cells towards either a Th1 or a Th2 response, achieved via M1 or M2b-secreted cytokines, respectively.[25]

A more recently discovered T lymphocyte sub-population, Th17 cells, are also responsible for defence against bacterial, viral and fungal infection, though are becoming increasingly recognised as key mediators in autoimmune disease and transplant rejection.[34]

The activity of Th1, Th2 and Th17 cells is modulated by Tregs, which are distinguished by their expression of transcription factor forkhead box P3 (Foxp3) alongside CD4 and CD25. Tregs limit inflammatory responses via cell-cell contact, deprivation of IL-2 required for T cell proliferation and release of anti-inflammatory cytokines IL-10 and TGF-β.[35–37] Additionally, Tregs are essential for the development of self-tolerance, prevention of autoimmune responses and immune protection of the fetus during pregnancy. The importance of Tregs is highlighted by Foxp3-deficient mouse models which develop lethal autoimmunity,[38] and insufficient activity is evident in multiple pathologies including cases of recurrent pregnancy loss.[4]

Unlike B cells, T cell activation is dependent upon stimulation by APCs expressing proteins of the major histocompatibility complex (MHC). MHC molecules allow for the binding and presentation of exogenous antigens to T cells, prompting their recognition and initiation of the immune response.[39]

The Major Histocompatibility Complex

The MHC, known as human leukocyte antigen (HLA) in humans, is a complex of over 200 genes encoding proteins capable of binding to pathogenic peptides.[39] The genetic diversity of HLA and polymorphism within the population as a whole allows for the presentation and recognition of a wide range of antigens and greatly limits the ability of a pathogen to evade immune detection.[39]

HLA molecules are split into classical and non-classical HLA, which exhibit differential expression throughout the body. Classical HLAs involved in antigen presentation mainly consist of Class I; HLA-A, -B and -C expressed on all nucleated cells of the body, and Class II; HLA-DR, -DP and -DQ whose expression is confined to APCs.[39] Class III HLAs also exist, though are not involved in antigen presentation and instead encode various other proteins including complement factors.[40]

Non-classical HLAs include HLA-E, -F and -G, which appear to have an immunomodulatory role in pregnancy and cancer, though compared to classical HLAs their function is considerably less well understood.[41–43]

Class I HLA process and present endogenous antigens (e.g. those from viruses) to activate $CD8^+$ cytotoxic T cells, whilst Class II facilitates presentation of exogenous antigens to $CD4^+$ T helper cells.[44] Alongside antigen presentation, HLA plays a central role in the development of tolerance towards self-antigens, where T cells reacting toward self-HLA are eliminated to prevent autoimmune responses.[39] The function of HLA in the recognition of self and non-self also holds relevance to solid organ transplantation, wherein differences in HLA genotype affect the likelihood of immune destruction of the allograft, known as rejection.

Organ Transplantation and Rejection

Within the field of transplantation, matching of antigens expressed on the donor graft is crucial for maximising recipient acceptance. Though other factors such as ABO blood group mismatch affect graft survival, HLA-incompatible renal transplants have a considerably higher rate of graft loss and patient death.[45] Exposure of individuals to non-self HLA, termed ‘sensitising events’, include prior transplants, transfusions and pregnancy, resulting in the production of anti-HLA antibodies and priming of memory B and T cell responses.[46] Antibodies can also be formed after transplantation in response to donor HLA mismatch, and so recipients are matched as closely as possible based on HLA genotype.[47]

Graft rejection can be characterised by the immune response involved, e.g. cellular (T cell mediated) or antibody-mediated rejection (AMR), though elements of both can overlap (Figure 4).[48]

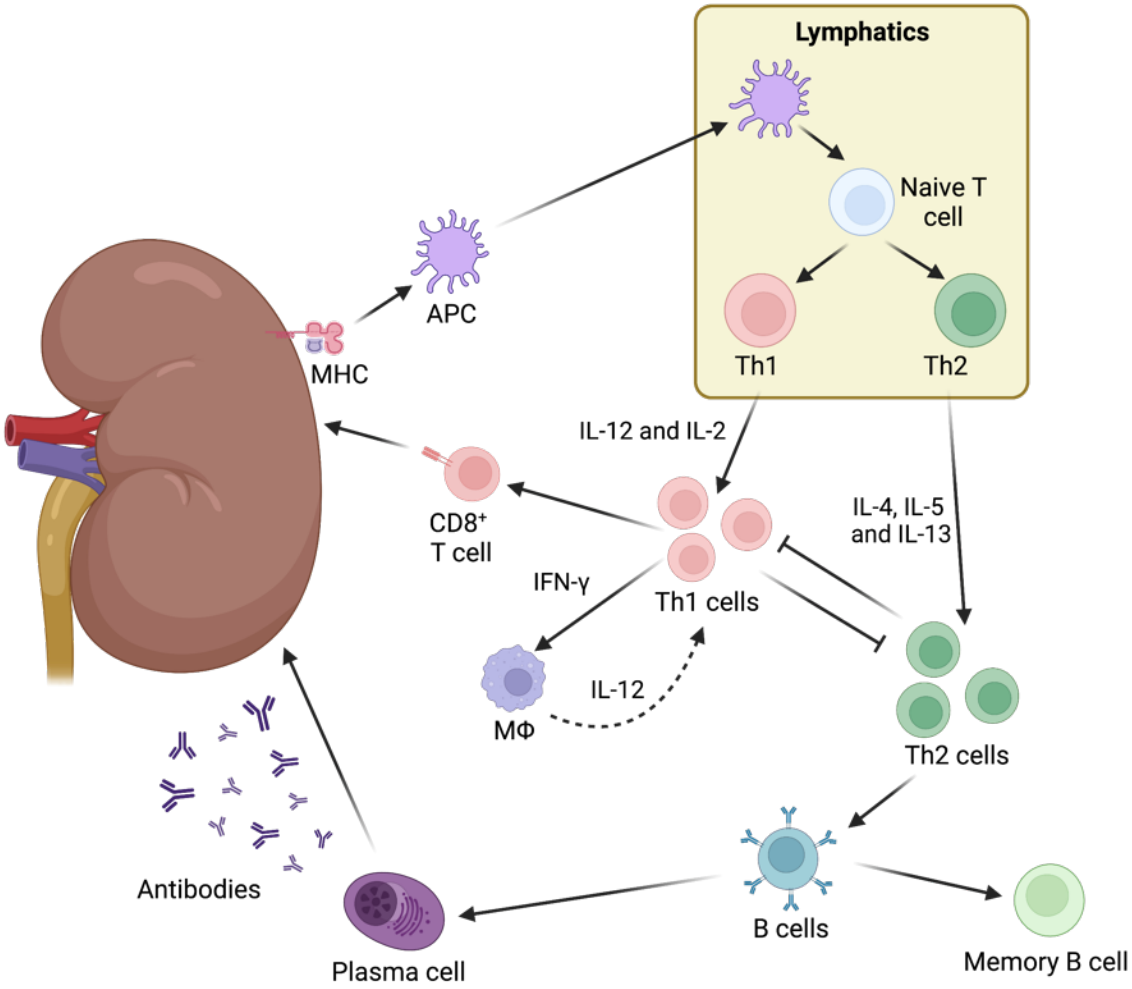

Figure 4. Mechanisms of allograft rejection. In response to allogeneic MHC expressed on the surface of the transplanted graft, antigen presenting cells (APCs) are stimulated and take up graft antigen before migrating to the lymphatics. Within the lymphatics, donor antigen is presented to naïve T cells, stimulating their differentiation into T helper 1 (Th1) and T helper 2 (Th2 cells). Th1 cells produce interleukins (IL) -12 and -2, creating a positive feedback loop and the proliferation of more Th1 cells. Interferon-gamma (IFN-γ) is also produced, stimulating the recruitment and activation of M1 macrophages (MΦ) which maintain the inflammatory response. Th1 cells activate cytotoxic CD8+ T cells, which directly attack the graft and cause apoptosis of donor cells via granzyme release. Th2 cells secrete IL-4, -5 and -13, and via contact with B cells stimulate them to produce donor-specific antibodies. Memory B cell proliferation occurs following B cell activation which confers a more rapid response to donor antigen following the first initial inflammatory event. Figure adapted from Jervis.[49] Created with BioRender.com.

Rejection is also classified by the timing of its onset, as reviewed by Moreau et al.[50]:

- Hyperacute – occurring within minutes post-transplant due to pre-formed donor-specific antibodies (DSAs). This type of rejection now rarely occurs as a result of pre-transplant techniques to determine ABO and HLA genotype and sensitisation.

- Acute – rejection within weeks to months post-transplant, driven by T cell responses and AMR.
- Chronic – long-term rejection due to formation of DSAs and stimulation of T cell responses. Chronic rejection is the most common cause of graft loss in modern medicine.

Allograft rejection is primarily diagnosed via biopsy in accordance with criteria established via the Banff Classification of Allograft Pathology, an international consensus for the diagnosis of T cell-mediated rejection and AMR.[48] Characteristic histological features of rejection include vascular inflammation, C4d deposition and fibrosis as a result of tissue injury ultimately leading to graft dysfunction.[48,51]

To predict and minimise the likelihood of allograft rejection by the recipient, a range of pre-transplant laboratory screening methods exist, known generally as crossmatching. Crossmatching techniques are divided into those assessing physical interaction between donor cells and recipient antibodies within serum e.g. complement dependent cytotoxicity (CDC) and flow cytometry crossmatch (FCXM),[47] or virtual crossmatching (VXM), wherein compatibility is determined via separate analysis of recipient anti-HLA antibodies and donor HLA genotype.[52] The latter has proven invaluable in the advancement of transplantation biology as a cheaper, more rapid method of crossmatching which reduces waiting time for organ allocation whilst still achieving successful graft survival.[53,54] The use of VXM also proves significant in informing the clinical management of patients, as DSAs can now be reduced both pre- and post-transplant using immunosuppressive medication, intravenous immunoglobulin (IVIG) and plasmapheresis, enabling long-term graft survival despite HLA mismatch.[47,55,56]

Pregnancy, which is itself classed as a sensitising event, has in the past been frequently compared to transplantation, where the placenta is described as a successful graft.[57] In this comparison, the paternally-inherited genes of the fetus are likened to donor HLA, tolerated by the recipient, in this case the mother, for the duration of pregnancy. However, unlike an allograft, the human placenta has developed multiple adaptations which allow for its tolerance from conception to birth.

Placental Development

Following fertilisation, the embryo undergoes a series of cell divisions which result in the production of the morula, and eventually by day 5, the blastocyst. The blastocyst consists of two cell layers, the outer of which is known as the trophectoderm and is responsible for forming the placenta and fetal membranes, whilst the inner cell mass develops into the embryo.[58] Around 8 days post-fertilisation, the blastocyst implants within the endometrium, stimulating stromal cells to differentiate and form the decidua – maternal tissue which provides nourishment to the embryo prior to placental development.[59,60] During decidualisation, cells of the trophectoderm fuse to create a syncytium, giving rise to two layers of trophoblast cell; the inner cytotrophoblast (CTB) and outer syncytiotrophoblast (STB).[61]

After implantation, placental villous development begins, and the underlying CTB proliferate and push the surrounding STB outwards, resulting in projections of stem and immature intermediate villi consisting of a CTB core with an outer syncytium.[61] At this stage, villi are surrounded by lacunae filled with uterine gland secretions, nourishing the conceptus and acting as a source of growth factors before onset of maternal blood flow into the intervillous space.[62] Around day 18-20, fetal capillaries originating from the inner cell mass begin to develop within villi, and by day 32 these vessels become connected to the fetal circulation by the umbilical cord.[59] Fetal villi possess their own specialised macrophages, named Hofbauer cells (HBCs), polarised towards an M2-like phenotype which support the development of the fetal vasculature and angiogenesis.[63] During this period of early pregnancy, villous development is characterised by branching angiogenesis, forming a network of finger-like extensions which constitute the villous tree.[64] In the first trimester, an invasive population of CTB, termed extravillous trophoblast (EVT), also infiltrate the decidua and remodel maternal spiral arteries to direct maternal blood into the placenta. After remodelling, lacunae join to become the intervillous space, filled with maternal blood in direct contact with the STB layer.[65] By the end of the first trimester, the initial blueprint of the haemochorial placenta has been established.[61]

Structure and Function of the Mature Human Placenta

Between 24-26 weeks' gestation, a switch from branching to non-branching villous angiogenesis occurs, in preparation to support a period of rapid fetal growth. Instead of the formation of new villous sprouts, existing capillaries elongate and loop, forming bulging terminal villi from mature intermediate villi, reducing the distance between the fetal circulation and the intervillous space.[64] Terminal villi act as the main site of gas and nutrient exchange between mother and fetus during late pregnancy, comprising 25% of total villous volume.[64] Villous maturation continues throughout gestation, and the CTB layer continually fuses with the overlying STB to replenish it.[59] At term, fewer CTB cells are present, though the STB layer is still distinctive.[58]

In the villous haemochorial placenta, fetal vessels are separated from intervillous maternal blood by the villous stroma and the overlying CTB and STB layers (Figure 5), constituting the maternal-fetal interface.[65] Exchange of gas and nutrients within the placenta occurs via active and passive mechanisms, both to meet the demand of the growing fetus and remove waste products via the maternal circulation. In addition to sustaining fetal metabolic processes, the placenta also has endocrine and immunological functions which are essential for the growth and survival of the fetus.

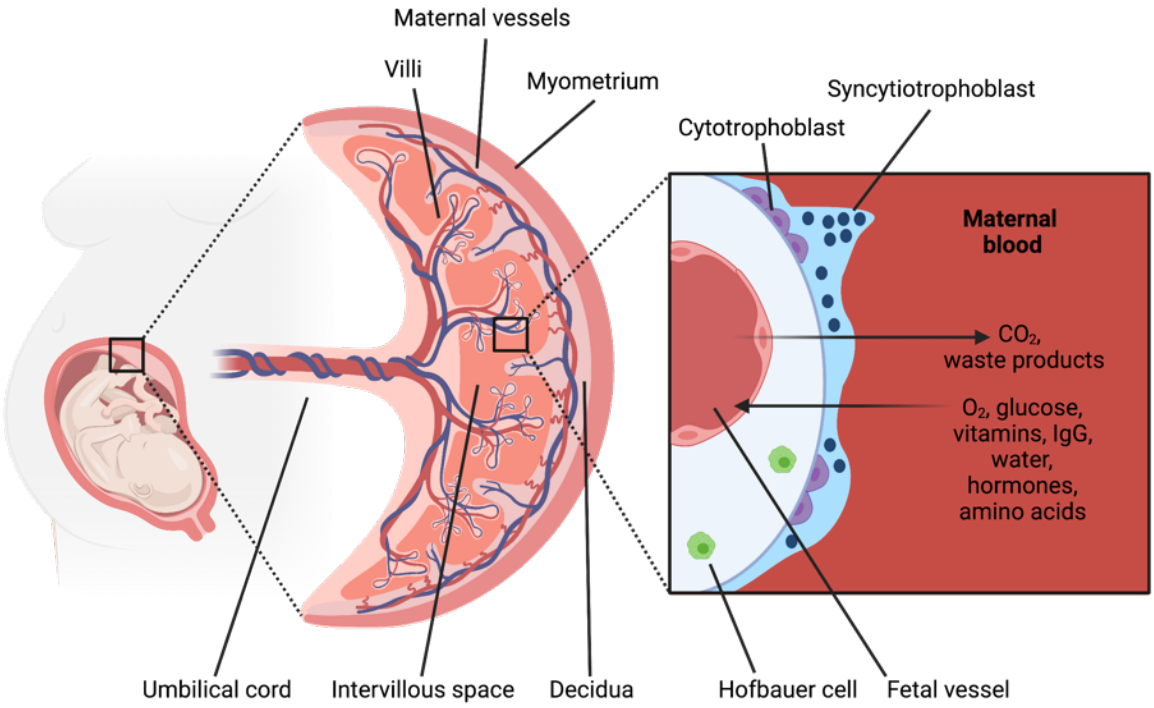

Figure 5. The macro and microstructure of the developed human placenta. CO_2 – carbon dioxide; IgG – immunoglobulin G; O_2 – oxygen. Figure adapted from Elad et al.[66] and created with BioRender.com.

The fetal circulation is connected to the placenta via the umbilical cord, usually consisting of two umbilical arteries and a vein surrounded by connective tissue. Via the umbilical cord, oxygen and carbon dioxide are transported to and from the fetus, respectively. Due to the lipophilic properties of these gases, they are able to diffuse passively across the placenta.[58] Fetal haemoglobin also has a higher affinity for oxygen compared to the maternal form, maximising the efficiency of transport across villi.[67] Vitamins are also able to diffuse across the placenta down a concentration gradient, due to their relatively low abundance in the fetal circulation compared to maternal blood.[58]

For the exchange of substances which are unable to cross the placenta via simple diffusion, the STB layer is rich in a range of transport proteins to facilitate their entry to the fetal circulation. During fetal development, glucose is the primary source of energy, however gluconeogenesis at this stage is limited. As such, the placenta displays high levels of expression of glucose transporters including glucose transporter isoform-1 (GLUT1) which move glucose from a high maternal concentration into fetal blood.[67] Development of the fetus also places large demand on the synthesis of protein, neurotransmitters, haem and nucleotides, dependent upon maternal supply of amino acids. To meet these requirements, more than 20 specialised amino acid transporters are expressed at the maternal-fetal interface to facilitate transport across sodium, electrical or concentration gradients.[68]

Alongside the transport of nutrients, the placenta produces chemical mediators including growth factors and hormones, each with varying endocrine, paracrine and autocrine effects.[67] The range of substances secreted by the placenta is vast, though several of note for the successful maintenance of pregnancy include progesterone, human chorionic gonadotropin (hCG) and placental growth hormone. The concentration of progesterone increases throughout gestation, initially produced by the corpus luteum and then the placenta once developed. Progesterone is essential for the

maintenance of pregnancy until term, inhibiting not only menstruation but also myometrial contraction, thus preventing preterm labour.[67,69,70] Like progesterone, hCG is produced in early pregnancy, and drives normal implantation and placentation.[71–73] Later, hCG produced by the placenta stimulates trophoblast differentiation, promoting maintenance of the STB layer by CTB fusion.[74,75] Hormones secreted by the placenta also regulate the maternal metabolic adaptation to pregnancy, required for sufficient nutrient delivery to the placenta. For example, placental growth hormone increases maternal insulin-like growth factor-1 (IGF-1) production and availability, which is associated with increased fetal glucose and amino acid supply, and consequent fetal growth.[76]

As the site of contact between semi-allogeneic fetal cells and the maternal circulation, the placenta plays a key role in modulating the *in utero* immune environment. Various adaptations allow for tolerance of the fetus, including those of maternal immune cells (to be discussed later), as well as mechanisms employed by the trophoblast and HBCs. Arguably the most important of trophoblast immune adaptations is their unique HLA expression profile. Both villous CTB and STB do not express most polymorphic HLA class I and II molecules, reducing the likelihood of detection by maternal immune cells.[77] Normally, a lack of HLA-A and -B expression renders cells prone to NK-mediated lysis in a phenomenon known as the 'missing-self' hypothesis.[78] However, EVT express HLA-C capable of binding to killer immunoglobulin-like receptors (KIRs) on NK cells, inhibiting their cytotoxicity.[79] As well as HLA-C, non-classical HLA-E and -G are present on the surface of EVT, which are known to modulate the activity of maternal immune cells, inhibiting deleterious effects of NK cells and stimulating T cells and DCs to adopt a more regulatory phenotype.[80,81] HLA-F is also expressed by EVT and though evidence shows it is also capable of binding to KIRs, its role in maternal-fetal tolerance is less well characterised compared to other placental HLA.[41,82]

Trophoblast express a variety of other immune checkpoint proteins, including those which modulate complement and influence immune cell polarisation. Complement regulatory proteins CD46, CD55 and CD59 are expressed on both EVT and STB, limiting excessive complement activation at the maternal-fetal interface.[83] CTB are also CD46-positive, however expression of CD55 and CD59 are reported less consistently between studies.[83,84] Instead, physical separation of the CTB from maternal blood by the STB layer likely provides protection from the complement system. Inflammation is further limited by the expression of ligands complementary to receptors on maternal immune cells, such as programmed death ligand 1 (PD-L1) which bind maternal macrophages and promote the adoption of an M2-like phenotype.[85] As resident fetal macrophages, HBCs are also anti-inflammatory and contribute toward healthy development via the secretion of angiogenic growth factors including VEGF, as well as the promotion of fetal tolerance. *In vitro*, HBCs appear resistant to pro-inflammatory mediators LPS and IFN-γ, and release high levels of anti-inflammatory IL-10, a cytokine associated with maternal immune cell tolerance during pregnancy.[86,87] HBCs also possess receptors for the Fc portion of IgG, which though currently poorly understood have been speculated to be involved in the sequestering and transfer of maternal IgG via the placenta, preventing the transfer of anti-fetal antibodies whilst allowing immunity towards pathogens to be conferred from mother to fetus before birth.[88] The placenta therefore serves not only to initiate and maintain maternal tolerance toward the fetus, but actively participates in the priming of the fetal immune system.

Placental Pathology

Taking into account the numerous vital roles of the placenta, it is perhaps unsurprising that disorders affecting its structure and/or function can have severe consequences for the survival of the fetus. After delivery, the placenta serves as a 'diary' of pregnancy, wherein pathological events are often visible as distinct histological features.[89,90] Placental lesions can be broadly categorised into those affecting the maternal or fetal vasculature and those mediated by inflammation, though these processes show a degree of overlap in some cases.[90] One such disorder diagnosed solely via placental histopathological examination is chronic histiocytic intervillositis (CHI), wherein immune cell infiltration is apparent within the maternal side of the placenta, resulting in placental dysfunction and subsequent FGR or fetal death.[91] Due to similarities shared with allograft rejection, CHI has been hypothesised as a disorder of excessive maternal inflammation and ultimately placental rejection.[92–94] Research into materno-fetal tolerance has drawn comparisons to the field of transplant biology, where maintenance of the semi-allogeneic fetus has been proposed to parallel the acceptance of a foreign graft by transplant recipient.[8] Similarities between both fields have encouraged questioning as to whether the same principles of tolerance and histocompatibility apply in pregnancy, and if so, how this can be utilised to inform the understanding and treatment of CHI and other obstetric conditions with immunological aetiology. This topic was explored in detail in a narrative literature review.

Chronic Histiocytic Intervillositis: A Breakdown in Immune Tolerance Comparable to Allograft Rejection?

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Abstract

Chronic Histiocytic Intervillositis (CHI) is a pregnancy disorder characterised by infiltration of maternal macrophages into the intervillous space of the human placenta, often with accompanying perivillous fibrin deposition. CHI is associated strongly with fetal growth restriction and increased risk of miscarriage and stillbirth. Although rare, affecting 6 in every 10000 pregnancies beyond 12 weeks' gestation, the rate of recurrence is high at 25-100%. To date, diagnosis of CHI can only be made post-delivery upon examination of the placenta due to a lack of diagnostic biomarkers, and criteria varies across publications. No treatment options have shown proven efficacy, and CHI remains a serious obstetric conundrum.

Although its underlying aetiology is unclear, due to the presence of maternal macrophages and the reported increased incidence in women with autoimmune disease, CHI is hypothesised to be an inappropriate immune response to the semi-allogeneic fetus. Given this lack of understanding, treatment approaches remain experimental with limited rationale. However, there is recent evidence that immunosuppression and antithrombotic therapies may be effective in preventing recurrence of associated adverse pregnancy outcomes. With similarities noted between the pathological features of CHI and acute rejection of solid organ transplants, further investigation of this hypothesis may provide a basis for tackling CHI and other immune-related placental conditions. This review will explore parallels between CHI and allograft rejection and identify areas requiring further confirmation and exploitation of this comparison.

Keywords

Pregnancy, stillbirth, miscarriage, macrophages, placenta, graft rejection, HLA

Introduction

From implantation of the embryo through to birth, successful pregnancy relies upon the promotion of a tolerogenic uterine environment to allow the fetus to thrive.[8] Simultaneously, and of equal importance, the maternal inflammatory response must be conserved to protect the mother and fetus against pathogens.[95] This 'paradox' of pregnancy, first described by Sir Peter Medawar in the 1950s,[8] has prompted investigation into the immunology of pregnancy, particularly where it may be dysfunctional.

Chronic histiocytic intervillositis (CHI), also known as chronic intervillositis (CI), chronic intervillositis of unknown etiology (CIUE) and massive chronic intervillositis (MCI),[96] is an example of a rare placental inflammatory disease which can occur during any trimester of pregnancy. Initially described by Labarrere and Mullen in 1987 as a placental lesion consisting of histiocyte (macrophage) infiltration within the intervillous space, fibrin deposition (Figure 6) and trophoblast necrosis,[91] CHI is strongly associated with fetal growth restriction (FGR),[97,98] miscarriage and stillbirth.[99,100] A systematic review by Bos et al.[96] found the rate of live birth in cases of CHI to be 54.9%, with only 32.4% of pregnancies reaching term, possibly related to FGR and consequent intervention. The same review also found a miscarriage rate of 24%, with half occurring between 12 and 22-weeks' gestation. FGR is reported to affect 42-61.5% of pregnancies diagnosed with CHI.[97,98] Importantly, CHI has a 25-100% chance of recurrence in subsequent pregnancies,[96,98,101] with more severe or diffuse CHI associated with poorer outcomes.[102] When infiltration of mononuclear cells and fibrin deposition have been graded in CHI cases,[103] placentas with the lowest score corresponded to live births and those with the highest grade have a 73% chance of *in utero* loss. A 2013 retrospective study from the Netherlands also correlated increased CHI severity with a shorter pregnancy duration and increased risk of miscarriage, stillbirth and neonatal death.[94] A report also suggests that CHI may mimic features of osteogenesis imperfecta, including bone fractures in the fetus, though this was unable to be confirmed genetically.[104] Three pregnancies studied in this report resulted in FGR and a small placenta, suggesting that fetal development may have been limited by uteroplacental insufficiency. CHI reportedly affects 6 in 10,000 second and third trimester placentas sent for histopathological examination[105] and has been identified in 4.4% of first trimester miscarriages with normal karyotype.[106] The suggested incidence of CHI in pregnancies with normal outcome is 0.2-0.4%.[107,108]

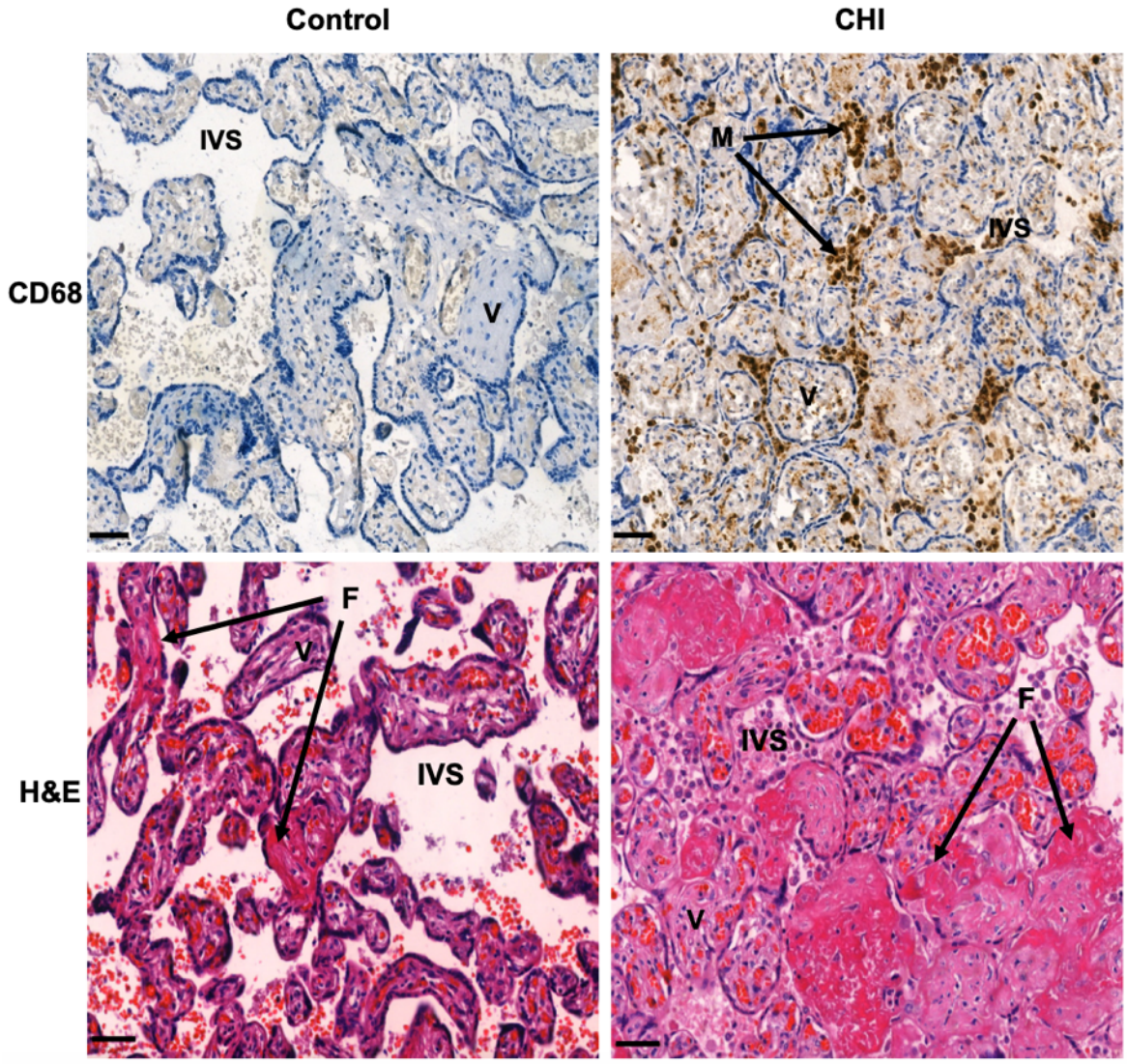

Figure 6. Histological characteristics of a placenta affected by chronic histiocytic intervillositis (CHI) compared with that of a healthy control pregnancy. Immunohistochemical staining demonstrates infiltration of maternal $CD68^{+}$ macrophages (M) into the intervillous space (IVS) of the placenta in CHI, surrounding fetal villi (V). Fibrin (F) shown by haematoxylin and eosin (H&E) staining as a shade of dark pink is present to a degree within the healthy term placenta, though is considerably increased in cases of CHI. Scale bars = 50μm.

The exact mechanism by which CHI causes adverse outcomes is unknown. However, Marchaudon et al.[100] found that CHI-affected pregnancies, complicated by spontaneous early miscarriage and FGR, were associated with more intense fibrin deposition within the placenta. In another comparison to healthy pregnancies, pregnancies with CHI demonstrated failure in physiological transformation of spiral arteries and a significantly higher presence of atherosclerotic-like lesions,[109] suggesting that nutrient and gas exchange across the placenta may be affected. Another study speculated that accumulation of cells within the intervillous space increases the oxygen diffusion distance between maternal erythrocytes and fetal villi,[93] a source of reduced placental efficiency and dysfunction.

The pathophysiology of CHI has been sparsely described in the literature and remains poorly understood, which is perhaps understandable given its relative rarity and description since the early 2000s. Investigations of the underlying mechanisms are mostly based on retrospective case series, allowing limited interpretation and extrapolation. Clinical and histological observations from

CHI patients indicate a disorder of immunological aetiology, though this is yet to be verified conclusively. This review appraises current literature regarding the pathophysiology, diagnosis and management of CHI. Furthermore, limitations in current evidence are considered and suggestions made for future research and its clinical management.

Immune Tolerance in Human Pregnancy

Historically, the ability of the mother to tolerate the semi-allogeneic fetus has led to the assumption that pregnancy is a state of immunosuppression.[110] However, more recent research has shed light on pregnancy as a unique state of tolerance, requiring a careful balance of fetal evasion of the maternal immune system with appropriate and proportionate modulation of maternal immune cell function.[111] Outside of pregnancy, non-self antigens, such as those on the surface of pathogens or a transplanted organ, result in an inflammatory response.[112] However, during pregnancy, immune tolerance results in the limitation of this response, allowing accommodation of the genetically different fetus across gestation.[111]

In brief, immune tolerance in pregnancy involves the expansion of immune modulating, anti-inflammatory cells and cell functions. Amongst these are regulatory T-cells (Tregs), which play an essential immunosuppressive role via secretion of anti-inflammatory cytokines IL-10 and TGF-β, and limitation of T cell responses towards fetal antigens.[111,113,114] Interestingly, Tregs demonstrate specificity towards fetal antigens, and exhibit accelerated proliferation during future pregnancies.[115] Following fertilisation and embryo implantation, uterine natural killer cells (uNKs) form the dominant component of the immune milieu, though their cytotoxic effects towards trophoblast cells are attenuated via decidual macrophages.[116] After uNKs, macrophages are the second most abundant decidual leukocyte and persist throughout pregnancy, with the majority polarised towards an M2 anti-inflammatory phenotype.[117] In rodent models, dendritic cells (DCs) are entrapped within the uterus preventing antigen presentation and may be responsible for the induction of Tregs from naïve T cells.[113,118] Stromal cells of the decidua may also contribute towards tolerance via silencing expression of genes related to chemokine production, as shown in mice.[119]

In addition to maternal adaptations, the trophoblast has evolved to evade immune detection, through limited expression of low-immunogenic non-classical self-antigens (human leukocyte antigens, HLA) and the immunomodulatory properties of expressed HLA-G.[42] Together, maternal immune cell adaptation and placental HLA expression demonstrate multiple protective mechanisms against both paternal antigen sensitisation and maternal anti-HLA antibody production; both known determinants of poor outcomes, including preterm delivery and chronic chorioamnionitis.[120,121] Control and appropriate timing of inflammation is crucial for all stages of healthy pregnancy, facilitating implantation, pregnancy maintenance and finally parturition.[6]

Inflammation in Implantation and Parturition

Normally, physiological inflammation within the local uterine environment occurs at specific stages in pregnancy and is required for adequate placental development, as well as the initiation of parturition. Healthy pregnancy is therefore a balance of inflammatory and tolerogenic processes, in which a breakdown in either may have pathogenic consequences, including recurrent pregnancy loss (RPL), Villitis of Unknown Etiology (VUE) and CHI.

