THE PREDICTION, DIAGNOSIS AND MANAGEMENT OF COMPLICATIONS IN MONOCHORIONIC TWIN PREGNANCIES

by

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A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

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November 2018

Abstract

Monochorionic twin pregnancies are high-risk and closely monitored antenatally. A systematic review revealed no existing predictive factors for twin-twin transfusion syndrome (TTTS), growth restriction, or intrauterine fetal death (IUFD). The Optimal Management of Monochorionic Twins (OMMIT) study found that first trimester inter-twin nuchal translucency discordance, crown-rump length discordance, β-hCG, PAPP-A, AFP, PlGF and sFlt-1 do not predict adverse outcome.

A difference was seen in novel second trimester biomarkers: in the recipient twin amniotic fluid metabolites pre- and post-fetoscopic laser ablation; and a relationship with recipient twin cardiac function was demonstrated. Discovery work on miRNA in second trimester maternal serum of TTTS pregnancies found no difference compared to uncomplicated monochorionic twin pregnancies.

A systematic review provided a more personalised risk prediction for the surviving co-twin in single IUFD, including that that the rate of abnormal brain imaging is 20% and the IUFDs occurring at 14-28 weeks are at higher risk.

A preliminary study of parent-fetal antenatal and postnatal attachment and depression in TTTS pregnancies found maternal attachment increased postnatally and depressive symptoms decreased, whereas paternal scores did not change. This

thesis has reported exciting findings which have clinical implications, and advance knowledge of complicated monochorionic twin pregnancies.

Executive Summary

Background

Monochorionic (MC) twin pregnancies are high risk and thus are closely monitored antenatally with ultrasound scans every 2 weeks from 16 weeks gestation. The aim of this intensive monitoring is to detect complications including twin-twin transfusion syndrome (TTTS) which affects 10-15% MC twin pregnancies and is thought to be related to abnormal inter-twin placental vascular anastomoses and without detection and treatment has a high mortality rate (>90%). Other serious complications include selective intrauterine growth restriction (sIUGR) and single intrauterine fetal demise (sIUFD) which can have serious sequelae for the surviving co-twin because of the placental anastomoses. At present it is not possible to predict which MC twins will develop these complications, thus all MC twins are closely monitored which has an impact on resources and patients. It is also not known how having a pregnancy affected by TTTS affects parents psychologically.

Aims

The aims of this thesis are to a) identify and evaluate first trimester potential prognostic factors of adverse outcome in MCDA twin pregnancies; b) perform discovery work to identify second trimester novel potential prognostic factors of TTTS (metabolomics in amniotic fluid, microRNA in maternal serum); c) examine the prognosis of surviving co-twins following spontaneous sIUFD to aid a personalised risk prediction; d) assess antenatal and postnatal parento-fetal/infant attachment and

depression in pregnancies complicated by TTTS, to help health care professionals identify which couples may need additional support.

Methods

The Optimal Management of Monochorionic Twins (OMMIT) study consisted of different groups of retrospectively and prospectively recruited participants with MC twin pregnancies. Different study designs were employed to assess first and second trimester potential prognostic factors, and the psychological impact of TTTS on recruited parents. To examine the prognosis of surviving co-twins following spontaneous sIUFD a systematic review and meta-analysis was performed.

To identify first trimester potential prognostic factors a systematic review and meta-analysis was performed. Inclusion criteria were studies that reported ultrasound measurements, maternal characteristics, or potential biomarkers, measured in the first trimester (i.e. up to 14 weeks gestation), in MCDA twin pregnancies that provided sufficient information to assess the association between the variable and outcome.

The results of the meta-analysis and previous second trimester work were used to inform a retrospectively recruited cohort study to assess the prognostic ability of first trimester potential prognostic factors. Stored first trimester maternal serum samples and ultrasound measurements from women who had undergone first trimester aneuploidy screening in the UK and Australia were used to investigate the association between nuchal translucency (NT) % discordance, crown-rump length

(CRL) % discordance, β-human chorionic gonadotropin (β-hCG), pregnancy-associated plasma protein-A (PAPP-A), alpha-fetoprotein (AFP), soluble fms-like tyrosine kinase-1 (sFlt-1), (placental growth factor) PlGF and adverse outcomes in MC twin pregnancies. Potential prognostic factors were analysed as continuous data, and adjusted odds ratios calculated.

As normal levels of AFP, sFlt-1 and PlGF in the first and second trimester in MC twin pregnancies were not known, a prospectively recruited case series was performed to determine maternal serum levels in uncomplicated MCDA twin pregnancies by performing repeated maternal blood sampling at 12, 16 and 20 weeks on MC twin pregnancies.

Discovery work was undertaken to explore novel potential prognostic factors in the second trimester using two different types of –omics technology. Untargeted metabolomic analysis on amniotic fluid samples taken during FLA from the recipient twin amniotic fluid sac was performed in a retrospectively recruited case series. The metabolite findings were correlated to the degree of cardiac dysfunction in the recipient twin.

Transcriptomics was conducted to explore the role of microRNAs as potential prognostic factors in a prospectively recruited case-control study. Microarrays were performed on second trimester maternal serum samples taken at diagnosis of TTTS and comparing the microRNA profiles to gestationally-matched serum samples taken

from women with uncomplicated MCDA twin pregnancies. The initial array findings were validated in a different cohort.

To examine the prognosis of surviving co-twins following spontaneous sIUFD a systematic review and meta-analysis was performed. The inclusion criteria were studies including twin pregnancies, irrespective of chorionicity, that reported the outcome of the surviving co-twin following spontaneous sIUFD after 14 weeks gestation. Outcomes evaluated were: co-twin death, preterm birth, abnormal antenatal and postnatal brain imaging, neurodevelopmental comorbidity and neonatal death. Sub-group analyses were performed based on pregnancy characteristics.

The final aspect of the OMMIT study was a prospectively recruited case series to explore antenatal and postnatal parento-fetal/infant attachment and depression in pregnancies complicated by TTTS. This was assessed quantitatively using questionnaires that mothers and fathers were asked to complete separately prior to fetoscopic laser ablation (FLA), 1 month after FLA, and postnatally.

Results

Main findings of first trimester prognostic factors meta-analysis:

48 studies were able to be included in the review. The commonest potential prognostic factors which were evaluated were ultrasound markers. Although first trimester NT >95th centile in ≥1 twin, and CRL discordance >10% demonstrated statistically significant associations with TTTS, their actual predictive abilities were

poor. Only one small study (n=51 pregnancies) looked at first trimester maternal serum biomarkers, they demonstrated a trend in association between β-hCG and PAPP-A and TTTS. One of the main issues was the variable way potential prognostic factors and outcomes were defined by different studies.

Main findings of first trimester prognostic factors retrospective cohort study:
First trimester maternal serum samples were analysed from 177 pregnancies. A significant association was found between NT % discordance and the fetal composite adverse outcome and TTTS; CRL % discordance and fetal composite, antenatal growth restriction, sIUFD; AFP and TTTS; PlGF and TTTS, sIUFD and double IUFD. When the aORs were converted into absolute risks based on extreme values for each potential prognostic factor, their prognostic abilities were too low to justify combining the factors in a prognostic model.

Main findings of first and second trimester prospective case series study:
There were 19 women with blood samples taken at all 3 time points. The longitudinal patterns of AFP, PlGF and sFlt-1 at 12, 16 and 20 weeks were demonstrated.

Main findings of amniotic fluid metabolomics retrospective case series:
A statistically significant difference was seen in 200 metabolites in amniotic fluid samples taken from the recipient twin pre-FLA compared to post-FLA in the 19 women who were included in the study. A correlation was also seen with the

metabolomic profile of the amniotic fluid samples taken pre-FLA and the cardiovascular function of the recipient twin prior to FLA.

Main findings of maternal serum microRNA prospective case-control study:
In the initial investigation cohort, 17 microRNAs were upregulated, and 14 microRNAS were downregulated in maternal serum samples taken from pregnancies complicated by TTTS compared to uncomplicated MC twin pregnancies. These differences did not remain significant after correction for multiple testing. Eight microRNAs were tested in the validation cohort, 6/8 were not significantly different between the 2 groups, and 2/8 microRNAs could not be assessed.

Main findings of co-twin prognosis systematic review:
The review confirmed that MC twins are at higher risk of co-twin IUFD and abnormal postnatal brain imaging than dichorionic twins. No difference was seen between the chorionicities regarding preterm birth, neurodevelopmental comorbidity or neonatal death. The risk of preterm birth was high in both chorionicities (dichorionic OR 53.7% [95%CI 40.8, 70.6] and MC OR 58.5% [95%CI 48.2, 70.9]). The risk of abnormal antenatal brain imaging could only be examined in MC twins, and a rate of 20% was calculated. MC twin pregnancies were at higher risk if the first twin IUFD occurred at 14-28 weeks compared to after 28 weeks.

Main findings of parental attachment depressive symptoms prospective case series:

Twenty-five couples were recruited and completed questionnaires at the first time point. Five couples completed questionnaires at all 3 time points. Maternal parento-fetal attachment increased pre-FLA to postnatally, and maternal depressive symptoms decreased, although no difference was seen 4 weeks after FLA. No difference was seen in the fathers, although mothers and fathers with a history of or current mental health problems did report significantly more depressive symptoms.

Conclusions

This thesis has progressed knowledge in the field of MC twin pregnancies. Although it is still not possible to predict which MC twin pregnancies will develop an adverse outcome based on factors measured in the first trimester, novel potential biomarkers have been explored in the second trimester and interesting findings have been reported which may improve understanding of the pathophysiology of TTTS.
New knowledge has been synthesised to produce a more personalised risk prediction for parents with a sIUFD, which has the clinical implications of improving patient counselling and decision making. The high risk of preterm birth, and the importance of offering antenatal brain imaging has been highlighted. Additional knowledge has been obtained to help referring centres identify mothers and fathers who may need additional psychological support following FLA.

The limitations of researching conditions which are relatively rare in the general obstetric population have been discussed: obtaining adequate sample sizes was difficult, including the recruitment of appropriate controls, and of obtaining different

tissue samples. The lack of an appropriate animal model impedes research in this area. The importance of having a defined set of core outcomes for future twin pregnancy research was highlighted.

A multitude of research ideas have been generated. Further work is required in larger cohorts looking at new prognostic factors, but this will require collaboration between centres. Women with complicated MC twin pregnancies should be recruited to the longitudinal prospective study of AFP, PlGF and sFlt-1 to compare levels at 12, 16 and 20 weeks with uncomplicated MC twin pregnancies. The metabolomics work should be continued by investigating the biological function of the significantly different metabolites by performing targeted assays to assess the phenotype. The microRNA work should be continued by performing the validation work in a different cohort, and trying to get the placenta-specific assays to work, particularly as TTTS may be considered a placental disease. It would be very interesting to explore microRNA in other tissue types, particularly placental tissue, but the logistics of sample collection makes this particularly challenging. The systematic review on sIUFD revealed a dearth of research regarding antenatal brain imaging in dichorionic twins, and very little research linking antenatal brain imaging with postnatal brain imaging. The psychological work should be continued in a larger cohort with additional strategies to improve questionnaire return rates. It would be fascinating to perform interviews to delineate the reasons behind the answers on the questionnaires.

MC twin pregnancies will remain at risk of complications unique to monochorionicity, but it is hoped that with continued research in this specialist area, adverse outcomes from these complications can be reduced.

Acknowledgements

I would like to thank Professor Mark Kilby and Dr Katie Morris for their assistance in securing the funding for this work, and their guidance as PhD supervisors. I am grateful to the Richard and Jack Wiseman Trust, British Maternal and Fetal Medicine Society and the Twins And Multiple Births Association for funding this work. I am extremely grateful to the women and men who agreed to participate in the research, particularly when they may have received bad news about their pregnancy, but were willing to participate to help other couples in the future. I would like to acknowledge the following people for their help with various aspects of my thesis:

Chapter 1

- Professor Dr Lopriore (Leiden University Medical Centre, The Netherlands) for providing the placental injection study images.

Chapter 2

- The work in the prognostic factors systematic review (CHAPTER 2) was included in the BMedSci dissertation of Mr Matthew Hall (University of Birmingham) who reviewed the papers and assisted with the data analysis. I was the principle investigator, second reviewer and assisted with data analysis, interpretation and wrote the manuscript.
- I thank the following authors for kindly providing additional information for which they received no payment or compensation: Dr DeKoninck (Monash Health,

Australia), Dr Bhide (St. George's Hospital, London), Professor Dr Lopriore (Leiden University Medical Center, The Netherlands), Dr Miura (Nagasaki University School of Medicine, Japan), Dr Paffoni (Ospedale Maggiore Policlinico, Italy), Dr Velayo (Tohoku University Graduate School of Medicine, Japan), and Dr Gao (The 1st Affiliated Hospital of Sun Yat-Sen University, China).

- I also thank Dr. Michael Chung Ming Chor (University Department of Chinese University of Hong Kong) for help with translation of articles, and Ms Lisa Xue (Royal College of Obstetricians and Gynaecologists) who provided many papers.

Chapter 3

- The statistical analysis in CHAPTER 3 was performed by Dr Rebecca Whittle (Keele University) under the supervision of Professor Richard Riley.
- I thank Professor Jon Hyett (Royal Prince Alfred Hospital, Sydney) for kindly allowing us to use his Australian blood samples, and Ms Jane Tooher for collecting all the outcome data for the Australian participants.
- I am extremely thankful to the data collectors in the UK, and Mr Ian Mills (Birmingham Women's and Children's NHS Foundation Trust) and Mr Naz Ahmed (University Hospital Birmingham) for their laboratory assistance.

Chapter 4

- The Birmingham Women's Hospital 'Multiples' antenatal clinic team, especially Mr Peter Thompson and Dr Tara Selman who assisted with recruiting participants for the work in CHAPTER 4 and CHAPTER 6.

- I am also grateful to Mrs Marguerite Usher-Somers, Mrs Jane Agnew and Mrs Jane Meredith (West Midlands Fetal Medicine Centre) for always thinking of the study when they did a monochorionic twin NT scan, and for their patience in teaching me how to scan.
- I am also very grateful to the team at West Midlands Fetal Medicine Centre who performed the fetoscopic laser ablations, and remembered that I needed to recruit and collect samples: Professor Mark Kilby, Mr Bill Martin, Dr Katie Morris, Mrs Veronica Donovan and all the fetal medicine midwives.

Chapter 5

- I am grateful to Dr Caroline Fox for allowing me to use the samples she collected and stored as part of her PhD for the metabolomics work in CHAPTER 5.
- Dr Will Allwood (University of Birmingham) and Professor Warwick Dunn (Birmingham Phenome Centre) who performed the metabolomics, statistical analysis and aided with interpretation of the metabolomics data.
- The cardiac measurements were performed by Professor Mark Kilby (University of Birmingham). Dr Tim Van Mieghem (University of Toronto, Canada) kindly provided his expertise to assist interpreting the findings.

Chapter 6

- I am grateful to Dr Bernadette Baker (University of Manchester) who assisted in the RNA extraction from my samples, and offered essential advice on study design and data interpretation.

- Dr Rasmus Sjørup Biensø (Exiqon) performed the arrays and assisted with interpreting the data.
- I thank Dr Andrew Beggs (University of Birmingham) who performed the Bayesian analysis and Ms Agata Stodolna (University of Birmingham) who performed the validation RT-PCR.

Chapter 7

- Dr Ashlee Rigby (Flinders University, Australia) was the second reviewer for CHAPTER 7 and assisted with data extraction, data interpretation, and amended the published manuscript.
- Dr Karla Hemming (University of Birmingham) provided her expert statistical help.
- Dr Sarah Hillman (University of Warwick) provided extremely helpful advice.
- Dr Rhona Mahony (National Maternity Hospital, Dublin) and Dr Boaz Weisz (Sheba Medical Center, Israel) kindly clarified information in their articles, and Miss Claire Hobby (Hobby Translations) and Dr Michael Chung Ming Chor (University Department of Chinese University of Hong Kong) provided translational expertise.

Chapter 8

- I am extremely grateful to Professor Helen Pattison (Aston University) for her advice regarding study design, psychological measures and data interpretation for CHAPTER 8.

- Dr Jelena Jankovic (Birmingham and Solihull Mental Health Foundation Trust) provided a helpful insight in to the interpretation of the findings from the view of a perinatal psychiatrist.

I am greatly indebted to my friends and family for their unwavering support throughout my PhD, especially my Dad who freely provided his wealth of academic experience, my Mum and Rhona for unfailingly believing in me, and Jonny for his patience and ensuring I remain balanced and properly fed.

My contribution to the research ideas

The following research ideas were mine: the systematic review in Chapter 2, which potential prognostic factors and outcomes to look at in Chapter 3 although the concept of exploring first trimester prognostic factors in MC twin pregnancies was Professor Kilby and Dr Katie Morris' idea. I also came up with the idea of the study in Chapter 4 as it developed from the work in Chapter 3, the use of metabolomics in Chapter 5 and microRNA in Chapter 6. The idea for the updated systematic review in Chapter 7 was Professor Kilby's idea, although I decided to include the new outcomes and perform the additional sub-group analysis. The study in Chapter 8 was my idea.

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AAA	arterioarterial anastomoses
AC	abdominal circumference
AFI	amniotic fluid index
AFP	alpha-fetoprotein
AGR	antenatally-detected growth restriction
ANOVA	analysis of variance
ANP	atrial natriuretic protein
AoPGR	antenatal or postnatal growth restriction
aOR	adjusted odds ratio
AREDF	absent or reversed end diastolic flow
ART	assisted reproductive technology
AUC	area under the curve
AVA	arteriovenous anastomoses
BCO	bipolar cord occlusion
BF	Bayes factor
β-hCG	beta-human chorionic gonadotropin
BMI	body mass index
BNP	brain natriuretic protein
BW	birthweight
BWD	birthweight discordance
BWH	Birmingham Women's Hospital

cDNA	complementary DNA
CHOP	Children's Hospital of Philadelphia
CI	confidence interval
CPAP	continuous positive airway pressure
CRL	crown-rump length
DC	dichorionic
DCDA	dichorionic diamniotic
dCt	delta Ct
dIUFD	double intrauterine fetal death
dIUGR	double intrauterine growth restriction
DNA	deoxyribose nucleic acid
DQASS	Down’s syndrome Quality Assurance Support Service
DV	ductus venosus
DVP	deepest vertical pocket
DZ	Dizygous
E/A	early passive/atrial contraction
EDF	end diastolic flow
EFW	estimated fetal weight
EFWD	estimated fetal weight discordance
EPDS	Edinburgh Postnatal Depression Scale
ET	ejection time
FASP	Fetal Anomaly Screening Programme
FLA	fetoscopic laser ablation

FMF	Fetal Medicine Foundation
fMRI	fetal magnetic resonance imaging
FSH	follicle-stimulating hormone
FT4	free thyroxine
FU	follow-up
GA	gestational age
GD	growth discordance
GDM	gestational diabetes mellitus
GHQ-30	General Health Questionnaire
GP	General Practitioner
HELLP	haemolysis, elevated liver enzymes, low platelets
HFEA	Human Fertilisation and Embryology Association
HSROC	hierarchical summary receiver operating characteristic
iAREDF	intermittently absent or reversed end diastolic flow
ICT	isovolumetric contraction time
IGFBP	insulin-like growth factor binding protein
IQR	interquartile range
IRT	isovolumetric relaxation time
ISUOG	International Society of Ultrasound in Obstetrics & Gynecology
IUFD	intrauterine fetal death
IUGR	intrauterine growth restriction
IVF	in-vitro fertilisation
IVH	intraventricular haemorrhage

LBW	low birthweight
LH	luteinising hormone
LV	left ventricle
MAAS	Maternal Antenatal Attachment Scale
MC	monochorionic
MCA-PSV	middle cerebral artery peak systolic velocity
MCDA	monochorionic diamniotic
MCMA	monochorionic monoamniotic
MeSH	medical subject heading
MgSO4	magnesium sulphate
miRNA	microRNA
MoM	multiples of the median
MOOSE	Meta-analyses and systematic reviews Of Observational Studies
MPAS	Maternal Postnatal Attachment Scale
MPD	maximum pool depth
MPI	myocardial performance index
MRI	magnetic resonance imaging
mRNA	messenger RNA
MTI	microRNA-Target Interaction
MZ	monozygous
m/z	mass/charge ratio
NA	not applicable
NEQAS	UK National External Quality Service

NICE	National Institute for Health and Care Excellence
NND	neonatal death
NNU	neonatal unit
non-sIUGR	non-selective intrauterine growth restriction
NP	not possible to calculate odds ratio
NS	not statistically significant
NT	nuchal translucency
OMMIT	Optimal Management of Monochorionic Twins
ONS	Office for National Statistics
OR	odds ratio
PAAS	Paternal Antenatal Attachment Scale
PAI	Prenatal Attachment Inventory
PAPP-A	pregnancy associated plasma protein-A
PCA	principal component analysis
PGR	postnatally-detected growth restriction
PlGF	placental growth factor
PPAS	Paternal Postnatal Attachment Scale
PPROM	preterm prelabour rupture of membranes
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
PROGRESS	Prognosis Research Strategy
PTB	preterm birth
PVH	periventricular haemorrhage
QC	quality control

QUADAS	Quality Assessment of Diagnostic Accuracy Studies
QUIPS	Quality in Prognosis Studies
RAS	renin-angiotensin system
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	randomised controlled trial
REMARK	REporting recommendations for tumour MARKer prognostic studies
RFA	radiofrequency ablation
RI	resistance index
RT-PCR	real-time polymerase chain reaction
RV	right ventricle
RVOT	right ventricular outflow tract
SD	standard deviation
sFlt-1	soluble fms-like tyrosine kinase-1
SGA	small for gestational age
sIUFD	single intrauterine fetal death
sIUGR	selective intrauterine growth restriction
SMD	standardised mean difference
SROC	summary receiver operating characteristic
STROBE	Strengthening the Reporting of Observational studies in Epidemiology
TAPS	twin anaemia polycythaemia sequence
TOP	termination of pregnancy
TOPS	twin oligo-polyhydramnios sequence
TRAP	twin reversed arterial perfusion sequence

TRIPOD	Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis
TSH	thyroid stimulating hormone
TTTS	twin-twin transfusion syndrome
UAD	umbilical artery Doppler
UHPLC-MS	ultra-high performance liquid chromatography-mass spectrometry
UKOSS	UK Obstetric Surveillance Survey
USS	ultrasound scans
UTR	untranslated region
UVVF	umbilical venous volume flow
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor 1
VVA	venovenous anastomoses
w	Week
WHO	World Health Organization

List of publications arising from this work

- Mackie FL, Hall MJ, Morris RK and Kilby MD (2018). “Early prognostic factors of outcomes in monochorionic twin pregnancy: systematic review and meta-analysis.” Am J Obstet Gynecol 219:436-46.
- Mackie FL, Morris RK and Kilby MD (2017). “The prediction, diagnosis and management of complications in monochorionic twin pregnancies: the OMMIT (Optimal Management of Monochorionic Twins) study.” BMC Pregnancy Childbirth 17(1):153.
- Mackie FL, Hall MJ, Hyett J, Mills I, Riley R, Morris RK and Kilby MD (2017). “First trimester prediction of adverse events in monochorionic diamniotic twins: The OMMIT study.” BJOG 124(S2):6.
- Dunn WB, Shek NW, Fox CE, Mackie FL, Van Mieghem T and Kilby MD (2015). “Non-targeted metabolomics in recipient amniotic fluid of monochorionic twin pregnancies complicated by severe twin to twin transfusion syndrome (TTTS) and treated by fetoscopic laser ablation (FLA).” BJOG 122(S2):8.
- Mackie FL, Dunn WB, Allwood JW, Van Mieghem T, Morris RK, Fox CE and Kilby MD (2016). “Metabolomic changes in amniotic fluid from Twin–twin transfusion syndrome recipient twins in relation to treatment and fetal cardiovascular risk.” Ultrasound Obstet Gynecol 48(S1):16-17.
- Dunn WB, Allwood JW, Van Mieghem T, Morris RK, Mackie FL, Fox CE and Kilby MD (2016). “Carbohydrate and fatty acid perturbations in the amniotic fluid of the

recipient twin of pregnancies complicated by twin-twin transfusion syndrome in relation to treatment and fetal cardiovascular risk." Placenta 44(8):6-12.

- Mackie FL, Rigby A, Morris RK and Kilby MD (2019). "Prognosis of the co-twin following spontaneous single intrauterine fetal death in twin pregnancies: a systematic review and meta-analysis." BJOG 126:569-78.
- Mackie FL, Pattison H, Jankovic J, Morris RK, Kilby MD (2019). "Parental attachment and depressive symptoms in pregnancies complicated by twin-twin transfusion syndrome." BMC Pregnancy Childbirth; (accepted for publication)
- Mackie FL, Whittle, R, Morris RK, Hyett, K, Riley, RD, Kilby MD (2019). "First trimester ultrasound measurements and maternal serum biomarkers as prognostic factors in monochorionic twins: a cohort study." J Diagn Progn Res; (accepted for publication).

CHAPTER 1 INTRODUCTION AND METHODS

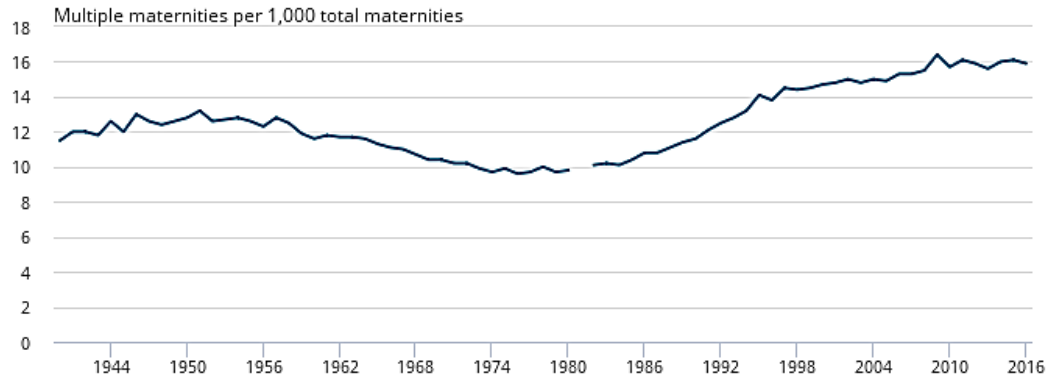
1.1 Overview of multiple pregnancy

1.1.1 Epidemiology

Multiple pregnancies, meaning twins, triplets and higher order births, have been increasing in incidence due to advancing maternal age, increased use of assisted reproductive technologies (ART), and immigration. The Office for National Statistics (ONS) reported in 2016 that 10,786 women in England and Wales gave birth to twins, 160 to triplets, and 5 to quadruplets or greater, equating to 1.59% of all women who gave birth, including live births and stillbirths, although rates have varied over time (Figure 1.1).

Figure 1.1 Multiple maternity rate, 1940-2016, England and Wales

(taken from Office for National Statistics (ONS 2017))



This rate is likely to be an underestimate due to the high rate of unrecognised first trimester miscarriages of multiple pregnancies, and “vanishing twins” whereby the pregnancy may start as a multiple pregnancy, but the second fetus is not viable and is absorbed into the mother’s body prior to 14 weeks gestation, usually with little adverse effect on the surviving fetus.

1.1.2 Aetiology of multiple pregnancy

Multiple pregnancy rates vary depending on setting. The lowest rates are reported in Asian countries such as Japan, Hong Kong and Singapore, with rates as low as 0.09% (Imaizumi 2005). The highest rates reported are in African countries such as Nigeria and Benin with rates as high as 6.65% (Nylander 1971). These discrepancies may be related to the levels of follicle-stimulating hormone (FSH) and luteinising hormone (LH) which are lower in Japanese women compared to American women, and Nigerian women (Soma 1975), and are involved in ovulation – a process implicated in the development of twin pregnancy (see section 1.1.3). Although recent accurate data from lower income countries is sparse, the rates in these countries are thought to be stable (Smits 2011). The increase in the multiple pregnancy rates in higher income countries is related to women child-bearing later in life, which in itself is a risk factor for multiple pregnancy (Hoekstra 2008), as well as the increased use of ART (Pison 2015). The Human Fertilisation and Embryology Association (HFEA) has successfully decreased the rate of multiple pregnancies created by ART as a result of the ‘single embryo transfer’ policy (HFEA 2018), but this guidance does not apply to countries outside the UK and women undergoing ART abroad still have a

higher risk of a multiple pregnancy, particularly higher order multiples. This thesis predominantly focuses on twin pregnancy.

1.1.3 Zygosity

Twin pregnancies arise from either two zygotes (dizygous, DZ), or one zygote (monozygous, MZ). DZ twins are formed when two oocytes are fertilised in the same menstrual cycle and are always genetically non-identical. This is related to altered endocrine milieu, including raised FSH levels (Lambalk 1998). MZ twins are formed when one single embryonic mass divides and are considered genetically identical, although there are reported cases of discordant anomalies in MZ twins, thought to be due to trisomy rescue, post-zygotic chromosomal non-disjunction, unequal blastomere allocation, anaphase lagging, point mutations in a single gene and epigenetic changes (Machin 2009, Saffery 2012). The reason for MZ splitting is not known.

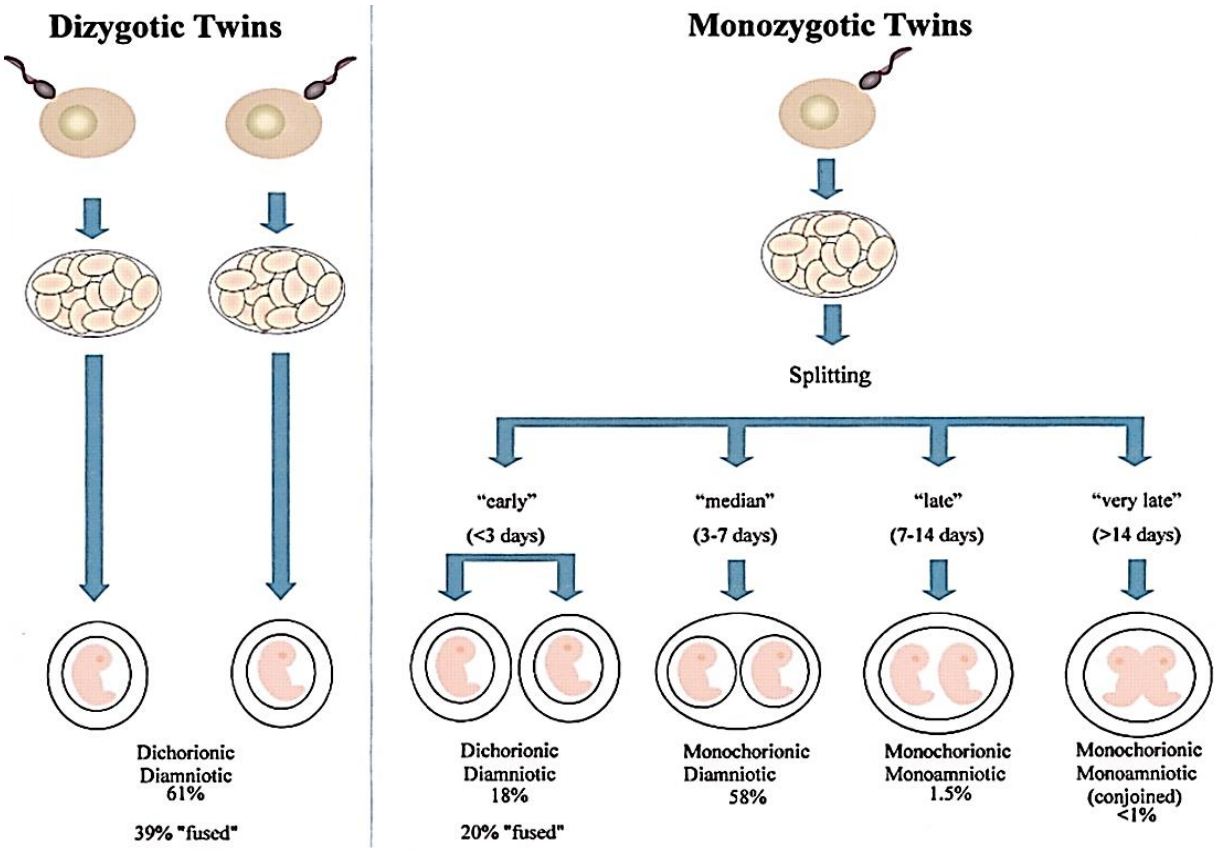
1.1.4 Chorionicity and amnionicity

In addition to classifying twins based on zygosity, twins can also be classified by chorionicity and amnionicity. The majority of twins (80%) are dichorionic diamniotic (DCDA) meaning that each twin has its own separate placenta and separate amniotic fluid sac and thus is the lowest risk type of twin pregnancy (Figure 1.2). DCDA twins are DZ, or MZ if cleavage occurs before day 4. The second commonest type of twins are monochorionic diamniotic (MCDA) meaning that the twins share a placenta, but have separate amniotic fluid sacs; the shared placenta makes them higher risk than

DCDA twins. MCDA twins are always MZ and form when cleavage occurs later at 4-8 days. If cleavage occurs later than 8 days, monochorionic monoamniotic (MCMA) twins form, and if cleavage occurs after 13 days conjoined twins will form.

Figure 1.2 Chorionicity and amnionicity of twin pregnancy according to zygosity

(taken from Greaves et al. (Greaves 2003))



As MC twins share a placenta, this can result in various complications unique to MC twin pregnancies either secondary to inter-twin placental vascular anastomoses or unequal placental territories (Sebire 1997, Denbow 2000, Acosta-Rojas 2007). Placental vascular anastomoses allow blood to flow freely between the twins and

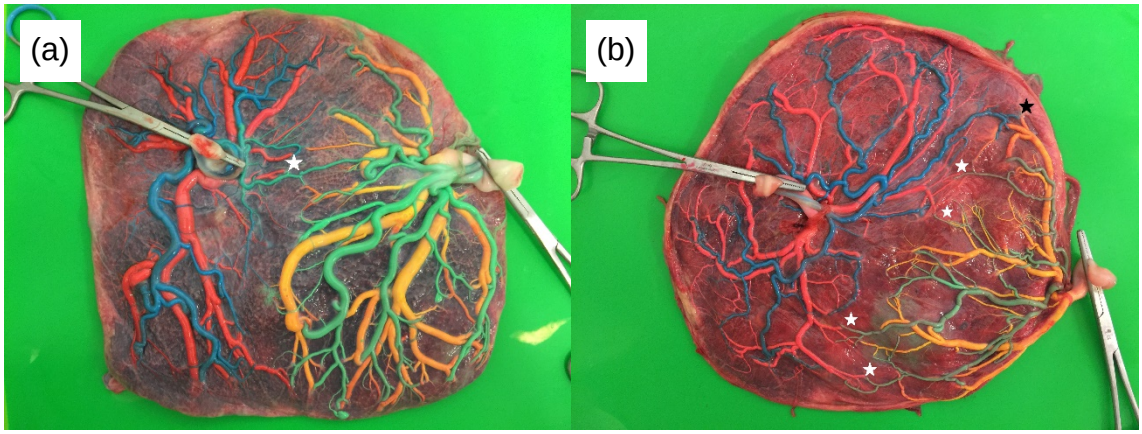
occur in the majority of MC twins (Denbow 2000). There are three different types of anastomoses: arteriovenous anastomoses (AVA), arterioarterial anastomoses (AAA) and venovenous anastomoses (VVA), which have different flow directions (see Figure 1.3(a) and Figure 1.3(b)).

Figure 1.3 Placenta injection study of (a) uncomplicated monochorionic twin pregnancy (b) monochorionic twin complicated by twin-twin transfusion syndrome

(pictures courtesy of Professor Dr Lopriore)

(a) Uncomplicated monochorionic twin pregnancy, delivered at 36 weeks gestation. Red and blue injected vessels are from one twin, and yellow and green injected vessels from the other twin. White star demonstrates an arterio-arterial anastomosis (AAAs)

(b) Quintero stage I, treated at 24 weeks with amnioreduction, delivered at 24+5 weeks. The recipient twin's placental share is on the left (red and blue injected vessels) and the donor twin's placental share is on the right with a smaller share (yellow and green injected vessels). White stars demonstrate arterio-venous anastomoses (AVAs) from donor to recipient twin. Black star demonstrates a cluster of AVAs from recipient to donor twin. There are no arterio-arterial anastomoses.



AVAs occur ‘deep’ at capillary level and are unidirectional: the anastomosis receives arterial blood from one twin and sends venous blood to the other twin (Denbow 2000). AAAs and VVAs are considered ‘superficial’ and are bidirectional: flow is allowed in either direction dependent on the gradient of inter-twin vascular pressure (Lewi 2013). As VVAs and AAAs form direct communications between the two fetal circulations, rather than at a capillary level as in AVAs, VVAs and AAAs have a much lower resistance. Anastomoses may be pathological or compensatory and different combinations of type, number and size of anastomoses are implicated in different complications unique to monochorionicity, including twin-twin transfusion syndrome (TTTS), growth restriction, and intrauterine fetal death (IUFD). This is why chorionicity determination dictates antenatal care (NICE 2011, Kilby 2016), and why MCDA twins are the focus of this thesis. These MC complications are investigated in this thesis and will now be outlined.

1.2 Twin-twin transfusion syndrome (TTTS)

1.2.1 Epidemiology and pathophysiology

Twin-twin transfusion syndrome (TTTS) can occur acutely in the intrapartum period (Lopriore 2014) although this is rare. The focus of this thesis is the more common “chronic TTTS” which occurs in the second, and less commonly the third, trimester. Where TTTS is referred to in the remainder of the thesis it is referring to chronic TTTS unless otherwise stated. TTTS affects approximately 10-15% of MC twin pregnancies (Berghella 2001, Lewi 2008) and without treatment has a perinatal

mortality rate of up to 90% (Haverkamp 2001). TTTS disease is due to abnormal placental vascular anastomoses creating a ‘recipient’ twin and a ‘donor’ twin which have different pathophysiology and sequelae, with serious adverse effects on the fetal cardiovascular and renal systems being demonstrated. There is a dearth of knowledge regarding the pathophysiology of TTTS because ethically-acceptable invasive in-utero sampling is limited as invasive sampling increases risks such as intrauterine infection and preterm birth (PTB). There are few occasions invasive sampling would be performed as part of clinical care in MC twin pregnancies, particularly uncomplicated MC twin pregnancies which also means that finding an appropriate control group is difficult. There are no adequate animal models of TTTS either (Shaw 2016, Wohlmuth 2016, Caloone 2017). The current knowledge will now be outlined.

1.2.1.1 Placental pathophysiology

No unique anastomotic pattern solely attributable to TTTS has been identified (Lewi 2013).

The general consensus is that AVAs allow the ‘donor’ twin to drain deoxygenated arterial blood into the shared chorionic villus tree, gaseous exchange occurs, but the ‘recipient’ twin then receives the oxygenated venous blood, not the donor twin. AVAs can exist in the opposite direction: arterial blood from the ‘recipient’ twin flows to venous blood in the ‘donor’ twin. However, when there is a net unbalanced inter-twin blood flow, for example if there are an odd number of AVAs, TTTS occur. Another contributing factor to the unbalanced inter-twin blood flow seen in TTTS is the lower

number of AAAs in TTTS placentas compared to unaffected MC placentas (Denbow 2000). AAAs are believed to be better at compensating for unbalanced blood flow than AVAs (De Villiers 2015, Zhao 2015) as AAAs have a much lower resistance than AVAs, according to a computer mode (Lewi 2013). VVAs are present in approximately 20-25% of MC placentas (Denbow 2000, De Villiers 2015) and are associated with TTTS although the reason for this is not clear (De Villiers 2015, Zhao 2015). There is debate as to whether TTTS is associated with angiogenesis whereby existing blood vessels are transformed, or vasculogenesis whereby new blood vessels are created.

The unbalanced inter-twin blood flow results in one twin, the ‘recipient’ twin, becoming hypervolaemic, which is associated with polyuria and polyhydramnios in all recipient twins, and cardiac dysfunction in 70% (Van Mieghem 2009c). The other twin, the ‘donor’ twin, becomes hypovolaemic and develops renal failure and oligohydramnios. There is no change in the inter-twin haemoglobin levels in TTTS (Saunders 1991), therefore the pathophysiology is linked to abnormal circulating volume and vascular resistance.

1.2.1.2 Fetal cardiac pathophysiology

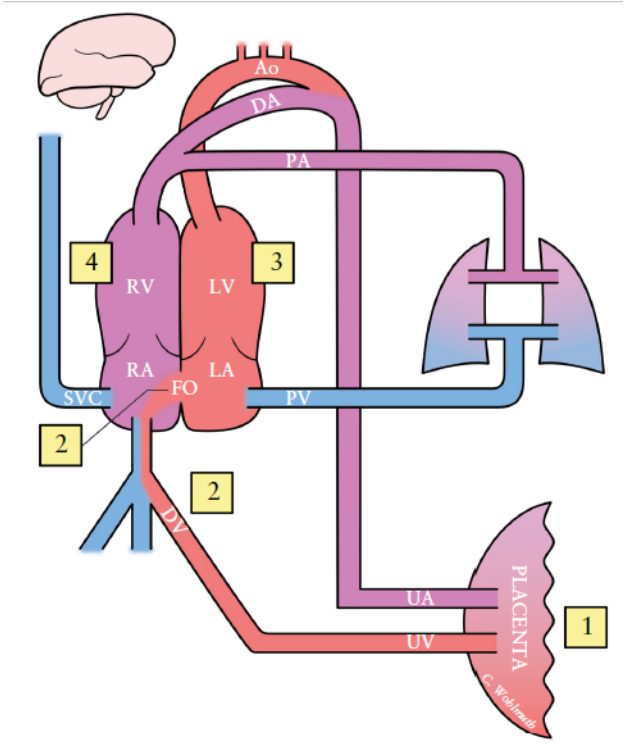
As TTTS is fundamentally a difference in inter-twin circulating volumes, the cardiac anomalies associated with TTTS are thought to reflect the physiological response to chronic unbalanced circulating volumes (Manning 2016). These anomalies can be both acquired structural anomalies and functional anomalies, and are mainly seen in the recipient twin (Michelfelder 2007). The recipient twin has an increased total

circulating volume, with increased preload and afterload, the latter demonstrated by an increased tricuspid regurgitation velocity, and associated with increased resistance and fetal hypertension (Mahieu-Caputo 2003). This can cause progressive biventricular hypertrophy in the recipient twin with associated atrioventricular valvular regurgitation, pulmonary valve atresia or stenosis, reduced myocardial compliance, and abnormal ductus venous Doppler velocimetry (Zosmer 1994, Stirnemann 2010b). Systolic and diastolic dysfunction may be present, with diastolic dysfunction being more common (Barrea 2005). Approximately 70% of recipient fetuses will have abnormal echocardiographic structural or functional findings at diagnosis of TTTS (Habli 2008a). The methods of assessing fetal cardiac function in TTTS pregnancies are outlined in section 1.2.4. There is debate whether the left or right side of the heart, and systolic or diastolic function is affected first. Previously it was thought that the right side of the heart was affected before the left, and ventricular hypertrophy and diastolic dysfunction occurred before subsequent systolic dysfunction (Barrea 2005). However, a more recent study has found that the left ventricle demonstrates abnormal filling pressure and greater systolic dysfunction before abnormalities in the right side of the heart (Wohlmuth 2017). See Figure 1.4 for proposed pathophysiology in the recipient twin (Wohlmuth 2017).

The donor twin on the other hand usually has a functional normal heart (Barrea 2005), with any right ventricular diastolic dysfunction due to increased placental resistance, possibly related to concurrent IUGR (Bajoria 2003, Van Mieghem 2010, Rychik 2012).

Figure 1.4 Proposed pathophysiology of recipient twin in twin–twin transfusion syndrome

(adapted from Wohlmuth et al. (Wohlmuth 2017)) (1) Neuroendocrine factors such as brain natriuretic peptide (BNP) and endothelin-1 are transferred via anastomoses from the donor twin to the recipient twin causing increased recipient blood pressure. (2) Decreased right-to-left shunting and altered ductus venosus (DV) filling times are observed. (3) Altered left ventricular (LV) function and, ultimately, (4) bilateral atrioventricular valve regurgitation and ventricular hypertrophy are seen.
Ao: aorta, DA: ductus arteriosus, FO: foramen ovale, LA: left atrium, PA: pulmonary arteries, PV: pulmonary veins, RA: right atrium, RV: right ventricle, SVC: superior vena cava, UA: umbilical artery, UV: umbilical vein.



1.2.1.3 Fetal renal pathophysiology

The hypervolaemia of the recipient twin causes the myocardial tissue to stretch, stimulating the release of atrial natriuretic protein (ANP) and brain natriuretic protein (BNP). ANP and BNP act on the kidneys and result in diuresis, polyuria and the polyhydramnios characteristic of TTTS (Bajoria 2001, Bajoria 2003). The fetal

hypertension of the recipient twin is secondary to vasoactive mediators produced by the donor twin to compensate the hypovolaemia that are transferred to the recipient twin via the anastomoses. The renin-angiotensin system (RAS) is upregulated in the donor twin in response to the chronic hypovolaemia and subsequent hypoperfusion, resulting in increased vascular resistance to compensate (Kilby 2001, Mahieu-Caputo 2005). Although the RAS is downregulated in the recipient's kidneys, the anastomoses allow the transfer of potent vasoconstrictors such as endothelin-1 that is involved in TTTS (Bajoria 1999a, Bajoria 2003). Consequently both the donor and recipient have hyperdynamic circulations.

1.2.1.4 Maternal pathophysiology

There is no substantive evidence to suggest that TTTS affects the mother's physiology. One observational study with no control group suggested that maternal hypoproteinaemia may play a role in TTTS by affecting amniotic fluid volumes (De Lia 2000). This led to a retrospective cohort study comparing 51 women with MCDA twin pregnancies who took nutritional supplement drinks three times a day, to 52 women with MCDA twins who did not take nutritional supplement drinks (Chiossi 2008). The gestational age at which supplementation was commenced is not clear, but a significantly lower incidence of TTTS in the supplemented group compared to the non-supplemented (15.5% and 38% respectively) was reported. The rate of TTTS in the non-supplemented is higher than would be expected, which is not commented on in the study. The maternal serum albumin and protein levels were measured in 16 and 15 of the supplemented and non-supplemented women and were significantly

different, with higher rates reported in the supplemented group. The authors state that the maternal nutritional status may affect the materno-fetal colloid osmotic pressure, although this hypothesis has not been investigated further.

1.2.2 Prediction of TTTS

First trimester

At present it is not possible to predict which pregnancies will develop TTTS, based on factors measured in the first trimester. CHAPTER 2 will systematically review and meta-analyse existing literature on first trimester potential prognostic factors. CHAPTER 3 will assess existing factors identified by the systematic review in a retrospective cohort, and CHAPTER 4 will look at the biomarkers in normal first and second trimester MC twins. CHAPTER 5 and CHAPTER 6 will take the work further by investigating novel potential biomarkers in the form of metabolites and microRNA (miRNA).

Second trimester and third trimester

Descriptive differences in maternal serum biomarkers have been demonstrated at diagnosis of TTTS in the second trimester. Maternal serum alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (β-hCG) in MC twins complicated by severe TTTS were significantly higher compared to gestationally-matched uncomplicated MC twin pregnancies (Figure 1.5(a) and (b)) (Fox 2009). Additionally, the ratio of soluble fms-like tyrosine kinase-1 (sFlt-1) to placental growth factor (PlGF) levels was significantly increased in TTTS (Figure 1.6) (Fox 2013), although no difference was

demonstrated when these biomarkers were examined individually. These biomarkers and their potential effects on the pathophysiology of TTTS and other MC twin complications will be explored in CHAPTER 3.

Figure 1.5 Concentration of (a) maternal serum alpha-fetoprotein (AFP) and (b) beta-human chorionic gonadotropin (β-hCG) in uncomplicated dichorionic twin pregnancies (DC), uncomplicated monochorionic twin pregnancies (MC) and twin-twin transfusion syndrome (TTTS) pregnancies

taken from Fox et al. (Fox 2009))

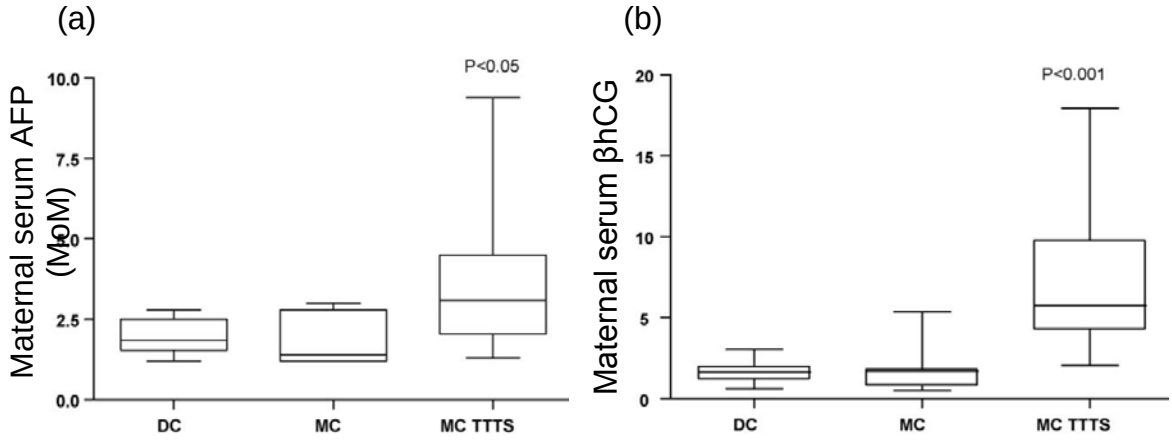
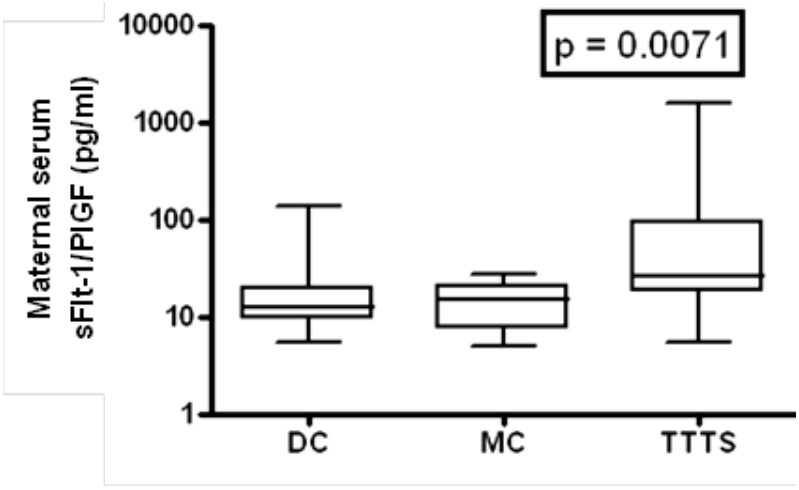


Figure 1.6 Ratio of soluble fms-like tyrosine kinase-1 (sFlt-1) to placental growth factor (PlGF) in uncomplicated dichorionic twin pregnancies (DC), uncomplicated monochorionic twin pregnancies (MC) and twin-twin transfusion syndrome (TTTS) pregnancies

(taken from Fox 2013)



Placental umbilical cord site insertion has been investigated by several studies but a systematic review and meta-analysis reported no association between velamentous cord insertion and TTTS (Kalafat 2017). Since the systematic review was performed, the first study to report cord insertion site based on antenatal assessment has been published (Couck 2017). This study demonstrated an association between velamentous cord insertion identified at 16 weeks gestation in one or both twins, and TTTS, but reported low positive and negative predictive values.

1.2.3 Signs and symptoms

The main clinical signs and symptoms of TTTS are a sudden increase in abdominal girth due to the polyhydramnios that can be associated with breathlessness and abdominal discomfort/pain. These signs may be difficult to recognise as a woman's abdominal girth will increase as part of a normal twin pregnancy, and this may be different to the rate of increase seen in singleton pregnancies. Women who develop TTTS later in pregnancy may also notice decreased fetal movements.

1.2.4 Diagnostic criteria and staging

The majority of TTTS cases are picked up on routine antenatal ultrasonography as the discordant amniotic fluid volumes are apparent on ultrasonography from 16 weeks gestation, see section 1.7. In Europe, TTTS is diagnosed when one twin has polyhydramnios defined as a maximum pool depth (MPD) >8cm at <20 weeks gestation, or MPD >10cm at >20 weeks gestation, and the other twin has oligohydramnios with a MPD <2cm irrespective of gestation (Quintero 1999). In North America, the gestation does not make a difference and the definition is MPD >8cm in one twin and <2cm in the other twin, irrespective of gestation of either twin. As TTTS can only occur in MC twins, the twins must be the same sex, and ideally monochorionicity should have been diagnosed in the first trimester (NICE 2011, Kilby 2016), based on a single placental mass, thin inter-twin membrane, and the presence of the 'T' sign (Sepulveda 1996).

TTTS is staged according to the Quintero staging to monitor disease progression and guide treatment (Table 1.1) (Quintero 1999). However, Quintero staging is not able to

predict outcome, including not being able to determine those at Stage I that will spontaneously resolve and those that will progress, and is criticised for not including cardiac function (Ville 2007, Stirnemann 2013, Djaafri 2017).

Table 1.1 Staging of twin-twin transfusion syndrome

(taken from Quintero et al. (Quintero 1999)
*Polyhydramnios: deepest vertical pocket (DVP) >8 cm; oligohydramnios: DVP <2 cm
†At least one of the following: a) Absent or reverse end diastolic flow velocity in the umbilical artery; b) Reverse flow in the ductus venosus; c) Pulsatile umbilical venous flow

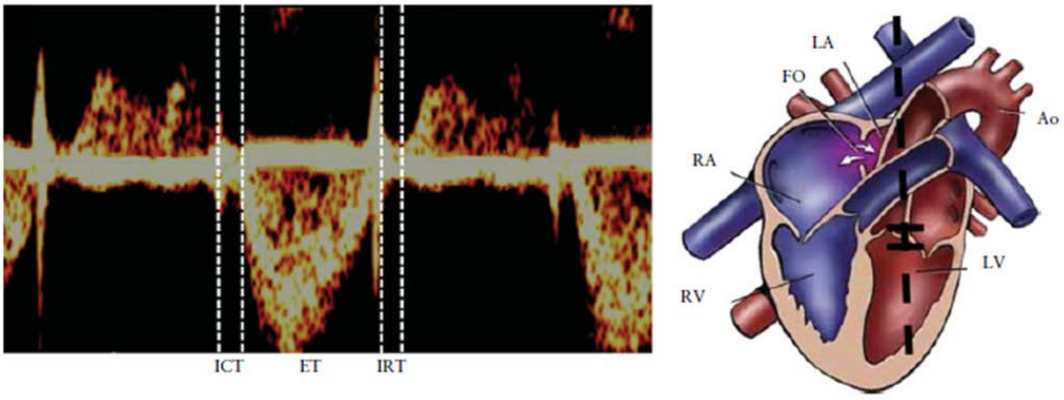
Stage	Poly- and oligo-hydramnios*	Absent bladder in donor	Critically abnormal Dopplers†	Hydrops: ascites, pericardial or pleural effusion, scalp oedema, or overt hydrops	Demise of one or both twins
I	+	-	-	-	-
II	+	+	-	-	-
III	+	+	+	-	-
IV	+	+	+	+	-
V	+	+	+	+	+

There are other scores that exist including the Children's Hospital of Philadelphia (CHOP) cardiovascular score (Rychik 2007), myocardial performance index (MPI) (Figure 1.7), also known as the 'Tei-index', and speckle tracking, that consider the cardiovascular dysfunction associated with TTTS, however there are conflicting results regarding the clinical utility of these scores (Rychik 2007, Stirnemann 2010a, Gapp-Born 2014, Zanardini 2014, Henry 2018). The measurement of the CHOP score is more time-consuming than the other two methods, thus MPI is more practical for clinical use. MPI and speckle tracking can both be performed on an ultrasound

machine used for standard fetal assessment, and no additional skills are needed other than the ability to acquire a four-chamber view of the heart. Van Mieghem et al. evaluated MPI and speckle tracking and stated MPI is preferred as it is highly feasible and reproducible in MC twins, whereas speckle tracking was only feasible in 80% of singleton pregnancies and would thus be even lower in twin pregnancies, and the inter- and intra-observer variation was higher in speckle tracking (Van Mieghem 2009a, Van Mieghem 2009b, Van Mieghem 2011a). Another group also stated MPI is the preferred assessment score (Villa 2014).