Endometrial studies have noted that implantation and placental development are associated with a strong pro-inflammatory Th1 type-response, characterised by increased pro-inflammatory cytokines, IL-6, IL-8 and TNFα.[122,123] The production of these cytokines by endometrial cells is responsible for the recruitment of uNKs, DCs and macrophages to the decidua.[122] An established inflammatory gradient has also been hypothesised for the production of increased adhesion molecules by endometrial epithelial cells in guiding the blastocyst to the implantation site and facilitating trophectoderm attachment.[122]

Following this inflammatory phase, recruited decidual leukocytes reportedly adopt a more immunomodulatory phenotype. uNKs are responsible for establishing instability within maternal vessels as a prerequisite to uterine vascular remodelling, promoting trophoblast invasion of the endometrium, via IFN-γ release and production of angiogenic factors to enhance maternal blood flow.[124–126] Like uNKs, DCs and macrophages begin to secrete angiogenic factors in addition to anti-inflammatory cytokines (IL-4, IL-10 and IL-13), and are responsible for the subsequent shift to an anti-inflammatory Th2 profile, which predominates for the remainder of pregnancy until parturition.[127] Macrophages also play an important role in phagocytosis of cellular debris, reducing contact between fetal antigens and the maternal immune system during vascular remodelling, and continued trophoblast release from the placenta through cell turnover and vesicle production.[128]

Though the initiation of parturition is poorly understood, an inflammatory component has been consistently identified, alongside the roles of myometrial stretch, hormones and prostaglandins.[129] An increase in circulating IL-1, IL-6 and TNF-α have been identified in both spontaneous term labour and preterm birth, and are thought to regulate prostaglandin release and activation via the cyclooxygenase-2 (COX-2) pathway.[130] The resulting cervical ripening, uterine contractions and placental detachment are all necessary components for normal birth.[130] The extent and timing of this cytokine cascade inevitably requires precise control, as excess inflammation (i.e. generated by autoimmune disease) may instigate early-onset of labour as epitomised in spontaneous preterm birth.[6,131,132]

Inflammation and Recurrent Miscarriage

Recurrent miscarriage (RM), defined as three or more consecutive miscarriages, is a diverse condition in which more than half of couples has no identifiable cause.[133] In such cases, immune dysfunction has been investigated as a possible explanation; low Treg levels, anti-HLA antibodies and NK cell levels have also been postulated as a causative factor,[4,5] with several groups reporting increased NK cell populations in pre-pregnancy endometrium of women with RM.[134–136] Nevertheless, studies have been contradictory.[137,138] Immunosuppression using prednisolone treatment has been trialled in RM and achieved depletion in uNKs,[139] but here again clinical efficacy is debated, especially given the recognised importance of uNK cells in the development of tolerance and healthy placentation.[140]

Inflammation and Villitis of Unknown Etiology

VUE is a placental inflammatory condition associated with FGR, stillbirth and possible neurological impairment.[13,141–143] Placentas with VUE exhibit infiltration of maternal $CD8^+$ T cells into the chorionic villous tree and a small number of maternal $CD68^+$ macrophages, in addition to the activation of resistant placental villous macrophages, Hofbauer cells.[144] Deposition of the

complement protein C4d, a marker of innate immune activation, is also evident, as well as a Th1 proinflammatory cytokine profile and upregulation of genes and chemokines associated with tissue rejection.[145,146] The primary cause of inflammation in VUE is unclear, however it is known that noxious insults to placental tissue induces release of damage-associated molecular patterns (DAMPs) which alter the chemokine profile and result in recruitment of maternal immune cells.[6,147] It may therefore be possible that VUE is a sterile inflammatory response to placental damage of unknown origin, occurring most commonly in the third trimester when tolerance begins to decline.[148]

Inflammation and Chronic Histiocytic Intervillositis

CHI is an inflammatory lesion of mononuclear cell accumulation within the intervillous space of the human placenta.[91] The predominant theory is it's a disorder of excessive maternal inflammation directed towards the placenta,[103,149] possibly occurring in early pregnancy, but exacerbated in the second and third trimester, where it is more readily identified. This theory is supported by the presence of fibrin deposition, complement activation and B and T cell responses directed towards paternal antigens in CHI.[94,149] CHI has also been previously linked to fetal and neonatal alloimmune thrombocytopaenia (FNAIT), a maternal immune response mounted against fetal platelet antigens.[150] Other studies have also reported increased incidence in women with pre-existing autoimmune disease.[151,152]

Pathophysiology of CHI

Proposed pathophysiological pathways in CHI are summarised in Figure 7. The infiltrate consists of $CD45^+$ and $CD68^+$ monocytes and a small proportion of $CD4^+$ and $CD8^+$ T cells (Figure 7).[93] $CD68^+$ macrophages form the predominant component of the cellular infiltrate in CHI, though despite their abundance it is largely undetermined what effect they exert on the placenta and how they contribute towards adverse pregnancy outcomes.[93]

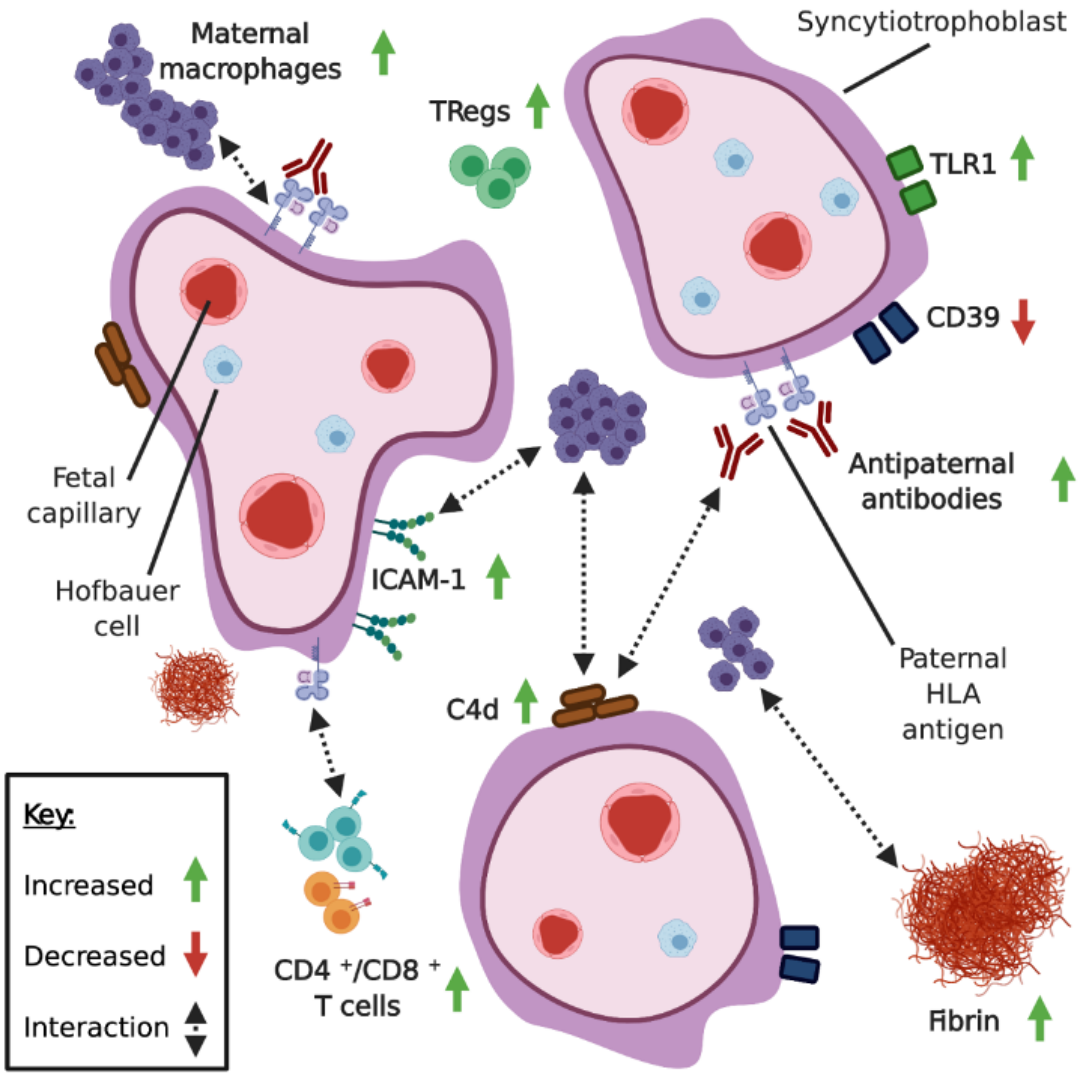

Figure 7. Suggested pathophysiology of chronic histiocytic intervillositis (CHI) of the placenta. Maternal monocytes, predominantly CD68+ M2-like macrophages, infiltrate the intervillous space of the placenta, in association with increased fibrin deposition.[153] CD4+ and CD8+ T cells are also increased within maternal blood surrounding the fetal villi, though they constitute a much smaller proportion of the infiltrate.[103] In addition to T cells directed towards paternal antigens, anti-HLA antibodies have been identified in women affected by CHI and are hypothesized to interact with antigens expressed on the placenta.[94] In some cases of CHI, deposition of the complement protein C4d, usually associated with an antibody-mediated immune response and macrophage recruitment, is increased along the surface of the syncytiotrophoblast.[149] Increased intercellular adhesion molecule-1 (ICAM-1) expression by syncytiotrophoblast is also hypothesized as a contributory factor in macrophage recruitment.[161] Expression of CD39, an ectonucleotidase responsible for hydrolysis of damage-associated molecular patterns (DAMPs), is decreased in CHI in areas of dense cellular infiltration, suggesting it may also be involved in the maternal inflammatory response.[163] The mechanism of fibrin deposition in CHI is unclear, though it is often present as a non-specific response to placental damage.[173] Macrophages in CHI have been found to express complement receptor CR4, which outside of CHI is capable of mediating monocyte adhesion to fibrinogen, though remains unexplored in this context.[153] Certain cases of CHI demonstrate increased toll-like receptor 1 (TLR1) expression, suggesting a possible bacterial component, though this is not always evident.[167] Figure created with BioRender.com.

Characterisation of the expression profile of maternal intervillous macrophages in CHI reveals an M2-like anti-inflammatory polarisation state indicated by expression of CD163.[153] This finding is consistent with previous data suggesting that macrophages in CHI are 'resting', without destructive infiltration into villous tissues.[93] In the same expression analysis, CHI macrophages showed an overexpression of CD11c and CD18, which together form the complement receptor CR4.[153] Though CR4's role in CHI is yet to be investigated, more general studies on its function suggest it mediates monocyte adhesion to fibrinogen, the soluble component in blood which is converted to insoluble fibrin during clotting.[154,155] As increased fibrin deposition is widely described as a histopathologic feature of CHI,[91,100,156] it is possible that its presence has an effect on monocyte accumulation and persistence in the intervillous space, but this proposal remains unexplored.

Reus et al.[94] detected a higher cytotoxic T lymphocyte precursor frequency (CTLpf) in women with CHI compared to controls, as well as the presence of partner-directed anti-HLA antibodies. In RM and preterm birth, the presence of anti-HLA antibodies is associated with the reduced chance of a livebirth,[5,120] but in CHI it is not yet known whether these antibodies have the ability to bind to the placenta and, if so, what effect they have on its structure and/or function. The generation of partner-directed anti-HLA antibodies, albeit to a lower degree, is also a normal physiological response to healthy pregnancy following exposure to paternal antigens during conception and birth.[157] Therefore, it remains to be confirmed whether these antibodies have clinical relevance in the development of CHI or are just a feature of the normal immune response in pregnancy.

Further evidence of a possible antibody-mediated component to CHI is suggested by the presence of complement cascade split product C4d along the apical surface of the syncytiotrophoblast.[149] C4d deposition is often used as a marker of activation of humoral immunity as it is produced following activation of the complement cascade classical pathway by both IgG and IgM.[158] Complement activation via anti-HLA antibody binding in other biological contexts, including *in vitro* models of solid organ rejection, appears to be responsible for increased adhesion of monocytes,[159] and other products of the cascade influence both macrophage and T cell activity.[160] Involvement of complement in CHI may represent a possible mechanistic link between antibodies in maternal serum and infiltration of macrophages and T cells into the placenta, though this remains speculative.

There are few studies of placental protein expression in CHI, but upregulation of intercellular adhesion molecule-1 (ICAM-1) on the syncytiotrophoblast has been noted.[161] ICAM-1 is responsible for the migration of leukocytes via the leukocyte function-associated antigen-1 (LFA-1) and increases monocyte adhesion to the syncytial surface.[162] *In vitro*, syncytiotrophoblast upregulate ICAM-1 in response to the cytokines IL-1α, IFN-γ and TNF-α, which are constitutively produced during inflammation by macrophages and T cells.[162] In CHI, the observed increase in ICAM-1 may be either a causative factor, contributing to maternal immune cell recruitment into the intervillous space, or a consequence of placental damage.

In addition to ICAM-1, a second protein implicated in immune cell recruitment in CHI is CD39, an ectonucleotidase normally expressed by trophoblast.[163] CD39 hydrolyses ATP (adenosine triphosphate) which is released from damaged or apoptotic cells and acts as a DAMP.[164] Therefore, ATP's hydrolysis by CD39 acts as an immunosuppressive mechanism in healthy tissue.[163] Sato et al.[163] found that CD39 expression was significantly decreased in 22 cases of CHI compared to controls. Furthermore, placentas from CHI pregnancies with poor outcomes (FGR and

fetal death) demonstrated a greater decrease in CD39 expression and a significantly greater level of $CD68^+$ cell infiltration compared to those resulting in livebirth. Regions of dense intervillous $CD68^+$ macrophage infiltration also corresponded with areas of trophoblast which displayed reduced CD39. Though the mechanism of CD39 downregulation was not investigated in this study, the findings indicate that it may be a contributory factor in CHI pathogenesis and certainly warrants further investigation.

Another immunomodulatory factor implicated in CHI is CD200 (Cluster of Differentiation 200), a cell surface protein with immunosuppressive function responsible for the promotion of anti-inflammatory M2 macrophages and Treg differentiation, as well as the inhibition of cytotoxic NK cell responses.[165,166] Preliminary data from a single case of CHI demonstrated reduced CD200 expression on syncytiotrophoblast compared to healthy term placenta.[165] This may suggest a mechanism for inadequate tolerance in CHI as theoretically, a lack of CD200 should result in fewer Tregs. However, a study by Capuani et al.[103] revealed that the condition was associated with increased Treg populations in the decidua and intervillous space. The Treg number was positively correlated with CHI severity, and although their role is unclear, it's possible that their expansion represents an effort to resolve inflammation and breakdown in tolerance at the maternal-fetal interface.[103]

As already described, the specific immunological trigger in CHI is unidentified, though a bacterial component has been speculated. Toll-like receptor-1 (TLR1) expressed by monocytes is involved in recognition of bacterial infections and lipopolysaccharide (LPS)-induced inflammation, and in a study by Hussein et al.[167] was upregulated in certain cases of CHI. In diagnosis of placental disease, cases are initially screened in order to rule out common infectious agents e.g. malaria, which exhibits a similar monocytic infiltrate;[168] however, the possibility of undetected bacteraemia has still been proposed.[167] Not all cases of CHI in this study exhibited increased TLR1 expression, and it is unlikely that the recurrent nature of CHI can be explained by a bacterial agent, so it is important that other main pathways of non-infectious pathogenesis are considered.

CHI and Other Inflammatory Placental Lesions

CHI can be associated with other inflammatory lesions of the placenta including VUE and massive perivillous fibrin deposition/maternal floor infarction (MPFD/MFI). Similarities and differences between these lesions are summarised in Table 1. Importantly, these lesions are distinguished from those with infectious cause, such as malaria and chorioamnionitis which result in placental infiltration of maternal macrophages and neutrophils, respectively. [169,170]

Table 1. Comparison of non-infectious inflammatory placental lesions.

	CHI	VUE	MPFD/MFI
Definition	Non-infectious infiltration of the intervillous space by maternal mononuclear cells, with or without associated fibrin deposition[171]	Non-infectious mononuclear cell infiltrate into the villous stroma, with destruction/necrosis of villous parenchyma[172]	Extensive deposition of fibrin within the intervillous space, or within and around the basal plate[173]
Incidence	0.01% of placentas[96]	5-15% of placentas[148]	0.028-0.4% of pregnancies[107,174,175]
Rate of recurrence	25-100%[96,98,101]	10-15%[148]	12-78%[174]
Associated adverse pregnancy outcomes	FGR, preterm birth, fetal death[96]	FGR,[176] stillbirth and neurological impairment[141,143]	FGR,[174] preterm birth,[174] fetal death,[177] fetal malformations and neurological impairment[178,179]
Histological features	• Trophoblast necrosis[91] • Infiltrate composed mainly of $CD68^+$ M2-like macrophages[93] • Fibrin deposition[96]	• Infiltrate composed mainly of maternal $CD8^+$ T cells[144] • Activation of fetal macrophages – Hofbauer cells[144]	• Fibrin deposition resulting in engulfment and atrophy of chorionic villi[173]
Evidence for immunological aetiology	• Association with autoimmune disease/autoantibodies[151,152] • C4d deposition[149] • Increased maternal CTLpf[94] • Increased anti-HLA antibodies[94] • Syncytiotrophoblast ICAM-1 upregulation[161] • Placental CD39 downregulation[163]	• Th1 proinflammatory cytokine profile[13] • C4d deposition[145] • Upregulation of graft rejection-associated genes and chemokines[146] • Upregulation of class I and II HLA in inflamed villi[146]	• Association with autoimmune disease/autoantibodies[173] • C4d deposition[180] • Increased anti-HLA antibodies[180,181] • Increased inflammatory chemokine expression[181]

Chronic histiocytic intervillositis (CHI), villitis of unknown etiology (VUE) and massive perivillous fibrin deposition (MPFD/MFI) have reported overlaps in pathology, with evidence of suggested immune involvement and rejection. CD – cluster of differentiation, CTLpf – cytotoxic T lymphocyte precursor frequency, FGR – fetal growth restriction, HLA – human leukocyte antigen, ICAM-1 – intercellular adhesion molecule-1.

In some instances of CHI, VUE is also evident, with combined lesions in 25-47% of cases.[102,161,182] Due to their concurrence, it has been suggested by Nowak et al.[102] that the two disorders are on

the same spectrum of disease, where CHI is the more extreme variant, causing higher morbidity and earlier presentation.[99,102] In support of this hypothesis, FGR is reported to be more frequent in CHI than VUE and is associated with a five-times higher risk of stillbirth (29.2% vs. 6.4%).[102] VUE also recurs in subsequent pregnancies, with an increasing risk of FGR and stillbirth.[13] There are important differences between CHI and VUE which cast doubt on the hypothesis that they are related disorders. Firstly, VUE is more frequent than CHI, affecting up to 15% of all placentas.[148] Inflammation in CHI is limited to the maternal placental intervillous compartment rather than infiltration of the chorionic villous tree and stroma, typical of VUE. There is also a lack of Hofbauer cell activation.[93] Although both disorders similarly exhibit a mononuclear inflammatory infiltrate, CHI is mainly composed of maternal macrophages with a smaller proportion of $CD8^+$ T cells.[102] Labarrere et al.[182] described both lesions as containing a higher proportion of $CD8^+$ T cells compared to $CD4^+$ T cells, however another study by Capuani et al.[103] found the ratio to be almost equal. Cytokine profiles in CHI and VUE have been compared by Freitag et al.[93] wherein CHI demonstrated lower expression of pro-inflammatory cytokines, including CCL2 compared to VUE. This finding has been hypothesised as an explanation for the lack of destructive action of mononuclear cells in CHI, though the study was limited in size (N=5), and thus requires further investigation and confirmation.[93]

Case reports have also described overlap of CHI with MPFD/MFI.[183,184] Like CHI, MPFD and MFI have been hypothesised as a possible manifestation of a maternal rejection-type response towards the placenta, with increased anti-HLA antibodies towards fetal antigens, C4d deposition and chemokine upregulation in certain cases.[181] Other similarities noted between MPFD and CHI include risk of recurrence and an association with autoimmune disease or autoantibodies.[185] The exact cause of MPFD is unknown, though studies have suggested a link to abnormalities in blood coagulation,[186] a theory which has also been proposed in CHI with regard to the observed increase in fibrin deposition.[185] Further work is required to establish whether these diseases are truly related, or separate entities with differing pathophysiology.

Diagnosis of CHI

Both VUE and CHI are asymptomatic for the mother and can occur across different maternal ages and in women with a range of obstetric histories.[93,96] Currently, they can only be diagnosed upon histopathological examination of the placenta after delivery. For CHI, there is no standardised classification by which it is graded; therefore scoring systems validated in several different patient populations are required.[96,102] Pathologists in two studies graded CHI according to level of severity observed: absent, focal (<10% of the slide), moderate (10-50%) and severe or massive (>50%).[94,101] Reus et al.[94] suggested this as a reliable technique for CHI detection, despite the inter-observer agreement being K=0.54 (moderate). A systematic review by Bos et al.[96] found that the only agreed criterion, amongst 18 studies on CHI, was the presence of intervillous infiltrate; with only 61% ruling out infectious cases, and many excluding cases with villitis. This suggests that the reported incidence of CHI may fluctuate depending on varied inclusion and exclusion criteria between centres. Based on these findings, Bos et al.[96] proposed alternative standardised criteria for diagnosing CHI:

I. An infiltrate present in the intervillous space (most important requirement).

II. Approximately 80% of the mononuclear cells in the intervillous space being $CD68^+$.
III. The occupied infiltrate being 5% or more of the total intervillous space.
IV. Exclusion of cases with clinical or histopathological signs of infection.

A standardised approach to the diagnosis of CHI is needed to define critical values to inform future patient care,[187] to reduce heterogeneity between studies, and allow more reliable comparisons in the underlying pathophysiology of CHI and its treatment. Besides the lack of robust diagnostic criteria, there is also a need to identify possible prenatal (or even preconception) biomarkers of CHI. Currently, reliance on histopathology for diagnosis means that intervention can only be initiated in subsequent pregnancies of women with poor outcomes attributed to CHI in a previous pregnancy. In particular, it is reported that serum alkaline phosphatase (ALP) is increased in some cases of CHI[97,100,188] as well as FGR and preterm delivery,[189,190] though whether these changes have any predictive value is unknown.[191] An observational study which investigated markers in CHI, including biochemical (e.g. Pregnancy Associated Plasma Protein-A (PAPP-A)) and radiological (placental dimension and uterine artery Doppler) features, found no assessment was consistently linked to diagnosis following delivery.[192] Nonetheless, such markers may help to elucidate pathophysiology and inform individual patient care, justifying closer prenatal monitoring.

Treatment of CHI

Due to CHI's high rate of recurrence, the use of pharmacological treatments including aspirin, low molecular weight heparin (LMWH) and/or steroids in future pregnancies, has been proposed.[98] Contro et al.[98] systematically reviewed the literature prior to 2010, which revealed 13 cases of CHI where immunosuppressive and/or thrombo-prophylactic intervention were trialled. There was no significant difference between livebirth rates in the treatment group compared to untreated CHI cases. However, the treatment regimens were inconsistent and small study sizes limited detailed analysis of therapeutic combinations. A case report by Ozawa et al.[97] described prophylactic treatments of a woman with previous CHI-related recurrent miscarriage; severe FGR and a stillbirth at 27 weeks' gestation. In this case, CHI occurred in her next pregnancy despite aspirin monotherapy, with diffuse presentation and moderate fibrin deposition.[97] For this woman, a combination of aspirin and heparin was given during her subsequent pregnancy; FGR occurred but the patient delivered a live infant at 33 weeks' gestation, with the placenta again showing diffuse macrophage infiltration and moderate fibrin deposition. Using a combination of prednisolone and low dose aspirin in another subsequent pregnancy, no FGR was noted and a live infant was delivered. The placenta showed focal CHI and mild fibrin deposition only. Although the pregnancy that used prednisolone and low dose aspirin in combination held the most favourable outcome and least severe CHI, conclusions should not be drawn from this single case report.
A prospective study by Mekinian et al.[152] investigated potential treatments for the prevention of CHI in 24 women with a previous diagnosis, and included a variety of treatment regimens; aspirin or LMWH monotherapy, aspirin/prednisone dual therapy, aspirin/LMWH/prednisone polytherapy and aspirin/LMWH/prednisone/hydroxychloroquine polytherapy. No particular treatment regimen was found to improve pregnancy outcomes, though again this study was limited by a small sample number in each treatment group and possible bias; given that polytherapy was used more frequently in women with more severe obstetric history.

Given the rarity of CHI, it is difficult to explore interventions and ethical issues surrounding randomized placebo-controlled studies in women who often have extremely poor obstetric histories. Evidence surrounding current therapies is limited, and no single treatment demonstrates clear beneficial effects.[97] The justification for use of immunosuppressive therapies in CHI is based on increasing evidence that it's a disease of maternal anti-fetal rejection, though the specific pathophysiology, as discussed already, is largely unknown. Comparing similarities between CHI and organ rejection could allow potential exploratory avenues and direct research into more targeted and effective treatments, once causative mechanisms are identified.

Similarities Between Allograft Rejection and CHI

As the fetus expresses paternal antigens, akin to a semi-allograft and requiring maternal immune tolerance, the state of pregnancy has long been likened to recipient acceptance of a transplanted donor organ.[8] In this comparison, the presence of anti-paternal T cells and antibodies in CHI patients[93,94] draws parallels with the donor-directed immune response in acute graft rejection; which unlike CHI has been better characterised, with attributed diagnostic criteria.[51] Histopathological features common to both allograft rejection and CHI include macrophage infiltration and deposition of fibrin and complement (Figure 8), ultimately resulting in dysfunction of the semi-allogeneic organ,[193] which in CHI, may be comparable to the failure of the placenta to maintain fetal growth and/or survival. Graft rejection can be classified as antibody mediated rejection (AMR) or cellular rejection, though it is possible for both to coexist.[194] In AMR, donor specific antibodies (DSAs) are either pre-formed following sensitisation events (e.g. pregnancy, blood transfusion or previous transplant)[46] or produced *de novo* by B cells and plasma cells in response to HLA antigens on the vasculature of the allograft.[47] AMR leads to graft loss via complement deposition, recruitment of NK cells, monocytes and macrophages which contribute towards fibrin formation, endothelial cell damage and eventually interruption of vascular function.[193,195] Anti-HLA antibodies identified in CHI could be likened to these DSAs, especially as complement, monocytes and macrophages also appear to play a key role in its pathophysiology.[91,149] However, as the placenta is known to express only the least polymorphic of HLA molecules with low immunogenicity, it is unclear how anti-HLA antibodies may be detrimental when not deemed so in normal healthy pregnancy.[157] On the other hand, in VUE, Enninga et al. noted increased placental expression of Class I and II HLA molecules along with upregulation of genes normally associated with graft rejection,[146] though this was within fetal villi as opposed to the syncytiotrophoblast in contact with the maternal circulation; where CHI is always focused. Such studies into VUE have strengthened the comparison with allograft rejection, though other groups have argued the disorder simultaneously resembles graft vs host disease given the coexistence of a fetal inflammatory response.[141,196] Currently, evidence for VUE as a form of rejection is greater compared to CHI (perhaps due to differences in incidence), though this hypothesis still remains to be proven. It may therefore be worthwhile to undertake similar studies in CHI, including the aforementioned genetic analysis, to determine whether these inflammatory processes are also common to this disorder.

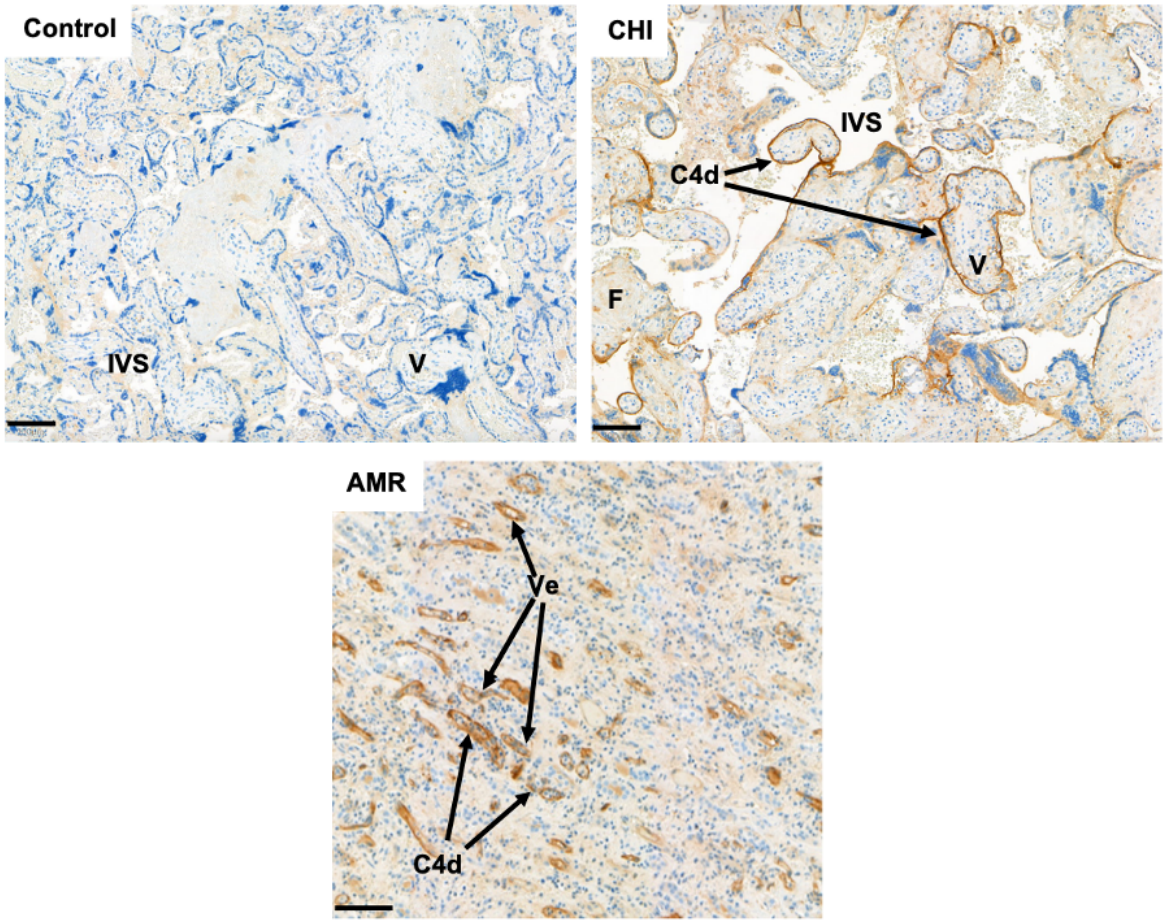

Figure 8. Immunohistochemical staining of complement cascade split product C4d in a healthy placenta and a case of chronic histiocytic intervillositis (CHI), compared to a biopsy of a kidney with confirmed antibody mediated rejection (AMR). In some cases of CHI, C4d is present along the apical membrane of the syncytiotrophoblast, similar to deposition within the vessels (Ve) of a rejected kidney allograft. Within placentas affected by CHI, terminal villi are often entrapped within deposits of fibrin (F). IVS = intervillous space, V = villi. Scale bars = 100μm.

Cellular rejection in a transplanted organ is characterised by $CD8^+$ T cell activation and the production of proinflammatory cytokines resulting in cytotoxic effects.[197] The action of T cells in cellular rejection can also be responsible for the recruitment of other effector cells, including macrophages, which further implement vascular injury.[198] $CD8^+$ T cells have been observed in CHI, though their role appears minor in comparison to that of the M2 macrophages which dominate the cellular infiltrate.[103] However, compared to graft rejection, the roles of T cells and macrophages and their possible interaction in CHI, remain poorly defined and require further study.

As some patients with CHI exhibit features consistent with either AMR or cellular rejection or both, it is possible that multiple pathological processes occur in the manifestation of CHI and may vary between individuals. This could explain the differing success of immunosuppressive therapies observed in CHI, and why particular therapies thought to target T and B cell responses, such as hydroxychloroquine, are reportedly effective in certain cases, but not others.[199] Similarities between rejection and CHI may suggest a potentially lucrative investigative avenue to frame future CHI research, though in comparison to VUE these have been explored to a much lesser extent. Considering the rarity of CHI, which limits current evidence base, the use of a model or framework on which to build future studies may expedite research and findings in the field.

Further Investigations

Much is still unknown about CHI, and current evidence is significantly limited by small sample sizes. Although rare diseases tend to have a weaker evidence-base, there are methods of overcoming these difficulties; encouragement to thoroughly investigate placental pathology in RM, FGR and stillbirth with subsequent multicentre studies would improve epidemiological knowledge and the robustness of studies performed. Moreover, prospective studies of unselective samples, determining the full spectrum of prognosis associated with CHI, as well as identifying a clear relationship between the intervillous infiltrate and pregnancy outcomes, would reduce the possibility of selection or reporting bias.

Important clinical questions remain, including which factors predict disease recurrence and the relationship between severity of histopathological findings and pregnancy outcomes, to assist in the assessment and management of patients with a history of CHI. Additionally, studies investigating the clinical and aetiological significance of CHI, VUE and MPFD in discrete and combined lesions are required, to ascertain whether these conditions are related or distinct entities.[102] It may be valuable to investigate whether all pregnancies of mothers with a history of CHI require closer monitoring, or whether this is the case only if the previous-defined disease was diffuse in the placenta or the pregnancy had a poor outcome.[102]

There is also an important need to identify risk factors for the development of CHI, in order to discriminate which women are likely to mount an inappropriate immune response to their fetus, prenatally or possibly even pre-conceptionally. In order to do so, future studies are required to characterise the full range of clinical presentations of CHI, as well as potential prognostic biomarkers. Utilisation of pre-transplantation testing may prove useful here, as advances in the prediction and prevention of rejection means graft survival can be maintained for years using medication even in recipients with a high degree of HLA mismatch to their donors, and may therefore also be possible in CHI.[55]

Aside from a lack of prognostic biomarkers and a treatment regime with proven efficacy, the fundamental background knowledge on the causes of CHI remains limited. Its similarities shared with organ rejection suggest that this may be used as a model of immune tolerance breakdown to direct research into CHI pathophysiology and management. In this model, CHI-affected placentas and maternal serum from such cases could be assessed in a similar fashion to other specialists in the investigation of organ rejection. For example, using the crossmatching process to pinpoint causative antibodies in sera of CHI patients. Additionally, undertaking assessments of organ function, such as imaging and serum analysis currently exercised in transplantation,[200,201] may help find indicators of placental dysfunction of value in all areas of placental research. Applying an established evidence base to a rare condition could accelerate the investigative process and allow for earlier detection, not only in CHI but also other obstetric conditions with a suspected immune component. Further to this, a strong rationale for the use of screening, immunosuppressive therapies and increased monitoring in CHI, could be provided to prevent subsequent adverse outcomes.

Conclusion

CHI is a rare but serious and recurrent cause of RM, FGR and stillbirth, and evidence for its aetiology, presentation, diagnosis and management is weak. Current research points towards a maternal immunopathological response as the underlying cause.

In tackling this placental inflammation, some evidence of improved pregnancy outcomes is seen using immunosuppressive therapies and closer monitoring of fetal growth.[152,202] There is a need for predictive or diagnostic antenatal testing to identify CHI and its severe outcomes, but the rarity and limitations of the current evidence-base restricts advancements in this area. A potential method to improve understanding of CHI is to model the disease on another process; one with a more robust evidence base, e.g. allograft rejection. To do so, more extensive study is required into the immunological mechanisms of CHI, on which knowledge is currently lacking. In defining any commonalities between CHI and rejection, such approaches could exploit the wealth of knowledge and technological advances already employed in transplant biology.

In future, collaborations between multiple centres that research and/or treat CHI is essential to expand sample sizes and identify mechanisms of pathogenesis. In doing so, the benefit of increased knowledge of CHI will likely extend to other obstetric disorders and reduce the high risk of recurrence and distress in affected women and their partners.

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None of the authors have a conflict of interest to declare.

Hypothesis

This thesis addressed the hypothesis that CHI results from a breakdown in immune tolerance and is a form of maternal-placental rejection, with affected placentas resembling a rejected organ. Due to CHI's inflammatory aetiology, treatment with immunomodulatory medications will be effective at reducing lesion severity and recurrence.

Project Aims and Objectives

1. Determine whether treatment including immunomodulatory medication (hydroxychloroquine and prednisolone) is effective at reducing lesion severity and improving outcomes in subsequent pregnancies following a diagnosis of CHI. Clinical data and placental histopathology reports from retrospective cases of index pregnancies and those following diagnosis will be extracted from participant medical records.