Figure 1.7 Measuring Myocardial Performance Index (MPI): Doppler trace and sample volume positioning

(taken from Van Mieghem et al. (Van Mieghem 2009b))
The isovolumetric contraction time (ICT), ejection time (ET) and isovolumetric relaxation time (IRT) are indicated. Ao: aorta, FO: foramen ovale, LA: left atrium, LV: left ventricle, RA: right atrium, RV: right ventricle.



Speckle tracking assesses ventricular function by tracking the velocity vectors generated from myocardial displacement (Van Mieghem 2011a). The MPI reflects global ventricular function and includes measures of systolic (isovolumetric contraction and ventricular ejection time) and diastolic (isovolumetric relaxation time)

function (Tei 1995). The MPI is calculated for each ventricle, with a higher score indicating greater dysfunction. It is calculated by the sum of the isovolumetric contraction time and the isovolumetric relaxation time, divided by ventricular ejection time.

In TTTS, the MPI is raised in at least 50% of stage I cases (Van Mieghem 2009c) and the proportion of TTTS pregnancies with a raised MPI increases as the Quintero stage worsens (Habli 2012, Gapp-Born 2014). Pre-fetoscopic laser ablation (FLA) the recipient twin's left MPI score is higher than the donor twin's left MPI, indicating the recipient has worse cardiac function (Ortiz 2018). When compared to MC twins with no TTTS, the recipient twin has a higher left MPI pre-FLA than control MC twins (Van Mieghem 2009c, Zanardini 2014, Wohlmuth 2017, Ortiz 2018).

1.2.5 Treatment

There are different treatment modalities that mainly depend on: severity of TTTS, gestation, and fetoscopic access.

1.2.5.1 Septostomy

Prior to the invention of FLA, amniotic septostomy was performed whereby a hole was created in the inter-twin membrane, converting the twins from MCDA to MCMA. Septostomy attempted to rebalance the differing amniotic fluid levels. However it makes diagnosis of TTTS recurrence more difficult, does not treat the underlying cause of TTTS, and may create the additional problems of preterm prelabour rupture

of membranes (PPROM), amniotic band syndrome and cord entanglement. As a result of the risk of the latter, earlier delivery at 32-34 weeks is required. Septostomy is no longer routinely practised, but does occur inadvertently in approximately 20% of pregnancies treated with FLA (Peeters 2014a).

1.2.5.2 Termination

Selective termination of the recipient twin was the main treatment for TTTS prior to the invention of FLA, particularly in severe TTTS (Taylor 2002), but is less common now due to the success of FLA. More recently, selective termination is usually only performed if there is evidence of a severe brain injury or concurrent severe growth discordance (see section 1.3.5 for more details) (Fisk 2009).

1.2.5.3 Fetoscopic laser ablation (FLA) and amnioreduction

For women diagnosed as stage II or worse, the gold standard of treatment prior to 26 weeks gestation is FLA (Roberts 2014). FLA involves the percutaneous insertion of a 2mm fetoscope through a 3.3mm port into the recipient twin's amniotic fluid sac under ultrasound guidance. A laser fibre of 500μm is inserted under direct fetoscopic guidance and used to coagulate all inter-twin AVAs, using the inter-twin membrane for guidance. A trial examining FLA technique reported that the Solomon Technique, whereby the ablated AVAs are "joined up" by ablating along the vascular equator, left fewer residual unablated anastomoses than the selective technique of only ablating the AVAs (Slaghekke 2014a), thus reducing the rate of twin anaemia polycythaemia sequence (TAPS) and recurrent TTTS (Slaghekke 2014b). No difference was seen in

perinatal mortality, and a subsequent study revealed no difference in neurodevelopmental comorbidity at 2 years of age (van Klink 2016) with the two techniques, but the Solomon technique is preferred. FLA is performed under local anaesthetic and sedation, or spinal anaesthetic. Following completion of FLA, amnioreduction is then performed in which the amniotic fluid is drained from the recipient twin's sac to a MPD of 5-6cm. Before the invention of FLA, amnioreduction was performed alone, and repeated as necessary. It is thought that amnioreduction reduces the intra-amniotic pressure thus reducing the risk of preterm labour, and allowing placental vessels to open further and improve placental blood flow (Garry 1998). However, as with any procedure involving the insertion of a needle into the uterus, it is associated with a risk of infection, preterm birth, and the polyhydramnios may recur.

The Eurofetus Trial, an international multicentre trial performed in 1999-2002, randomised 172 women to FLA or amnioreduction) and reported higher survival rates, and lower neurological comorbidity, in those who underwent FLA (Senat 2004). Amnioreduction is currently performed if the patient is unable to travel to a Centre that performs FLA, or is a therapeutic option if TTTS is diagnosed after 26 weeks gestation. In the latter, the risks of continuing the pregnancy and performing serial amnioreductions need to be weighed against the risks of preterm iatrogenic delivery, and the administration of antenatal corticosteroids for fetal lung maturation should be considered.

Effect of FLA on cardiac function

The recipient twin cardiac function slightly worsens during FLA, but improves following FLA (Gratacós 2002a, Barrea 2006, Habli 2008b, Papanna 2011, Gapp-Born 2014, Finneran 2017, Wohlmuth 2017, Henry 2018, Ortiz 2018), with improvement in function demonstrable within 48 hours of FLA (Van Mieghem 2009c, Wohlmuth 2017). The donor twin may exhibit temporary signs of cardiac overload with tricuspid insufficiency and abnormal ductus venosus (DV) flow (Van Mieghem 2009c), likely due to the sudden hypervolaemia on a background of vasoconstriction that can cause transitory hydrops (Gratacós 2002b) and increased umbilical artery pulsatility index following FLA (Wohlmuth 2017). By 4 weeks post-FLA many recipient and donor twins display a normal cardiac function (Van Mieghem 2009c). This is thought to be due to the interruption of transfer of fluid and vasoactive mediators between the twins through the ablated anastomoses (Wohlmuth 2017). This remodelling is quicker in-utero than after birth which may be a consequence of fetal cardiomyocytes having plasticity and being able to replicate in-utero, whereas this ability is lost after birth (Ahuja 2007, Drenckhahn 2008). Cardiac dysfunction is not resolved in all twins and acquired structural anomalies can develop, most commonly in the form of right ventricular outflow tract (RVOT) anomalies in recipient twins (Michelfelder 2015), and coarctation of the aorta in donor twins (van den Boom 2010), although the latter is less well substantiated (Manning 2016). RVOT anomalies include valvular dysplasia, stenosis and regurgitation and may require postnatal intervention (Bajoria 2002, Moon-Grady 2011, Michelfelder 2015). MC twins have a higher background risk of congenital heart disease compared to singletons,

with estimates around 4-11% for at least one twin. Often these defects are discordant between the twins. In TTTS pregnancies, the rate of postnatal RVOT anomalies is above 20%.

In the long term, although the systolic and diastolic blood pressure of both the recipients and donors is still raised at 2 years of age (Pruetz 2015), by 10 years no significant cardiac dysfunction is evident (Gardiner 2014, Herberg 2014).

Effect of FLA on renal function

Measuring fetal levels of biomarkers related to renal function, such as ANP, BNP or endothelin-1, post-FLA is difficult for ethical reasons as it would require an invasive procedure for which there is rarely a concurrent clinical indication. Samples post-delivery have been collected but this is a logistical challenge as often deliveries are preterm and vaginal, and women deliver at their referring hospital which is usually not the hospital they attended for FLA. One group that did collect amniotic fluid samples at caesarean section to assess ANP, BNP and endothelin-1 also collected amniotic fluid samples at time of fetal blood sampling and combined the groups, thus the range of time at which samples were collected was 20-36 weeks gestation and it is difficult to interpret these results (Bajoria 2003). Consequently the best measure of renal function in-utero is the MPD, which does increase in the donor twin following successful FLA.

A study from the Netherlands found that in the neonatal period, twins with TTTS who underwent FLA had a better renal function than twins with TTTS who underwent

expectant management or serial amnioreduction (Verbeek 2017). The definition of renal function used was based on: urine output on days 1-3, creatinine and urea levels at days 3-7 of life, although the bodily fluid measured was not clear. Only 7.1% of survivors treated with FLA had a creatinine level >100μmol/L, compared to 37.9% of survivors who did not undergo FLA ($p<0.01$). As the study highlights, large, long-term studies are required, with a clear explanation as to how renal function was assessed.

When to perform FLA

For women with Quintero stage I TTTS there is debate as to whether conservative or active management is best. Quintero stage I TTTS progresses in 27% [95%CI 16-39%) according to a meta-analysis of 7 studies (172 pregnancies) (Khalil 2016a) thus three quarters will regress or remain stable, but it is not currently possible to identify those pregnancies that will regress and those that will progress. Consequently it is not clear whether stage I should be managed conservatively with continued surveillance, or with intervention (FLA). An international multicentre cluster randomised controlled trial (RCT) (NCT01220011) is being performed to answer this question and is due to report in Spring 2019 (Ville 2010).

1.2.6 Outcome

Without FLA, the perinatal mortality rate is up to 90% (Haverkamp 2001). With FLA the rates of adverse outcomes have decreased over time (Akkermans 2015), and are thought to be related to operator experience (Morris 2011, Peeters 2014b). A

systematic review of 37 studies which described 3868 women who underwent FLA between 1990 and 2015 reported a rate of perinatal survival of at least one twin in 81% (SD 8.3), both twins in 52% (SD 14.8) and only one twin in 20% (SD 10.5) (Akkermans 2015). A sub-group analysis was performed to compare the rate of perinatal survival pre-2011 to post-2011 and revealed a significant increase in the perinatal survival rate of both twins (31% to 62%) and at least one twin (70% to 88%) over time. This does still mean that even with treatment at least 12% of pregnancies will result in a double fetal loss.

A major contributor to perinatal mortality and long-term outcome in TTTS pregnancies is PTB, with PTB occurring in up to 17% <28 weeks gestation, 30% <32 weeks gestation, and 50% <34 weeks gestation after FLA for TTTS (Robyr 2005, Yamamoto 2005). A review of 1092 pregnancies treated with FLA for TTTS found that fetal survival and PPROM rates had risen significantly over the 16 year study period (Stirnemann 2018). The authors argue that the focus should move from IUFD prevention, to strategies to prevent PTB and treat PPROM as these are now the current main postoperative complications. There are no effective prediction or prevention methods at present and research in this area is ongoing, but beyond the scope of this thesis.

Neurodevelopmental comorbidity is decreased by FLA (Salomon 2010), but even with FLA still affects approximately 10% of pregnancies (Rossi 2011). A core

outcome set to stipulate which outcomes should be reported in TTTS research is being developed (Perry 2018).

TTTS is viewed as one disease on a spectrum of feto-fetal transfusional disorders. TAPS is a gradual process that may occur following treatment for TTTS (2-13% TTTS pregnancies) or spontaneously (3-5% MC twins) (Slaghekke 2010, Tollenaar 2016). It is defined as a large difference in the inter-twin haemoglobin levels, without twin oligo-polyhydramnios sequence (TOPS) and is due to tiny (<1mm) AVAs. Post-FLA pregnancies should be monitored for TAPS (Kilby 2016) and the anaemic fetus may require cordocentesis and in-utero transfusion.

1.2.7 Psychological impact of TTTS on parents

Given the high-risk nature of TTTS pregnancies, and the wide range of possible outcomes, there has been little research on the psychological and emotional effects TTTS has on parents. Only one study was identified which has explored antenatal materno-fetal attachment in TTTS pregnancies, and they found that whilst attachment increased in mothers of uncomplicated MC and DC twins, it did not increase in mothers whose pregnancies were complicated by TTTS (Beauquier-Maccotta 2016). The same study also reported greater antenatal and postnatal depressive symptoms in mothers with pregnancies affected by TTTS. The effect on fathers does not appear to have been examined. CHAPTER 8 will look at the impact of TTTS on attachment and depressive symptoms in mothers and fathers as these factors are known to affect future child development.

As FLA is a relatively new treatment, long-term follow up studies looking at effects on subsequent pregnancies are only just able to be published. FLA appears to have no adverse effect on future obstetric and gynaecological health (Le Lous 2018, Vergote 2018). However, adverse psychological and emotional effects on mothers at a median of 7 years post-FLA are still apparent (Vergote 2018).

1.2.8 Prediction of adverse outcome following diagnosis of TTTS

Some studies have attempted to create a model to predict the outcome following diagnosis and treatment of TTTS, although none are suitable for clinical application at present (Stirnemann 2013). As TTTS can be viewed as a predominantly cardiovascular disorder, the ability to include measures of cardiac disease may aid prediction of outcome of TTTS. This is particularly important given that severe cardiac dysfunction can result in poor end-organ perfusion and long-term neurological comorbidity. Current studies are conflicting whether the severity of cardiac impairment can predict survival (Stirnemann 2010b, Van Mieghem 2013, Delabaere 2016). As there are other factors that influence the outcome of TTTS, including technical surgical factors, and placental territory proportions, it is likely that cardiac function assessment alone is unable to predict outcome. There is a line of thought that duration rather than severity of cardiac disease is more influential on recovery (Van Mieghem 2013). Functional cardiovascular changes are present early in the disease process (Baschat 2011), prior to the diagnosis of TTTS (Barrea 2005,

Van Mieghem 2011b, Zanardini 2014), therefore the ability to predict which pregnancies will develop TTTS with severe cardiac sequelae may be possible.

A potential way to improve the prognostic ability of these scores is to add biomarkers to the ultrasound measures. As well as possibly improving prognostic ability, the use of biomarkers is not as time-consuming for clinicians compared to detailed echocardiography, and inter- and intra-observation variation is reduced. Furthermore, additional training is not required, or scanning resources. ANP and BNP are used as serum biomarkers of cardiac failure in adults and are present in the amniotic fluid of TTTS recipient twins (Bajoria 2001, Habli 2010), with BNP levels higher in TTTS recipient twins than in normal singleton pregnancies (Bajoria 2003, Van Mieghem 2010). Individually, although increasing levels of amniotic fluid N-terminal prohormone BNP (NT-proBNP) is associated with worsening cardiomyopathy in the recipient twin, the sensitivities and specificities are sub-optimal (Bajoria 2002, Habli 2010). Plasma BNP in women with pregnancies complicated by TTTS and MC twins without TTTS are not different (Bajoria 2002), therefore the use of BNP as a prognostic marker would require an invasive procedure. Endothelin-1 is used as a biomarker of adult cardiac failure with higher levels associated with higher mortality (Zhang 2017), and is present in the amniotic fluid of TTTS pregnancies. As with BNP, endothelin-1 is higher in the amniotic fluid of recipient twins than singletons and differences are also seen in fetal plasma (Bajoria 2002, Bajoria 2003). Cardiac troponin, another serum biomarker used in adults to denote myocardial damage, is also present in the amniotic fluid of recipient twins (Van Mieghem 2010), but again

the clinical utility is yet to be determined. New biomarkers need to be identified and CHAPTER 5 investigates the potential use of metabolomics in predicting cardiac dysfunction.

1.3 Growth restriction

1.3.1 Epidemiology and pathophysiology

Although not unique to MC twins, growth restriction, particularly selective intrauterine growth restriction (sIUGR), is more common in MC twins than DC twins affecting 10-25% of MC twins (Lewi 2008a). As MC twins are MZ, differences in growth cannot be explained by genetic factors (Gao 2012). The factor believed to be most important in growth discordance is placental territory (Lewi 2013), which correlates strongly with birthweight discordance (BWD) (Denbow 2000). Unequal placental sharing equates to a decreased area for nutrient transfer, gaseous exchange and removal of waste products and thus affecting fetal growth. Velamentous cord insertion is often associated with abnormal placental sharing, and growth discordance (Konno 2018) (see section 1.3.2 for more details). Placental anastomoses also play a role. Unlike TTTS which is associated with unbalanced unidirectional AVAs and a lack of AAAs, growth discordance and unequal placental sharing is associated with large bidirectional AAAs, AVAs with a large net flow, and a large total diameter of anastomoses that appear to equalise inter-twin blood flow (Lewi 2013, De Villiers 2015). Thus it is believed that twins with greater inter-twin blood flow will be associated with milder growth restriction and a better outcome, than growth restricted

fetuses with less inter-twin blood flow (Bennasar 2017). It is thought that blood is ‘transfused’ from the larger twin to the growth restricted twin, ameliorating the placental insufficiency and ‘rescuing’ the growth restricted twin (Denbow 2000). This may explain why 20% of the larger twins with Gratacós Type III growth restriction develop hypertrophic cardiomyopathy, compared to 2.5% of Type I and II, because they are acting as a “pump” to rescue the smaller twin (Munoz-Abellana 2007). The large AAAs produce an umbilical artery Doppler waveform unique to MC twins with ‘cyclical’ intermittent absent/reversed end diastolic flow (AREDF) and positive EDF, this is discussed further in section 1.3.4. Differences in placental characteristics have been noted between those with early-onset growth restriction, defined as prior to 20 weeks gestation, and late-onset growth discordance (Lewi 2008a). Placentas from early-onset growth discordance MCDA twin pregnancies demonstrate more unequal placental territories, larger AAAs and a larger total diameter of anastomoses compared to those with late-onset discordant growth. The latter group was not significantly different to placentas taken from concordant MCDA twin pregnancies, suggesting that different pathophysiological mechanisms may be involved in growth restriction.

Growth restriction in MC twins can also be non-selective (non-sIUGR) in which both fetuses are IUGR. This is less commonly reported than sIUGR (2.7% vs 34.5% respectively in a Chinese cohort) (Gao 2012), and is more difficult to diagnose as outlined in section 1.4.4. The pathophysiology of non-sIUGR is likely to be different to that of sIUGR, however there has been little research on non-sIUGR as the majority

has focused on sIUGR (Gao 2012). In theory, the pathophysiological process should affect the whole placenta, so the cause may be genetic or viral, and environmental factors known to affect fetal growth in singleton pregnancies such as maternal diet, smoking status and drug taking could also play a part (Denbow 2000), although evidence in twin pregnancies is conflicting (Schwendemann 2005, Fox 2011). The sparse literature that does exist either makes no distinction between non-sIUGR and sIUGR (Chang 2009, Fox 2011), or the number of non-sIUGR twin pregnancies included have been too small (Gao 2012).

1.3.2 Prediction of growth restriction

First trimester

At present it is not possible to predict which pregnancies will develop growth restriction, based on factors measured in the first trimester. CHAPTER 2 will systematically review and meta-analyse existing literature on first trimester potential prognostic factors. CHAPTER 3 will assess existing factors identified by the systematic review in a retrospective cohort.

Second and third trimester

The same systematic review that reported no association between velamentous cord insertion and TTTS did report a significant association between velamentous cord insertion and selective fetal growth restriction, and BWD >20%. The authors suggest this is due to velamentous cord insertions being more likely to be compressed, thus reducing the blood flow (Kalafat 2017). When this relationship was examined in the

study that used antenatal assessment of umbilical cord insertion site, selective growth restriction was associated with velamentous cord insertion in one twin but poor positive and negative predictive values were reported (Couck 2017). The Gratacós classification divides twins into three types of sIUGR based on umbilical artery Doppler findings and is associated with clinical outcome, this is outlined in Table 1.2 in section 1.3.4.

1.3.3 Signs and symptoms

The measurement of symphysis-fundus height in multiple pregnancy is not recommended. Women who develop growth restriction later in pregnancy may notice decreased fetal movements but the majority of growth restriction is diagnosed on routine ultrasonography, see section 1.7.

1.3.4 Diagnostic criteria and staging

As alluded to, growth restriction in twin pregnancies is difficult to diagnose for various reasons. As in singletons, it is important to delineate between small for gestational age (SGA) fetuses which are constitutionally small, and those that are pathologically growth restricted. Another problem common to both singleton and twin pregnancies is that ultrasound measurement of fetal growth is not always correct. The accuracy of ultrasound assessment of fetal growth in twin pregnancies is even lower than in singleton pregnancies however it is the best imaging modality most widely available (Leombroni 2017, Neves 2017, Kadji 2018).

A particular issue specific to growth restriction in twin pregnancy is the myriad of definitions used to define pathological growth. The results of a Delphi procedure to formulate a consensus definition of sIUGR were published in January 2018 (Khalil 2018). The definition for MC twins was: (i) estimated fetal weight (EFW) <3rd centile in one twin; or (ii) two out of the following four parameters: EFW <10th centile in one twin, abdominal circumference (AC) <10th centile in one twin, estimated fetal weight discordance (EFWD) ≥25%, umbilical artery pulsatility index >95th centile in the smaller twin. This differed to the definition in DC twins which did not include AC as an acceptable measure. These definitions require validation prior to clinical use. Although a consensus definition has been decided, the issue remains that there are no published validated twin-specific growth charts available at present. The expert panel in the Delphi procedure did not state twin-specific or customised growth charts as essential parameters, despite studies demonstrating a significant difference in singleton and twin growth, particularly later in pregnancy (Ong 2002, Liao 2012, Odibo 2013, Stirrup 2014). The use of twin-specific growth charts may change in the future as groups in the UK (Kalafat 2018), Spain (Torres 2017), Italy (Ghi 2017), and Slovenia (Bricelj 2017) have recently developed twin-specific growth charts. These charts require vigorous validation before they can be used in a routine clinical context.

Prior to the publication of the Delphi procedure, the most widely accepted definition of sIUGR was EFWD ≥25% with one twin concurrently below the 10th centile. MC pregnancies fitting this definition can be sub-divided based on umbilical artery

Doppler findings in the smaller twin as described by Gratacós et al. (Gratacós 2007) (Table 1.2). The different types reflect different placental angioarchitecture and unlike the Quintero staging of TTTS, pregnancies do not progress through Gratacós classification types (Lopriore 2012).

Table 1.2 Gratacós classification of selective intrauterine growth restriction

EDF: end diastolic flow, GA: gestational age. *taken from Bennasar et al. (Bennasar 2017). †taken from a systematic review of 610 MC twin pregnancies by Buca et al. (Buca 2017)

Type	Umbilical artery Doppler	Typical placental features*	Proportion of Type (%) (95%CI)†	GA at diagnosis (weeks) mean (95%CI)†
I	positive EDF	Milder discordance in placental territories and/or large number of inter-twin anastomoses	39.0 (35.1, 43.0)	26.5 (22.2, 30.9)
II	persistently absent/ reversed EDF	Substantial discordance in placental territories and number and diameter of placental anastomoses smaller than type I	38.2 (34.3, 43.0)	21.1 (19.4, 22.8)
III	intermittent absent/reversed EDF	Large discordance in placental territories, large placental AAAs, short distance between placental cord insertion sites	22.8 (19.5, 26.3)	20.2 (15.2, 25.3)

1.3.5 Treatment

There is no specific guidance on how to treat, and when to deliver growth restricted twin pregnancies (Buca 2017). Management may vary depending on gestational age, Gratacós classification and if there is evidence of fetal deterioration as assessed by

DV Doppler. Expectant management is often employed for sIUGR Type I, with weekly umbilical artery Doppler assessment, and bi-weekly ultrasonography fetal growth measurement. The management of Type II and III is less clear.

FLA of anastomoses may be an option before viability, but is not routinely offered for growth restriction at present, and choosing candidates is difficult (Bennasar 2017). Survival rates are not as good as in TTTS, a study published in 2015 reported that at least one twin survived in 71.8% (102/142), and both twins survived in 34.5% (49/142) (Peeva 2015). FLA for IUGR is technically more difficult than in TTTS as there is no polyhydramnios (Quintero 2001). There is a train of thought that as some anastomoses may be beneficial to the growth restricted twin, ablating them may in fact worsen the growth restricted twins prognosis which is an additional complexity of the procedure (Lewi 2013). The National Institute for Health Research has issued a commissioned call for a Health Technology Assessment Programme grant to perform a feasibility study of an RCT of intervention or expectant management for early-onset fetal growth restriction which will determine whether an RCT in this complex area is feasible (NIHR 2017).

If the prognosis is extremely poor, termination of the whole pregnancy may be discussed. This is performed by injecting potassium chloride. Selective feticide of the growth restricted fetus is also an option and is used in an attempt to save the normally grown twin. This is performed by ultrasound guided bipolar cord occlusion (BCO), or intrafetal radiofrequency ablation (RFA). The overall survival rate of the co-

twin following BCO is 79.1% (95%CI 71.3-87.5) (459 procedures), and 76.8% (95%CI 67.6, 87.2) (310 procedures) following RFA although the systematic review reporting these rates was unable to perform sub-group analysis according to those performed for sIUGR (Gaerty 2015). A study that performed BCO for Type II and III sIUGR reported a survival rate of 93.4% (84/90), and 92.9% (78/84) delivered after 32 weeks gestation (Parra-Cordero 2016).

1.3.6 Outcome

The outcomes of sIUGR pregnancies can vary and may be linked to Gratacós classification/placental anastomoses (Table 1.3).

Table 1.3 Outcome of selective intrauterine growth restricted pregnancies according to Gratacós classification

(taken from a systematic review of 610 monochorionic twin pregnancies by Buca et al. (Buca 2017), does not include pregnancies with concurrent TTTS that underwent fetal therapy) GA: gestational age, IUFD: intrauterine fetal death

Type	IUFD (%) (95%CI)	Overall perinatal mortality (%) (95%CI)	Abnormal brain imaging (%) (95%CI)	GA at delivery (weeks) mean (95%CI)
I	3.1 (1.6, 5.0)	4.1 (1.2, 8.7)	3.82 (0.5, 10.0)	33.7 (33.0, 34.3)
II	11.0 (4.1, 20.6)	16.1 (4.6, 32.7)	14.15 (6.7, 23.8)	30.9 (30.0, 31.8)
III	9.6 (6.2, 13.6)	11.5 (7.7, 16.0)	11.88 (4.6, 22.0)	32.0 (31.3, 32.8)

Pregnancies classified as sIUGR Type II have a significantly worse perinatal mortality rate (OR 4.1 [95%CI 1.6, 10.3]) and higher rate of abnormal postnatal brain imaging (OR 4.9 [95%CI 1.9, 12.9]) than Type I (Buca 2017). No significant difference is seen

between Type II and III in these outcomes. When smaller twins were compared to larger twins, in Type II the smaller twins had a significantly higher perinatal mortality rate than the larger twin (OR 2.4 [95%CI 1.3-4.4]), but no difference was seen between the larger and smaller twins in Type I and Type III. This fits with Type II being associated with smaller anastomoses, and thus “rescue” transfusion is unable to occur. The large AAAs associated with Type III mean that any change in blood pressure, for example during a bradycardia, has a substantial effect on the other twin and thus sIUGR Type III has an unpredictable clinical course (Buca 2017). This may explain why neurological comorbidity is seen in the normally grown twin from a sIUGR twin pregnancy with 2 survivors. The mean gestational age at delivery demonstrates that prematurity is a major problem for sIUGR pregnancies.

The outcome of non-sIUGR pregnancies are worse than sIUGR pregnancies which is perhaps not surprising considering both twins are pathologically growth restricted (Gao 2012, Ananicz 2016).

1.4 Intrauterine fetal death (IUFD)

1.4.1 Epidemiology and pathophysiology

Single IUFD (sIUFD) after 14 weeks gestation is thought to affect 4% of MC twins, whereas double (dIUFD) occurs in 6%, however these rates are from a study over 20 years old, during which time antenatal care of twin pregnancies has changed, and all IUFDs were included irrespective of cause (Sebire 1997). An ongoing study by the

UK Obstetric Surveillance System (UKOSS) aims to report the rate of spontaneous single MC twin demise in the UK, and subsequent adverse outcomes (UKOSS 2016). sIUFD and dIUFD may be secondary to underlying aforementioned pathology: TTTS, TAPS, growth restriction, but the pathophysiology surrounding spontaneous IUFD is less clear. What is known is that shared circulation means sIUFD may lead to brain injury and/or dIUFD due to the acute exsanguination of the surviving co-twin and consequent hypoperfusion, secondary to the extreme hypotension that occurs at the death of the first twin resulting in hypoxic-ischaemic injury to the surviving co-twin's central nervous system and potentially IUFD. The presence of large bidirectional anastomoses, particularly VVAs, appears related to a worse prognosis (Denbow 2000, De Villiers 2015), although there are no specific placental characteristics unique to IUFD.

1.4.2 Prediction of IUFD

First trimester

Currently, it is not possible to predict which pregnancies will develop IUFD based on factors measured in the first trimester. CHAPTER 2 will systematically review and meta-analyse existing literature on first trimester potential prognostic markers. CHAPTER 3 will assess existing factors identified by the systematic review in a retrospective cohort.

Second and third trimester

One study has attempted to create a score to predict IUFD and neonatal death (NND) from 26 weeks gestation onwards, but did not demonstrate acceptable predictive ability to aid clinical care (Oohashi 2014).

1.4.3 Signs and symptoms

As with singleton pregnancies, IUFD may occur with no warning and be detected at the woman's next ultrasound scan. Some women report decreased fetal movements, but this can be difficult to delineate if the other twin is still alive and moving.

1.4.4 Diagnostic criteria

This is confirmed by the absence of a fetal heartbeat on ultrasound scan.

1.4.5 Management

There is no specific guidance for the management of the surviving co-twin. Immediate preterm delivery of the surviving co-twin at diagnosis of the sIUFD is not advised unless the surviving co-twin is in fetal distress. Middle cerebral artery peak systolic velocity (MCA-PSV) measurement can be performed to assess for the presence of fetal anaemia, indicating major exsanguination and a worse prognosis for the surviving co-twin (Senat 2003). There is no evidence at present that in-utero transfusion improves outcome. Detailed brain imaging is advised for all co-twin

survivors, ideally by fetal MRI 4 weeks after the IUFD, to assess for brain injury (Kilby 2016, Mackie 2016).

1.4.6 Outcome for surviving co-twin

If the sIUFD occurs prior to 14 weeks there is no effect on the health of the surviving co-twin and the dead fetus is reabsorbed into the mother's body. If the sIUFD occurs after 14 weeks gestation the outcome for the surviving co-twin may be worse, depending on the type and direction of blood flow in the inter-twin anastomoses. A systematic review published in 2011 reported that subsequent co-twin death occurred in 15% of MC twins, PTB in 68%, and neurodevelopmental problems in 25% (Hillman 2011). This systematic review and meta-analysis has been updated in CHAPTER 7 with a focus on spontaneous IUFD, and additional outcomes have been explored to enable more tailored patient counselling.

1.5 Preterm birth (PTB)

PTB is a major cause of perinatal morbidity and mortality in twin pregnancies, with the majority of MC twins delivering before 37 weeks, according to international guidance (Khalil 2016b, Kilby 2016). The causes of PTB are multi-factorial and beyond the scope of this thesis.

1.6 Maternal complications

Women with twin pregnancies are at higher-risk of various pregnancy complications compared to women with singleton pregnancies, including: gestational diabetes mellitus (GDM), gestational hypertension, pre-eclampsia, eclampsia, post-partum haemorrhage, and caesarean section (Roach 1998, Conde-Agudelo 2000, Campbell 2004, Rao 2004, Rauh-Hain 2009). When these complications are examined in the context of chorionicity, no difference has been demonstrated between MC and DC twins in incidence of GDM and caesarean section, and although studies are conflicting regarding pre-eclampsia, the general consensus is that there is no difference in chorionicities (Carter 2015). This supports that these complications are related to the presence of greater placental mass, rather than characteristics inherent to MC or DC twins specifically.

1.7 Monochorionic twins antenatal care surveillance

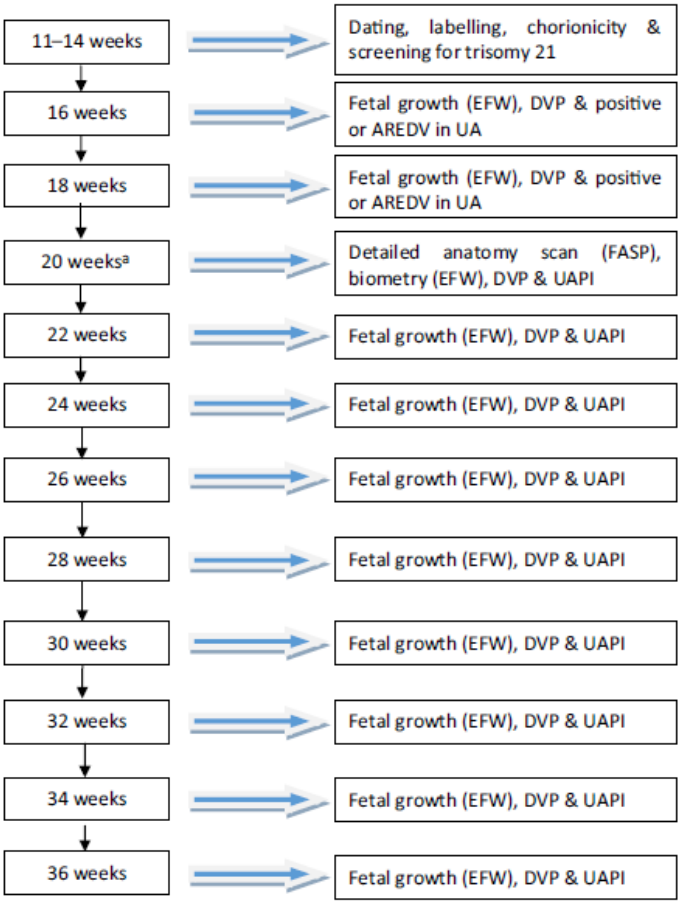
1.7.1 Uncomplicated monochorionic twins

In the first trimester, all twin pregnancies should undergo ultrasound examination to assess: gestation of pregnancy based on crown-rump length (CRL), chorionicity, the position and labelling of the twins, and major congenital malformations (NICE 2011, Kilby 2016). Screening for aneuploidy should be offered (see CHAPTER 3 for more detail). Risks of MC twins should be outlined to parents. As it is currently not possible to predict which pregnancies will develop TTTS, growth restriction or IUFD, all MC

twins are closely monitored. According to national and international guidance, all MC twin pregnancies should undergo ultrasonographic assessment of fetal growth, liquor volume, and umbilical artery Doppler assessment every 2 weeks, from 16 weeks gestation to assess for signs of TTTS and/or sIUGR (Figure 1.8) (ACOG 2016, Khalil 2016b, Kilby 2016, RANZCOG 2017). It is not known if this is the best model of care, but it is not ethically acceptable to decrease the level of antenatal surveillance, despite the fact that the majority of MC twins will not develop TTTS or growth restriction, and the resources and patient and health care professional time involved in this monitoring is intensive (McDonald 2017). A Cochrane review (Woolcock 2017) attempted to look at ultrasound surveillance regimes in twin pregnancies, but was only able to include one study (Giles 2003) which compared performing umbilical artery Doppler assessment at 25, 30 and 35 weeks gestation to not performing umbilical artery Doppler assessment, but was inadequately powered to demonstrate a statistically significant difference in outcome.

Figure 1.8 Antenatal surveillance in uncomplicated monochorionic twins

(taken from RCOG Green-top guidance (Kilby 2016) and ISUOG (Khalil 2016b))
AREDV: absent or reversed end-diastolic velocities, DVP: deepest vertical pocket, EFW: estimated fetal weight, FASP: Fetal Anomaly Screening Programme, UA: umbilical artery, UAPI: umbilical artery pulsatility index



1.7.2 Complicated monochorionic twins

If TTTS or EFWD >20% is suspected, referral to a tertiary fetal medicine centre is advised (Kilby 2016). If a woman has undergone FLA for TTTS, she should be monitored for TAPS by MCA PSV measurement. Women with a sIUFD after 14 weeks gestation should also be referred to a fetal medicine centre for further assessment, particularly of potential brain injury.

1.7.3 Mode of delivery

In uncomplicated MC twins, to lower the risk of IUFD, delivery is recommended at 36+0-37+0 weeks gestation, with antenatal corticosteroid cover (Kilby 2016). Mode of delivery is dependent on the presentation of the 1st twin and maternal choice. If the 1st twin is breech presentation, caesarean section is recommended. If the 1st twin is cephalic, vaginal delivery or caesarean section may be chosen. Women should be counselled regarding the higher risk of post-partum haemorrhage associated with all multiple pregnancies and thus active management of the third stage of delivery. The possibility of acute intrapartum TTTS should be discussed and continuous electronic fetal monitoring used to aid detection. If vaginal delivery is chosen, the possibility of requiring an emergency caesarean section for the 2nd twin should also be conferred. MC twins with a history of treated TTTS should be delivered between 34+0-36+6 weeks gestation (Kilby 2016). The guidance for timing of delivery of pregnancies affected by sIUGR is less clear, and should be decided on an individual case basis, based on fetal growth velocity, umbilical artery and DV Doppler velocity waveforms.

1.8 Prediction and prognosis of complications

1.8.1 Prediction, prognosis and risk factors

Prediction is a general term that encompasses diagnosis (the presence of a disease at that time) and prognosis (the future outcome risk). As this thesis is investigating the risk of developing a complication in the future, not the presence or absence of a complication at that present moment in time, this is prognostic research, not

diagnostic research. According to the international Prognosis Research Strategy (PROGRESS) Group, in clinical medicine “prognosis refers to the risk of future health outcomes in people with a given disease or health condition” (Hemingway 2013). It is thus the examination of the relationship between a baseline health state (startpoint) and a future outcome (endpoint). This may be from a startpoint of a clinical context such as pregnancy and examining the natural course of pregnancy and future development of complications, or it may be from the startpoint of the diagnosis of a pregnancy complication and the future outcome as a result of different treatments. This thesis will focus on the former as it does not investigate the effectiveness of treatment. In CHAPTER 2 and CHAPTER 3 the startpoint is MC twin pregnancy, and the endpoint is diagnosis of an adverse complication e.g. TTTS, growth restriction or IUFD. In CHAPTER 7 the startpoint is spontaneous sIUFD and the endpoint is the outcome for the surviving co-twin e.g. co-twin death, PTB, abnormal brain imaging. The first step in prognostic research is investigating potential prognostic factors.

1.8.2 Prognostic factors

A prognostic factor is any measure present in the startpoint population that is associated with a future outcome (Riley 2013). The term ‘risk factor’ is often used interchangeably with ‘prognostic factor’, but the former term is more appropriately reserved to indicate causal factors of disease onset. Whilst some prognostic factors are causal, the majority are not. The term ‘predictive factor’ may also be used, but as described previously, this is a more generic term and is not specific to prognosis, therefore the term ‘prognostic factor’ will be used in this thesis. Prognostic factors

may be: maternal characteristics such as body mass index (BMI); biomarkers such as metabolites or ultrasound measures; or symptoms or behavioural characteristics such as concurrent depression. These factors may already be known to be prognostic in clinical situations, or studies may be performed to discover novel potential prognostic factors based on biological feasibility and hypotheses of pathophysiology. To assess a potential prognostic factor, the association between the factor and outcome should be calculated, and its prognostic ability above existing prognostic factors ascertained (see section 3.4.8 for more details).

Establishing individual prognostic factors may inform diagnosis, highlight new disease sub-types, indicate disease progression, identify modifiable targets for intervention, and aid prediction of treatment response (see section 1.8.4). Prognostic factors can also be used in further research to inform sample size, randomisation procedure, and analysis strategy in randomised trials. However, a combination of multiple prognostic factors in a prognostic model often has a better prognostic ability than individual prognostic factors (Riley 2013).

1.8.3 Prognostic models

The aim of a prognostic model is to accurately calculate the risk an individual has of reaching a specific endpoint. Prognostic models are usually developed using data from cohort studies, and performing multivariable logistic regression. A prognostic model should be developed according to a robust statistical methodology, be easy to

use in a clinical setting, acceptable by patients, externally validated in a different cohort, and its implementation should have a positive impact on patient care.

To develop a prognostic model, several steps should be taken and various aspects should be reported, as outlined by the Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis (TRIPOD) statement (Moons 2015). The performance of the model is indicated by the *discrimination* that describes the ability of the model to discriminate between patients with TTTS and those without TTTS for example. The *calibration* of the model is another indicator of performance and reports the agreement between the predictive outcome and the observed outcome. Once the initial model has been developed, this requires *internal validation* to demonstrate the reproducibility of the model in the setting where the model was originally developed. Internal validation may be performed by boot-strapping whereby random samples are taken from the original dataset to create an additional dataset. Following internal validation, *external validation* should be performed to demonstrate the applicability of the model to different patient populations, ideally this should be performed by independent investigators in a different geographical area. This step is important as most models perform well when they are internally validated, however their prognostic ability is often optimistic, thus external validation is required (Smith 2014).

To produce a clinically useful prognostic model, several criteria need to be fulfilled as highlighted by Cheong-See et al. (Cheong-See 2016). There should be a suitable

sample size, which is particularly hard for conditions considered relatively rare in the general obstetric population such as TTTS. The choice of prognostic factors is important and should be based on a systematic review of existing factors, and evaluation of individual prognostic factors, prior to being combined in a prognostic model (Kleinrouweler 2016). The measurement of the prognostic factors and outcome(s) must be reliable so that the model can be replicated in various clinical settings.

As Cheong-See et al. highlight, many models, particularly in obstetrics, are not able to be used in clinical practice due to insufficient/non-existent external validation in a different cohort (Cheong-See 2016) and even fewer have been evaluated with regards to their impact on health outcome (Steyerberg 2013). This has been echoed by Kleinrouweler et al. who stated that there is “a rise in the number of prognostic models being published, including for obstetric outcomes, without the corresponding increase in the number of models being applied in practice”(Kleinrouweler 2016). Models should ultimately improve patient outcome by allowing timely prediction of adverse outcome and enabling subsequent prevention or treatment. A systematic review of obstetric prognostic models by Kleinrouweler et al. demonstrated that the majority of obstetric models do not meet these criteria and more work is required (Kleinrouweler 2016).

If a validated prognostic model is created, they produce more accurate risk predictions than clinical intuition (Grove 2000), and ideally allow the implementation of stratified medicine.

1.8.4 Stratified medicine

Stratified medicine is targeting care or treatment based on the risk shared by a sub-group of patients with similar characteristics (Hingorani 2013). For patients this means they receive more tailored care based on their individual risk prediction determined by either the presence of prognostic factors or the output of a prognostic model, with the aim of achieving an improved outcome. This may be receiving the best treatment option for them, or preventing the use of a treatment that will not be beneficial to them. As well as improving individual patient care, stratified medicine also has financial implications by allowing treatments to be used more appropriately.

In the setting of MC twin pregnancies, being able to stratify pregnancies as high-risk or low-risk in the first trimester could have several important effects. For those considered low-risk, patients could be reassured, and it may allow decreased antenatal surveillance. As previously highlighted, the majority of MC twin pregnancies will not develop TTTS or growth restriction for which additional ultrasonography is performed to detect. Decreased surveillance may improve patient satisfaction as they would not be required to attend antenatal clinic as frequently and have to arrange childcare or work cover. It would also decrease the impact MC twin pregnancies have on health care professionals' time and clinic resources. However, the prognostic factor/model would have to be highly accurate, as the consequence of classifying a patient incorrectly as low-risk and decreasing surveillance may mean that TTTS is not detected until too late, and fetal death has occurred. Alternatively, low-risk

patients could continue with the current antenatal surveillance, and high-risk patients could receive additional surveillance such as more frequent ultrasonography, or earlier/additional assessment by a fetal medicine centre. This does have financial implications, but may prevent fetal death as TTTS is a highly morbid and rapidly progressive condition, and even with current surveillance every 2 weeks, fetuses still die before FLA. Identifying high-risk pregnancies may enable the development of new therapies, and prophylactic treatments that at present are not possible.

The ability to predict which twins will develop a poor cardiac outcome following FLA may allow the targeted use of drugs such as nifedipine that has been associated with improved survival in recipient twins (Crombleholme 2010), and is currently being evaluated in an ongoing study (Gardiner 2018). It may also allow the opportunity to explore the use of other drugs such as digoxin (De Lia 1985, Arabin 1998) or sildenafil (Zaretsky 2014) either as adjuvants to FLA, novel pharmacological agents, or even first trimester prophylaxis.

1.9 First trimester potential prognostic factors

The first trimester potential prognostic factors examined in CHAPTER 3 are outlined in the chapter.

1.10 Potential prognostic factors from novel technologies

Potential prognostic factors may be found using novel technologies. '-omics' technologies are an evolving area of research that study the molecules of the whole biology of a system whether it is an organism, tissue, or cell. This is referred to as a holistic view, or high-dimensional biology (Horgan 2011). The molecular mechanisms of normal biological processes or disease processes can be studied. Another benefit of –omics technologies is that they are an ideal method to discover potential biomarkers. A large number of molecules can be explored simultaneously, various biofluids can be used, and experiments are hypothesis-generating i.e. no hypothesis is required before the study so the data are analysed and hypotheses are formed based on the results.

1.10.1 Genomics

Genomics covers the study of all DNA in an organism or cell. This can be in the form of the whole genome down to individual genes, or fragments of DNA such as cell-free fetal DNA. Although there are no published whole-genome arrays in TTTS pregnancies, there has been targeted gene work, for example examining renin gene expression in the fetal kidneys that is useful for understanding more regarding the pathophysiology (Kilby 1994).

1.10.2 Transcriptomics

Transcriptomics investigate the transcriptome, which are factors involved in translation of genes for protein synthesis. Most commonly this is messenger RNA (mRNA), but miRNA is also included in this category. No published studies have looked at changes in miRNA in TTTS and this will be explored in CHAPTER 6. The data derived from transcriptomics can then be related to which genes they are associated with, and which biological functions they affect.

A pilot study in 2005 demonstrated that cell-free fetal mRNA is present in the cell-free supernatant of amniotic fluid of TTTS pregnancies, and allows the investigation of fetal gene expression (Larrabee 2005). In 2013 the same group performed whole transcriptome analysis of cell-free RNA from amniotic fluid and reported a significant difference in the expression of 801 genes in samples from TTTS pregnancies prior to FLA compared to gestationally-matched singleton controls (Hui 2013). The group validated their findings in 4/6 genes they chose to validate: *FLT1, NTRK3, NRXN3* and *AVPR1A*. These genes are involved in cardiac and neurological development, and angiogenesis thus supporting that transcriptomics may be a useful tool to improve knowledge of TTTS pathophysiology, and provide potential candidates for fetal therapies in the future. Targeted work has also been performed based on initial transcriptomic studies. Miura et al. targeted 9 placenta predominant mRNAs in maternal plasma they had identified in singleton pregnancy transcriptomics. They found a significant difference in 6/9 mRNAs: *hPL, PSG2, PSG3 syncytin, syncytin2, ADAM12*, and suggested they may act as potential biomarkers for the development

of TTTS (Miura 2014). Fox et al. reported a significant difference in cell-free fetal mRNA in maternal plasma of: *VEGF-A, Endoglin, and Ang-2* between women with TTTS and uncomplicated twin pregnancies (Fox 2012). Another study reported a higher fold count of mitochondrial RNA in placental tissue from pregnancies with TTTS and concurrent sIUGR compared to those with sIUGR but no TTTS (Chang 2018). All these studies demonstrate that the transcriptome appears altered in TTTS and further research in this area is warranted.

1.10.3 Proteomics

Proteomics explore all the proteins expressed in the organism, tissue or cell and reflect the interaction between genes and the environment. Although many individual proteins have been examined in TTTS (Bajoria 1999a, Bajoria 2003, Adama van Scheltema 2005, Fox 2010, Van Mieghem 2010, Fox 2014a), see section 1.2.1.3, no hypothesis-generating studies were found where non-targeted proteomics were performed in the setting of TTTS.

1.10.4 Metabolomics

Metabolomics investigate metabolites, the phenotypes of metabolism, which represent the dynamic interaction between the genotype and its environment, and are the final downstream product. Consequently metabolomics may produce prognostic factors able to be used for prediction of diagnosis or prognosis, to stratify care, and improve knowledge of underlying molecular mechanisms (Beger 2016).

In singleton pregnancies, research using metabolomics is becoming increasingly popular with studies using the technique on various biofluids, including amniotic fluid, to examine IUGR (Horgan 2010, Diaz 2013, Stevens 2014), pre-eclampsia (Dunn 2009, Kenny 2010, Bahado-Singh 2015, Bahado-Singh 2017, Chen 2017) PTB (Thomas 2015, Baraldi 2016, Cecatti 2016, Virgiliou 2017), GDM (Dani 2014, Lehmann 2015, Chorell 2017), maternal smoking (Fischer 2017), also specifically in the first-trimester for prediction of aneuploidy (Bahado-Singh 2013a) and pre-eclampsia (Bahado-Singh 2012, Bahado-Singh 2013b, Austdal 2015, Koster 2015). In non-pregnant adults, relevant to this work it has been used to profile functional and metabolic changes associated with heart failure (Deidda 2015, Wang 2015). Metabolomics have also been investigated in first trimester maternal serum samples to predict congenital heart disease (Bahado-Singh 2014) and abnormal lipid metabolism appears to play a role.

In twin pregnancies there are fewer metabolomics studies with only one untargeted metabolomics study published in humans (Cosmi 2013). This study used liquid chromatography-high resolution mass spectrometry to examine the metabolome in cord blood from 4 sIUGR MC pregnancies and compare it to the metabolome in 4 appropriately grown MC twins (Cosmi 2013). They reported a trend of increased phenylalanine, and decreased isoleucine, proline, tryptophan and valine in sIUGR twins and theorise that the results did not reach statistical significance due to the small sample size. No hypothesis-generating metabolomics studies in TTTS could be found, but this is explored further in CHAPTER 5.

1.11 Conclusion

MC twins share a placenta which can result in various complications including highly morbid TTTS, growth restriction and IUFD. Currently it is not possible to predict which MC twin pregnancies will develop these complications. One way to improve care in these pregnancies is by identifying first trimester prognostic factors capable of determining which MC twin pregnancies will develop an adverse outcome. A further way to potentially improve care is to examine the relationship between FLA and parento-fetal/infant attachment and depression in pregnancies complicated by TTTS.

1.12 Hypothesis

I hypothesise that prognostic factors to predict the development of complications in MC twin pregnancies do exist, and that mothers and fathers cope with having a pregnancy affected by TTTS in different ways.

1.13 Thesis rationale and aims

This thesis aims to evaluate first trimester maternal serum and ultrasound measures as potential prognostic factors of complications in MC twins; investigate metabolite and miRNA changes in pregnancies with TTTS as novel second trimester potential prognostic factors; examine the prognosis of surviving co-twins following sIUFD to aid

counselling of parents regarding the risk of potential outcomes; and assess antenatal and postnatal parento-fetal/infant attachment and depression in pregnancies complicated by TTTS to help health care professionals identify couples needing additional support.

1.13.1 Aims

The aims of this thesis are to:

1) Identify first trimester potential prognostic factors of adverse outcome in MCDA twin pregnancies by systematically reviewing existing literature. (CHAPTER 2) (Systematic review and meta-analysis)
2) Evaluate first trimester potential prognostic factors (NT % discordance, CRL % discordance, β-hCG, Pregnancy-associated plasma protein-A (PAPP-A), AFP, sFlt-1, PlGF) of adverse outcome in MCDA twin pregnancies. (CHAPTER 3) (Retrospectively recruited cohort study)
3) Determine maternal serum levels of first trimester potential prognostic factors (AFP, sFlt-1, PlGF) in uncomplicated MCDA twin pregnancies. (CHAPTER 4) (Prospectively recruited case series)
4) Perform discovery work to identify second trimester novel potential prognostic factors (metabolomics in amniotic fluid, miRNA in maternal serum) of TTTS. (CHAPTER 5 and CHAPTER 6) (Retrospectively recruited case series, and prospectively recruited case-control study respectively)
5) Examine the prognosis of surviving co-twins following sIUFD by systemically reviewing existing literature. (CHAPTER 7) (Systematic review and meta-analysis)

6) Assess antenatal and postnatal parento-fetal/infant attachment and depression in pregnancies complicated by TTTS. (CHAPTER 8) (Prospectively recruited case series)

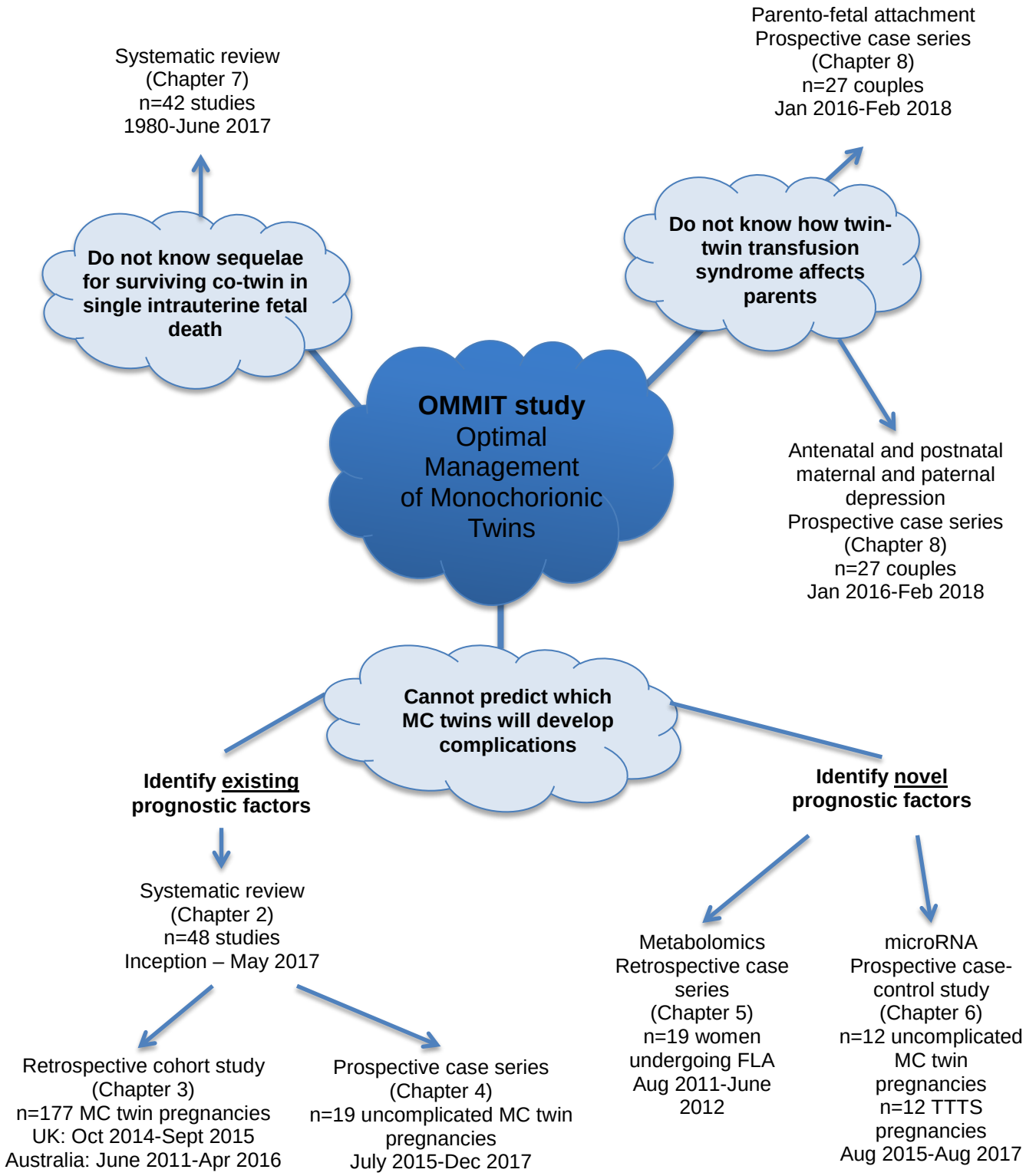
1.14 Methods

To identify first trimester potential prognostic factors a systematic review and meta-analysis was performed. The OMMIT (Optimal Management of Monochorionic Twins) study was designed to assess the prognostic ability of first trimester potential prognostic factors identified by the systematic review and meta-analysis, and by previous work performed in the second trimester that demonstrated a significant difference in various serum biomarkers in TTTS pregnancies (Figure 1.9). The prognostic ability was investigated in a retrospectively recruited cohort of women with MCDA twin pregnancies for whom there were stored first trimester maternal serum samples and ultrasound measures. Discovery work was undertaken to explore novel potential prognostic factors in the second trimester using two different types of –omics technology. Untargeted metabolomics on amniotic fluid samples taken during FLA from the recipient twin amniotic fluid sac was performed. The metabolite findings were correlated to the degree of cardiac dysfunction in the recipient twin. Transcriptomics was conducted to explore the role of miRNAs as potential prognostic factors by performing microarrays on second trimester maternal serum samples taken at diagnosis of TTTS and comparing the miRNA profiles to gestationally-matched serum samples taken from women with uncomplicated MCDA twin

pregnancies who were also recruited as part of the OMMIT study. The prognosis of surviving co-twins following spontaneous sIUFD was examined by systematic review and meta-analysis with sub-group analyses based on pregnancy characteristics to aid a personalised risk prediction. Antenatal and postnatal parento-fetal/infant attachment and depression in pregnancies complicated by TTTS was examined quantitatively using questionnaires that mothers and fathers were asked to complete separately prior to FLA, 1 month after FLA, and postnatally, to help health care professionals identify which couples may need additional support. See each chapter for more detailed methods.

Figure 1.9 The 'Optimal Management of Monochorionic Twins' (OMMIT) study

Dates refer to sample collection, or search date for systematic reviews



CHAPTER 2 EARLY PROGNOSTIC FACTORS OF OUTCOMES IN MONOCHORIONIC TWIN PREGNANCY: SYSTEMATIC REVIEW AND META-ANALYSIS

- These findings have been published in full article form [Mackie FL, Hall MJ, Morris RK and Kilby MD (2018). “Early prognostic factors of outcomes in monochorionic twin pregnancy: systematic review and meta-analysis.” Am J Obstet Gynecol; 219:436-46.]

2.1 Introduction

Monochorionic (MC) twin pregnancies are considered high-risk because of the potential to develop the morbid conditions of TTTS, TAPS, or TOPS (Sebire 1997, Moldenhauer 2015). Additionally, MC twins have a greater likelihood of developing sIUGR, and sIUFD and dIUFD compared to DC twins (Hillman 2011). Consequently, international professional guidelines advise that all MC twin pregnancies undergo ultrasound assessment of fetal growth, amniotic fluid volume, and umbilical artery Doppler velocimetry every 2 weeks from 16 weeks gestation (ACOG 2016, Khalil 2016b, Kilby 2016). However, the majority of MC twins will not develop any of these complications (Lewi 2008). At present, no screening test is available to predict which MC twin pregnancy will develop complications and therefore all MC twins undergo this intensive antenatal surveillance that has an impact on patients and healthcare resources.

2.1.1 Objectives

To assess the ability of first trimester pregnancy related factors (ultrasound measurements, maternal characteristics, and biomarkers) to predict complications in MC twin pregnancies.

2.2 Materials and methods

The systematic review was performed according to an *a priori* protocol and complied with recommended guidance including the 'Meta-analyses and systematic reviews Of Observational Studies' (MOOSE) and 'Preferred Reporting Items for Systematic reviews and Meta-Analyses' (PRISMA) guidelines (Stroup 2000, Moher 2009). Previous work by our group has shown a correlation between comparing methodological and reporting quality in diagnostic test accuracy, and prognostic factor accuracy (Selman 2011). Ethical approval was not required.

2.2.1 Eligibility criteria

Studies that reported ultrasound measurements, maternal characteristics, or potential biomarkers, measured in the first trimester (i.e. up to 14 weeks gestation), in MCDA twin pregnancies that provided sufficient information to assess the association between the variable and outcome were eligible for inclusion. Monochorionicity had to have been confirmed either by the presence of the 'T' sign or absence of the 'lambda [λ]' or 'twin peak' sign on first trimester ultrasound (Sepulveda 1996), or postnatally by placental examination. If DC twin pregnancies were included, these

were removed prior to analysis; if it was not possible to identify and remove the DC twin pregnancies, the study was not considered eligible for inclusion. Pregnancies affected by the following were excluded: major structural or chromosomal anomalies, twin-reversed arterial perfusion (TRAP), miscarriage, sIUFD <14 weeks gestation, higher order multiple or monoamniotic pregnancies. All study designs were included, although case series needed to include at least 5 MCDA twin pregnancies.

2.2.2 Potential prognostic factors

All first trimester potential prognostic factors were included. Data were extracted using the same cut-offs as reported by the authors. For the meta-analysis thresholds were not combined (i.e. CRL discordance >10% was not combined with CRL discordance >20%). Maternal age and BMI were analysed as continuous variables. Maternal ethnicity was dichotomised to 'Caucasian' and 'non-Caucasian' to enable meta-analysis, parity was dichotomised to 'multiparous' and 'nulliparous', maternal smoking was dichotomised to current 'smoker' and 'non-smoker' with ex-smokers included in the 'non-smoker' group, and mode of conception was dichotomised to 'spontaneous' or 'ART'.

2.2.3 Outcomes

The outcomes evaluated were:

- TTTS, irrespective of whether treatment was required/performed, and according to definitions used by authors of individual studies (see Table 2.1 in

section 2.3.3) including significant discrepancy in inter-twin amniotic fluid volumes as per Quintero (Quintero 1999).

- Antenatally-detected growth restriction only (AGR), based on EFW (irrespective of the presence of umbilical artery Doppler abnormalities), as defined by each study. Regardless of definition used within individual studies, the consensus definition of ≥20% inter-twin EFW discordance as per the American College of Obstetricians and Gynecologists, the International Society of Ultrasound in Obstetrics & Gynecology (ISUOG) and the Royal College of Obstetricians and Gynaecologists in the UK (RCOG) (ACOG 2016, Khalil 2016b, Kilby 2016) was adopted.
- Postnatally-detected growth restriction only (PGR), based on birthweight (BW) as defined by each study, but if reported as inter-twin discordance, must be ≥20%
- Antenatal and postnatal growth restriction within the same pregnancy i.e. antenatal growth restriction was diagnosed and confirmed postnatally.
- Antenatal or postnatal growth restriction (AoPGR) which includes all the growth restricted pregnancies in the other three growth restriction groups (AGR, PGR, antenatal and growth restriction within the same pregnancy)
- sIUFD after 14 weeks gestation
- dIUFD after 14 weeks gestation

The definitions of the outcomes were not pre-specified to allow for variation of definitions. Where a definition exists e.g. Quintero for TTTS, ≥20% for antenatal growth discordance, this was adopted for decisions regarding inclusion of studies for

meta-analysis. For those analyses where there was variation in definitions, sensitivity analysis was employed where possible to determine the effects of the definition on the results.

2.2.4 Information sources

Electronic databases were searched: MEDLINE, EMBASE, ISI Web of Science, CINAHL, the Cochrane Central Registration of Controlled Trials and Research Registers, and Google Scholar, from inception to 12 May 2017. Grey literature was hand searched and bibliographies of articles checked.

2.2.5 Search strategy

Keywords and MeSH terms relating to the following were used: TTTS, TAPS, TOPS, fetal death, IUGR, diseases in twins, amniotic fluid, placenta, biomarkers, ultrasonographic markers and prediction; and combined with “monochorionic” and “twins” (Appendix 10.1).

2.2.6 Study selection and data extraction

Manuscripts to be included in the review were selected by two reviewers (FLM, MJH) independently in a two stage process; the first being review of titles and abstracts for selection for the second stage of full manuscript review. Where there was disagreement consensus was reached by a third reviewer (RKM). There was no restriction on language or study design. Abstracts were included if there was

sufficient information to assess the study quality and association between the variable and the outcome. Data were extracted independently by FLM and MJH using a purposely-designed data collection form (Appendix 10.2). Any discrepancies were resolved by RKM and MDK. Authors were contacted to clarify information as required.

2.2.7 Quality assessment of included studies

The quality of the studies was assessed by FLM and MJH using the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) tool (von Elm 2007) as this was considered most appropriate as the majority of studies were observational. It was not possible to use the recommended quality checklists for prognostic factor research (Riley 2013) e.g. Quality in Prognosis Studies (QUIPS) (Hayden 2013), REporting recommendations for tumour MARKer prognostic studies (REMARK) (McShane 2005), nor diagnostic studies Quality Assessment of Diagnostic Accuracy Studies (QUADAS) (Whiting 2011) due to the large number of included studies that were not focused on the prognostic value of factors.

2.2.8 Assessment of heterogeneity

Forest plots were created to visually assess outliers and any unusual results were investigated with sensitivity analysis. The I^2 value was calculated for each meta-analysis. A measurement ≥50% indicated a substantial risk of heterogeneity. Where there was significant heterogeneity (visually or statistically), a sensitivity analysis was performed to assess the effect.

2.2.9 Assessment of reporting bias

In meta-analyses with >10 studies, a funnel plot was generated using the *metafunnel* command (Sterne 2003) in Stata (Stata, 2015 Release 13.1 StataCorp, Texas, USA), and Egger's test was performed using the *metabias* command (Harbord 2000), with a significance level of 10%.

2.2.10 Data synthesis

Meta-analyses were reported per pregnancy, not per fetus for two reasons. Firstly, reporting at the fetus level would require an adjustment for clustering, but more importantly when considering prognostic factors for pregnancy related diseases in multiple pregnancy, any change in antenatal management due to a prognostic test/model would be effected at the pregnancy level.

2.2.11 Data synthesis for factors reported as means and medians

For continuous data with a normal distribution, medians were converted to means to enable meta-analysis. When the interquartile range (IQR) was reported, the standard deviation (SD) was calculated as IQR/1.35 (Cochrane 2011). When medians were not reported with IQRs, the mean and SD was estimated (Hozo 2005). For non-normally distributed data (NT discordance, CRL discordance and parity) where only the median was reported, it was not possible to convert the median to means, therefore these results could not be included in meta-analysis.

2.2.12 Data synthesis for association

Data were extracted to create 2x2 contingency tables to compare: a) disease vs. no disease but where other complications may be present, and b) disease vs. normal pregnancy where no complications were present at all. For outcomes with more than three included studies, odds ratios (OR) were calculated using the *metan* command (Harris 2009) in Stata, and pooled using the DerSimonian-Laird random effects model to account for expected clinical heterogeneity. ORs >2 were considered to demonstrate a moderate association (Morris 2014), thus the prognostic ability of the factor was subsequently investigated. For continuous variables reported as, or converted into, means and SDs, the standardised mean difference (SMD) was calculated using the *metan* command in Stata. SMDs ≥0.5 were considered to demonstrate a moderate effect (Cohen 1977) and the prognostic ability of the factor was investigated.

2.2.13 Data synthesis for prognostic ability

Bivariate meta-analysis using a random effects model was performed in analyses of more than three studies to calculate the summary sensitivity, specificity, positive likelihood ratio and negative likelihood ratio using the *metandi* command (Harbord 2008) in Stata. Hierarchical Summary Receiver Operating Characteristic (HSROC) curves were generated using the *metandiplot* command (Harbord 2008) to represent the level of uncertainty of sensitivity and specificity for bivariate analyses. Univariate analysis was carried out for analyses with less than four studies using MetaDiSc (v1.4116, Madrid, Spain)(Zamora 2006), with symmetrical Summary Receiver

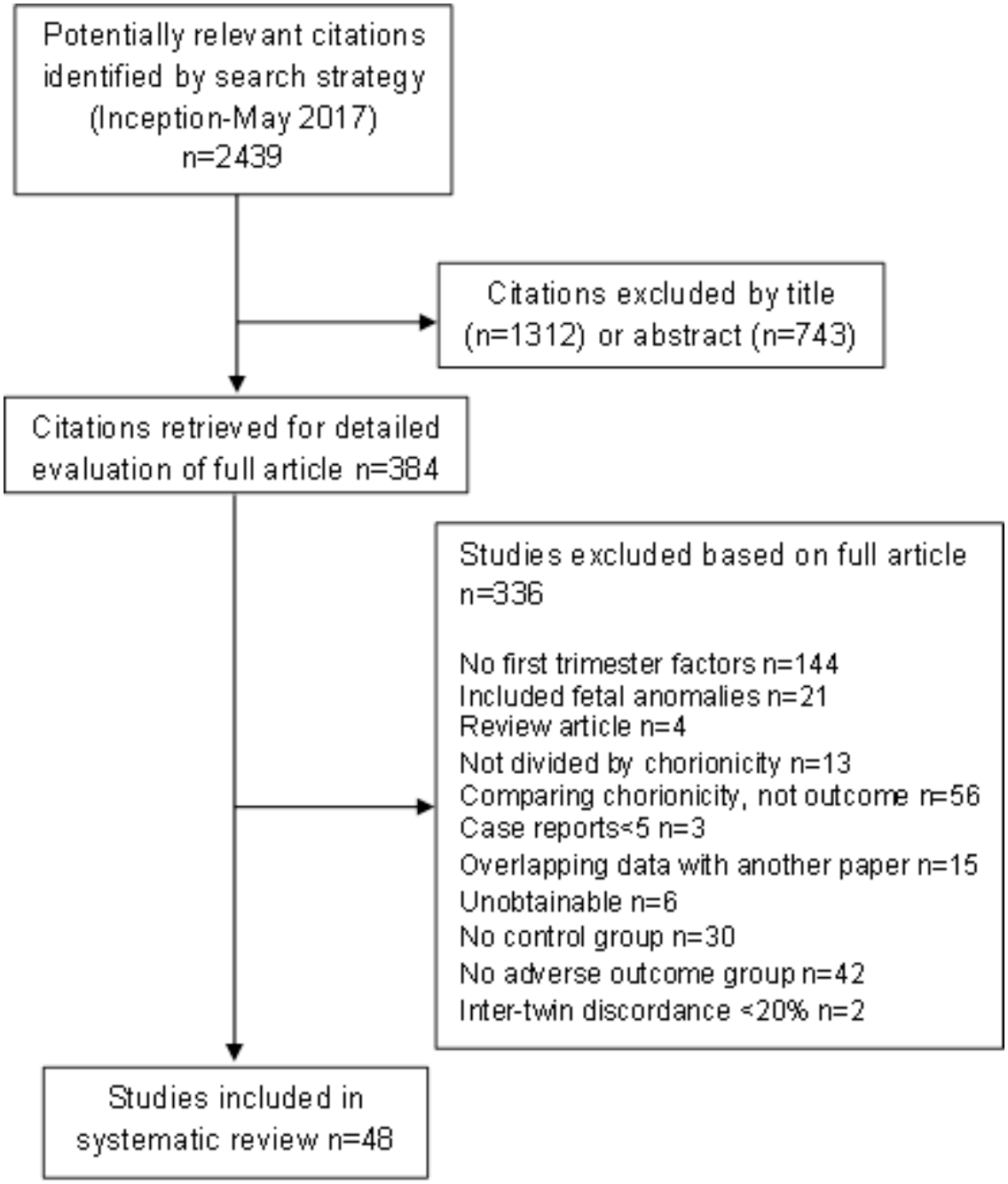
Operating Characteristic (SROC) curves generated. 0.5 was added to cells of 0 to perform univariate meta-analysis, but not bivariate meta-analysis, due to the necessary use of different computer programs. When a prognostic factor was found to have a moderate association with the outcome, the predictive ability was investigated and the post-test probability using Fagan's nomogram (Fagan 1975), which accounts for pre-test probability, was calculated.

2.3 Results

2.3.1 Study selection

Electronic searches identified 2439 citations of which 1312 titles were excluded after review of titles and 743 after abstract review. The full papers of the remaining 384 full articles were assessed (Figure 2.1) of which 48 studies (Sebire 2000, Sooranna 2001, Matias 2005, Bajoria 2006, Sueters 2006, Bajoria 2007, El Kateb 2007, Kagan 2007, Tai 2007, Kusanovic 2008, Lewi 2008a, Lewi 2008b, Chang 2009, Linskens 2009a, Maiz 2009, Linskens 2010, Matias 2010, Torres-Torres 2010, Carver 2011, Fratelli 2011, Matias 2011, Murakami 2011, Memmo 2012, Velayo 2012, Ashoor 2013, Chai 2013, Cosmi 2013, Flock 2013, Ghalili 2013, Moriichi 2013, Schrey 2013, Taylor-Clarke 2013, Zhao 2013a, Baraa Allaf 2014, Fujioka 2014, Johansen 2014, Miura 2014, Yinon 2014, Zanardini 2014, Zoppi 2014, Sarais 2015, Zhang 2015, Ben-Ami 2016, Chang 2016, Stagnati 2016, Chang 2017, McDonald 2017, Sun 2017) met the inclusion criteria equating to the evaluation of 5365 MC twin pregnancies. See Table 2.1 for the study characteristics of all included studies.

Figure 2.1 Flow diagram of study inclusion



2.3.2 Studies and potential prognostic factors not included in meta-analyses

Studies that met the inclusion criteria but were unable to be included in meta-analysis are described below, as are individual prognostic factors that were unable to be included in meta-analysis.

Studies

In calculating discordance between ultrasound measures, all studies used the larger measure as the denominator, apart from Memmo et al. (Memmo 2012) who used the smaller NT as the denominator, however as they only reported the median NT discordance, there were too few studies to include this variable in meta-analysis. Chang et al. (Chang 2009) could not be included in the analysis because they divided their AGR group into those with and without umbilical artery Doppler abnormalities and as the variable of maternal age was reported as a mean, the groups could not be combined. Cosmi et al. (Cosmi 2013) was not included in the maternal age analysis for the same reason as Chang et al. Sun et al. (Sun 2017) divided their control group into two sub-groups that could not be combined and thus could not be included in the maternal age analysis. Velayo et al. (Velayo 2012) could not be included in this analysis because they did not report the standard deviations.

Flöck et al. (Flock 2013) was not included in the TTTS outcome as the participants were also included in the larger cohort reported by Ben-Ami et al. (Ben-Ami 2016). Zhao et al. (Zhao 2013a) was not included in the TTTS analysis because they excluded those with TTTS who had undergone FLA and the group would have been

heterogeneous. El Kateb et al. (El Kateb 2007) could not be included in the PGR group because the variables and outcomes were only reported per fetus. The 'early onset' growth discordance group in the study by Lewi et al. (Lewi 2008a) was not included as their definition of the outcome and variable both included difference in CRL. Kagan et al. (Kagan 2007) could not be included in the IUFD outcome because they only reported sufficient information for a 2x2 contingency table on their sub-group of IUFD at 13-18 weeks. Nine studies were unable to be included in any meta-analysis due to an insufficient number of studies reporting the same variable and outcome (Tai 2007, Lewi 2008a, Lewi 2008b, Maiz 2009, Linskens 2010, Matias 2010, Matias 2011, Johansen 2014, Zoppi 2014).

Potential prognostic factors

The following factors were reported for the outcomes under examination, but were unable to be included in meta-analysis:

Ultrasound measurements

The following factors were reported by less than 3 studies thus could not be included in meta-analysis: abnormal DV Doppler in one/both fetuses (Maiz 2009, Matias 2010); umbilical venous volume flow in fetus 1 ≥ fetus 2 (Zoppi 2014). NT discordance ≥10%, ≥30%, ≥40%, ≥50% (Kagan 2007); NT discordance >3.5 mm (Linskens 2009a); NT difference (Matias 2010, Flock 2013); NT ratio (Matias 2010); NT larger twin (Memmo 2012, Zoppi 2014); NT smaller twin (Memmo 2012, Zoppi 2014); NT any twin (Matias 2010). CRL discordance >4%, ≥5%, ≥5.5%, ≥7%, ≥10%,

≥15%, ≥20% (Kagan 2007, Fratelli 2011, Johansen 2014); CRL discordance >11% (Tai 2007); CRL discordance ≥6 mm, ≥12 mm (Lewi 2008b); CRL difference (Matias 2010); CRL ratio (Matias 2010); CRL larger twin (Memmo 2012, Zoppi 2014); CRL smaller twin (Memmo 2012, Zoppi 2014); CRL any twin (Matias 2010).

Maternal characteristics

Gravida (Kusanovic 2008, Yinon 2014), height, and weight (Kusanovic 2008).

First trimester serum markers

Four first trimester maternal serum markers were investigated: β-hCG, PAPP-A, thyroid stimulating hormone (TSH) and free thyroxine (FT4), but meta-analysis was not possible. Linskens et al. (Linskens 2010) reported a trend in difference in β-hCG multiples of the median (MoM) in pregnancies with TTTS, and uncomplicated MC twins (median 1.99 vs 1.53 respectively, p=0.32, 51 pregnancies) and PAPP-A (median 1.94 vs 1.69 respectively, p=0.51, 51 pregnancies). Ashoor et al. (Ashoor 2013) reported no difference in TSH, FT4 or free β-hCG MoM in 17 pregnancies with TTTS and 'normal outcome twins', although the authors do not state if the control group included DC twins thus the number of MC twin pregnancies in the analysis is not clear. The following levels are reported in the TTTS and 'normal outcome twins' respectively: TSH (median 1.38 [IQR 0.52–2.05] vs 1.00 [IQR 0.26–1.36] respectively, p=0.424, number of pregnancies not clear), FT4 MoM (median 0.94 [IQR 0.90–1.16] vs 0.98 [IQR 0.91–1.08], p=0.773, number of pregnancies not clear), free β-hCG MoM (median 0.95 [IQR 0.51–2.22] vs 1.00 [IQR 0.69–1.36], p=0.997,

number of pregnancies not clear). These serum markers were only reported in the context of TTTS, and not the other outcomes.

2.3.3 Study characteristics

Table 2.1 displays the study characteristics of the included articles and details regarding individual measurements such as definitions of growth restriction. 26/48 studies were published in the 5 years prior to May 2017 when the search was performed. The oldest study was from 2000. 37 studies were cohort studies, 4 studies were case-controls, 2 were case series, and 5 were unclear in their study design. Data collection was a retrospective search of a prospectively recorded database in 15 studies, 20 performed prospective data collection, 6 were retrospective data collection, and it was not clear in 7 studies. Enrolment was consecutive in 16 studies, and not stated in 32 studies. 32 groups of patients were from 9 different European countries, the UK being the commonest (n=10), 6 from the USA and Canada, 4 from China, 4 from Japan, 3 from Taiwan, 2 from Australia, 2 from Israel, 1 from Mexico. The longest study ran for 16 years, with the modal time period being 2 years and shortest being 1 year. The smallest study analysed 12 women, and the largest analysed 470 women.

The ultrasound measurements reported were: NT, CRL, the presence of reversed ‘a’ wave in the DV, and umbilical venous flow velocity. The maternal characteristics reported in eligible studies were: maternal age, ethnicity, BMI, parity and smoking. Mode of conception and fetal gender were also reported. The first trimester biomarkers reported were all from maternal serum, and included TSH, FT4, β-hCG

and PAPP-A. The most frequently investigated outcomes were TTTS (n=31 studies), AGR (n=14 studies), and PGR (n=12 studies).

Table 2.1 Characteristics of studies eligible for inclusion

Abbreviation: 1st T: 1st trimester, AC: abdominal circumference, AFI: amniotic fluid index, ART: assisted reproduction technology, AUC: area under (receiver operating characteristic) curve, β-hCG: beta-human chorionic gonadotropin, BMI: body mass index, BWD: birthweight discordance, C-section: Caesarean section, CRL: crown-rump length, DA: diamniotic, DC: dichorionic, DV: ductus venosus (Doppler), DVP: deepest vertical pocket, EFWD: estimated fetal weight discordance, EXC: exclusion criteria, FLA: fetoscopic laser ablation, GD: growth discordance, INC: inclusion criteria, IUFD: intrauterine fetal demise, IUGR: intrauterine growth restriction, IVF: in vitro fertilisation, LBW: low birthweight, MA: monoamniotic, MC: monochorionic, MoM: multiple of median, NT: nuchal translucency, PAPP-A: pregnancy-associated plasma protein A, proBNP: prohormone of brain natriuretic peptide, sIUGR: selective intrauterine growth restriction, TAPS: twin anaemia-polycythaemia sequence, TOP: termination of pregnancy, TRAP: twin reversed arterial perfusion syndrome, TTTS: twin-twin transfusion syndrome, UAD: umbilical artery Doppler, USS: ultrasound scan, UVVF: umbilical venous volume flow A
*additional information/clarification obtained by contacting the authors

Author year	Study design, data collection, enrolment	Study population (Location, years, inclusion and exclusion criteria)	Total pregnancies eligible for study	Total MC pregnancies analysed in study	Outcomes used in review	Outcome definition used by study	Control group(s) definitions used by study	Potential prognostic factors
Ashoor 2013 (Ashoor 2013)	Case series, prospective, not stated	*Location:* ?1 centre in UK *Years:* 2006-2011 *INC:* twin pregnancies with live fetuses at 11-13 weeks 2 livebirths at ≥33 weeks, or severe TTTS necessitating FLA *EXC:* Maternal history of hypo/ hyperparathyroidism or diabetes, fetal abnormalities, pre-eclampsia, BW<5th centile	Not stated	77	TTTS	'polyhydramnios surrounding the recipient fetus whose bladder was enlarged, oligo-/anhydramnios around the smaller donor fetus whose bladder was collapsed.'	Normal pregnancies with 2 livebirths at >33 weeks	Thyroid hormones, mode of conception (spontaneous or use of ovulation inducing drugs), maternal age (median), BMI (median), ethnicity
Bajoria 2006 (Bajoria	Unclear, not stated, not stated	*Location:* 1 centre in UK *Years:* not stated *INC:* MC pregnancies	Not stated	32	BWD and sIUGR in same	BWD ≥20% with no polyhydramnios in	BWD ≤10% and normal liquor volume in both twins	Maternal age (median), ethnicity

2006)		with/without discordant growth *EXC:* chronic TTTS, single/double IUFD, intrapartum stillbirth, aneuploidy, structural abnormalities, pregnancies with: diabetes, hypertension, renal disease, cardiac disease			pregnancy	the larger twin, and smaller twin must have AC ≤5th centile with abnormal UAD, in same pregnancy		
Bajoria 2007 (Bajoria 2007)	Unclear, not stated, not stated	*Location:* ? centres in UK *Years:* not stated *INC:* MC pregnancies with/without TTTS *EXC:* single/double IUFD, intrapartum stillbirth, aneuploidy, structural abnormalities, pregnancies with: diabetes, hypertension, renal/cardiac disease	Not stated	30	TTTS	AFI ≥40cm in larger twin and ≤4cm in smaller twin. The smaller twin must also have IUGR, and the EFWD ≥15%, all in same pregnancy.	Concordant growth and AFI ≤24cm	Maternal age (median)
Baraa Allaf 2014 (Baraa Allaf 2014)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database and study	*Location:* 9 centres in USA *Years:* 2007-2011 *INC:* 2 live fetuses at 11-13^{+6} weeks *EXC:* chromosomal abnormalities, major congenital malformations, single/double IUFD in 1st T	Not stated	177	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	NT >95th centile in one/both fetuses, NT discordance ≥20% (AUC), CRL discordance (AUC), combined NT and CRL discordance (AUC)
					IUGR	EFW<10th centile in either fetus	No IUGR	NT >95th centile in one/both fetuses

Ben-Ami 2016 (Ben-Ami 2016)	Cohort, not stated, not stated	*Location:* 7 centres in Israel, Spain, Germany and Canada *Years:* 1997-2013 *INC:* MCDA twin pregnancies undergoing NT scan at 11-14 weeks with 2 live fetuses *EXC:* fetal congenital/structural abnormalities, single/double IUFD, higher order multiple reductions, non-IVF fertility treatments	337	327	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	Mode of conception (spontaneous or IVF/ICSI), maternal age (mean)
Carver 2011 (Carver 2011)	Cohort, retrospective collection of prospectively recorded data, consecutive	*Location:* 1 centre in USA *Years:* 2000-2009 *INC:* MCDA twins delivered at hospital and who underwent antenatal care at that hospital *EXC:* No sonographic examinations in 2nd T, no antenatal records	151	145	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	Maternal age (mean), ethnicity
Chai 2013 (Chai 2013)	Cohort, retrospective, not stated	*Location:* 1 centre in China *Years:* 2005-2012 *INC:* MCDA pregnancies *EXC:* Aneuploidy/fetal anomalies, TTTS, TRAP, TAPS, LBW in both twins	Not stated	113	LBW	BW <10th centile in 1 twin, BW>10th centile in the other twin	No LBW	Maternal age (mean)
Chang 2009 (Chang 2009)	Cohort, prospective, not stated	*Location:* 1 centre in Taiwan *Years:* 2006-2008 *INC:* live-born MCDA twins with a placenta able to be studied postnatally *EXC:* fetal anomalies, single IUFD, TTTS	53	51	sIUGR	EFW <10th percentile in 1 twin, with and without UAD abnormalities	No TTTS or sIUGR	Maternal age (mean)

Chang 2016 (Chang 2016)	Cohort, unclear, consecutive	*Location:* 1 centre in Taiwan *Years:* 2013-2015 *INC:* MC twins delivered by C-section with cord blood samples *EXC:* women who went into labour, TTTS, TAPS, congenital/structural or genetic malformations	Not stated	32	sIUGR	The definitions are not clear. The terms sIUGR, fetal weight and BWD are used inter-changeably. Authors state: BWD >20% and BW <10th centile in 1 twin according to pregnancy birthweight chart, which is subdivided to those with and without UAD abnormalities	"Normal" MC twins, definition not stated	Fetal gender
Chang 2017 (Chang 2017)	Cohort, unclear, unclear	*Location:* 1 centre in Taiwan *Years:* 2013-2014 *INC:* MC twins delivered at centre with cord blood samples *EXC:* TTTS, congenital/structural or genetic malformations	Not stated	24	BWD and sIUGR in same pregnancy	EFW <10th centile and BWD >20% in same pregnancy	No sIUGR	Maternal age (mean), parity (mean)
Cosmi 2013 (Cosmi 2013)	Unclear, prospective, selected but unclear how	*Location:* 1 centre in Italy *Years:* 2009-2011 *INC:* MCDA pregnancies selected from previously published cohort but inclusion criteria not stated *EXC:* unknown last menstrual period, unknown chorionicity, triplets, TTTS or related conditions, MCMA, 1st T	Not stated	12	sIUGR	EFW<10th centile in smaller twin, >10th centile larger twin, with and without UAD abnormalities	EFW >10th centile in both twins and confirmed after birth, and normal UAD	Maternal age (median), parity

		discrepancy in CRL>5 days, structural/ chromosomal abnormalities, single IUFD, selective feticide, maternal history of cardiovascular disease, endocrine disorders, clinical chorioamnionitis, maternal consumption of: alcohol, drugs of abuse						
El Kateb 2007 (El Kateb 2007)	Cohort, prospective, consecutive	*Location:* 1 centre in France *Years:* 2002-2006 *INC:* MCDA pregnancies with 1st T NT and CRL measurements *EXC:* chromosomal abnormalities or congenital malformations	Not stated	103	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	NT >95th centile (per fetus), CRL discordance ≥10% (per pregnancy)
					LBW	BW <5th percentile (per twin analysis)	No LBW	NT >95th centile (per fetus), CRL discordance ≥10% (per fetus)
Flöck 2013 (Flock 2013)	Cohort, retrospective search of database with prospectively recorded data, enrolment to database and study not stated	*Location: ?* centre in Germany *Years:* 2004-2010 *INC: '*unaffected*'* twins on perinatal database *EXC:* structural fetal malformations, aneuploidy, vanishing twin, embryo reduction	849 fetuses (does not state how many pregnancies)	706 fetuses, equating to 353 pregnancies in total, 73/353 MCDA, 280 DCDA	sIUGR	Not stated	No sIUGR	Mode of conception (spontaneous or IVF/ICSI)
Fratelli 2011	Cohort, retrospective,	*Location:* 1 centre in Italy *Years:* 2001-2009	136	135	TTTS	Polyhydramnios (DVP ≥8cm	No TTTS	NT discordance

(Fratelli 2011)	retrospective search of database with prospectively recorded data, consecutive enrolment to database but unclear to study	*INC:* 1st T viable MC twin at 11-13+6 weeks and follow-up at that centre *EXC:* pregnancies referred at later gestation, aneuploidy				before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin		≥20%, NT >95th centile in one, NT >95th centile in both, NT >95th centile in one/both fetuses, NT discordance (median), NT discordance (AUC), CRL discordance ≥10%, CRL discordance (median), CRL discordance (AUC)
					sIUGR	EFW<10th percentile and abnormal UAD in same pregnancy	No sIUGR	As for TTTS outcome
					IUFD	Miscarriage <24 weeks or spontaneous death of at least 1 fetus	No IUFD	As for TTTS outcome
Fujioka 2014 (Fujioka 2014)	Cohort, prospective, not stated	*Location:* 1 centre in Japan *Years:* 2007-2010 *INC:* MCDA twins with N-terminal proBNP levels measured at delivery *EXC:* congenital/ chromosomal abnormalities, TTTS, referred to centre >26 weeks	124	73	sIUGR	EFW <10th percentile at 18-26 weeks	No sIUGR	Mode of conception (spontaneous or ART), fetal gender, maternal age (median)

Ghalili 2013 (Ghalili 2013)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database and study	*Location*: 2 centres in Australia *Years:* 2006-2010 *INC:* MCDA twins undergoing 1st T scan, *EXC*: higher order multiples, non-viable or lethal structural anomalies at 12 week scan, unable to determine mode of conception or pregnancy outcome	312	294	LBW in one/ both twins	BW <10th centile in one/both twins	BW >10th centile in both twins	Mode of conception (spontaneous or IVF)
					TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	Mode of conception (spontaneous or IVF)
Johansen 2014 (Johansen 2014)	Cohort, retrospective search of database with prospectively recorded data, enrolment to database and study unclear	*Location:* 14 centres in Denmark *Years:* 2004-2006 *INC:* 2 live DA fetuses and chorionicity determined in 1st T *EXC:* unknown chorionicity, MCMA, reduction from higher order multiple, selective feticide or termination due to severe malformation/ chromosomal anomaly	281	260	BWD	BWD ≥20%	BWD <20%	CRL discordance ≥10%, CRL discordance ≥10% (AUC), CRL discordance ≥4% (OR), CRL discordance ≥5.5% (OR), CRL discordance ≥7% (OR), CRL discordance ≥10% (OR)

					Fetal loss of at least 1 fetus	Miscarriage ≤23+6 weeks, IUFD >23+6 weeks	2 livebirths	As for BWD outcome
Kagan 2007 (Kagan 2007)	Cohort, retrospective search of database with prospectively recorded data, enrolment to database and study unclear	*Location:* 1 centre in UK *Years:* 2001-2006 *INC:* MCDA pregnancies undergoing combined 1st T aneuploidy screening *EXC:* chromosomal or structural defects, pregnancies with missing outcome data	560	470	TTTS	Severe TTTS requiring FLA. TTTS defined as: polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	NT discordance ≥20%, NT discordance (median), NT discordance (AUC), CRL discordance ≥10%, CRL discordance (median), CRL and NT discordance (AUC)
					IUFD ('early')	Single/double IUFD at 13-18 weeks with no intervention	Not clear	NT discordance (AUC), CRL and NT discordance (AUC)
Kusanovic 2008 (Kusanovic 2008)	Case-control, database with prospectively recorded data, enrolment to database and study unclear	*Location:* ? centres in USA and China *Years:* not stated *INC:* MCDA twin pregnancies 16-26 weeks *EXC:* pre-eclampsia at time of venepuncture, fetal congenital anomalies	Not stated	69	TTTS	Polyhydramnios (DVP ≥8cm) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	Maternal age (median), gravida, height (median), weight (median), BMI (median), smoking

Lewi 2008a (Lewi 2008a)	Cohort, prospective, not stated	*Location:* 2 centres in Belgium and Germany *Years:* 2004-2007 *INC:* MCDA twin pregnancies *EXC:* TTTS, spontaneous miscarriage, IUFD <16 weeks, structural anomalies that influence biometry	208	163	GD ('late' onset)	BWD ≥25% but EFWD<20% at 20 weeks	BWD <25%	CRL difference (mean)
Lewi 2008b (Lewi 2008b)	Cohort, prospective, not stated	*Location:* 2 centre in Belgium and Germany *Years:* 2002-2007 *INC:* MCDA twin pregnancies with 2 live fetuses at 11-14 weeks *EXC:* single/double IUFD, TRAP, structural anomalies	202	200	TTTS	polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	CRL discordance ≥6mm
Linskens 2009 (Linskens 2009a)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database and study	*Location:* 1 centre in Netherlands *Years:* 2004-2008 *INC:* MCDA twins undergoing combined 1st T aneuploidy screening and followed up *EXC:* single/ double IUFD, PTB	61	55	TTTS	polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS and birth >26 weeks	NT discordance ≥20%, NT discordance ≥3.5mm, NT discordance (median), NT discordance (AUC), CRL discordance (median), maternal age (median), ethnicity, smoking, parity, mode of conception (spontaneous or ART)

Linskens 2010 (Linskens 2010)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database and study	*Location:* 1 centre in Netherlands *Years:* 2004-2009 *INC:* MCDA twins undergoing combined 1st T aneuploidy screening and followed up *EXC:* not stated	56	51	TTTS	polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS or single or double IUFD	PAPP-A (median MoM), β-hCG (median MoM)
Maiz 2009 (Maiz 2009)	Cohort, prospective, unclear enrolment	*Location:* 1 centre in UK *Years:* 2006-2008 *INC:* MCDA and DCDA twin pregnancies with 2 live fetuses at 11-13+6 weeks *EXC:* unable to measure DV in both fetuses, outcome data missing	733 MCDA and DCDA (does not state number of MCDA)	179 (MCDA but we exclude 4 from analysis with aneuploidy/major defects, therefore 175)	Severe TTTS	Severe TTTS requiring FLA or in which the fetus(es) died prior to FLA: 'ultrasound diagnosis of hydramnios in one twin and anhydramnios in the other, and absent or reversed end diastolic flow in either the umbilical artery or ductus venosus in one or both fetuses'	No TTTS or aneuploidy/major fetal defects	DV abnormal in one, DV abnormal in both, DV abnormal in one/both fetuses
					sIUGR	Severe sIUGR requiring FLA, definition of sIUGR not stated	No sIUGR or aneuploidy/major fetal defects	DV abnormal in one, DV abnormal in both, DV abnormal in one/both fetuses
					Single IUFD or	Gestation at IUFD not stated but	No single IUFD or aneuploidy/major fetal	DV abnormal in one, DV

					miscarriage	must be >14 weeks	defects	abnormal in both, DV abnormal in one/both fetuses
Matias 2005 (Matias 2005)	Cohort, prospective, not stated	*Location:* 1 centre in Portugal *Years:* not stated *INC:* MCDA pregnancies referred to unit for 'routine 1st T ultrasonographic assessment' *EXC:* not stated	Not stated	50	TTTS	Anhydramnios and nonvisible bladder in the donor in combination with polyhydramnios and dilated bladder in the recipient.	No TTTS, 2 livebirths	Raw NT values, NT discordance ≥20%, DV abnormal in one, DV abnormal in both, DV abnormal in one/both fetuses, maternal age (median)

Matias 2010 (Matias 2010)	Cohort, prospective, not stated	*Location:* 1 centre in Portugal *Years:* 1997-2008 *INC:* MCDA pregnancies undergoing 1st T assessment *EXC:* malformations (e.g. megacystis), single IUFD	Not stated	99	TTTS	Oligohydramnios and non-visible bladder in the donor in combination with polyhydramnios and dilated bladder in the recipient	No TTTS	NT per fetus (mean), NT difference (mean), NT ratio (mean), NT difference (AUC), NT ratio (AUC), CRL per fetus (mean), CRL difference (mean), CRL ratio (mean), CRL difference (AUC), CRL ratio (mean), DV abnormal in one, DV abnormal in both, DV abnormal in one/both fetuses, DV abnormal (AUC)
Matias 2011 (Matias 2011)	Cohort, prospective, not stated	*Location:* 2 centres in Portugal and UK *Years:* 2006-2009 (UK), 1998-2009 (Portugal) *INC:* MC pregnancies that did not require antenatal interventions, and resulted in 2 healthy livebirths *EXC:* major fetal abnormalities, single/double IUFD, FLA for TTTS or sIUGR	326	237	BWD	BWD ≥20%	BWD <20%	DV abnormal in one/both fetuses

***McDonald 2017 (McDonald 2017)**	Case series, prospective, consecutive	*Location:*1 centre in Australia *Years:* 2011-2014 *INC:* All MCDA twins attending for antenatal care at centre *EXC:* patients referred to centre for FLA, but remainder of care elsewhere	162	156	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS or chromosomal/ structural anomalies (parity, ethnicity). No TTTS, sIUGR, IUFD, TAPS, chromosomal/structural anomalies (maternal age, BMI)	Maternal age (mean), BMI (mean), ethnicity, parity
					sIUGR	EFW ≤10th centile in one or both twins, and/or EFWD >20%	No sIUGR or chromosomal/ structural anomalies (parity, ethnicity). No TTTS, sIUGR, IUFD, TAPS, chromosomal/structural anomalies (maternal age, BMI)	Maternal age (mean), BMI (mean), ethnicity, parity
					Single or double IUFD	Gestation at IUFD not stated, but median 22.0 (IQR 20.1-30.0 weeks) thus presumed 2nd trimester	No IUFD or chromosomal/ structural anomalies	Maternal age (mean), BMI (mean), ethnicity, parity
Memmo 2012 (Memmo 2012)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database and study	*Location:* 1 centre in UK *Years:* 2000-2010 *INC:* MC pregnancies with 1st T CRL and NT measurements *EXC:* TTTS Stage I managed conservatively who did not undergo FLA, MCMA, aneuploidy, fetal structural anomalies, spontaneous pregnancy loss <16 weeks	279	242	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	2 healthy livebirths at >34 weeks with no TTTS or sIUGR for all outcomes except parity which was compared to no TTTS.	NT discordance (median), NT larger twin (median), NT smaller twin (median), CRL discordance (median), CRL larger twin (median), CRL smaller twin (median), CRL discordance (AUC),

								maternal age (median), parity
					sIUGR 'early'	1 twin EFW <10th centile, before 26 weeks gestation and no signs of TTTS	2 healthy livebirths at >34 weeks with no TTTS or sIUGR except parity which was compared to no sIUGR.	As for TTTS outcome
***Miura 2014 (Miura 2014)**	Cohort, not stated, not stated	*Location:* 1 centre in Japan *Years:* not stated *INC:* MC pregnancies who visited centre at 12-21 weeks *EXC:* not stated	Not stated	28	TTTS	Polyhydramnios (DVP ≥8cm) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS or chromosomal/ structural anomalies	Maternal age (mean), parity
Moriichi 2013 (Moriichi 2013)	Cohort, prospective, not stated	*Location:* 1 centre in Japan *Years:* 2007-2011 *INC:* MC pregnancies with 2 livebirths *EXC:* chromosomal aberrations, congenital anomalies, IUFD, TTTS	Not stated	36	BWD	BWD ≥20%	BWD <20%	Maternal age (mean), parity
Murakami 2011 (Murakami 2011)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database and study	*Location:* 1 centre in Japan *Years:* 2006-2010 *INC:* Twins pregnancies attending for antenatal care at centre *EXC:* congenital anomalies associated with IUGR, multifetal pregnancy reduction	125 (51 MCDA, 74 DCDA)	42	TTTS	Polyhydramnios (DVP ≥8cm) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	Mode of conception (spontaneous or IVF/ ovulation induction)
					IUFD	Single/double IUFD >16 weeks	No IUFD	Mode of conception (spontaneous or IVF/ ovulation induction)

***Sarais 2015 (Sarais 2015)**	Cohort, retrospective search of database with prospectively recorded data, enrolment to database and study unclear	*Location:* 1 centre in Italy *Years:* 2007-2011 *INC:* MC pregnancies undergoing antenatal care at centre, which progressed >16 weeks *EXC:* higher order multiples, those presenting >16 weeks	Not stated	145	TTTS	Not stated	No TTTS or chromosomal/ structural anomalies	Mode of conception (spontaneous or IVF)
					IUFD	Single and double IUFD, >16 weeks	No IUFD or chromosomal/ structural anomalies	Mode of conception (spontaneous or IVF)
Schrey 2013 (Schrey 2013)	Cohort, not stated, not stated	*Location:* ? centres in Canada *Years:* not stated *INC:* 'potential discordant MC twin pregnancies as candidates for placental sampling were identified in the antenatal period' *EXC:* fetal abnormalities, syndromes or infections, pre-eclampsia, diabetes, placental tumours	Not stated	15	BWD	BWD ≥20% (all smaller twins also below the 10th centile)	BWD <20%	Fetal gender
Sebire 2000 (Sebire 2000)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database unclear, but consecutive to study	*Location: ?* centre in UK *Years:* unclear start date but delivered prior to June 1999 *INC:* MCDA pregnancies with 2 live fetuses at 10-14 weeks gestation, with outcome information available *EXC:* structural/chromosomal anomalies, TOP	303	287	Severe TTTS	Anhydramnios and no-visible bladder in the donor fetus and polyhydramnios and a dilated bladder in the recipient fetus, which resulted in either miscarriage or fetal death or required intrauterine treatment or post-mortem evidence that the cause of death was TTTS.	No TTTS	NT >95th centile per fetus, NT >95th centile in one/both fetuses

Sooranna 2001 (Sooranna 2001)	Unclear, prospective, not stated	*Location:* ? centres in UK *Years:* not stated *INC:* MC and DCDA pregnancies *EXC:* chronic TTTS, single IUFD, structural/chromosomal abnormalities, selective feticide, embryo reduction, maternal complications: hypertension, pre-eclampsia, renal/cardiac disease	Not stated	29	sIUGR	EFWD ≥20% with smaller twin's AC ≤5th centile and abnormal UAD in same pregnancy, with absence of polyhydramnios in the larger twin's sac	EFWD ≤10% and normal AFI throughout pregnancy	Fetal gender
Stagnati 2016 (Stagnati 2016)	Cohort, retrospective search of database with prospectively recorded data, consecutive enrolment to database and study	*Location:* 1 centre in Italy *Years:* 2008-2013 *INC:* MCDA pregnancies of with 1st T scan and antenatal care at centre *EXC:* referral after 1st T and/or incomplete follow-up, fetal structural abnormalities or abnormal karyotype, TTTS, TAPS, sIUGR or EFWD ≥25% at <28 weeks	172	136	sIUGR	EFW <5th centile	No sIUGR	Maternal age (median), BMI (median), mode of conception (spontaneous or ART), parity
					BWD	BWD >25%	BWD ≤25%	As for sIUGR outcome
Sueters 2006 (Sueters 2006)	Cohort, prospective, consecutive	*Location:* 1 centre in Netherlands *Years:* 2002-2004 *INC:* MCDA pregnancies, <16 weeks at referral, no signs of TTTS at initial USS *EXC:* fetal abnormalities	25	23	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	NT >95th centile in one/both fetuses, maternal age (median), parity (median)
Sun 2017 (Sun 2017)	Case-control, retrospective, not stated	*Location:* 1 centre in China *Years:* not stated *INC:* MCDA twins	Not stated	14	BWD	BWD >20%	BWD <10% and normal UAD	Maternal age (mean), fetal gender

		EXC: pre-eclampsia, TTTS, TAPS, fetal structural/chromosomal anomalies, maternal or pregnancy complications						
Tai 2007 (Tai 2007)	Cohort, prospective, not stated	*Location:* 1 centre in USA *Years:* 2000-2006 *INC:* twin pregnancies undergoing 1st T aneuploidy screening with 2 fetal heartbeats detected *EXC:* chromosomal/major congenital anomalies, 1st/2nd T TOP, MCMA	Not stated	43	TTTS	Not stated	No TTTS	CRL discordance ≥11%
Taylor-Clarke 2013 (Taylor-Clarke 2013)	Case-control, prospective, consecutive	*Location:* 1 centre in UK *Years:* not stated *INC:* MCDA pregnancies referred for ultrasound assessment from local maternity units *EXC:* not stated	Not stated	55	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS	Maternal age (median)
Torres-Torres 2010 (Torres-Torres 2010)	Cohort, retrospective, consecutive	*Location:* 1 centre in Mexico *Years:* 2008-2009 *INC:* MCDA twins undergoing antenatal care at centre *EXC:* not stated	Not stated	34 (but we excluded 4 from analysis with aneuploidy/major defects, therefore 30)	TTTS	Polyhydramnios (DVP ≥8cm) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	'Normal' with no complications, structural abnormalities, TTTS or sIUGR	Maternal age (median)
					sIUGR	EFW >10th centile in one fetus	'Normal' with no complications, structural abnormalities, TTTS or sIUGR	Maternal age (median)
***Velayo 2012 (Velayo 2012)**	Case-control, retrospective, not stated	*Location:* ? centre in ? country *Years:* 2008-2009 *INC:* MCDA twins *EXC:* not stated	Not stated	35	TTTS	Polyhydramnios in 1 twin, oligohydramnios in the other twin. FLA performed for	No TTTS or chromosomal/ structural anomalies. EFWD <15%, no polyhydramnios or	Maternal age (mean), BMI (mean), ethnicity, parity, mode of

						all TTTS patients	oligohydramnios, UAD, MCA and DV Dopplers normal.	conception (spontaneous or not stated)
Yinon 2014 (Yinon 2014)	Cohort, prospective, not stated	*Location:* 1 centre in Israel *Years:* 2010-2012 *INC:* MCDA twins *EXC:* chronic hypertension, pre-gestational diabetes, congenital/chromosomal abnormalities, single IUFD at presentation	60	45	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	Normal: appropriately grown, EFWD <25%, normal amniotic fluid volumes, normal UADs, similar MCA-PSV in both twins for all outcomes, except smoking which is no TTTS.	Maternal age (median), gravida, BMI (median), smoking
				37	sIUGR	EFW <10th centile in one fetus and EFWD ≥25% in same pregnancy	Normal: appropriately grown, EFWD <25%, normal amniotic fluid volumes, normal UADs, similar MCA-PSV in both twins for all outcomes, except smoking which is no sIUGR	Maternal age (median), gravida, BMI (median), smoking
Zanardini 2014 (Zanardini 2014)	Cohort, prospective, not stated	*Location:* 1 centre in Italy *Years:* 2009-2012 *INC:* MCDA pregnancy attending centre for antenatal care *EXC:* MCMA, congenital cardiac anomaly/arrhythmia, fetal anomaly, TRAP, IUFD at presentation, maternal age <18 years, higher order multiples, patients lost to follow-up, TTTS diagnosed at <17 weeks	139	100	TTTS	Polyhydramnios (DVP ≥8cm before 20 weeks, or ≥10cm after 20 weeks) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	'Uncomplicated' throughout pregnancy, plus 4 with sIUGR (not defined)	Mode of conception (spontaneous or not stated), maternal age (median), parity

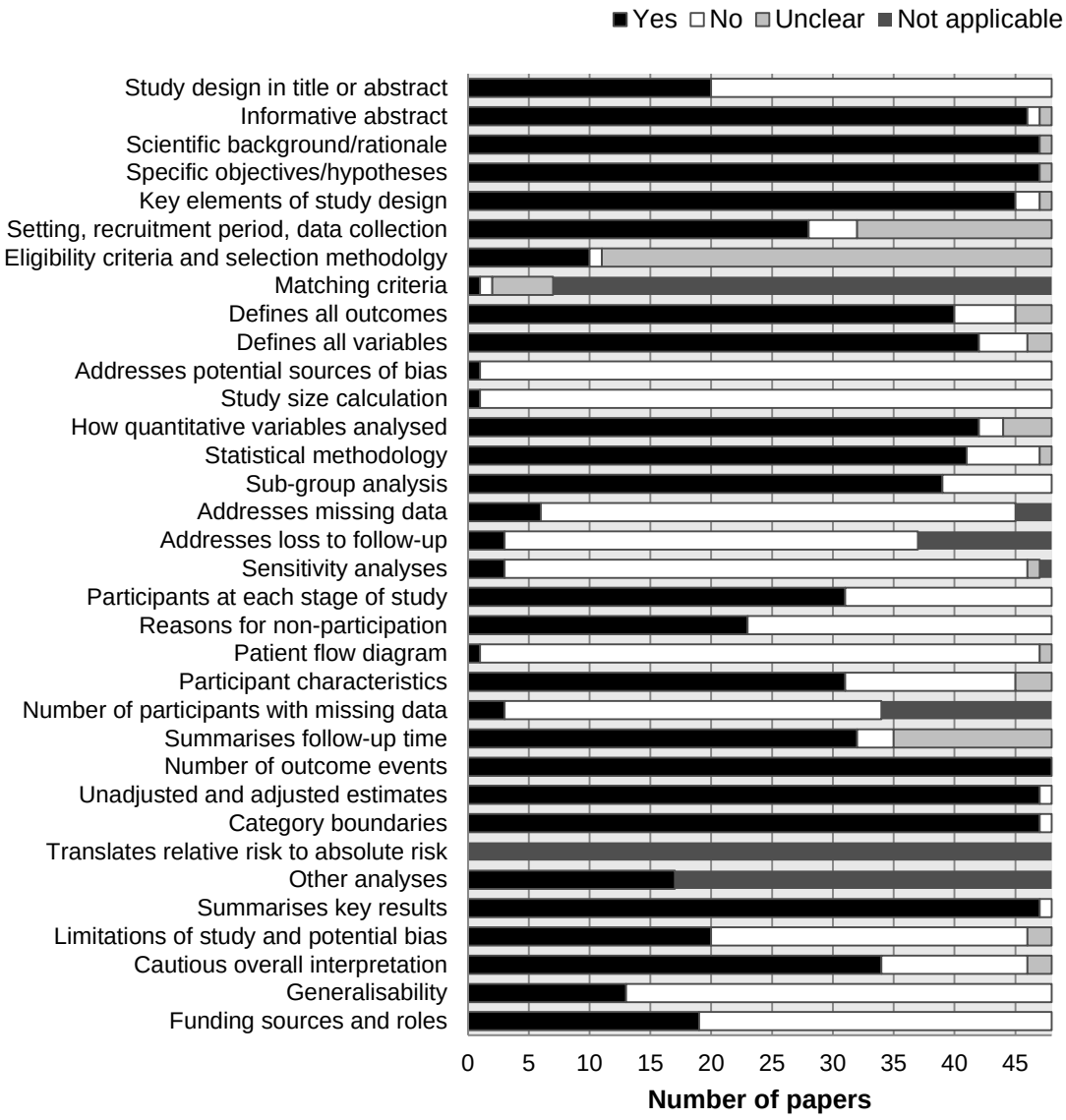
Zhang 2015 (Zhang 2015)	Not stated, retrospective, not stated	*Location:* ? centres in China *Years:* 2009-2013 *INC:* MCDA twins *EXC:* severe maternal complications, TTTS, IUFD	Not stated	24	LBW	BW <10th centile in one twin	No LBW	Maternal age (mean), BMI (mean), fetal gender
***Zhao 2013 (Zhao 2013a)**	Cohort, retrospective, consecutive	*Location:* 1 centre in The Netherlands *Years:* 2002-2012 *INC:* MCDA twins with stored placentas *EXC:* TRAP, IUFD, higher order multiples, if underwent FLA or selective feticide	Not stated	235	TTTS	Polyhydramnios (DVP ≥8cm) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS or chromosomal/ structural anomalies	Fetal gender
					BWD	BWD ≥25%	No BWD or chromosomal/ structural anomalies	Fetal gender
					TAPS	Antenatally MCA-PSV >1.5 MoM in the donor and MCA-PSV <1.0 MoM in the recipient, and/or postnatally inter-twin haemoglobin difference >8.0 g/dl, and at least one of the following: reticulocyte count ratio >1.7 and placenta with only small (diameter < 1mm) vascular anastomoses	No TAPS or chromosomal/ structural anomalies	Fetal gender

Zoppi 2014 (Zoppi 2014)	Cohort, prospective, not stated	*Location:* ? centre in Italy *Years:* 2010-2012 *INC:* MCDA pregnancies 11-14 weeks gestation *EXC:* malformations, single or double IUFD <16 weeks, TOP <16 weeks	87	71	TTTS	Diagnosed <26 weeks. Polyhydramnios (DVP ≥8cm) in the recipient twin, oligohydramnios (DVP ≤2cm) in the donor twin	No TTTS, sIUGR, amniotic fluid discordance	UVVF larger twin (median), UVVF smaller twin (median), NT larger twin (median), NT smaller twin (median), CRL larger twin (median) CRL smaller twin (median)
				70	sIUGR	AC <5th centile in one fetus, and EFWD >25% in same pregnancy	?No TTTS, sIUGR, amniotic fluid discordance	As for TTTS outcome

2.3.4 Risk of bias of included studies

Most studies were not designed for the recruitment of participants to examine first trimester potential prognostic factors, consequently, the quality assessment should be interpreted with caution. The quality assessment results are likely to be reflective of the ability of the studies to demonstrate a difference in potential prognostic factors between groups, however the quality assessment of the ability of the individual studies to demonstrate prognostic ability for each factor is likely to be sub-optimal, as the QUIPS checklist could not be used as previously mentioned. The different aspects of the ‘STROBE’ classification are demonstrated in Figure 2.2.