2. Apply unbiased, systematic computerised quantification to archived placental tissue from the aforementioned cases of CHI to characterise inflammatory features in the disorder. Inflammation will then be compared to placentas collected prospectively from healthy control pregnancies and treated subsequent pregnancies in other women with a prior history of CHI to identify any changes in inflammatory features.

3. Investigate the possible role of maternal anti-fetal antibodies in CHI by utilising crossmatching techniques commonly employed for the prediction of allograft rejection. Archived placental tissue from the cohort of index pregnancies with CHI will be immunostained for evidence of C4d deposition as a marker of AMR. Serum from the prospectively recruited women with a history of CHI will be screened for antibodies against fetal HLA and the placenta and compared to healthy controls.

Ethical Permission

Informed and written consent was obtained from all study participants. For archived samples from index pregnancies, ethical approval was granted by the National Research Ethics Service (NRES) Committee London-City & East (REC ref: 14/LO/1352). For prospectively collected banked tissue samples from participants gathered prior to July 2018, approval was granted by NRES Committee Northwest-Greater Manchester West (REC ref: 14/NW/1149). Between February and July 2018, permission was granted by the NRES Committee South East Coast-Surrey Research Ethics Committee (REC ref: 16/LO/1666). Samples collected after this period were recruited under the Tommy's Project Ethics (REC ref: 15/NW/0829).

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Chapter 2: Materials and Methods

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Retrospective Placental Tissue Collection from Index Cases of CHI

Study participants were identified via the Paediatric Histopathology Department at Saint Mary's Hospital, Manchester, UK, by searching placental histopathology reports for a diagnosis of CHI in a previous pregnancy; samples were sent for histopathological analysis following poor outcome e.g. fetal growth restriction, miscarriage, stillbirth or neonatal death. Diagnosis was made by a specialist perinatal pathologist in accordance with the initial description of CHI by Labarrere and Mullen as a placental lesion consisting of histiocyte (macrophage) infiltration into the intervillous space.[1] Where archived placental tissue samples from a participant's first pregnancy diagnosed with CHI (henceforth referred to as 'index' cases) were available, tissue was provided as formalin-fixed paraffin-embedded (FFPE) blocks processed automatically in 10% formalin. Identically processed placental tissue from uncomplicated term pregnancies was also supplied for use as controls by Paediatric Histopathology.

Accompanying hospital records were obtained and participant demographics collected including: maternal age, BMI, ethnicity, presence of autoimmune disease and antibody profile. Participant's obstetric history, including gravidity (number of pregnancies), parity (number of pregnancies reaching >24 weeks' gestation regardless of outcome) and the number of pregnancies prior to the diagnosis of CHI were recorded where applicable. Outcomes of pregnancies were classified as liveborn and still living, stillbirth (fetal death ≥24 weeks' gestation), miscarriage (fetal death <24 weeks' gestation, including spontaneous abortion), termination of pregnancy for fetal anomaly (TOPFA) and neonatal death (death of an infant within 28 days of birth). In addition to positive CHI diagnosis, the presence of increased fibrin, villitis and a small for gestational age placenta was noted.

Informed and written consent was obtained from study participants and a favourable ethical opinion was given by National Research Ethics Service (NRES) Committee London-City & East (REC ref: 14/LO/1352).

Prospective Blood and Tissue Collection from Subsequent and Healthy Control Pregnancies

Participant Recruitment

Women attending Saint Mary's Hospital for care in a subsequent pregnancy following a previous diagnosis of CHI were recruited to the study from the Tommy's Rainbow Clinic. For healthy control pregnancies, women delivering vaginally or via Caesarean section at Saint Mary's Hospital consented to participate in the study on the date of delivery. Healthy women were selected to match the demographics of participants with CHI where possible, and recruitment excluded smokers, women on immunosuppressant medication, advanced maternal age (>37 years) and those with ongoing infection, a history of autoimmune disease or known SARS-CoV-2 infection (active or past). Informed written consent was obtained from all study participants following ethical approval. For samples from participants gathered before July 2018, approval was granted by NRES Committee Northwest-Greater Manchester West (REC ref: 14/NW/1149). Between February and July 2018, permission was granted by the NRES Committee South East Coast-Surrey Research Ethics Committee (REC ref: 16/LO/1666). Samples collected after this period were recruited under the Tommy's Project Ethics (REC ref: 15/NW/0829).

Maternal Blood Sampling and Processing

For prospective maternal blood collection, Vacutainer™ serum and EDTA (anticoagulated) samples were taken from previously diagnosed CHI patients during the second or third trimester and from healthy control patients prior to delivery of their baby. Blood samples were first centrifuged (Sigma 3-16KL, St Louis, USA) at 2500rpm for 10 minutes at 4°C, before serum and plasma supernatants were retained and aliquoted. From the EDTA centrifuged blood, the buffy coat (leukocyte layer) was also retrieved at the red cell/plasma interface. All processed samples were subsequently stored at -80°C and thawed for use when required.

Placental Tissue Collection and Processing

Samples of villous tissue for storage as FFPE blocks were taken as $1cm^3$ full thickness cubes from the corresponding point of cord insertion on the maternal side, edge and middle (between cord insertion and edge) of the placenta and briefly rinsed in phosphate buffered saline (PBS). Chorionic plate and decidua were trimmed from the sample. Tissue was fixed by manual immersion in 4% neutral buffered formalin (NBF) for 24 hours on a roller mixer at room temperature according to standard in-house biobank protocol. Samples were then removed from NBF and placed into PBS on a roller mixer for 24 hours at 4°C to rinse out excess fixative. Following this, samples were loaded into an automated Leica TP1020 tissue processor (Leica Biosystems, Wetzlar, Germany) for dehydration over 18 hours in a series of increasing alcohol concentrations and xylene before submersion in paraffin wax. Processed tissue was then manually embedded into paraffin wax moulds and cooled to produce blocks.

At the point of placental tissue sampling, additional $1cm^3$ cubes from the point of cord insertion, middle and edge were taken, washed in PBS and cut into 3mm pieces before equal distribution between aliquots to give tissue from a mix of placental areas in each tube. Umbilical cord samples were also taken as approximately 3mm thick discs, rinsed in PBS to reduce maternal blood contamination and placed into aliquots. Both villous tissue and umbilical cord samples were frozen and stored at -80°C for later use.

Placental Histology

Slide Preparation

For analysis of histological features in placentas from healthy pregnancies and index and subsequent cases of CHI, slides were prepared from FFPE blocks. Using a Leica RM2245 microtome (Leica Biosystems), 5µm sections of placental tissue were cut and mounted onto both uncoated slides (VWR International, Pennsylvania, USA) and SuperFrost (Thermo Fisher, Massachusetts, USA) slides coated with poly-l-lysine solution (Sigma-Aldrich, Missouri, USA). Slides were dried in an incubator at 38ºC overnight before long-term storage at room temperature.

Haematoxylin and Eosin Staining for Fibrin

For visualisation of fibrin deposition in placentas, haematoxylin and eosin (H&E) staining was utilised. Uncoated slides were incubated at 60°C for 10 minutes prior to dewaxing in Histoclear (Fisher Scientific, UK) (1x8 minute immersion, 2x1 minute immersion) and rehydration in industrial methylated spirit (IMS, Fisher Scientific) (2x1 minute immersion in 100%, 2x1 minute immersion in 70%) before rinsing in tap water. Slides were incubated in filtered haematoxylin (Sigma-Aldrich) for

10 minutes to counterstain nuclei and rinsed in warm water for colour development followed by a quick immersion in 1% (v/v) acid alcohol. To stain fibrin a distinct shade of pink, slides were incubated with eosin (Sigma-Aldrich) for 10 minutes and rinsed quickly in cold water to avoid loss of colour. Dehydration was then carried out in solutions of 70%, 95% and 100% industrial methylated spirit (IMS) for 1 minute each, followed by incubation in Histoclear solutions for 3x1 minutes and mounting with DPX medium (Thermo Fisher Scientific) and coverslips.

Immunohistochemistry

$CD68^+$ Macrophages

CD68 staining for macrophage detection was carried out in-house at the Maternal and Fetal Health Research Centre, Manchester. Coated slides prepared previously were warmed at 60°C for 10 minutes before dewaxing and rehydration as described above.

Antigen retrieval was then performed using 0.01M sodium citrate (Sigma-Aldrich) (pH 6.0) and heating in a microwave twice, each for 5 minutes at 800W. Slides were cooled in the citrate buffer for 20 minutes before rinsing in tap water. Sections were circled using a PAP pen (VWR International) and covered with 3% (v/v) hydrogen peroxide (VWR International) for 10 minutes at room temperature to quench endogenous peroxidase activity. Following two 5 minute washes in tris-buffered saline (TBS) (Fisher Scientific), blocking of non-specific antibody binding was conducted using 10% (w/v) normal goat serum (Sigma-Aldrich) in 0.1%TBS-Tween (Biotium, UK) for 30 minutes at room temperature. Sections were then incubated with mouse monoclonal anti-human CD68 (Table 2) diluted in 10% normal goat serum/TBS solution.

After overnight incubation in primary antibody at 4°C, sections were washed in TBS for 5 minutes, TBS-Tween (0.6%) twice, each for 5 minutes and TBS alone for 5 minutes. To detect bound anti-CD68, sections were then incubated with biotinylated polyclonal goat anti-mouse secondary antibody (Agilent Technologies, 3.3µg/ml) for 30 minutes at room temperature followed by washes in TBS and TBS-Tween (0.6%) as previously described. Avidin-peroxidase (Sigma-Aldrich, 5µg/ml in TBS) was then applied for 30 minutes at room temperature to amplify the secondary antibody signal before two 5 minute washes in TBS-Tween (0.6%) and one 5 minute wash in TBS. Diaminobenzidine (DAB) chromogen (BD Biosciences, New Jersey, USA) was reconstituted according to manufacturer's instructions and dropped onto each section for secondary antibody detection. Following a 4-minute incubation and 5-minute wash in distilled water, placental sections were finally placed in haematoxylin for 5 minutes as a nuclear counterstain before rinsing in cold water and dunking in 1% (v/v) acid alcohol. Slides were then left in warm water for 5 minutes and then transferred into cold water. Dehydration of sections was conducted by sequential washing in solutions of 70% (2x3 minutes), 95% (2x3 minutes) and 100% IMS (3x3 minutes) before one 2 minute and two 10 minute immersions in Histoclear. DPX mounting medium was dropped onto each section before coverslip application and storage. Negative control sections utilised to ensure antibody specificity were treated identically except for omission of primary antibody which was replaced with non-immune mouse IgG (Sigma-Aldrich) at the same working concentration.

$CD3^+$ T Cells

Placental sections on coated slides from control and subsequent pregnancies were stained in-house for T cells according to the immunohistochemical protocol detailed previously, using anti-

CD3 antibody (Table 2). DAB incubation was shortened to 2 minutes to optimise specific staining to background ratio. As previous, negative controls consisted of primary antibody substitution with non-immune mouse IgG at the same working concentration.

C4d Deposition

Staining for C4d deposition was carried out by the Department of Adult Histopathology, Manchester Royal Infirmary, Manchester, to replicate the protocols used in the processing of archived tissue for pilot studies. Slides of tissue from the point of cord insertion and edge were initially deparaffinised in EZ Prep Volume Adjust (Ventana Co.) according to manufacturer's instructions. Slides were then washed in TRIS-based Reaction Buffer (pH 7.6) and antigen retrieval achieved using heat and TRIS-EDTA-boric acid (pH 8.4, Ventana Co., Arizona, USA) for 60 minutes. Ultraviolet inhibitor blocking solution was then applied for 4 minutes before a further 30-minute incubation at room temperature with rabbit polyclonal anti-human C4d antibody (Table 2). Slides were then incubated in horseradish peroxidase-linked secondary antibody for 8 minutes before an 8-minute incubation in DAB chromogen. To amplify positive staining, a copper enhancer was applied for 4 minutes, before a 12-minute counterstain in Haematoxylin II and 4 minutes in bluing reagent. Slides were dehydrated and mounted with coverslips, as described above.

Immunofluorescence

Characterisation of Macrophage Phenotype

To determine the polarisation of intervillous macrophages in CHI, sections of placental tissue from index cases on coated slides were stained using immunofluorescence. Sections from the area of placenta with the greatest number of intervillous macrophages as determined by CD68 staining were chosen where possible. Anti-CD68 was used as a pan-macrophage marker in combination with either anti-CD80 or anti-CD86 on two separate serial 5µm sections for identification of M1 macrophages. For visualisation of M2 macrophages, two separate serial sections were stained, one with anti-CD68 in combination with anti-CD206, and another with anti-CD163 alone (Table 2). Sections were warmed for 10 minutes at 60ºC before dewaxing, rehydration and outlining with a PAP pen as detailed previously. 0.1% (w/v) fish gelatine (SLS Ltd, Nottingham, UK) and 10% (v/v) donkey serum (Sigma-Aldrich) in 0.3% (v/v) TBS-Triton (Sigma-Aldrich) was used to block non-specific antibody binding by incubation on slides for 30 minutes at room temperature. Following blocking, slides were incubated with primary antibody (Table 2) for one hour at room temperature. Three 5 minute PBS washes were then used to remove bound antibody, before incubation in both donkey anti-mouse Alexa-Fluor (AF) 488 and donkey anti-rabbit AF568-conjugated secondary antibodies (Abcam, Cambridge, UK, at 2µg/ml each in TBS) (except for CD163 staining which required only AF488) for 30 minutes at room temperature. Slides were then washed again as previous and incubated with AF647-conjugated rabbit monoclonal anti-human cytokeratin 7 (CK7) (Abcam, 0.5µg/ml in fish gelatine/PBS-Triton block) for one hour at room temperature for visualisation of syncytiotrophoblast to allow distinction between intervillous and villous macrophages. Following another washing step, autofluorescence was quenched with TrueView (Vector Laboratories, California, USA) according to kit instructions, and slides dried and mounted with ProLong Diamond Antifade Mountant (Life Technologies Ltd, California, USA). Negative

sections were processed identically but with omission of primary antibody and replacement with mouse and rabbit non-immune IgG at the same working concentration.

Table 2. Primary antibodies used in immunohistochemistry and/or immunofluorescence staining.

Antigen	Supplier	Working Concentration	Antibody Host and Type	Positive Control Tissue
CD3	Dako	1.4µg/ml	Ms mAb	Tonsil
CD68	Dako	0.8µg/ml	Ms mAb	Tonsil
CD80	Abcam	10µg/ml	Rb pAb	Tonsil
CD86	Abcam	5.6µg/ml	Rb mAb	Omental carcinoma
CD163	Bio-Rad	5µg/ml	Ms mAb	Tonsil
CD206	Abcam	2µg/ml	Rb pAb	Mouse lung
C4d	Bio-Rad	1:75 dilution*	Rb pAb	Rejected kidney

Optimum working antibody concentrations were determined on positive control tissue according to manufacturer's recommendations. CD – cluster of differentiation; mAb – monoclonal antibody; Ms – mouse; pAb – polyclonal antibody; Rb – rabbit. *Stock antibody concentration not determined by supplier.

Screening for Placental-Directed Antibodies in Maternal Plasma

To determine whether antibodies directed towards the placenta were present in women with a history of CHI, placental tissue was incubated with autologous maternal plasma in a method adapted from published methods on the diagnosis of autoimmune encephalitis.[2] To avoid masking of placental antigens which may occur with formalin fixation, staining was carried out on unfixed snap frozen tissue embedded in optimal cutting temperature compound (OCT) frozen at -20°C. 5µm villous sections were then cut using a Leica RM2235 cryostat (Leica Biosystems) and captured onto SuperFrost slides and allowed to air dry at room temperature before storage at -20°C until staining. After outlining with a PAP pen, sections were blocked with 10% v/v goat serum and 0.1% fish gelatine in 0.3% PBS-Triton for 30 minutes at room temperature. Simultaneously, autologous maternal EDTA plasma samples were heated for 30 minutes at 56°C to reduce complement activity, as recommended by standard Transplantation Laboratory protocol at Manchester Royal Infirmary, UK. Plasma was then diluted 1 in 10 in blocking buffer and incubated on corresponding placental tissue for each participant overnight at 4°C. Plasma was washed from slides using PBS three times for 5 minutes each. Goat polyclonal anti-human IgG FITC-conjugated secondary antibody (Abcam, 1µg/ml in PBS) was then applied for 30 minutes at room temperature for detection of any bound maternal antibody. Washing steps were repeated as previous before incubation with AF647-conjugated anti-CK7 for one hour at room temperature to allow visualisation of syncytiotrophoblast. Following washing steps, autofluorescence on placental tissue was quenched using TrueView according to kit instructions, before slides were mounted with ProLong Diamond Antifade Mountant and coverslips.

Image Analysis and Quantification

Stained slides were visualised and scanned at the University of Manchester Bioimaging Facility using brightfield microscopy and a 3D Histech Panoramic 250 Flash Slide Scanner. CaseViewer and QuPath (v.0.3.0)[3] were then used to open and analyse image files where applicable.

Maternal $CD68^+$ Macrophages and $CD3^+$ T Cells

To reduce bias and gain a more representative measure of placental staining, a systematic random sampling method was adapted from Mayhew.[4] Firstly, a 1000x1000µm grid was superimposed onto each section and grid squares selected using a coin flip and random number generator to determine the starting row (heads for 1st or tails for 2nd) and grid square respectively (Figure 9). Areas of tissue aside from the villous placenta (e.g. decidua or membranes) and dead space on the slide were not included. Square annotations equal to $1mm^2$ were then drawn to highlight regions of interest (ROI).

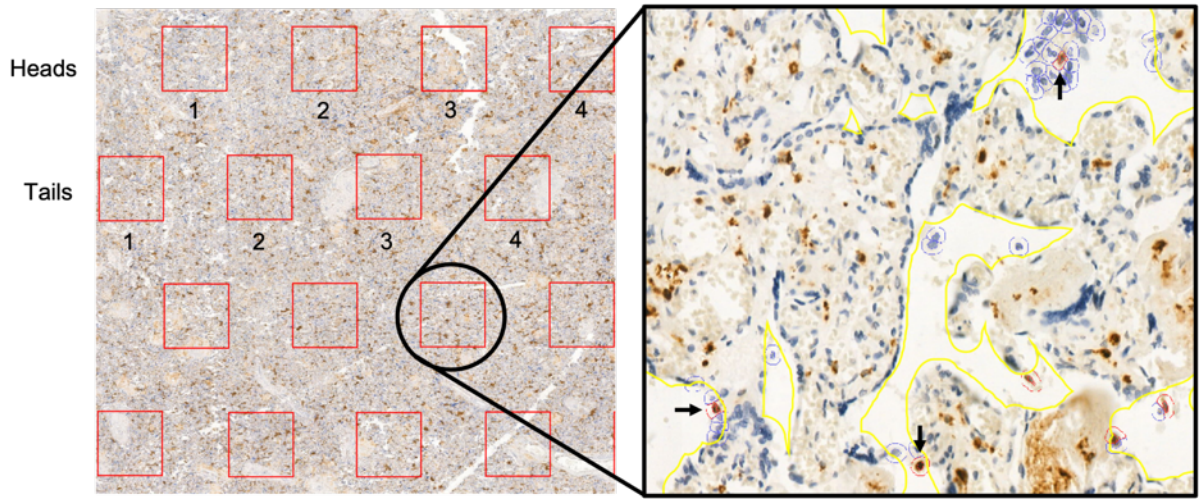

Figure 9. Example of systematic positive cell detection on immunohistochemically stained placental tissue in QuPath software.[3] A $1mm^2$ grid is superimposed onto the image and square annotations drawn on every other row. A coin flip is then used to choose the starting row, first or second (heads or tails, respectively). Each square is then assigned a number from left to right, and a random number generator used to determine the square which will be analysed. The brush tool is used to draw around villous tissue and exclude it from the area measurement, leaving only intervillous space. Finally, a positive cell detection is run within the intervillous area, giving a positive cell count per mm^2 intervillous space and highlighting negative cells in blue and positive cells in red (shown by arrows).

To establish the minimum number of ROI required for analysis, preliminary macrophage analysis data from 8, 16 and 24 ROI in three placentas were analysed via Friedman test in GraphPad Prism (v9.2.0) (Figure 10). Data from 8 regions and above did not significantly differ from one another, thus 8 was chosen as the minimum number of ROI for each placenta.

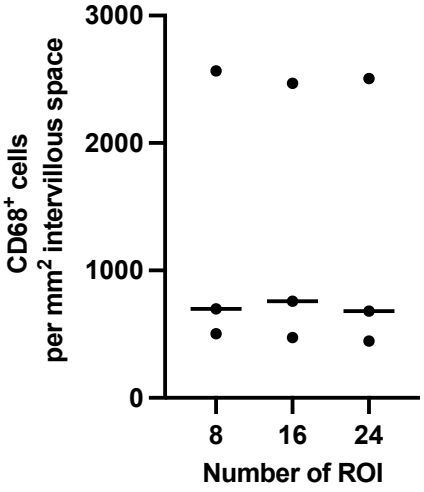

Figure 10. Determination of minimum number of regions of interest (ROI) required for CD68$^+$ cell quantification in QuPath software. Preliminary intervillous CD68$^+$ cell counts from 8, 16 and 24 regions of interest in three placentas were analysed to determine whether increased number of ROIs influenced average measurements.

Following selection of regions of interest on each slide, villous tissue and fibrin was manually drawn around and excluded from the annotation to ensure only intervillous space was selected for analysis of maternal immune cells (Figure 9). A positive cell detection was then run within the selected intervillous area to detect brown DAB-positive (CD68$^+$ or CD3$^+$) cells, expressed per mm^2 of intervillous area. Cell counts were averaged for each slide, then these measurements averaged again for each placenta to give an overview of organ pathology.

Fibrin Deposition

To quantify fibrin deposition in placental samples, ROI on H&E-stained slides were selected as described above using QuPath software. For the purpose of analysis, the 1mm^2 annotation was split into three classifications of tissue area: villous tissue, fibrin and intervillous space. Fibrin was then manually selected to produce an area measurement and expressed as a percentage of ROI area to give the proportion of placental area occupied by fibrin deposition. As for quantification of maternal immune cells as detailed previously, data from each slide was averaged to give a final measurement for each placenta.

C4d Deposition

After viewing of C4d-stained slides, semi-quantitative analysis was chosen over QuPath quantification given the relatively low frequency of positive staining of villi and high background staining which prevented reliable computerised detection. Eight regions of interest were randomly chosen as described above and positive staining graded according to criteria previously published by Bendon et al.[5]; 0 = 0% to 5% of villi affected; 1 = 5% to 25%; 2 = 25% to 75%; and 3 ≥ 75%. Scores for all eight regions were then averaged per placenta.

Macrophage Phenotyping

Slides were processed in CaseViewer using the inbuilt filter to reduce autofluorescence resulting from paraffin-fixation and endogenous fluorescence. Representative images of areas with maternal

macrophage infiltration, identified by CD68 or CD163 outside of positive CK7 staining borders, were taken for each macrophage marker and compared to a selection of healthy control pregnancies.

Maternal Placental-Directed Antibodies in Plasma

Immunofluorescent slides stained with autologous plasma were scanned and analysed in QuPath using inbuilt pixel classifiers (Figure 11). The threshold for positive staining was determined by establishing the value which resulted in the detection of the least background staining on the negative control slide. This threshold was then applied on the remainder of slides as an automated script. Area of positive staining was given in μm^2 and expressed as a proportion of tissue area, before calculating a mean value for each section and sample. Values were then normalised to those from the negative control.

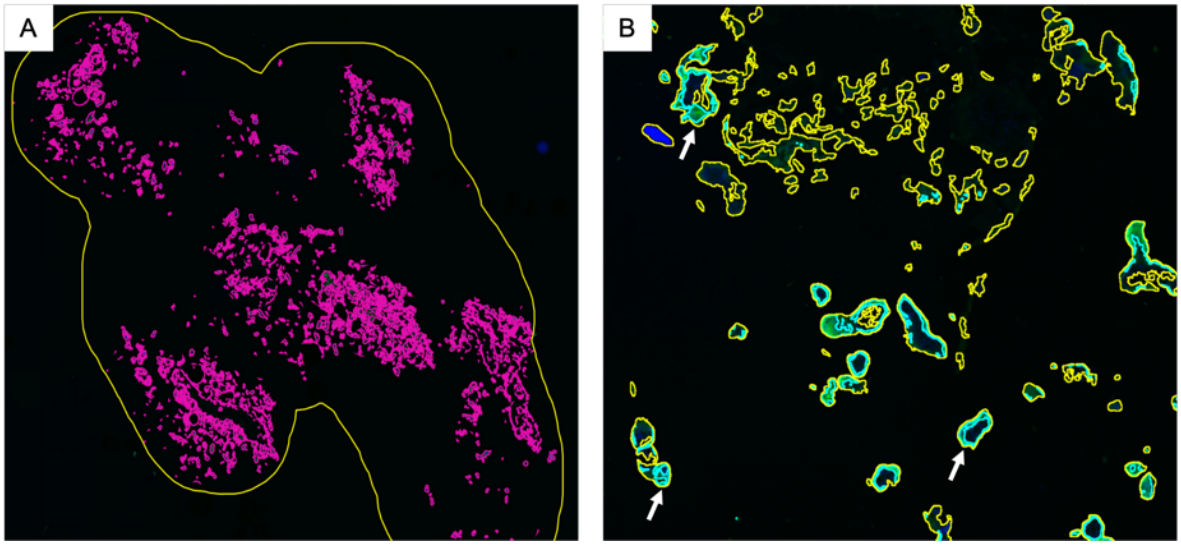

Figure 11. Detection of positive fluorescence area on sections of stained placental tissue in QuPath. A) An annotation is drawn around the section of tissue and a pixel classifier run on all fluorescence channels to identify and create an annotation of tissue area (pink). B) Detected tissue area is then selected (yellow), and another pixel classifier run on the FITC-fluorescent channel only, giving a measurement of green fluorescence area above a predetermined threshold value (cyan, shown with arrows).

Transplant Laboratory Crossmatching

HLA Antibody Screening

To detect classical class I and II anti-HLA antibodies, maternal EDTA plasma samples were processed by the Transplantation Laboratory at Manchester Royal Infirmary. A mixed Luminex assay was used to initially determine any presence of anti-HLA antibodies, consisting of LABScreen Mixed Beads (One Lambda Inc, Thermo Fisher) coated in several HLA antigens and each possessing a fluorochrome specific to surface antigens (Figure 12). Maternal plasma was incubated with the mixed beads at 22°C for 15 minutes before washing in LABScreen wash buffer (One Lambda Inc, Thermo Fisher) according to manufacturer's protocol. R-phycoerythrin (PE)-conjugated goat anti-human IgG and PE-conjugated donkey anti-human IgM secondary antibodies (One Lambda Inc, Thermo Fisher) were then added for 5 minutes at 22°C to allow detection of bound antibody. After a final wash in wash buffer, beads were resuspended in buffer before

transfer to a 96-well PCR plate for analysis using the Luminex LabScan3D. The Luminex analysis detects antibodies using a dual laser system which firstly identifies antibody specificity by a unique combination of dyes within individual beads coated with groups of antigen and secondly via the presence of antibody in patient plasma binding to beads given by a measurement of mean fluorescence intensity (MFI). Its readout is input into HLA Fusion One Lambda Software (One Lambda Inc, Thermo Fisher), where results are checked with established criteria from the Manchester Royal Infirmary Transplantation Laboratory. At this stage, false negatives and positives are detected. As the mixed bead assay gives information only on the presence of antibodies in the plasma sample and not antibody specificity, samples testing positive were then retested in the same manner but with mixed beads replaced with specific LABScreen beads (One Lambda Inc, Thermo Fisher) possessing only a single antigen each with a unique dye combination. This allows for the identification of anti-HLA antibodies and their specificity.

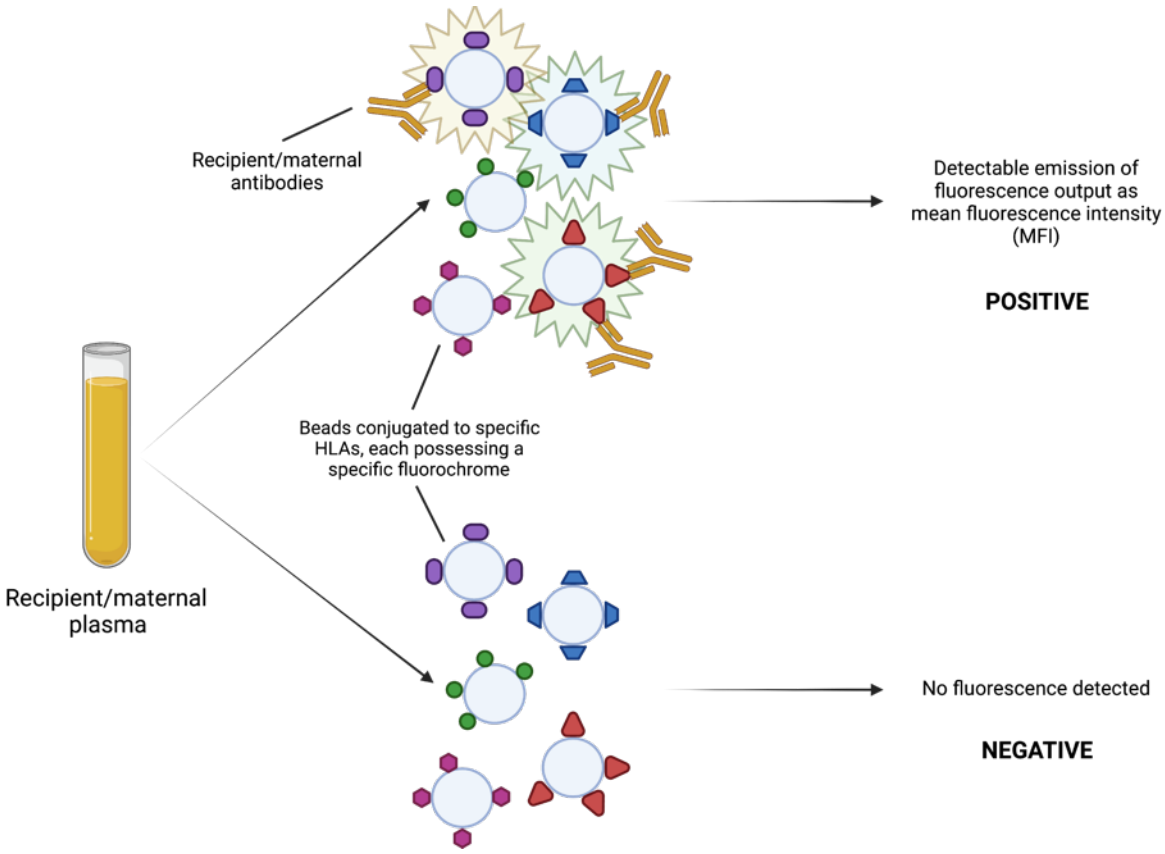

Figure 12. Luminex flow crossmatch assay for anti-HLA antibodies in recipient plasma (or in the case of pregnancy, maternal plasma). HLA – Human Leukocyte Antigen. Figure created with BioRender.com.

DNA Extraction

In the case of a positive anti-HLA antibody screen, the HLA genotype for both mother and fetus (or mother alone where fetal DNA was not available) was determined. Maternal genotype was used to establish the cut-off for positive results, as only specificities with an MFI above the self-HLA genotype are likely to be true positives. Fetal HLA genotypes were determined to identify whether maternal antibodies specific to the fetus' HLA were present. Maternal DNA was obtained using thawed EDTA buffy coat samples taken during gestation as starting material. Umbilical cord was chosen as the starting material for fetal DNA extraction over placenta in order to reduce maternal DNA contamination. Snap frozen umbilical samples were thawed on ice and blended at room

temperature using a Stuart handheld homogeniser (Cole-Parmer, UK). Both maternal buffy coats and homogenised umbilical cord samples were then processed using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to kit instructions. The concentration of extracted DNA in the resulting sample was tested using a NanoDrop™ 2000 (Thermo Fisher Scientific) to ensure the DNA concentration was sufficient for genotyping (≥20ng/µl), before storage at -20°C until testing.

Maternal and Fetal HLA Genotyping

Extracted DNA samples for mother and fetus were processed at the Manchester Royal Infirmary Transplantation Laboratory to determine HLA genotype via the Luminex LABType™ Sequence Specific Oligonucleotide (SSO) HLA Typing protocol. This protocol uses SSOs bound to fluorescently tagged microspheres to identify HLA alleles encoded by the DNA in the sample. Isolated DNA was added to a mixture of D-mix, Amplification Primer and Taq Polymerase before centrifugation at 1000rpm in a Thermo IEC CL30 Centrifuge (Thermo Fisher Scientific). PCR amplification was then carried out in a Veriti Thermal Cycler (Thermo Fisher Scientific). The resulting amplified DNA was denatured using Denaturation Buffer and spun at 1000rpm in the Thermo IEC CL30 Centrifuge. After 10 minutes, Neutralization Buffer was added to each sample and centrifuged again at 1000rpm. Locus-specific Bead Mixture and Hybridization Buffer were mixed and added to the samples for a 15-minute incubation at 60°C. After 2 washes in Wash Buffer for 5 minutes at 2900rpm in the centrifuge, R-Phycoerythrin-conjugated Streptavidin (SAPE) Solution was then added to the samples for fluorescent labelling and incubated for 5 minutes at 60°C. After a further 5-minute wash in Wash Buffer, samples were processed using the Post-PCR LABScan 3D (One Lambda Inc, Thermo Fisher) to identify fluorescently-tagged strands of DNA and the corresponding allele. A State Registered Clinical Scientist was then required to review the results and confirm HLA genotype. Data on the number of antibodies against fetal HLA alleles and their MFI was then collected.

Calculated Reaction Frequency and Prediction of Crossmatch Results

Using maternal anti-HLA antibody specificities and HLA genotype, calculated Percentage Reaction Frequency (%cRF) was established for each participant testing positive via input of the results of Luminex screen into the NHS Blood and Transplant Kidney cRF tool.[6] %cRF reflects the percentage of donors who would be anticipated to have an unsuitable HLA profile in the context of organ transplantation. %cRF is based on a panel of lymphocytes from 100 blood donors to represent the HLA type of the general population of the United Kingdom. Organ recipients reacting with 0 out of 100 lymphocyte samples in the panel are assigned a %cRF of 0% and are classed as unsensitised, with 100% of donors proving suitable. On the other hand, those with a score of >80% are classed as highly sensitised, as >80% of donors are considered unsuitable for the patient.

Statistical Analysis

GraphPad Prism v9 (GraphPad Software, USA) was used for statistical analysis of clinical, histological and transplant crossmatching data. Shapiro-Wilk test was initially run to establish the distribution of all variables. Categorical variables were analysed via Chi-square analysis or Fisher's Exact test where sample size was limited. Non-normally distributed data was analysed via Mann-Whitney test to compare two groups, or Kruskal-Wallis test with Dunn's multiple comparisons for

more than two experimental groups. Differences between normally distributed data were determined via ordinary one-way ANOVA with Tukey's multiple comparisons. Spearman's correlation was used to investigate relationships between variables. For all tests, statistical significance was set at $p<0.05$.

Chapter 2 References

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Chapter 3: Immunomodulatory Therapy Reduces the Severity of Placental Lesions in Chronic Histiocytic Intervillositis

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Author Contributions

C.A.B collected, analysed and interpreted data and wrote the manuscript. C.W, G.B and E.C contributed toward data collection. C.A.B, I.P.C and A.E.P.H contributed toward study conception and design. G.B, E.C and C.L.T assisted with the preparation and revision of the manuscript.

Abstract

Chronic histiocytic intervillositis (CHI) is a rare, but highly recurrent inflammatory placental lesion wherein maternal macrophages infiltrate the intervillous space. Pregnancies with CHI are at high risk of fetal growth restriction, miscarriage or stillbirth. Presently, the diagnosis can only be made after histopathological examination of the placenta. Given its proposed immunological aetiology, current treatments include aspirin, heparin, and immunomodulatory agents. However, the rationale for these medications is largely based upon small case series and reports as there is a lack of larger studies investigating treatment efficacy. Therefore, this study sought to determine whether inclusion of immunomodulatory medications was effective at reducing the severity of lesions and improving pregnancy outcomes in subsequent pregnancies. Thirty-three women with a history of CHI in at least one pregnancy (index case) were identified retrospectively through medical records. Twenty-eight participants presented with a first subsequent pregnancy and a further eleven with a second subsequent pregnancy at a specialist clinic for pregnancy after loss. Data on maternal demographics, medical history, medication, pregnancy outcome and placental pathology was collected and compared between pregnancies. Twenty-seven (69%) subsequent pregnancies were treated with at least one or both of prednisolone and hydroxychloroquine. Inclusion of at least one immunomodulatory agent in treatment regimen resulted in an almost 25% increase in overall livebirth rate (61.5% vs 86.2%). In women treated with immunomodulatory medication a greater proportion of placentas had reduced severity of lesions compared to those treated without (86.7% vs 33.3% respectively). A reduction in CHI severity was associated with a 62.3% improvement in livebirth rate compared to those where severity remained unchanged in relation to the index case. These data provide preliminary evidence that the use of immunomodulatory medication in the management of CHI improves histopathological lesions and the chance of livebirth in subsequent pregnancies. Due to CHI's rarity and ethical and feasibility issues, randomised controlled trials in affected women are challenging to conduct. As a result, collaboration between centres is required in future to increase study sample sizes and elucidate the mechanisms of hydroxychloroquine and prednisolone in reducing pathology.

Keywords

Placental histopathology, prednisolone, hydroxychloroquine, stillbirth, treatment, miscarriage, outcomes

Introduction

Chronic histiocytic intervillositis (CHI), also known as chronic intervillositis or chronic intervillositis of unknown etiology,[1] is a pregnancy disorder strongly associated with fetal growth restriction, miscarriage, stillbirth and neonatal death.[2–5] Estimated to affect 6 in every 10,000 pregnancies over 12 weeks' gestation, CHI is characterized by maternal macrophage infiltration into the intervillous space of the placenta and has a 25-100% risk of recurrence in subsequent pregnancies.[1,3,6,7] Many cases also exhibit marked perivillous and/or intervillous fibrin deposition and trophoblast necrosis.[8] Due to its asymptomatic nature and a lack of reliable associated biomarkers, currently a diagnosis of CHI can only be made following delivery by histopathological examination of the placenta. Management of CHI is further complicated by a lack of standardized treatment options proven to prevent recurrence.

Owing to the presence of maternal macrophages and the reported increased incidence in women with autoimmune disease, CHI has been hypothesized to be a disorder of failed maternal-fetal tolerance and excessive inflammation.[9] On this basis and due to the presence of intervillous and perivillous fibrin, current treatments include thromboprophylactic agents such as aspirin and low-molecular-weight heparin (LMWH) as well as those aimed at suppressing inflammation e.g. corticosteroids and hydroxychloroquine.[10] A systematic review of six observational studies conducted in 2010 found no significant improvement in pregnancy outcome with aspirin and LMWH alone. Though a growing number of case reports detail use of immunomodulatory agents such as prednisolone and hydroxychloroquine,[10,11] there remains a striking lack of larger studies supporting the efficacy of any treatment regime in reducing severity of CHI and improving pregnancy outcomes. Notably, a prior case series has highlighted that women with worse obstetric histories tend to be prescribed more therapeutic agents, despite their unproven efficacy.[10] Due to the high rate of recurrence of CHI and severe consequences of the disorder, studies are urgently needed to determine effective therapies.

By retrospectively identifying women with a previous diagnosis of CHI, we aimed to investigate pregnancy outcomes and placental pathology in subsequent pregnancies referred to a specialist service following poor perinatal outcome, stillbirth or neonatal death. We hypothesized, due to the immunological nature of CHI, that treatment regimens where immunomodulatory agents were included would decrease the severity of the condition and consequently improve the chance of livebirth.

Materials and Methods

Participant Recruitment and Data Collection

Women with a previous histopathological diagnosis of CHI between 2009 and 2021 were identified retrospectively from medical records at Manchester University NHS Foundation Trust, UK. The majority of these cases were identified after the death of a baby or late miscarriage when histopathological evaluation of the placenta is recommended practice. In other cases (following the birth of a live infant) the placenta is sent away for examination for a variety of clinical indications (e.g. FGR, fetal compromise at birth, placental abruption, previous late pregnancy loss). CHI was diagnosed by a specialist perinatal pathologist in accordance with its initial description by Labarrere

and Mullen as a placental lesion consisting of histiocytic (macrophage) infiltration into the intervillous space.[8] Data on maternal demographics, medical history including results of tests for autoantibodies (lupus anticoagulant, anti-phospholipid, antinuclear and anticardiolipin antibodies) and obstetric history were collected from the woman's case record. The first pregnancy diagnosed with CHI was classified as the 'index' case, with data on any subsequent pregnancies recorded where applicable from retrospective medical records. Pregnancy outcomes consisted of liveborn and still living, liveborn at term and still living (>37 weeks' gestation), miscarriage (fetal death <24 weeks gestation, including spontaneous abortion), stillbirth (fetal death ≥24 weeks gestation) and termination of pregnancy for fetal anomaly (TOPFA). Cases of neonatal death (death of an infant within 28 days after birth) were also recorded. Fetal growth restriction (FGR) was defined as growth below the 3rd percentile, and small for gestational age (SGA) as between 3rd and 10th centile.[12] Centiles were calculated for pregnancies >20 weeks' gestation using the GROW centile calculator, for cases where maternal demographics, pregnancy outcome and fetal sex were known.[13]

The therapeutic agents used in our service evolved over time following publications from other researchers,[3,10,11,14] and following discussion with colleagues with expertise in lupus in pregnancy. Initially, pregnancies with CHI were managed using aspirin and LMWH, but this evolved to a combination of aspirin given at a dose of 75-150mg once a day, a prophylactic dose of LMWH (e.g. Tinzaparin 4,500iu) once a day, hydroxychloroquine 200mg twice a day and prednisolone 20mg once a day in the morning. Drug therapy was started from a viability scan at 6-7 weeks' gestation. Women underwent ultrasound assessment of uterine artery Doppler at 17 weeks' gestation, if this showed no abnormality then prednisolone was reduced by 5mg per week. If there was evidence of uterine artery notching or raised pulsatility index, prednisolone was continued at 20mg and then uterine artery Dopplers were reassessed after 2 weeks. If there was no improvement by 21 weeks' gestation the prednisolone was reduced at this stage. Women underwent regular ultrasound assessment of fetal growth, amniotic fluid volume and umbilical artery Doppler after 23 week's gestation at a minimum frequency of three-weekly intervals.

Where available, placental histopathology reports were analyzed for detail on histopathological features including the presence of villitis, increased fibrin deposition and recurrence and severity of CHI. Change in severity of CHI in subsequent pregnancies was in comparison to the severity of the index case of CHI. Focal CHI without accompanying fibrin deposition was classified as 'mild', those with accompanying fibrin as 'moderate', and diffuse, high-grade lesions classified as 'severe'.

Informed and written consent was obtained from all study participants and ethical approval granted by the NRES Committee North West – Greater Manchester West and NRES Committee London – City & East.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism Version 9.1.0. Chi-square and Kruskal-Wallis tests were performed for categorical and continuous variables respectively. For statistical analysis, first and second subsequent pregnancies were combined, and pregnancy outcomes

divided into liveborn and still living or liveborn at term and still living versus adverse outcome (TOPFA, miscarriage, stillbirth and neonatal death). Where sample number precluded analysis by Chi-square, Fisher's Exact test was performed instead. Statistical analysis of treatment regimen was performed by dividing participants into those which received immunomodulatory therapy (one or both of prednisolone and hydroxychloroquine) and those without (one or both of aspirin and heparin, or untreated).

Results

Participant Demographics

Thirty-three women with a history of at least one pregnancy affected by CHI between 2009 and 2021 were identified retrospectively from medical records. Twenty-eight women presented with a subsequent pregnancy and eleven of these with a further second subsequent pregnancy. Participant demographics, medical and obstetric history are shown in Table 3. Four Dichorionic-Diamniotic (DCDA) twin pregnancies were included in the study. Twenty-six women were White British (78.8%), with a smaller proportion of Asian (*n*=6) and Black African women (*n*=1). Four index CHI pregnancies (12.1%) were conceived using ART (Assisted reproductive technology), compared to only one first subsequent pregnancy (3.6%) and no second subsequent pregnancies. Underlying autoimmune disease was present in four cases (12.1%), consisting of coeliac disease, hypothyroidism, autoimmune thrombocytopenia and hypermobility syndrome respectively. Seventeen women had been tested for antinuclear antibodies, three of which were positive (17.7%) and 25 and 22 women had testing for anti-cardiolipin antibodies and anti-phospholipid antibodies respectively, none of which were positive. Of 26 women tested for lupus anticoagulant, one was positive (3.9%). Pre-existing hypertension was present in two women (6.1%).

Table 3. Demographic characteristics and obstetric and medical history of women with a diagnosis of chronic histiocytic intervillositis (CHI).

	Index CHI	1st Subsequent Pregnancy	2nd Subsequent Pregnancy
***N* participants**	33	28	11
***N* fetuses**	34	30	12
Maternal age (years)	32 (19-38)	34 (22-41)	37 (26-40)
BMI	26 (20-47)	27 (20-47)	27 (20-40)
Ethnicity			
White British	26 (78.8%)		
Asian	6 (18.2%)		
Black African	1 (3.0%)		
Lifestyle			
Smoker	3 (9.4%)	1 (3.6%)	0
Unknown	1		
Alcohol consumption	0		
Unknown	1		
Obstetric History			
Previous livebirths	0 (0-5)		
Previous losses	0 (0-4)		
Primigravida	11 (33.3%)		
ART pregnancy	4 (12.1%)	1 (3.6%)	0
Twin pregnancy	1 (3.0%)	2 (7.1%)	1 (9.1%)
Medical History			
Autoimmune disease	4 (12.1%)		
Pre-existing hypertension	2 (6.1%)		
Anti-nuclear antibodies	3 (17.7%)		
Untested	16		
Lupus anticoagulant	1 (3.9%)		
Untested	7		

Index pregnancy was defined as a participant's first pregnancy diagnosed with CHI by placental histopathological examination following poor outcome. Subsequent pregnancies refer to those following diagnosis. Continuous variables are presented as median (range) and categorical variables *N* (percentage). ART – assisted reproductive technology; BMI – body mass index.

Pregnancy Outcomes

Outcomes of index pregnancies diagnosed with CHI and subsequent pregnancies are listed in Table 4. The proportion of infants born by Caesarean section increased from 27.3% (6/33) in index pregnancies to 59.1% (13/28) and 45.5% (5/11) in first and second subsequent pregnancies respectively, though this was not statistically significant. Fetal sex did not differ significantly across pregnancies, and in two subsequent pregnancies fetal sex had not been determined due to loss early in gestation.

The outcome of subsequent pregnancy/ies improved significantly, with 70% of first and 100% of second subsequent pregnancies resulting in a liveborn and still living infant, compared to 11.8% of index pregnancies (p<0.0001). Gestation at delivery also increased significantly across pregnancies (p=0.002), from a median of 26 weeks' gestation in index cases to 37 weeks' gestation in first subsequent (p=0.04) and 38 weeks in second subsequent pregnancies (p=0.002). Of infants who were liveborn, rates of neonatal death were significantly reduced from 50% (4/8) in index cases to 3.0% (1/33) of subsequent pregnancies (p=0.003). TOPFA occurred in five index pregnancies (14.7%) and one first subsequent pregnancy (3.3%), after diagnosis of skeletal dysplasia (n=1), severe FGR (n=3), triploidy (n=1) and neurological disorder (n=1). In one case of stillbirth occurring in an index pregnancy, Trisomy 18 was diagnosed at post-mortem. For infants whose birthweight centiles could be calculated, rates of FGR <3rd centile decreased significantly between index and subsequent pregnancies from 52.0% (13/25) to 9.1% (3/33) (p=0.0007). There were no significant differences in the incidence of SGA across pregnancies. Overall birthweight centiles increased significantly across pregnancies from a median of 2.8 in index cases to 22.8 and 23.2 in first and second subsequent pregnancies, respectively (p<0.0001). In subsequent pregnancies, 23.5% of livebirths were preterm (8/34), significantly reduced from 75.0% (6/8) of index cases (p=0.01). One participant was diagnosed with gestational diabetes in their index pregnancy (3.0%), and there were no cases of preeclampsia. Chorioamnionitis occurred in one index case (3.0%) alongside CHI, however since CHI recurred without infection in the participant's subsequent pregnancy this case was included in the study.

Table 4. Outcomes of index and subsequent pregnancies in women with a diagnosis of chronic histiocytic intervillositis (CHI).

	Index CHI	1st Subsequent Pregnancy	2nd Subsequent Pregnancy
***N* participants**	33	28	11
***N* fetuses**	34	30	12
Caesarean Section (>24 weeks)	6 (27.3%)	13 (59.1%)	5 (45.5%)
Male fetus	13 (38.2%)	5 (53.6%)	7 (58.3%)
Unknown fetal sex	0	2	0
Pregnancy Outcome			
Liveborn and still living	4 (11.8%)	21 (70.0%)	12 (100%)
Stillbirth	16 (47.1%)	1 (3.3%)	0
Miscarriage	5 (14.7%)	6 (20.0%)	0
TOPFA	5 (14.7%)	1 (3.3%)	0
Neonatal death	4 (11.8%)	1 (3.3%)	0
Gestation at delivery (weeks)	26 (17-41)	37 (12-39)	38 (35-39)
Birthweight centile	2.8 (0-68.4)	22.8 (0.4-99.9)	23.2 (3.8-89.6)
Complications			
FGR <3rd centile	13 (52.0%)	3 (13.6%)	0
SGA 3rd-10th centile	5 (20.0%)	2 (9.1%)	1 (9.1%)
Preterm <37 weeks (% of livebirths)	6 (75.0%)	5 (22.7%)	3 (25.0%)
Maternal comorbidities			
Gestational diabetes	1 (3.0%)	0	0

Index pregnancy was defined as a participant's first pregnancy diagnosed with CHI by placental histopathological examination following poor outcome. Subsequent pregnancies refer to those following diagnosis. Miscarriage was defined as fetal death <24 weeks gestation, and stillbirth as fetal death >24 weeks gestation. Neonatal death refers to the death of an infant within 28 days after birth. Continuous variables are presented as median (range) and categorical variables *N* (percentage). FGR – fetal growth restriction; SGA – small for gestational age; TOPFA – termination of pregnancy for fetal anomaly.

Placental Pathology

Findings of placental histopathology are shown in Table 5. By design, all index pregnancies had an accompanying placental pathology report. The number of cases with histopathological examination of the placenta decreased to 76.7% (23/30) and 66.7% (8/12) in first and second subsequent pregnancies as a result of placentas not having been sent for histopathological examination. The incidence of CHI significantly decreased from index pregnancies, with an overall recurrence rate of 41.9% (13/31) in subsequent pregnancies (p<0.0001). There were no significant differences in the incidence of villitis, increased fibrin deposition and placentas classified as small for gestational age.

Pregnancies with concurrent CHI and villitis in the placenta exhibited no differences in outcome compared to those with CHI alone.

Table 5. Histopathology of placentas from index and subsequent pregnancies in women with chronic histiocytic intervillositis (CHI).

	Index CHI	**1st Subsequent Pregnancy**	**2nd Subsequent Pregnancy**
N placentas	34	30	12
N placentas with pathology reports	34 (100%)	23 (76.7%)	8 (66.7%)
CHI	34 (100%)	11 (47.8%)	2 (25.0%)
Chronic villitis	5 (14.7%)	6 (26.1%)	2 (25.0%)
Increased fibrin deposition	18 (52.9%)	7 (30.4%)	2 (25.0%)
Small for gestational age	7 (20.6%)	4 (17.4%)	1 (12.5%)

Index pregnancy was defined as a participant's first pregnancy diagnosed with CHI by placental histopathological examination following poor outcome. Subsequent pregnancies refer to those following diagnosis. Variables are expressed as *N* (percentage).

The severity of CHI lesions in placentas from index and subsequent pregnancies as determined by histopathological examination is shown in Figure 13. Detail of CHI lesion severity was available for 28/34 (82.4%) and 30/31 (96.8%) placentas from index and subsequent pregnancies, respectively. Placentas from index cases exhibited mainly mild or moderate CHI (39.3% (11/28) and 39.3% (11/28)), followed by a smaller proportion of severe cases (21.4% (6/28). In comparison, CHI was absent in the majority of placentas from subsequent pregnancies (60% (18/30)), whilst 23.3% displayed mild lesions (7/30), 10.0% moderate (3/30) and 6.7% severe (2/30). Overall, severity of CHI was significantly reduced in subsequent pregnancies compared to index ($p<0.0001$).

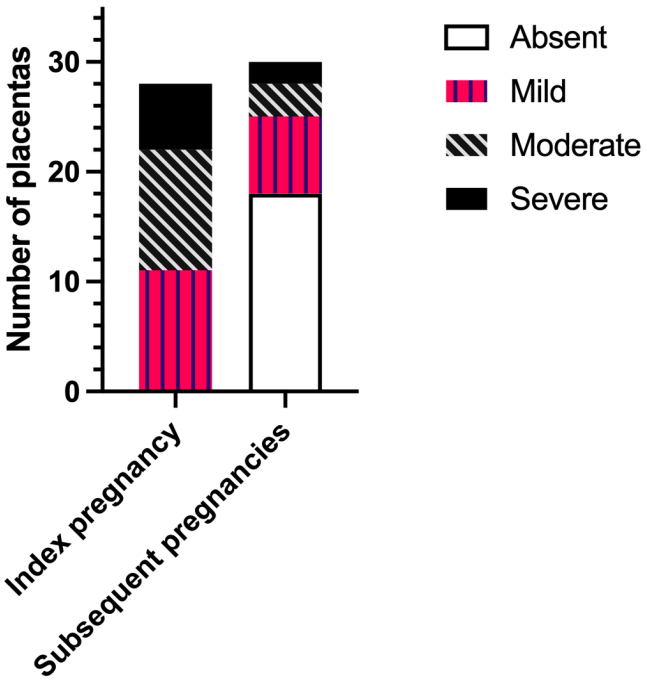

Figure 13. Severity of chronic histiocytic intervillositis (CHI) lesions in placentas from index and subsequent pregnancies according to pathologist's report. Index CHI refers to a participant's first pregnancy diagnosed with CHI by placental histopathological examination following poor outcome. Subsequent pregnancies refer to those following diagnosis.

Treatment Effect

Treatment regimen across pregnancies is shown in Figure 14. The majority of index pregnancies had no medication (24/33, 72.7%), compared to 10.7% (3/28) of first subsequent pregnancies. No second subsequent pregnancies were untreated. In index pregnancies, the nine treated participants were on medication for pre-existing medical conditions or risk factors and took aspirin alone (*n*=4), in combination with LMWH (*n*=4) or hydroxychloroquine (*n*=1). There was no significant difference in the livebirth rate of index pregnancies between women on medication and those without. In subsequent pregnancies, 69.2% (27/39) of all participants received immunomodulatory therapy with at least one or both of hydroxychloroquine or prednisolone, with the majority of participants (53.9% (21/39)) receiving all four medications. All four women with a positive autoantibody screen were treated in their subsequent pregnancies (*n*=5) with aspirin and LMWH (*n*=1); aspirin, low-molecular-weight heparin and hydroxychloroquine (*n*=1); aspirin, prednisolone and hydroxychloroquine (*n*=1) or a combination of all four medications (*n*=2). These pregnancies all resulted in the birth of a live infant surviving past 28 days of life.

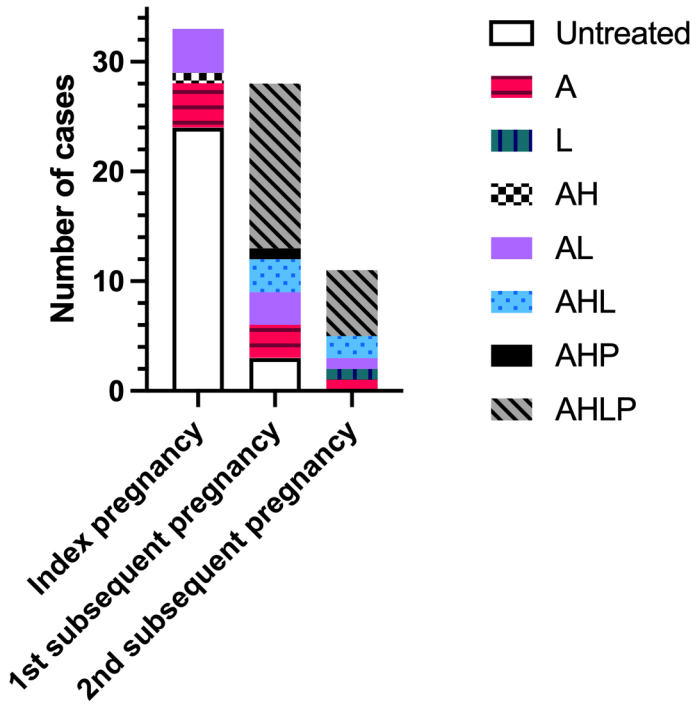

Figure 14. Treatment regimen across index pregnancies with chronic histiocytic intervillositis (CHI) and first and second subsequent pregnancies. Index pregnancy was defined as a participant's first pregnancy diagnosed with CHI by placental histopathological examination following poor outcome. Subsequent pregnancies refer to those following diagnosis. A – aspirin; H – hydroxychloroquine; L – low-molecular-weight heparin; P – prednisolone.

The overall rate of infants liveborn and still living (including infants born <37 weeks' gestation) was almost 25% higher in women receiving immunomodulatory treatment with one or both of hydroxychloroquine and prednisolone compared to those without (25/29 (86.2%) versus 8/13 (61.5%)) (Figure 15A). However, this did not reach statistical significance due to the number of pregnancies included in the study (p=0.11). Inclusion of immunomodulatory medication in treatment regimen also resulted in a 19% increase in the proportion of infants liveborn at term (>37 weeks) (Figure 15B) (65.5% (19/29) with immunomodulators versus 46.2% (6/13) without), though again this was not statistically significant. There were no significant effects of immunomodulatory treatment on the incidence of FGR (8.33% (2/24) with versus 12.5% (1/8) without), SGA (12.5% (3/24) with versus 12.5% (1/8) without) or on overall birthweight centiles (median 20.9 with versus 19.5 without). Administration of immunomodulatory medication similarly had no significant effect on gestation at delivery (median 37 with versus 37 without) (Figure 15C).

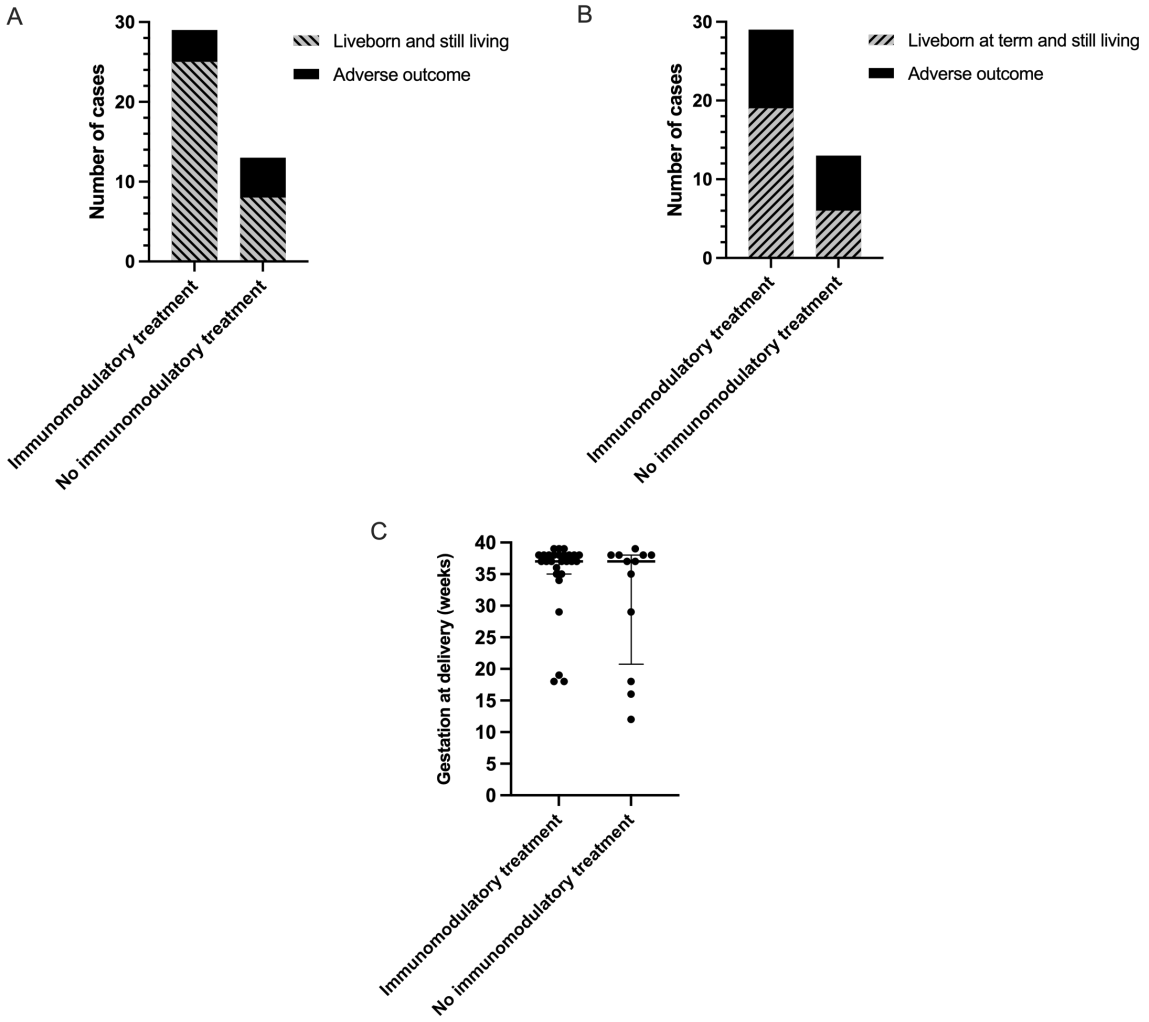

Figure 15. Outcomes of subsequent pregnancies in women with a previous diagnosis of chronic histiocytic intervillositis (CHI) following treatment with or without immunomodulators. A) Number of pregnancies resulting in a liveborn and still living infant (including those born <37 weeks' gestation) or adverse outcome (miscarriage, termination of pregnancy, stillbirth or neonatal death). B) Number of pregnancies resulting in a liveborn and still living infant at term (>37 weeks), or adverse outcome (miscarriage, termination of pregnancy, stillbirth, preterm birth or neonatal death). C) Gestation at delivery in subsequent pregnancies treated with or without immunomodulatory medication. Bars show median and error bars interquartile range. Immunomodulatory treatment refers to a regimen including one or both of prednisolone and hydroxychloroquine in combination with either aspirin, heparin or both. Pregnancies without immunomodulatory treatment were untreated or received aspirin or heparin or both.

Change in CHI severity following treatment regimen is shown in Figure 16A. 7/13 (53.8%) placentas from women treated without immunomodulatory medication were sent for histopathological examination, compared to 23/29 of those who were treated with it (82.8%). Change in severity of CHI in one subsequent pregnancy treated without immunomodulatory medication could not be determined due to a lack of detail on severity of the index case. The majority of remaining placentas from pregnancies treated without immunomodulatory medication exhibited no change in CHI severity compared to their index case (66.7% (4/6)). In comparison, 86.7% (20/23) of cases treated with immunomodulators displayed a reduction in CHI severity or a lack of recurrence (p=0.02). Decreased severity of CHI in subsequent pregnancies was associated

with a 62.3% increase in livebirth rate compared to pregnancies where severity was unchanged (20/22 (90.9%) vs 2/7 (28.6%) respectively) (p=0.003) (Figure 16B).

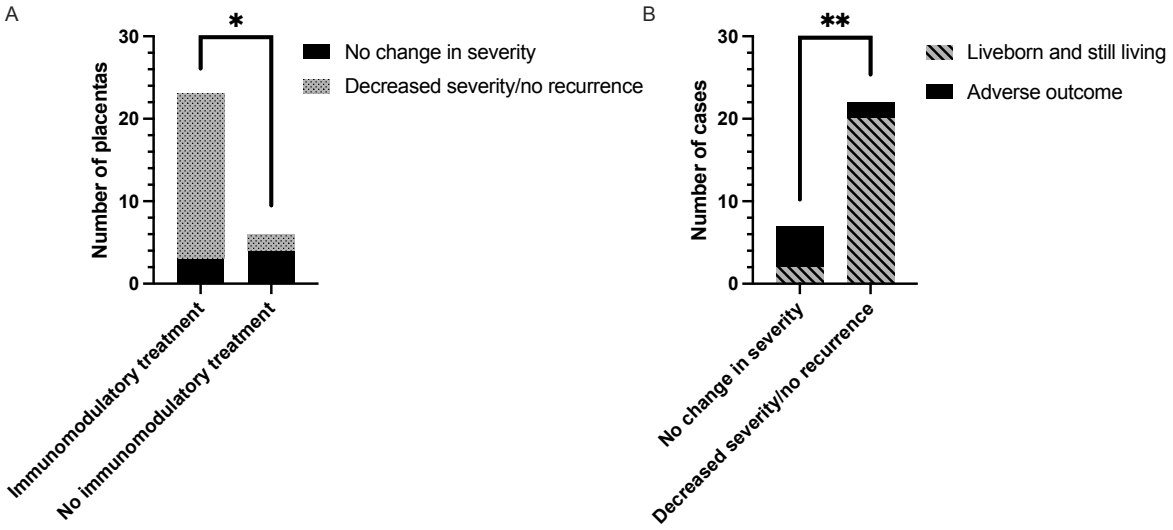

Figure 16. The effect of immunomodulatory medication in subsequent pregnancies after a diagnosis of chronic histiocytic intervillositis (CHI). A) Change in CHI lesion severity in pregnancies treated with or without immunomodulatory medication, compared to the participant's first placenta diagnosed with CHI from a previous pregnancy. Severity of CHI was determined by pathologist's report. Immunomodulatory treatment refers to a regimen including one or both of prednisolone and hydroxychloroquine in combination with either aspirin, heparin or both. Pregnancies without immunomodulatory treatment were untreated or received aspirin or heparin or both. B) Rate of infants liveborn and still living (past 28 days of life) in subsequent pregnancies related to change in CHI severity. * - p<0.05; ** - p<0.01.

Discussion

These data suggest that the use of one or both of prednisolone and hydroxychloroquine in the treatment of CHI resulted in a reduction of disease severity and a trend towards an increase in livebirth rate. Overall, a decrease in CHI severity was associated with a 62.3% reduction in pregnancy loss. These findings suggest that management of CHI in a specialist centre for women pregnant after loss improves outcomes, with all second subsequent pregnancies resulting in the birth of a healthy infant.

Strengths and Limitations

Previously, treatment of CHI has been informed by case reports and small studies detailing successful pregnancy outcome following the use of prednisolone and other immunomodulatory agents,[11] due to a lack of randomized controlled trials informing management. Given CHI's rarity and ethical and feasibility issues surrounding recruitment of women with such poor obstetric histories, it is unlikely that there will be a study where treatment is compared to placebo or a combination of agents known to be ineffective (e.g. aspirin and LMWH alone). As such, case series prove invaluable in guiding the care of women with a history of CHI. To date, this series is the largest to investigate the efficacy of immunomodulatory medication in the treatment of CHI and proposes a standardized protocol of medication to increase the chance of positive outcome in subsequent pregnancies.

Through analysis of medical records, we have been able to follow women through subsequent pregnancies following diagnosis of CHI and formulate a well-characterized cohort of a rare condition. However, we recognize that women who decided to become pregnant or became pregnant could differ from all women who have CHI. The demographic characteristics of our sample were similar to those from other case series,[5,15] suggesting that this potential selection bias had not significantly altered the nature of the sample.

In previous investigations, the rate of recurrence of CHI and accompanying outcome in treated pregnancies has been relatively well documented, though there is a paucity of evidence regarding improvement in lesion severity. For the majority of pregnancies we included, detail of lesion severity was available in placental pathology reports, allowing any change to be related to an individual's index case. To our knowledge, this is the first time such an approach has been taken in a case series of treated pregnancies following CHI. Here, it was shown that although overall recurrence of CHI in participants treated with immunomodulatory treatment did not differ significantly compared to those treated without, the severity of lesions was greatly reduced. This indicates that inclusion of disease severity as an outcome in future studies on CHI may provide better insight into treatment effect rather than simple recurrence rate alone.

Due to the retrospective nature of this study, there were certain variables for which data was not available within medical records. For instance, many women whose index case of CHI occurred less recently had not received screening for all autoantibodies as this was a more recent practice. Additionally, the range of antibodies tested was limited, which may explain the low incidence of autoantibodies in this cohort in comparison to studies which looked more specifically at antibody status and have reported incidences between 29% and 58%.[9,10] Since this suggestion of an autoimmune component to CHI, it has become standard practice within our center to offer women an antibody screen following referral. In future, this will allow better characterization of any possible role pre-existing antibodies may have within CHI and may inform immunosuppressive treatment if underlying conditions are present.

Several subsequent pregnancies included were without an accompanying placental histopathology report as a direct result of having not been sent for examination. In all these cases, all respective pregnancies had resulted in a livebirth. This is suggestive that there may have been a bias towards reporting pathology only in pregnancies resulting in poor outcome. In addition, pathologists were informed of patient's medical history which may have influenced diagnosis. In response to decreased rates of pathological examination in subsequent pregnancies, the importance of histopathological examination following a history of CHI is becoming increasingly recognized amongst midwives and clinicians within our center. Consequently, placental histopathology is now a routine requirement and is anticipated to improve sample size for prospective studies.