Figure 2.2 Quality assessment of included studies according to 'Strengthening The Reporting of Observational studies in Epidemiology' (STROBE) checklist



Of note is that the studies were poor at stating how they addressed missing data, and which data were missing meaning that attrition bias may have affected the results of the meta-analysis. Another notable finding is that the method of enrolment was not stated in the majority of studies, thus the level of selection bias is not clear. One aspect of the study design that may increase the risk of heterogeneity was that different control groups were used: (i) MC twin pregnancies with no maternal or fetal

complications, (ii) MC twins with no fetal complications, (iii) other MC twin pregnancies in the study who did not have the condition being examined but did have other MC complications. For the growth restriction outcomes, studies were classified according to the time that the growth measurement was performed, meaning that 5 studies (El Kateb 2007, Chai 2013, Ghalili 2013, Zhao 2013a, Zhang 2015) despite calling their outcome IUGR, were included in the PGR group as their definition was based on birthweights, not antenatal ultrasound measurements. In calculating discordance between EFWs, or BWs, all studies used the larger measure as the denominator. Four studies included in the PGR meta-analyses only measured abnormal growth by BWD, Moriichi et al. (Moriichi 2013), Stagnati et al. (Stagnati 2016), Sun et al. (Sun 2017), Zhao et al. (Zhao 2013a), meaning that both babies may have weighed >10th centile. All other studies that reported abnormal growth as an outcome had to have at least one fetus/baby <10th centile, except for three studies that were not able to be included in meta-analyses due to being the only studies which measured their potential prognostic factor (Lewi 2008a, Matias 2011, Johansen 2014). Not all the participants in the study by Murakami et al. (Murakami 2011) had delivered at the time the study was published, therefore only those who had delivered were included in the meta-analysis. Only one funnel plot and Egger's test was required, that did not suggest significant publication bias in the maternal age and TTTS analysis (Figure 2.7(b)).

2.3.5 Synthesis of results

Meta-analysis could be performed for the following prognostic factors:

a) Ultrasound measurements: NT >95th centile in one/both fetuses, NT discordance ≥20%, CRL discordance ≥10%.
b) Maternal characteristics: age, ethnicity, BMI, parity, smoking, mode of conception, fetal gender.

In total, 20 separate meta-analyses were performed; of these, 3 demonstrated a moderate association (OR >2) (Table 2.2, section 2.3.6, 2.3.7 and 2.3.8), but none demonstrated a prognostic ability for any outcome under investigation. The other 17 analyses that did not demonstrate an association are reported in section 2.3.9. Meta-analysis was unable to be performed on first trimester biomarkers because of the way the data were presented. An insufficient number of studies reported antenatal and postnatal growth restriction within the same pregnancy (Bajoria 2006, Chang 2017), and IUFD as outcomes to include in meta-analysis. Only 4 studies (Memmo 2012, Ashoor 2013, Yinon 2014, Chang 2016) used a control group of 'normal' pregnancies, the other 44 studies used a control group of 'no disease under investigation' e.g. TTTS vs no TTTS, therefore the results should be considered as comparing to 'no disease'.

Table 2.2 Summary of accuracy of prognostic factors with a significant association with twin-twin transfusion syndrome

*Analysed by bivariate analysis. †Analysed by univariate analysis. CRL: crown-rump length, NT: nuchal translucency

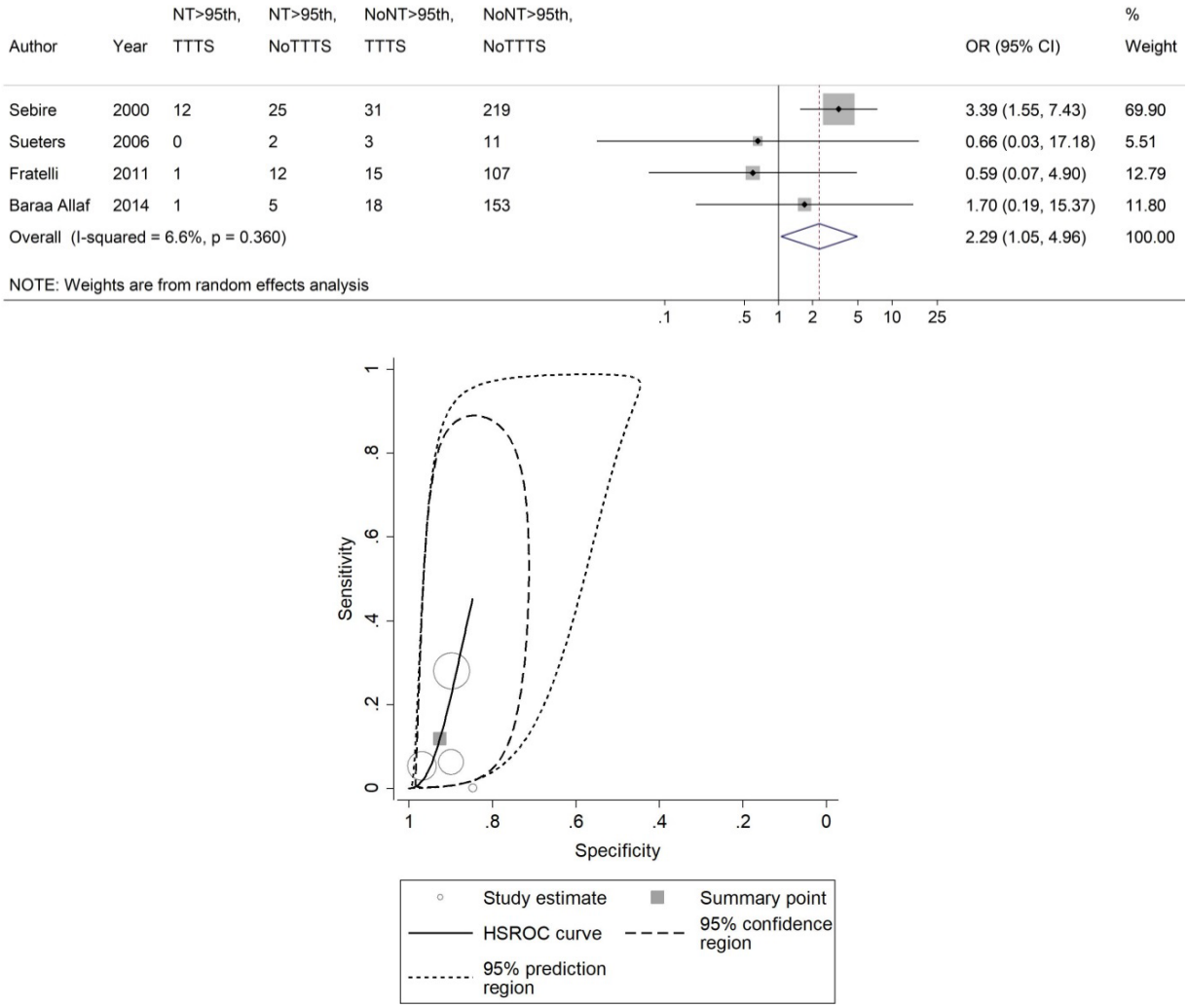
Prognostic factor	Sensitivity (95%CI)	Specificity (95%CI)	Positive likelihood ratio (95%CI)	Negative likelihood ratio (95%CI)
NT>95th centile in one/both fetuses*	0.118 (0.035, 0.330)	0.926 (0.882, 0.954)	1.589 (0.589, 4.290)	0.953 (0.830, 1.094)
CRL discordance ≥10%†	0.203 (0.120, 0.308)	0.908 (0.882, 0.929)	2.180 (1.147, 4.142)	0.904 (0.794, 1.030)
Maternal ethnicity*	0.826 (0.672, 0.917)	0.278 (0.135, 0.917)	1.145 (0.941, 1.394)	0.624 (0.349, 1.117)

2.3.6 NT>95th centile in one/both fetuses and TTTS

A significant association between NT>95th centile in one/both fetuses and TTTS was found (OR 2.29 [95%CI 1.05, 4.96] I^2=6.6%, 4 studies, 615 pregnancies) (Figure 2.3(a)). Bivariate meta-analysis results are in Table 2.2. The post-test probability of a positive result was 0.22 (95%CI 0.13, 0.35), and a negative result was 0.14 (95%CI 0.13, 0.15), assuming pre-test probability of 0.176 based on a prevalence of 15%. See Figure 2.3(b) for the HSROC that shows reasonable specificity but poor sensitivity.

Figure 2.3 (a) Forest plot of association between NT>95th centile (NT>95th) in one/both fetuses and twin-twin transfusion syndrome (TTTS) and (b) Hierarchical summary receiver operating characteristic curves (HSROC)

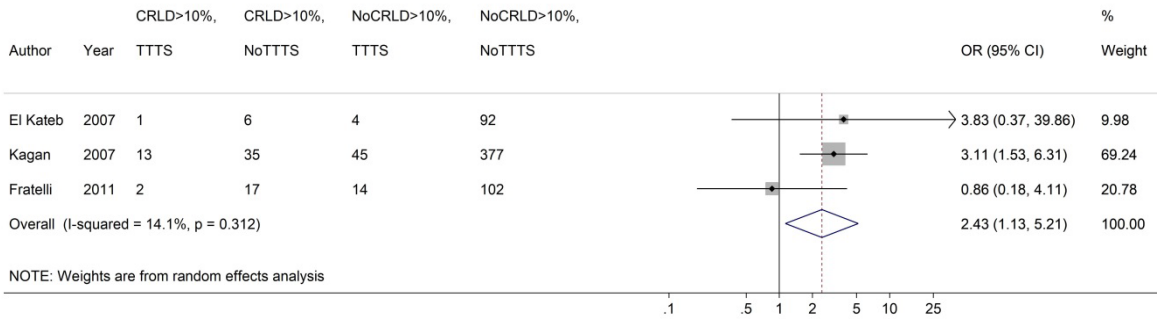
This visually represents the global summary of prognostic factor performance by plotting the mean sensitivity against the reversed mean specificity produced by the bivariate analysis. The ellipses represent the 95% confidence intervals of the mean sensitivities and specificities and 95% prediction region. The closer the values are to the top left corner, the greater the accuracy of the prognostic factor.

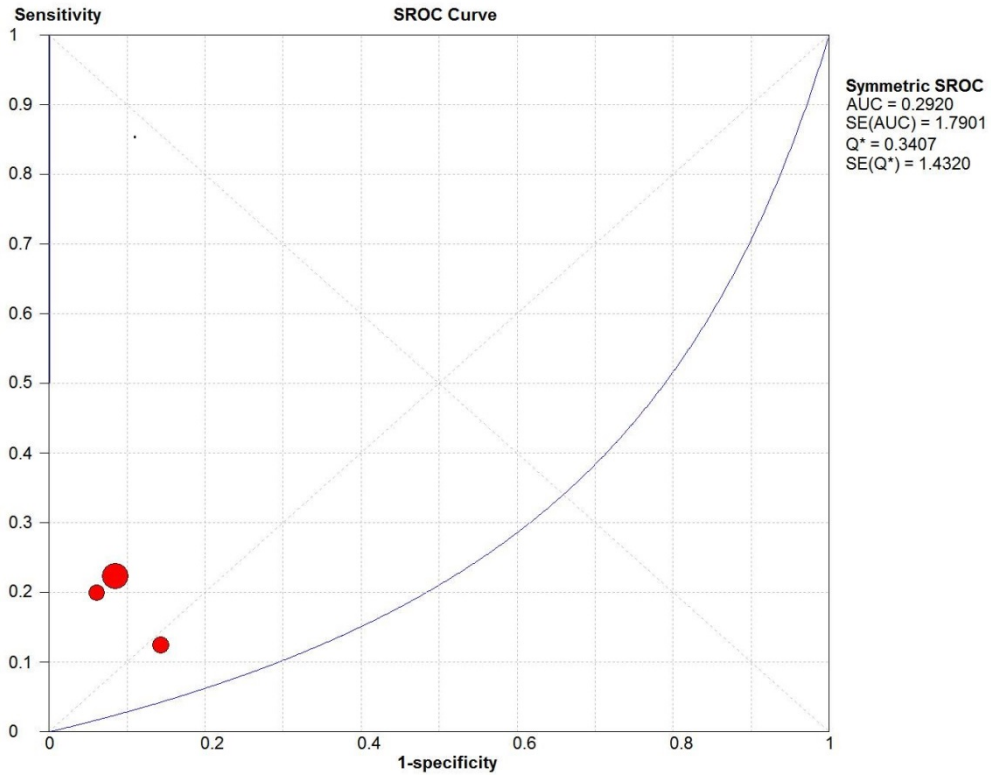


2.3.7 CRL discordance ≥10% and TTTS

A significant association between CRL discordance ≥10% and TTTS was found (OR 2.43 [95%CI 1.13, 5.21] I^2=14.1%, 3 studies, 708 pregnancies) (Figure 2.4(a)). Univariate meta-analysis results are in Table 2.2. The post-test probability of a positive result was 0.28 (95%CI 0.20, 0.38), and a negative result was 0.13 (95%CI 0.12, 0.15), assuming pre-test probability of 0.176 based on a prevalence of 15%. See Figure 2.4(b) for the SROC.

Figure 2.4 (a) Forest plot of association between crown-rump length discordance ≥10% (CRLD>10%) and twin-twin transfusion syndrome (TTTS) and (b) Summary receiver operating characteristic curves



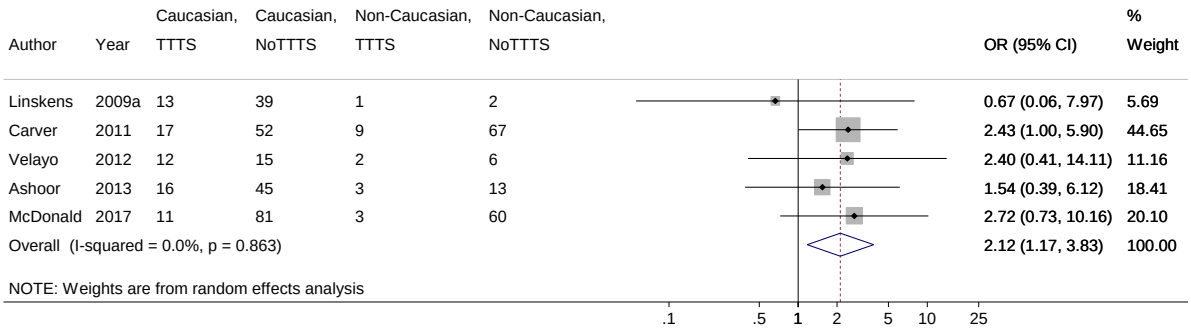


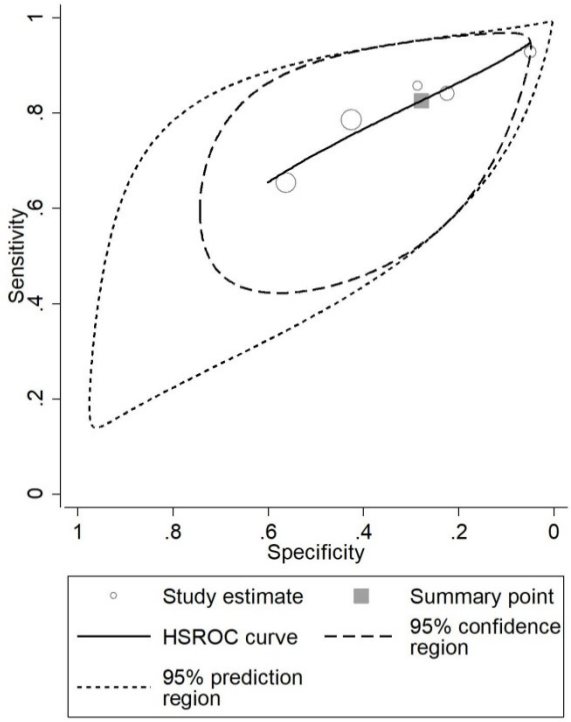
2.3.8 Maternal ethnicity and TTTS

An OR >1 indicated a higher-risk of TTTS if the woman was Caucasian, and an OR <1 indicated a higher-risk of TTTS if the woman was non-Caucasian. A significant association between maternal ethnicity and TTTS was found (OR 2.12 [95%CI 1.17, 3.83] I^2=0.0%, 5 studies, 467 pregnancies) (Figure 2.5(a)). Bivariate meta-analysis results are in Table 2.2. The post-test probability of a positive result was 0.17 (95%CI 0.15, 0.19), and a negative result was 0.10 (95%CI 0.06, 0.16), assuming pre-test probability of 0.176 based on a prevalence of 15%. See Figure 2.5(b) for HSROC that shows moderate sensitivity but poor specificity.

Figure 2.5 (a) Forest plot of association between maternal ethnicity and twin-twin transfusion syndrome (TTTS) (b) Hierarchical summary receiver operating characteristic curves

This visually represents the global summary of prognostic factor performance by plotting the mean sensitivity against the reversed mean specificity produced by the bivariate analysis. The ellipses represent the 95% confidence intervals of the mean sensitivities and specificities and 95% prediction region. The closer the values are to the top left corner, the greater the accuracy of the prognostic factor.



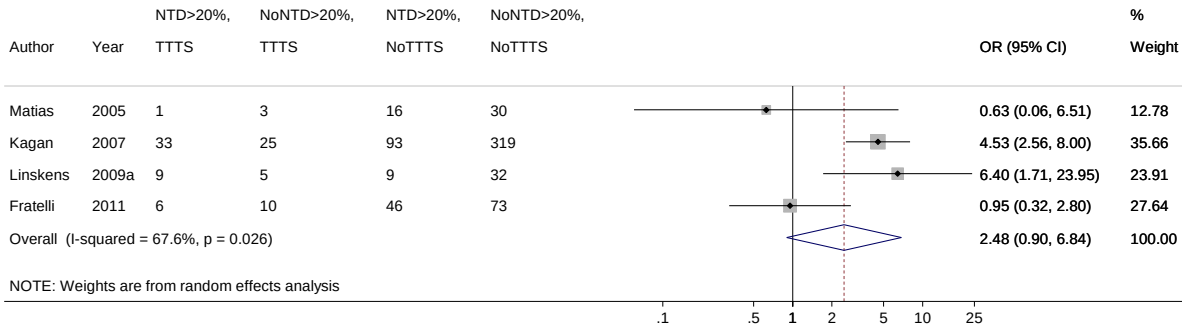


2.3.9 Meta-analyses with no moderate/strong prognostic association

NT discordance ≥20% and TTTS

There was a trend towards a significant association between NT discordance ≥20% and TTTS, however there was a high-risk of heterogeneity (OR 2.48 [95%CI 0.90, 6.84] I^2=67.6%, 4 studies, 710 pregnancies) (Figure 2.6). To investigate the heterogeneity a sensitivity analysis was performed removing Kagan et al. (Kagan 2007) as it only included those with severe TTTS requiring FLA in their group, whereas others included those with a diagnosis of TTTS irrespective of intervention. Removing Kagan et al. made no difference to the results or level of heterogeneity (results not shown) therefore this study was included. On visual inspection of the forest plot, Matias et al. was noted to be an outlier, however there was no reason to remove the study based on study design or characteristics, therefore this study was included.

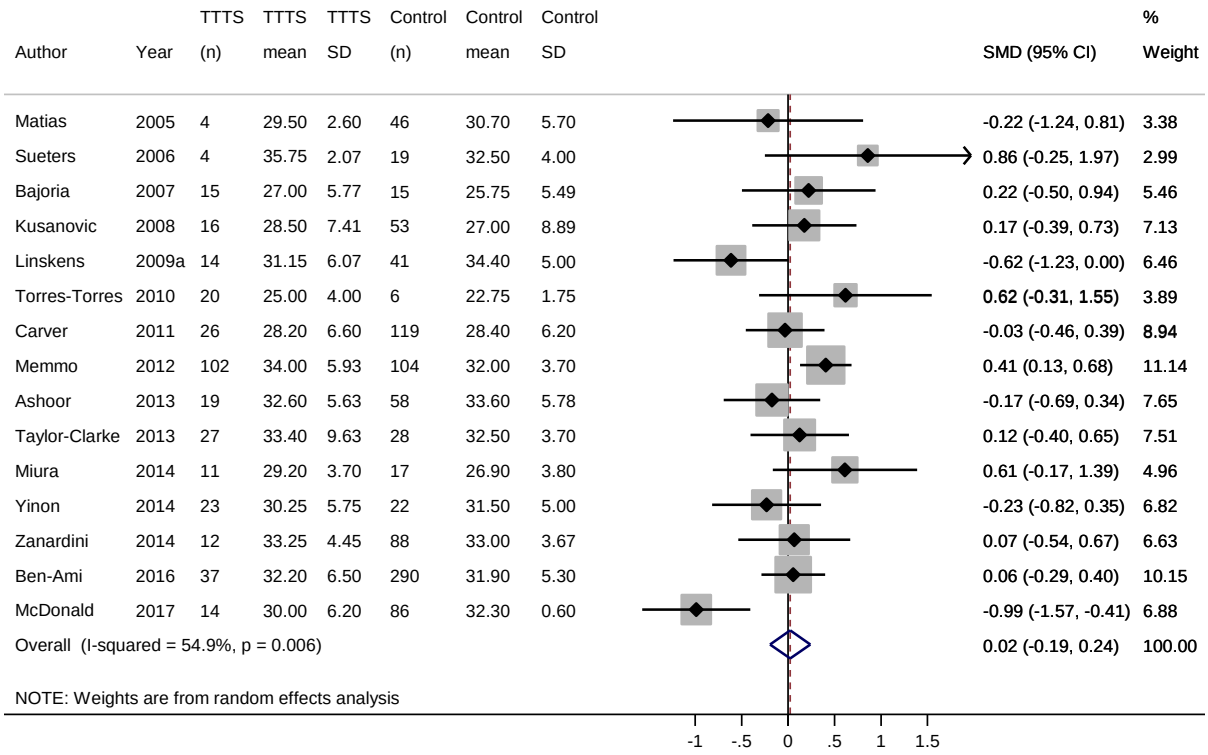
Figure 2.6 Forest plot of association between NT discordance ≥20% and TTTS

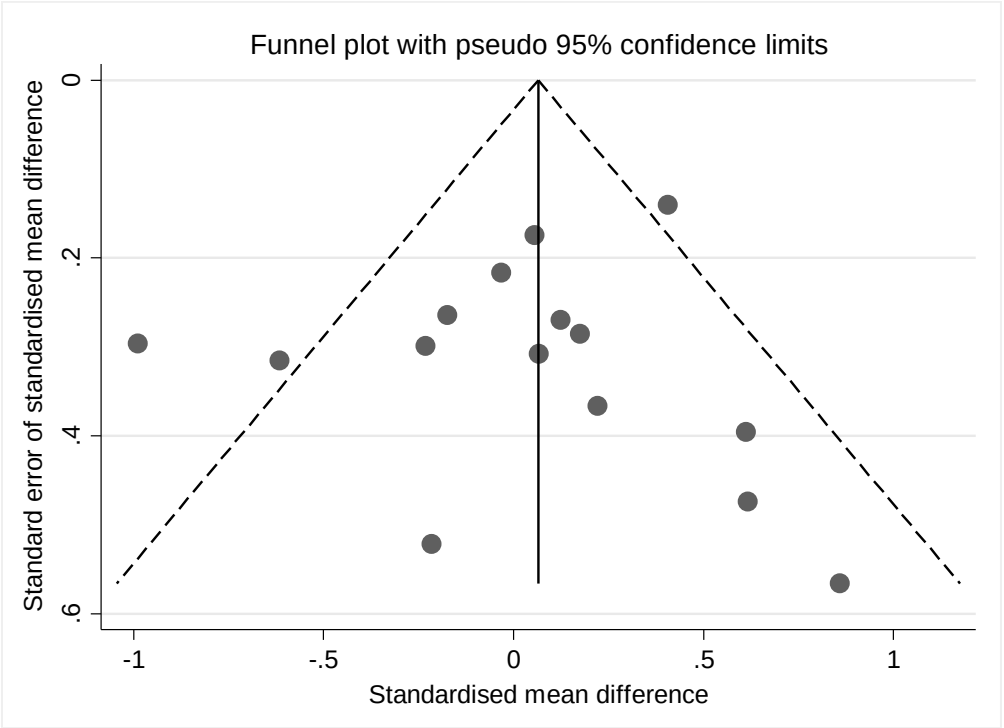


Maternal age and TTTS

No significant association between maternal age and TTTS was found (SMD 0.02 [95%CI -0.19, 0.24] I^2=54.9%, 15 studies, 1336 pregnancies) (Figure 2.7(a)). Although the I^2 suggests a high-risk of heterogeneity, there were no obvious outliers from visual inspection of the forest plot or in study design. The funnel plot (Figure 2.7(b)) does appear asymmetrical, although Egger's test does not suggest small-study effects with p=0.576.

Figure 2.7(a) Forest plot of association between maternal age and TTTS and (b) Funnel plot of maternal age and TTTS studies

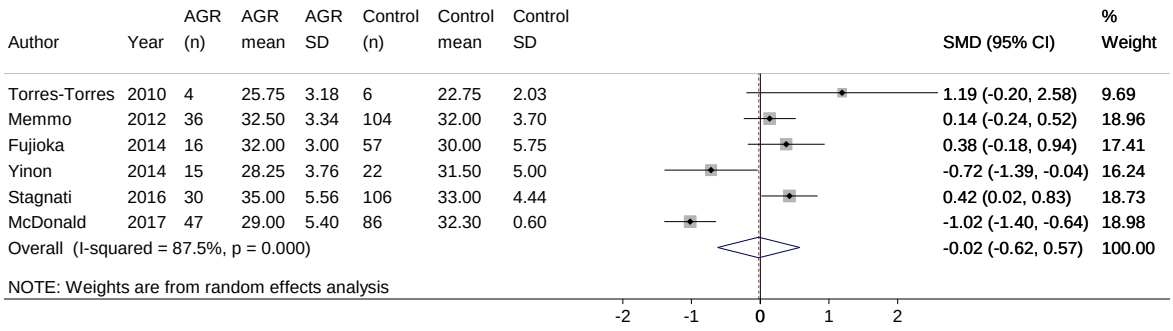




Maternal age and AGR

No significant association between maternal age and AGR was found (SMD -0.02 [95%CI -0.62, 0.57] I^2=87.5%, 6 studies, 529 pregnancies) (Figure 2.8). To investigate the high-risk of heterogeneity, a sensitivity analysis was performed removing Memmo et al. (Memmo 2012), Fujioka et al. (Fujioka 2014) and Stagnati et al. (Stagnati 2016) as these studies restricted their definitions of AGR based on gestation. Removing these studies made no difference to the results or level of heterogeneity (results not shown) therefore the studies were included. An additional sensitivity analysis was performed by removing Yinon et al. (Yinon 2014) and McDonald et al. (McDonald 2017) following visual assessment of the forest plot. This decreased the I^2 to 0.0% and produced a significant association between maternal age and AGR (SMD 0.32 [95%CI 0.08, 0.56], 4 studies, 359 pregnancies) (forest plot not shown), however as the effect of maternal age was small, the prognostic ability was not further investigated. When the studies were examined in more detail, these were the only 2 studies in the meta-analysis that included twins with EFWD in their abnormal growth group, whereas the definition used in the other 4 studies required the EFW of 1 twin to be <10^{th} centile (Torres-Torres 2010, Memmo 2012, Fujioka 2014) or 5^{th} centile (Stagnati 2016).

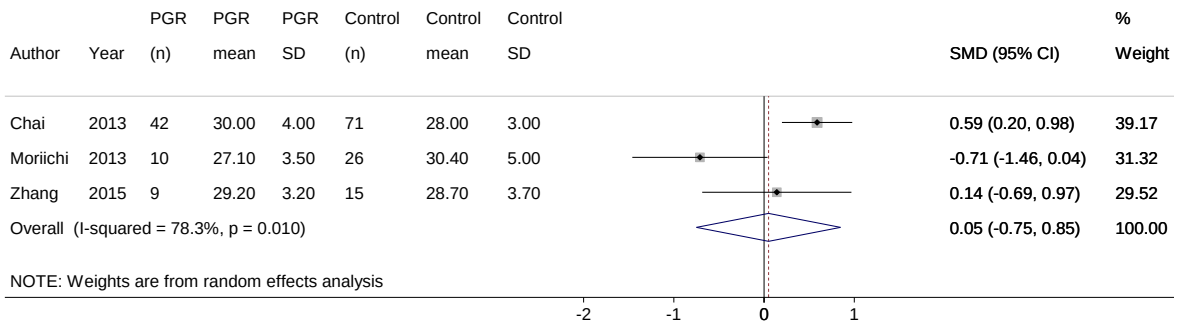
Figure 2.8 Forest plot of association between maternal age and antenatal growth restriction (AGR)



Maternal age and PGR

No significant association between maternal age and PGR was found (SMD 0.05 [95%CI -0.75, 0.85] I^2=78.3%, 3 studies, 173 pregnancies) (Figure 2.9). It was not possible to investigate the high-risk of heterogeneity as there were too few studies.

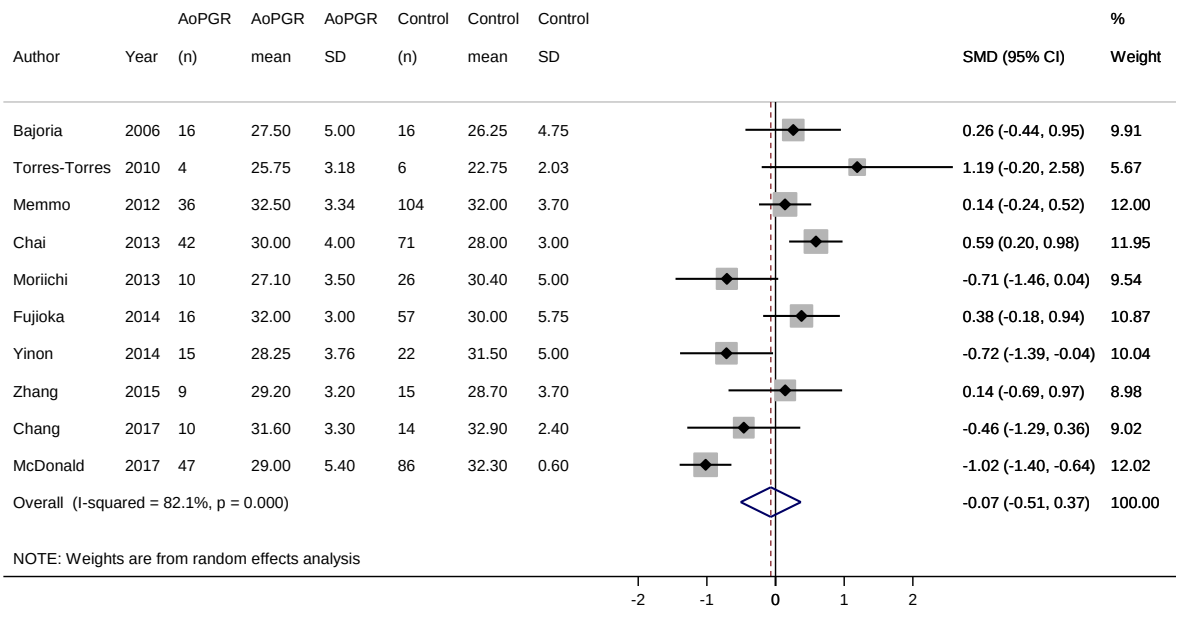
Figure 2.9 Forest plot of association between maternal age and postnatal growth restriction (PGR)



Maternal age and AoPGR

No significant association between maternal age and AoPGR was found (SMD -0.07 [95%CI -0.51, 0.37] I^2=82.1%, 10 studies, 622 pregnancies) (Figure 2.10). To investigate the high-risk of heterogeneity, a sensitivity analysis was performed removing Memmo et al. (Memmo 2012) and Fujioka et al. (Fujioka 2014) as these studies restricted their definitions of AGR based on gestation. However, removing these studies made no difference to the results or level of heterogeneity (results not shown) therefore these studies were included.

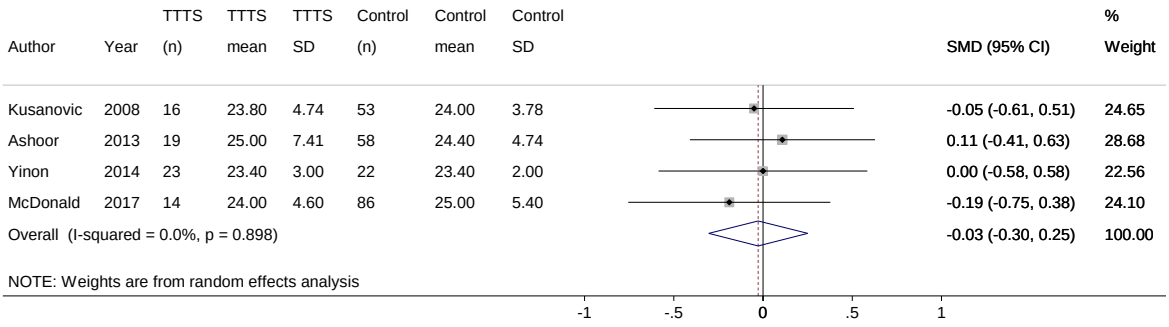
Figure 2.10 Forest plot of association between maternal age and antenatal or postnatal growth restriction (AoPGR)



Maternal BMI and TTTS

No significant association between maternal BMI and TTTS was found (SMD -0.03 [95%CI -0.30, 0.25] I^2=0.0%, 4 studies, 291 pregnancies) (Figure 2.11).

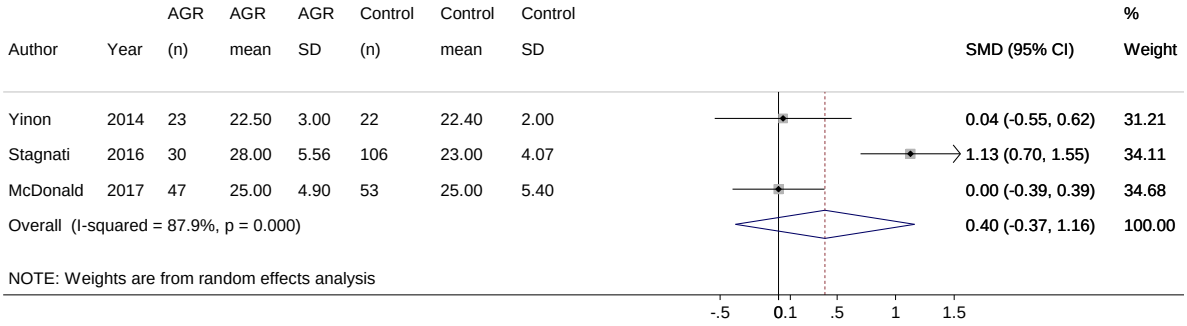
Figure 2.11 Forest plot of association between maternal BMI and TTTS



Maternal BMI and AGR

No significant association between maternal BMI and AGR was found (SMD 0.40 [95%CI -0.37, 1.16] I^2=87.9%, 3 studies, 281 pregnancies) (Figure 2.12). Following visual assessment of the forest plot, Stagnati et al. (Stagnati 2016) was noted to be an outlier, which was the only study in the meta-analysis that did not include EFWD in their definition of abnormal growth. It was not possible to investigate this further as there were too few studies.

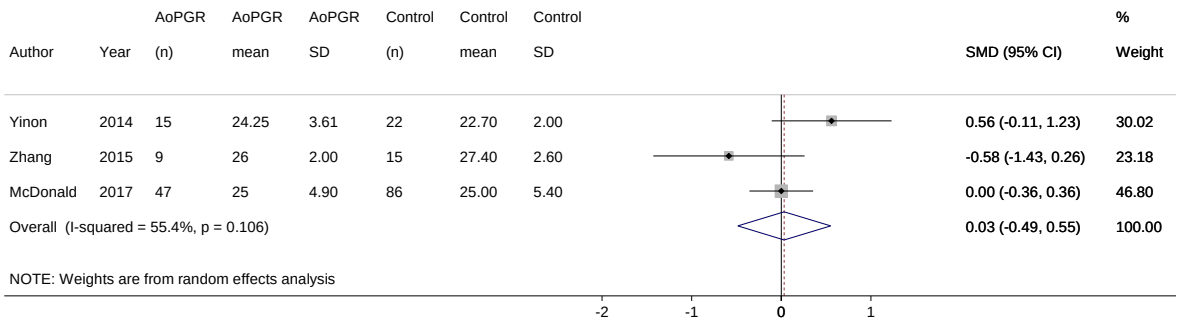
Figure 2.12 Forest plot of association between maternal BMI and antenatal growth restriction (AGR)



Maternal BMI and AoPGR

No significant association between maternal BMI and AoPGR was found (SMD 0.03 [95%CI -0.49, 0.55] I^2=55.4%, 3 studies, 194 pregnancies) (Figure 2.13). It was not possible to investigate the high-risk of heterogeneity as there were too few studies.

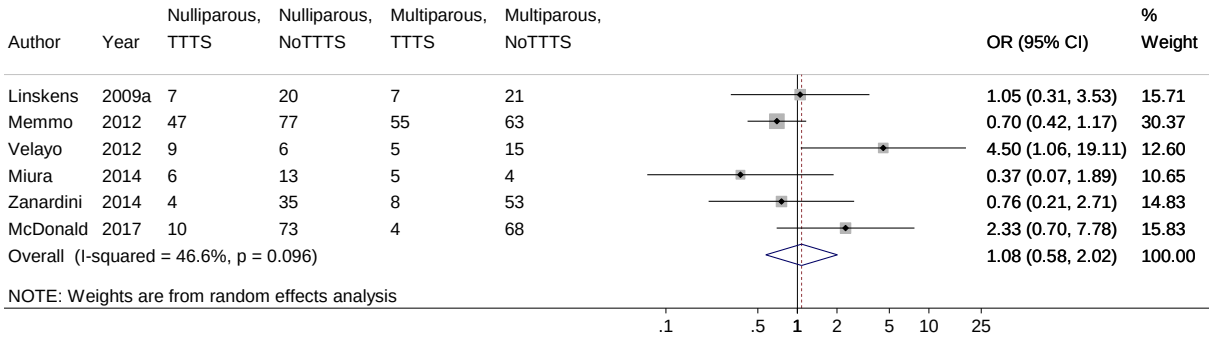
Figure 2.13 Forest plot of association between maternal BMI and antenatal or postnatal growth restriction (AoPGR)



Parity and TTTS

An OR >1 indicated a higher-risk of TTTS if the woman was nulliparous, and an OR <1 indicated a higher-risk of TTTS if the woman was multiparous. No significant association between parity and TTTS was found (OR 1.08 [95%CI 0.58, 2.02] I^2=46.4%, 6 studies, 615 pregnancies) (Figure 2.14).

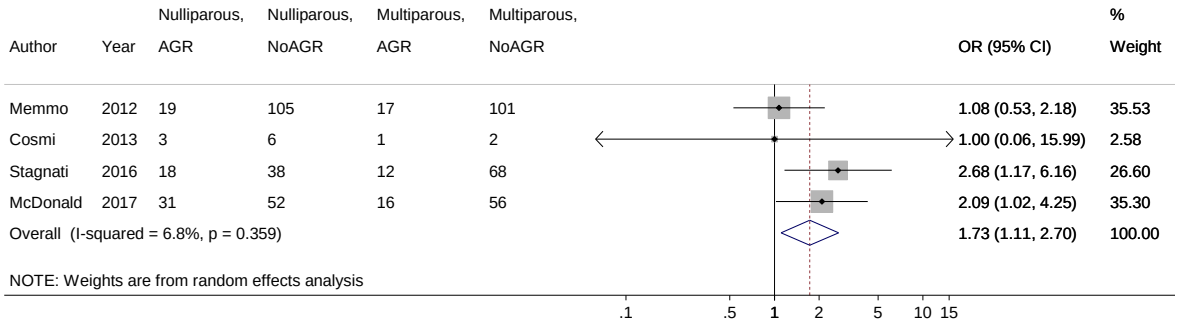
Figure 2.14 Forest plot of association between parity and TTTS



Parity and AGR

An OR >1 indicated a higher-risk of AGR if the woman was nulliparous, and an OR <1 indicated a higher-risk of AGR if the woman was multiparous. There appears to be an association between parity and AGR (OR 1.73 [95%CI 1.11, 2.70] I^2=6.8%, 4 studies, 545 pregnancies) (Figure 2.15) although as it was a weak association with an OR <2 this was not further investigated.

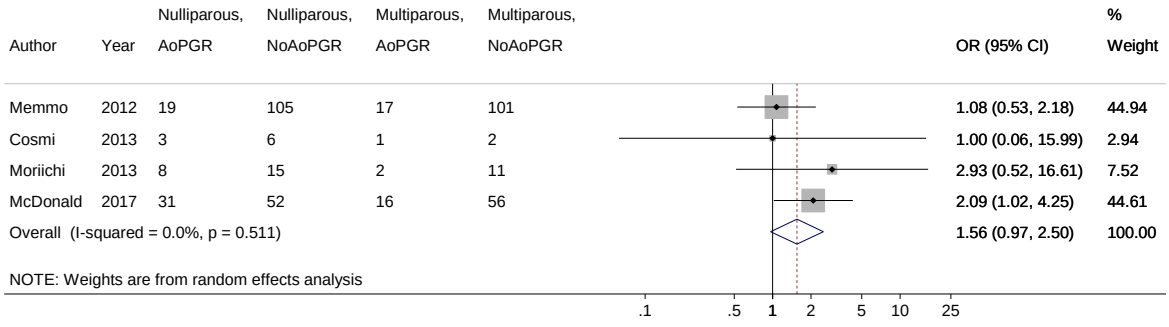
Figure 2.15 Forest plot of association between parity and antenatal growth restriction (AGR)



Parity and AoPGR

An OR >1 indicated a higher-risk of AoPGR if the woman was nulliparous, and an OR <1 indicated a higher-risk of AoPGR if the woman was multiparous. A trend towards an association between parity and AoPGR was found (OR 1.56 [95%CI 0.97, 2.50], I^2=0.0%, 4 studies, 445 pregnancies) (Figure 2.16).

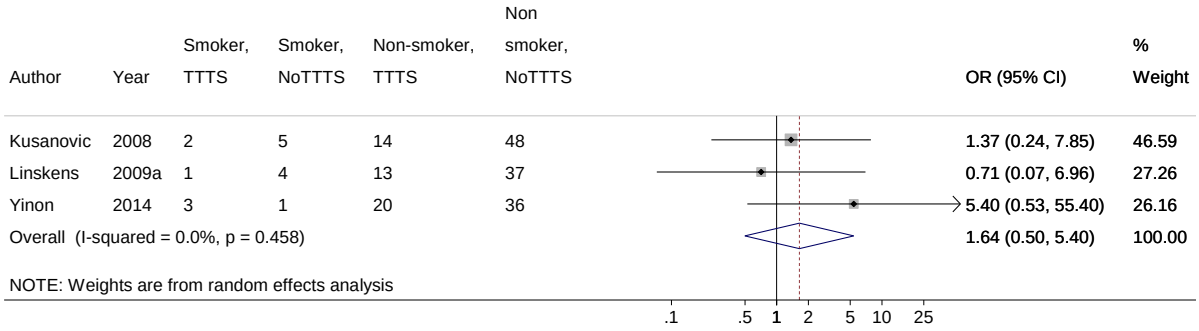
Figure 2.16 Forest plot of association between parity and antenatal or postnatal growth restriction (AoPGR)



Maternal smoking and TTTS

An OR >1 indicated a higher-risk of TTTS if the woman was a smoker, and an OR <1 indicated a higher-risk of TTTS if the woman was a non-smoker. No significant association between maternal smoking and TTTS was found (OR 1.64 [95%CI 0.50, 5.40] I^2=0.0%, 3 studies, 184 pregnancies) (Figure 2.17).

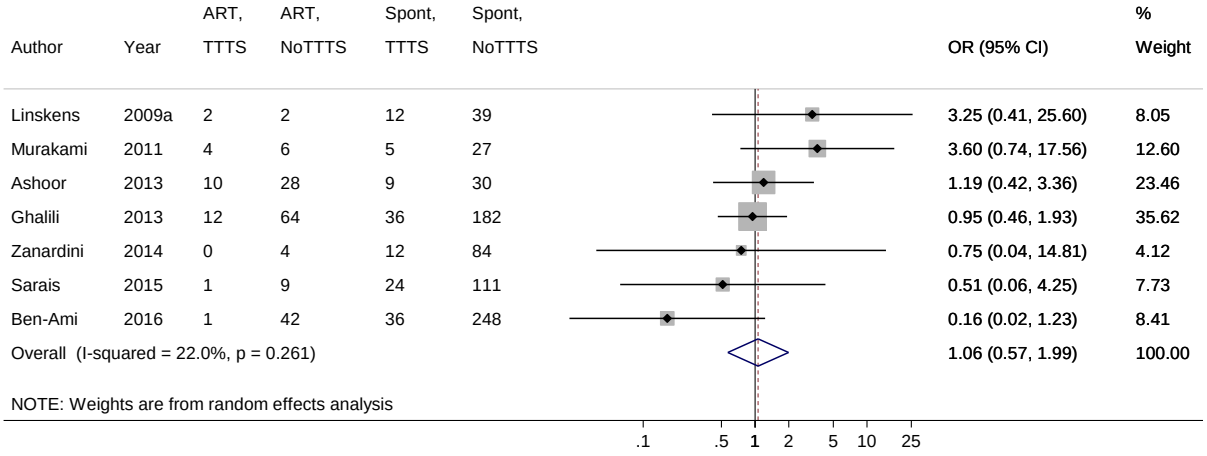
Figure 2.17 Forest plot of association between maternal smoking and TTTS



Mode of conception and TTTS

An OR >1 indicated a higher-risk of TTTS if the pregnancy was conceived by ART, and an OR <1 indicated a higher-risk of TTTS if the pregnancy was conceived spontaneously. No significant association between mode of conception and TTTS was found (OR 1.06 [95%CI 0.57, 1.99] I^2=22.0%, 7 studies, 1040 pregnancies) (Figure 2.18).

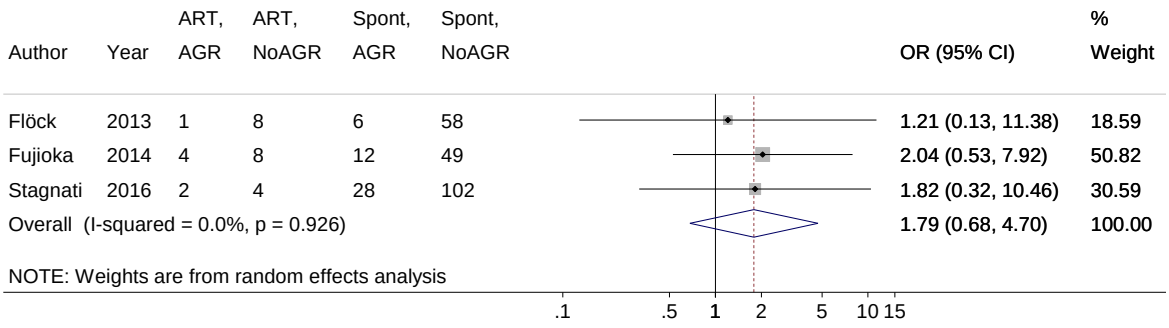
Figure 2.18 Forest plot of association between mode of conception and TTTS



Mode of conception and AGR

An OR >1 indicated a higher-risk of AGR if the pregnancy was conceived by ART, and an OR <1 indicated a higher-risk of AGR if the pregnancy was conceived spontaneously. No significant association between mode of conception and AGR was found (OR 1.79 [95%CI 0.68, 4.70] I^2=0.0%, 3 studies, 282 pregnancies) (Figure 2.19).

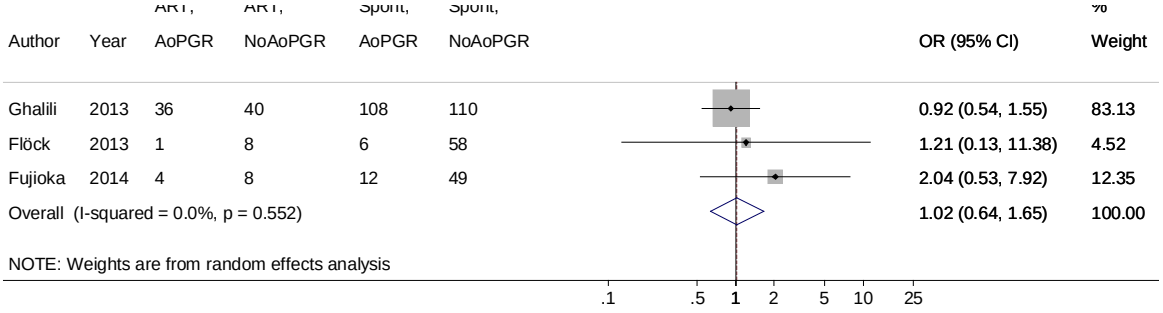
Figure 2.19 Forest plot of association between mode of conception and antenatal growth restriction (AGR)



Mode of conception and AoPGR

An OR >1 indicated a higher-risk of AoPGR if the pregnancy was conceived by ART, and an OR <1 indicated a higher-risk of AoPGR if the pregnancy was conceived spontaneously. As Ghalili et al. (Ghalili 2013) presented both BWD and LBW in one baby, and in two babies, the latter two measures were combined to reflect LBW in at least one twin. No association between mode of conception and AoPGR was found (OR 1.02 [95%CI 0.64, 1.65], I^2=0.0%, 3 studies, 440 pregnancies) (Figure 2.20).

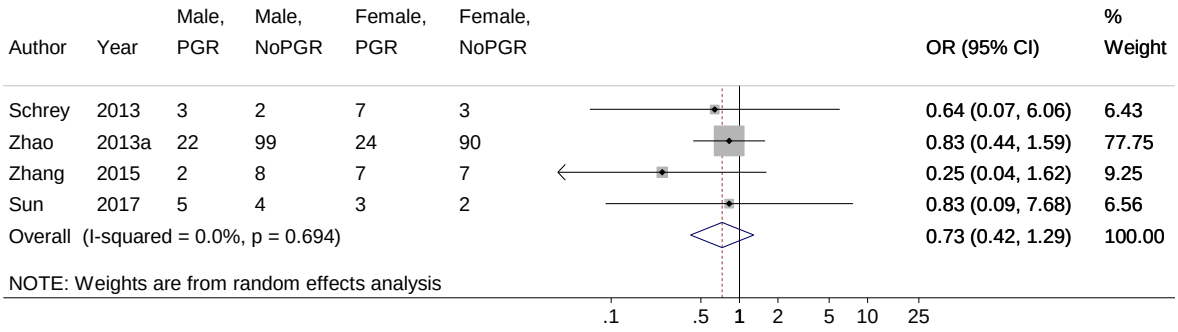
Figure 2.20 Forest plot of association between mode of conception and antenatal or postnatal growth restriction (AoPGR)



Fetal gender and PGR

An OR >1 indicated a higher-risk of PGR if the fetuses were male, and an OR <1 indicated a higher-risk of PGR if the fetuses were female. No significant association between fetal gender and PGR was found (OR 0.73 [95%CI 0.42, 1.29] I^2=0.0%, 4 studies, 288 pregnancies) (Figure 2.21).

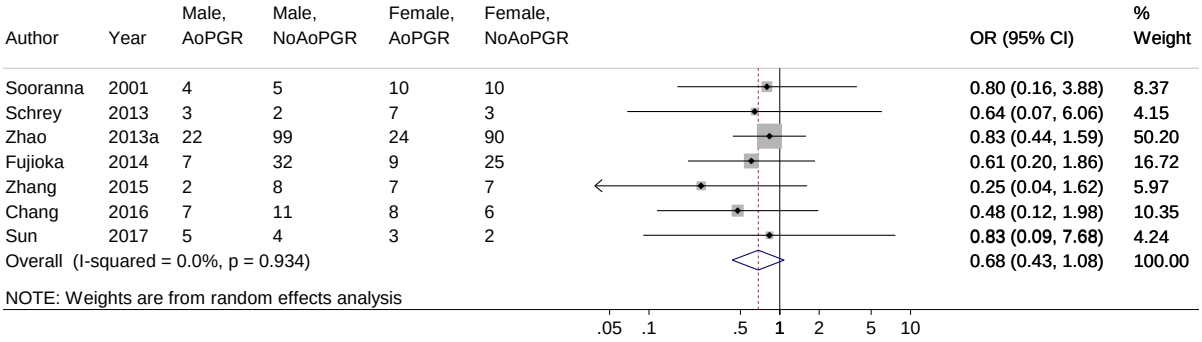
Figure 2.21 Forest plot of association between fetal gender and postnatal growth restriction (PGR)



Fetal gender and AoPGR

An OR >1 indicated a higher-risk of AoPGR if the fetuses were male, and an OR <1 indicated a higher-risk of AoPGR if the fetuses were female. No significant association between fetal gender and AoPGR was found (OR 0.68 [95%CI 0.43, 1.08], I^2=0.0%, 7 studies, 422 pregnancies) (Figure 2.22).

Figure 2.22 Forest plot of association between fetal gender and antenatal or postnatal growth restriction (AoPGR)



2.4 Comment

2.4.1 Main findings

This is the first systematic review to look at first trimester potential prognostic factors for growth restriction in MC twins, and explore maternal characteristics and first trimester maternal serum biomarkers as prognostic factors for TTTS. Although a significant association was found between NT>95th centile in one/both fetuses and TTTS, and CRL discordance ≥10% and TTTS, both demonstrated poor individual prognostic ability. A moderate significant association between maternal ethnicity and TTTS was found, with Caucasian women more likely to develop TTTS, but as there is no plausible biological mechanism for this association this may reflect the lack of diversity within the study populations and publication bias. The other first trimester ultrasound measurements and maternal characteristics demonstrated no association with adverse outcomes.

Only 2 studies examined first trimester maternal serum biomarkers, with Ashoor et al. (Ashoor 2013) finding no significant difference in TSH, FT4 or β-hCG in those pregnancies that developed TTTS, and Linskens et al. (Linskens 2010) noting a trend towards increased β-hCG and PAPP-A levels in those pregnancies that developed TTTS. As Linskens only reported the median and not the IQR these studies could not be combined in meta-analysis, but this warrants further investigation due to the small study sizes and biological plausibility of β-hCG and PAPP-A being implicated in TTTS as markers of placental function.

2.4.2 Strengths and limitations

A major strength of this study was to include all possible prognostic factors and perform a robust statistical analysis to look at the association and prognostic ability of the factors. The search strategy was as inclusive as possible, and there was no limit on language. It was particularly important to look at modifiable factors such as smoking and maternal BMI where lifestyle changes may be associated with a lower risk for adverse outcome.