Clinical Context

Though the specific effects of prednisolone and hydroxychloroquine could not be individually determined here, our data is suggestive that inclusion of at least one of these medications in the

treatment of CHI can significantly reduce the severity of macrophage infiltration into the intervillous space. Further to this, a reduction in CHI severity increased the likelihood of a live birth. This is in line with previous evidence that pregnancies where CHI is severe or diffuse in the placenta are more strongly associated with poor outcome.[16] Pregnancy outcomes in CHI and severity following hydroxychloroquine use have not been well documented previously, although a single study stated that four out of six pregnancies where it was included in treatment regimen resulted in a liveborn infant.[10] Prednisolone has also in several case reports been associated with improved pregnancy outcome and reduced severity of both CHI and fibrin deposition compared to treatment with aspirin or heparin alone.[2,11,17] Within our cohort, there was no significant difference in fibrin deposition between index and subsequent pregnancies, and the majority did not have an increase in fibrin noted in their pathology report. This is perhaps unsurprising as fibrin deposition has been classified previously as an accompanying feature which may or may not occur alongside CHI.[1]

Whilst the exact cause of pathology in CHI is unknown, current evidence suggests that it is driven by excessive maternal inflammation. Inflammatory features characterized so far include the presence of partner-directed T lymphocytes and antibodies[18] and deposition of complement in the placenta.[19] Our observation that administration of prednisolone and hydroxychloroquine aimed at suppressing the maternal immune response reduces CHI severity is therefore consistent with this hypothesis. The specific mechanism of hydroxychloroquine and prednisolone's anti-inflammatory effects in CHI are unknown, though in mouse and *in vitro* models of antiphospholipid syndrome, it has been suggested that hydroxychloroquine is able to reduce complement activation and antibody binding to the syncytiotrophoblast.[20] In addition, hydroxychloroquine does not appear to have negative effects on placental explants and increases release of anti-inflammatory cytokine interleukin-10.[20] As complement deposition and excessive inflammation is characteristic of CHI, further investigation into the mechanisms of immunomodulatory therapy in the condition is warranted.

Evidence of CHI as an alloimmune condition has also provided rationale for the use of other immunomodulatory and immunosuppressive routes of treatment, including intravenous immunoglobulin (IVIG) therapy, tumor necrosis factor (TNF) antagonists and tacrolimus.[21] In a case report by Abdulghani et al.[14] IVIG was used following the failure of prednisolone to produce successful outcome in a previous pregnancy, and resulted in two subsequent healthy pregnancies. Histopathological examination of both placentas revealed that CHI had not recurred in either case. Similarly, use of TNF antagonist adalimumab was reported in the pregnancy of a woman with recurrent intervillositis and has been proposed as a possible agent in the prevention of recurrent miscarriage.[22] Though these case reports provide anecdotal evidence hinting at the benefits of these agents, there is still a lack of larger studies to justify their use and as such much controversy remains.

Despite the lack of comparative studies where the efficacy of immunomodulatory medication in treating CHI has been specifically investigated, a livebirth rate of 66.7% in 21 women receiving various combinations of aspirin, heparin, prednisolone and hydroxychloroquine has previously been reported by Mekinian et al.[10] Conversely, in the only systematic review of intervention in CHI

to date, treatment was suggested to correlate with worse outcome, with a reported livebirth rate of 30.8%.[3] Here, livebirth rate with inclusion of one or both of hydroxychloroquine and prednisolone was markedly higher than both studies at 86.2%. Importantly, the systematic review did not include any studies wherein hydroxychloroquine was administered, and treatment regimen was less consistent than that used within this case series, both of which may be factors in the differences observed. As in the aforementioned studies, differences in livebirth rates within this cohort were not proven to be significantly different between treatment groups, likely due to limited sample size considering that data did show a trend towards statistical significance. Given that the only systematic review is now a decade old, it is probable that an updated study of the literature is required to better characterize treatment combinations and their effect on pregnancy outcome.

In subsequent pregnancies, gestational age at delivery, fetal growth and rates of preterm birth improved greatly, though these effects could not be significantly attributed to immunomodulatory treatment. It is possible that this is due to limited numbers, or an effect of treatment using LMWH and aspirin. Adverse outcomes may also have been reduced as a consequence of increased fetal monitoring following specialist care after previous pregnancy loss. As there were insufficient numbers of untreated women and all participants had attended the specialist clinic, it was not possible to distinguish whether these outcomes were a result of intervention or treatment. Increased fetal monitoring is unable to influence fetal growth, but delivery via Caesarean section showed a trend towards increasing across subsequent pregnancies despite lacking statistical significance. Other centers have reported low levels of spontaneous labor in cases of CHI and early delivery of a liveborn infant has been detailed in a case report following observations of fetal growth plateau.[10,11] Therefore, it is possible that electing for early delivery may have had a beneficial effect in select cases.

Recurrence rates of CHI in subsequent pregnancies vary widely between studies and have been reported between 25 and 100%.[1,3,7] Within this cohort, recurrence of CHI was 41.9%, though the majority of pregnancies were treated which in twenty placentas reduced severity, some to the point where CHI was absent. In agreement with observations made by other groups, recurrence of CHI was not always associated with adverse outcome.[10,23] Bos et al. in their systematic review stated that incidence and recurrence of CHI may vary due to differing inclusion and exclusion criteria across studies.[1] A controversial criterion noted was the exclusion of cases with chronic villitis or villitis of unknown etiology alongside CHI. As concurrent CHI and chronic villitis lesions have been reported in 25-47% of placentas,[16,24] those with combined lesions were not excluded here as they represented a similar and considerable proportion of cases. Of particular interest was the observation that the incidence of chronic villitis did not differ across pregnancies and therefore seemed unaffected by intervention within the participant group studied. In addition, though chronic villitis is itself associated with FGR,[25] outcomes of pregnancies with concurrent CHI and chronic villitis were not significantly different, suggesting that chronic villitis in the presence of CHI may not have been a strong factor in adverse outcome.

Conclusion

The data from this retrospective study suggest that including at least one of hydroxychloroquine or prednisolone in treatment regimen of pregnancies following diagnosis of CHI is effective at reducing placental lesion severity. In turn, decreased severity of CHI improves the likelihood of livebirth in subsequent pregnancies. From this, we propose that a standardized treatment protocol including aspirin, heparin, prednisolone and hydroxychloroquine may prove beneficial in the management of CHI. Studies into the efficacy of both hydroxychloroquine and prednisolone as well as other immunomodulatory agents used within the condition is extremely limited, and feasibility of randomized controlled trials in affected women is low given their poor obstetric history. To overcome this challenge, collaboration between centers specializing in the management of CHI is required to increase sample sizes to allow sufficient evaluation of treatment regimen.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 4: Characterising Histopathological Features in Pregnancies with Chronic Histiocytic Intervillositis

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C.A.B, I.P.C and A.E.P.H contributed toward study design and planning. C.A.B performed experiments, collected, analysed and interpreted data and wrote the manuscript. T.R assisted with anti-CD68 and CD3 staining.

Abstract

Context

Chronic histiocytic intervillositis (CHI) is a rare condition characterised by maternal immune cell infiltration into the human placenta. CHI is strongly associated with fetal growth restriction, miscarriage and stillbirth, and knowledge of its aetiology and consequently effective treatment is limited. Currently, diagnosis is largely subjective and varies widely between centres, making comparison between studies challenging.

Objective

This study aimed to objectively quantify inflammatory cells and features in healthy placentas and those with CHI, and determine how pathology may be altered in subsequent pregnancies following diagnosis. Analysis of macrophage phenotype was also undertaken in untreated cases of CHI.

Design

Unbiased software analysis was applied to immunohistochemically stained untreated (index) cases of CHI, subsequent pregnancies and healthy control pregnancies. Placentas from index cases were additionally stained by immunofluorescence for M1 (CD80 and CD86) and M2 macrophage markers (CD163 and CD206).

Results

Quantification revealed a median 28-fold increase in macrophage infiltration in index cases versus controls, with CHI recurring in only 2 of 11 (18.2%) subsequent pregnancies. 3 out of 14 (21.4%) placentas initially diagnosed with CHI did not exhibit infiltration above controls. Macrophages in index pregnancies strongly expressed CD163. In QuPath-confirmed index cases, fibrin deposition showed a 51% increase in comparison to healthy placentas, which improved significantly in subsequent pregnancies. $CD3^+$ T cells were also elevated in subsequent pregnancies compared to controls.

Conclusions

In CHI, intervillous macrophages expressed CD163, indicating they may be resolving inflammation. Non-subjective computerised analysis of inflammation in CHI may be useful alongside pathologist expertise in assessment of lesion severity, and determining how treatment affects recurrence in subsequent pregnancies.

Introduction

Chronic histiocytic intervillositis (CHI) is an inflammatory lesion of the human placenta affecting 6 in every 10,000 pregnancies past 12 weeks' gestation,[1] wherein maternal macrophages infiltrate the intervillous space.[2] Though rare, recurrence in subsequent pregnancies varies from 25-100%, and the condition is strongly linked to fetal growth restriction (FGR), miscarriage and stillbirth.[3–5] Management of CHI is complicated by a lack of understanding into its cause and a lack of standardisation in diagnosis and treatment between centres, with intervention remaining largely experimental.[1] As such, CHI poses a distressing problem both for expectant parents and clinicians overseeing their care.

The cause of CHI is largely unknown, though involvement of maternal immune cells (namely $CD68^+$ macrophages), fibrin deposition and the reported presence of autoantibodies has given rise to the hypothesis that it is a disorder of failed maternal-fetal tolerance and is primarily caused by an excessive inflammatory immune response.[6] Despite constituting the majority of cellular infiltrate in CHI, it is unclear how macrophages contribute towards its pathophysiology. A single-centre study of fourteen cases suggested they are of an anti-inflammatory phenotype, due to expression of M2 marker CD163.[7] This finding appears consistent with a separate study of five placentas wherein a lack of pro-inflammatory chemokine upregulation and villous infiltration was evident in CHI.[8] Currently, the observation of an inflammatory infiltrate within the placenta following histopathological examination is the only method to diagnose CHI, due to a lack of reliable biomarkers before or during pregnancy.[9] A recent systematic review revealed that diagnostic criteria for CHI varied widely across eighteen included studies, with large discrepancies in inclusion and exclusion criteria between groups.[1] The single universally agreed criterion for diagnosis of CHI was the presence of an intervillous infiltrate, with only half of included studies specifying that the infiltrate must be composed of macrophages. Variation in diagnostic criteria from this study and publications since are summarised in Table 6. Reporting of lesion severity and fibrin deposition is also largely based upon estimation of coverage area and described generally as 'focal' or 'diffuse'.[1] Consequently, diagnosis remains largely subjective, making comparison between studies difficult and limiting research advances into the aetiology of the condition. Additionally, changes in immune cell profile and lesion severity in subsequent pregnancies receiving specialist care or therapeutic intervention have been difficult to establish given the lack of standardised diagnostic criteria.

With a view to reduce subjectivity, this study aimed to utilise computerised image quantification software to better characterise and interrelate inflammatory features in placentas with CHI. Following this, changes in immune cell profile in subsequent pregnancies receiving specialist care after a previous diagnosis of CHI were determined to investigate the effect of treatment on pathology. Finally, descriptive investigation of macrophage polarisation in untreated cases of CHI was undertaken to elucidate their role in the pathophysiology of the condition.

Table 6. Variation in diagnostic criteria of chronic histiocytic intervillositis across published studies.

	Labarrere and Mullen, 1987[2]	Jacques and Qureshi, 1993[10]	Boyd and Redline, 2000[4]	Rota et al., 2006[11]	Parant et al., 2009[5]	Traeder et al., 2010[12]	Marchaudon et al., 2011[13]	Heller, 2012[14]	Capuani et al., 2013[15]	Freitag et al., 2013[8]	Reus et al., 2013[16]	Labarrere et al., 2014[17]	Bendon et al., 2015[18]
Inclusion criteria													
Intervillositis/intervillous infiltrate	X	X	X	X	X	X	X	X	X	X	X	X	X
Mononuclear infiltrate	X	X		X	X		X		X		X		
Histiocytic monomorphic infiltrate			X			X	X	X				X	
Massive/widespread/diffuse infiltrate	X		X							X		X	
Fibrin deposition	X	X			X				X				
Trophoblast necrosis	X	X		X									
Maternal origin of infiltrate											X		
Exclusion criteria													
Sign of infection	X			X	X				X	X		X	
Villitis		X	X			X			X				X
Chorioamnionitis				X								X	
Cytomegalovirus infection					X					X			
Presence of ‘other obvious placental lesions”				X	X		X						
Malaria													
Polymorphic infiltrate			X			X							
Infiltration/destruction of placental tissue										X			
Lymphocytic vasculitis				X									
Congenital malformations													

X denotes usage of a particular inclusion or exclusion criterion. Adapted and updated from Bos et al.[1]

Table 6. Variation in diagnostic criteria of chronic histiocytic intervillositis across published studies (cont.).

	Labarrere et al., 2015[19]	Mekinian et al., 2015[20]	Revaux et al., 2015[21]	Nowak et al., 2016[22]	Sabra et al., 2016[23]	Koby et al., 2018[9]	Sato et al., 2019[24]	Bos et al., 2020[25]	Homatter et al., 2020[26]	Matuizzi et al., 2020[27]	Sauvestre et al., 2020[28]	Simula et al., 2020[29]	Nedberg et al., 2021[30]
Inclusion criteria													
Intervillositis/intervillous infiltrate	X	X	X	X	X	X	X	X	X	X	X	X	X
Mononuclear infiltrate	X	X	X	X	X	X	X	X		X	X	X	
Histiocytic monomorphic infiltrate		X	X	X	X	X			X			X	
Massive/widespread/diffuse infiltrate	X	X	X										
Fibrin deposition													
Trophoblast necrosis													
Maternal origin of infiltrate			X		X								
Exclusion criteria													
Sign of infection	X	X	X	X	X		X	X		X		X	
Villitis							X						
Chorioamnionitis													
Cytomegalovirus infection		X				X							
Presence of ‘other obvious placental lesions”													
Malaria		X			X								
Polymorphic infiltrate													
Infiltration/destruction of placental tissue													
Lymphocytic vasculitis													
Congenital malformations					X				X				

Materials and Methods

Participant Recruitment and Sample Collection

For the investigation of first (index) cases of CHI who were not receiving specialist care, participants were retrospectively identified via searching of placental histopathology reports at the Paediatric Histopathology Department, Saint Mary's Hospital, Manchester, UK, for a diagnosis of CHI following poor pregnancy outcome e.g. FGR, termination of pregnancy (TOP), miscarriage (fetal death <24 weeks' gestation), stillbirth (fetal death >24 weeks' gestation) or neonatal death (death within the first 28 days after birth). Diagnosis had been made by a specialist perinatal pathologist according to the initial description of CHI by Labarrere and Mullen as a lesion consisting of "massive infiltrate of the intervillous space by mononuclear cells".[2] Individuals with accompanying archived placental tissue samples available for analysis with permission for research studies were then included for participation in the study. An initial subset of healthy term control pregnancies with no evidence of CHI were also provided by the Paediatric Histopathology Department. All tissue was received as formalin-fixed paraffin embedded (FFPE) blocks.

Participants with a diagnosis of CHI in a previous pregnancy were also prospectively recruited whilst attending Tommy's Rainbow Clinic at Saint Mary's Hospital for care in a subsequent pregnancy. Further healthy control pregnancies were recruited and consented on the planned date of delivery by Caesarean Section, induction of labour or in early labour. Healthy controls were selected to match the demographic characteristics of participants with a history of CHI where possible. Women who smoked cigarettes, who were on immunosuppressant medication or with a history of autoimmune disease, advanced maternal age (>37 years), those with any ongoing infection or previous known SARS-CoV-2 infection were excluded. Following consent, placentas were collected and 1cm^3 cubes sampled from the centre (point of cord insertion), edge and middle (between centre and edge) of the tissue and rinsed in phosphate buffered saline (PBS). Samples were then fixed in neutral buffered formalin (NBF) for 24 hours at room temperature before rinsing in PBS, automated dehydration in a Leica TP1020 tissue processor (Leica Biosystems, Wetzlar, Germany) and embedding in paraffin wax.

Informed and written consent was obtained from all study participants. For archived tissue from Paediatric Histopathology, ethical approval was granted by NRES Committee London-City & East (REC ref: 14/LO/1352). For samples from patients gathered before July 2018, approval was granted by NRES Committee Northwest-Greater Manchester West (REC ref: 14/NW/1149). Between February and July 2018, the NRES Committee South East Coast-Surrey Research Ethics Committee approved the study (REC ref: 16/LO/1666). Samples collected after this period were recruited under the Tommy's Project Ethics (REC ref: 15/NW/0829).

Unless otherwise stated reagents were obtained from Sigma-Aldrich (Poole, UK).

Immunohistochemical Staining of Maternal Immune Cells

For detection of $CD68^+$ and $CD3^+$ cells in the intervillous space, immunohistochemistry was performed on 5μm sections of placental tissue mounted on poly-l-lysine coated slides (Fisher Scientific). After dewaxing and rehydration, antigen retrieval was performed via microwave heating in 0.01M sodium citrate buffer (pH 6) at 800W for 10 minutes. Endogenous peroxidase activity was quenched using 3% (v/v) hydrogen peroxide solution and non-specific staining blocked with 10% (v/v) normal goat serum in TBS-Tween (Biotium). Sections were then incubated in primary antibody; anti-human CD3 (mouse monoclonal, Dako, 1.4 μg/ml), or anti-CD68 (mouse monoclonal, Dako, 0.8μg/ml) overnight at 4°C. Following washes in TBS and 0.6% (v/v) TBS-Tween, slides were incubated with goat anti-mouse biotinylated IgG (Agilent, 3.3μg/ml) for 30 minutes at room temperature. Slides were washed as previous and signal amplified by incubation in avidin-peroxidase (5μg/ml) for 30 minutes and subsequent washes. Diaminobenzidine was applied for 4-minutes for detection of positive staining, sections washed and then counterstained with filtered Harris' haematoxylin, dehydrated and mounted with coverslips. Negative sections were treated identically with the substitution of primary antibody for non-immune mouse IgG at the same working concentration.

Haematoxylin and Eosin Staining of Fibrin Deposition

5μm sections of placental tissue mounted on uncoated slides (VWR International) were dewaxed and rehydrated as previous before incubation with filtered Harris' haematoxylin for 10 minutes. To stain fibrin a distinct shade of pink, sections were incubated in eosin solution for 10 minutes and rinsed quickly in cold tap water to avoid loss of colour. Slides were then dehydrated and mounted with DPX and coverslips.

Immunofluorescence for Analysis of Macrophage Polarisation

For determination of macrophage polarisation, index cases of CHI and representative healthy controls were stained for anti-CD68 (mouse monoclonal, Dako, 0.8μg/ml) and M1 markers anti-CD80 (rabbit polyclonal, Abcam, 10μg/ml) or anti-CD86 (rabbit polyclonal, Abcam, 5.6μg/ml). For staining of M2 markers anti-CD68 (concentration as above) was used in combination with anti-CD206 (rabbit polyclonal, Abcam, 2μg/ml) or anti-CD163 (mouse monoclonal, Bio-Rad, 5μg/ml) applied alone.

Sections were initially treated as previous apart from blocking which was carried out with 0.1% (w/v) fish gelatine (SLS Ltd) and 10% v/v donkey serum in 0.3% TBS-Triton. Primary antibodies were incubated for 1 hour at room temperature to minimise background staining before thorough washing in PBS. Donkey anti-mouse AF488 and donkey anti-rabbit AF568-conjugated secondary antibodies (Abcam, 2μg/ml) were applied for 30 minutes at room temperature. For visualisation of syncytiotrophoblast to allow discrimination between maternal macrophage and Hofbauer cell staining, AF647-conjugated rabbit anti-human cytokeratin 7 (CK7) was applied for one hour at room temperature. Slides were then washed, autofluorescence quenched with TrueView according to manufacturer's instructions and mounted with ProLong Diamond Antifade Mountant (Life

Technologies Ltd). Negative controls were treated identically with substitution of primary antibodies for mouse and rabbit non-immune IgG at the same working concentration.

Image Analysis and Quantification

Stained slides were scanned at the University of Manchester Bioimaging Facility using brightfield microscopy and a 3D Histech Panoramic 250 Flash Slide Scanner. CaseViewer was used to open files, reduce autofluorescence via inbuilt filters and take representative images of macrophage polarisation staining. For analysis of CD68, CD3 and fibrin staining, quantification was carried out in QuPath (v.0.3.0)[31] by superimposing a 1mm^2 grid onto whole slides and using a coin flip and random number generator to determine starting row and regions of interest, respectively. For calculation of positive cell counts, villous tissue was highlighted and excluded from the annotation, and a positive cell detection run within the remaining tissue to give a measure of cell infiltration per mm^2 of intervillous area. Fibrin deposition was quantified in the same manner by highlighting areas of pink staining to produce a measure of fibrin expressed as a percentage of region of interest area. For all data, measurements were averaged per placental area (centre, middle, edge) then averaged again to give an estimate of whole organ pathology. M1 and M2 macrophage marker expression was determined within CaseViewer by viewing whole slides from index cases of CHI to establish the presence of staining and its localisation qualitatively compared to representative control sections.

Statistical Analysis

All statistical analysis was undertaken in GraphPad Prism v9. Miscarriage, stillbirth, TOP and neonatal death were grouped and compared to pregnancies that ended in live birth by Fisher's Exact test between groups due to small sample size. Initially, the Shapiro-Wilk test was used to establish normality of data. Ordinary one-way ANOVA with Tukey's multiple comparisons and Kruskal-Wallis with Dunn's multiple comparisons were run for normally and non-normally distributed data, respectively. For analysis of CD3 data the Mann-Whitney test was run as only two experimental groups were compared. Relationships between variables were analysed using Spearman correlation. Statistical significance was set at $p<0.05$ for all analysis.

Results

Participant Demographics

Twenty-four healthy control participants, fourteen index cases of CHI and eleven participants returning for care in a subsequent pregnancy following CHI diagnosis were recruited to the study. Participant demographic characteristics are shown in Table 7. Of index pregnancies, one participant had no accessible clinical data to accompany available placental tissue due to having given birth at another centre, but was nevertheless included in the study considering the rarity of samples.

As a result of study design, rate of livebirth was significantly lower in index pregnancies compared to controls ($p<0.0001$). On the contrary, livebirth rate was greater in subsequent pregnancies

compared to index (p<0.0001). Gestation at delivery, birthweight and individualised birthweight centiles (IBC) were significantly lower in index pregnancies than control values (p=0.045, p<0.0001 and p<0.0001, respectively). Between index and subsequent pregnancies, only birthweight showed a significant increase (p=0.001).

Two participants (15.4%) were taking aspirin in their index pregnancies for pre-existing risk factors.[32] In subsequent pregnancies, most participants (8/11, 72.7%) were administered a combination of aspirin, LMWH, prednisolone and hydroxychloroquine, with the remainder on a similar treatment protocol without prednisolone.

Table 7. Participant demographic characteristics of healthy control pregnancies, index pregnancies with chronic histiocytic intervillositis (CHI) and subsequent pregnancies in women with a previous diagnosis.

	Control	Index CHI	Subsequent pregnancy
N	24	13	11
Maternal age (years)	32.5 (±4.8)	29.6 (±6.6)	31.3 (±7.0)
Maternal BMI	26.3 (±4.5)	28.6 (±5.4)	25.4 (±3.5)
Ethnicity			
Asian	2 (8.3%)	2 (15.4%)	3 (27.3%)
Black African	3 (12.5%)	1 (7.7%)	0
Eastern European	1 (4.2%)	0	0
White British	17 (70.8%)	10 (76.9%)	8 (72.7%)
White Irish	1 (4.2%)	0	0
Gravidity	3 (1-11)	4 (2-10)	3 (2-8)
Parity	2 (0-8)	3 (1-7)	1 (0-2)
Maternal comorbidities			
Autoimmune disease	0	1 (7.7%)	1 (9.1%)
Diabetes	0	0	1 (9.1%)
Hypertension	0	1 (7.7%)	0
Pregnancy outcomes			
Livebirth	24 (100%)	2 (15.4%)	11 (100%)
Miscarriage	0	1 (7.7%)	0
TOP	0	1 (7.7%)	0
Stillbirth	0	9 (69.2%)	0
Neonatal death	0	0	0
Caesarean section (>24 weeks)	17 (70.8%)	1 (7.7%)	7 (63.6%)
Gestation at delivery (weeks)	39 (35-41)	36 (23-40)	38 (26-39)
Birthweight (g)	3491 (±409.1)	1691 (±1077)	2886 (±890.7)
IBC	66.7 (18.8-95.2)	4.35 (0-45.3)	17.4 (1.1-100)
Fetal sex (*n* males)	11 (45.8%)	7 (58.3%)	9 (81.8%)
Unknown fetal sex	0	1	0

Index pregnancy refers to a participant's first pregnancy to be diagnosed with CHI by histopathological examination of the placenta following poor pregnancy outcome. Normally and non-normally-distributed variables were expressed as median (range) and mean (±standard deviation) respectively. Categorical variables are shown as *N* (percentage). Miscarriage and stillbirth were defined as fetal death below or above 24 weeks gestation, respectively. Neonatal death refers to death of an infant within 28 days after birth. BMI – body mass index; IBC – individualised birthweight centile; TOP – termination of pregnancy.

CD68$^+$ Macrophage Infiltration

Staining for maternal CD68$^+$ macrophages was evident within the intervillous space (IVS) of placentas in all three experimental groups (Figure 17). Within control placentas (*n*=24), maternal macrophages were rare (Figure 17A), compared to index CHI cases (*n*=14) where CD68$^+$ cells were distinctly more evident within the intervillous space either diffusely or as focal lesions of multiple cells (Figure 17B). As expected, placentas from index pregnancies with a histopathological diagnosis of CHI exhibited an almost 28-fold increase in CD68$^+$ macrophage infiltration compared to controls (358.5 [interquartile range (IQR) 62-2581] vs 13 [IQR 9-33] per mm^2 IVS respectively, $p<0.0001$) (Figure 17D). After analysis, certain index cases of CHI did not exhibit CD68$^+$ cell counts above controls. Following this finding, a more quantitative approach to CHI diagnosis was suggested, taking into account levels of macrophage infiltration in healthy placentas. The 95th percentile of macrophage counts from controls (80 macrophages per mm^2 intervillous space) was used as a minimum threshold for CHI classification, with any placentas displaying a measurement above this classified thereon as QuPath-defined CHI. After application of this threshold, three placentas diagnosed with CHI by histopathological criteria were excluded from the refined group. In subsequent pregnancies, macrophage infiltration was reduced to levels similar to that of control placentas (median 28 [IQR 16-51] vs 13 [IQR 9-32.5] respectively). Compared to index cases, subsequent pregnancies exhibited an almost 13-fold decrease in median macrophage infiltration (28 [IQR 16-51] vs 358.5 [IQR 62-2581] respectively), though this was not statistically significant. Placental pathology reports were available for nine subsequent pregnancies, and all detailed no histopathological recurrence of CHI in tissue (Figure 17C). However, following QuPath analysis, two subsequent pregnancies exhibited macrophage infiltration above control values, suggesting possible recurrence of CHI in these cases (Figure 17D).

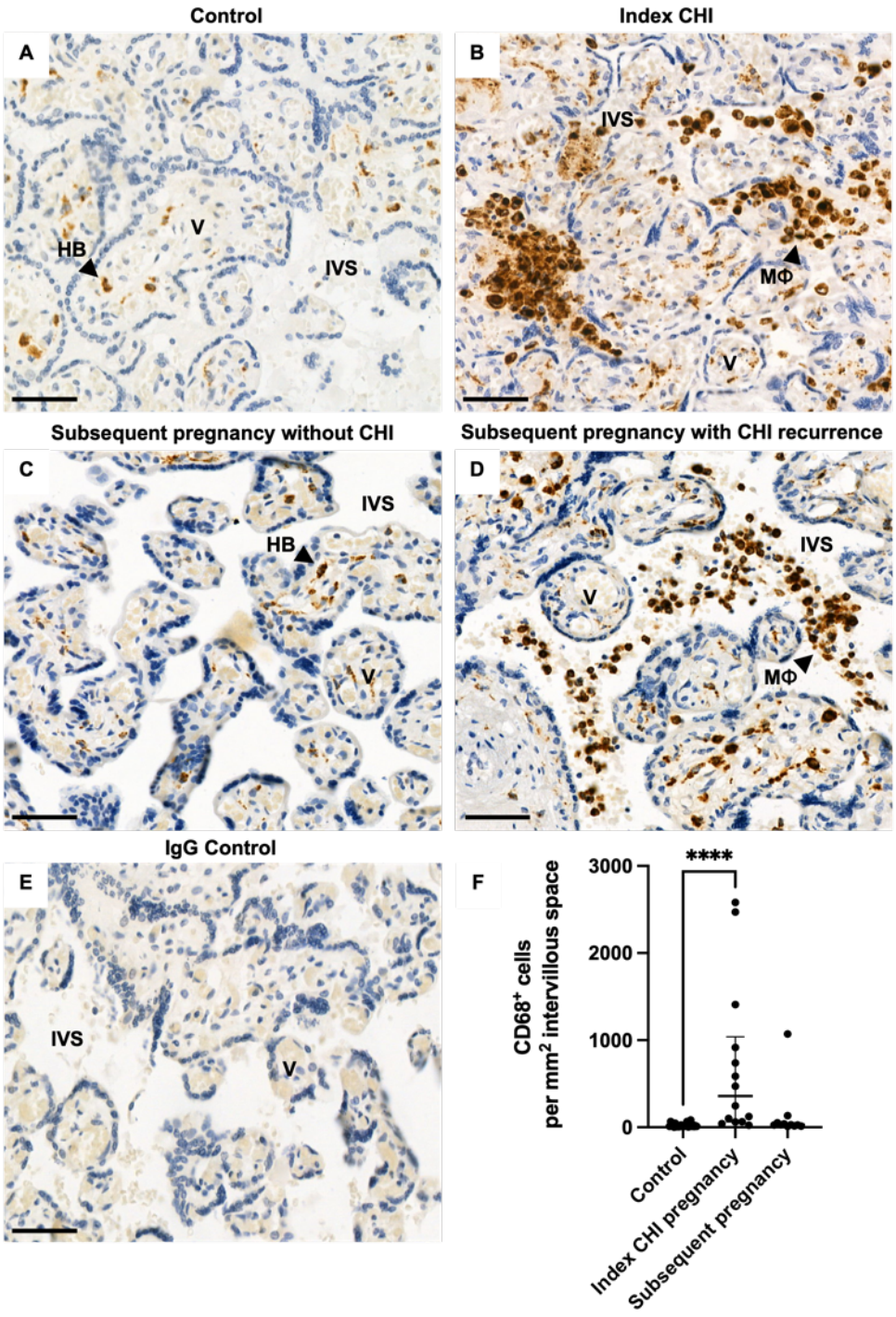

Figure 17. Maternal CD68⁺ macrophage infiltration in healthy control placentas and placentas with a diagnosis of chronic histiocytic intervillositis (CHI). A) In placentas from healthy control pregnancies, the intervillous space (IVS) is clear, with some CD68⁺ staining in Hofbauer cells (HB) within fetal villi (V). B) An index case of CHI, characterised by intervillous macrophage (MΦ) infiltration. Subsequent pregnancies may exhibit fewer intervillous CD68⁺ macrophages (C), or CHI may recur (D). E) Negative control placental tissue, where anti-CD68 antibody was substituted for mouse non-immune IgG at the same working concentration. Scale bars = 50μm. F) Quantification of intervillous CD68⁺ cells in healthy control pregnancies, index and subsequent cases of CHI following diagnosis via QuPath software. Bars represent median and error bars interquartile range. Kruskal-Wallis with Dunn's post-hoc multiple comparisons test, **** - $p<0.0001$. Index CHI refers to the first pregnancy diagnosed by histopathological examination.

Fibrin Deposition

Upon gross histological examination of placentas stained with H&E, fibrin deposition was present in control placentas to a degree (Figure 18A), however appeared to be elevated in certain areas of placentas with CHI compared to controls (Figure 18B). Both perivillous and intervillous fibrin was present, with engulfment of villi and macrophage accumulation evident around fibrotic areas (Figure 18B). In both control and index pregnancies, levels of fibrin deposition varied widely amongst placentas, occupying between 1.4-12.5% of placental area (Figure 18C). Histopathologist-defined cases of CHI displayed a slightly higher mean proportion of fibrin deposition compared to controls (6.1 ±3.4 vs 4.5 ±2.3, respectively), though this lacked statistical significance. Following refinement of index cases using the 95th percentile threshold for CD68^{+} cells, mean fibrin deposition in the remaining QuPath-defined cases increased compared to controls (6.8 ±3.3 vs 4.5±2.3, respectively), nearing the threshold of statistical significance (p=0.08). In subsequent pregnancies receiving specialist care, the mean proportion of villous tissue occupied by fibrin was similar to controls and did not differ significantly, with a value slightly below that of healthy controls (3.1 ±1.2 vs 4.5 ±2.3). Compared to both histopathologically diagnosed and QuPath-defined cases of CHI, subsequent pregnancies displayed a significant reduction in the proportion of placental fibrin (p=0.04 and p=0.01 respectively). Within index cases of CHI, the extent of fibrin deposition was not related to the severity of macrophage infiltration (Figure 18D).

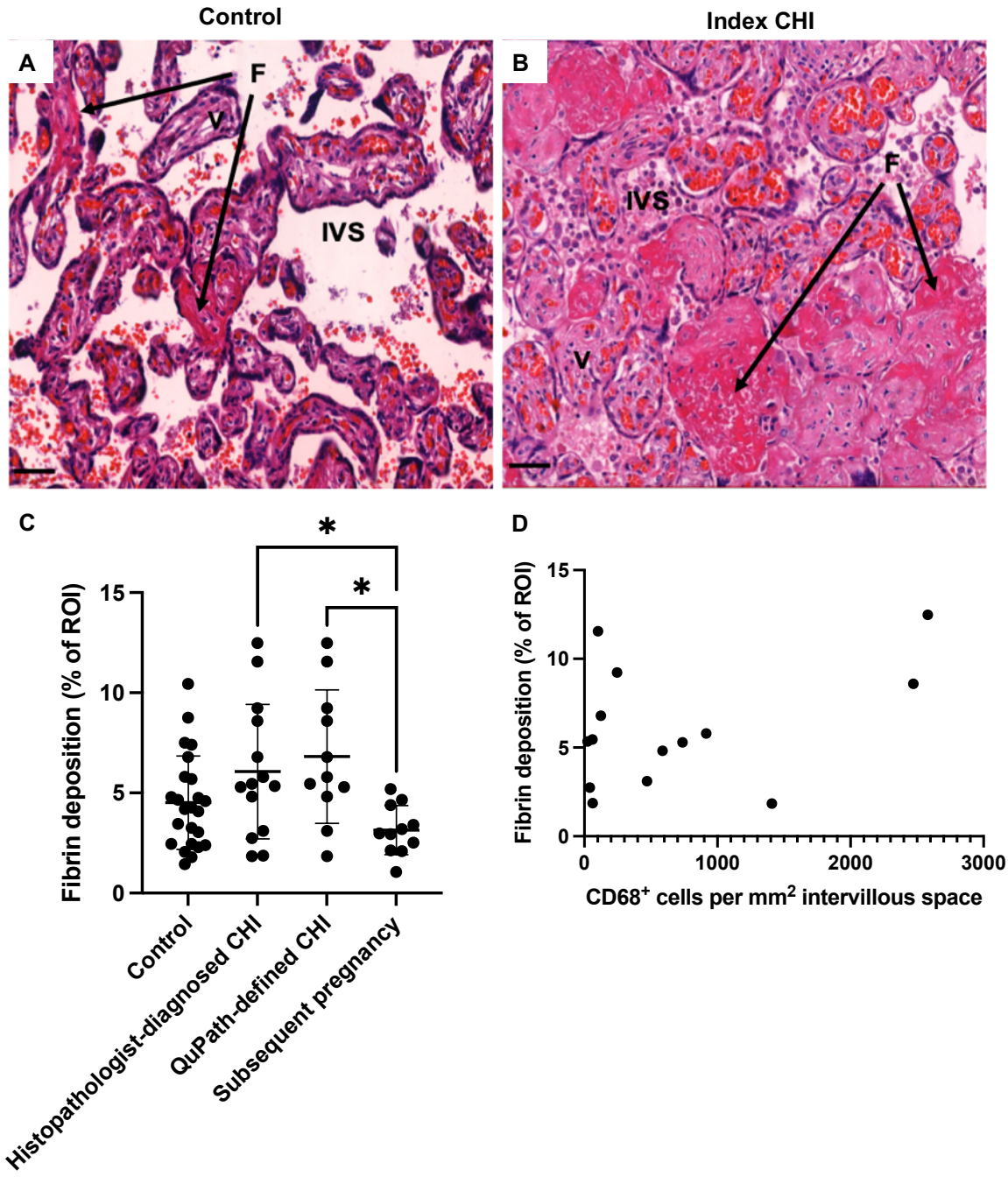

Figure 18. Haematoxylin and eosin stain for fibrin deposition in A) a healthy control placenta and B) a case of chronic histiocytic intervillositis (CHI). Within the healthy term placenta, fibrin (F) is present to a degree within the intervillous space (IVS), but in certain cases of CHI can occupy large areas of the placenta. Scale bars = 50μm. C) QuPath software was used to quantify fibrin deposition as a percentage of regions of interest (ROI) in control pregnancies as well as index cases of CHI and subsequent pregnancies following diagnosis. Index cases of CHI were further split into pathologist-diagnosed cases, and those which exhibited macrophage infiltration above the 95th percentile of control values described as QuPath-defined cases. Bars represent mean and error bars standard deviation. Ordinary one-way ANOVA with Tukey's multiple comparisons, * - p<0.05. D) Plot comparing CD68^{+} macrophage infiltration and fibrin deposition within placentas from index cases of CHI.