One limitation of this review was the different definitions that studies used for their control groups, variables and outcomes, which is why individual definitions were not rigidly stipulated prior to commencing the search. This was an issue for growth restriction as there is currently no validated standard definition of abnormal growth in MC twins. Consequently, the included studies defined growth restriction in a myriad of ways: AC ≤5th centile, EFW <10th centile, EFW <5th centile, EFWD >20%, LBW <10th centile, LBW <5th centile, BWD ≥20%, BWD ≥25%, and in different combinations of in one twin, or both twins when not measuring discordance, all of which can be associated with adverse outcome. This issue has attempted to be investigated by a recent Delphi consensus looking at selective fetal growth restriction in twin pregnancy (Khalil 2018), and will be addressed by the creation of a 'Core Outcome Set' for 'selective fetal growth restriction in twin pregnancies'(Gordijn 2017) which is due to be completed by August 2019. In this systematic review, the problem was attempted to be addressed by creating different growth restriction groups so as to be as inclusive as possible. The AGR group reflects real life and is what obstetricians base their management on. However, ultrasound scanning and

calculation of EFWD only has a moderate ability to detect BWD with a recent systematic review reporting a sensitivity of 65.4% (95%CI 57.9, 72.3) and specificity of 90.8% (95%CI 87.1, 93.5%) for EFWD ≥20% predicting BWD ≥20%, although the analysis did include DC and MC twins (Leombroni 2017). Therefore, the PGR group was included as an absolute measure, which avoids scan error. However, there is controversy whether BWD is reflective of pathological growth, and indeed what the cut-off should be. A recent meta-analysis, that also highlighted the problem of different definitions of abnormal growth in twins, demonstrated that MC twins with BWD ≥20% (which also included EFWD ≥20%) had a higher risk of IUFD than concordant MC twins (OR 2.8 [95%CI 1.3,5.8] 6 studies, 1286 pregnancies, I^2 not reported) (D'Antonio 2017a). Currently, MC twins with isolated BWD are not managed differently neonatally as this is guided by the actual birthweight. Additionally, BWD is not always reflective of IUGR with 21.1% of pregnancies with BWD ≥20% not including at least one fetus with an EFW <10th centile, and 21% of pregnancies with at least one fetus with an EFW <10th centile not demonstrating concurrent BWD (Neves 2017). However, most studies in the search that reported growth based on postnatal measures used BWD as opposed to LBW, therefore it was decided to include BWD in the systematic review. The use of inter-twin growth discordance in isolation, whether EFWD or BWD, also presents the problem of missing pregnancies where both twins are growth restricted, but irrespective of choice of definition and cut-offs, all growth outcomes have the common problem of not being based on specific twin growth charts, which until July 2017 did not exist. Since performing this review, twin growth charts have been launched in the UK to enable more accurate assessment of twin fetal growth (TAMBA 2017). Another issue

was that of cut-offs for the variables as the cut-offs have not been adequately validated and may not be appropriate for the study's patient population, or the conditions being explored. The initial aim was to compare a) disease vs. no disease but where other complications may be present, and b) disease vs. normal pregnancy where no complications were present at all. However as most studies used the former control group, it was not possible to perform a separate analysis for the latter comparison. This has only allowed the evaluation of the ability of each potential prognostic factor to predict a specific condition, and not any condition (and thus cannot predict the chance of the pregnancy being completely 'normal').

2.4.3 Comparison with existing literature

A systematic review evaluating prognostic factors up to 16 weeks for TTTS has recently been published, however they only looked at ultrasound prognostic markers, and their search was up to April 2014 (Stagnati 2017). They stated that an increased risk of TTTS was associated with inter-twin NT discrepancy, NT>95th centile, and CRL discrepancy, but similar to the results in this chapter, the prognostic ability of these factors was low. In addition to the inclusion of other MC twin complications, there are other differences between this review and the review by Stagnati et al., including that Stagnati et al. did not exclude pregnancies with chromosomal/structural anomalies that affect first trimester ultrasound measurements. D'Antonio et al. performed a systematic review examining the ability of first trimester CRL discordance ≥10% to predict BWD ≥20%, PTB, fetal anomalies, IUFD and NND, and found it also had a low prognostic ability for all outcomes (D'Antonio 2014).

2.4.4 Implications

The main clinical implication of the results of this systematic review is that they support the guidance from the Royal College of Obstetricians and Gynaecologists (RCOG) that 'screening for TTTS by first trimester NT measurements should not be offered'(Kilby 2016). Although significant associations between first trimester variables and subsequent pregnancy outcome have been shown, the prognostic ability of each individual variable is poor, thus the results do not suggest their use to screen MC pregnancies in clinical practice.

A gap in knowledge has been identified which has implications for research as most studies able to be included in the systematic review were not designed with the intention of assessing first trimester prognostic factors for subsequent outcomes in MC twins. Consequently, the OMMIT study (ISRCTN13114861) was designed: a large cohort study, purposefully-designed to investigate potential prognostic factors and explore novel prognostic markers that have not previously been evaluated: including AFP, PAPP-A, and sFlt-1 (Mackie 2017). To avoid the problem of using non-validated cut-offs, variables should be kept continuous and not dichotomised.

2.5 Conclusion

The association and prognostic ability of first trimester factors, including maternal characteristics, associated with TTTS, growth restriction and IUFD were investigated. It is not currently possible to predict adverse outcomes in MC twin pregnancies, and a lack of research investigating first trimester biomarkers in MC twin pregnancies has

been revealed. Different assessment methods and definitions of each variable and outcome were an issue and this highlights the need for a large cohort study to evaluate these factors.

CHAPTER 3 IDENTIFYING FIRST TRIMESTER PROGNOSTIC FACTORS OF COMPLICATIONS IN MONOCHORIONIC TWIN PREGNANCIES

- These findings were presented as an oral presentation at the British Maternal and Fetal Medicine Society 18th Annual Conference, March 2017, Amsterdam, The Netherlands, and as a platform poster presentation at the Royal College of Obstetricians and Gynaecologists Annual Academic Meeting, February 2018, London, both presented by F Mackie.
- The protocol for this study has been published [Mackie FL, Morris RK and Kilby MD (2017). "The prediction, diagnosis and management of complications in monochorionic twin pregnancies: the OMMIT (Optimal Management of Monochorionic Twins) study." BMC Pregnancy Childbirth 17(1):153].
- These findings have been published in abstract form [Mackie FL, Hall MJ, Hyett J, Mills I, Riley R, Morris RK and Kilby MD (2017). "First trimester prediction of adverse events in monochorionic diamniotic twins: The OMMIT study." BJOG 124(S2):6].
- These findings have been accepted for publication in full article form [Mackie FL, Whittle R, Morris RK, Hyett J, Riley RD and Kilby MD (2019). "First trimester ultrasound measurements and maternal serum biomarkers as prognostic factors in monochorionic twins." J Diagn Progn Res]

3.1 Overview

Monochorionic (MC) twins are high-risk pregnancies. At present it is not possible to delineate which MC twins will develop complications. The systematic review in CHAPTER 2 demonstrated that first trimester ultrasound measurements are associated with adverse outcome, and that there is a dearth of research into biomarkers in MC twin pregnancies with the only validated biomarkers in MCDA twins being those used in the combined and quadruple aneuploidy screening tests. After a systematic review of current research, the next step in prognostic research is to assess the predictive ability of individual prognostic factors. This chapter will outline potential first trimester ultrasound measurements and maternal serum biomarkers, and will evaluate their ability to predict adverse outcomes in MCDA twin pregnancies in an international retrospectively recruited cohort of MCDA twin pregnancies undergoing first trimester aneuploidy screening.

3.2 Potential prognostic factors

In this section, unless stated otherwise, the ultrasound measurements and biomarkers referred to were measured in the first trimester, and the biomarkers were evaluated in maternal serum or plasma samples. Studies in twin pregnancies were used where possible, but if no evidence was found in twin pregnancies, research in singleton pregnancies was presented. Every attempt was made to report results according to chorionicity, but if this was not possible using the information provided in the published article, MC and/or DC twins were referred to as ‘twin pregnancies’.

3.2.1 Nuchal translucency (NT)

NT is an ultrasound measurement of subcutaneous fluid in the nuchal fold at the back of the fetal neck. It is performed in a mid-sagittal view, with the fetus at 90° to the angle of insonation and in a neutral position with the neck neither hyperextended nor flexed (FMF 2004). There should be adequate magnification, the callipers should be placed on the outer borders at the widest part of the nuchal fold, and the mean of 2 measurements should be used. The commonest association with a raised NT, defined as ≥3.5mm, is chromosomal anomalies (Montenegro 1997, Snijders 1998, Souka 1998, Souka 2001). Consequently NT is one component of first trimester combined aneuploidy screening test that consists of: NT, maternal age, free β-hCG and PAPP-A which are detailed elsewhere in this section. There is a strict time frame in which combined screening can be performed, 11+0 to 14+1 weeks, which is calculated based on the CRL (see below) (FASP 2015). The time frame and process is the same when combined aneuploidy screening is performed in twin pregnancies as in singleton pregnancies, but the NT measurement of each twin in included in the algorithm, and an individual risk of each DC twin may be calculated. However, the accuracy of the combined aneuploidy screening test in twin pregnancies is lower than in singletons (Wald 2003). One study compared the screening accuracy of combined screening in singleton pregnancies, MC twins, and DC twins (Wald 2003). They found a detection rate of 85%, 84% and 70% respectively based on a 5% false positive rate. Accuracy is lower in twins because of inter-twin differences in the biochemical levels of β-hCG and PAPP-A. The substantial decrease in accuracy in DC twins reflects aneuploidy often being discordant in DC twins, thus the alteration in the affected twin's biochemical levels is compensated by the other twin's normal

levels and the detection rate is lower (Garchet-Beaudron 2008). Additionally, MC twins have a higher prevalence of an increased NT in euploid fetuses than DC twins, and singleton fetuses (8.4% vs 5.4% vs 5.2% respectively) which also increases the false positive rate (Sebire 1996). A recent Cochrane review reported that at a cut-off point of 1:250 risk of trisomy 21, the sensitivity and specificity of combined aneuploidy screening in all pregnancies is 85% (95%CI 81, 87) and 95% (95%CI 95, 96) (25 studies, 174,712 fetuses including 1032 fetuses affected by Down's syndrome) (Alldred 2017). Two of the 25 studies were performed in multiple pregnancies, but when the authors removed these 2 studies as part of a sensitivity analysis, it made no difference to the results. Prats et al. performed a meta-analysis of first trimester combined screening in twin pregnancies only (5 studies, 12,974 fetuses, 69 cases of trisomy 21) and reported a higher sensitivity (89.3% [95%CI 79.7, 94.7]) and similar specificity (94.6% [95%CI 93.3, 95.7]) compared to Alldred et al. (Prats 2014). The reason for the discrepancy in included studies in the two meta-analyses is not clear, but despite Alldred et al. being published in 2017, the search was run up to 2011, whereas Prats et al. performed their search up to 2013. Prats et al. also performed an analysis comparing MC with DC twins, but found no difference in test accuracy.

In addition to aneuploidy, a raised NT measurement is also associated with fetal structural abnormalities particularly cardiac defects (Souka 1998, Hyett 1999). The pathogenesis of how an increased NT measurement is linked to cardiac defects is not clear, but one hypothesis is that the raised fluid in the nuchal fold is a result of cardiac failure (Montenegro 1997). Other hypotheses include compressed umbilical

veins due to a nuchal cord (Maymon 1999), increased type VI collagen in the skin of the nuchal fold (von Kaisenberg 1998), or delayed endothelial remodelling causing jugular lymphatic sac distension (Haak 2002), which also links to cardiovascular function.

The relationship between NT and growth restriction in twin pregnancies, was not able to be explored in CHAPTER 2 due to too few studies being able to be grouped together for meta-analysis. Individual studies report conflicting findings. One study suggested NT discordance has excellent test accuracy to predict sIUGR in MC twin pregnancies with an AUC of 0.93 (95%CI 0.87, 1.00) although this is based on 4 (2.96%) MC sIUGR pregnancies out of a total cohort of 136 MC twin pregnancies (Fratelli 2011). Other studies have found no relationship between growth restriction and NT measurements in twin pregnancies in the first trimester (El Kateb 2007, Memmo 2012, Zoppi 2014).

The systematic review in CHAPTER 2 did find an association between a first trimester NT measurement >95th centile in one/both fetuses and TTTS (OR 2.29 [95%CI 1.05, 4.96] I^2=6.6%, 4 studies, 615 pregnancies), but it had a low predictive ability. A trend towards a significant association between NT discordance ≥20% and TTTS was also reported, although there was a high-risk of heterogeneity (OR 2.48 [95%CI 0.90, 6.84] I^2=67.6%, 4 studies, 710 pregnancies). No studies could be meta-analysed that had not dichotomised the NT data which is important to note as by dichotomising the data important data is lost, and it may be that by examining the data as continuous variables the predictive ability may be improved. The connection

between a raised NT and TTTS is interesting given the association between a raised NT and cardiac defects, and the high rate of cardiac dysfunction seen in TTTS, especially in the recipient twin. A substantial proportion of the initial work on cardiac defects and raised NT measurements was performed in fetuses with aneuploidy (Haak 2003). However euploid fetuses with a raised NT and abnormal DV Doppler are often found to have cardiac defects as well (Matias 1999). In TTTS, Sebire et al. hypothesise that the raised NT is because of hypervolaemic congestion which the developing fetal heart is less able to cope with in the first trimester compared to later in pregnancy (Sebire 2000). No other meta-analyses of NT measurement and its association with adverse outcome in MC twins could be performed in CHAPTER 2. The study that reported a high AUC for predicting sIUGR by NT discordance (Fratelli 2011) suggests a relationship may exist. In view of the lack of research, and the possible link between NT and TTTS, this warrants further investigation as a potential prognostic factor of adverse outcome in MC twin pregnancies.

3.2.2 Crown-rump length (CRL)

CRL is the ultrasound measurement that assesses the length of the fetus from the top of the fetal head to the bottom of the buttocks. It is performed in a mid-sagittal view, with the fetus at 90° to the angle of insonation and in a neutral position (Intergrowth-21st 2010). The image should fill ≥30% of the monitor screen and the callipers to perform the measurement should be placed on the outer borders. The CRL is routinely used to date the pregnancy so that antenatal care can be planned accordingly. The correct dating of twin pregnancies is vital to ensure management plans are performed in a timely manner, such as delivery at 36 weeks gestation for

normal MCDA twins to avoid perinatal death. The use of the last menstrual period is not as reliable as ultrasound due to the natural biological variation in menstrual cycle length and frequency, and the reliance on self-reporting by women (Mongelli 1996, Taipale 2001, Savitz 2002). There are various formulae to convert the CRL into gestational age (Sladkevicius 2005), but the differences between the formulae are not considered clinically significant. The formulae used for singleton charts are able to be used for twin pregnancies (Dias 2010) because the rate of growth in singletons and twins is similar in the first trimester (Martins 2009, Morin 2011). In twin pregnancies there is the additional complexity of which twin's CRL measurement to use. The most accurate method of dating twin pregnancies by ultrasound has been debated with some studies supporting the use of the smallest twin CRL (Salomon 2005, Dias 2010, Chaudhuri 2013), others the largest twin CRL (Simpson 2013), and others the mean inter-twin CRL (Dias 2010), although the majority of these studies have been performed on DC twins. As advised by various national bodies (Morin 2011, NICE 2011, Khalil 2016b, Kilby 2016), the consensus is to use the largest twin CRL (Simpson 2013) as it is rare for a twin to be pathologically large, and this means that the pregnancy is not dated based on a potentially pathologically small fetus affected by aneuploidy (Nakamura 2015), or a fetus which may already be demonstrating signs of IUGR (Memmo 2012, Zoppi 2014). CRL measurements including both twins, such as the use of the mean inter-twin CRL in MC twins is controversial due to the reported association between inter-twin CRL discordance, and subsequent adverse outcomes such as TTTS.

The CRL is important if the woman opts to undergo aneuploidy screening as the normal values of the NT measurement and serum biomarkers depend on gestation at

measurement. A CRL of 45-84mm is equivalent to 11+0 to 14+1 weeks (Robinson 1975) which is considered the optimum time for combined first trimester aneuploidy screening (FASP 2015). If the woman presents after the first trimester as indicated by a CRL >84mm, the head circumference should be used to date the pregnancy as the CRL after this time is less accurate (NICE 2011). Combined aneuploidy screening is not an option at this stage due to decreased accuracy; if the woman would like to undergo aneuploidy screening she will need to have the quadruple test. The quadruple test may be performed up to 20+0 weeks gestation and consists of serum inhibin-A, unconjugated oestriol, β-hCG and AFP, the latter two biomarkers are described below.

The association between TTTS and CRL was explored by the meta-analysis in CHAPTER 2 that found that CRL discordance ≥10% was significantly associated with TTTS (OR 2.43 [95%CI 1.13, 5.21] I^2=14.1%, 3 studies, 708 pregnancies) but had a low prognostic ability. However, studies dichotomised the data using a non-validated cut-off, and it may be that when examined as continuous data, the prognostic ability is improved. Other cut-offs and calculations involving CRL were investigated, but there were insufficient studies to group together to perform meta-analyses.

With regards the relationship between CRL and growth restriction in twin pregnancies, two studies have suggested fair to good test accuracy with AUCs of 0.77 (95%CI 0.37,1.00) (Fratelli 2011) and 0.89 (95%CI 0.83, 0.95) (Memmo 2012) when using CRL to predict sIUGR in MC twin pregnancies. Another study reported a possible link between CRL discordance ≥10% and SGA fetuses in MC and DC twins

as a higher proportion of twins in the CRL discordance group had a SGA fetus compared to the proportion of SGA fetuses in the CRL concordant group (Grande 2016). This study also found a possible link between CRL discordance and BWD, but only in MC twins. Another study found no relationship between growth restriction and CRL discordance in twin pregnancies (El Kateb 2007).

3.2.3 β-human chorionic gonadotropin (β-hCG)

hCG is fundamental for a healthy pregnancy. It is a non-covalent heterodimer comprised of α- and β- subunits common to the 3 isoforms of hCG. hCG is a member of the glycoprotein hormone family and is structurally similar to LH, TSH and FSH. This means that hCG can bind to LH receptors and there is an overlap in function at the start of pregnancy. The β-subunit is 80% homologous to LH, whereas the α-subunit is almost completely homologous to LH, TSH and FSH; therefore it is difficult to distinguish the α-subunit in most commonly used assays, thus β-hCG is most frequently measured (Stenman 2006). There are three dimeric isoforms of hCG that are a result of post-translational modifications, and the three isoforms have different sites of production and distinct functions: “regular” hCG, sulphated hCG and hyperglycosylated hCG (Nwabuobi 2017). Sulphated hCG is only produced by the anterior pituitary gland and mainly acts during the menstrual cycle. Hyperglycosylated hCG is produced by cytotrophoblasts and is an autocrine/paracrine factor, as opposed to a hormone, and promotes cell growth and differentiation. Hyperglycosylated hCG levels are also high in germ cell tumours such as testicular cancer (Milose 2011), but this thesis will focus on pregnancy. In the first 11 weeks of

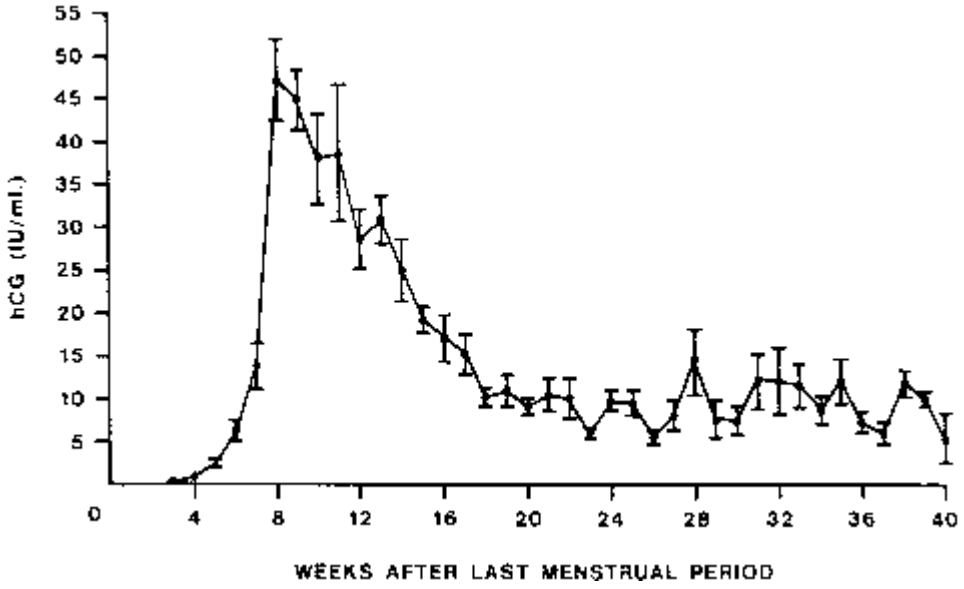
gestation, invasive extravillous trophoblasts secrete hCG, is mainly in the hyperglycosylated form (Guibourdenche 2010).

Since its discovery in the early 1900s, hCG has been found to be involved in a plethora of processes crucial for pregnancy development, as summarised by Cole et al. (Cole 2010), including promoting blastocyst signalling to the endometrium prior to implantation, progesterone secretion from the corpus luteum, uterine vasculature angiogenesis, immunosuppression and blockage of phagocytosis of invading trophoblast cells, and myometrial quiescence. hCG is mainly produced by syncytiotrophoblast during pregnancy (Handschuh 2007, Fournier 2015) and has a half-life of 37 hours, thus making it a useful pregnancy-specific biomarker (Faiman 1968). Prior to conception, hCG injections are used in ART to stimulate oocyte maturation and ovulation before oocyte retrieval (Filicori 2005). In pregnancy, maternal serum β-hCG levels increase in the first trimester and decrease for the remainder of pregnancy (Figure 3.1), with levels related to the rate of cytotrophoblast differentiation into syncytiotrophoblast, as opposed to placental mass (Hay 1988). The same pattern is seen in twin pregnancies, although maternal serum levels are higher in twin than singleton pregnancies in the first trimester (Spencer 2000, Spencer 2008, Madsen 2011) and second trimester (Nebiolo 1991, Neveux 1996, O'Brien 1997, Räty 2000, Xie 2008, Ren 2016, Svirsky 2016). When chorionicities were compared MC twins had higher maternal serum β-hCG levels than DC twins in the second trimester (Muller 2003), although findings in the first trimester are conflicting with the majority of studies reporting no difference (Spencer 2001, Niemimaa 2002, Gonce 2005, Spencer 2008, Koster 2010, Madsen 2011, Svirsky

2016), and two studies reporting higher levels in DC than MC twin pregnancies in the first trimester (Linskens 2009b, Prats 2012).

Figure 3.1 Maternal serum levels of human chorionic gonadotropin (hCG) in singleton pregnancy

(n=443 pregnancies) (Braunstein 1976). Mean and Standard error of the mean displayed.



Due to the sharp increase in the first trimester, maternal serum β-hCG levels are used to diagnose pregnancy itself, and to monitor early pregnancy complications including miscarriage (Stenman 2006) and ectopic pregnancy (Silva 2006), in conjunction with ultrasound assessment (Seeber 2006). Low maternal serum levels, or those with a sub-optimal rise are more likely to result in miscarriage in singleton pregnancies (Stenman 2006), although no studies were found supporting this in twin pregnancies (Rosner 2015). Maternal serum levels are part of the first trimester combined, and second trimester quadruple test as part of the aneuploidy screening programme (FASP 2015) as increased first and second trimester β-hCG is associated with aneuploidy (Bogart 1987, Wapner 2003). This increase is also seen

in twin pregnancies affected by aneuploidy (Noble 1997, Madsen 2011). Another focus of research on first trimester hCG is on pre-eclampsia prediction. The biological basis behind this hypothesised link is that low first trimester hyperglycosylated hCG levels are associated with non-invasive, and thus dysfunctional, syncytiotrophoblasts (Keikkala 2013). Although initial studies showed promise in singletons, a combination of first trimester biomarkers, including decreased first trimester β-hCG, and ultrasound measures with sufficient predictive ability for clinical practice has not yet been discovered (Zhong 2015). Results are more encouraging from second trimester work (Spencer 2006) with a combination of β-hCG and PAPP-A improving the current predictive ability of maternal characteristics which the current National Institute for Health and Care Excellence (NICE) guidance uses to indicate which women should receive low dose aspirin to reduce their risk of pre-eclampsia (Wright 2016).

In singletons, increased second trimester β-hCG is associated with the following complications as summarised by Gagnon et al. (Gagnon 2008): IUGR, PTB, miscarriage, stillbirth and pregnancy-induced hypertension. In twins, one study that only included DCDA twins reported an association between increased second trimester β-hCG and PTB, and neonatal unit (NNU) admission, but these associations were not present with first trimester β-hCG (Rosner 2015). Another study that did not perform sub-group analysis according to chorionicity reported increased first trimester β-hCG was associated with pregnancy-induced hypertension, IUGR and SGA in twin pregnancies, but there was no association with β-hCG levels and miscarriage, PTB <34 weeks, GDM and sIUGR (Iskender 2013). Another study did find an association between first trimester β-hCG and PTB <32

weeks gestation, although chorionicity and predictive ability were not reported (Laughon 2009). The evidence in singleton pregnancies shows poor predictive ability of first trimester hCG to predict PTB, and SGA fetuses (Zhong 2015).

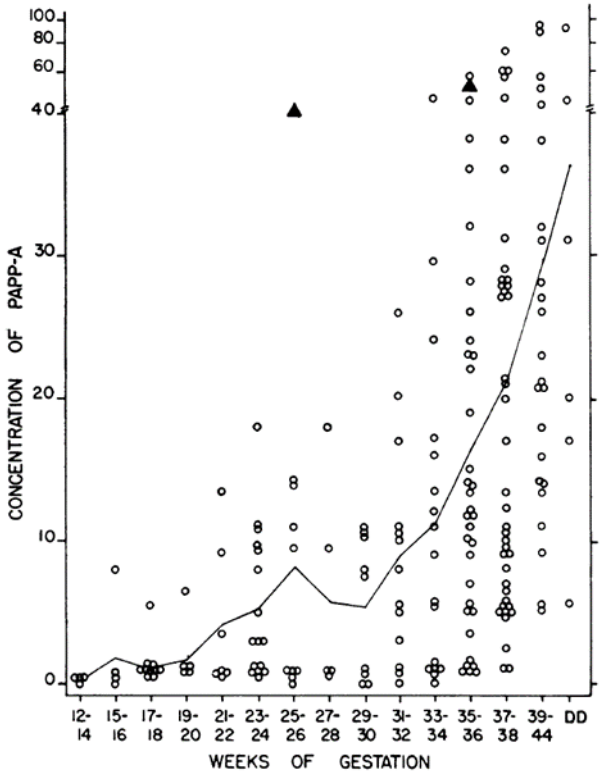
Two studies have investigated β-hCG levels in second trimester maternal serum at diagnosis of TTTS and both studies found significantly higher β-hCG levels in women with pregnancies affected by TTTS than matched MC and DC controls (Fox 2009, Sermondade 2009). Following FLA, there was no difference in maternal serum β-hCG 1 week post-FLA (Fox 2009), but another study reported a decrease at 2 and 4 weeks post-FLA (Hanaoka 2011) although this study did not perform blood sampling 1 week post-FLA. A difference between Quintero stages I and II, compared to III and IV was also reported, with the worse Quintero stages associated with higher β-hCG levels (Hanaoka 2011). Linskens et al. (Linskens 2010) examined first trimester β-hCG in the context of TTTS and although they reported a trend in increasing β-hCG in 12 TTTS pregnancies compared to 39 uncomplicated MC twin pregnancies, the p value was 0.32 suggesting there was actually no difference between the groups. Ashoor et al. also looked at first trimester β-hCG in 19 TTTS pregnancies and found no difference compared to 58 uncomplicated MC twin pregnancies (Ashoor 2013). Given the relationship between β-hCG and TTTS in the second trimester, and the lack of first trimester research, β-hCG merits further investigation in the first trimester in MC twin pregnancy complications.

3.2.4 Pregnancy-associated plasma protein A (PAPP-A)

PAPP-A is a metalloprotease that cleaves insulin-like growth factor binding protein (IGFBP) -4 and -5 (Byun 2001). It is produced by the syncytiotrophoblast and decidua (Schindler 1984, Barnea 1986) and has a half-life of 53±26 hours (Bischof 1981). When PAPP-A releases insulin-like growth factors (IGFs) from their IGFBPs by its protease activity, the IGFs are able to aid trophoblast invasion and thus placental development (Jones 1995). Consequently low PAPP-A is associated with the adverse outcomes detailed below. Maternal serum PAPP-A levels increase throughout gestation (Figure 3.2).

Figure 3.2 Maternal serum levels of pregnancy-associated plasma protein A (PAPP-A) in singleton and twin pregnancy

n=204 singleton pregnancies, represented by circles and n=2 twin pregnancies, represented by triangles. (Lin 1974). Individual values and mean line displayed.



In twin pregnancies, PAPP-A levels are higher in the first trimester (Spencer 2000, Niemimaa 2002, Spencer 2008, Madsen 2011, Sung 2017) and second trimester (Svirsky 2016) compared to singleton pregnancies, no studies were found comparing third trimester levels. When chorionicities are compared results are conflicting; some studies report no difference in first trimester PAPP-A (Niemimaa 2002, Koster 2010, Svirsky 2016), and other studies report lower levels in MC compared to DC twins in the first trimester (Spencer 2008, Linskens 2009b, Madsen 2011, Prats 2012).

First trimester PAPP-A levels are used clinically as part of the combined aneuploidy screening programme for twin and singleton pregnancies (FASP 2015) as low

PAPP-A is associated with aneuploidy in singleton and twin pregnancies (Madsen 2011). Low first trimester PAPP-A is considered a “major risk factor for delivery of a SGA neonate” in singletons according to RCOG guidance (Robson 2013). Maternity units have their own local guidance for low PAPP-A results. There is little evidence to support a significant association between low first trimester PAPP-A and growth restriction in twin pregnancies. Other associations between low first trimester PAPP-A and adverse outcome in singleton pregnancies, as reviewed by Gagnon et al. (Gagnon 2008) include: IUGR, PTB, pregnancy-induced hypertension, miscarriage and stillbirth, although the predictive ability for low birthweight (LBW), pre-eclampsia and stillbirth is poor (Morris 2017). In twin pregnancies research is sparse and conflicting. Rosner et al. only included DCDA twins and reported an association with low first trimester PAPP-A and PTB <37 weeks (relative risk 5.56 [95%CI 1.53-20.22]), but no association with PTB <34 weeks, LBW, hypertensive disorders of pregnancy or IUFD (Rosner 2015). Iskender et al. did not divide results according to chorionicity and did not find any difference in first trimester PAPP-A and miscarriage, PTB <34 weeks, pregnancy-induced hypertension, GDM, discordant growth, IUGR, SGA, or sIUGR in twin pregnancies (Iskender 2013). Another study also found no association between first trimester low PAPP-A and PTB at <37 or <32 weeks gestation (Laughon 2009). The relationship between pre-eclampsia and low PAPP-A in twin pregnancies is also inconsistent with one study reporting no difference in PAPP-A levels in twin pregnancies with pre-eclampsia and twin pregnancies without pre-eclampsia (Francisco 2017), and another study reporting increased PAPP-A in 12 twin pregnancies with pre-eclampsia whereas the singleton pre-eclampsia pregnancies were associated with decreased PAPP-A (Svirsky 2016). This appears

to be the only study in which increased PAPP-A is associated with an adverse outcome as Gagnon et al. reported that no studies have found any relation between high PAPP-A in the first trimester and adverse outcome (Gagnon 2008). Nevertheless, in singletons, when first trimester PAPP-A is combined with first trimester PlGF, mean arterial pressure, and uterine artery pulsatility index, a recent study demonstrated that the combination has better predictive ability than the maternal factors currently used in the NICE guidance to determine which women should be prescribed prophylactic low dose aspirin (Tan 2018).

Linskens et al. (Linskens 2010) was the only study to examine first trimester PAPP-A in TTTS and reported no difference in maternal serum PAPP-A in 12 TTTS pregnancies compared to 39 uncomplicated MC twin pregnancies. Considering the inconsistent associations between low PAPP-A and adverse outcomes in twin pregnancies, further research is required, including in TTTS.

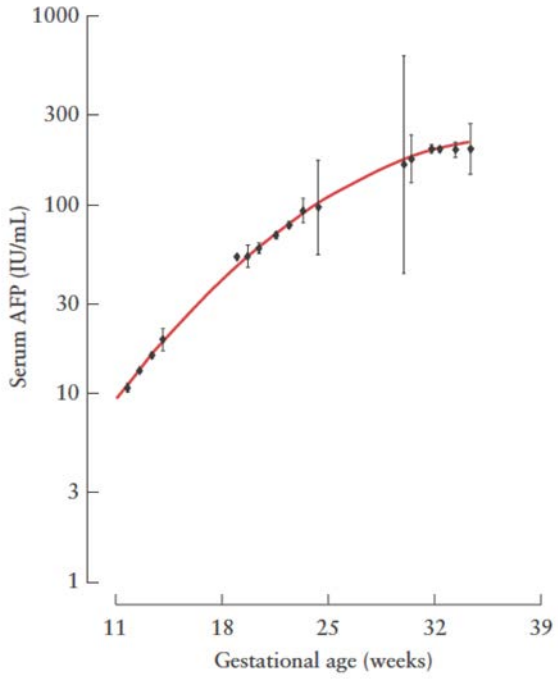
3.2.5 Alpha-fetoprotein (AFP)

AFP is a large plasma protein produced by the yolk sac, and hepatocytes in the fetal liver (Gitlin 1975). The exact action of AFP is not known but it is considered a marker of placental function (Mizejewski 2001). Altered serum levels are linked to various conditions outside pregnancy, including nonseminomatous germ cell tumours and hepatocellular carcinomas, but this thesis will focus on pregnancy. AFP is found in the amniotic fluid as the fetus urinates it out (Gitlin 1975). It is transferred from the fetus to the mother by different methods: paracellular diffusion across the fibrinoid deposits which act as breaks in the normally continuous syncytiotrophoblast

(Mizejewski 2001, Sharony 2016); or by entering the maternal blood vessels in the basal plate of the decidua basalis. Some attribute high maternal serum AFP levels to increased fibrin deposition which is associated with adverse outcome (Katzman 2002), and thought to create a “leaky” and dysfunctional placenta. AFP has a half-life of 2.5-5.5 days (Bredow 1985), and increases throughout gestation (Figure 3.3).

Figure 3.3 Maternal serum levels of alpha-fetoprotein (AFP) in singleton pregnancy

(n=17071 at 11+0 to 13+6 weeks, n=8583 at 19+0 to 24+6 weeks, n=8607 at 30+0 to 34+6 weeks) (Bredaki 2015). Median and 95%CI displayed.



Wald et al. first highlighted that second trimester AFP levels in 11 euploid multiple pregnancies were significantly higher than in 22 matched singleton pregnancies (Wald 1975). These findings have been replicated by other studies (Barnabei 1995, Neveux 1996, O'Brien 1997, Perenc 1998, Räty 2000, Xie 2008, Ren 2016, Svirsky 2016) and demonstrate that singleton levels cannot be used for twin pregnancies,

although no distinction was made by the studies of MC or DC twins. There is less research on first trimester AFP levels, particularly in twin pregnancies, as only one study could be found comparing first trimester AFP levels in twins and singletons (Berry 1995). They found “a less marked rise” in the first trimester than second trimester but highlight that they were not able to capture all the twin pregnancies in the first trimester, with half the “true rate” being identified as twin pregnancies in the first trimester, and again no distinction between chorionicities was made. When chorionicities are compared results are conflicting; one study reported no difference in second trimester maternal serum AFP (Muller 2003), but another study reported higher AFP levels in DC than MC twin pregnancies (Svirsky 2016), although it is not clear what gestation the blood samples were taken in the latter study.

The lack of focus on first trimester AFP in twin pregnancies may be because of its clinical ineptness in first trimester singleton pregnancies (Alldred 2015) with no studies found exploring first trimester AFP in twins in view of future growth restriction. In singleton pregnancies in the first trimester, one study has shown raised AFP is associated with spontaneous PTB but the predictive ability is poor (Beta 2011). Second trimester maternal serum AFP levels are widely used as part of the national aneuploidy screening programme (FASP 2015) as decreased second trimester AFP is associated with trisomy 21 (Merkatz 1984). Elevated second trimester AFP also has additional associations with a host of complications in singleton pregnancies, as summarised by Gagnon et al. (Gagnon 2008), including: IUGR, placental abruption, PTB, miscarriage, and pregnancy-induced hypertension, but AFP levels are not used clinically to predict or diagnose any of them. Decreased AFP is also associated with

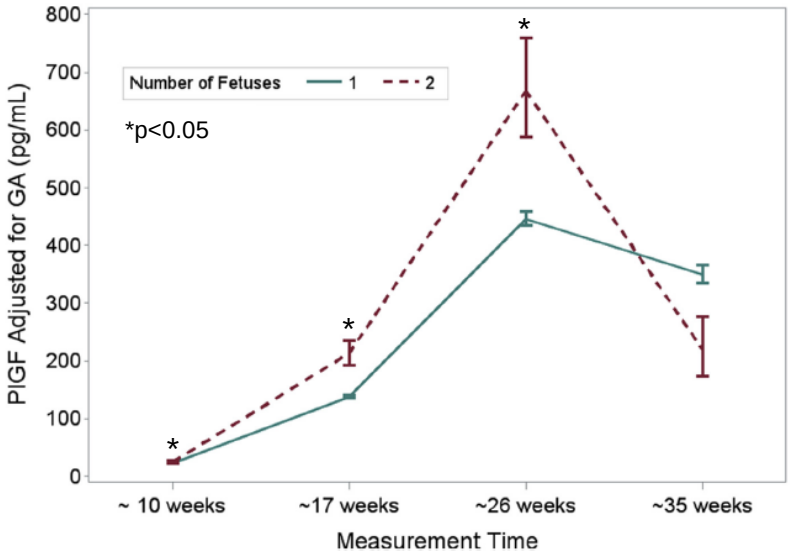
adverse outcomes such as triploidy, PTB, miscarriage and stillbirth (Thomas 1990, Zarzour 1998, Krause 2001). The enigma of high and low levels of AFP resulting in the same adverse outcomes suggests that its function is integral to the development of a healthy pregnancy. In twin pregnancies, abnormal second trimester AFP is linked to PTB, LBW, miscarriage and NNU admission (Wald 1978, Ghosh 1982, Hong 1996, Rosner 2015).

Both amniotic fluid and maternal serum AFP levels have been examined in TTTS, but only in samples taken at diagnosis of TTTS in the second trimester. Fox et al. (Fox 2009) reported higher AFP levels in TTTS pregnancies (n=21) compared to matched uncomplicated MC (n=6) and DC (n=12) twin pregnancies. AFP levels increased even further following FLA (Fox 2009). Sermondade et al. (Sermondade 2009) found no significant difference in maternal serum AFP levels in pregnancies affected by TTTS (n=60) compared to MC twins without TTTS (n=380). Huber et al. (Huber 2004) was the only study to explore amniotic fluid AFP, which was taken from the recipient twin's sac; when compared to singleton pregnancies with normal karyotypes, no difference was seen. A positive correlation was demonstrated between AFP and the DV pulsatility index in the recipient twin which the authors state reveals an association between AFP and severity of congestive heart failure. Considering the lack of information on AFP in twin pregnancies, particularly in the first trimester, this warrants further investigation.

3.2.6 Placental growth factor (PlGF)

PlGF is a dimeric glycoprotein and a member of the vascular endothelial growth factor family (Carmeliet 2001, Sung 2017), promoting angiogenesis and vasculogenesis. It is produced mainly by trophoblasts, placental villi and human umbilical vein endothelial cells. Levels are higher in twin pregnancies than singleton pregnancies (Maynard 2008, Sánchez 2012, Cowans 2013, Faupel-Badger 2015, Sung 2017), and increase in pregnancy up to the third trimester, then decrease in both singleton and twin pregnancies (Maynard 2008, Romero 2008, Wataganara 2012, Faupel-Badger 2015, Tsiakkas 2015a) (Figure 3.4). The difference between twin and singleton pregnancy levels in the first trimester is much smaller, but remains significant (Cowans 2013, Faupel-Badger 2015). A recent study reported that first trimester maternal serum PlGF levels were higher in DC twin pregnancies compared to singleton pregnancies, but that there was no difference in MC twin pregnancies compared to singletons; they did not directly compare MC with DC twin pregnancies (Francisco 2017). This is supported by another study that found that first trimester maternal serum PlGF levels were significantly higher in DC twin pregnancies compared to singletons (41% higher AFP level), and although MC twin levels were significantly higher than singletons (16%), the difference was not as big (Cowans 2013). This study also did not directly compare MC with DC twin pregnancies. Other studies found no difference in first trimester (Sánchez 2012) or third trimester PlGF (Faupel-Badger 2015) when MC twins were compared to DC twins, although there were only 5 MC twin pregnancies in the latter study. Another study combined first and second trimester samples and reported no difference between chorionicities (n=126 DC, 18 MC twin pregnancies) (Svirsky 2016).

Figure 3.4 Maternal serum levels of placental growth factor (PlGF) in twin pregnancy (n=91) compared to singleton pregnancy (n=2193)
(Faupel-Badger 2015). Information on chorionicity not available.



PlGF, placental growth factor.

[a] Mean (95% confidence interval) adjusted for gestational age at blood collection and presented by week of gestation; BIRTH cohort.

Faupel-Badger. Maternal angiogenic factors in twin and singleton pregnancies. Am J Obstet Gynecol 2015.

The relationship of PlGF with placental mass is controversial (Shibata 2005, Maynard 2008, Wataganara 2012) and serum levels are likely to be affected by additional factors. For example in trophoblast-like BeWo cells and cultured cytotrophoblasts, hypoxia downregulates PlGF mRNA and decreases PlGF production, thus decreasing angiogenesis (Lash 2002, Nagamatsu 2004). This has created a biological conundrum as it is unclear whether low PlGF occurs first, or hypoxia. It is thought that hypoxia secondary to the failure of extravillous trophoblast invasion might decrease placental output of PlGF thus contributing to decreased circulating free PlGF (Shibata 2005). This is one hypothesis regarding the link between PlGF and pre-eclampsia. Since its initial identification in 1991, there has been increasing interest in the use of PlGF for the prediction of pre-eclampsia. Studies in singletons

have shown that PlGF is decreased in pregnancies affected by pre-eclampsia (Maynard 2003, Levine 2004b, Shibata 2005, Staff 2005, Romero 2008, Wikström 2008, Nanjo 2017). This decrease is present in the first trimester, prior to the appearance of the clinical signs of pre-eclampsia, although the predictive ability of first trimester PlGF as a single marker is insufficient for clinical use. However, second trimester PlGF levels can be used as a single marker for pre-eclampsia. NICE evaluated the use of the 'Triage PlGF test' for the diagnosis of pre-eclampsia, and the recommendations made in May 2016 were that it could be used as a "rule out" test for pregnancies 20-34+6 weeks gestation, in addition to clinical information, but stated that there was insufficient evidence to use it as a "rule-in" test (NICE 2016). The guidance does not state whether this evaluation includes use in multiple pregnancies. Research on PlGF levels and pre-eclampsia in twin pregnancies indicate that different levels need to be used in twin pregnancies as PlGF levels in pre-eclamptic twin pregnancies are significantly higher than those in pre-eclamptic singleton pregnancies, irrespective of gestation (Boucoiran 2013, Faupel-Badger 2015, Sung 2017). PlGF levels in twin pregnancies affected by pre-eclampsia follow the same pattern as singletons and are lower than twin pregnancies not affected by pre-eclampsia (Boucoiran 2013, Dröge 2015, Svirsky 2016) including in the first trimester, prior to the appearance of clinical signs and symptoms (Francisco 2017).

The association between PlGF and pathologically abnormal twin fetal growth has been explored superficially, although the results are conflicting as Boucoiran and Yinon (Boucoiran 2013, Yinon 2014) found combined first and second trimester PlGF to be lower in twin pregnancies affected by SGA and sIUGR, whereas Kusanovic

found no difference in second trimester PlGF levels between twins pregnancies in which at least one twin had a birthweight below the 10th centile (Kusanovic 2008). This conflict may be due to the different definitions of abnormal growth, and a reflection of the small numbers of twin pregnancies in the study (69, 60, 69 pregnancies respectively) although the gestation at testing was similar in all 3 studies. Studies of abnormal growth in singleton pregnancies have demonstrated decreased second and third trimester PlGF is associated with SGA fetuses in normotensive pregnancies i.e. with no concurrent pre-eclampsia (Bersinger 2004, Shibata 2005, Romero 2008) thus supporting that PlGF is related to fetal growth, and the conflicting results in twin pregnancies may reflect different growth restriction phenotypes and pathogenesis, as discussed in section 1.3. Unlike in pre-eclampsia, the decrease in PlGF in normotensive SGA pregnancies is not accompanied by an increase in sFlt-1 (see section 3.2.7), suggesting that decreased PlGF is not a result of increased sFlt-1 “mopping up” PlGF, and that there is an unrelated mechanism.

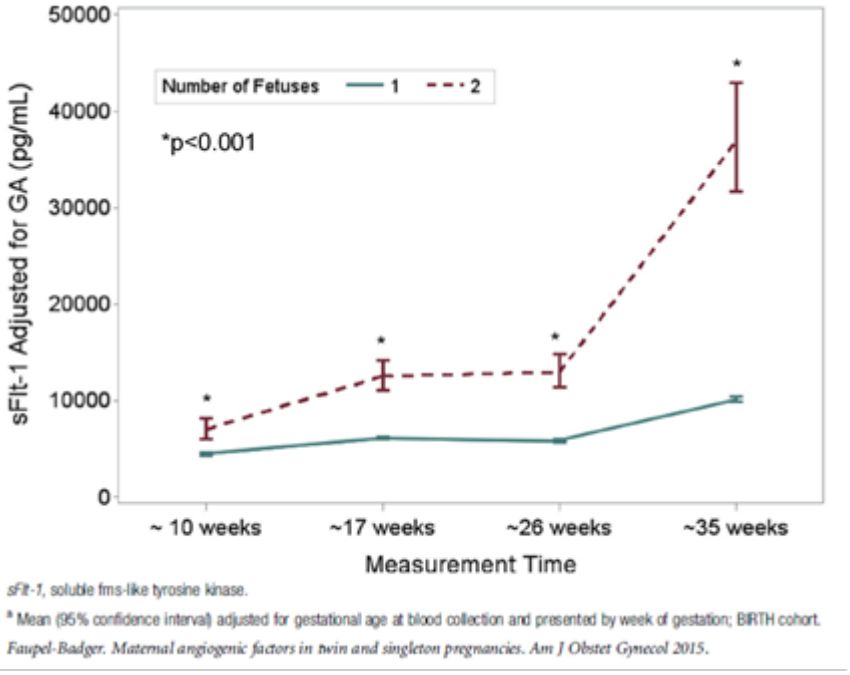
Very little research has looked at the relationship between PlGF and TTTS. Fox et al. demonstrated no difference in second trimester maternal serum PlGF in twin pregnancies affected by TTTS, and uncomplicated MCDA and DCDA pregnancies (Fox 2010). However other studies have found significantly lower PlGF levels in maternal serum on the day prior to FLA in TTTS pregnancies compared to uncomplicated MCDA twins (Kusanovic 2008, Chon 2017). Interestingly Chon et al. demonstrated that this difference was no longer apparent at 1 day post-FLA, and 3 weeks post-FLA (Chon 2017). Yinon et al. also found lower PlGF in TTTS pregnancies compared to uncomplicated MCDA twins at 15-20, and 21-28 weeks

gestation (Yinon 2014). No studies have looked at first trimester PlGF and future TTTS risk with the earliest sample being taken at 15 weeks gestation when the clinical signs of TTTS have appeared (Yinon 2014). Given the role that PlGF plays in angiogenesis and that changes in twin pregnancies that will go on to develop pre-eclampsia are present in the first trimester, one could hypothesise that PlGF is altered in the first trimester in pregnancies that will go on to develop TTTS and other adverse outcomes.

3.2.7 Soluble fms-like tyrosine kinase-1 (sFlt-1)

Soluble feline McDonough sarcoma (fms)-like tyrosine kinase is also known as soluble vascular endothelial growth factor receptor 1 (sVEGFR1). When circulating it binds to vascular endothelial growth factor (VEGF) and PlGF in target tissues with high affinity, thus antagonising the two angiogenic molecules and producing an anti-angiogenic state (Kendall 1996, Clark 1998). It is produced by human villous and extravillous trophoblast (Clark 1998). sFlt-1 increases throughout pregnancy (Wataganara 2012, Faupel-Badger 2015) (Figure 3.5), and levels fall within 24-48 hours of delivery, supporting that it is produced predominantly by the placenta (Maynard 2003, Reddy 2009). Maternal serum levels are higher in twin pregnancies than singleton pregnancies (Maynard 2008, Sánchez 2012, Dröge 2015, Faupel-Badger 2015), and also increase throughout gestation (Figure 3.5). When MC twins were compared to DC twins, levels were higher in MC twins in the third trimester (Faupel-Badger 2015), although no difference was present in the first trimester between chorionicities in another study (Sánchez 2012).

Figure 3.5 Maternal serum levels of soluble fms-like tyrosine kinase-1 (sFlt-1) in twin pregnancy (n=91) compared to singleton pregnancy (n=2193)
(Faupel-Badger 2015). Information on chorionicity not available.



sFlt-1, soluble fms-like tyrosine kinase.

[a] Mean (95% confidence interval) adjusted for gestational age at blood collection and presented by week of gestation; BIRTH cohort.

Faupel-Badger. Maternal angiogenic factors in twin and singleton pregnancies. Am J Obstet Gynecol 2015.

As with PlGF, the relationship between sFlt-1 and placental mass is debated (Shibata 2005, Nevo 2008, Wataganara 2012, Ruiz-Sacedón 2014, Dröge 2015, Faupel-Badger 2015, Manthati 2017) and serum levels are likely to be affected by other factors. Hypoxia plays an important role but has the opposite effect on sFlt-1 compared to PlGF as hypoxia upregulates sFlt-1 mRNA expression in cytotrophoblasts and increases sFlt-1 production (Nagamatsu 2004, Nevo 2006, Padavala 2006). Increased sFlt-1 creates the same ultimate state as decreased PlGF in hypoxic conditions: decreased angiogenesis. Interestingly, this effect on sFlt-1 mRNA expression and production appears cell specific as human umbilical vein endothelial cells and villous fibroblasts do not demonstrate increased sFlt-1 mRNA expression and production in hypoxic conditions. The same conundrum exists as with

PlGF and hypoxia: does hypoxia lead to high sFlt-1, or vice versa (Karumanchi 2004). Irrespective of whether high levels of sFlt-1 are the cause or consequence, the association between increased levels of sFlt-1 and pre-eclampsia is widely reported (Maynard 2003, Park 2005, Staff 2005, Romero 2008, Wikström 2008, Nanjo 2017). This is supported by the vasoconstrictive effect that exogenous sFlt-1 has on rats that leads to hypertension and glomerular endotheliosis (Maynard 2003), the latter of which is specific to pre-eclampsia and supports that sFlt-1 has a direct action on the maternal endothelium.

The increase in sFlt-1 is present 5 weeks before the clinical symptoms of pre-eclampsia appear (Levine 2004b). NICE evaluated the use of the 'Elecsys immunoassay sFlt-1/PlGF' test for the diagnosis of pre-eclampsia, and the recommendations made in May 2016 were that it could be used as a "rule out" test for pregnancies 20-34+6 weeks gestation, in addition to clinical information, but stated that there was insufficient evidence to use it as a "rule-in" test (NICE 2016). The guidance does not state whether this evaluation includes use in multiple pregnancies. As sFlt-1 levels in twin pregnancies with pre-eclampsia are significantly higher than in singleton pregnancies with pre-eclampsia, different cut-offs need to be used (Boucoiran 2013, Dröge 2015, Faupel-Badger 2015). sFlt-1 levels in twin pregnancies affected by pre-eclampsia are still higher than twin pregnancies not affected by pre-eclampsia (Dröge 2015). Fascinatingly, when placental sFlt-1 mRNA expression was examined in DCDA twins with pre-eclampsia but concordant growth, there was a significant difference when one twin's placenta was compared to the other twin's placenta within the same pregnancy (Nevo 2008). There was no

difference in the control twin pairs, implying that pre-eclampsia in twin pregnancy may be mediated by one twin, and partially explaining why serum levels are not just doubled in twin pregnancies compared to singletons.

The relationship between sFlt-1 and pathologically abnormal twin fetal growth is less clear. Boucoiran et al. reported no difference in sFlt-1 levels at 12-18 weeks or 24-26 weeks in SGA pregnancies, defined as at least one twin with a birthweight <10th centile based on Canadian growth charts (Boucoiran 2013). Ruiz-Sacedon et al. also reported no difference in sFlt-1 in the first, second or third trimester in DCDA twins with BWD >20% (Ruiz-Sacedón 2014). Yinon et al. found that gestation affected sFlt-1 significance as in MCDA twin pregnancies with sIUGR there was no difference in samples taken at 13-20 weeks, but at 21-28 weeks the sIUGR pregnancies had a significantly higher sFlt-1 level compared to uncomplicated MCDA twins (Yinon 2014). They also found that in cord blood samples taken at delivery, sFlt-1 was significantly higher in the growth restricted twin, compared to the normally grown twin in the same pregnancy. Nevo et al. compared sFlt-1 mRNA expression in placentas from sIUGR MC and DC twins, and found that in both the MC and DC growth-restricted twins placental territory, there was increased sFlt-1 mRNA expression, compared to the internal control of the normally grown twin in the same pregnancy (Nevo 2008). The group also compared sFlt-1 placental mRNA expression in singleton pregnancies with (i) severe IUGR defined as birthweight <5th centile and abnormal umbilical artery Dopplers (ii) SGA pregnancies defined as birthweight ≤10th centile with normal Dopplers, (iii) age-matched healthy with normal fetal growth and no signs of placental dysfunction. They found that the IUGR group had significantly

higher sFlt-1 mRNA expression than the SGA group, and the control group, but there was no difference between the SGA and control groups. As highlighted by the studies above, definition of abnormal growth may impact on findings. Concurrent pre-eclampsia may also play a role, but this has been explored in more detail in singleton pregnancies. In singletons there is no difference in sFlt-1 levels in normotensive pregnancies with SGA babies (Shibata 2005, Romero 2008, Nanjo 2017, Yoshida 2018), although SGA babies in pre-eclamptic pregnancies did have higher sFlt-1 levels than controls (Nanjo 2017).

There has been little research investigating sFlt-1 in TTTS, although all 4 studies that have explored it reported significantly higher sFlt-1 levels at diagnosis of TTTS compared to uncomplicated MC twins (Kusanovic 2008, Fox 2010, Yinon 2014, Chon 2017). Chon et al. found that the sFlt-1 levels decreased 3 weeks after FLA. No studies have looked at first trimester sFlt-1 and future TTTS with the earliest sample being taken at 15 weeks gestation when the clinical signs of TTTS had begun to appear (Yinon 2014). Given the role that sFlt-1 plays in angiogenesis and the antagonistic effect it has on PlGF, one could hypothesise that sFlt-1 is altered in the first trimester in pregnancies that will go on to develop TTTS or other MC twin pregnancy complications.

3.3 Existing prognostic factors

Existing prognostic factors are confounding factors that affect both the dependent and independent variables. It is important to identify these factors so that the results

can be adjusted accordingly, and the additional prognostic ability of the first trimester ultrasound measurements and biomarkers can be calculated on top of the prognostic ability of these existing prognostic factors. In this study, the existing prognostic factors are maternal age, BMI, smoking status, ethnicity, parity and mode of conception.

It is difficult to know if NT is associated with the existing prognostic factors as any associations are likely to be due to the link between the existing prognostic factor and the adverse outcome, such as maternal age and aneuploidy of which an increased NT is indicative, rather than there being a direct association between increasing maternal age and increasing NT. It is also not possible to know if CRL is affected by existing prognostic factors as all fetuses will have an increasing CRL throughout pregnancy: it will be assumed that they are a different gestational age rather than that anything abnormal is occurring.

3.3.1 Maternal age

The systematic review in CHAPTER 2 did not find any association between maternal age and TTTS, growth restriction or IUFD.

In singleton pregnancies, first trimester β-hCG is not associated with maternal age, however in the second and third trimester increased β-hCG is associated with increased maternal age (Wright 2015a). PAPP-A and AFP are not associated with maternal age in singleton pregnancy at any gestation (Bredaki 2015, Wright 2015b). Maternal age did not appear to affect PlGF in 61 twin pregnancies (Sánchez 2012), although a study in 7066 first trimester serum samples, and 8078 second trimester serum samples from singleton pregnancies reported that PlGF increased with

maternal age, but this association was not seen with the 10,464 third trimester samples (Tsiakkas 2015a). Another study found a difference in the first trimester, but not second trimester PlGF levels in singletons (Faupel-Badger 2011). sFlt-1 in uncomplicated singletons is not associated with maternal age in first or second trimester (Faupel-Badger 2011, Tsiakkas 2015b).

3.3.2 Maternal BMI

Raised maternal BMI is implicated in many obstetric complications in twin pregnancies including GDM, pregnancy-induced hypertension and pre-eclampsia (Fox 2014b). The systematic review in CHAPTER 2 did not find any association between maternal BMI and TTTS, growth restriction or IUFD.

In singleton pregnancies, decreased β-hCG was associated with increased maternal weight (Wright 2015a), as was PAPP-A throughout gestation (Wright 2015b).

AFP should be adjusted for maternal weight (Thomas 1990) as it decreases with increasing maternal weight in singleton pregnancies (Bredaki 2015). Maternal BMI may affect PlGF with a higher BMI associated with lower PlGF throughout singleton gestation (Zera 2014) although other studies found no difference in first trimester PlGF in normal singleton pregnancies (Faupel-Badger 2011, Kosiński 2014), and another study found that increasing maternal weight is associated with decreasing PlGF throughout gestation in singleton pregnancies (Tsiakkas 2015a). In singleton pregnancies BMI has an inverse relationship with sFlt-1 throughout gestation (Zera 2014, Tsiakkas 2015b, Manthati 2017), although another study in first and second trimester singleton pregnancies demonstrated a positive correlation (Faupel-Badger 2011) and another study demonstrated no relationship (Staff 2005), although the

latter study dichotomised BMI to <30 or >30kg/m^2 which may explain why no difference was seen.

3.3.3 Maternal smoking status

The systematic review in CHAPTER 2 did not find any association between maternal smoking and TTTS, and there were insufficient studies to investigate growth restriction or IUFD although maternal smoking is a well-known risk factor for growth restriction and IUFD in singleton pregnancies.

In singleton pregnancies, smokers had lower β-hCG and PAPP-A compared to non-smokers in the first and second trimester (Wright 2015a). AFP is higher in smokers compared to non-smokers throughout gestation (Bredaki 2015). Smoking appears to be associated with higher first trimester PlGF in twin pregnancies (Boucoiran 2013, Kosiński 2014) and singleton pregnancies (Tsiakkas 2015a). Smokers have lower sFlt-1 levels than non-smokers in singleton pregnancies (Tsiakkas 2015b).

3.3.4 Maternal ethnicity

The systematic review in CHAPTER 2 found that Caucasian women were at higher risk of TTTS than non-Caucasian women. It was not possible to investigate growth restriction and IUFD and the association with ethnicity in the systematic review due to too few studies being eligible for inclusion. However, ethnicity is associated with a woman's risk of developing complications, with "black" women more likely to deliver a twin with a smaller birthweight compared to "white" women (Cuff 2017).

In twin pregnancies, second trimester β-hCG is affected by ethnicity (O'Brien 1997).

In singleton pregnancies, Afro-Caribbean and East Asian women had higher β-hCG in the first trimester compared to Caucasian women, whereas South Asian women had lower levels (Wright 2015a). AFP is affected by maternal ethnicity in singleton pregnancies (Thomas 1990) with lower levels reported in South Asian and East Asian women, and higher levels in Afro-Caribbean women compared to Caucasian women throughout gestation (Wald 1997, Bredaki 2015). Second trimester AFP is also affected by ethnicity in twin pregnancies (O'Brien 1997). PAPP-A and PlGF was higher in Afro-Caribbean, South Asian and East Asian women compared to Caucasian throughout gestation in singleton pregnancies (Tsiakkas 2015a, Wright 2015b). sFlt-1 was also higher in Afro-Caribbean women than Caucasian women throughout gestation in singleton pregnancies, although was not different in South Asian and East Asian women (Tsiakkas 2015b).

3.3.5 Parity

The systematic review in CHAPTER 2 did not find any association between parity and TTTS. There was a weak association between AGR and nulliparity. It was not possible to investigate parity in the context of IUFD.

First trimester β-hCG and PAPP-A in singleton pregnancies were not affected by parity (Wright 2015a, Wright 2015b). First trimester AFP in singletons is higher in nulliparous woman than multiparous women (Bredaki 2015), whereas the relationship may be the opposite in PlGF: nulliparous women have lower PlGF than multiparous women (Tsiakkas 2015a). Other studies dispute this as no difference was found in first or second trimester PlGF in singleton pregnancies (Faupel-Badger 2011) or in first trimester twin pregnancies (Sánchez 2012). sFlt-1 in first trimester normal

singletons had no association with parity, however in the second trimester sFlt-1 levels were higher in nulliparous women compared to multiparous women (Faupel-Badger 2011), although another study reported that sFlt-1 levels were higher in nulliparous throughout gestation (Tsiakkas 2015b).

3.3.6 Mode of conception

The systematic review in CHAPTER 2 did not find any association between mode of conception and TTTS or growth restriction. There were insufficient studies to investigate IUFD.

In euploid uncomplicated MC twins, the first trimester NT measurement was higher in ART conceived twins compared to spontaneously conceived twins, although no difference was seen according to mode of conception in DC twins (Flock 2013). In singleton pregnancies, there was increased β-hCG in ART pregnancies compared to spontaneously conceived singletons throughout gestation (Wright 2015a). In singletons conceived by (in-vitro fertilisation) IVF, maternal serum PAPP-A was lower in the first trimester compared to those conceived spontaneously, and was higher in the second and third trimester compared to spontaneously conceived singleton pregnancies (Wright 2015b). When first and second trimester women were combined PAPP-A was higher in ART conceived twins than spontaneously conceived twins, but β-hCG, AFP and PlGF did not demonstrate a difference (Sánchez 2012, Svirsky 2016). Second trimester β-hCG and AFP in twin pregnancies were not affected by mode of conception (Räty 2000). The lack of difference in AFP and PlGF is echoed in other studies in singletons (Bredaki 2015, Tsiakkas 2015a). First trimester sFlt-1 is higher in ART conceived DCDA twin pregnancies compared to spontaneously

conceived DCDA twin pregnancies, MCDA twins were not included in the analysis (Sánchez 2012). In singleton pregnancies, no difference was reported in sFlt-1 levels and mode of conception (Tsiakkas 2015b).

3.3.7 Gestational age at delivery

This existing prognostic factor was only used to adjust the neonatal composite outcome as it is known to affect outcome.

3.3.8 Steroids administration

This existing prognostic factor was only used to adjust the neonatal composite outcome as it is known to affect outcome.

3.3.9 Magnesium sulphate administration

This existing prognostic factor was only used to adjust the spontaneous PTB and neonatal composite outcomes as it is known to affect these outcomes.

Although the majority of outcomes were not affected by the existing prognostic factors according to my systematic review, previous studies have reported conflicting findings and most maternal serum potential prognostic factors were affected by existing prognostic factors, therefore the results were adjusted accordingly.

3.3.10 Aims

The aims of this study are to investigate if in MC twin pregnancies, the following first trimester ultrasound measurements and maternal serum biomarkers individually are (i) associated with adverse outcome, (ii) act as prognostic factors of adverse outcome:

1) NT
2) CRL
3) β-hCG
4) PAPP-A
5) AFP
6) PlGF
7) sFlt-1

Hypotheses

In first trimester MC twin pregnancies:

1) Increased NT discordance is associated with adverse outcome and has improved predictive ability above existing prognostic factors.
2) Increased CRL discordance is associated with adverse outcome and has improved predictive ability above existing prognostic factors.
3) Increased β-hCG is associated with adverse outcome and has improved predictive ability above existing prognostic factors.
4) Decreased PAPP-A is associated with adverse outcome and has improved predictive ability above existing prognostic factors.

5) Increased AFP associated with adverse outcome and has improved predictive ability above existing prognostic factors.
6) Decreased PlGF is associated with adverse outcome and has improved predictive ability above existing prognostic factors.
7) Increased sFlt-1 is associated with adverse outcome and has improved predictive ability above existing prognostic factors.

3.4 Methods

3.4.1 Participants

An existing multicentre, international cohort of MC twin pregnancies was used. All women with a MCDA twin pregnancy in the West Midlands and North Thames regions who had undergone first trimester aneuploidy screening between October 2014 and September 2015, or women with a MCDA twin pregnancy who booked at the Royal Prince Alfred Hospital, Sydney between June 2011 and April 2016, and for whom there was a stored first trimester maternal serum sample, were eligible for inclusion. Therefore there was retrospective consecutive enrolment. The data used had been prospectively recorded as part of routine clinical care. Chronicity had to have been determined in the first trimester based on: a single placental mass, a thin inter-twin membrane, the presence of the 'T' sign and absence of Lambda sign (Sepulveda 1996). If the twins were a different sex, or postnatally the pregnancy was diagnosed as DC based on placental assessment, these pregnancies were excluded, but placental histology was not routinely checked for all pregnancies. As participants in the study had MCDA pregnancies, the majority were spontaneous conceptions

therefore dates relating to ART could not be used. Consequently, the gestational age was calculated based on the largest twin CRL, as this is the measurement advised by various national bodies (Morin 2011, NICE 2011, Khalil 2016b, Kilby 2016, RANZCOG 2017), and is the gestational age upon which clinicians will have based management decisions.

Women were booked at 29 different secondary and tertiary care maternity units, depending on geographical area, and were under consultant-led care due to the high-risk nature of multiple pregnancies. Women were not eligible for inclusion if they had a: miscarriage prior to 14 weeks gestation, MCMA pregnancy, higher order multiple pregnancy, or the pregnancy was affected by serious structural or congenital anomalies, whether concordant or discordant, as the aetiology of their adverse outcomes, such as growth restriction, PTB or IUFD would be different to pregnancies not affected by structural or congenital anomalies, and would therefore increase heterogeneity within the cohort. Where outcome data were missing, due to the time period and setting diversity women were not contacted for further details and these pregnancies were excluded. Pregnancies were cared for according to local and national guidelines. Postnatal outcome data were collected until discharge from hospital. No further follow-up data were collected. Anonymised data were collated using a purpose-designed data collection form (Appendix 10.3), input and coded in an Excel spreadsheet. Any discrepancies were clarified with the data collector and the original data source checked.

3.4.2 Sample collection and storage

Maternal serum samples had to have been taken when the largest twin CRL was between 45-84mm, thus corresponding to the first trimester, as directed by the Fetal Anomaly Screening Programme (FASP) (FASP 2015). Venepuncture was performed by trained professionals at each hospital site and samples were taken in bottles containing sodium heparin, with or without a gel separator. The manufacturer of the bottle depended on hospital site.

The UK samples were couriered to Birmingham Women's Hospital (BWH) biochemistry laboratory at room temperature, the same day. At least one hour was allowed for clotting. Within 4 hours of arrival in the laboratory, the whole blood samples were centrifuged at 3000 *g* for 10 minutes at room temperature and the serum was removed and loaded onto the automated platform to measure β-hCG and PAPP-A (see section 3.4.4). After completion of the β-hCG and PAPP-A assays the samples were stored at -80°C until the AFP, sFlt-1 and PlGF analyses were performed, between 12 months and 1 year 11 months after initial blood sampling.

The samples from Sydney were collected from one hospital and were centrifuged and transferred to the automated assay platform within 4 hours of sample collection in the same hospital to measure β-hCG and PAPP-A for first trimester aneuploidy screening. These values were not included in the results as they were converted in MoM and it was not possible to access the raw values. The residual serum from the samples was frozen at -80°C within 4 hours of completion of β-hCG and PAPP-A assays. The samples remained at -80°C before being transferred to BWH on dry ice, where they were immediately transferred to -80°C freezers.

Prior to the AFP, sFlt-1 and PlGF analyses, the samples had been stored for between 4 months and 5 years 3 months.

3.4.3 Prognostic factors: ultrasound measurements (NT, CRL)

NT and CRL measurements were performed using standard practice (FASP or Fetal Medicine Foundation [FMF] protocols with UK National External Quality Service [NEQAS] quality assessment) in women who consented to first trimester aneuploidy screening (FMF 2004, FASP 2015). These were performed by sonographers and fetal medicine doctors in the local units who were approved by the FMF to perform these scans.

NT discordance (%) was calculated as the smallest NT subtracted from the largest NT, divided by the largest NT multiplied by 100. CRL discordance (%) was calculated as per NT discordance. These measurements were treated as continuous data within analyses, no cut-offs were applied (Royston 2006).

3.4.4 Prognostic factors: biomarkers (β-hCG, PAPP-A, AFP, PlGF, sFlt-1)

The 5 biomarkers were measured on one serum sample per woman. β-hCG and PAPP-A had been prospectively measured in these samples as part of the UK and Australian aneuploidy screening programmes according to the manufacturers' instructions (see Table 3.1 and below for more details). The laboratories performing these analyses were CPA (UK) Ltd. accredited and are externally quality assessed by UK National External Quality Service (NEQAS) and Down's syndrome Quality Assurance Support Service (DQASS). Consequently the people performing the

assays were blinded to the subsequent future outcomes of the pregnancies. The β-hCG and PAPP-A measures were not repeated, therefore the measurements used in the analysis were the same as those used to guide clinical care. Due to the Australian laboratory reporting the β-hCG and PAPP-A results as MoM instead of the raw values, it was not possible to include the 64 Australian samples in the analysis for these two markers; however the measurements of the other factors (NT, CRL, AFP, PlGF and sFlt-1) were included from the Australian samples.

The PlGF and sFlt-1 assays were performed in one batch using an assay approved by the UK's NICE for ruling-out pre-eclampsia (Roche Diagnostics Limited, Sussex, UK) (20, 21). These assays were performed at the Biochemistry Department at University Hospital Birmingham by people blinded to the outcome and the samples were loaded on to the automated platform at random. The AFP assays were commenced immediately following the sFlt-1 and PlGF assays, thus the samples only went through one freeze-thaw cycle. These assays were performed at the Biochemistry Department at BWH by people blinded to the outcome and the samples were loaded on to the automated platform at random.

The machines were successfully calibrated and the quality control procedures were met. The biomarker measurements were not repeated as the assays and laboratory have been validated for clinical use therefore the levels were believed to be accurate, albeit that the results have not been used for this purpose previously. The inter-assay coefficient of variation for β-hCG, PAPP-A, AFP, PlGF and sFlt-1 were all <5%.

In order to measure the AFP, PlGF and sFlt-1, the samples were gradually defrosted from -80°C to room temperature, inverted twice and vortexed at 2700 *g* for 3

seconds, three times at room temperature. All reagents were brought up to room temperature and the assays were performed as detailed below.

β-hCG

Free β-hCG was measured using time-resolved fluoroimmunoassay manufactured by PerkinElmer (PerkinElmer Wallac Oy, Turku, Finland) on the AutoDELFIA® immunoanalyser, according to the manufacturer's instructions. The assay is based on the sandwich principal: a two-site, non-competitive immunoassay, in which free β-hCG is "sandwiched" between two monoclonal antibodies directed against two separate antigens, on the β-hCG molecule. One antibody is also bound to samarium which remains following the incubation with the antibodies and the washes. Enhancement solution is added which liberates the samarium from the antibody sandwich, and is detected fluorimetrically in proportion to the β-hCG concentration.

PAPP-A

PAPP-A was measured using time-resolved fluoroimmunoassay manufactured by PerkinElmer (PerkinElmer Wallac Oy, Turku, Finland) on the AutoDELFIA® immunoanalyser, according to the manufacturer's instructions. The assay is based on the sandwich principal: a two-site, non-competitive immunoassay, in which PAPP-A is "sandwiched" between two monoclonal antibodies directed against two separate antigens, on the PAPP-A molecule. Initially, biotin-labelled PAPP-A-specific antibodies are incubated with streptavidin-coated microparticles. There is then a wash step and the samples are added with another PAPP-A specific antibody labelled with europium and there is another wash step. Enhancement solution is

added to liberate the europium from the complex and the fluorescence from each well is measured using time resolved fluorimetry which is proportional to the concentration of PAPP-A.

AFP

Total AFP was measured using time-resolved fluoroimmunoassay manufactured by PerkinElmer (PerkinElmer Wallac Oy, Turku, Finland) on the AutoDELFIA® automatic immunoassay system, according to the manufacturer's instructions. The assay is based on the sandwich principal: a two-site, non-competitive immunoassay, in which AFP is "sandwiched" between two monoclonal antibodies directed against two separate antigens on the AFP molecule. 25µl of each sample was incubated with 200µl buffer and incubated for 1 hour. 200µl monoclonal AFP-specific antibody labelled with europium (diluted 1:75) was added and incubated for one hour. The plate was washed 6 times and incubated with 200µl Enhancement Solution for 5 minutes. The liberated europium was measured fluorimetrically in proportion to the AFP concentration.

PlGF

Free PlGF was measured using an electrochemiluminescence immunoassay (ECLIA) manufactured by Roche (Roche Diagnostics Limited, Sussex, UK) on the 'Elecsys 2010, Modular E170' immunoassay analyser, according to the manufacturer's instructions. The assay is based on the sandwich principal: a two-site, non-competitive immunoassay, in which PlGF is "sandwiched" between two antibodies via two separate antigens. 500µl aliquots of the samples were loaded into the analyser,

50µl of each sample was incubated with biotinylated monoclonal PlGF-specific antibody and a monoclonal PlGF-specific antibody labelled with a ruthenium complex (Tris(2,2'bipyridyl)ruthenium(II)-complex) to form the sandwich complex. A second incubation was performed with streptavidin-coated microparticles that bind the complex to the plate via the interaction of biotin and streptavidin. The mixture was aspirated into the measuring cell and the microparticles were magnetically captured onto the surface of the electrode. The unbound substances were washed away by the ProCell wash (Roche). A voltage was applied to the electrode causing a chemiluminescent emission that was measured by a photomultiplier. The results were determined using a calibration curve generated by a 2-point calibration and master curve provided by the reagent barcode.

sFlt-1

Total sFlt-1 was measured in combination with the PlGF assay. The only differences were that 20µl of each sample was used, and the antibodies were sFlt-1 specific.

Table 3.1 Potential prognostic factors of primary interest

Factor	How measured	When measured	Biological role and clinical use	Lower limit of detection	Upper limit of detection	Manufacturer
Nuchal translucency (NT)	By accredited sonographers as part of the National Aneuploidy screening programme	11+0 – 14+1 weeks, calculated based on the CRL which must be 45-84mm	Measurement of lymphatic fluid at the back of the fetus's neck. High measurement indicates high risk of chromosomal or cardiac problems. Discrepancy between twins may indicate adverse outcome.	0.1cm	Infinite	NA
Crown-rump length (CRL)	By accredited sonographers as part of the National Aneuploidy screening programme	11+0 – 14+1 weeks, calculated based on the CRL which must be 45-84mm	Measurement of the length of the baby used to date the pregnancy. Discrepancy between twins may indicate adverse outcome.	1mm	Infinite	NA
β-human Chorionic Gonadotropin (β-hCG)	Clinical laboratory accredited to perform testing as part of National Aneuploidy screening programme	Sample taken at 11+0 -14+1 weeks gestation, analysed within 24 hours of the sample being taken	Produced by the syncytiotrophoblast and maintains the corpus luteum during early pregnancy. High level indicates increased risk of aneuploidy, low levels associated with miscarriage.	1.5ng/mL	200ng/mL	PerkinElmer Wallac Oy, Turku, Finland

Factor	How measured	When measured	Biological role and clinical use	Lower limit of detection	Upper limit of detection	Manufacturer
Pregnancy associated plasma protein A (PAPP-A)	Clinical laboratory accredited to perform testing as part of National Aneuploidy screening programme	Sample taken at 11+0 -14+1 weeks gestation, analysed within 24 hours of the sample being taken	Produced by the syncytiotrophoblast and encodes a gene which cleaves insulin-like growth factor binding proteins. Low level indicates increased risk of aneuploidy, and intra-uterine growth restriction.	5mU/L	10000mU/L	PerkinElmer Wallac Oy, Turku, Finland
Alpha-fetoprotein (AFP)	Clinical laboratory accredited to perform testing as part of National Aneuploidy screening programme	Sample taken at 11+0 -14+1 weeks gestation, stored at -80°C and analysed 4 months to 5 years 3 months later	Human function is unknown, but abundant in fetuses. Low level indicates increased aneuploidy risk in twin pregnancies in the 2nd trimester as part of the Quad test. High level indicates increased risk of neural tube defects in 1st and 2nd trimester. Marker of placental function.	1U/mL	1000U/mL	PerkinElmer Wallac Oy, Turku, Finland
Placental growth factor (PlGF)	Clinical laboratory with experience of performing these assays for pre-eclampsia prediction	Sample taken at 11+0 -14+1 weeks gestation, stored at -80°C and analysed 4 months to 5 years 3 months later	Produced by the syncytiotrophoblast and promotes angiogenesis. Low level indicates pre-eclampsia in singleton pregnancies prior to the appearance of signs and symptoms.	3pg/mL	10000pg/mL	Roche Diagnostics Limited, Sussex, UK

Factor	How measured	When measured	Biological role and clinical use	Lower limit of detection	Upper limit of detection	Manufacturer
Soluble fms-like tyrosine kinase-1 (sFlt-1)	Clinical laboratory with experience of performing these assays for pre-eclampsia prediction	Sample taken at 11+0 -14+1 weeks gestation, stored at -80°C and analysed 4 months to 5 years 3 months later	Produced by the syncytiotrophoblast and prevents angiogenesis. High level indicates pre-eclampsia in singleton pregnancies prior to the appearance of signs and symptoms.	10pg/mL	85000pg/mL	Roche Diagnostics Limited, Sussex, UK

3.4.5 Adjustment factors

The existing prognostic factors used as adjusted factors for the individual potential prognostic factors are detailed in Table 3.2. It was not possible to adjust for gestational age at blood sampling as gestational age was calculated based on CRL which is one of the potential prognostic factors under evaluation.

Table 3.2 Standard clinical information used as adjustment factors (i.e. existing prognostic factors)

Prognostic factor	How measured	When measured	Categorical or continuous data	Outcomes it may affect
Maternal age	From date of birth	Booking	Continuous	All
Maternal body mass index (BMI)	Height and weight measured and calculated (kg/m^2)	Booking	Continuous	All
Maternal smoking status	Maternal reporting as documented in the medical notes	Booking	Categorical: -Never smoked (reference) -Current smoker -Ex-smoker	All
Maternal ethnicity	Maternal reporting as documented in notes	Booking	Categorical: -White (reference) -Non-white: other/mixed, Oriental, South Asian, African-Caribbean	All
Parity	Maternal reporting as documented in notes	Booking	Categorical: -Nulliparous (reference) -1 previous delivery after 24 weeks gestation -2 or more previous deliveries after 24 weeks gestation	All

Prognostic factor	How measured	When measured	Categorical or continuous data	Outcomes it may affect
Mode of conception	As documented in the notes	Booking	Categorical: -Natural conception (reference) -Assisted conception	All
Gestational age at delivery	As documented in the notes	Delivery	Continuous	Neonatal composite
Steroid administration	As documented in the notes	Throughout pregnancy	Categorical: -Yes steroids -No steroids (reference)	Neonatal composite
Magnesium sulphate (MgSO4) administration	As documented in the notes	Throughout pregnancy	Categorical: -Yes MgSO4 -No MgSO4 (reference)	Neonatal composite Spontaneous PTB

3.4.6 Outcomes

The primary outcome was a composite of adverse fetal events defined as the presence of at least one of the following: TTTS, AGR, PGR, TAPS, or IUFD. PGR was included in the fetal adverse outcome composite as the definition of PGR used does result in a change in neonatal clinical care.

Secondary outcomes and their definitions are listed below.

a) TTTS: polyhydramnios (>8cm MPD in the recipient at <20 weeks of gestation or >10cm from >20 weeks of gestation) in combination with oligohydramnios in the donor (<2 cm MPD irrespective of gestation) in same sex twins diagnosed as MCDA twins in the first trimester. It was staged as per Quintero staging criteria (Quintero 1999). Pregnancies affected by TTTS with concurrent growth restriction were not included in the AGR or PGR groups as the aetiology of the growth restriction is different and would make the growth restriction group heterogeneous.

b) AGR: AC or EFW <10th centile in either/both fetus(es), and/or growth discordance >20% recorded at least twice over ≥2 week period. As a result of different measures and definitions of twin growth, for which there is at present no validated international consensus, the term ‘growth restriction’ was used to denote a twin which demonstrated pathologically abnormal growth. It was believed to be important to use the different measures, as even within UK units, despite the RCOG Green-top guidelines for the Management of Monochorionic Twin Pregnancy (Neilson 2008, Kilby 2016), the measurements and definitions used to make clinical decisions were different. Pregnancies were not considered to have a growth problem if there was only one abnormal ultrasound scan, and

the subsequent scans were normal. When growth was examined as an individual outcome, it was divided into antenatally- and postnatally-detected growth problems. From both growth restriction sub-groups pregnancies with TTTS and TAPS were excluded as the abnormal growth seen is these conditions is thought to be linked to the pathology of TTTS or TAPS and thus has a different aetiology to those with solely growth problems. Twins with an IUFD were also excluded if the IUFD was not preceded by evidence of growth restriction in the pregnancy. By including these patients it would make the growth group heterogeneous. Included in the 'AGR' group are pregnancies with:

- SGA fetus. The definition of SGA was an EFW <10th centile in English cohort, and an AC <10th centile in the Australian cohort. This was based on the growth chart each unit used as when these pregnancies were being cared for, and clinical decisions were being made, this was not on a specific and validated growth chart for twin pregnancies. Different definitions were required because of differing guidance in the two countries for clinical decisions.

- IUGR. As per SGA but with abnormal umbilical artery Dopplers. This may occur in one (see selective IUGR below) or both of the twins.

- sIUGR. One twin EFW <10th centile or AC <10th centile and with abnormal umbilical artery Doppler as classified by Gratacós et al. (Gratacós 2007) as below:

  - Type I positive end-diastolic flow (EDF) in the umbilical artery

- Type II persistently absent or reversed end-diastolic flow (AREDF)
- Type III intermittent absent or reversed end-diastolic flow (iAREDF) in the absence of fetal breathing.

- Inter-twin growth discordance. Antenatal growth discordance was defined as >20% difference in the EFW using Hadlock's formula. This was calculated by subtracting the weight of the smallest twin from the biggest twin, then dividing by the weight of the biggest twin, and multiplying by 100.

c) Postnatally-detected growth restriction: birthweight <9th centile World Health Organization (WHO) charts. In the 'postnatally-detected growth restriction' group are fetuses with a birthweight <9th centile on the WHO growth chart (0-2 years or PTB charts depending on gestation at delivery) (RCPCH 2016). Although these charts were designed for singletons, no specific validated charts for twin pregnancies exist at present. It was not possible to use a specific weight cut-off for NNU admission as this varies between NNUs. Unlike isolated antenatal EFW discordance which was considered a sign of growth restriction as it changes the clinical management of the pregnancy, isolated postnatal actual BWD was not included as it is not used clinically postnatally, and it was felt too far to extrapolate postnatal BWD to equate to antenatal EFW discordance. For pregnancies in which only one twin had a birthweight <9th centile and abnormal growth was not detected antenatally, the naming of twin 1 and twin 2 antenatally and postnatally had to be assumed to be consistent.

d) IUFD: fetal death after 14 weeks gestation. This may be a single IUFD (sIUFD) whereby one twin died or a double IUFD (dIUFD) whereby both twins died. The

pregnancy was considered a miscarriage if the pregnancy loss occurred at 14-23+6 weeks, and a stillbirth if the loss occurred at ≥24 weeks. Pregnancies with a loss <14 weeks gestation were not eligible for inclusion in the study as the first trimester ultrasound scan and biomarkers are measured at this time, and the pathology and consequences of a loss at <14 weeks gestation is different to that of a loss >14 weeks gestation.

e) Spontaneous PTB: if it occurred between 24-33+6 weeks gestation. Any births less than 34 weeks were sub-classified as spontaneous or iatrogenic as it would be clinically most useful if there were factors to predict spontaneous PTB, whereas iatrogenic PTB is more reflective of disease severity as it is a treatment rather than a pathological process. Only spontaneous PTBs were included in this outcome.

f) Neonatal composite outcome: NND, respiratory distress syndrome, assisted ventilation (continuous positive airway pressure [CPAP] or endotracheal tube) for >24 hours, intraventricular haemorrhage or other brain injury, necrotising enterocolitis, neonatal encephalopathy, chronic lung disease, severe jaundice requiring phototherapy, severe infection e.g. septicaemia, meningitis, exchange transfusion, cardiac impairment, neurological impairment.

g) Maternal morbidity composite outcome: GDM, severe infection, hypertensive disorders (pregnancy induced hypertension requiring medication, pre-eclampsia, eclampsia or HELLP [haemolysis, elevated liver enzymes, low platelets] syndrome), placental abruption, venous thromboembolism, disseminated intravascular coagulopathy, High-Dependency or Intensive Care Unit admission,

cerebrovascular event, renal or liver failure, pulmonary oedema, massive obstetric haemorrhage (>2L estimated blood loss), acute fatty liver.

3.4.7 Missing data

As the proportion of patients with missing data was greater than 5% for maternal BMI and smoking status, to replace the missing values for these two factors, multiple imputation was performed by using a chained equation approach with predictive mean matching for continuous variables based on maternal age, BMI, log(β-hCG), log(PAPP-A), log(AFP), log(sFlt-1), log(PIGF), ethnicity, assisted conception, steroid use, magnesium sulphate (MgSO4), smoking status, country of antenatal care, parity, gestation at delivery, NT discordance (%), CRL discordance (%) and fetal adverse outcome composite. Ten imputed datasets were created for missing maternal BMI and smoking status that were then combined across all datasets using Rubin's rule to obtain final model estimates. The neonatal outcome was missing for 14 babies, but this was not imputed due to the large number of imputations needed for missing outcome data so these pregnancies were not included in the neonatal composite outcome, but were included in the other outcomes.

3.4.8 Statistical analysis

All analyses were performed using Stata/MP 14.0 (Stata Corporation, TX, USA).

The inter-twin discordance as a percentage, calculated as the largest NT or CRL, minus smallest NT or CRL, divided by the largest NT or CRL multiplied by 100 were used as prognostic factors for outcomes reported per pregnancy (fetal adverse

outcome composite, maternal antenatal and postnatal composite, AGR, PGR, TTTS, sIUFD, dIUFD, spontaneous PTB). Each individual twin's NT measurement was used for outcomes reported per fetus/baby (AGR, PGR, IUFD, neonatal composite). The individual twin's CRL was not investigated as a potential prognostic factor as it was used as a proxy for gestational age. The inter-twin discordance in NT or CRL, and the five biomarkers were not investigated with regards the neonatal composite due to the potential prognostic factors being measured per pregnancy and the neonatal composite outcome being per baby.

The five serum biomarkers (β-hCG, PAPP-A, AFP, PlGF and sFlt-1) were log transformed as they were highly skewed. All potential prognostic factor measurements remained as continuous variables during analysis, and no cut-offs were applied as there are no established and validated cut-offs for twin pregnancies, and data is lost by dichotomising variables (Altman 2006). The association between continuous prognostic factors and the outcome was included in the regression models as a linear term. The use of fractional polynomials were considered as part of a sensitivity analysis but the best fitting models were those with the linear term only included.

The association between each of the seven prognostic factors of interest (NT, CRL, β-hCG, PAPP-A, AFP, sFlt-1, PIGF) and the primary and secondary outcomes was assessed by univariable logistic regression models fitted individually to each prognostic factor and each outcome. The unadjusted association was reported as the OR with 95% confidence intervals (95%CI). A random intercept term at the level of the mother was included in the logistic regression models, to account for clustering of

multiple babies per woman, where it was sensibly estimable, for outcomes relating to individual babies. The clustering of babies within mothers for the outcome of IUFD was not able to be accounted for using a random effects model due to small numbers and minimal variation within the clusters which created convergence issues, hence a fixed effect model was used.

A multivariable logistic regression model was fitted, with random effects as necessary as described previously, to produce adjusted odds ratios (aOR). The potential prognostic factors were adjusting for standard characteristics defined *a priori* to be likely to be prognostic of adverse outcome: maternal age, BMI, smoking status, ethnicity, parity, mode of conception. The spontaneous PTB outcome was additionally adjusted for administration of MgSO4. The neonatal composite outcome was adjusted for the gestation at delivery, administration of steroids, and MgSO4. It was not possible to adjust for all these factors for the sIUFD and dIUFD per pregnancy outcome due to convergence issues, therefore this outcome was adjusted for maternal age, BMI, ethnicity and mode of conception.

The aOR represents the prognostic ability of each individual potential prognostic factor above the prognostic ability of the existing prognostic factors. The p-valve presented is for the aOR, <0.05 was considered statistically significant.

The c-statistic was calculated when a significant association was found between the prognostic factor and adverse outcome. When developing a prognostic model, the discrimination represents the ability of the model to separate cases from controls. The discrimination can be represented by the c-statistic. More specifically, the c-statistic is the probability that for any randomly selected pair of individuals, the model assigns a higher probability to the individual who experiences the outcome. As the

aim was to evaluate individual prognostic factors, not the combination of prognostic factors in a prognostic model, how each individual potential prognostic factor of interest improved the discrimination of the existing prognostic factors was assessed, by reporting the change in c-statistic (the area under the curve [AUC]).

The data are a convenience cohort of women and therefore no power calculation was used to determine a sample size. A typical rule of thumb is at least ten events are required for every candidate prognostic factor of interest (Peduzzi 1996) thus as 7 potential prognostic factors were examined, a sample size of ≥70 women was required.

3.4.9 Translating the prognostic effect into clinical utility

To demonstrate how the odds of developing the fetal adverse outcome would change across the distribution of each potential prognostic factor, two values for each potential prognostic factor were chosen *a priori.* The values for the ultrasound measurements were based on cut-offs used in existing literature for NT % discordance (0% and 20%) and CRL % discordance (0% and 10%). The values used for the maternal serum biomarkers were based on centiles in the study cohort: for AFP and sFlt-1 the 50^{th} centile and 95^{th} centile were used, for PlGF the 50^{th} and 5^{th} centiles were used. The results were adjusted for using the mean values of the adjustment factors in the study cohort. The patient characteristics remained the same and were based on the mean and most common options in the patient cohort: 30 years old, BMI 25, non-smoker, Caucasian, nulliparous and natural conception.

3.4.10 Ethical approval

Ethical approval was granted from East Midlands – Derby REC (15/EM/0240) on 09 June 2015 and the 1st amendment to include the Australian samples was approved on 21 April 2016, and the 2nd amendment to extend the time period of the project was approved on 22 February 2016. Confidential Advisory Group (CAG) approval was granted (15/CAG/0142) on 24 June 2015. Ethical approval was granted by the Royal Prince Alfred Hospital Research and Ethics Governance Office (HREC/11/RPAH/472) in January 2016. As the study is being conducted following the completion of the participants' pregnancies, the results of the study will not affect patient care and patients whose blood samples were used were not informed of the results. This work is part of a larger study, which was registered on 22nd April 2016: ISRCTN 13114861 (www.isrctn.com/ISRCTN13114861). The protocol was published prior to analysis (Mackie 2017). Since submitting the protocol for publication, it should be noted that a collaboration with the Royal Prince Alfred Hospital, Sydney, Australia was formed to generate an international cohort.

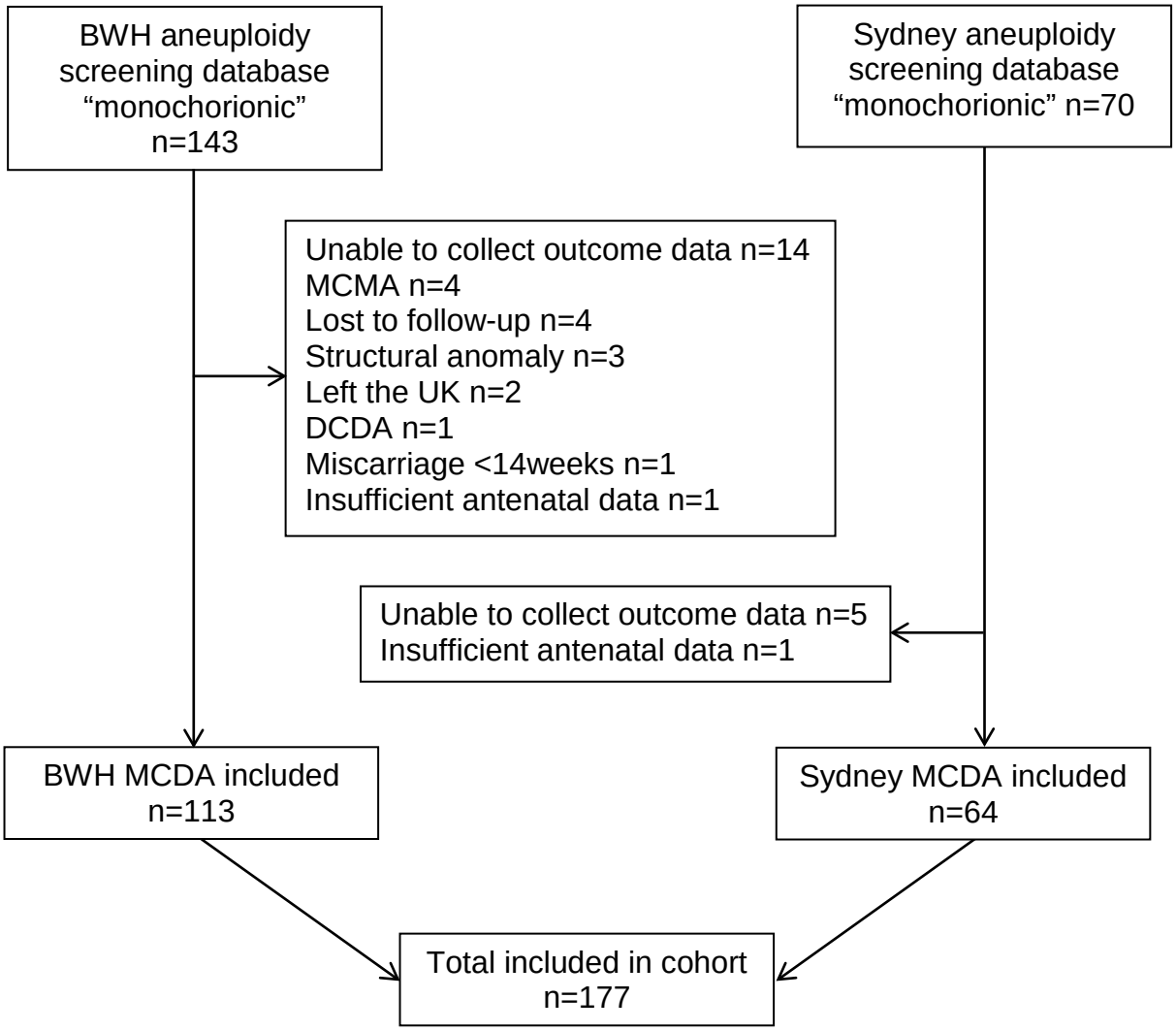
3.5 Results

There were 213 women identified as MC twin pregnancies on the BWH and Royal Prince Alfred Hospital, Sydney first trimester aneuploidy screening databases. Of these women 177 pregnancies (354 fetuses/babies) (83.1%) were included in the study (Figure 3.6). No woman was included twice. The commonest reason for being unable to include a woman in the study was being unable to collect the outcome

data. One DCDA pregnancy was incorrectly identified as a MCDA pregnancy in the first trimester and was not included.

Figure 3.6 Flow diagram of patient inclusion

BWH: Birmingham Women's Hospital



3.5.1 Existing prognostic factors

The maternal characteristics that were considered existing prognostic factors and were used to calculate aORs are summarised in Table 3.3. There was no or minimal missing data in the majority of the factors, with the exception of BMI which had 19% missing and maternal smoking status which was missing in 7.34%.

Table 3.3 Maternal characteristics used as adjustment factors

BMI: body mass index kg/m^2, * fetal complications: twin-twin transfusion syndrome, twin anaemia polycythaemia sequence, growth restriction, intrauterine fetal death.

	Total cohort (n=177)	**≥1 fetal complication* present (n=94)**	**No fetal complication (n=83)**
Maternal age; mean (SD)	30.38 (5.43)	30.73 (5.30)	29.99 (5.53)
Maternal BMI; mean (SD)	24.87 (5.41)	24.71 (5.08)	25.05 (5.77)
Maternal smoking status; n (%)			
Never	127 (77.44)	64 (75.29)	63 (79.75)
Current smoker	12 (7.32)	8 (9.41)	4 (5.06)
Ex-smoker	25 (15.24)	13 (15.29)	12 (15.19)
Maternal ethnicity; n (%)			
White	112 (64.74)	60 (64.52)	52 (65.00)
Mixed	10 (5.78)	3 (3.23)	7 (8.75)
Oriental	22 (12.72)	11 (11.83)	11 (13.75)
South Asian	19 (10.98)	12 (12.90)	7 (8.75)
African-Caribbean	10 (5.78)	7 (7.53)	3 (3.75)
Parity; n (%)			
0	107 (60.45)	61 (64.89)	46 (55.42)
1	48 (27.12)	23 (24.47)	25 (30.12)
2	18 (10.17)	8 (8.51)	10 (12.05)
3	2 (1.13)	1 (1.06)	1 (1.20)
4	2 (1.13)	1 (1.06)	1 (1.20)
Assisted conception; n (%)	24 (13.95)	14 (15.22)	10 (12.50)
Gestational age at delivery; median (IQR)	35.43 (33.00, 36.57)	34.36 (30.00, 36.43)	36.00 (35.00, 36.57)
Steroids administration	125 (71.02)	67 (71.28)	58 (70.73)
Magnesium sulphate administration	12 (6.82)	10 (10.64)	2 (2.44)

3.5.2 Adverse outcomes

There were 55/177 (31.07%) participants who did not experience an adverse outcome and delivered 2 healthy twins after 34 weeks gestation. The fetal adverse outcome composite affected 94/177 (53.11%) pregnancies (Table 3.4). The rate of AGR, TTTS and IUFD is in-keeping with the incidence reported in the literature, therefore the cohort is representative of the UK and Australian general MC twin population. Of the 23 pregnancies with TTTS, 10 had concurrent AGR, but were only included in the TTTS and fetal adverse outcome composite, not the AGR outcome. The causes of the 11 sIUFDs were: 7 TTTS, 2 sIUGR, 1 unexplained IUFD at 20 weeks, 1 intrapartum stillbirth at 23^{+6} weeks following spontaneous PTB. The causes of the 12 dIUFDs were: 7 TTTS, 2 sIUGR, 2 unexplained dIUFDs at 16 weeks, 1 unexplained dIUFD at 21 weeks. 19/41 (46.3%) pregnancies diagnosed as AGR were also diagnosed as PGR, with 3 pregnancies not included in the PGR outcome due to dIUFDs. 24/43 (55.8%) pregnancies diagnosed as PGR were not diagnosed antenatally. Only 2 pregnancies had isolated BWD (i.e. no other sign of abnormal growth) and were not considered growth restricted. The other pregnancies with BWD had additional signs of growth restriction. The majority of pregnancies with AGR had an inter-twin EFWD >20% (27/41, 65.85%), of which 23 pregnancies had 1 fetus <10^{th} centile, and 1 pregnancy had both fetuses below the 10^{th} centile.

Table 3.4 Number of events

(per pregnancy unless otherwise stated)

Outcome	N (%)
Uncomplicated monochorionic diamniotic twin pregnancy, delivered >34 weeks gestation	55 (31.07)
Fetal adverse outcome composite	94/177 (53.11)
Twin-twin transfusion syndrome	23/177 (12.99)
Antenatal growth restriction	41/177 (23.16)
Antenatal growth restriction (per fetus)	73/354 (20.6)
Postnatal growth restriction	43/177 (24.29)
Postnatal growth restriction (per baby)	54/354 (15.25)
Intrauterine fetal death (single)	11/177 (6.21)
Intrauterine fetal death (double)	12/177 (6.78)
Maternal antenatal and postnatal composite	46/177 (25.99)
Spontaneous preterm birth	12/177 (6.78)
Neonatal composite	91/340 (26.76)

3.5.3 Potential prognostic factors

The median gestational age at blood sampling was 12+6 weeks (IQR: 12+3, 13+2). The average values of the prognostic factors for the total cohort are displayed in Table 3.5 and scatterplots of individual data points are in Appendix 10.4. There was no or minimal missing data in the majority of the factors, with the exception of β-hCG and PAPP-A which had 36.2% missing due to not being able to be included from the 64 Australian women; sFlt-1, PlGF and AFP had 0.56% missing data which equated to one blood sample that was not transferred from Australia to the UK, but for which the woman was included in the NT and CRL assessment. The potential prognostic factor values in the UK cohort were compared to the Australian cohort, and the NT

measurement per fetus and sFlt-1 levels were significantly higher in the Australian samples than the UK sample (Table 3.5).

There was no difference in the CRL measurement per fetus which demonstrates that there was no difference in the gestation at blood sampling.

Table 3.5 Average values of potential prognostic factors in cohort

(per pregnancy unless otherwise stated) CRLD: crown-rump length discordance, NA: not applicable as Australian measures not used, NTD: nuchal translucency discordance.

Potential prognostic factor	Measures in total cohort (n)	Average value in total cohort	Average value in UK cohort	Average value in Australian cohort
NTD %; median (IQR)	177	11.76 (5.55, 21.15)	11.11 (5.48, 20.00)	13.81 (5.66, 24.38)
CRLD %; median (IQR)	177	4.22 (1.75, 7.02)	3.71 (1.77, 6.95)	5.08 (1.86, 7.04)
NT (mm); median (IQR) (per fetus)	354	1.6 (1.4, 1.9)	1.6 (1.3, 1.8)	1.8 (1.5, 2.1)
CRL (mm); mean (SD) (per fetus)	354	64.02 (8.49)	64.11 (9.60)	63.86 (9.99)
β-hCG (ng/mL); median (IQR)	113	64.15 (45.90, 83.91)	64.15 (45.90, 83.91)	NA
PAPP-A (U/L); median (IQR)	113	5354.06 (3515.85, 7629.06)	5354.06 (3515.85, 7629.06)	NA
AFP (U/mL); median (IQR)	176	29.29 (23.40, 41.50)	29.26 (24.04, 41.06)	29.32 (22.26, 41.81)
sFlt-1 (pg/mL); median (IQR)	176	2163 (1645, 2945.5)	2109 (1600, 2761)	2455 (1830, 3115)
PlGF (pg/mL); median (IQR)	176	60.45 (40.89, 89.02)	56.75 (38.84, 86.85)	62.66 (45.785, 90.19)

3.5.4 Nuchal translucency

Table 3.6 demonstrates the unadjusted OR, aOR and 95%CIs for the association between inter-twin NT % discordance and each adverse outcome. The change in c-statistic shows the added value of NT % discordance over standard previously identified prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception). There was an unadjusted significant association between increased NT % discordance and the fetal adverse outcome composite, and TTTS. These associations remained after adjustment demonstrating that increased NT % discordance is prognostic of the fetal adverse outcome composite, with an estimated 3% (aOR 1.03 95%CI 1.01, 1.06) increase in the odds of experiencing an outcome in the fetal adverse outcome composite, for each 1% increase in NT % discordance, and an estimated 6% (aOR 1.06 95% CI 1.03, 1.10) increase in the odds of developing TTTS, for each 1% increase in NT % discordance. The change in the c-statistic for fetal adverse outcome composite was quite high (0.05, baseline c-statistic 0.594) as it was for TTTS (0.14, baseline c-statistic 0.617). NT % discordance was not able to predict any other adverse outcome.

Table 3.6 Association between NT % discordance and adverse outcome

(n=177 pregnancies) Outcome per pregnancy. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity and mode of conception. sIUFD and dIUFD was only able to be adjusted for maternal age, BMI, ethnicity and mode of conception. †p value of the adjusted OR. Bold denotes significant associations. Change in c-statistic represents the additional prognostic value of each individual potential prognostic factor above the existing standard prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception).

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Fetal composite	**1.03 (1.01, 1.05)**	**1.03 (1.01, 1.06)**	**0.011**	**0.05**
TTTS	**1.05 (1.02, 1.08)**	**1.06 (1.03, 1.10)**	**<0.001**	**0.14**
Antenatal growth restriction	1.01 (0.99, 1.03)	1.01 (0.99, 1.04)	NS	-
Postnatal growth restriction	1.01 (0.98, 1.03)	1.01 (0.98, 1.03)	NS	-
sIUFD	1.01 (0.98, 1.05)	1.02 (0.98, 1.06)	NS	-
dIUFD	1.02 (0.98, 1.06)	1.02 (0.98, 1.06)	NS	-
Maternal composite	1.01 (0.99, 1.03)	1.01 (0.98, 1.03)	NS	-
Spontaneous preterm birth	1.00 (0.64, 1.04)	0.99 (0.95, 1.04)	NS	-

Table 3.7 Association between individual twin NT measurement (mm) and adverse outcome

(n=354 fetuses/babies) Outcome per fetus/baby. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity, mode of conception. Neonatal composite outcome additionally adjusted for gestation at delivery, administration of steroids, and magnesium sulphate. †p value of the adjusted OR.

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Antenatal growth restriction	0.62 (0.15, 2.56)	0.67 (0.13, 3.35)	NS	-
Postnatal growth restriction	0.81 (0.39, 1.68)	0.75 (0.36, 1.55)	NS	-
IUFD	1.46 (0.35, 6.08)	1.20 (0.62, 2.30)	NS	-
Neonatal composite	0.93 (0.30, 2.89)	1.07 (0.33, 3.50)	NS	-

An individual twin's first trimester NT measurement was not predictive of any adverse outcome (Table 3.7).

3.5.5 Crown-rump length

There was an unadjusted significant association between increased CRL % discordance and the fetal adverse outcome composite, AGR, and sIUFD (Table 3.8). These associations remained after adjustment demonstrating that increased CRL % discordance is a prognostic factor of adverse outcome in MC twin pregnancies. The change in the c-statistic was quite high (0.10, 0.12 and 0.09 for TTTS, AGR and sIUFD respectively. The baseline c-statistics for TTTS, AGR and sIUFD 0.617, 0.616 and 0.625 respectively. CRL % discordance was not able to predict any of the other adverse outcomes.

Table 3.8 Association between CRL % discordance and adverse outcome

(n=177 pregnancies) Outcome per pregnancy. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity and mode of conception. sIUFD and dIUFD was only able to be adjusted for maternal age, BMI, ethnicity and mode of conception. †p value of the adjusted OR. Bold denotes significant associations. Change in c-statistic represents the additional prognostic value of each individual potential prognostic factor above the existing standard prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception).

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Fetal composite	**1.16 (1.06, 1.27)**	**1.17 (1.07, 1.29)**	**0.001**	**0.10**
TTTS	1.07 (0.96, 1.20)	1.09 (0.97, 1.23)	NS	-
Antenatal growth restriction	**1.17 (1.06, 1.30)**	**1.20 (1.08, 1.34)**	**0.001**	**0.12**
Postnatal growth restriction	1.05 (0.95, 1.15)	1.04 (0.94, 1.15)	NS	-
sIUFD	**1.17 (1.00, 1.36)**	**1.19 (1.01, 1.40)**	**0.035**	**0.09**
dIUFD	1.06 (0.91, 1.24)	1.12 (0.94, 1.33)	NS	-
Maternal composite	0.96 (0.87, 1.06)	0.97 (0.87, 1.07)	NS	-
Spontaneous preterm birth	0.93 (0.77, 1.11)	0.92 (0.76, 1.11)	NS	-

3.5.6 β-human chorionic gonadotropin

First trimester maternal serum β-hCG was not prognostic for any adverse outcome (Table 3.9).

Table 3.9 Association between first trimester maternal serum β-hCG and adverse outcome

(n=113 pregnancies) Outcome per pregnancy. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity and mode of conception. sIUFD and dIUFD was only able to be adjusted for maternal age, BMI, ethnicity and mode of conception. †p value of the adjusted OR. Bold denotes significant associations. Change in c-statistic represents the additional prognostic value of each individual potential prognostic factor above the existing standard prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception).

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Fetal composite	1.41 (0.77, 2.57)	1.35 (0.68, 2.68)	NS	-
TTTS	1.11 (0.47, 2.63)	1.04 (0.41, 2.65)	NS	-
Antenatal growth restriction	1.21 (0.61, 2.41)	1.25 (0.56, 2.79)	NS	-
Postnatal growth restriction	1.04 (0.56, 1.93)	0.95 (0.47, 1.94)	NS	-
sIUFD	1.40 (0.45, 4.36)	1.52 (0.44, 5.25)	NS	-
dIUFD	1.66 (0.50, 5.55)	1.91 (0.48, 7.61)	NS	-
Maternal composite	1.30 (0.69, 2.45)	1.23 (0.58, 2.62)	NS	-
Spontaneous preterm birth	0.87 (0.30, 2.59)	0.81 (0.24, 2.72)	NS	-

3.5.7 Pregnancy-associated plasma protein A

First trimester maternal serum PAPP-A was not prognostic for any adverse outcome (Table 3.10).

Table 3.10 Association between first trimester maternal serum PAPP-A and adverse outcome

(n=113 pregnancies) Outcome per pregnancy. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity and mode of conception. sIUFD and dIUFD was only able to be adjusted for maternal age, BMI, ethnicity and mode of conception. †p value of the adjusted OR. Bold denotes significant associations. Change in c-statistic represents the additional prognostic value of each individual potential prognostic factor above the existing standard prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception).

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Fetal composite	1.06 (0.63, 1.81)	1.10 (0.55, 2.18)	NS	-
TTTS	1.50 (0.61, 3.65)	1.26 (0.40, 3.91)	NS	-
Antenatal growth restriction	1.09 (0.56, 2.12)	1.21 (0.53, 2.78)	NS	-
Postnatal growth restriction	0.82 (0.43, 1.58)	1.03 (0.44, 2.42)	NS	-
sIUFD	0.94 (0.33, 2.69)	1.09 (0.30, 3.93)	NS	-
dIUFD	1.36 (0.38, 4.91)	0.63 (0.11, 3.65)	NS	-
Maternal composite	0.82 (0.45, 1.49)	0.68 (0.32, 1.47)	NS	-
Spontaneous preterm birth	0.75 (0.26, 2.17)	0.68 (0.20, 2.37)	NS	-

3.5.8 Alpha-fetoprotein

There was an unadjusted significant association between increased AFP and TTTS (Table 3.11). This association became borderline after adjustment for existing prognostic factors. AFP was not associated with any other adverse outcome.

Table 3.11 Association between first trimester maternal serum AFP and adverse outcome

(n=176 pregnancies) Outcome per pregnancy. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity and mode of conception. sIUFD and dIUFD was only able to be adjusted for maternal age, BMI, ethnicity and mode of conception. †p value of the adjusted OR. Bold denotes significant associations. Change in c-statistic represents the additional prognostic value of each individual potential prognostic factor above the existing standard prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception).