CD3$^+$ T Cell Infiltration

For quantification of T cell infiltration, analysis was limited to placentas from control (*n*=11) and subsequent pregnancies (*n*=9) (Figure 19). With regard to overall numbers of infiltrating maternal CD3$^+$ T cells, placentas from subsequent pregnancies exhibited varying levels with an almost 6-fold increase in median compared to controls (28.4 [IQR 23.0-86.9] vs 4.9 [3.0-6.1] respectively, $p<0.0001$). T cell infiltration was not correlated with the extent of macrophage infiltration in subsequent pregnancies (Figure 19E).

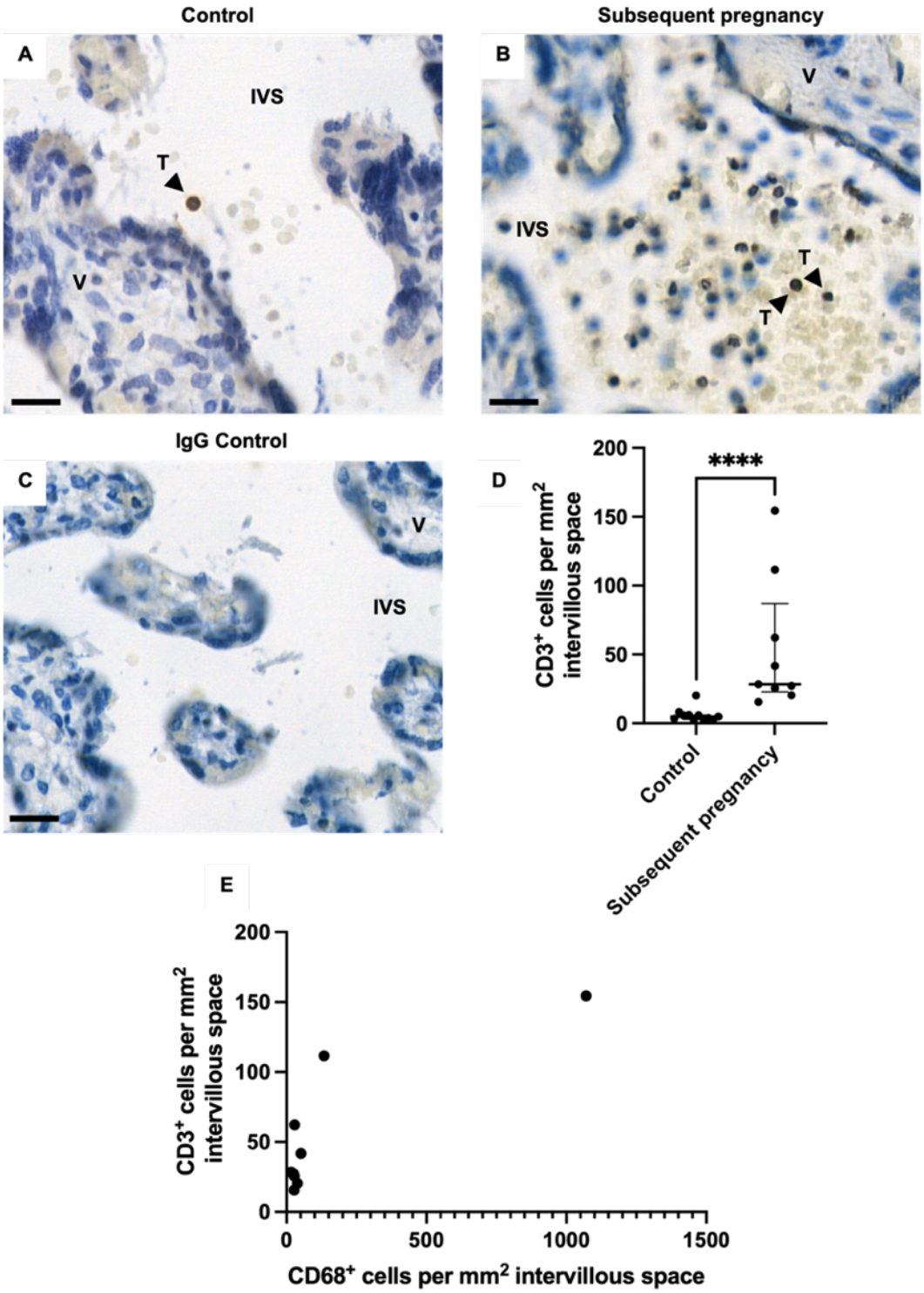

Figure 19. CD3⁺ T cell infiltration in A) a healthy control placenta and B) a placenta from a subsequent pregnancy following diagnosis of chronic histiocytic intervillositis (CHI). Within healthy controls, CD3⁺ T cells (T) are occasionally visible in the intervillous space (IVS) surrounding fetal villi (V). However, in some subsequent pregnancies following a diagnosis of CHI, maternal T cells are abundant within the IVS. C) Placental tissue stained with mouse IgG at the same working concentration as primary antibody as a negative control. Scale bars = 20μm. D) Quantification of maternal T cell infiltration undertaken in QuPath Software. Bars represent median and error bars interquartile range. Mann-Whitney test, **** - p<0.0001. E) Plot comparing intervillous CD3⁺ T cells and CD68⁺ macrophages in subsequent pregnancies.

Macrophage Polarisation in Index Cases of CHI

To determine the polarisation of macrophages in CHI and how they may contribute to pathology, immunofluorescence staining for M1 and M2 markers was utilised as shown in Figure 20 and Figure 21, respectively.

All placental sections from QuPath-confirmed index cases of CHI studied displayed more positive CD68 staining within the intervillous space compared to controls, though neither CD80 or CD86-positive cells were evident in either group (Figure 20C and D). Staining for CD68 and M2 marker CD206 revealed distinct co-staining of Hofbauer cells within villi of control placentas and those with CHI (Figure 21A and C). Within control placentas, villous Hofbauer cell staining for CD206 was more abundant compared to those with CHI. Outside of villi, no $CD68^{+}/CD206^{+}$ double-positive intervillous cells were visible within sections from index CHI cases. However, intervillous macrophages in CHI were strongly positive for the M2 marker CD163 across all cases studied (Figure 21D).

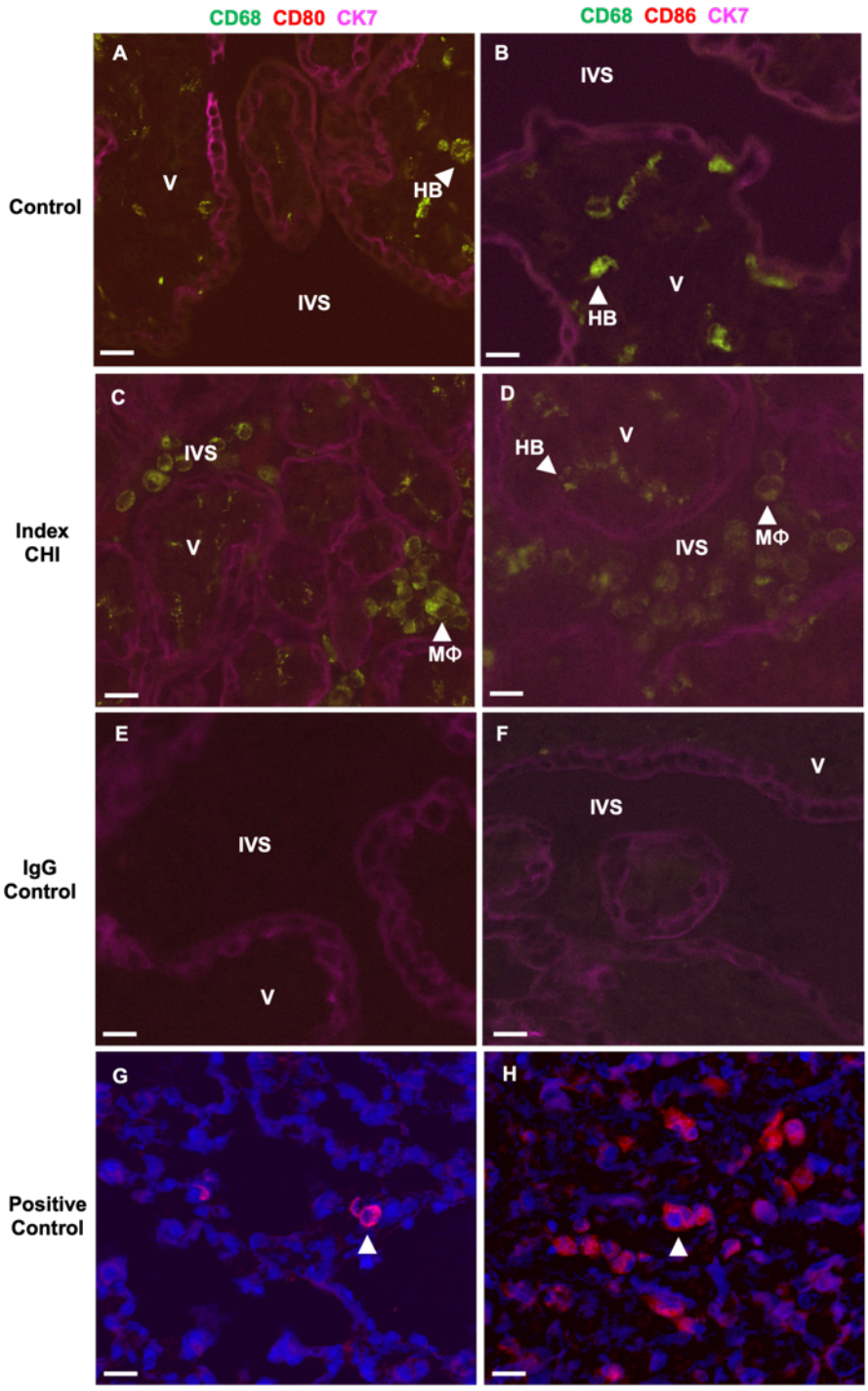

Figure 20. Representative immunofluorescence staining for M1 macrophages (MΦ) in a healthy control placenta and an index case of chronic histiocytic intervillositis (CHI). Placentas (A-D) were stained for CD68 (green) as a pan-macrophage marker, together with either CD80 or CD86 (red) as M1 macrophage markers. Cytokeratin 7 (CK7) (pink) was used as a trophoblast marker for distinction between maternal macrophages in the intervillous space (IVS) and Hofbauer cells (HB) which are fetal macrophages within villi (V). Non-immune rabbit and mouse IgG was used as a negative isotype control for CD68 and CD80/86 (E and F). Mouse lung and omental carcinoma tissue were used as positive controls for CD80 (G) and CD86 (H), respectively and nuclei stained with DAPI (blue). Index CHI refers to a participant's first diagnosed case of CHI by histopathological examination of the placenta. Arrows indicate positive cells. Scale bars = 20μm.

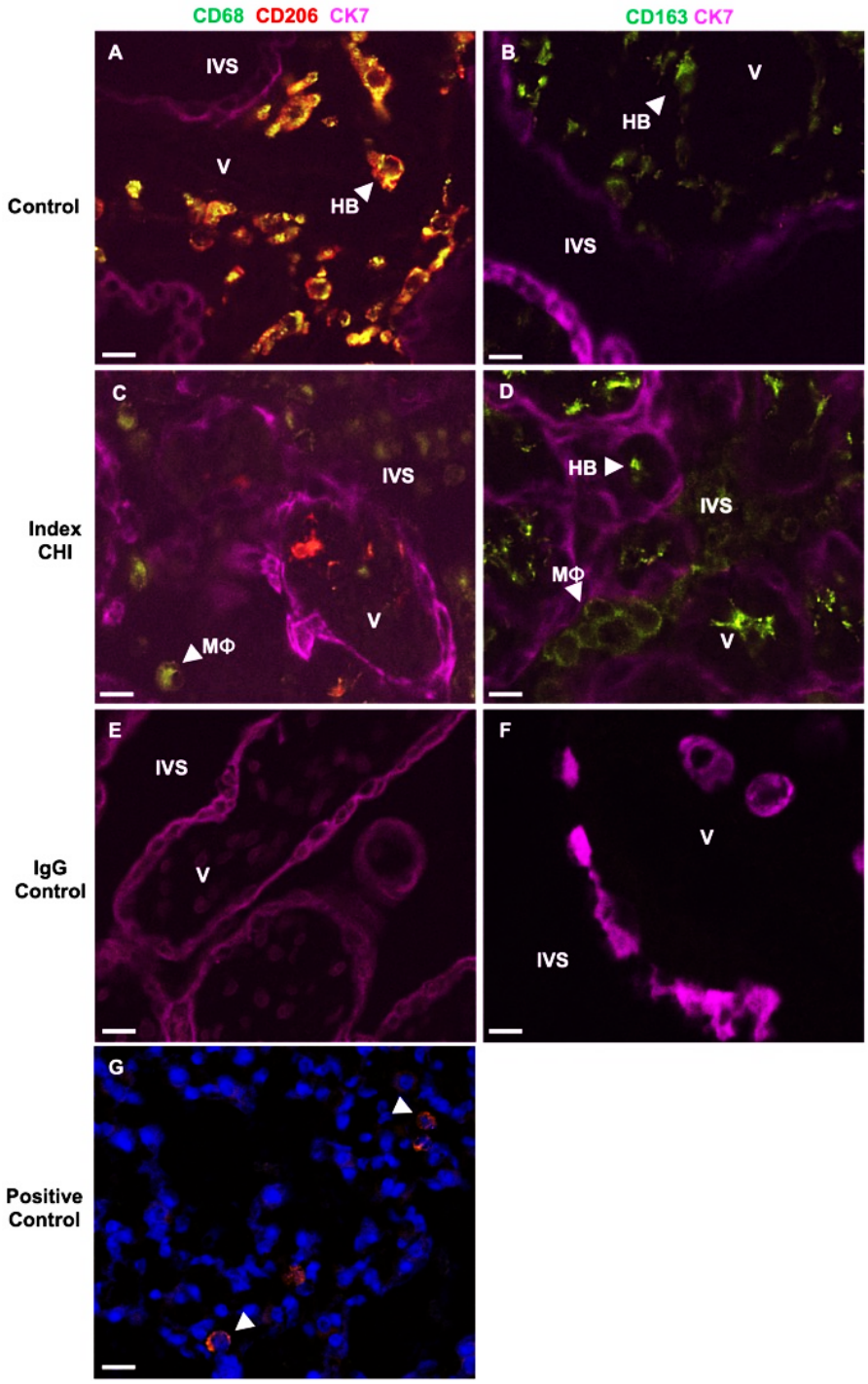

Figure 21. Representative immunofluorescence staining for M2 macrophages (MΦ) in a healthy control placenta and an index case of chronic histiocytic intervillositis (CHI). CD68 (green) was used as a pan-macrophage marker in combination with CD206 (red) as an M2 macrophage marker (A and C), and stained cells within the intervillous space (IVS) as well as Hofbauer cells (HB) within fetal villi (V). Sections were also stained with anti-CD163 (green) as a second M2 marker (B and D). Cytokeratin 7 (CK7) (pink) was used to label trophoblast and allow distinction between maternal intervillous macrophages and fetal Hofbauer cells. For isotype controls, anti-macrophage primary antibodies were substituted for mouse and/or rabbit IgG at the same working concentration (E and F), and nuclei stained with DAPI (blue). Index CHI refers to a participant's first diagnosed case of CHI by histopathological examination of the placenta. Mouse lung tissue was used as a positive control for CD206 staining (G). Arrows indicate positive cells. Scale bars = 20μm.

Discussion

To date, the diagnosis of CHI and profiling of the inflammatory infiltrate has been made by histopathological assessment, which is largely subjective or based upon manual counting and estimation, making comparison between published studies challenging. In order to improve understanding of the condition and the effect of treatments, there is a need for a standardised method of diagnosis and assessment of lesion severity to ensure reproducibility amongst publications. This approach would also provide a basis for collaboration between centres to address the need for larger studies into CHI's pathophysiology. In this cohort study, QuPath image quantification software was applied to cases of CHI with an aim to reduce subjectivity and systematically quantify immunopathological features in affected placentas.

Following QuPath analysis, placentas with CHI exhibited large variability in the quantity of $CD68^+$ macrophage infiltration, fibrin deposition and T cell infiltration between cases, suggesting varying lesion severity which was detected using computerised quantification. Within these placentas, maternal macrophages showed strong CD163 staining, suggestive of an M2-like anti-inflammatory phenotype. With the exception of $CD3^+$ T cells, the inflammatory profile of placentas from subsequent pregnancies were similar to those of controls, indicating that specialist care and therapeutic intervention following diagnosis may be associated with reduced severity of macrophage infiltration and fibrin deposition.

As expected by definition, index pregnancies diagnosed by histopathological examination demonstrated large increases in intervillous $CD68^+$ macrophage infiltration compared to healthy placentas. However, following QuPath analysis, a subset of placentas initially diagnosed as CHI did not exhibit elevated macrophage infiltration above the level of controls. As a result the 95^{th} percentile of control data was adopted as a threshold for defining CHI similar to the manner by which laboratory reference ranges are routinely established,[33] with placentas reaching this value termed QuPath-defined CHI pregnancies. Importantly, this threshold is remarkably similar to the mean value of 80 $CD68^+$ cells per field of view determined by Heller, after manual counting in placentas with CHI.[14] Notably, compared to Heller's report, our control values exhibited a larger range (0-24 reported by Heller vs 0-84 here), which may be a reflection of the larger sample size of control placentas analysed in this investigation (n=11 vs n=24). Following adoption of the criterion, three placentas were excluded from the index group. In two subsequent pregnancies, placental histopathology reports were not available as a result of tissue not being sent for examination, and the remainder detailed no presence of CHI despite QuPath analysis suggesting recurrence in two pregnancies. There are several possible explanations for these findings, the first being that pathologists are not blinded to pregnancy outcome when receiving samples for examination, which may result in bias when reporting lesion presence and severity. Alternatively, CHI may be diagnosed following observation of 'focal' lesions, rather than those diffusely spread across the whole placenta.[5] Thus, the standard method of placental sampling could miss focal lesions present outside the sampled tissue.[34] Though analysis of multiple regions of interest as utilised within this

study aimed to reduce the likelihood of foci of CHI being excluded, there is a possibility that focal lesions were not within the areas randomly chosen for analysis. Additionally, smaller focal areas may not have been reflected in overall macrophage infiltration values for each placenta after data from different sampling areas were averaged. With this in mind, future studies on larger cohorts are warranted to determine the true incidence of discrepancies between computerised quantification and histopathological diagnosis. If computerised quantification is reproducible, the threshold provided by Heller[14] and this study could be employed to aid in the diagnosis of CHI.

In index pregnancies diagnosed by pathologists, the extent of fibrin deposition did not differ significantly from controls, though following refinement of the group using QuPath thresholding fibrin deposition showed a trend towards a significant increase, likely limited by sample size. This finding is perhaps unsurprising given that increased perivillous or intervillous fibrin has been previously noted to be an accompanying feature in certain cases of CHI but is not always an essential criterion for diagnosis.[1] Moreover, fibrin deposition increases with gestational age due to turbulent blood flow within the intervillous space.[35,36] Although the application of QuPath quantification software as detailed within this study is novel to CHI research, several other groups have adopted grading systems for macrophage infiltration and fibrin deposition. In the largest study to date of pregnancies complicated by CHI, Sauvestre et al.[28] graded lesion severity in 122 cases according to the criteria of Rota et al.[11], classifying infiltrate covering 5-10% of the intervillous space as grade 1, 10-50% as grade 2, and >50% as grade 3. Within the cohort, placentas with grade 3 CHI were more likely to have concurrent fibrin deposition, suggesting an association between the two pathological features. In addition, increased intensity of fibrin deposition has been previously linked to spontaneous miscarriage and FGR in cases of CHI.[13] In contrast, here the extent of macrophage infiltration was not correlated with the proportion of tissue occupied by fibrin. Again, this is likely reflective of the fact that fibrin deposition was only elevated in specific cases. Within the context of CHI, the role of fibrin deposition has not been investigated, though more generally the formation of placental fibrin is recognised as a non-specific response to tissue damage.[37,38] In other pregnancy complications such as FGR and preeclampsia, perivillous fibrin is increased; this is hypothesised to be a result of damage to villous tissue and acts as a mechanism of villous repair to protect areas of denuded syncytiotrophoblast.[37] In its initial description in 1987, trophoblast necrosis was reported in cases of CHI,[2] which may act as a trigger for increased fibrin formation, though a relation between these two inflammatory features in the condition is yet to be studied. In massive perivillous fibrin deposition (MPFD) which is another recurrent placental lesion occasionally showing overlap with CHI, maternal auto-reactive antibodies have been suggested as a causative factor, with several affected women displaying anti-HLA or antiphospholipid antibodies.[36,39] Similarly, these antibodies have been implicated in the development of CHI, particularly anti-HLA antibodies, given that the disorder reportedly shares histopathological features common to graft rejection including C4d and fibrin deposition as well as macrophage infiltration.[6,18,40] However, an antibody mediated component to CHI is yet to be confirmed, and is explored in Chapter 5.

In several cases of CHI studied here, the cellular infiltrate was especially evident surrounding areas of fibrin deposition, an observation which has been reported previously.[29] Colocalisation of macrophages and fibrin tempts speculation as to whether there is an interaction between these features of inflammation. Fibrin is capable of modulating macrophage behaviour *in vitro*, stimulating polarisation toward an M2-like profile, which is responsible for tissue repair, phagocytosis of apoptotic cells and resolution of inflammation.[41] Here, intervillous macrophages in index cases of CHI appeared to be of an M2 phenotype, with no apparent expression of M1 markers CD80 and CD86 or M2 marker CD206, but strong positivity for CD163. This is consistent with previous evidence from an immunohistochemical study on immune cell polarisation in CHI which also suggested maternal macrophages were $CD163^+$, 'resting' within the intervillous space.[7] The anti-inflammatory phenotype of intervillous macrophages is further implied by a lack of pro-inflammatory cytokine upregulation in cases of CHI, suggesting that macrophages do not contribute toward tissue damage.[8] Currently, it is not known what attracts maternal immune cells to the placenta in CHI, though M2-like polarisation of macrophages hints at the possibility of an initial inflammatory insult stimulating their recruitment. A similar M2-dominated macrophage infiltrate into the decidua in oocyte donation pregnancies has been described, and is associated with HLA mismatches between mother and fetus.[42] As previously mentioned, anti-HLA antibodies have been implicated in CHI, with small studies noting increased overall anti-HLA antibody positivity in affected women compared to controls without CHI, including those directed towards the fetus.[16,40] In a study of two women with CHI by Benachi et al.,[40] upregulation of class I and class I HLA at the maternal-fetal interface was evident, though the significance of this remains unclear. To investigate the possible role of HLA further and determine whether mismatches may be responsible for macrophage recruitment similar to allogeneic oocyte donation pregnancy, larger scale studies are required to robustly establish the prevalence of anti-HLA antibodies in affected women.

An interesting observation made in this case series was that of decreased Hofbauer cell staining for the M2 marker CD206 in cases of CHI compared to controls. In CHI, Hofbauer cell polarisation, like intervillous macrophage polarisation, is poorly characterised likely due to the fact that inflammation appears localised to the intervillous space with a lack of villous involvement.[8] However, more generally CD206 is recognised as a mediator of antigen clearance and presentation in both macrophages and dendritic cells, and $CD206^+$ decidual macrophages have been implicated in successful implantation in rodent models.[43,44] Furthermore, the soluble form of CD206 fluctuates at differing stages of inflammation and can be utilised as a prognostic marker.[45,46] More extensive profiling of CD206 and other macrophage cell markers, e.g. via emerging technologies such as imaging mass cytometry and multiplex immunofluorescence, may therefore be worthwhile in cases of CHI to further characterise inflammation and identify potential inflammatory biomarkers.

Following a diagnosis of CHI, subsequent pregnancies are often treated with medication aimed at reducing coagulation, including aspirin, heparin, and those which modulate the immune response

e.g. steroids, hydroxychloroquine and intravenous immunoglobulin.[20,39,47] Despite the use of immunomodulatory medication, there is a distinct lack of study into their effect on the frequency and severity of CHI lesions, and current evidence is largely based upon case reports and small series.[20,39] Additionally, a systematic review in 2010 concluded that intervention (largely aspirin and heparin-based) had no significant effect on pregnancy outcome or CHI recurrence.[3] In this study, pregnancies treated at a specialist clinic for care after previous CHI were studied to determine the effect on placental pathology. Eight of eleven subsequent pregnancies were treated with a combination of aspirin, heparin, prednisolone and hydroxychloroquine, and the remainder with aspirin, heparin and hydroxychloroquine. Overall, macrophage infiltration appeared to reduce, and QuPath quantification suggested CHI had returned in only 18.2% of cases, though pathologists reported no recurrence. However, in subsequent pregnancies, macrophage infiltration was not significantly reduced compared to index cases, though this is likely to be due to the two placentas which exceeded the QuPath threshold for diagnosis of CHI. Importantly, all of these pregnancies resulted in livebirth, and there were no neonatal deaths. Reported recurrence rates of CHI vary widely between studies, though within our cohort recurrence was below the estimated range which is reported between 25-100%.[1] Although there was no untreated group with which to compare placental pathology, nevertheless, decreased macrophage infiltration in subsequent pregnancies is consistent with our previous study wherein immunomodulatory medication reduced lesion severity.[47] Anecdotal evidence from case reports has proposed a similar effect.[39] As well as a reduction in macrophage infiltration, pregnancies receiving specialist care also demonstrated significantly lower fibrin deposition than both pathologist and QuPath-defined index cases indicating that treatment may also impact this aspect of CHI. In contrast to other pathological features however, maternal T cells remained elevated above controls in placentas from subsequent pregnancies. Due to availability of tissue, immunohistochemistry for $CD3^+$ T cells could not be carried out on samples from untreated index pregnancies, therefore it was not possible to determine T cell infiltration in this group relative to controls. In spite of this limitation, a $CD3^+$ component of the maternal infiltrate in CHI has been reported by other groups, mainly comprising of equal proportions of $CD4^+$ and $CD8^+$ cells with very few regulatory T cells.[15,19,48] A significant $CD3^+$ infiltrate in subsequent pregnancies following a diagnosis of CHI implies that these pregnancies, though resulting in good outcome, still differ from controls. Additionally, it indicates that administered treatment may not have as profound an effect on T cells compared to macrophages. The mechanism of prednisolone and hydroxychloroquine's action in CHI is uninvestigated, though an explant model has shown that hydroxychloroquine treatment promoted syncytiotrophoblast regeneration and release of IL-10, a cytokine known to regulate T cell activity.[49] Here, only CD3 was used to detect the presence of T cells, which is recognised as a general T cell marker, and does not distinguish between subsets. Consequently, further investigation of these placentas using markers for $CD4^+$, $CD8^+$ and Foxp3 will likely be useful in determining how each population may be affected by treatment with immunomodulatory medication.

Strengths and Limitations

To date, this study is the first to use systematic, semi-automated image quantification software to measure and interrelate immune features in CHI. Quantification of cells and fibrin using this method arguably reduces subjectivity and bias which can arise with manual counting. Furthermore, the protocol is easily reproducible, facilitating comparison between studies. This is especially important in a rare disease such as CHI where consolidating data between groups is required to reach sufficient sample size. In our protocol, manual highlighting of villous tissue was required before commencing cell counting, however there is scope to develop tissue detection within QuPath software which would allow the process to be fully automated and expedited. Following this, the software could then be used alongside pathologist's expertise as a tool to aid in diagnosis and the assessment of lesion severity in other inflammatory placental disorders as well as CHI.

As a result of CHI's rarity, research is scarce and knowledge of its pathophysiology lacking. With this in mind, this study represents a well-characterised cohort of the rare inflammatory lesion. Immunofluorescence staining for determination of macrophage phenotype in index cases of CHI undertaken in this study can be used to inform the development of an antibody panel in future studies. For example, techniques such as imaging mass cytometry now allow for the spatial and phenotypic analysis of immune cells which will prove invaluable in CHI research.[50] Characterisation of placentas from subsequent pregnancies has also given insight into how treatment may affect placental inflammation and can be used to direct future studies on interactions between medication and immune cell populations.

Due to the high rates of preterm birth, miscarriage and stillbirth in CHI, index pregnancies were of significantly lower gestation compared to controls. It is therefore possible that this may account for the lack of differences between these groups. Tackling this limitation in future is challenging, as non-CHI pregnancies which do not reach term are likely not normal and any inflammatory features may be a result of other complications (such as chorioamnionitis[51,52]) or maternal comorbidities. The exception to this would be to collect placental tissue from gestational age-matched uncomplicated terminations of pregnancy for socioeconomic reasons, which has been undertaken previously by groups studying other aspects of placental biology.[53] Collection of placental tissue from these cases is not without its own feasibility issues, however doing so in future may prove a more valid comparison of fibrin deposition and other inflammatory features with cases of CHI. 70.8% of control placentas here were also collected following Caesarean section delivery in the absence of labour, and may exhibit reduced inflammation compared to those sampled following labour as described by Vega Sanchez et al.[54] Further expanding collection of healthy placentas to balance those from labouring and non-labouring deliveries in future will therefore help to address this confounding factor.

Though placentas from subsequent pregnancies of women with a previous diagnosis of CHI were collected for this study, these samples were not all from the same participants who donated index

placental tissue. On account of this limitation, changes in placental pathology could only be made overall for experimental groups as opposed to paired analysis of individuals. The reason for this was primarily due to the large time difference between the sampling of placental tissue from archived index cases and prospective subsequent pregnancies. Secondly, several placentas from women with a history of CHI attending the hospital for care in a subsequent pregnancy had not been sent for histopathological examination, likely due to good pregnancy outcome and a lack of awareness of CHI. Within Saint Mary's Hospital, awareness around the importance of histopathological examination of placental tissue from women diagnosed with CHI has been raised by the group and is anticipated to increase the sample size of future studies on the condition. Collection of multiple placentas from single participants is therefore anticipated to further improve conclusions made on treatment efficacy and changes in inflammatory features at the individual level.

Conclusion

This study proposes a systematic quantification method to quantify inflammatory features in placentas with CHI, which used alongside histopathological expertise may help to reduce subjectivity in diagnosis and assessment of lesion severity. Additionally, analysis of pathological features via QuPath software enables comparison between untreated and subsequent pregnancies to give insight into how treatment may affect particular immune cell populations in the condition. In CHI, maternal macrophages appear to adopt an M2 anti-inflammatory profile, though further investigation of their phenotype is required to determine their role in its pathophysiology.

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Chapter 5: Investigating Chronic Histiocytic Intervillositis as a Form of Maternal Anti-Fetal Rejection

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Author Contributions

C.A.B, I.P.C and A.E.P.H contributed toward study design and planning. C.A.B performed experiments and data collection, analysis and interpretation. L.B.F assisted with experiments and revision and preparation of the manuscript.

Abstract

Problem

Chronic histiocytic intervillositis (CHI) is a recurrent placental lesion wherein maternal macrophages infiltrate the intervillous space. Currently, its cause is unknown, though due to similarities to rejected allografts, including fibrin deposition and immune cell infiltration, CHI is hypothesised to represent maternal-fetal rejection.

Method of Study

Virtual crossmatching was applied to pregnancies from healthy control women and those with a history of CHI. Anti-HLA antibodies in maternal plasma were measured via Luminex, and percentage calculated reaction frequency (%cRF) determined. Maternal antibodies were compared to fetal HLA to identify fetal specific antibodies (FSAs). Immunohistochemical staining was used to identify C4d in placentas from untreated CHI, subsequent pregnancies and healthy controls. Anti-placental antibodies were investigated by immunofluorescence using matched maternal plasma and placenta in controls and subsequent pregnancies.

Results

There were no significant differences in the median level of placental C4d deposition between healthy control pregnancies and index pregnancies with CHI and those following diagnosis, (control 1 [IQR 0-1.4] vs index 1 [0.5-1.9] vs subsequent pregnancies 1 [0.9-2]). Anti-HLA antibodies were present in slightly more controls than cases of CHI (8/17 (47.1%) vs 6/15 (40.0%), respectively), but there was no significant difference in the level of sensitisation or %cRF. There was no difference in FSAs between controls and CHI cases (median 2 [IQR 0-3.8] vs 5 [1.0-6.0]). The percentage area of anti-placental antibody fluorescence did not differ significantly between groups (control 2 [IQR 0.5-5.1] vs previous CHI 0.5 [IQR 0.1-4.7].

Conclusions

Though cases of CHI share similarities with allograft rejection including fibrin deposition, macrophage infiltration and organ dysfunction, an antibody mediated component could not be confirmed. These data suggest that CHI may not result from antibodies directed at HLA antigens or from anti-placental antibodies, but treatment in subsequent pregnancies and lack of recurrence is likely an important confounding factor. Further investigation of antibodies outside of treated pregnancies is required to determine any possible role in the pathophysiology of CHI.

Introduction

Throughout healthy human pregnancy, the fetus is tolerated by the maternal immune system despite possessing genetically foreign paternal HLA. This ability of the semi-allogeneic fetus to evade mounting an inflammatory response has led the conceptus to be coined as the 'most successful graft'.[1] In recent years, several mechanisms in pregnancy have come to light which promote maternal-fetal tolerance, including specialised placental HLA expression and maternal immune cell adaptations.[2–5] The failure of any one of these tolerogenic processes is becoming increasingly linked to the pathogenesis of multiple inflammatory placental disorders resulting in adverse pregnancy outcomes such as preterm birth, miscarriage and stillbirth.[6–8]

A distinct example of a placental lesion with a strongly alleged immune aetiology is chronic histiocytic intervillositis (CHI), wherein maternal macrophages infiltrate the intervillous space.[9] CHI is associated with failure of the fetus to grow, and in severe cases can result in miscarriage or stillbirth.[10] Though the cause of CHI is largely unknown, previous case reports and series have suggested the possibility of an antibody-mediated component, given its increased incidence in women with autoimmune disease,[11,12] high recurrence rate,[13,14] evidence of increased anti-HLA antibodies,[15,16] and in selected cases deposition of complement split product C4d.[15,17] In addition, certain cases of CHI also exhibit accompanying fibrin deposition,[10] and anti-paternal T cells have been detected in several women with the disorder.[16] Importantly, many of these pathological features are also common to rejected allografts, and as a result CHI has been likened to maternal-fetal rejection and treated with immunosuppressive medication.[12,18–20] Despite suggestions of antibody involvement from case reports, CHI's rarity has made conducting larger scale studies into its pathophysiology difficult. Research into the condition is further complicated by the fact its diagnosis can only be made after delivery of the placenta, in most cases when the health or survival of the fetus has already been compromised.

For decades, predicting those at risk of organ rejection has been possible via laboratory crossmatching techniques which can inform clinical management and facilitate the maintenance of a foreign graft for a sustained period, usually years. The success of these methods and similarities shared between rejected allografts and placentas with CHI begs the question as to whether crossmatching may be applied to pregnancy to improve knowledge of the pathophysiology of CHI, and provide a screening tool to determine women who may be at risk. In this study, we aimed to apply virtual crossmatching techniques commonplace in transplantation biology to cases of CHI to investigate the possibility of placental rejection. In doing so, we hypothesised that affected women would exhibit increased evidence of humoral involvement including high anti-HLA and anti-placental antibodies and deposition of C4d, similar to that observed in the rejection of a transplanted organ.

Materials and Methods

Participant Recruitment and Sample Collection

For investigation of C4d deposition in untreated cases of CHI, participants were retrospectively identified by searching medical records at Saint Mary's Hospital, Manchester, UK, for a diagnosis of CHI in a previous pregnancy. Cases were then refined into those with archived placental tissue available for analysis from the Paediatric and Perinatal Histopathology Department. Placental tissue from cases of CHI and healthy controls were received as formalin-fixed paraffin embedded (FFPE) blocks.

To determine the presence of anti-HLA antibodies within maternal plasma, blood was collected prospectively both from women in their second or third trimester attending Tommy's Rainbow Clinic for care in a subsequent pregnancy following a previous diagnosis of CHI, and healthy control pregnancies on the date of delivery at Saint Mary's Hospital. Vacutainer™ EDTA samples were obtained and centrifuged at 2500rpm for 10 minutes at 4°C, and plasma supernatant retrieved and stored as aliquots at -80°C. The buffy coat layer was also retrieved at the red cell/plasma interface for maternal DNA analysis and stored in the same manner. Where participants were giving birth at Saint Mary's Hospital, placental tissue was collected and fixed in formalin before embedding in paraffin wax to produce FFPE blocks. Umbilical cord tissue was also sampled, rinsed and frozen at -80°C for later use.

Informed and written consent was obtained from all study participants. For archived tissue from Paediatric Histopathology, ethical approval was granted by NRES Committee London-City & East (REC ref: 14/LO/1352). For samples gathered before July 2018, approval was granted by NRES Committee Northwest-Greater Manchester West (REC ref: 14/NW/1149). Between February and July 2018, the NRES Committee South East Coast-Surrey Research Ethics Committee approved the study (REC ref: 16/LO/1666), and samples collected after this period were obtained under the Tommy's Project Ethics (REC ref: 15/NW/0829).

C4d Immunohistochemistry and Grading

Staining for C4d in healthy control placentas and those from index cases of CHI and subsequent pregnancies was carried out by the Department of Adult Histopathology, Manchester University NHS Foundation Trust. 5μm sections of FFPE tissue were cut from the point of cord insertion and edge of the placenta, mounted onto SuperFrost slides (ThermoFisher Scientific) and dried overnight at 38°C before long-term storage at room temperature. A tissue biopsy section from a rejected kidney with confirmed antibody-mediated rejection was provided by Manchester Royal Infirmary Transplantation Laboratory, both for use as a positive control and to investigate similarities with healthy control placentas and those with CHI. Slides were deparaffinised in EZ Prep Volume Adjust (Ventana Co., Arizona, USA) according to manufacturer's instructions and washed in TRIS-based Reaction Buffer (pH 7.6). Antigen retrieval was achieved using heat and TRIS-EDTA-boric acid (pH 8.4, Ventana Co.) for 60 minutes. Ultraviolet inhibitor blocking solution was applied for 4 minutes before a further 30-minute incubation at room temperature with rabbit

polyclonal anti-human C4d antibody (Cell Marque, Sigma Aldrich, Missouri, USA, 1:75 dilution). Slides were then incubated in horseradish peroxidase-linked secondary antibody for 8 minutes before an 8-minute incubation in diaminobenzidine chromogen. To amplify positive staining, a copper enhancer was applied for 4 minutes, before a 12-minute counterstain in Haematoxylin II and 4 minutes in bluing reagent. Finally, slides were dehydrated by sequential washes in solutions of 70% (2x3 minutes), 95% (2x3 minutes) and 100% industrial methylated spirit (ThermoFisher) (3x3 minutes) before 1x2 minute and 2x10 minute immersions in Histoclear (Thermo Fisher). After staining, placental slides were mounted with coverslips and left to dry before scanning at the University of Manchester Bioimaging Facility using brightfield microscopy and a 3D Histech Panoramic 250 Flash Slide Scanner.

Scanned slides were opened using QuPath computerised image analysis software [21] and a 1mm^2 grid superimposed upon the scanned slide image. A random number generator was then used to select eight regions for blinded semi-quantitative analysis of staining intensity. Intervillous C4d positivity was graded on a scale of 0 to 3; 0 = 0% to 5% of villi affected; 1 = 5% to 25%; 2 = 25% to 75%; and 3 ≥ 75%.[17] C4d scores from the point of cord insertion and edge placental regions were finally averaged to provide an overall score for each placenta.

Anti-HLA Antibody Screening of Maternal Plasma

For detection of class I and II anti-HLA antibodies, maternal EDTA plasma samples were processed by the Transplantation Laboratory at Manchester Royal Infirmary, Manchester. A mixed Luminex assay was used to initially determine any presence of anti-HLA antibodies, consisting of LABScreen Mixed Beads (One Lambda Inc, Thermo Fisher) coated in several HLAs and each possessing a fluorochrome specific to surface antigens. Maternal plasma was incubated with the mixed beads at 22°C for 15 minutes before washing in LABScreen wash buffer according to manufacturer's protocol. R-phycoerythrin (PE)-conjugated goat anti-human IgG and PE-conjugated donkey anti-human IgM secondary antibodies were then added for 5 minutes at 22°C to allow detection of bound antibody. After a final wash in wash buffer, beads were resuspended in buffer before transfer to a 96-well PCR plate for analysis using the Luminex LabScan3D. The Luminex analysis detects antibodies using a dual laser system which firstly identifies the antibody specificity by the unique combination of dyes of individual beads coated with groups of antigen and secondly the presence of antibody in patient plasma binding to beads, giving a measurement of mean fluorescence intensity (MFI). Its readout is input into HLA Fusion One Lambda Software, where results are checked with established criteria from the Manchester Royal Infirmary Transplantation Laboratory. At this stage, false negatives and positives were detected.

As the mixed bead assay gives information only on the presence of antibodies in the plasma sample and not antibody specificity, samples testing positive were then retested in the same manner but with mixed beads replaced with specific LABScreen beads possessing only a single antigen with a unique internal dye combination. Thereby, facilitating the identification of HLA antibodies and their specificity.

Maternal and Fetal HLA Genotyping

In the case of a positive anti-HLA antibody screen, maternal and fetal DNA (or maternal DNA alone where fetal tissue was not available) was extracted for determination of HLA genotype. Maternal and fetal DNA was extracted from thawed EDTA buffy coat and snap frozen umbilical cord samples, respectively. Samples were processed using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to kit instructions.

Extracted DNA samples were analysed at the Manchester Royal Infirmary Transplantation Laboratory to determine HLA genotype via the Luminex LABType™ Sequence Specific Oligonucleotide (SSO) HLA Typing protocol. This protocol uses SSOs bound to fluorescently tagged microspheres to identify HLA alleles encoded by DNA in the sample. Results were reviewed by a State Registered Clinical Scientist (Laura B Ford) to confirm HLA genotype and establish any false positive results from the anti-HLA antibody screen. The number of fetal-specific anti-HLA antibodies (FSAs) and their MFI was then noted in positive cases where fetal DNA was available for analysis.

Percentage Calculated Reaction Frequency

Using maternal anti-HLA antibody specificities and HLA genotype, percentage calculated Reaction Frequency (%cRF) was established for each participant testing positive via input of the results of Luminex screen into the NHS Blood and Transplant Kidney %cRF tool.[22] %cRF reflects the percentage of donors who would be anticipated to have an unsuitable HLA profile in the context of renal transplantation. %cRF is based on a panel of lymphocytes from 100 blood donors to represent the HLA type of the general population of the United Kingdom. Organ recipients reacting with 0 out of 100 lymphocyte samples in the panel are assigned a %cRF of 0% and are classed as unsensitised and 100% of donors would be considered suitable, whereas those with a score of >80% are classed as highly sensitised and prove more challenging to find a suitable donor, as >80% of donors are considered unsuitable for the patient.

Prediction of Crossmatch Results

In participants with FSAs, results of T and B cell flow cytometry crossmatch (FXCM) and complement-dependent cytotoxicity (CDC) crossmatch were predicted using a formula developed by the Manchester Royal Infirmary Transplantation Laboratory.[23] HLA specificities and their respective FSA MFI values were inputted to give an estimation of positive or negative result. Where results were in between the in-house cut-off values for a positive or negative result, these were described as equivocal, and could not be reliably predicted as positive or negative.

Immunofluorescence for Maternal Anti-Placental Antibodies

To determine whether antibodies directed towards the placenta were present in subsequent pregnancies of women with a history of CHI, placental tissue was incubated with autologous maternal plasma in a method adapted from that used in the diagnosis of autoimmune encephalitis.[24] Approximately 3mm^3 snap frozen placental tissue was fixed in 4%

paraformaldehyde for two hours at room temperature and washed in PBS overnight at 4°C before embedding in OCT (ThermoFisher Scientific) and freezing at -20°C. Once frozen, three 5µm sections from each sample were cut with a cryotome. Villous tissue samples were then incubated with blocking buffer (5% (v/v) bovine serum albumin (BSA) in PBS) for 30 minutes at room temperature. Autologous maternal EDTA plasma samples were simultaneously heated for 30 minutes at 56°C to reduce complement activity, diluted 1 in 10 in BSA blocking buffer and incubated with corresponding placental tissue overnight at 4°C. Plasma was then washed from tissue in PBS via four 30-minute washes. Goat polyclonal anti-human IgG FITC-conjugated secondary antibody (Abcam, 1µg/ml in PBS) was then applied for one hour at room temperature for detection of any bound maternal antibody. Washing steps were repeated as previous and tissue mounted with ProLong Diamond Antifade Mountant (Life Technologies Ltd, California, USA) and coverslips. A negative control sample was included and treated identically with the omission of maternal plasma.

Quantification of Immunofluorescence

Slides were scanned by the University of Manchester Bioimaging Facility using brightfield microscopy and a 3D Histech Panoramic 250 Flash Slide Scanner. Scanned images were opened and analysed in QuPath software (v3.0).[21] Inbuilt pixel classifiers were run on all slides for DAPI (blue) and FITC (green) channels to give a measure of tissue area on the slide. Another classifier was then run on the FITC channel only to determine the area of positive staining. The minimum threshold for positive FITC intensity was determined using the negative control slide to minimise the detection of background staining. Positive tissue area was expressed as a percentage of total tissue area for each slide. Values were normalised to the negative control value for each staining run, given that the amount of detected area in this sample could not be adjusted to completely zero.

Statistical Analysis

All statistical analysis was undertaken using GraphPad Prism v9 (GraphPad Software, USA). The Shapiro-Wilk test was run initially to establish whether variables had a normal distribution. Continuous demographic data was analysed via ordinary one-way ANOVA with Dunn's multiple comparisons test or Kruskal-Wallis test for normally distributed and non-normally distributed data, respectively. Grading of C4d staining was analysed via Kruskal-Wallis test. Categorical demographic data and proportions of antibody-positive participants were analysed using Fisher's Exact test. For %cRF values and positive fluorescent tissue area, the Mann-Whitney test was run to determine statistical differences. Statistical significance was set at $p<0.05$ for each test.

Results

Participant Demographic Characteristics

Seventeen index cases of CHI had available placental tissue as FFPE embedded blocks, with eight healthy controls provided by the Department of Paediatric Histopathology. A further nineteen

healthy controls and sixteen women in subsequent pregnancies after CHI were recruited to the study. For two index cases and one subsequent pregnancy, demographic information and pregnancy outcomes could not be obtained as records were unavailable or participants gave birth at another centre. Despite this, these pregnancies were included in the study due to availability of placental tissue and the rarity of CHI. Demographic characteristics of remaining study participants and pregnancy outcomes are shown in Table 8. Gravidity and parity were significantly higher in index pregnancies compared to controls (p=0.0096 and p=0.012, respectively). In women returning for care in a subsequent pregnancy following diagnosis, parity was significantly lower compared to index cases of CHI (p=0.0053). By definition, all index cases had a diagnosis of CHI listed on their accompanying placental histopathology report. Placental tissue was available in ten subsequent pregnancies, though two placentas had not been sent for histopathological examination. There was no histopathological diagnosis of recurrent CHI in any subsequent pregnancies.

Table 8. Participant demographic characteristics of healthy control pregnancies, index pregnancies with chronic histiocytic intervillositis (CHI) and subsequent pregnancies of women with a previous diagnosis of CHI.

	Control	Index CHI	Subsequent pregnancy
N	27	15	15
Maternal age (years)	32.9 (±4.7)	29.5 (±6.1)	30.6 (±6.1)
Maternal BMI	25.9 (±4.5)	27.9 (±4.3)	26.5 (±5.2)
Unknown	0	5	0
Ethnicity			
Asian	3 (11.1%)	3 (21.4%)	4 (26.7%)
Black African	4 (14,8%)	1 (7.1%)	0
Eastern European	1 (3.7%)	0	1 (6.7%)
White British	18 (66.7%)	10 (71.4%)	10 (66.7%)
White Irish	1 (3.7%)	0	0
Unknown	0	1	0
Gravidity	3 (1-11)	4 (2-10)	3 (2-8)
Unknown	0	0	1
Parity	2 (0-8)	3 (1-7)	1 (0-3)
Maternal autoimmune disease	0	2 (13.3%)	1 (6.7%)
Pregnancy outcomes			
Livebirth	27 (100%)	2 (12.5%)	14 (93.3%)
Miscarriage	0	1 (6.3%)	1 (6.7%)
TOP	0	1 (6.3%)	0
Stillbirth	0	12 (75.0%)	0
Oocyte donor pregnancy	0	0	1 (6.7%)
Caesarean section (>24 weeks)	20 (74.1%)	2 (14.3%)	8 (57.1%)
Gestation at delivery (weeks)	39 (35-41)	33.5 (23-40)	37 (17-39)
Birthweight (g)	3442 (±417.6)	1578 (±1040)	2969 (±808.5)
Unknown	0	1	0
IBC	63.6 (11-95.2)	4.4 (0-45.3)	33.1 (1.1-100)
Unknown	0	1	2
Fetal sex (*n* males)	12 (44.4%)	7 (50.0%)	11 (78.6%)
Unknown	0	1	1
CHI in placenta	N/A	15 (100%)	0 (100%)*
No histopathology report	N/A	0	2

Index pregnancy refers to a participant's first pregnancy to be diagnosed with CHI by histopathological examination of the placenta following poor pregnancy outcome. Normally and non-normally-distributed variables are expressed as median (range) and mean (±standard deviation) respectively. Categorical variables are shown as *N* (percentage). Miscarriage and stillbirth were defined as fetal death below or above 24 weeks gestation, respectively. BMI – body mass index; IBC – individualised birthweight centile; TOP – termination of pregnancy. *Matched placental tissue was available in ten of fifteen subsequent pregnancies.

C4d Deposition in Index Cases of CHI

FFPE tissue was available for analysis in pregnancies from twenty-five healthy controls, seventeen index cases of CHI and eleven subsequent pregnancies. Immunohistochemistry for C4d deposition is shown in Figure 22. In healthy control placentas (Figure 22A), those from index cases of CHI (Figure 22B) and subsequent pregnancies (Figure 22C), positive C4d staining was apparent to a degree along the apical surface of the syncytiotrophoblast, similar to a rejected kidney (Figure 22D). Semi-quantitative grading of C4d deposition revealed no significant difference between placentas from healthy controls (1 [IQR 0-1.4]), index cases of CHI (1 [IQR 0.5-1.9]) and subsequent pregnancies (1 [IQR 0.9-2]) (Figure 22E).

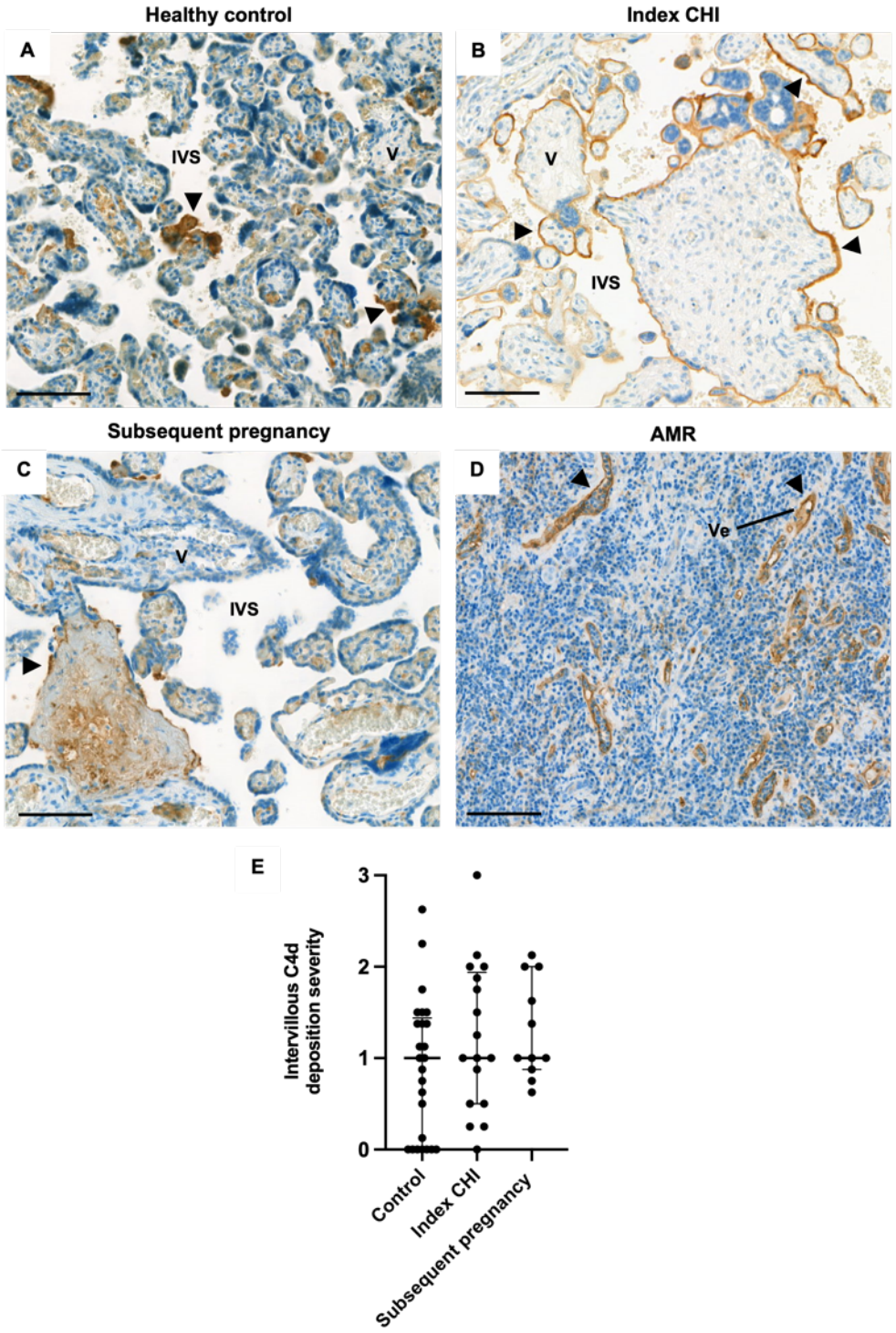

Figure 22. Deposition of complement split product C4d in placental tissue and a biopsy of a kidney graft with confirmed antibody mediated rejection (AMR). A) In placentas from healthy control pregnancies, a degree of C4d is evident in along the syncytiotrophoblast layer (shown with arrows). B) Certain index cases of chronic histiocytic intervillositis (CHI) exhibit strongly positive C4d staining along the apical surface of syncytiotrophoblast covering fetal villi (V) in contact with the maternal intervillous space (IVS). C) Subsequent pregnancies following diagnosis also show some staining in villi appearing to be surrounded with fibrin. D) C4d staining in tissue from a kidney with AMR, wherein complement is similarly present surrounding vessels (Ve). Scale bars = 100μm. E) Semi-quantitative grading of C4d staining. Bars represent median and error bars interquartile range. Index pregnancy refers to a participant's first pregnancy to be diagnosed with CHI by histopathological examination following poor pregnancy outcome.

Maternal Anti-HLA Antibody Screening

Seventeen controls and fifteen participants with previous CHI had plasma available for analysis by Luminex for the presence of anti-HLA antibodies. A slightly higher proportion of healthy control participants tested positive for anti-HLA antibodies (Class I and II) compared to those with a history of CHI (8/17 (47.1%) vs 6/15 (40.0%), respectively) but this was not statistically significant (Figure 23A).

There was no significant difference in the median level of sensitisation between antibody positive healthy controls and women with a diagnosis of CHI (99.0 [interquartile range (IQR) 36.3-100] vs 83.5 [IQR 51.0-99.3], respectively) (Figure 23B). Five of eight (62.5%) anti-HLA antibody positive controls were classed as highly sensitised with a %cRF >80%, compared to four out of six (66.7%) participants with previous CHI.

Amongst anti-HLA positive participants, a greater proportion of those with a history of CHI displayed Class I positivity compared to controls (5/6 (83.3%) vs 6/8 (75.0%), respectively) (Figure 23C). Conversely, fewer participants with CHI tested positive for Class II anti-HLA antibodies (4/6 with previous CHI (66.7%) vs 7/8 controls (87.5%)) (Figure 23D). It should be noted that these variations in proportions were not statistically significant.

Six antibody positive control participants and three participants with previous CHI had matched placental tissue available for analysis. Amongst all those testing positive for anti-HLA antibodies, %cRF was not related the severity of intervillous C4d deposition (Figure 23E).

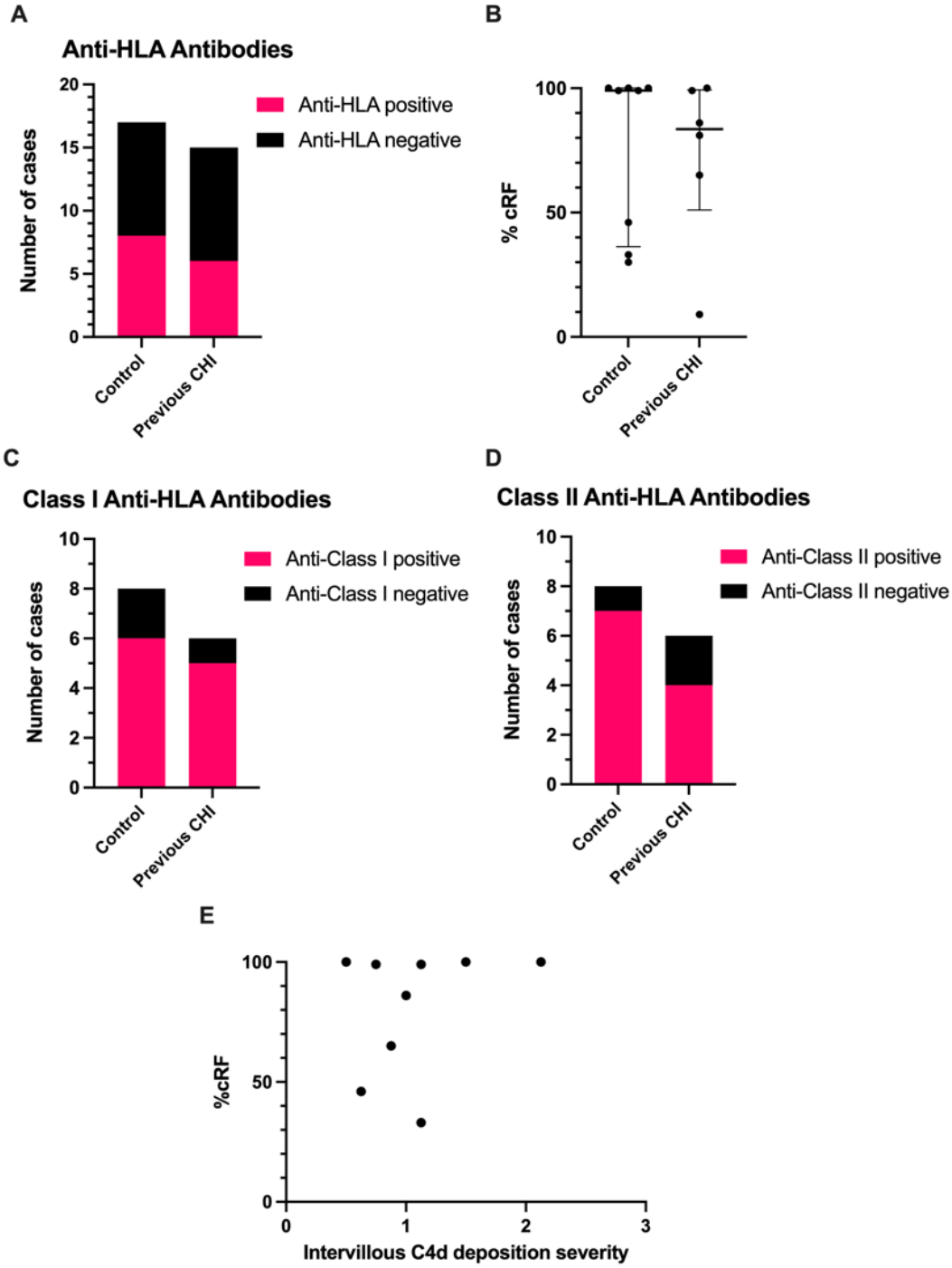

Figure 23. Anti-HLA antibody positivity in healthy control pregnancies and subsequent pregnancies of women with a previous diagnosis of chronic histiocytic intervillositis (CHI). A) Frequency of anti-HLA antibody positivity determined via Luminex screening. B) Percentage calculated reaction frequency (%cRF) of antibody-positive study participants. %cRF reflects the percentage of donors an individual would be likely to reject based on the general surrounding population. A score of 0% is classed as 'unsensitised' and easy to match to a suitable transplant, whilst a score of >80% is defined as 'highly sensitised' and proves more challenging to find a suitable donor. Bars represent median and error bars interquartile range. C) Class I Anti-HLA (HLA-A, B and C) antibody positivity. D) Class II Anti-HLA (HLA-DP, DQ, DR) antibody positivity. E) Plot comparing %cRF values of antibody-positive participants and placental intervillous C4d staining where matched tissue was available for analysis, showing no relationship between the variables.

Screening for Fetal-Specific Antibodies

Umbilical cord tissue matched to maternal plasma was available for HLA genotype analysis in all eight antibody-positive healthy controls and three of six women with a diagnosis of CHI in a previous pregnancy. Fetal DNA was unavailable in the remainder of pregnancies from women with previous CHI as two participants had delivered at another centre, and one placenta had not been sent for research.

FSAs were evident in five of eight (62.5%) anti-HLA antibody positive control pregnancies compared to all three cases of previous CHI with available fetal DNA (100%) (Figure 24). There was no statistically significant difference in the number of FSAs in healthy controls compared to cases of previous CHI (2 [IQR 0-3.8] vs 5 [1.0-6.0], respectively) (Figure 24A). Furthermore, in participants diagnosed with CHI there was no difference in the MFI value compared to those found in control pregnancies (23884 [IQR 4723-28628] vs 17881 [IQR 8345-26500], respectively) (Figure 24B). FSA specificities are listed in Supplementary Table 1.

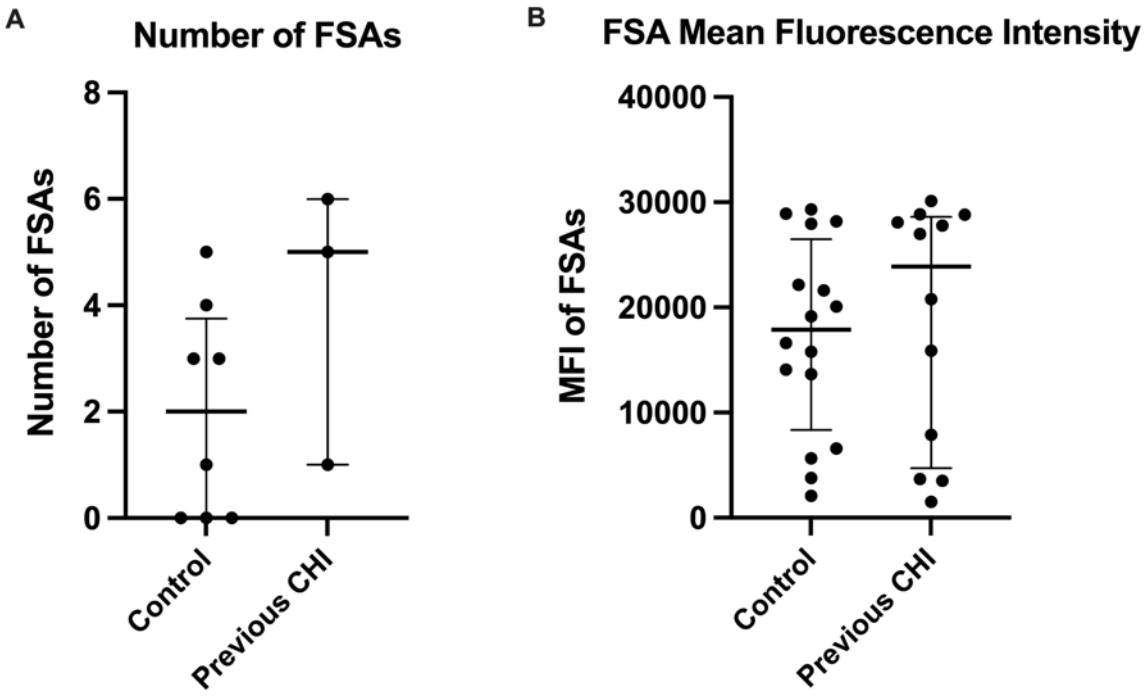

Figure 24. Fetal-specific anti-HLA antibodies (FSAs) in healthy control pregnancies and subsequent pregnancies of women with a previous diagnosis of chronic histiocytic intervillositis (CHI). A) Number of FSAs was determined via Luminex screening of maternal plasma and HLA genotyping of the fetus to identify antibodies towards paternally-inherited HLA. B) Mean fluorescence intensity (MFI) of FSAs obtained via Luminex screening of maternal plasma. Bars represent median and error bars interquartile range.

Using the MFI values of FSAs, the results of FXCM and CDC crossmatch were predicted, as shown in Table 9. In one case of previous CHI (33.3%), the B cell FXCM result was equivocal.

Between control participants and those with a history of CHI, there were no significant differences in predicted T or B cell positivity for either FXCM or CDC crossmatch.

Table 9. Crossmatch prediction results of participants with fetal specific antibodies in healthy control pregnancies and those with a previous diagnosis of chronic histiocytic intervillositis (CHI).

		FCXM		CDC	
	N	**Positive T cell**	**Positive B cell**	**Positive T cell**	**Positive B cell**
Control	5	4 (80.0%)	5 (100%)	4 (80.0%)	4 (80.0%)
Previous CHI	3	2 (66.7%)	2 (66.7%)	2 (66.7%)	2 (66.7%)

CDC – complement-dependent cytotoxicity; FXCM – flow cytometry crossmatch. Results predicted via the formula composed by Said et al.[23] For one participant with a history of CHI, the B cell FXCM result was equivocal, meaning neither a reliable positive or negative result could be established.

Immunofluorescence for Maternal Anti-Placental Antibodies

Matched frozen placental tissue and autologous maternal plasma was available for immunofluorescence analysis of placental antibody binding in seventeen healthy controls and ten subsequent pregnancies following a diagnosis of CHI (Figure 25). In the negative control sample stained without maternal plasma, a small degree of staining was present around the apical side of the syncytiotrophoblast layer, which may represent residual autofluorescence (Figure 25C). However, this positive staining appeared to cover less area compared to both healthy control and previous CHI placentas (Figure 25A and B). Upon quantification of staining, fluorescent area varied widely in both groups; there was no statistically significant difference in percentage positive tissue area between control placentas and placentas from women with a diagnosis of CHI (2 [IQR 0.5-5.1] vs 0.5 [IQR 0.1-4.7], respectively) (Figure 25D).