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Fetal composite	1.91 (0.93, 3.94)	2.08 (0.94, 4.59)	NS	-
TTTS	**3.04 (1.05, 8.78)**	**3.24 (1.00, 10.48)**	**0.057**	**0.07**
Antenatal growth restriction	1.55 (0.67, 3.55)	2.10 (0.82, 5.40)	NS	-
Postnatal growth restriction	0.88 (0.39, 1.98)	0.88 (0.35, 2.20)	NS	-
sIUFD	0.68 (0.16, 2.87)	0.80 (0.17, 3.90)	NS	-
dIUFD	1.33 (0.34, 5.21)	0.97 (0.18, 5.33)	NS	-
Maternal composite	0.56 (0.25, 1.26)	0.55 (0.21, 1.42)	NS	-
Spontaneous preterm birth	0.96 (0.24, 3.81)	0.76 (0.15, 3.80)	NS	-

3.5.9 Placental growth factor

There was an unadjusted significant association between decreased PlGF and TTTS, sIUFD and dIUFD (Table 3.12). These associations remained after adjustment demonstrating that decreased PlGF is a prognostic factor of adverse outcome in MC twin pregnancies. The change in the c-statistic was quite high for all the significantly associated adverse outcomes (the baseline c-statistics for TTTS, sIUFD and dIUFD

were 0.617, 0.625 and 0.783 respectively). PlGF was not able to predict any of the other adverse outcomes.

Table 3.12 Association between first trimester maternal serum PlGF and adverse outcome

(n=176 pregnancies) Outcome per pregnancy. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity and mode of conception. sIUFD and dIUFD was only able to be adjusted for maternal age, BMI, ethnicity and mode of conception. †p value of the adjusted OR. Bold denotes significant associations. Change in c-statistic represents the additional prognostic value of each individual potential prognostic factor above the existing standard prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception).

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Fetal composite	0.73 (0.44, 1.22)	0.65 (0.37, 1.13)	NS	-
TTTS	**0.43 (0.20, 0.91)**	**0.42 (0.19, 0.93)**	**0.033**	**0.07**
Antenatal growth restriction	0.91 (0.50, 1.66)	0.88 (0.44, 1.76)	NS	-
Postnatal growth restriction	1.55 (0.84, 2.85)	1.62 (0.80, 3.29)	NS	-
sIUFD	**0.35 (0.13, 0.97)**	**0.34 (0.12, 0.98)**	**0.045**	**0.06**
dIUFD	**0.23 (0.08, 0.63)**	**0.18 (0.05, 0.58)**	**0.003**	**0.07**
Maternal composite	0.78 (0.44, 1.39)	0.70 (0.34, 1.42)	NS	-
Spontaneous preterm birth	0.69 (0.26, 1.82)	0.70 (0.25, 1.98)	NS	-

3.5.10 Soluble fms-like tyrosine kinase-1

First trimester maternal serum sFlt-1 was not prognostic for any adverse outcome (Table 3.13).

Table 3.13 Association between first trimester maternal serum sFlt-1 and adverse outcome

(n=176 pregnancies) Outcome per pregnancy. *Adjusted for maternal age, BMI, smoking status, ethnicity, parity and mode of conception. sIUFD and dIUFD was only able to be adjusted for maternal age, BMI, ethnicity and mode of conception. †p value of the adjusted OR. Bold denotes significant associations. Change in c-statistic represents the additional prognostic value of each individual potential prognostic factor above the existing standard prognostic factors (maternal age, BMI, smoking status, ethnicity, parity and mode of conception).

Adverse outcome	Unadjusted OR (95% CI)	Adjusted* OR (95% CI)	p-value†	change in c-statistic
Fetal composite	1.12 (0.52, 2.40)	1.03 (0.42, 2.50)	NS	-
TTTS	1.91 (0.62, 5.88)	1.64 (0.44, 6.03)	NS	-
Antenatal growth restriction	1.25 (0.50, 3.13)	1.47 (0.49, 4.35)	NS	-
Postnatal growth restriction	0.63 (0.25, 1.59)	0.65 (0.22, 1.88)	NS	-
sIUFD	1.27 (0.27, 6.04)	1.79 (0.30, 10.64)	NS	-
dIUFD	4.13 (0.92, 18.58)	8.21 (1.02, 66.24)	NS	-
Maternal composite	1.36 (0.57, 3.27)	1.26 (0.44, 3.58)	NS	-
Spontaneous preterm birth	0.38 (0.08, 1.84)	0.30 (0.05, 1.90)	NS	-

3.5.11 Translating the prognostic effect into clinical utility

The odds of developing the fetal adverse outcome based on 2 values for each potential prognostic factor were translated to demonstrate the prognostic effect for each individual potential prognostic factor (Table 3.14).

Table 3.14 Probability of developing a fetal adverse outcome according to potential prognostic factor measurements determined a priori

*Adjusted for using the mean values of the adjustment factors in the study cohort. The patient characteristics remained the same and were based on the mean and most common options in the patient cohort: 30 years old, BMI 25, non-smoker, Caucasian, nulliparous and natural conception.

Potential prognostic factor measurement	Adjusted probability*
NT (% discordance) 0%	0.446
NT (% discordance) 20%	0.604
CRL (% discordance) 0%	0.379
CRL (% discordance) 10%	0.750
AFP 50th centile (29.3 U/mL)	0.549
AFP 95th centile (54.7 U/mL)	0.658
PlGF 50th centile (60.4 pg/mL)	0.535
PlGF 5th centile (23.6 U/mL)	0.634
sFlt-1 50th centile (2169pg/mL)	0.545
sFlt-1 95th centile (4089pg/mL)	0.549

3.6 Discussion

3.6.1 Nuchal translucency

This study has demonstrated that increased first trimester inter-twin NT % discordance is a prognostic factor for the fetal adverse outcome composite, and TTTS. The ability of NT % discordance to predict TTTS is supported in part by a meta-analysis by Stagnati et al. (Stagnati 2017) of 7 studies, 128/1087 TTTS pregnancies, which found that NT discrepancy, defined as a combination of NT discordance >10%, >20%, >0.5mm, ≥0.6mm depending on the definition each study

used, had low sensitivity (52.8 [95% CI 43.8, 61.7] I^2=48.7%) but better specificity (72.5 [95%CI 61.7, 82.0] I^2=84.3%) although perhaps unsurprisingly these results were at high-risk of heterogeneity. The systematic review and meta-analyses in CHAPTER 2 found a trend towards a significant association between NT discordance ≥20% and TTTS, and that NT>95th centile in one/both fetuses significantly increased the risk of developing TTTS. When individual NT measurements were examined in this cohort, no significant association was found, however the group was intentionally not dichotomised as in the studies in the meta-analysis as there are no validated cut-offs for twin pregnancies. These findings suggest that there may be a link between an abnormal NT measurement and TTTS, although it may be more specific, for example in a sub-set of TTTS who will go on to develop cardiac dysfunction as demonstrated by the connection between a raised NT and cardiac defects outside TTTS (Souka 1998, Hyett 1999).

3.6.2 Crown-rump length

This work has found that increased first trimester inter-twin CRL % discordance is a prognostic factor of the adverse fetal composite outcome, AGR and sIUFD. The ability of CRL % discordance to predict the fetal adverse outcome may reflect its ability to predict AGR as 41/94 (43.6%) pregnancies in the fetal adverse outcome composite were complicated by AGR, and 27/41 (65.85%) had an EFWD >20%. Individual twin CRL measurements were not assessed as potential prognostic factors as they were used to indicate gestational age. It was not possible to examine the association between CRL and growth restriction in the meta-analysis in CHAPTER 2, but two studies did demonstrate CRL discordance to have good test accuracy for

predicting sIUGR (Fratelli 2011, Memmo 2012). This suggests that the pathological mechanisms contributing to abnormal antenatal fetal growth are present in the first trimester. No association was seen with TTTS in the cohort study, despite the significant association in the meta-analysis in CHAPTER 2, but the predictive ability was weak. As >60% TTTS pregnancies also have an element of growth restriction (Lewi 2014), it may be that the association is more reflective of concurrent growth restriction, rather than TTTS, however this was not examined in this cohort as including TTTS pregnancies in the growth restriction growth would make the group too heterogeneous, and the sub-group of TTTS with concurrent growth restriction (n=10 pregnancies) would be too small. Given the different causes of sIUFD it is difficult to ascertain why CRL discordance is associated with sIUFD. One could hypothesise that if the pathological process has begun in the first trimester and is sufficient to cause a difference in CRL, then either protective/compensatory mechanisms are not in place, or the disease severity is severe – thus more likely to lead to sIUFD.

3.6.3 β-human chorionic gonadotropin

First trimester β-hCG was not associated with any adverse outcome. This is in line with the results of the systematic review as neither of the two studies included reported a difference in first trimester β-hCG levels in pregnancies affected by TTTS and uncomplicated MC twin pregnancies (Linskens 2010, Ashoor 2013). Given the differences seen in the second trimester in TTTS pregnancies (Fox 2009, Sermondade 2009), it may be that it is too early to see a significant difference in maternal serum levels in the first trimester, or that the β-hCG levels fail to decrease

in the second trimester in TTTS and it is this inability to decrease that is involved in the pathogenesis of TTTS. Given the myriad of biological roles that β-hCG plays in pregnancy, this would require further investigation. With regards growth restriction, there is little existing literature to compare the finding of no association with first trimester β-hCG. The only study on first trimester β-hCG in twin pregnancies did not perform sub-group analysis according to chorionicity as only 4/104 included pregnancies were MCDA (Iskender 2013). The authors divided their cohort into those with a first trimester β-hCG result >90th centile, and those <90th centile. They found that a higher proportion of women with "IUGR & SGA" had a first trimester β-hCG result >90th centile, compared to the proportion of women with "IUGR & SGA" in the <90th centile group. There was no difference in the first trimester β-hCG result centiles in women with sIUGR, or "disconcordant fetal growth".

3.6.4 Pregnancy-associated plasma protein A

First trimester PAPP-A was not associated with any adverse outcome. This finding is supported by Linskens et al. who found no difference in first trimester PAPP-A levels in MC twin pregnancies with and without TTTS (Linskens 2010). The lack of significant association between first trimester PAPP-A and growth restriction is slightly surprising given that low PAPP-A is used clinically in singleton pregnancies to indicate those at high risk of delivery of a SGA neonate. However, Iskender et al. (Iskender 2013) reported no difference in the proportion of twin pregnancies with a first trimester PAPP-A >90th centile or <90th centile for any of their growth outcomes: growth discordance, IUGR, SGA or sIUGR.

3.6.5 Alpha-fetoprotein

This study has found that increased first trimester maternal serum AFP is a prognostic factor for TTTS, although this became borderline significant when adjusted for existing prognostic factors. The wide 95%CIs should also be taken into consideration when interpreting this finding. As second trimester work has previously shown a difference in AFP levels in MC twins with and without TTTS (Fox 2009), it is credible that there is an association between first trimester AFP and TTTS. However other second trimester work did not find a difference in TTTS pregnancies (Sermondade 2009). The lack of associations between first trimester AFP and adverse outcome in twin pregnancies may reflect the pattern seen in singletons that most associations have been demonstrated in second trimester AFP levels, and first trimester AFP is not used clinically.

3.6.6 Placental growth factor

Decreased first trimester PlGF is a prognostic factor for TTTS, sIUFD and dIUFD. As PlGF is involved in angiogenesis, the association with decreased PlGF and TTTS may be biologically plausible. Studies have shown that decreased PlGF levels are also present in the second trimester in TTTS pregnancies (Yinon 2014, Chon 2017). Although another study disputes there is any difference between MC twin pregnancies with and without TTTS in the second trimester (Fox 2010). As with pre-eclampsia, which is also associated with decreased first trimester PlGF, these findings suggest that the pathological process of TTTS may be occurring before clinical signs are visible on ultrasound scan. The association between PlGF and

sIUFD and dIUFD may be related to the high proportion of IUFDs that were affected by TTTS (at least 7/11 and 7/12 respectively) but it was not possible to perform a sub-group analysis as the numbers would be too small. Consequently, PlGF could be viewed as a biomarker of severity of TTTS, particularly as the aOR worsens in accordance with the outcome: 0.42 (95%CI 0.19, 0.93) for TTTS, 0.34 (95%CI 0.12, 0.98) for sIUFD, and 0.18 (95%CI 0.05, 0.58) for dIUFD. First trimester PlGF was not a prognostic factor for antenatal or postnatal growth restriction. Results from twin pregnancy studies in the second trimester have been conflicting, thus further research is needed.

3.6.7 Soluble fms-like tyrosine kinase-1

First trimester sFlt-1 was not associated with any adverse outcome. This is slightly unexpected given its relationship with PlGF and the fact that this study has found that PlGF is a prognostic factor for certain adverse outcomes in twin pregnancies. However, this does echo the findings that normotensive SGA singleton pregnancies only demonstrate decreased PlGF and are not accompanied by increased sFlt-1, thus sFlt-1 and PlGF findings do not necessarily go hand in hand, and there may be an unrelated mechanism. Existing research does demonstrate that changes in sFlt-1 may be present in the second trimester in the context of abnormal growth, and it may be that it is too early to see these changes in the first trimester. This is also the case with TTTS as all 4 studies that investigated maternal serum sFlt-1 levels at diagnosis of TTTS in the second trimester have reported significantly higher sFlt-1 in TTTS pregnancies compared to uncomplicated MC twin pregnancies. As sFlt-1 is anti-angiogenic, it may be that these effects are more apparent in the second trimester

when the pregnancy is established, as opposed to in the first trimester where vasculogenesis plays an important role (Flamme 1997). Or it may be that unlike in pre-eclampsia whereby the mother exhibits pathophysiological abnormalities, for example changes in maternal blood pressure and maternal kidneys, TTTS and sIUGR are fetal conditions and do not affect the mother, therefore although there may be fetal changes, these may not be reflected in maternal serum. Particularly as in pregnancy there is obvious capillary growth within the placenta villi but little angiogenesis occurs in maternal tissue (Clark 1998). There is the suggestion that the major role for sFlt-1 is through systemic effects on maternal circulation rather than on the local placental vasculature (Hirashima 2003) thus effects are seen in pre-eclampsia but not TTTS or sIUGR. This is compounded in MC twin pregnancy complications as the pathophysiological processes are often discordant between the twins for example with a hypervolaemic recipient twin, and hypovolaemic donor twin in TTTS. When placental villi from recipient and donor twins were compared sFlt-1 mRNA was upregulated in the donor villi, but not the recipient villi which the authors hypothesise is due to hypoperfusion of the donor villi and subsequent hypoxia and ischaemia (Kumazaki 2002). Thus the donor and recipient twins may contribute different amounts of sFlt-1 to the maternal circulation and the level may not appear abnormal. An alternative explanation is that genetic variants in the offspring genome may play a role. A recent study of 4,380 women with pre-eclampsia demonstrated certain genetic variants near the *FLT1* gene are associated with an increased risk of pre-eclampsia (McGinnis 2017), thus it may be that a genetic susceptibility is needed for pre-eclampsia and the associated abnormal sFlt-1 levels, which may not have been present in this TTTS group.

3.6.8 Strengths and limitations

This is the first study to explore the individual prognostic value of first trimester maternal serum AFP, PlGF and sFlt-1 for predicting adverse outcome in MC twins. It is also the largest study to evaluate first trimester β-hCG and PAPP-A, and to investigate the independent prognostic value of first-trimester NT and CRL % discordance using such robust statistical methodology, to the authors' knowledge. The potential prognostic factors were examined as continuous data, without the use of non-evidence based, non-validated cut-offs making the study more statistically robust compared to other studies. The study was designed and analysed according to a vigorous prognostic research methodology; and has been reported in-keeping with REMARK which is a great strength of this study. The results were adjusted using a comprehensive list of existing prognostic factors and whilst the evidence was sparse for some adjustment factors, the adjusted and unadjusted results are presented. Sample size was an issue with this study due to the relative scarcity of MCDA twins in the UK and Australian general obstetric populations, however there were a sufficient number of events (n=94) if you apply the widely accepted rule of thumb of requiring 10 events for each 7 potential prognostic factors being examined (Peduzzi 1996), and there was little missing data. The rate of fetal adverse outcome composite (94/177 pregnancies, 53.1%)) demonstrates how high-risk MCDA twin pregnancies are, and how important research in this area is, particularly given the potentially fatal outcome of these complications. Although the use of a composite outcome is less desirable than individual outcomes, they were created to enable meaningful statistical analysis within this relatively specialised area of obstetrics. The conditions included in the primary outcome of the fetal adverse outcome composite

are monitored in the same way: with at least 2-weekly ultrasound scans, and the potential sequelae of TTTS, TAPS and growth restriction are the same: IUFD. The conditions were examined individually as well, but if viewed pragmatically, the clinical action for being higher-risk for one of the conditions in the fetal adverse outcome composite group could be similar for all conditions, for example increased monitoring, and a lower threshold to refer to a tertiary fetal medicine centre. As the study included women who underwent antenatal care at 28 different UK maternity units, the results are generalisable to the UK obstetric population and possibly to other high-income countries, however the study should be repeated in other cohorts to account for different obstetric populations. The results may be less applicable to those in developing countries, who lack the resources to conduct prognostic testing and such intensive antenatal surveillance.

Another major strength of this study is that the potential prognostic factors evaluated were evaluated by appropriately accredited NHS laboratories, and the assays are readily accessible, easily and reliably measurable on an automated platform, and only require a small amount of maternal blood thus presenting no risk to the mother or fetuses. The ultrasound measurements used are easily calculable and although ultrasound may be subject to inter-operator variability, all health care professionals performing these assessments require additional certification, which is a national programme in the UK and thus the variability should be negligible. Consequently any prognostic factors with sufficient predictive ability would be clinically useful and feasible to measure at a national level, as with first trimester aneuploidy screening.

Examining maternal blood in twin pregnancy does present some problems. The main issue is that maternal blood contains biomarkers from both twins and each twin may

be affected differently by the same adverse outcome, therefore abnormal levels of a biomarker from one twin may be disguised by the levels of the other twin. This was an issue with aneuploidy screening but has been overcome by using the most accurate cut-offs for twin pregnancies (Wald 2005) as opposed to simply doubling singleton values (Muller 2003). However this screening test is still less accurate than in singletons. Cell-free fetal DNA testing for aneuploidy in twin pregnancy was also less accurate than in singleton pregnancy when it was initially introduced, however the technology has improved and the test accuracy is now comparable to singletons (Mackie 2018).

One of the main issues with this study was the absence of internationally agreed definitions and outcomes for multiple pregnancies, particularly with regards to abnormal growth. Therefore pragmatic decisions were made, based on clinical utility, and how patients would be managed if factors were found to be prognostic. Since performing this study, a Delphi consensus was published to focus definitions and outcomes in fetal growth restriction in twins (Khalil 2018), but this requires validation and was published too late to be used in this study. Although the growth restriction definitions were as pragmatic as possible, different pathophysiological mechanisms (Lewi 2008a) may be included as the sample size was too small to do further sub-group analysis, possibly explaining why biomarkers were not significant for the growth restriction outcomes. It may also be that “true” or “confirmed” growth restriction was not represented due to the discrepancy between antenatally- and postnatal-detected growth restriction not being concordant, although the growth restriction definitions were chosen as the antenatal measurements are those that guide clinicians’ management decisions. Whether to include isolated BWD as an

outcome was a dilemma. Interestingly, the experts who participated in the Delphi process included BWD in the list of potential parameters to define fetal growth restriction in DC twins, but not MC twins, thus supporting that BWD is not important in MC twin pregnancies. A recent meta-analysis reported that BWD ≥20% in MC twins is not associated with an increased risk of NND (D'Antonio 2017b), but is associated with an increased risk of IUFD compared to concordant MC twins. This risk increased further when at least one of the twins was SGA, although the authors do not comment on how many pregnancies developed isolated BWD with the absence of antenatal signs of growth restriction, therefore it is not possible to know how many of these higher risk pregnancies would have been detected antenatally, and thus would have been included in the growth restriction outcomes. The authors also do not state whether they included pregnancies affected by sIUFD which will affect the results and it is not possible to delineate if they have included or excluded these pregnancies as they have not presented their 2x2 data. As only 2/177 pregnancies in this current cohort developed isolated BWD it is unlikely they would have a substantial impact on the findings, but it was thought more appropriate not to include these pregnancies in the fetal adverse outcome composite or postnatally-detected growth restriction group given the dearth of evidence demonstrating that it is clinically significant.

LBW was included in the PGR and fetal adverse outcome composite outcomes as the reported accuracy of predicting fetal growth restriction on ultrasound using Hadlock's formula in twin pregnancies is sub-optimal with a sensitivity of 70.1% (95%CI 62.2, 77.1), specificity of 86.4% (95%CI 82.5, 89.6), positive predictive value of 67.9% (95%CI 60.1, 75.0) and negative predictive value of 87.5% (95%CI 83.7, 90.7). Therefore by including LBW this would allow the inclusion of pathologically

growth restricted babies who were missed on ultrasound scan, thus allowing complete reporting of pathology (Harper 2013). If viewed pragmatically, it was felt that if a prognostic factor was able to predict which pregnancies would result in a LBW (<9th centile), antenatally this pregnancy would be managed differently to a pregnancy with no signs of abnormal growth.

The neonatal outcomes were difficult to collect due to in-utero and ex-utero transfers, and the results are difficult to interpret due to the myriad of confounding factors involved in neonatal outcomes. The latter point is also true of PTB and the maternal composite with a plethora of confounding factors that were beyond the scope of this study to adjust for, and may explain why none of the potential prognostic factors were predictive of these outcomes.

There is debate as to whether these biomarkers, particularly those related to angiogenesis, should be measured in serum or plasma (Levine 2004a, Staff 2005, Romero 2008) as it is thought that free VEGF released from platelets may affect sFlt-1 levels in serum (Jelkmann 2001). However many studies have used serum and have demonstrated a difference in pre-eclamptic pregnancies (Koga 2003, Maynard 2003, Tsatsaris 2003, Levine 2004b, Staff 2005). Serum was used in this study for several reasons: the assays that the laboratories use and that are recommended by NICE require serum samples, the VEGF levels were not measured which is the focus of concern regarding the use of plasma or serum samples, and the stored first trimester aneuploidy screening samples were serum samples.

3.6.9 Clinical implications and future research

The aim of this study was to identify individual prognostic factors of complications in MC twin pregnancies. For those markers where there was a statistically significant association with adverse outcome translating this into clinical scenarios using common values demonstrated that the clinical utility of individual biomarkers was poor i.e. the change in risk of adverse outcome in an individual was not clinically useful to allow a change in management. The RCOG MC twin pregnancy guidance recommends that “screening for TTTS by first trimester nuchal translucency measurements should not be offered” (Kilby 2016) and the findings of this study support that NT % discordance, and individual NT measurements alone should not be used to predict TTTS.

Although the changes in *individual* biomarkers do not accurately predict outcome, and their individual predictive ability was thought to be too low to justify combination in a prognostic model, the findings are exciting from a pathophysiological perspective as they suggest that physiological changes occur before the appearance of the ultrasound signs of polyhydramnios and oligohydramnios, and IUFD. This supports that first trimester prognostic factors may exist, and warrant further investigation. Interestingly no potential prognostic factors affected both growth restriction and TTTS supporting that they have different pathological mechanisms. The lack of animal models and scarcity of MCDA twin pregnancies makes the pathophysiology difficult to investigate. No longitudinal studies have been performed prospectively recruiting women with MCDA pregnancies in the first trimester, prior to the appearance of the clinical signs of MC twin complications, and comparing those who subsequently

develop a complication. This would help determine whether the differences in the biomarkers are because the biomarker is abnormal earlier in pregnancy, or that it does not increase.

3.7 Conclusion

This study has demonstrated the predictive ability of individual first trimester ultrasound measurements and maternal serum biomarkers. Individually the prognostic factors have insufficient ability to predict complications in MC twin pregnancies, but the findings suggest that physiological changes occur in the first trimester, before the appearance of clinical signs, thus further research is warranted.

CHAPTER 4 FIRST AND SECOND TRIMESTER MATERNAL SERUM AFP, PLGF AND SFLT-1 IN UNCOMPLICATED MONOCHORIONIC TWIN PREGNANCIES

4.1 Overview

First trimester maternal serum AFP, PlGF and sFlt-1 levels were evaluated as potential prognostic factors of complications in MC twin pregnancies in CHAPTER 3 because differences had been demonstrated in these biomarkers between MC twins with TTTS and uncomplicated MC twins in the second trimester. When first trimester concentrations of these biomarkers were evaluated in CHAPTER 3 AFP was found to have a borderline significant association with adverse outcome in MC twin pregnancies, PlGF was found to have a significant association with adverse outcome, and sFlt-1 was found to have no association. Consequently it was hypothesised that the first trimester may be too early to see changes in the biomarkers. As stated in CHAPTER 3 there is little research examining AFP, PlGF and sFlt-1 levels in MC twins longitudinally, thus this chapter will explore AFP, PlGF and sFlt-1 levels at 12, 16 and 20 weeks gestation in prospectively recruited women with MC twin pregnancies.

4.2 Background

4.2.1 AFP, PlGF, sFlt-1

Detailed information on these biomarkers can be found in sections 3.2.5, 3.2.6 and 3.2.7.

4.2.2 Aims

The aims of this study are to investigate the maternal serum concentrations of AFP, PlGF and sFlt-1 in MC twin pregnancies at 12, 16 and 20 weeks gestation.

Hypotheses

In the context of repeated blood sampling at 12, 16 and 20 weeks in MC twin pregnancies:

1) AFP will increase over time.
2) PlGF will increase over time.
3) sFlt-1 will increase over time.

4.3 Methods

4.3.1 Participants

All women with MCDA twin pregnancies who booked at BWH were approached about the study. The recruitment period was 1 July 2015 – 31 December 2017. Women were approached either in antenatal clinic or in the West Midlands Fetal

Medicine Department if they were attending for first trimester aneuploidy screening. Recruitment was prospective. Women were eligible if they booked at less than 14 weeks gestation. Chronicity had to have been determined in the first trimester based on: a single placental mass, a thin inter-twin membrane, the presence of the 'T' sign and absence of Lambda sign (Sepulveda 1996). If the twins were a different sex, or postnatally the pregnancy was diagnosed as DC based on placental assessment, these pregnancies were excluded. As participants in the study had MCDA pregnancies, the majority were spontaneous conceptions therefore dates relating to ART could not be used. Consequently, the gestational age was calculated based on the largest twin CRL, as this is the measurement advised by various national bodies (Morin 2011, NICE 2011, Khalil 2016b, Kilby 2016), and is the gestational age upon which clinicians will have based management decisions. Women were not eligible for inclusion if they had a: miscarriage prior to 14 weeks gestation, MCMA pregnancy, higher order multiple pregnancy. Women with pregnancies affected by serious structural or congenital anomalies were also excluded, whether concordant or discordant, as the aetiology of their adverse outcomes, such as growth restriction, PTB or IUFD would be different to pregnancies not affected by structural or congenital anomalies, consequently increasing heterogeneity within the cohort. If a structural or chromosomal abnormality was diagnosed later in pregnancy, the woman was withdrawn from the study and none of her data were included. MCDA twin pregnancies at BWH were cared for in a specialised multiple pregnancy antenatal clinic, and managed according to the most recent NICE and RCOG guidance (NICE 2011, Kilby 2016). There was a specific local proforma detailing the risks of MCDA pregnancies, including TTTS, sIUGR and preterm labour, which clinicians completed

in the booking appointment i.e. the first appointment in antenatal clinic in the first trimester. After completing the proforma, the clinician asked if the participant would allow the researcher to discuss the study with them; if so the researcher explained the study and recruited participants who provided written informed consent (see Appendix 10.12 and 10.13 for patient information sheet and consent form). Depending on gestation, blood sampling was performed then, or at a later date. Serial blood sampling was performed at 12, 16 and 20 weeks gestation and coincided with antenatal clinic appointments.

MCDA twin pregnancies were monitored with ultrasound assessment of fetal growth, liquor volume and umbilical artery Doppler at least every 2 weeks from 16 weeks gestation. Some participants were also recruited to the STOPPIT-2 trial (ISRCTN 98835694) (Norman 2018) and underwent a cervical length scan. Based on the results of the cervical length scan the woman may have been randomised to an Arabin pessary or normal management. As PTB was not an outcome in this study, being in both studies was not an issue. Postnatal outcome data were collected until discharge from hospital (see Appendix 10.3 for data collection form). No further follow-up data were collected. Anonymised data were collated, imputed and coded in an Excel spreadsheet by a single researcher.

4.3.2 Sample collection and storage

Maternal venous blood samples were collected in 7.5ml serum gel tubes (Sarstedt, Nümbrecht, Germany) from the antecubital fossa, allowed to clot for 1 hour at room temperature, centrifuged at 3000*g* for 10 minutes at room temperature, and

sediment-free serum aliquots were stored immediately at -80°C prior to analysis. Serum sample aliquots were stored for a maximum of 32 months prior to analysis.

4.3.3 Biomarkers (alpha-fetoprotein, placental growth factor and soluble fms-like tyrosine kinase-1)

The biomarkers were measured as described in section 3.4.4.

4.3.4 Adjustment factors

Measurements were not adjusted as the aim of the work is to demonstrate the normal values for the cohort across time.

4.3.5 Missing data

To be eligible for inclusion, all participants had to have provided a blood sample at all 3 time points, with measurable levels of AFP, PlGF and sFlt-1. The only missing data was the smoking status of one participant which was assumed to be the modal status of non-smoker. No other imputations were performed.

4.3.6 Statistical analysis

All statistical analysis was performed in Stata (Stata, 2015 Release 13.1 StataCorp, Texas, USA). As this was a sample of convenience, no power calculation was performed.

The skewing and kurtosis of the data was assessed using the *sktest* command (StataCorp. 2013i), and the Shapiro-Wilk test of normality was performed using the *swilk* command (StataCorp. 2013k). Descriptive data are reported as medians and IQRs.

A line plot was generated using the *twoway connected* command (StataCorp. 2013d) to visually display the data for each biomarker longitudinally. To assess any difference between the three time points, the Shapiro-Wilk test of normality was performed, and the presence of outliers was examined using box plots. All data were not normally distributed consequently the Kruskall-Wallis test was performed using the *kwallis* command (StataCorp. 2013e). If a significant difference was found, this was investigated by Dunn's test using the *dunntest* command (Dinno 2015).

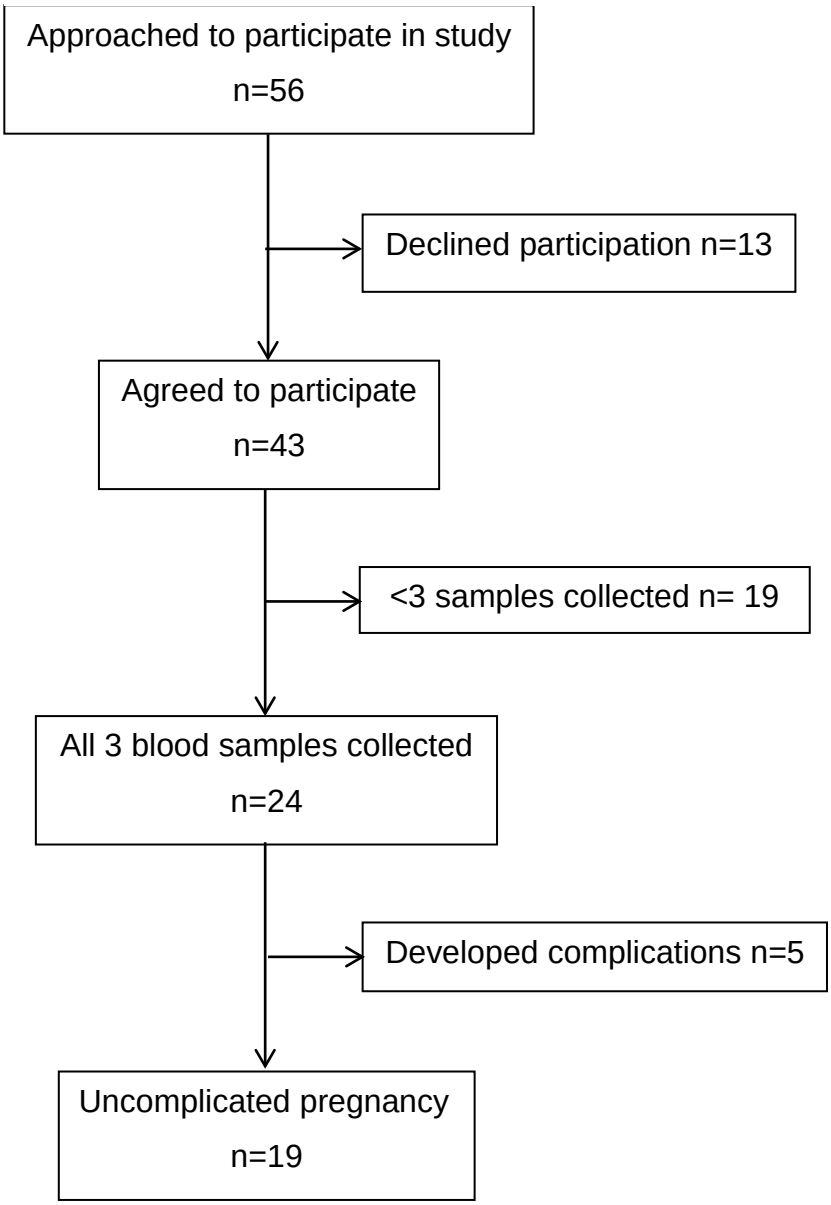
4.3.7 Ethical approval

Ethical approval was granted from East Midlands – Derby REC (15/EM/0244) on 01 July 2015 and the 1st amendment to include all MC twin pregnancies not just those undergoing first trimester aneuploidy screening was approved on 28 September 2015, the 2nd amendment to include parental attachment and depression assessment in the complicated sub-group was approved on 22 December 2015, the 3rd amendment to extend the study period was approved on 08 March 2017. This work is part of a larger study, which was registered on 22nd April 2016: ISRCTN 13114861 (www.isrctn.com/ISRCTN13114861). The protocol was published prior to analysis (Mackie 2017).

4.4 Results

Fifty-six women with MCDA twin pregnancies were approached to participate in the study, 43/56 (76.8%) consented to participate (Figure 4.1). Of the participants, 24 had blood sampling at all 3 time points, and 19/24 (79.2%) women had uncomplicated pregnancies. Five women developed complications antenatally (3 growth restriction, 1 TTTS, 1 spontaneous sIUFD of unknown cause) and were thus not included in the study. No woman was included twice.

Figure 4.1 Flow diagram of patient inclusion



4.4.1 Maternal demographic data

All data were able to be collected except for one participant's smoking status. The demographic data are displayed in Table 4.1.

Table 4.1 Maternal demographic data

(n=19 pregnancies). Gestation in weeks+days.

	Uncomplicated MC twin pregnancies
Maternal age; median (IQR) years	28 (26, 33)
Maternal BMI; median (IQR) kg/m^2	25 (22, 29)
Maternal smoking status; n (%)	
Never	14 (73.7)
Current smoker	3 (15.8)
Ex-smoker	2 (10.5)
Maternal ethnicity; n (%)	
White	11 (57.9)
Mixed	2 (10.5)
Oriental	1 (5.3)
South Asian	3 (15.8)
African-Caribbean	2 (10.5)
Parity; n (%)	
0	9 (47.4)
1	6 (31.6)
2	3 (15.8)
≥3	1 (5.3)
Assisted conception; n (%)	1 (5.3)
Gestation at 1st blood collection median (IQR)	12+5 (12+0, 13+4)
Gestation at 2nd blood collection median (IQR)	16+1 (15+6, 16+4)
Gestation at 3rd blood collection median (IQR)	20+1 (19+6, 20+4)
Gestation at delivery; median (IQR)	36+2 (36+0, 36+3)

4.4.2 Biomarkers at each time point

The median values of each of the 3 biomarkers are displayed in Table 4.2. As demonstrated by the individual line plots (Figure 4.2, Figure 4.3 and Figure 4.4) and the IQRs in Table 4.2, the spread of concentrations of each biomarker appears to increase at each time point.

Table 4.2 Alpha-fetoprotein, placental growth factor and soluble fms-like tyrosine kinase-1 levels in the first and second trimester in monochorionic twin pregnancies

(n=19 pregnancies)

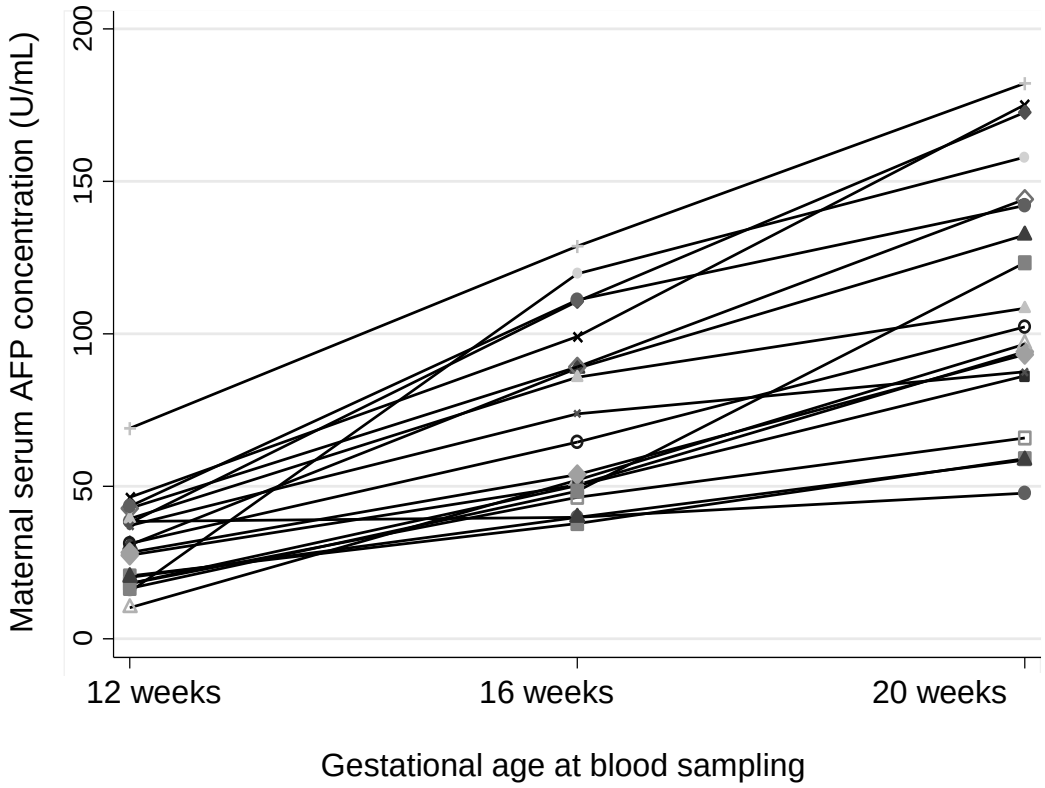
Biomarker, time point at blood sampling	Median (IQR)
AFP (U/mL)	
12 weeks	30.9 (19.25, 38.97)
16 weeks	64.6 (49.48, 94.14)
20 weeks	102.3 (86.89, 143.15)
PlGF (pg/mL)	
12 weeks	65.29 (54.36, 129)
16 weeks	284.10 (183.5, 443)
20 weeks	558.3 (428.5, 1011)
sFlt-1 (pg/mL)	
12 weeks	2231 (1918, 3363.5)
16 weeks	2271 (1837.5, 3466)
20 weeks	2552 (1892, 3696)

4.4.3 Alpha-fetoprotein

There was a significant change in AFP over time using the Kruskal Wallis test, $\chi^2(2)=36.424$, p=0.0001 (Figure 4.2). Post hoc Dunn's test revealed a significant increase in AFP concentrations at each time point.

Figure 4.2 Line plot of individual maternal serum AFP concentrations at 12, 16 and 20 weeks

(n=19 women with uncomplicated monochorionic twin pregnancies) p=0.0001 12 vs 16 weeks, p=0.0000 12 vs 20 weeks, p=0.0174 16 vs 20 weeks

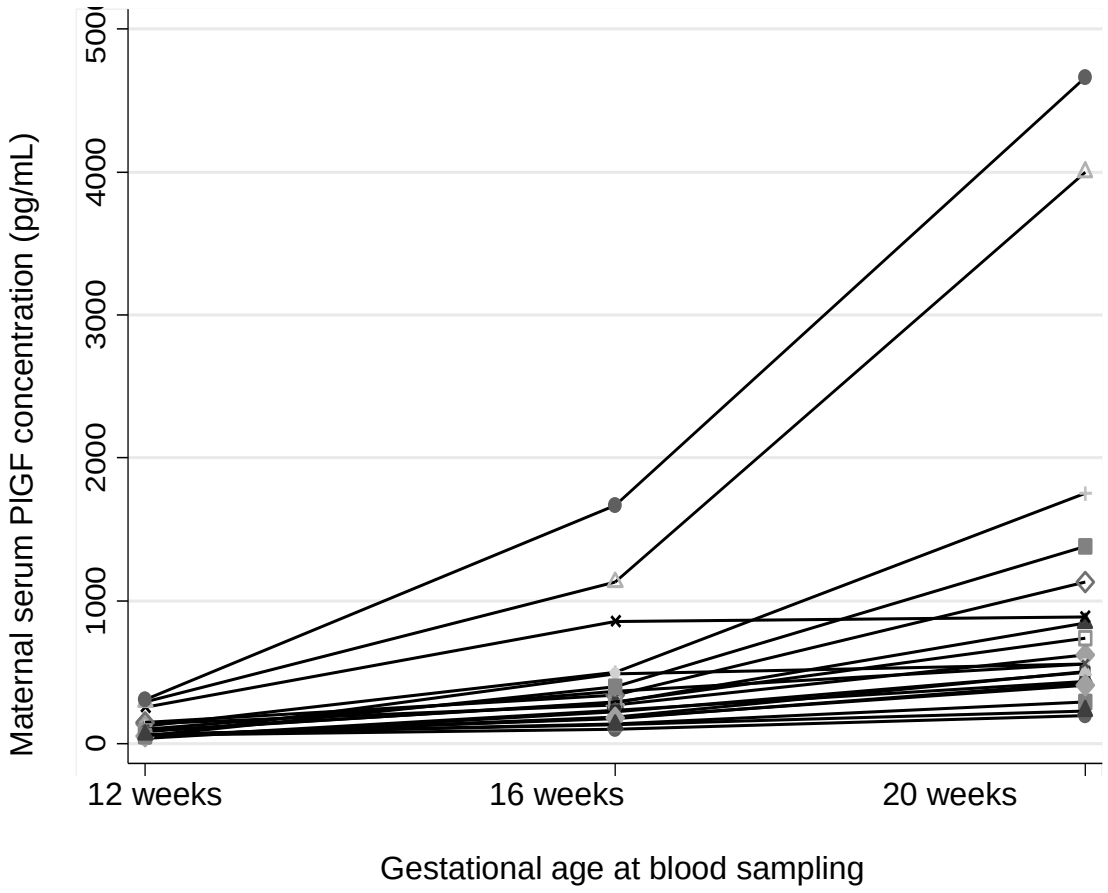


4.4.4 Placental growth factor

There was a significant change in PlGF over time using the Kruskal Wallis test, $\chi^2(2)=34.709$, p=0.0001 (Figure 4.3). Post hoc Dunn's test revealed a significant increase in PlGF concentrations at each time point.

Figure 4.3 Line plot of individual maternal serum PlGF concentrations at 12, 16 and 20 weeks

(n=19 women with uncomplicated monochorionic twin pregnancies) p=0.0002 12 vs 16 weeks, p=0.0000 12 vs 20 weeks, p=0.0093 16 vs 20 weeks

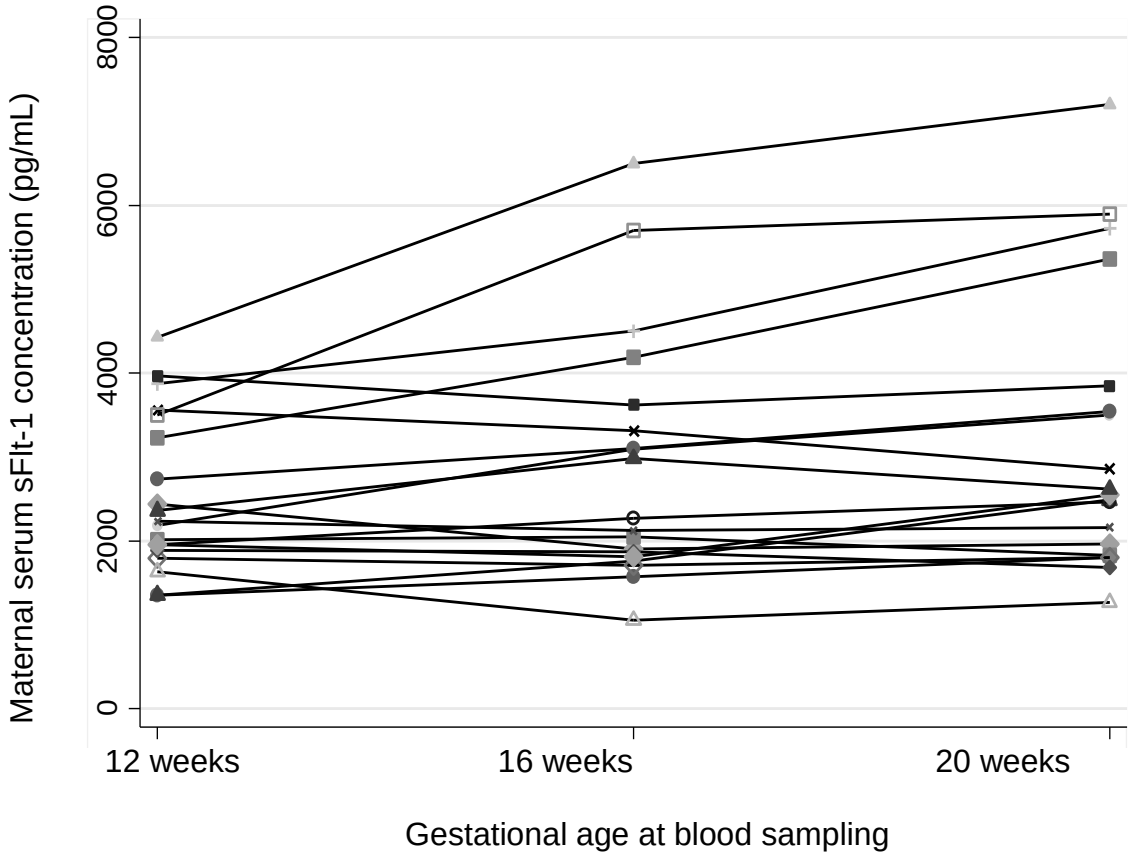


4.4.5 Soluble fms-like tyrosine kinase

There was no significant change in sFlt-1 over time using the Kruskal Wallis test, $\chi^2(2)=0.819$, p=0.664 (Figure 4.4).

Figure 4.4 Line plot of individual maternal serum sFlt-1 concentrations at 12, 16 and 20 weeks

(n=19 women with uncomplicated monochorionic twin pregnancies) No significant difference seen.



4.5 Discussion

4.5.1 Alpha-fetoprotein and placental growth factor

The increase in AFP and PlGF over time echoes the increases seen in singleton pregnancies. The concentrations reported in this work are substantially higher than reported in studies of singleton pregnancies (Bredaki 2015, Faupel-Badger 2015), supporting that singleton levels cannot be used for twin pregnancies.

4.5.2 Soluble fms-like tyrosine kinase-1

There was no significant change in sFlt-1 over time which is interesting given its relationship with PlGF, and that PlGF did significantly increase over time. This is supported by a study by Faupel-Badger et al. who demonstrated a greater increase in sFlt-1 between 26 weeks to 35 weeks gestation, compared to 10-17 weeks gestation in both singleton and twin pregnancies (Faupel-Badger 2015).

4.5.3 Strengths and limitations

This is the first piece of work to perform repeated measures of AFP, PlGF and sFlt-1 in uncomplicated MC twin pregnancies at 12, 16 and 20 weeks gestation. The biomarker concentrations were not adjusted for factors known to affect measures such as ethnicity which is a limitation of this work, however as the focus of the work was to look at the pattern in the biomarkers overtime, these factors will have remained the same at each time point.

4.5.4 Clinical implications and future research

The findings have no immediate clinical implications as this is only a descriptive study, and further research is required. The work should be repeated in a larger cohort of MC twins in order to recruit sufficient numbers of uncomplicated MC twin pregnancies to power the study, and also complicated MC twin pregnancies which will then allow comparison between the two groups. Longitudinal analysis of the biomarkers complements the work on first trimester maternal serum biomarkers as it may provide gestational cut-offs for when each biomarker can be accurately measured. It may also improve knowledge surrounding the pathogenesis of MC twin complications as by recruiting complicated MC twin pregnancies this will enable investigation as to whether the biomarkers are low only in the first trimester, or remain low throughout the first and second trimester when signs and symptoms begin to appear. It would be interesting to compare these levels in DC twin pregnancies, although this may have less clinical utility as DC twins develop fewer, and different complications as MC twins. As the IQRs increase over time, this would support that first trimester measures of the biomarkers may be best as there is less deviation from the median.

4.6 Conclusion

This study improves knowledge of the longitudinal pattern of maternal serum AFP, PlGF and sFlt-1 in MC twin pregnancies in the first and second trimester. Further work is required, including recruiting a sufficient number of women with complicated MC twin pregnancies so as to compare the change over time and delineate whether

the association between AFP and PlGF and adverse outcome is due to a lower concentration of these factors only in the first trimester, or throughout pregnancy.

CHAPTER 5 AMNIOTIC FLUID METABOLITES IN TWIN-TWIN TRANSFUSION SYNDROME AND ASSOCIATION WITH FETAL CARDIAC FUNCTION

- These findings were presented as a platform poster at British Maternal and Fetal Medicine Society 17th Annual Conference, London, UK, and as an oral presentation at the 26th World Congress on Ultrasound in Obstetrics and Gynaecology, Rome, Italy, where Dr F Mackie won the prize for best free communication.
- These findings have been published in abstract form [Dunn WB, Shek NW, Fox CE, Mackie FL, Van Mieghem T and Kilby MD (2015). "Non-targeted metabolomics in recipient amniotic fluid of monochorionic twin pregnancies complicated by severe twin to twin transfusion syndrome (TTTS) and treated by fetoscopic laser ablation (FLA)." BJOG 122(S2):8.] and [Mackie FL, Dunn WB, Allwood JW, Van Mieghem T, Morris RK, Fox CE and Kilby MD (2016). "Metabolomic changes in amniotic fluid from Twin–twin transfusion syndrome recipient twins in relation to treatment and fetal cardiovascular risk." Ultrasound Obstet Gynecol 48(S1):16-17.]
- These findings have also been published in full article form [Dunn WB, Allwood JW, Van Mieghem T, Morris RK, Mackie FL, Fox CE and Kilby MD (2016). "Carbohydrate and fatty acid perturbations in the amniotic fluid of the recipient twin of pregnancies complicated by twin-twin transfusion syndrome in relation to treatment and fetal cardiovascular risk." Placenta 44(8):6-12.]

5.1 Overview

In addition to the existing prognostic factors examined in CHAPTER 3 and CHAPTER 4, new potential biomarkers were sought by attempting to learn more about the pathophysiology of TTTS. This chapter explores the possibility of using metabolomics in the context of TTTS. Work examining the relationship between amniotic fluid metabolites and cardiac function in recipient twins is described. The reasons why amniotic fluid was chosen are outlined in the discussion. There was a focus on cardiac function as TTTS is predominantly a cardiovascular disease, and mortality of the recipient twin may be related to cardiac dysfunction. There is still a lot that is not known regarding the pathophysiology of TTTS, including the associated cardiac dysfunction, therefore a non-targeted metabolomics study was performed.

5.2 Metabolomics

As described in section 1.10.4 metabolomics investigates the final downstream product of the interaction between the genotype and its environment and has not been explored in the setting of TTTS before. Metabolomics has been used to explore cardiovascular disease in non-pregnant adults and does demonstrate functional and metabolic changes in heart failure (Deidda 2015, Wang 2015). Metabolomics have also been investigated in first trimester maternal serum samples to predict congenital heart disease (Bahado-Singh 2014) which demonstrated that abnormal lipid metabolism appears to play a role. In twin pregnancies only one metabolomics study has been published in humans (Cosmi 2013). This study used liquid

chromatography-high resolution mass spectrometry to examine the metabolome in cord blood from 4 sIUGR MC pregnancies and compared it to the metabolome of 4 appropriately grown MC twins (Cosmi 2013). They reported a trend of increased phenylalanine, and decreased isoleucine, proline, tryptophan and valine in sIUGR twins, and theorise that the results did not reach statistical significance due to the small sample size.

5.2.1 Aims

The aims of this study are to investigate if in amniotic fluid samples taken from the recipient twin affected by TTTS there is a:

1) difference in the metabolomic profile pre-FLA and post-FLA.
2) relationship between the recipient twin cardiovascular function at diagnosis of TTTS (pre-FLA) and metabolomic profile.

Hypotheses

The metabolomic profile of the amniotic fluid may reflect the severity of cardiac dysfunction in the recipient twin in TTTS. As the amniotic fluid profile of TTTS pregnancies has not been explored before, this is hypothesis-generating work and no specific metabolites were specified.

5.3 Methods

5.3.1 Participants

Women with MCDA twin pregnancies attending the West Midlands Fetal Medicine Centre between August 2011-June 2012 for assessment and treatment of TTTS were consecutively recruited by Dr Caroline Fox as part of her MD (Fox 2013). TTTS was diagnosed and prospectively staged according to the Quintero definitions (Quintero 1999) as previously outlined (Section 1.2.2). Those who required amnio-infusion prior to and/or during FLA were not included as the concentration of the metabolites would have been affected.

5.3.2 Sample collection

At FLA to treat TTTS an amniotic fluid sample (10ml) was taken at insertion of the trocar into the recipient amniotic sac. The post-FLA amniotic fluid sample (10ml) was removed at the end of the FLA, prior to amniodrainage. Samples were stored at -80°C before preparation and analysis in March 2015.

5.3.3 Cardiac function assessment

High-resolution fetal echocardiography was performed in the recipient twin with curvilinear array transducers (7–3.5 MHz) on a Siemens S3000 ultrasound machine (Siemens Ltd, Erlangan, Germany) by a single operator (Professor Mark Kilby). The MPI was calculated for each ventricle by adding the isovolumetric contraction time to the isovolumetric relaxation time, and dividing by ventricular ejection time (Tsutsumi

1999, Van Mieghem 2009c). A higher score indicated worse cardiac function. Cardiac dysfunction was indicated by the presence of tricuspid regurgitation, reversed flow in the DV during atrial contraction, and a tricuspid early passive/atrial contraction (E/A) ratio of >95% CI outside the normal limits for gestation. This was performed 24 hours prior to FLA, and repeated within 6 hours post-FLA.

5.3.4 Fetoscopic laser ablation (FLA)

Prophylactic tocolysis (indomethacin 100mg per rectum) was given 2 hours before FLA and intravenous cefuroxime (1.5g) was given peri-operatively. FLA was performed using local anaesthesia (1% lignocaine skin/myometrial infiltration) and maternal Remifentanil sedation by one operator (Professor Mark Kilby). Using continuous ultrasound guidance a 3.3mm port with trocar was inserted and a 2.0mm curved or straight fetoscope (Storz, Germany) was introduced, depending on placental site. The vasculature on the placental surface was carefully mapped and inter-twin anastomoses identified. A selective sequential FLA technique using a Diode laser system (30–50 W) was used, with an additional “Solomon” procedure in cases that were recruited to the Solomon Trial (Slaghekke 2014b). Amnio-infusion was not performed due to affecting the concentration of the metabolites. Women undergoing repeat FLA were not included.