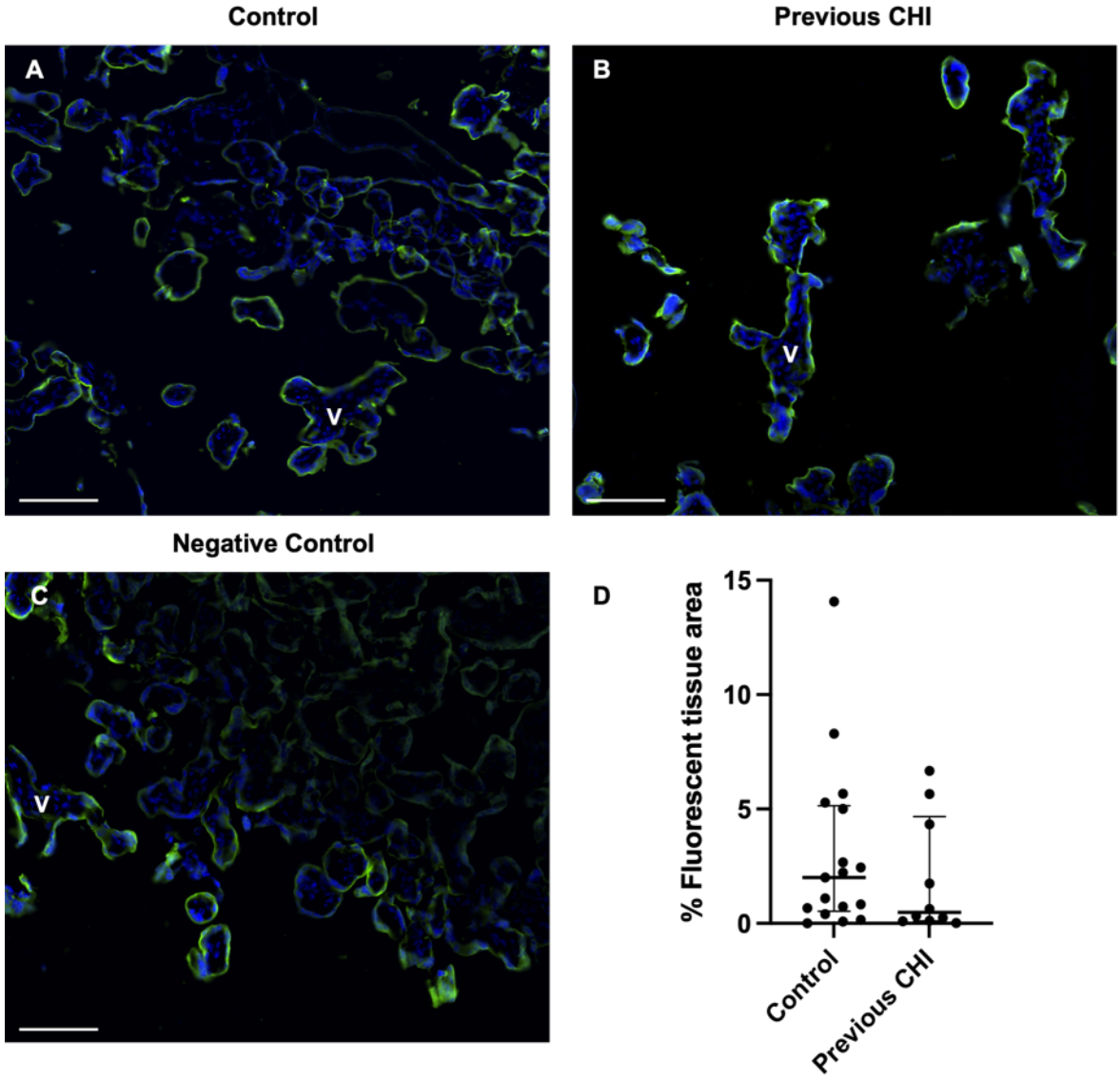

Figure 25. Immunofluorescence staining of intact placental tissue for maternal anti-placental antibodies in healthy control pregnancies and those with a previous diagnosis of chronic histiocytic intervillositis (CHI). In both placentas from A) healthy control women and B) women with a previous diagnosis of CHI, IgG from maternal plasma (green) is evident around the apical layer of the syncytiotrophoblast surrounding fetal villi (V). A degree of background staining is evident in the negative control (C), incubated without maternal plasma. Nuclei stained with DAPI (blue), scale bars = 100μm. D) Quantification of positive green staining area in placentas from control pregnancies and those following a diagnosis of CHI by QuPath software. Values are normalised to those of the negative control slide. Bars represent median and error bars interquartile range.

Discussion

Currently, the pathophysiology of CHI is poorly understood, though pathological similarities shared with rejected allografts, its high recurrence rate and reported association with maternal autoimmune disease have resulted in the hypothesis that it is a disorder of maternal anti-fetal rejection.[10–13,17,20] Increased maternal anti-HLA antibodies have also been described in some case reports.[15,16] Despite this evidence, extensive transplant-style crossmatching is yet to be applied to cases of CHI in a case-control study design. Here, virtual crossmatching and tissue-based assays were used to determine whether CHI resembles organ rejection with respect to C4d deposition and the presence of antibodies including those directed towards fetal HLA or other placental antigens.

Previously, C4d deposition has been described in several published reports of placentas with CHI,[15,17,25] reminiscent of the appearance of a rejected kidney biopsy wherein complement deposition is evident surrounding vessels.[26,27] However, within this study there were no significant increases in the magnitude of C4d deposition in index cases of CHI or subsequent pregnancies compared to healthy controls. Here, C4d staining was graded by a single investigator only. In future, grading from multiple independent reviewers should be undertaken to confirm the results and determine the level of scoring agreement. Bendon et al.[17] also noted that C4d deposition in placentas with CHI was localised to areas of macrophage infiltration. As inflammatory placental lesions can often be focal in presentation, it may be justified to explore whether a similar relationship between C4d deposition and the presence of macrophages was apparent within this cohort, as this was not specifically investigated here. In organ transplantation, positive C4d staining is considered indicative of antibody interaction with graft tissue, and is included in the Banff Criteria for diagnosing allograft rejection.[26] Consistent with a lack of intervillous C4d deposition, the proportion of participants with anti-HLA antibodies did not differ significantly between controls and women with previous CHI, at 47.1% versus 40.0%, respectively. Additionally, there were no differences in the proportion of positive predicted crossmatch results or %cRF value. With regard to anti-HLA antibodies, pregnancy is considered as both the most common and strongest sensitising event, and anti-HLA positivity is reported in 29-49% of parous women.[28–31] The mechanism of HLA sensitisation in healthy pregnancy appears to be a result of fetal cells, cell free fetal DNA and microvesicles crossing the placenta into the maternal circulation and providing a source of fetal antigen.[32–34] Recent evidence from murine models also suggests that circulating maternal lymphocytes phagocytose fetal antigen.[35] Sensitisation by the placenta itself is thought to be minimal, due to the lack of HLA expression by cytotrophoblast and syncytiotrophoblast at the maternal-fetal interface, and invasive trophoblast populations also downregulate the more polymorphic HLA-A, -B and class II molecules, instead expressing HLA-C and regulatory HLAs including -E, -F and -G which modulate the activity of maternal immune cells.[2,36,37] However, in previous studies of CHI maternal anti-HLA antibody positivity has been reported to be as high as 75%, though a total of only six women have been tested in published studies undertaken by two separate groups.[15,16] In contrast, evidence from this study instead suggests that anti-HLA antibody sensitisation in women with previous pregnancies complicated by the condition is comparable to

healthy controls. As a result of CHI's rarity, sample size within this study was limited, and gravidity and parity differed significantly between index and control pregnancies. With this in mind, these results should be interpreted with caution until anti-HLA antibody screening is replicated in larger scale studies with more closely matched control samples. In order to confirm or refute involvement of anti-HLA antibodies, there is a requirement for centres both to collaborate to increase sample size and standardise blood and tissue sampling timepoints to allow comparison between studies.

Due to CHI's asymptomatic nature, diagnosis can currently only be made retrospectively following histopathological analysis of the placenta, in most cases following poor pregnancy outcome, e.g. fetal growth restriction, miscarriage or stillbirth. Therefore, the collection of plasma in this study was limited to subsequent pregnancies following diagnosis, all of which were receiving a form of thromboprophylactic and immunomodulatory treatment that may have prevented CHI recurrence and impacted antibody screening results. Following solid organ transplantation and in autoimmune disease, both hydroxychloroquine and prednisolone are used routinely as maintenance immunosuppression to prolong graft survival or suppress disease. It is therefore possible that any anti-HLA antibodies which may have been present in previous pregnancies were reduced as a result of medication. This limitation extends beyond this study and into research on CHI as a whole, as plasma is rarely collected at the time of index pregnancies and women are increasingly beginning to take immunosuppressive medications even before conception, making screening for maternal antibodies in this period and pregnancy before treatment difficult. Furthermore, ethical issues surrounding randomised control trials using placebo or less effective medications in women with multiple previous poor pregnancy outcomes means that obtaining plasma from untreated pregnant women is unlikely. To address the confounding factor of medication, it may be worthwhile in future to obtain ethical permission to screen anti-HLA antibodies outside of pregnancy and before pre-conception treatment has commenced, perhaps soon after women receive a diagnosis of CHI at routine postnatal visits after a perinatal death or pregnancy complication. Preliminary data from two women with CHI has suggested that fetal-specific anti-HLA antibodies are stable for up to 17 months, further justifying the use of sampling following the first affected pregnancy.[15]

In the transplant setting, results of recipient anti-HLA antibody screening must be compared to donor genotype to determine the clinical relevance of any positive antibody specificities. Similarly, in this study we were able to compare maternal anti-HLA antibody repertoire to corresponding fetal genotype in the majority of participants to identify FSAs and predict crossmatch results. Between healthy pregnancies and those in women with a history of CHI, there were no significant differences in the number of FSAs, their MFI or the predicted result of FXCM and CDC crossmatch. Again, this may be a reflection of immunomodulatory therapy, though sample size was also limited for these variables as only three and five cases of CHI and controls had antibodies toward their fetus, respectively. In a study by Benachi et al.[15] of two anti-HLA antibody positive women with pregnancies complicated by CHI, a C1q binding assay was undertaken to determine whether the detected antibodies were able to interact with complement. In both women, complement-fixing

antibodies directed toward the fetus were present, suggesting possible relevance in CHI pathophysiology as C4d and C5b-9 complement split products were also observed in the placentas. In healthy pregnancy, trophoblast HLA expression is restricted, so the significance of anti-HLA antibodies in maternal blood is unclear. However, trophoblast necrosis has been noted in CHI since its initial description by Labarrere and Mullen in 1987.[9] It may therefore be possible that exposure of the maternal immune system toward underlying fetal HLA within villi, e.g. that expressed by fetal Hofbauer cells,[38] occurs in CHI allowing antibody interaction, though this has not yet been investigated. Alternatively, the HLA profile of trophoblast may be altered in CHI, as suggested by the aforementioned study, where upregulation of class I HLA and HLA-DR expression was observed in the syncytiotrophoblast of both placentas.[15] Investigating alterations in placental HLA expression and the ability of any anti-HLA antibodies to bind complement in future could therefore be useful in confirming HLA involvement in CHI.

As well as anti-HLA antibodies, CHI has been associated with maternal autoimmune disease and fetal and neonatal alloimmune thrombocytopenia (FNAIT).[11,12,39] The link between autoantibodies and development of CHI remains unclear, and rates of autoimmune disease in women with CHI vary widely, from 12% (as reported by our previous study in Chapter 3) to 58%.[11,12,18] Here, we incubated maternal plasma with matched placental tissue to determine the possibility of an anti-placental antibody; there were no differences between controls and women with CHI. As control participants were not taking any medication during their pregnancy, this result again may have arisen from the use of hydroxychloroquine and/or prednisolone in women with previous CHI. In studies of women with antiphospholipid syndrome, hydroxychloroquine is effective at reducing titres of autoantibodies which *in vitro* bind to trophoblast and initiate release of pro-inflammatory cytokines.[40,41] It is therefore conceivable that maternal antibodies may have been present in index pregnancies affected by CHI but without accompanying C4d deposition, similar to a phenomenon termed as C4d-negative rejection, recently acknowledged in AMR wherein complement is not detected in graft biopsies.[27] In investigating this possibility, applying the same experimental method in index pregnancies is limited by the fact that maternal plasma taken during pregnancy from these cases has often not been stored as diagnosis is made only after delivery. An alternative method would be to directly apply anti-human IgG or IgM to stored placental tissue from these cases to highlight any bound anti-placental antibody, similar to that used in the diagnosis of lupus via skin biopsies, known as the lupus band test.[42] Obtaining plasma from women with antiphospholipid syndrome would also likely serve as a suitable positive control to ensure antibodies are detected via the assay.

Strengths and Limitations

To date, this study represents the largest attempt to apply crossmatching techniques in samples from women with a previous diagnosis of CHI. In collaboration with the Manchester Royal Infirmary Transplantation Laboratory, extensive anti-HLA antibody screening was undertaken to a high standard as used in the clinical setting to determine donor-recipient suitability pre-transplant. In the

majority of cases, matched placental tissue and fetal DNA was also available, which allowed any fetal or placental-directed antibodies to be identified and for the prediction of crossmatch results.

As CHI has previously been hypothesised as an inappropriate immune response toward fetal HLA inherited from the father, both previous studies to investigate anti-HLA antibodies had paternal DNA available for genotyping.[15,16] In this study, analysis was restricted to fetal DNA due to the fact that collection of paternal plasma samples was not covered by ethical agreements. This meant that only anti-HLA antibodies directed toward the fetus of the current pregnancy could be identified, as opposed to other specificities which may have been present in past pregnancies with CHI. In future, expansion of study ethical agreements could be expanded to include collection and analysis of paternal DNA. This will not only allow for identification of more general anti-paternal antibodies, but also of any effect a change in partner may have on CHI recurrence and severity, as anecdotal evidence from affected women suggests that CHI persists in subsequent pregnancies despite different paternity.[43]

In a recent study which identified CHI in 40.7% of placentas from women immunised against fetal platelet antigens, antibodies in plasma were tested at four separate timepoints from 16-20 weeks' gestation.[39] Antibody-positive women were defined as those with a positive screen in at least one of four samples, as antibodies may be transient. Here, plasma from only one time point during gestation was available for analysis, which could have affected antibody titre and %cRF values. Further investigations using longitudinal sampling of maternal plasma are therefore warranted to confirm antibody screening results, and will also give insight into how anti-HLA antibodies may fluctuate throughout gestation.

In preparation for solid organ transplantation, recipients are routinely screened for antibodies against classical HLA. With regard to pregnancy, several other HLA molecules including HLA-E, -F and -G are relevant in modulating the immune response and promoting maternal tolerance toward the fetus.[44–46] Due to the fact that this study was carried out using Luminex antibody screening at Manchester Royal Infirmary Transplantation Laboratory, only antibodies toward classical HLAs relevant to transplantation could be analysed. As maternal tolerance appears to fail in CHI, screening for anti-HLA-E, -F and -G antibodies would be a logical next step in identifying any possible role of an antibody-mediated component to its pathophysiology.

Conclusion

Though CHI has been likened to maternal rejection of the placenta, an antibody-mediated component to the disorder could not be confirmed via C4d immunohistochemistry or analysis of maternal plasma from subsequent pregnancies. In investigating any possible role of antibodies in CHI, treatment using immunosuppressive medication is a likely confounding variable. Further study is therefore required to determine whether antibodies are present above controls outside of

pregnancy, and any possible role of antibodies toward non-classical HLAs which are not detected via standard crossmatching techniques.

Supplementary Data

Supplementary Table 1. Fetal-specific anti-HLA antibody (FSA) specificities in healthy control pregnancies and those following a previous diagnosis of chronic histiocytic intervillositis (CHI).

	FSA Specificity					
	Class I			**Class II**		
Control	HLA-A	HLA-B	HLA-C	HLA-DP	HLA-DQ	HLA-DR
Case 1						+
Case 2	+	+			+	+
Case 3		+	+			+
Case 4	+	+			+	+
Case 5	+					+
Previous CHI						
Case 1		+	+	+		+
Case 2					+	
Case 3	+	+	+		+	+

Antibody positivity against particular HLAs are indicated with '+'.

Chapter 5 References

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Chapter 6: General Discussion

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Summary

CHI represents a serious obstetric condition which although rare, is highly recurrent in nature and results in extremely poor pregnancy outcomes including fetal growth restriction, miscarriage, stillbirth and perinatal death.[1] Currently, the aetiology and pathophysiology of CHI is largely unknown, and consequently there is a paucity of evidence guiding effective prevention or treatment. This thesis sought to address these gaps in knowledge by investigating the effect of immunomodulatory treatment on CHI severity, characterising the inflammatory profile of affected placentas, and applying transplant crossmatching to cases to determine whether there is evidence of maternal-fetal rejection. Analysis of clinical data of women attending Saint Mary's Hospital Rainbow Clinic for care in a subsequent pregnancy following a previous diagnosis of CHI concluded that the inclusion of at least one of hydroxychloroquine or prednisolone was effective at reducing severity of lesions, potentially improving the rate of livebirth.[2] Variation in lesion severity between index and subsequent pregnancies was also demonstrated via automated quantification of intervillous $CD68^+$ cells and fibrin, and allowed for the development of a threshold for diagnosis based upon the numbers of macrophages present in the intervillous space in control placentas. In untreated index cases of CHI, intervillous macrophages expressed M2 marker $CD163^+$, though C4d deposition was not elevated above levels seen in controls. Placentas from index cases of CHI exhibited some features reminiscent of a rejected organ including fibrin deposition, M2 macrophage accumulation and organ dysfunction, however an antibody-mediated component could not be confirmed.

In this overarching discussion, the findings of this study are discussed in the context of the wider literature on both CHI and organ rejection, and the comparison between these two conditions explored. Potential avenues for future research are also proposed.

Inflammation in CHI

Role of Macrophages

During gestation, macrophages have numerous essential roles including the production of anti-inflammatory cytokines and phagocytosis of fetal antigens which may leak into the maternal environment during trophoblast turnover.[3,4] In healthy pregnancy, macrophages are predominantly contained within the decidua,[5] in contrast to CHI where they accumulate in large numbers within the intervillous space. The cause of macrophage infiltration in CHI is currently unknown, though immunofluorescence staining of placentas from index cases in this study revealed that the infiltrate consisted of $CD163^+$ cells, indicative of an M2-like phenotype. This observation is consistent with data from Freitag et al.[6] who concluded that there is a lack of pro-inflammatory cytokine upregulation in placentas with CHI, which has been hypothesised as the cause of their non-destructive behaviour. Select cases of CHI also express increased ICAM-1 in syncytiotrophoblast, which may be acting as an attractant factor for monocytes, though this is yet to be investigated on a wider range of samples.[7] Together, these observations are suggestive that there may be an initial

insult or chemotactic signal which stimulates systemic monocyte recruitment to the placenta and their differentiation into M2 macrophages to resolve inflammation. Presently, such an inflammatory trigger remains unidentified.

Normally, macrophages are also cleared following the resolution of inflammation, either via migration into draining lymph nodes or apoptosis locally at the site of injury.[8] In CHI however, macrophages aggregate within the placenta and are evident upon histopathological examination after delivery, suggesting that macrophage clearance does not occur to a sufficient degree, or inflammation is sustained and unresolved. It is important to consider however that currently only a snapshot of macrophage phenotype can be studied via histology, likely long after placental inflammation has been initiated earlier in gestation. Considering this, it is therefore also plausible that macrophage polarisation may change during the course of CHI given their ability to demonstrate high phenotypic plasticity.

In the field of reproduction, it is becoming increasingly recognised that non-infectious, 'sterile' inflammation resulting from tissue injury and cell death within the placenta may be involved in various poor pregnancy outcomes including miscarriage, preterm birth and stillbirth; all of which are more common in cases of CHI.[1,9] In the first published description of CHI by Labarrere and Mullen in 1987,[10] trophoblast necrosis was noted as a histological feature, and has been described in two further studies since.[11,12] In their review, Nadeau-Vallée et al.,[9] describe that damage to trophoblast may result in the release of DAMPs including uric acid and cell free fetal DNA, which subsequently act as a signal for monocyte recruitment. It is therefore conceivable that necrosis in CHI may expose the maternal immune system to fetal antigens and DAMPs, triggering an inflammatory response which M2 macrophages later attempt to resolve. Fibrin deposition, as observed within our cohort and widely reported by other groups,[13] may also be relevant in this hypothesis, as H&E staining showed numerous villi which appeared to be surrounded by fibrinoid material. Within the placenta, fibrin deposition is known to act as a protective barrier around areas of damaged syncytiotrophoblast,[14] and therefore its increased presence in certain cases of CHI provides further evidence that there is a level of tissue damage which may contribute toward DAMP release. To our knowledge, there have not yet been any studies to investigate whether trophoblast necrosis is causally linked with macrophage recruitment and fibrin deposition or the release of DAMPs in CHI, and therefore this may represent a worthwhile avenue for future studies into inflammation in the disorder.

Maternal Antibodies

In order for damage to placental tissue to occur and monocytes to be recruited, logically a prior event to stimulate the initiation of an immune response must have occurred. Multiple groups have speculated that there is an antibody-mediated component to CHI, citing evidence of C4d deposition, autoantibodies and those directed toward fetal HLA.[15–17] Within our cohort, there was no evidence to suggest C4d involvement in index cases compared to controls, or increased antibodies toward HLA or other placental antigens in subsequent pregnancies. As subsequent

pregnancies were treated, there is a possibility that these findings were directly impacted by immunomodulatory medication. Additionally, recurrent CHI was not identified in any of these subsequent pregnancies studied via histopathological examination, meaning conclusions on pathophysiology could not be reliably made in these cases. However, due to the rarity of CHI and high degree of recurrence it is not possible to prospectively collect antenatal samples from untreated pregnancies. In index cases of CHI, C4d deposition was present along the syncytiotrophoblast, however this was not significantly different to healthy controls. Due to the fact CHI can only be diagnosed retrospectively, material from these pregnancies was limited to placenta only. Recently, it has become recognised that AMR can occur without evidence of C4d deposition,[18] and in light of this an AMR-like process cannot yet be fully excluded especially as maternal serum was not available for analysis of antibodies in index pregnancies. Importantly, data suggests that antibodies generated during pregnancy e.g. those toward fetal HLA,[16,19,20] persist for some time afterward, and so it is possible that sampling maternal serum in the postpartum period after an initial diagnosis of CHI may allow for insight into any possible role of antibodies in the disorder.

In pregnant women with antiphospholipid syndrome; an example of a disorder with known anti-placental antibodies, IgG bind to beta$_2$-glycoprotein I (β2-GPI) expressed on trophoblast, activating the complement cascade and the NLRP3 inflammasome.[21] This results in the recruitment of both monocytes and neutrophils at the maternal-fetal interface via release of IL-1β and TNF-α. In contrast, the profile of the cellular infiltrate in CHI is primarily composed of $CD68^+$ macrophages, with only minor involvement of other cell types including $CD4^+$ and $CD8^+$ T cells.[22] If there is an antibody-mediated component to CHI, it remains to be confirmed which specific signals result in the recruitment and polarisation of macrophages in particular, as opposed to other immune cells which usually also respond to antibody interaction with tissue. Generally, the polarisation of macrophages toward an M2 phenotype is induced by anti-inflammatory signals including IL-4, -10, -13 and -21.[23] To date, there is only a single study wherein the cytokine and chemokine profile of CHI has been explored.[6] Within this investigation, the only cytokine associated with the stimulation of M2 macrophage polarisation to be measured was IL-13, and was not detected within the five placental samples included. In future, larger scale studies of placentas using cytokine arrays are warranted to investigate the expression of a wide range of signalling molecules which may be dysregulated in CHI and contribute toward macrophage recruitment.

CHI as a Form of Maternal Anti-Fetal Rejection

As the human placenta is semi-allogeneic and must be tolerated by the maternal immune system throughout pregnancy, it is often likened to recipient acceptance of a solid organ allograft.[24] In this analogy, inflammatory disorders of the placenta where maternal tolerance appears to fail, e.g. CHI, VUE and MPFD, have been hypothesised as forms of anti-fetal rejection.[25–27] Consistent with this, index cases of CHI in this study met several of the Banff Criteria used to diagnose rejection,[28]

including: $CD68^+$ macrophage infiltration, deposition of fibrin and subsequent graft dysfunction, in this case placental insufficiency presenting as FGR, miscarriage or stillbirth.

In addition to their vital role in pregnancy, macrophages are also strongly implicated in rejection of solid organ transplants with their phenotype and function tightly linked to graft survival. In the pathogenesis of chronic rejection, the polarisation of infiltrating recipient macrophages appears to be predominated by those skewed toward an M2 phenotype positive for CD163, with very few M1 cells.[29,30] This profile is comparable to the findings of this study which demonstrated CD163 staining in intervillous macrophages within cases of CHI. In chronic allograft rejection, more severe $CD68^+$ macrophage infiltration is associated with both increased fibrosis and decreased length of graft survival.[31] Here, macrophage counts could not be related to the level of fibrin deposition, though Reus et al.,[17] concluded that increased severity of CHI was associated with earlier pregnancy loss. As fibrin deposition was not always present, it is possible that there are separate inflammatory pathways contributing toward macrophage infiltration and coagulation in CHI. This may also explain CHI's occasional overlap with MPFD,[32,33] though more research is required to investigate the relationship between these two pathological features.

Within the field of solid organ transplantation, recipient and donor are matched as closely as possible based upon HLA genotype to reduce the likelihood of *de novo* anti-HLA antibody formation. Similarly, several groups have discussed the possibility of HLA mismatch in the pathophysiology of CHI.[16,17,34] In support of this hypothesis, discordant CHI has been described in the placentas of three dizygotic twin pregnancies,[34] suggestive of a fetal genetic component (e.g. in HLA type) to the disorder. Following virtual crossmatching applied here to women with a history of CHI, there were no differences in FSAs or anti-placental antibodies compared to healthy controls. However, as only fetal DNA was analysed in this study as opposed to paternal, conclusions could not be made regarding any FSAs which may have been present in prior pregnancies. CHI also did not recur in subsequent pregnancies studied which could explain the lack of antibody binding observed on the surface of placental tissue. Furthermore, analysis of antibodies and fetal DNA was limited to classical HLA only, which represents only a small proportion of paternally-inherited allogeneic antigens expressed at the maternal-fetal interface. In transplantation, minor histocompatibility antigens as well as HLA influence graft acceptance, albeit to a lesser degree. These antigens are also expressed on the placenta, and can elicit formation of fetal-specific T cells.[35,36] During healthy pregnancy, the function of these cells is normally attenuated by modified antigen presentation pathways which restrict anti-fetal cytotoxic and helper T cell responses.[37] As elevated T cell infiltration in CHI has been reported by several other groups, [22,34,38] and an antibody-mediated component could not be confirmed here, investigation of T cell-driven rejection and any role of mismatches in other placental antigens represents a potential area for future investigation.

Though this study focused mainly on idiopathic CHI, i.e. those without obvious concurrent infection, the lesion is often identifiable in cases of maternal infectious disease, including malaria and more recently the novel coronavirus SARS-CoV-2.[39–41] An increased incidence of CHI has also been reported in oocyte donation pregnancies with HLA mismatch and FNAIT.[42–44] The occurrence of CHI in this range of conditions is suggestive that there may be multiple pathways in its pathogenesis, and causes of inflammation may differ in each case. In chronic rejection, macrophages have been described as 'end-effector cells in a final common pathway' towards vasculopathy, independent of T or B cell mediated rejection.[30] Similarly, it could be stipulated that CHI is the end result of multiple different possible inflammatory processes, for example viral infection, sterile inflammation or that initiated by autoantibodies, which lead to macrophage accumulation and placental dysfunction. In this hypothesis, it would therefore be expected that not all women with CHI would be positive for the same single antibody. There was a much lower incidence of autoimmune disease and autoantibody positivity within the cohort of women with CHI studied here than reported elsewhere, though a smaller range of antibody specificities was tested compared to previously published data.[15,45] Expanding our antibody testing panel in future will allow for more valid comparison with other studies and give insight into the incidence of antibodies which may be associated with placental pathology.

Whilst tolerance of both the placenta and a solid organ allograft undoubtedly share similarities, there are important differences that limit this comparison. Firstly, within the human haemochorial placenta, fetal and maternal circulations are anatomically separated, unlike in transplantation where surgical anastomosis is required to establish recipient blood supply to the organ. At the maternal-fetal interface within the placenta, there also exist a wide range of evolutionary mechanisms which reduce the likelihood of trophoblast eliciting an inflammatory response (Reviewed in Chapter 1). Likewise, maternal cells exhibit adaptations in order to allow tolerance of the fetus. On the contrary, allograft tolerance following transplantation is not intrinsic and is instead reliant upon neutralisation of pre-existing recipient antibodies and immunosuppressive medication.[46,47] Moreover, as opposed to transplantation where differences in HLA genotype between recipient and donor are a barrier to a successful graft, diversity in HLA between reproductive partners is thought to convey an immunological advantage to offspring.[48] It therefore still remains to be confirmed if HLA disparity is implicated in untreated cases of CHI, and if so, why it adversely affects pregnancy outcome in CHI when it does not do so in healthy pregnancy.

Despite fundamental differences between organ transplantation and pregnancy, shared features between rejected allografts and placentas with CHI may at least provide direction for future study, especially given that the mechanisms of rejection are better understood. For instance, animal models of rejection have allowed for investigation into agents allowing manipulation of macrophage phenotype and subsequent effects on inflammation and graft survival.[49,50] Confirmation of macrophage polarisation in CHI, their function and any relationship with fibrin in larger sample groups may therefore allow for the investigation of similar anti-rejection therapies in the disorder.

With the advancement of research into targeted liposomal drug delivery to the placenta,[51] it is also possible that these methods could be applied to CHI to administer local immunomodulatory therapy as opposed to current medication which instead aims to affect the systemic maternal immune response.

Treatment of CHI

Evidence of CHI as an inflammatory condition has provided rationale for the use of a range of therapies in subsequent pregnancies, aimed both at reducing activation of the coagulation cascade and modulating the maternal immune response. Data from our study is in line with this hypothesis of CHI as a disorder of aberrant maternal inflammation, as treatment with at least one immunomodulatory medication (of hydroxychloroquine and prednisolone) was more effective at reducing lesion severity compared to aspirin and LMWH alone.[2] Similarly, the only systematic review of treatment efficacy in CHI by Contro et al.[52] concluded that aspirin and heparin had no beneficial effect. This may be due to the fact that not every placenta affected with CHI demonstrates increased coagulation and fibrin deposition as highlighted within this study. Within this systematic review, prednisolone was also used in three pregnancies but did not demonstrate any improvement in livebirth rate.[52] However, in this review and our study, limited sample size meant that the efficacy of individual agents could not be determined. With this in mind, in future it would be beneficial to expand the size of our cohort to allow comparison of pregnancy outcome between those treated with prednisolone and those with hydroxychloroquine to determine which medication has the most effect. This is especially important considering that there is controversy surrounding glucocorticoids such as prednisolone and their association with maternal side effects and risks to the developing fetus, whereas the safety profile of hydroxychloroquine has been well documented in pregnant women undergoing treatment for lupus.[53–56] Additionally, the aforementioned systematic review is now considerably outdated, justifying the requirement for up-to-date analysis into treatment effect across published studies.

Between specialist centres worldwide, the treatment regimen for managing women in their subsequent pregnancies after a diagnosis of CHI varies widely. Immunomodulatory therapies detailed in published case reports and studies include hydroxychloroquine and prednisolone similar to the protocol used within our centre, as well as TNF-α antagonists, IVIG and the anti-rejection medication tacrolimus.[32,57,58] In other centres where CHI is not well recognised, it is also not uncommon for women to remain untreated, unfortunately suffering multiple pregnancy losses following their initial diagnosis before self-referral to a specialist clinic.[59] As the feasibility of randomised controlled trials in CHI is likely to be low, it may instead be possible to consolidate outcome data from multiple centres using different protocols in the clinical management of CHI to compare the efficacy of certain regimens. In doing so, the initiation of collaboration between international centres treating women with CHI would be necessary, as well as the establishment of a shared biobank of placental and serum samples to accompany clinical data analysis. Because of CHI's rarity, such a collaboration is likely required to increase sample size and infer meaningful

results on its pathophysiology, effective prevention and treatment. Larger scale studies may also facilitate identification of possible biomarkers including raised serum alkaline phosphatase, which has been described in several published cases but its diagnostic significance unconfirmed.[60–62]

Conclusion

Our data supports previous suggestions that index pregnancies with CHI share some features common to rejected solid organ allografts, including deposition of fibrin, infiltration of M2-like $CD163^{+}$ macrophages and ultimate organ dysfunction. However, there was no evidence for increased placental C4d deposition above controls, and antibodies toward HLA or other placental antigens were not elevated in subsequent pregnancies. A likely confounding variable in the investigation of an antibody-mediated component to CHI is immunomodulatory treatment in subsequent pregnancies, which is associated with reduced severity of placental lesions and consequently an improved livebirth rate. Further study into the pathophysiology of CHI in untreated pregnancies and the efficacy of varying treatment regimens is required to reduce poor pregnancy outcomes associated with the condition, and is reliant upon collaborative efforts between specialist centres.

Future Work

From this project, several areas of CHI pathophysiology and potential treatments requiring further investigation have been identified.

Within this study, the efficacy of individual treatments following analysis of clinical data could not be determined due to limited sample size.[2] Collaboration between specialist centres treating women in subsequent pregnancies and the establishment of a biobank of samples from pregnancies with CHI is required, which will allow evaluation of the impact varying treatment regimen has on pregnancy outcome. An updated systematic review on the efficacy of treatment of CHI is also warranted, given that the only published example is over a decade old and therefore does not include more recent publications.[52]

In order to identify potential biomarkers and risk factors associated with CHI, a better understanding of its immunological pathophysiology is required. To facilitate this, centres undertaking research into CHI in future should aim to standardise the criteria used for diagnosis and study inclusion and exclusion to enable comparison and improve consistency between groups. Furthermore, demographic and medical history collected from study participants could be expanded to give a more comprehensive dataset, for example including information on changes in paternity and family history of autoimmune disease or pregnancy complications to investigate any possible association with CHI. Results from routine clinical tests carried out during pregnancy could also be utilised to identify potential changes and trends in women with CHI, for example in serum alkaline phosphatase, which has previously been implicated in the disorder but its significance unconfirmed.[60]

Following QuPath analysis, placentas with CHI demonstrated large variance in $CD68^+$ macrophage counts and fibrin deposition, which may be reflective of disease severity, and suggested possible discrepancies with histopathological diagnosis. Application of the QuPath cell quantification methods to a larger cohort of samples, including those from other centres, is required to assess reproducibility of results. In future, development of analysis scripts within QuPath could allow for automated quantification of slides to expedite the process, as here manual exclusion of villi was required to exclude fetal macrophages from analysis. Additionally, sample collection should be extended to gestational age-matched control placentas e.g. those from elective terminations, to allow for more valid comparison between placentas with CHI from losses early in gestation.

Index pregnancies with CHI exhibited a $CD163^+$ macrophage infiltrate suggestive of an M2-like polarisation state, and $CD3^+$ T cells were present to a level above controls in subsequent pregnancies despite treatment. To confirm the M2-like phenotype of intervillous macrophages in CHI, more extensive immune cell profiling should be applied to samples from index cases. For example, by utilising imaging mass cytometry which now allows for the staining of a large panel of immune cell markers.[63] Similarly, $CD3^+$ T cell staining and quantification is required in index pregnancies, as well as further characterisation of T cell subtypes. Markers for $CD4^+$, $CD8^+$ and Foxp3 can be used via placental immunohistochemistry to identify T helper, cytotoxic and Treg populations, respectively. Finally, in order to determine the attractive signals for leukocyte recruitment and stimulation of M2 polarisation, an extensive cytokine and chemokine array may be used in placental explants from pregnancies with CHI, given that this has only been studied by one group on five placentas to date.[6]

In pregnancies of women with a diagnosis of CHI, an increased incidence of C4d deposition, maternal autoantibodies, anti-HLA antibodies and those directed toward the placenta could not be confirmed. As immunomodulatory treatment was a likely confounding factor in this study, in future maternal serum should be sampled outside of pregnancy during routine postnatal appointments following a diagnosis of CHI. Maternal serum should also be collected at multiple timepoints to allow longitudinal analysis of antibody titre, as employed in other studies of the condition.[16,44] To facilitate comparison with other studies reporting on the incidence of autoimmune disease, the panel of autoantibodies tested following CHI should be expanded to include those not covered by this study, e.g. anti-phosphatidylserine/prothrombin and anti-β2-GPI antibodies.[15,45] The range of antibody specificities tested in women with CHI could be further extended to those which hold specific relevance to pregnancy, including HLA-E, -F and -G, as well as minor histocompatibility antigens also expressed on trophoblast.[36] Binding of these, or other unidentified antibodies to the placenta in index cases may be assessed by incubating placental tissue from these pregnancies with maternal serum obtained outside of pregnancy, or via the direct application of anti-human IgG similar to that undertaken in the diagnosis of lupus.[64]

Overall, this study supports the hypothesis of CHI as a disorder of aberrant maternal inflammation, which without immunomodulatory treatment and specialist management has devastating effects on pregnancy outcome. Greater awareness of the condition and collaboration between specialist centres is vital to expedite research into CHI's likely cause, as well as preventative measures to reduce the impact on subsequent pregnancies after diagnosis.

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