5.3.5 Sample preparation

Sample preparation, ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) and biostatistical data analysis were performed by

Professor Warwick Dunn and Dr William Allwood at the Birmingham Phenome Centre. All samples were randomised to ensure the order of preparation was not affected by the subject, TTTS severity or date of sample collection. Deproteinisation was performed by vortex-mixing 250µL of amniotic fluid with 1000µL of methanol for 15 seconds to precipitate proteins and DNA. Centrifugation (15 minutes, 13,000 g) drying to induce metabolite stability was performed and the samples were stored at -80°C prior to analysis. A pooled quality control (QC) sample was prepared by combining 80µL aliquots of each of the 38 amniotic fluid samples as is standard practice (Dunn 2011). The pre-FLA samples were used to assess the relationship with cardiac dysfunction.

5.3.6 UHPLC-MS analysis

All chemicals and solvents applied were of HPLC analytical grade (JT. Baker, UK). UHPLC-MS analysis of amniotic fluid extracts and QC samples was performed by passing the samples through a Dionex™ UltiMate™ 3000 metabolite separation system coupled to an electrospray LTQ-FT Ultra™ mass spectrometer (Thermo Scientific Ltd. UK), a hybrid of the Linear Ion Trap mass spectrometer (LTQ) and Fourier Transform (FT) mass spectrometer. Samples were reconstituted in 100µL of 50:50 methanol:water, vortex-mixed for 15 seconds, centrifuged (15 minutes, 13,000 g) and transferred to vials with 200µL fixed inserts (Thermo-Fisher Ltd UK). All samples were stored in the autosampler at 5°C and analysed separately in negative and positive electrospray ionisation (ESI) modes within 72 hours of reconstitution. UHPLC separations were performed applying a Hypersil Gold C_{18} reversed phase column (100 x 2.1mm, 1.9µm) at a flow rate of 400µL.min^{-1}, at a column temperature

of 40°C, and with two solvents: solvent A (HPLC grade water + 0.1% formic acid) and solvent B (HPLC grade methanol + 0.1% formic acid). A gradient elution was performed as follows: hold 100% A 0-1.5 min, 100% A - 100% B 1.5-6 min curve 3, hold 100% B 6-12 min, 100% B – 100% A 12-13 min curve 3, hold 100% A 13-15 min. Injection volume was 5µL. UHPLC eluent was introduced directly in to the electrospray LTQ-FT Ultra™ mass spectrometer with source conditions as follows: spray voltage -4.5 kV (ESI-) and +5 kV (ESI+), sheath gas 30 arbitrary units, aux gas 15 arbitrary units, capillary voltage 35 V, tube lens voltage -100 V (ESI-) and +90 V (ESI+), capillary temperature 280°C, ESI heater temperature 300°C. Data were acquired in the FT mass spectrometer in the mass/charge ratio (m/z) range 100-1000 at a mass resolution of 50,000 (FWHM defined at m/z 400), with a scan speed of 0.4 sec and an AGC setting of $1x10^6$. Analysis order was composed of 10 QC sample injections for system conditioning followed by a QC sample injection every 6th injection with two QC sample injections at the end of the analytical run. Amniotic fluid extracts for each subject were analysed in a random order, but the pre-FLA and post-FLA samples for each participant were analysed sequentially.

5.3.7 Data pre-processing

UHPLC-MS raw data profiles were converted into a Network Common Data Form (NetCDF) format within the Xcalibur™ (Thermo Fisher Scientific) software's File Converter program. Each NetCDF based three-dimensional data matrix (intensity × m/z × retention time [one per sample]) was converted into a vector of peak responses, defined as the “sum of intensities over a window of specified mass and time range” using the freely available XCMS software

(http://masspec.scripps.edu/xcms/xcms.php) as described previously (Dunn 2008). Data were exported from XCMS as a .csv file for further data analysis. Metabolite annotation was performed applying the PUTMEDID_LCMS workflow (www.mcisb.org/resources/putmedid.html) as previously described (Brown 2011). All metabolite annotations are reported at level 2 (putatively annotated compounds) according to Metabolomics Standards Initiative (MSI) reporting standards (Sumner 2007). In cases where a single metabolite is detected as multiple metabolite features (i.e. where multiple types of ions are detected for the same metabolite thus overestimating the number of metabolites detected (Brown 2009)), only a single feature is reported that was chosen as having a p-value nearest to 0.05).

5.3.8 Statistical analysis

As no existing studies have explored metabolomics and TTTS, untargeted metabolomics was performed, for which a sample of convenience is considered acceptable, so no power calculation was performed (Nyamundanda 2013). Medians and IQRs were described. Intergroup comparisons for continuous variables with a non-parametric distribution were made using the Mann-Whitney U test to determine significant differences between the data sets. Categorical data were analysed using Fisher's exact test and OR and 95%CI were reported. Metabolomics processed data were analysed in ‘R’ (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria) applying the unsupervised multivariate principal components analysis (PCA), supervised multivariate Partial Least Squares-Discriminant Analysis (PLS-DA), univariate non-parametric Wilcoxon Signed Rank test and Spearman rank correlation analysis. The fold change (median

peak area pre-FLA/median peak area post-FLA) was calculated including 95%CI. Metabolites were manually clustered into classes defining similar chemical structure or metabolic pathway to identify biologically relevant and robust metabolic changes. Results were considered significant if $p<0.05$.

5.3.9 Ethical approval

Ethical approval was granted by Birmingham Black Country Local Research Ethics Committee (No: 06/Q2702/71 accepted in 2006) with written consent obtained from all participants.

5.4 Results

5.4.1 Participant characteristics

Amniotic fluid samples from 19 women with MCDA twins complicated by TTTS undergoing FLA were collected and analysed. The characteristics of the women at TTTS diagnosis are reported in Table 5.1. The Quintero stage at FLA was: stage I 1/19 (5.2%), stage II 3/19 (15.8%), and stage III 15/19 (79%).

Table 5.1 Demographic and clinical data for all participants

(n=19 TTTS pregnancies; n=38 fetuses) BMI: body mass index, EFW: estimated fetal weight, IUFD: intra-uterine fetal death. *Perinatal survival defined as total number of survivors (all fetuses) who survived until at least 28 days of age

Patient characteristics	**Median (IQR)**
Maternal age (years)	29.0 (25.0, 31.5)
Maternal BMI	24.4 (21.5, 26.65)
Gestational age at FLA (weeks)	20+2 (19+4, 21+3)
Inter-twin difference in EFW at FLA (%)	25.2 (19.2, 30.5)
Fetoscopic laser ablation (FLA) variables	**Median (IQR)**
Duration of FLA (minutes)	19 (13, 25)
Number of arteriovenous anastomoses ablated	8 (7.5 – 8.5)
Amniodrainage post-FLA (ml)	2600 (2250, 3100)
Pregnancy outcome	
Gestational age at delivery (weeks, days) Median (IQR)	32+3 (29+6, 34+0)
At least one survivor *n* (%)	18/19 (94.7)
Two survivors *n* (%)	12/19 (63.2)
Single IUFD *n* (%)	6/19 (31.5)
Double IUFD *n* (%)	1/19 (5.3)
Perinatal survivor at 28 days *n (%)* (total fetuses)	30/38 (78.9)

5.4.2 Recipient twin cardiac function

The cardiac function measurements of the recipient twin are reported in Table 5.2.

Table 5.2 Recipient twin cardiac function

DV: ductus venosus, E/A: early passive/atrial contraction, EFW: estimated fetal weight, IUFD: intra-uterine fetal death, FLA: fetoscopic laser ablation, LV: left ventricle, MPI: myocardial performance index, RV: right ventricle. *p<0.05 pre-FLA vs post-FLA

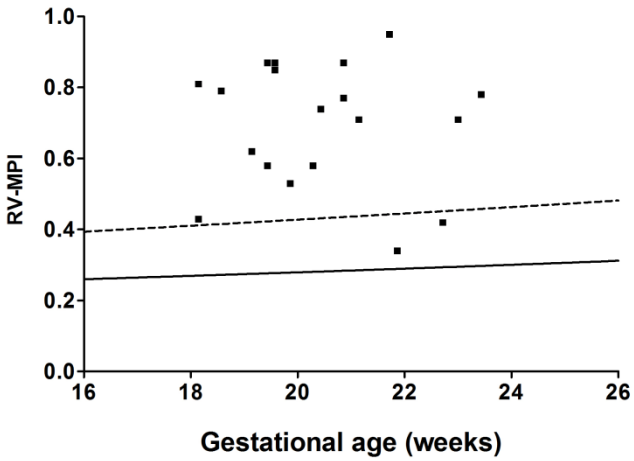
	Pre-FLA	**Post-FLA**
RV MPI (z-score) Median (IQR)	4.96 (3.36, 6.31)	2.90 (1.71, 4.50)*
LV MPI (z-score) Median (IQR)	2.74 (1.78, 3.73)	2.07 (0.56, 3.22)*
Absent/reversed "A-wave" in DV *n* (%)	7/19 (36.8)	2/19 (10.5)
Tricuspid regurgitation *n* (%)	15/19 (78.9)	12/19 (63.2)
RV E/A ratio >95%CI for gestation *n* (%)	11/19 (57.9)	6/19 (31.2)

Pre-FLA the recipient twin right ventricle (RV) and left ventricle (LV) MPI was elevated (>95%CI for gestation) in 89.4% (17/19) and 73.7% (14/19) respectively (Figure 5.1(a) and Figure 5.1(b). Of the two recipient twins with a RV MPI within 95%CI for gestation, one was Quintero stage I and one stage III. In the 5 recipient twins with a LV MPI within 95%CI for gestation, all had stage III.

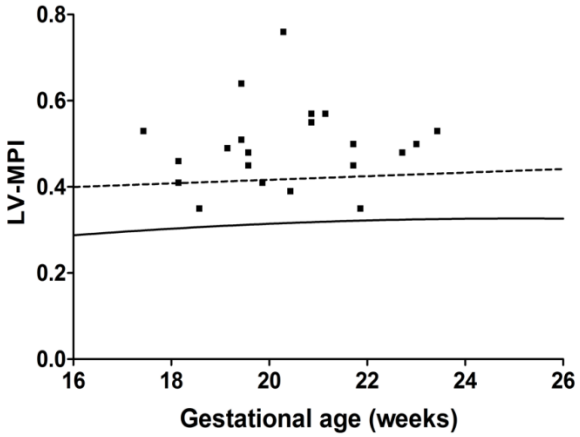
Figure 5.1(a) Right ventricular myocardial performance index (RV-MPI) (b) Left ventricular myocardial performance index (LV-MPI) in recipient fetuses at diagnosis of twin-twin transfusion syndrome

The graph demonstrates individual fetal values against gestational age. The solid black line depicts the median MPI for uncomplicated monochorionic twins, and the dashed line depicts the upper 95% confidence interval (Van Mieghem 2009c).

(a)



(b)

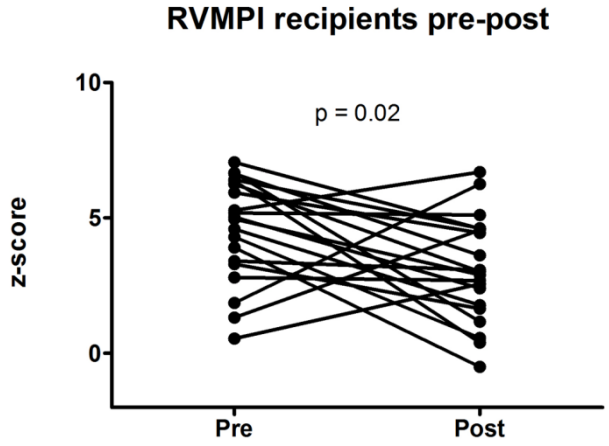


The recipient twins' RV MPI (p=0.02) and LV MPI (p=0.03) decreased significantly after FLA Figure 5.2(a) and Figure 5.2(b).

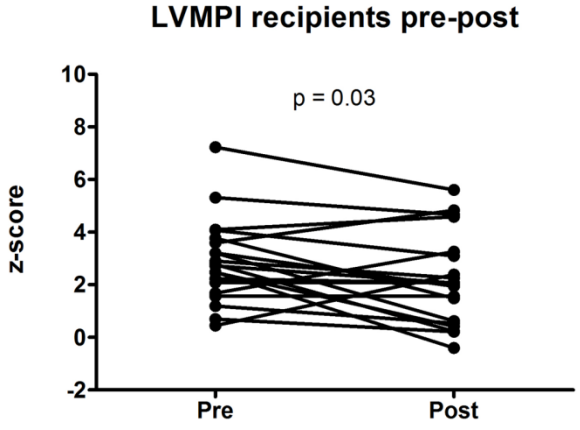
Figure 5.2 Changes in recipient twin (a) Right Ventricular Myocardial Performance Index (RVMPI) and (b) Left Ventricular Myocardial Performance Index (LVMPI) pre- and post-fetoscopic laser ablation

RV and LV MPI z-scores before and 6 hours post- fetoscopic laser ablation (individual matched data shown).

(a)



(b)



5.4.3 Metabolome of recipient twin amniotic fluid

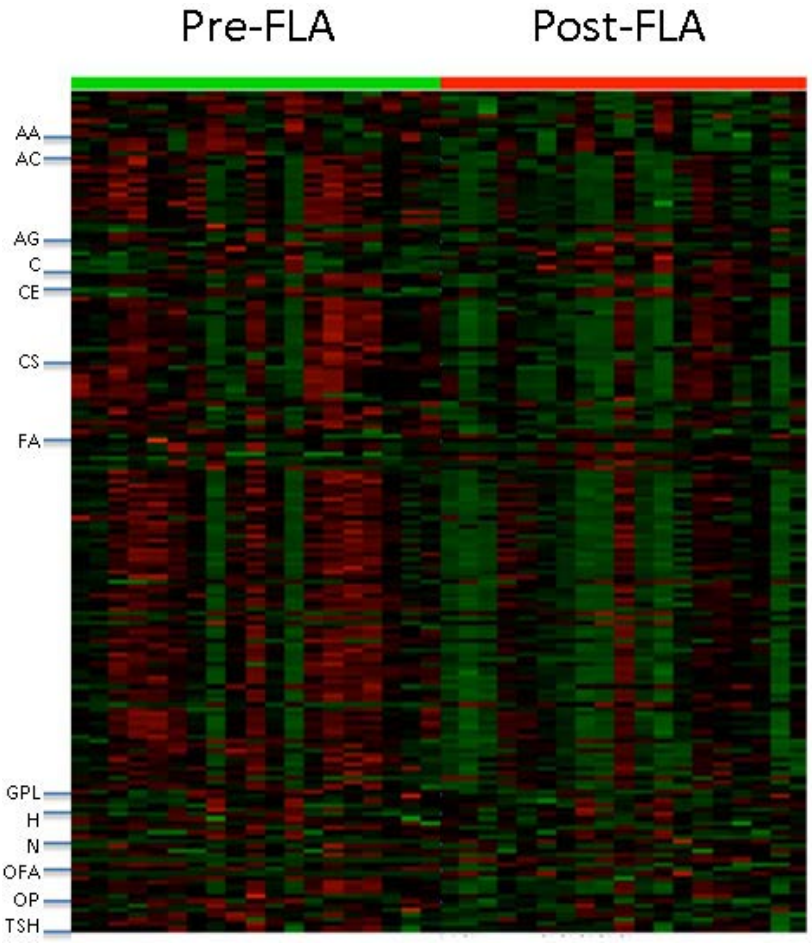
38 paired amniotic fluid samples were collected pre- and post-FLA. Following quality assurance of the data, 2694 and 1510 metabolite features remained in positive and negative ion modes respectively.

5.4.4 Metabolomic changes pre- and post-FLA

There was a significant difference in the relative concentration of 200 metabolites when the pre-FLA amniotic fluid samples were compared to the matched post-FLA samples ($p<0.005$). There was no difference if the Solomon FLA technique was used (data not shown). Figure 5.3 visually displays these data as a “heat map” of relative concentration changes pre-FLA and post-FLA for all metabolite classes containing three or more metabolites.

Figure 5.3 Heat map showing the distribution of concentrations for individual metabolites (rows) for samples collected pre- and post-fetoscopic laser ablation (FLA) (columns)

Green represents a low concentration whereas red represents a high concentration in the range of concentrations for each metabolite. Abbreviations are: amino acid metabolism (AA), acyl carnitines (AC), acyl glycerides (AG), carbohydrates (C), cholesterol esters (CE), ceramides and sphingolipids (CS), fatty acids (FA), glycerophospholipids (GPL), haem metabolism (H), nucleotides (N), oxidised fatty acids (OFA), oxidative phosphorylation (OP) and thyroid/steroid hormones (TSH)



There were 13 metabolite “classes” consisting of ≥3 metabolites which demonstrated significant fold changes: acyl carnitines, acyl glycerides, amino acid metabolism, carbohydrate metabolism, cholesterol esters, ceramides and sphingolipids, fatty acid

metabolism, glycerophospholipids, haem metabolism, nucleosides, oxidised fatty acids, oxidative phosphorylation (electron transport chain) and thyroid/steroid hormone metabolism. Particularly interesting findings include higher levels of carbohydrates pre-FLA compared to post-FLA, and higher levels of fatty acids (shown by higher levels of acyl carnitines, acyl glycerides, fatty acids, oxidised fatty acids and TCA/oxidative phosphorylation metabolites) post-FLA compared to pre-FLA (Appendix10.5).

5.4.5 Association between recipient twin pre-FLA metabolites and cardiac function

There were 102 pre-FLA amniotic fluid metabolites significantly correlated with the recipient twin RV MPI (Appendix 10.6), and 118 metabolites significantly correlated with the LV MPI (Appendix 10.7). A summary of the findings grouped according to metabolite class are displayed in Table 5.3.

When the individual metabolites were grouped into metabolite classes, the overall correlation of each class to the RV or LV MPI was calculated. The acyl carnitines, acyl glycerides, ceramides, sphingolipids, glycerophospholipids, fatty acids and oxidised fatty acids classes were negatively correlated with the RV and LV MPI, meaning that an increased concentration in these classes was associated with better cardiac function. Carbohydrates were positively correlated with the RV and LV MPI cardiac function meaning that an increased concentration in these classes was associated with worse cardiac function. Two oxidative phosphorylation metabolites were negatively correlated with LV MPI alone and not RV MPI; no metabolites were correlated just with the RV MPI and not LV MPI.

Table 5.3 Correlation of recipient twin amniotic fluid metabolic profiles and cardiac function according to metabolite class

LV: left ventricle, MPI: myocardial performance index, RV: right ventricle.
The metabolites identified as having a significant association with RV MPI or LV MPI (denoted as '+' correlation coefficient shows that as MPI increases so does the metabolite concentration) or a negative correlation (denoted as '-' shows that as MPI increases the metabolite concentration decreases). *The range reported is for all individual metabolites grouped in the class.

Metabolite Class	RV MPI		LV MPI	
	Number of Metabolites	Correlation coefficient range*	Number of Metabolites	Correlation coefficient range*
Acyl amino acids	2	+0.30 to +0.37	2	+0.34 to +0.39
Acyl carnitine	6	-0.43 to -0.31	5	-0.46 to +0.41
Acyl glycerides	9	-0.49 to +0.60	12	-0.52 to +0.54
Bile acid metabolism	3	+0.31 to +0.36	3	-0.38 to +0.44
Carbohydrates	4	-0.33 to +0.45	3	-0.57 to +0.65
Ceramides & sphingolipids	9	-0.49 to +0.42	8	-0.43 to -0.30
CoA metabolism	2	-0.40 to -0.30	2	-0.34 to +0.36
Fatty acid metabolism	13	-0.52 to +0.46	10	-0.67 to +0.54
Glycerophospholipids	28	-0.64 to +0.51	41	-0.53 to +0.50
Nucleoside	4	-0.36 to +0.54	3	-0.48 to +0.34
Oxidised fatty acids	7	-0.48 to +0.57	4	-0.31 to -0.30

5.5 Discussion

5.5.1 Metabolome of recipient twin amniotic fluid

This work has demonstrated that it is possible to perform metabolomics on amniotic fluid taken during FLA from the recipient twin amniotic sac. There are no other studies on the amniotic fluid metabolome in twin pregnancies to compare the findings, and for ethical and practical reasons outlined below it was not possible to recruit a MC twin control group.

5.5.2 Metabolomic changes pre- and post-FLA

Following FLA, a variety of different metabolite classes were different in the recipient twin amniotic fluid. Post-FLA, higher levels of fatty acids and lower levels of carbohydrates were seen compared to pre-FLA suggesting that there is a switch from fetal or placental use of fatty acids as precursors for energy metabolism to carbohydrates post-FLA. Of five acyl carnitines, it is the medium chain derivatives (hexanoyl, octanoyl and decanoyl) that dominated the increase seen in acyl carnitines post-FLA, suggesting a specific perturbation in medium chain fatty acid oxidation. Also changes related to oxidative phosphorylation were noted, again supporting that a switch in energy production. Another change seen post-FLA was a decrease in the concentrations of thyroid and steroid hormones, potentially related to an attenuated “stress” response post- FLA as it is known that FLA may temporarily worsen the cardiac function for example. A final noteworthy finding was that pseudouridine was 20% higher pre-FLA compared to post-FLA. This metabolite is increased in heart failure in adults and is considered as good a marker as BNP for cardiac failure (Dunn 2007). Interestingly pseudouridine is also raised in renal failure (Dzúrik 1992) and it may be that the level in the recipient twin decreases post-FLA as the high pseudouridine levels in the donor twin can no longer be transferred to the recipient twin through the ablated anastomoses.

As alluded to, the metabolite changes seen pre- and post-FLA may be related to changes in cardiac function as a significant decrease in the RV and LV MPI was seen post-FLA compared to pre-FLA, and correlations were found between metabolites and MPI.

5.5.3 Association between recipient twin pre-FLA metabolites and cardiac function

The shift from fatty acids to carbohydrates pre- to post-FLA may be related to recipient twin cardiac function (as measured by LV and RV MPI) as fatty acids are negatively correlated and carbohydrates are positively correlated with increasing MPI score. Put another way, this means that pre-FLA, when the MPI is higher and cardiac function is worse, more fatty acids are used for energy metabolism, so the fatty acids concentrations are low and carbohydrates concentrations are high because the carbohydrates are not being metabolised. Post-FLA, when the MPI decreases and cardiac function improves, metabolism switches so carbohydrates are low and fatty acids are high. These results do not confirm causality, but a link is apparent. Interestingly, metabolic modulators prescribed in adult cardiac disease, including hypertrophic cardiomyopathy which is also observed in recipient twins, aim to shift the use of fatty acids as substrates for metabolism to glucose (Drury 2015) as it is a more efficient way to produce energy, and produce symptomatic improvement (Abozguia 2010).Ceramides, sphingolipids and glycerophospholipids were also negatively correlated with MPI score and warrant further investigation due to their known involvement in adult cardiac failure (Park 2012). Changes in N,N-dimethylarginine and the structurally similar N,N-diacetylspermine are also worthy of further investigation as N,N-dimethylarginine is a known inhibitor of nitrous oxide synthesis from arginine and therefore reduces vasodilation which is believed to play a role in the pathogenesis of TTTS. Symmetrical and asymmetrical N,N-dimethylarginine are implicated in cardiac function and cardiovascular health (Gorenflo 2001, Gore 2013).

5.5.4 Strengths and limitations

In this relatively small cohort study a substantial proportion of the recipient twins had evidence of cardiac dysfunction with an elevated LV and RV MPI. Such data are consistent with those previously described in the literature (Van Mieghem 2010) thus the cohort represents a general TTTS population. The rapid improvement in the recipient RV and LV MPI post-FLA has also been previously reported in the literature (Papanna 2011) suggesting that FLA was effective, as is also demonstrated by the high survival rate. Another strength is that the cardiac function assessments were only performed by a single observer, thus avoiding inter-observer variability. Significant differences have been found, however it is difficult to delineate whether the source of the metabolites is fetal or placental as at that gestation amniotic fluid is derived from fetal and placental metabolite secretion. As amniotic fluid sampling was repeated after a median time of 19 minutes, it is possible that the changes seen when the pre-FLA samples were compared to the post-FLA samples are secondary to a combination of trophoblast/vessel destruction from the ablation and recipient twin cardiovascular change, but there is some biological plausibility in the findings. It is important to consider that maternal metabolites may be present in amniotic fluid. Although the mechanism is not known, ‘maternal mirror syndrome’, maternal oedema in the presence of fetal oedema, can occur in TTTS Quintero Stage IV or V and thus it may be that maternal metabolites are present amniotic fluid, however there were no pregnancies with TTTS Stage IV or V at FLA included in the cohort thus it is unlikely to have affected the results. Due to the sample size, it was not possible to adjust for the Quintero Stage of TTTS, as Stages I-III may have different effects on the metabolome. There is also the possibility multiple testing has affected the findings,

which is an issue with all –omics technologies due to the large amount of data produced (Broadhurst 2006). As this was untargeted mechanistic discovery work, no statistical correction for a false discovery rate was applied as this can increase the number of false negatives, restricting biological interpretation and mean that potentially important metabolites are missed and not investigated in future work (McDonald 2014). Instead, individual metabolites were grouped together according to metabolic function, pathway or structure; thus if multiple individual metabolites are significant in the same group, the pathway can be considered mechanistically significant, as opposed to a random finding due to statistical chance.

When designing a metabolomics study, one important consideration is the choice of biofluid and timing of sample collection. As the focus was to explore the pathophysiology of cardiac dysfunction in TTTS prior to FLA treatment which can affect cardiac function, amniotic fluid was chosen because it is the most ethically-acceptable fetal biofluid able to be collected at the time of TTTS diagnosis. In-utero fetal blood sampling has serious associated risks. Amniotic fluid comprises mainly of fetal urine at this gestation and thus is reflective of fetal metabolism (Palmas 2016). Ideally a gestationally-matched MC twin control group would be recruited, however it is rare to perform an invasive procedure in normal MC twins at this gestation thus it was not possible. It was also not possible to collect donor twin amniotic fluid as it would be technically too difficult and dangerous and often the recipient twin has worse cardiac function than the donor twin. As this is the first work to be conducted in this area, samples were collected at diagnosis of TTTS in the second trimester. To collect first trimester samples would have meant the recruitment and collection of

many MC twin samples because the condition is relatively rare. Although metabolomics could have been performed on first trimester or second trimester maternal serum samples, as it is the first time the technology has been used in TTTS and there is little known about metabolomics in twin pregnancies, amniotic fluid was chosen to decrease the risk of maternal metabolites affecting the results.

5.5.5 Clinical implications and future research

These findings represent preliminary mechanistic work but cannot be used clinically at present. The results need to be validated, ideally by another metabolomics centre, with amniotic fluid samples from different pregnancies. It would be interesting to collect amniotic fluid samples and placental samples at delivery and to correlate these samples with fetal cardiac function prior to delivery. However this would be difficult as the Fetal Medicine Centre treats patients from a wide geographical area, and the majority of patients will deliver at their local centre. Additionally many pregnancies treated by FLA will have a spontaneous PTB and caesarean section is the best way to collect “clean” amniotic fluid at delivery. The study would also require a large sample number as cardiac function often normalises after FLA (Van Mieghem 2009c).

In adults with cardiac failure, prediction of survival is improved when cardiac ultrasound measurements are combined with serum-derived biomarkers (Latini 2007). In recent years there has been a move to a metabolomic approach for profiling functional and metabolic changes in adults with heart failure (Deidda 2015). This could be applicable in fetal cardiac disease in TTTS, with ultrasound measurements being combined with other biomarkers to improve prognostic ability (Van Mieghem

2010). The work presented here suggests that metabolomics may identify candidate biomarkers worthy of future prognosis research. Future work should include targeted metabolomics to evaluate specific individual metabolites and assess their prognostic ability.

5.6 Conclusion

This study has demonstrated a difference in the recipient twin's amniotic fluid metabolome pre- and post-FLA, suggesting that it is affected by FLA. A relationship between recipient twin cardiac function at diagnosis of TTTS (pre-FLA) and metabolomic profile has been reported, and hypotheses have been generated. The correlation of the balance between fatty acid and carbohydrate use in energy metabolism and measures of recipient twin cardiac dysfunction warrant further investigation. The limitations in assessing in-utero fetal metabolism have been discussed. Further targeted metabolomics studies in different biofluids and tissues are now required to improve mechanistic knowledge, and identify potential prognostic 'biomarkers'.

CHAPTER 6 MICRORNA CHANGES IN MATERNAL SERUM FROM PREGNANCIES COMPLICATED BY TWIN-TWIN TRANSFUSION SYNDROME: A DISCOVERY STUDY

- These findings were presented as a poster presentation at the Royal College of Obstetricians and Gynaecologists Annual Academic Meeting, February 2018, London, by F Mackie.

6.1 Overview

In addition to the prognostic factors examined in CHAPTER 3, new potential prognostic factors were sought by attempting to learn more about the pathophysiology of TTTS. This chapter explores the possibility of using miRNA in the context of TTTS. The miRNA profiles of maternal serum samples taken at diagnosis of TTTS are compared to matched maternal serum samples taken from women with uncomplicated MC twin pregnancies. The reasons why maternal serum samples were used, and why the samples were taken at diagnosis of TTTS, are outlined. As this is the first time that miRNAs have been explored in TTTS, an array was performed, and the results were validated in a different cohort.

6.2 microRNA

As described in section 1.10.2 miRNA are a sub-group of transcriptomics as they are involved in the translation of genes for protein synthesis. miRNAs are highly

ubiquitous noncoding RNAs, consisting of a single strand of around 21–25 nucleotides that are conserved across many species (Ke 2003). They are involved in post-transcriptional regulation of gene expression and function through base-pairing with the 3' untranslated region (UTR) of the complementary mRNA molecules, which then silences the mRNA by degradation of the target mRNA, or repression of translation (Lai 2002, Bartel 2004). These endogenous regulatory miRNAs are present in many human bodily fluids and tissues, including human placenta (Maccani 2011, Flor 2012, Morales-Prieto 2012, Wang 2012b, Chen 2013b, Higashijima 2013, Wen 2017). Their potential role as biomarkers in detecting and defining prognosis in heart disease and cancer, among many other diseases, has been reported (Wang 2008). There is less miRNA research in pregnancy, particularly twin pregnancy, however changes have been demonstrated.

In pregnancy, changes in miRNAs may be in placenta-specific miRNAs i.e. those expressed only in the placenta, or non-placental-specific miRNAs. There is a group of miRNAs, known collectively as the chromosome 19 miRNA cluster (C19MC), that are expressed almost exclusively in the placenta (Noguer-Dance 2010). These miRNAs are some of the most abundant miRNAs in maternal serum and human placental tissue (Mouillet 2010, Donker 2012). Placental miRNA are present, and stable, in maternal blood (Chim 2008), and are believed to be released from the syncytiotrophoblast and carried in plasma in at least two forms: protein-bound miRNA and vesicular miRNA (Mouillet 2015). They are cleared from the maternal circulation soon after delivery (Morisaki 2015) and consequently may act as biomarkers for diseases related to pregnancy. miRNA changes in pregnancy conditions are not only

seen in placenta-specific miRNA, but also in non-placental specific miRNA. These changes will be outlined below.

A variety of biofluids and tissues are used to investigate changes in miRNA in pregnancy-related conditions, including placental tissue, maternal plasma, maternal serum, chorioamniotic membrane, cervical cells, and in-vitro cell lines including HUVEC and BeWo cells. miRNA changes within the same woman may be different depending on the tissue/biofluid sampled (Timofeeva 2018), thus when interpreting miRNA studies, it is imperative that findings are compared with those in the same tissue type, and also the same animal species (Barchitta 2017). Logically, some pregnancy miRNA investigative work is performed on placental tissue and cell lines in order to better understand pathophysiological mechanisms. However, changes seen in placental tissue do not necessarily translate to changes in maternal blood (Timofeeva 2018), thus it is vital to consider the aim of the miRNA work and acknowledge that differences seen in placental tissue have limited clinical utility as a prognostic factor due to the risk of obtaining a placental sample antenatally. As the aim of this work is to explore future potential prognostic factors, maternal serum was chosen, and the background information in this section will focus on miRNA changes in human maternal serum samples where possible. This is different to the metabolomics work in CHAPTER 5 as placenta-specific miRNA are identifiable in maternal serum, however metabolites in maternal serum are not placenta-specific as there is a combination of maternal and placental metabolites present in maternal serum. Additionally it was not possible to compare amniotic fluid samples from pregnancies with TTTS to those without TTTS for ethical reasons, however by using maternal serum it is possible to compare those with TTTS and those without TTTS

and thus the presence of maternal miRNAs in maternal serum can be compared between the two groups.

The majority of miRNA work in pregnancy has been performed in singleton pregnancies, with a particular focus on pre-eclampsia, but miscarriage, PTB, LBW, and macrosomia have also been explored as reviewed by Barchitta et al (Barchitta 2017). A cornucopia of studies has demonstrated miRNA changes in maternal serum and plasma taken at various time points in pre-eclampsia (Gunel 2011, Yang 2011, Agarwal 2012, Hromadnikova 2012, Wu 2012, Zhang 2012, Li 2013, Ura 2014, Akehurst 2015, Munaut 2016, Sandrim 2016b, Gunel 2018), including in miRNAs related to angiogenesis. Certain miRNAs, including plasma hsa-miR-195-5p, may be linked to the increase in sFLT-1 seen in pre-eclamptic pregnancies (Sandrim 2016a). Thus miRNAs may act as 'biomarkers' of placental and vascular function in pregnancy. A recent proof of concept study has shown that miRNA changes in the buffy coat removed from first trimester maternal plasma samples may be able to predict pre-eclampsia in singleton pregnancies (Winger 2018).

Studies have also demonstrated miRNA changes in various tissues including placental tissue in pre-eclampsia (Zhang 2010, Choi 2013, Lykoudi 2018), miscarriage (Wang 2012c, Dong 2014, Li 2016) and LBW (Song 2013, Wang 2014); the chorioamniotic membrane (Montenegro 2009, Enquobahrie 2016), and cervical cells (Elovitz 2014, Sanders 2015) in PTB, but as mentioned previously the focus of this work is on maternal serum samples.

Care should be taken when translating findings in singleton pregnancies to twin pregnancies. There are significant differences in types and quantities of third trimester maternal plasma miRNA in uncomplicated twin pregnancies, compared to uncomplicated singleton pregnancies (Ge 2011). Differences have also been demonstrated in spent blastocyst medium from ART procedures between twin and singleton pregnancies (Noli 2016), and in singleton and twin pregnancy sheep undernutrition models (Lie 2016). However there is a dearth of research looking at miRNA differences in complications in twin pregnancies. Wen et al. examined placental tissue from 14 MC twin pregnancies with sIUGR and found a difference in 14 miRNAs when the miRNA profile of the larger twin was compared to the smaller twin, thus miRNA may play a role in the pathology of MC twin pregnancy complications, however the authors did not look at MC twin pregnancies without sIUGR or maternal serum samples (Wen 2017). No other studies were found looking at growth restriction in twin pregnancy, and there have been no reports of miRNAs in TTTS pregnancies, in any bodily fluid. As changes in miRNAs associated with trophoblast proliferation, angiogenesis and fetal growth have been demonstrated (Fu 2013, Zhao 2013b) this suggests that miRNAs are important in human placentation, and related conditions, and thus warrant investigation in TTTS.

6.2.1 Aims

The aim of this study is to investigate if there is a difference in the miRNA profile of maternal serum samples taken at the time of diagnosis of TTTS compared to matched maternal serum samples from women with uncomplicated MCDA twin pregnancies.

Hypothesis

There is a difference in human maternal serum miRNAs in pregnancies complicated with TTTS compared to uncomplicated matched MCDA twin pregnancies.

6.3 Methods

6.3.1 Participants

No woman was included in both the investigation and validation cohorts.

Investigation cohort

Patients were recruited prospectively as part of the OMMIT study (Mackie 2017). Maternal blood samples were obtained the day prior to FLA from patients attending the West Midlands Fetal Medicine Centre for FLA for the treatment of TTTS from August 2015 to August 2017. TTTS was defined as a MPD >8cm in the recipient twin at <20 weeks gestation, or MPD >10cm at >20 weeks gestation, in combination with a MPD <2cm in the donor twin. Patients were prospectively staged using the Quintero classification (Quintero 1999). A control group of pregnant women with uncomplicated MCDA twin pregnancies who booked at BWH were recruited over the same time period. The control group underwent serial maternal blood sampling at 12, 16 and 20 weeks gestation to coincide with their antenatal clinic appointments (see section 4.3.1 for more details of the participants). Chorionicity was confirmed by the presence of the ‘T’ sign on first trimester ultrasound (Sepulveda 1996). Women whose pregnancies were affected by chromosomal/structural anomalies were not eligible for inclusion. Participants in the control group had no serious adverse

maternal or fetal outcome and delivered two healthy babies which did not require NNU admission. Patients were matched based on: maternal age, ethnicity, parity, BMI, gestation at blood sampling, and fetal sex.

Validation cohort

The validation cohort was recruited as part of the same OMMIT study within the same time period. Women whose pregnancies were complicated by TTTS were matched to women with uncomplicated MC twin pregnancies, based as on the same factors as in the investigation cohort. No woman was included twice.

6.3.2 Sample collection and storage

Venous blood samples were collected in 7.5ml serum gel tubes (Sarstedt, Nümbrecht, Germany) from the antecubital fossa, allowed to clot for 1 hour at room temperature, centrifuged at 3000*g* for 10 minutes at room temperature, and sediment-free serum aliquots were stored immediately at -80°C prior to analysis and during transit. Serum sample aliquots were stored for a maximum of 13 months prior to analysis in the investigation cohort, and a maximum of 31 months for the validation cohort, which is considered acceptable practice in a recent best practice paper (Khan 2017). Serum was chosen as it has higher miRNA concentrations than plasma (Wang 2012a).

6.3.3 RNA extraction for profiling array

Total RNA was extracted from serum using the miRCURY™ RNA Isolation Kit – Biofluids (Exiqon, Vedbaek, Denmark). Serum was thawed on ice to room temperature and centrifuged at 3000*g* for 5 minutes. 200µL of supernatant from each sample was transferred to new tubes, and 62.5µL of a well-mixed master mix of Lysis buffer (60µL), RNA Spike-in template mixture (1µL) (miRCURY LNA™ Universal RT microRNA PCR, RNA Spike-in kit [Exiqon]) and carrier-RNA (1.5µL) (MS2 RNA, [Roche, West Sussex, UK]) was added. The samples were vortexed and incubated for 3 minutes at room temperature. The Protein Precipitation Solution BF (20µL) was added to each sample, the samples were vortexed and incubated for 1 minute at room temperature, then centrifuged at 11,000*g* for 3 minutes. The clear supernatant was then transferred to a new tube and isopropanol (270µL) was added, and the sample vortexed to adjust the binding conditions. A miRNA Mini Spin Column BF was placed in a collection tube and the sample loaded onto the column, incubated for 2 minutes at room temperature and centrifuged at 11,000*g* for 30 seconds. The flow-through was discarded and the column placed back in the collection tube. To remove residual DNA which may affect downstream applications, Wash Solution 2 BF (700µL) was added to the spin column BF and centrifuged at 11,000*g* for 30 seconds. The flow-through was discarded and the column returned to the collection tube. This was repeated with 250µL Wash Solution 2 and was centrifuged at 11,000*g* for 2 minutes. rDNase (50µL) was added to the membrane of the spin column and incubated for 15 minutes at room temperature to aid digestion of the DNA. The columns were washed with Wash Solution 1 BF (100µL), then Wash Solution 2 BF (700µL), then Wash Solution 2 BF (250µL). Following each wash the samples were

centrifuged at 11,000*g* for 30 seconds, apart from the final wash following which the samples were centrifuged for 2 minutes, to ensure complete drying of the membrane. The spin column was placed in a new collection tube and RNase free H_2O (50µl) was added directly onto the membrane, incubated for 1 minute at room temperature, and centrifuged at 11,000*g* for 1 minute to elute the samples. The extracted RNA was stored at -80°C prior to transfer to Exiqon, Denmark, 2 months later, on dry ice.

6.3.4 Quality control of profiling array samples

Haemolysis was assessed by 2 miRNAs known to correlate with the degree of haemolysis: hsa-miRNA-451 which is expressed on red blood cells, and hsa-miRNA-23a which is not affected by haemolysis. A ratio >7.0 of those 2 miRNAs indicates that the sample may be affected by haemolysis. Spike-ins were added to each sample as technical controls: UniSp2 and UniSp4 are RNA isolation controls and assess RNA extraction; and UniSp6 is a complementary DNA (cDNA) synthesis control to assess reverse transcription and qPCR. A negative control, “blank” sample, was included in the reverse transcription step to detect RNA contamination.

6.3.5 Quantitative RT-PCR miRNA profiling array

miRNA profiling was performed by Exiqon using the miRCURY LNA™ Universal RT microRNA PCR Human panel I+II. These panels contain primer sets for the 752 human miRNA most commonly differentially expressed in disease and/or cited in the scientific literature, including miRNAs associated with cardiovascular disease, renal disease, angiogenesis, and placenta-specific miRNA, in-keeping with the

pathophysiological mechanisms of TTTS (see Appendix 10.8 for full list of miRNAs tested). All the miRNAs were polyadenylated and reverse transcribed into cDNA. The cDNA and ExiLENT SYBR® Green master mix were transferred to the qPCR panels with 384 well plates pre-loaded with the specified primers, using a pipetting robot. The amplification was performed in a Lightcycler® 480 Instrument (Roche).

6.3.6 Identification of candidate miRNAs

The candidate miRNAs were decided upon based on:

- Being significantly different in the TTTS compared to control samples, prior to Benjamini-Hochberg correction for multiple testing.
- Being present in all investigation cohort samples, irrespective of whether they were TTTS or control samples.
- Having validated targeted functional genes with strong evidence of microRNA-Target Interaction (MTI) reported on MiRTarBase (version 7.0) (Chou 2018).
- Biological plausibility for association with TTTS based on a literature search using keywords: placenta, pregnancy, twin, maternal, gestation, cardio-$, renal-$, vascul-$, angio-$, trophoblast, fetal.
- Including a mixture of upregulated and downregulated miRNAs.

6.3.7 Validation with RT-PCR of candidate miRNAs

The validation of the candidate miRNAs was performed by Dr Andrew Beggs and Miss Agata Stodolna at the University of Birmingham. miRNA was extracted from serum with miRNeasy Serum/Plasma Kit (Qiagen, Manchester, UK) according to

manufacturer's instructions (see Appendix 10.9). The quality and the concentration of the samples was investigated with High Sensitivity RNA TapeStation (Agilent, Stockport, UK). Two endogenous controls were used: miR-361-5p and miR-451a. Samples were converted to cDNA, and miRNA was amplified using TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher Scientific, Warrington UK) according to the manufacturer's instructions. RT-PCR was performed with TaqMan Advanced miRNA Assays (Applied Biosystems) according to the manufacturer's instructions. 5µl of 1:10 dilution of cDNA template was used for the RT-PCR reaction with 10µl of 2X TaqMan Fast Advanced Master Mix (Applied Biosystems), 1µl of 20X TaqMan Advanced miRNA Assay and 4µl of RNase-free water. The reaction was run on the QuantStudio 5 instrument (Applied Biosystems) under conditions: 1 cycle for 20 seconds at 95ºC, then 40 cycles of 1 second at 95ºC and 20 seconds at 60ºC.

6.3.8 Statistical analysis

Normalisation was performed based on the average of the assays detected in all 10 samples. A Heatmap with red denoting miRNA expression above the mean and green denoting miRNA expression below the mean, and a PCA plot were drawn to assess the miRNA expression profiles of the samples by depicting the normalised dCq values for the top 50 miRNAs with the widest variation in expression in the samples. If any samples appeared to be outliers, this was further investigated as to whether the cause was due to pathology, sample quality, an error in processing, or natural miRNA variation. To compare the miRNAs in different groups, a volcano plot was drawn to allow easy identification of miRNAs upregulated and downregulated in

each group. To quantitatively compare the groups, normality of the data was assessed using the Shapiro-Wilk test. The t-test was used when data were parametric, and the Wilcoxon test or Mann-Whitney U test was used when data were non-parametric to compare matched samples, or groups accordingly. Benjamini-Hochberg correction was applied to control for the false discovery rate in the context of multiple testing. $p<0.05$ was considered significant. In order to further investigate the differential expression differences and correct for population substructure, raw dCt data on a probe wise level was imported into R 3.3.1 (R Core Team (2013). R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria) and multivariate logistic regression analysis was performed with case control as the outcome and age, ethnicity, gestation, parity and maternal BMI as independent variables along with dCt using *limma*. Moderated t-statistics were then calculated using Empirical Bayesian shrinkage (*eBayes)* and the *topTable* command used to rank significant probes by descending Bayes Factor (BF). To assess the validation cohort, individual data points were plotted using the *dotplot* command on Stata (StataCorp. 2013b). The ΔΔCts of the control group were compared to the matched ΔΔCts of the TTTS group by the Wilcoxon Signed Rank test using the *signrank* command on Stata (StataCorp. 2013h). Findings with $p<0.05$ were considered significant.

For the initial profiling array no power calculation was performed as it was discovery work, thus a sample size of 10 is considered adequate, based on existing literature. The results of the investigation cohort were used to perform a power calculation based on the observed dCt values, standard deviations and correcting for multiple

testing (using Stata 12.1) and revealed that 16 samples (n=8 TTTS, n=8 control) are needed in the validation cohort to demonstrate a statistically significant difference between the TTTS and uncomplicated MC twin pregnancy samples, with close matching of demographic variables in subjects, as in the investigation cohort.

6.3.9 Ethical approval

This study received ethical approval from East Midlands Research Ethics Committee (15/EM/0244) in July 2015 and all patients provided written informed consent (see Appendix 10.12 and 10.13 for patient information sheet and consent form).

6.4 Results

6.4.1 Participant characteristics

Investigation cohort

Five patients were included in the TTTS group, and were matched to five from the control group. 2001 was paired with 2047, 2002 with 2020, 2018 with 2027, 2022 with 2039, 2043 and 2023 (Table 6.1). TTTS patients were selected pragmatically and matched as closely with the control group as possible. The median gestational age at blood sampling in the TTTS group was 20+0 weeks (IQR 19+4-20+0 weeks) compared to 20+2 weeks (20+0-20+2 weeks) in the control group.

Table 6.1 Patient demographic data of investigation profiling array cohort

†This patient was excluded from analysis as a biological outlier due to natural variation in maternal miRNA. BMI: body mass index, GA: gestational age, TTTS: twin-twin transfusion syndrome

Patient number	Complication (Quintero stage)	Maternal age (years)	Maternal ethnicity	Parity	Maternal BMI (kg/m^2)	GA at blood sampling (weeks)	Fetal sex
2001	Nil	24	White European	0	31.9	19+1	Female
2047	TTTS (Stage II)	29	White European	1	29.4	20+0	Female
2002	Nil	27	White European	1	25.2	19+4	Female
2020	TTTS (Stage IIIR)	33	White European	1	15.8	19+5	Female
2018	Nil	24	White European	0	24.7	20+0	Female
2027	TTTS (Stage IIIR)	23	White European	1	32	20+3	Male
2022†	Nil	28	White European	0	29.4	20+0	Female
2039	TTTS (Stage II)	28	White European	0	19.6	20+2	Female
2043	Nil	29	White European	0	18.5	21+1	Male
2023	TTTS (Stage II)	35	White European	0	25.1	20+2	Male

Validation cohort

An independent cohort of 19 women in total was included in the validation cohort. The sample from participant 2124 was used as the reference sample as it most closely matched the median characteristics of the validation control group. Samples 2102 and 2013 (TTTS samples) were removed from analysis as there was insufficient miRNA in the samples, thus there were 8 control samples and 8 TTTS samples (Table 6.2). 2015 was paired with 2067, 2045 with 2025, 2068 with 2071, 2094 with 2069, 2104 with 2012, 2108 with 2101, 2112 with 2009, 2086 and 2017. TTTS patients were selected to match the control group as closely as possible. The median gestational age at blood sampling in the TTTS group was 20+1 weeks (IQR 19+6-20+5 weeks) compared to 20+2 weeks (20+0-20+6 weeks) in the control group.

Table 6.2 Demographic data of participants included in the validation cohort

†Patient excluded from analysis as insufficient miRNA. BMI: body mass index, GA: gestational age, TTTS: twin-twin transfusion syndrome

Patient number	Complication (Quintero stage)	Maternal age (years)	Maternal ethnicity	Parity	Maternal BMI (kg/m^2)	GA at blood sampling (weeks)	Fetal sex
2015	Nil	42	White European	4	44.5	20+0	Female
2067	TTTS (Stage III)	27	White European	1	35.7	19+4	Female
2045	Nil	20	White European	2	19.0	21+5	Male
2025	TTTS (Stage II)	22	White European	2	18.3	20+4	Male
2068	Nil	36	White European	1	25.5	19+6	Female
2071	TTTS (Stage I)	35	White European	1	22.7	20+0	Female
2094	Nil	30	White European	0	27.1	20+3	Female
2069	TTTS (Stage III)	28	White European	1	23.5	20+1	Female
2104	Nil	36	White European	1	25.6	20+4	Male
2012	TTTS (Stage III)	27	White European	3	23.5	20+1	Male
2108	Nil	34	White European	0	29.1	19+2	Male
2101	TTTS (Stage II)	24	White European	1	23.9	21+0	Female
2112	Nil	36	White European	1	24.6	20+0	Male

Patient number	Complication (Quintero stage)	Maternal age (years)	Maternal ethnicity	Parity	Maternal BMI (kg/m^2)	GA at blood sampling (weeks)	Fetal sex
2009	TTTS (Stage II)	24	White European	1	27.6	19+4	Male
2086	Nil	34	White 'Other'	0	22.4	21+3	Female
2017	TTTS (Stage III)	28	White European	4	29.8	21+3	Female
2124 (reference)	Nil	28	White European	1	31.7	20+2	Female
2102†	TTTS (Stage III)	21	White European	0	28.6	19+1	Male
2013†	TTTS (Stage III)	20	White European	1	19.8	19+5	Female

6.4.2 Quality control of profiling array samples

There was a low level of haemolysis in all 10 samples as demonstrated by the hsa−miR−23a−3p to hsa−miR−451a ratio being less than 7.0 in all the samples (Figure 6.1). The steady level of spike-ins in the samples (Figure 6.2) demonstrates high quality input RNA and successful reverse transcription and qPCR. The similar level in the blank sample demonstrates no RNA contamination in the PCR.

Figure 6.1 hsa−miR−23a−3p to hsa−miR−451a ratio as an indicator of haemolysis in all individual profiling array samples

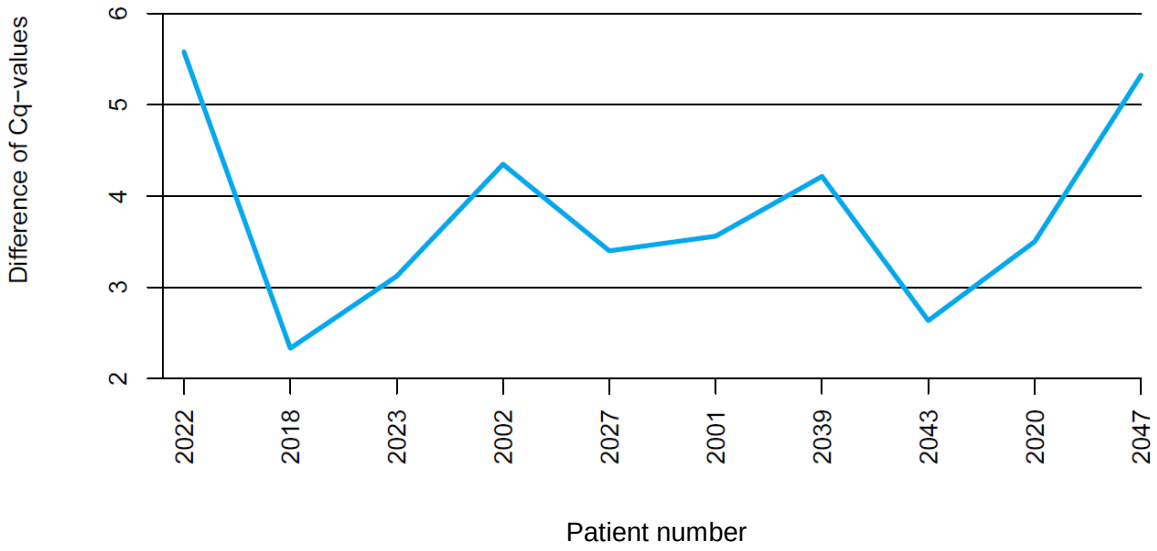
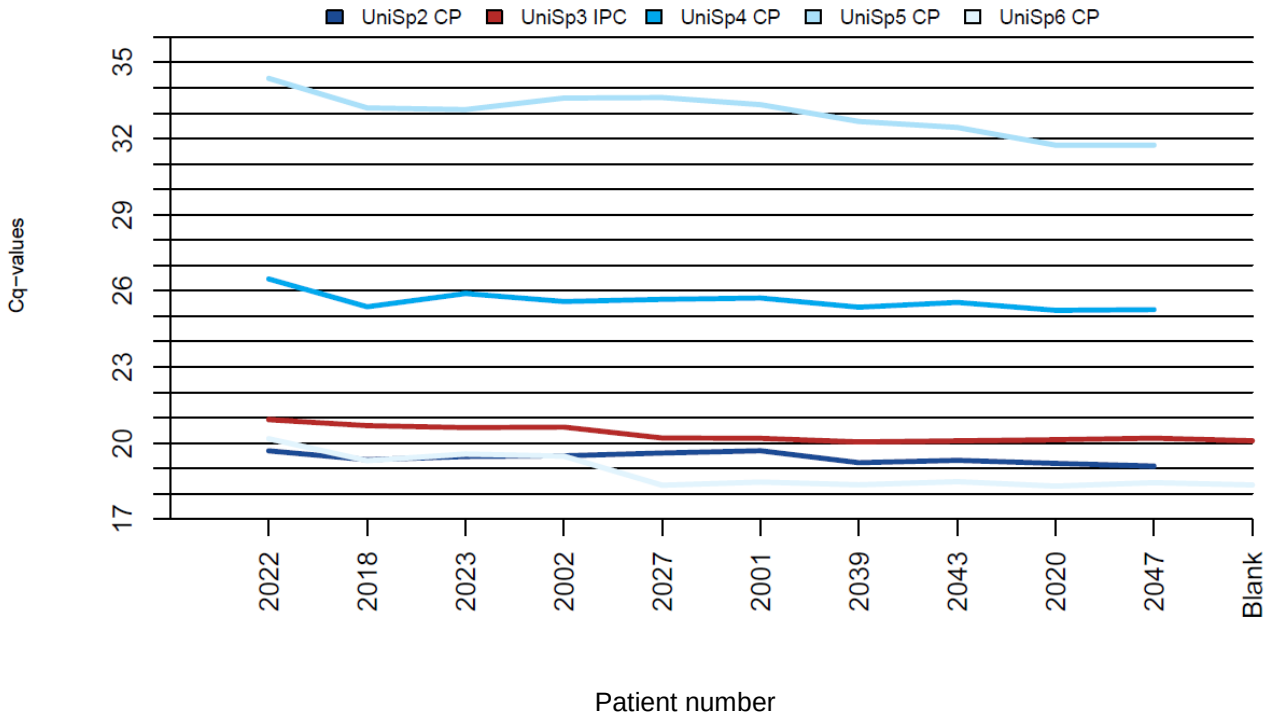


Figure 6.2 Spike-in raw Cq-values in all individual profiling array samples



From the Heatmap, and PCA plot of the top 50 miRNA with largest variation in expression across all samples (Figure 6.3 and Figure 6.4), sample 2022 (control) appeared to be an outlier. When this was further investigated, the quality of the sample was the same as the other samples and there was no clinical reason why this sample should be different, therefore the difference was believed to be due to biological variation, and the sample was excluded from further analysis.

Figure 6.3 Heatmap of miRNA expression in all profiling array samples

Maternal serum samples from pregnancies with twin-twin transfusion syndrome (TTTS) (n=5) compared to matched controls with uncomplicated monochorionic diamniotic twin pregnancies (n=5)

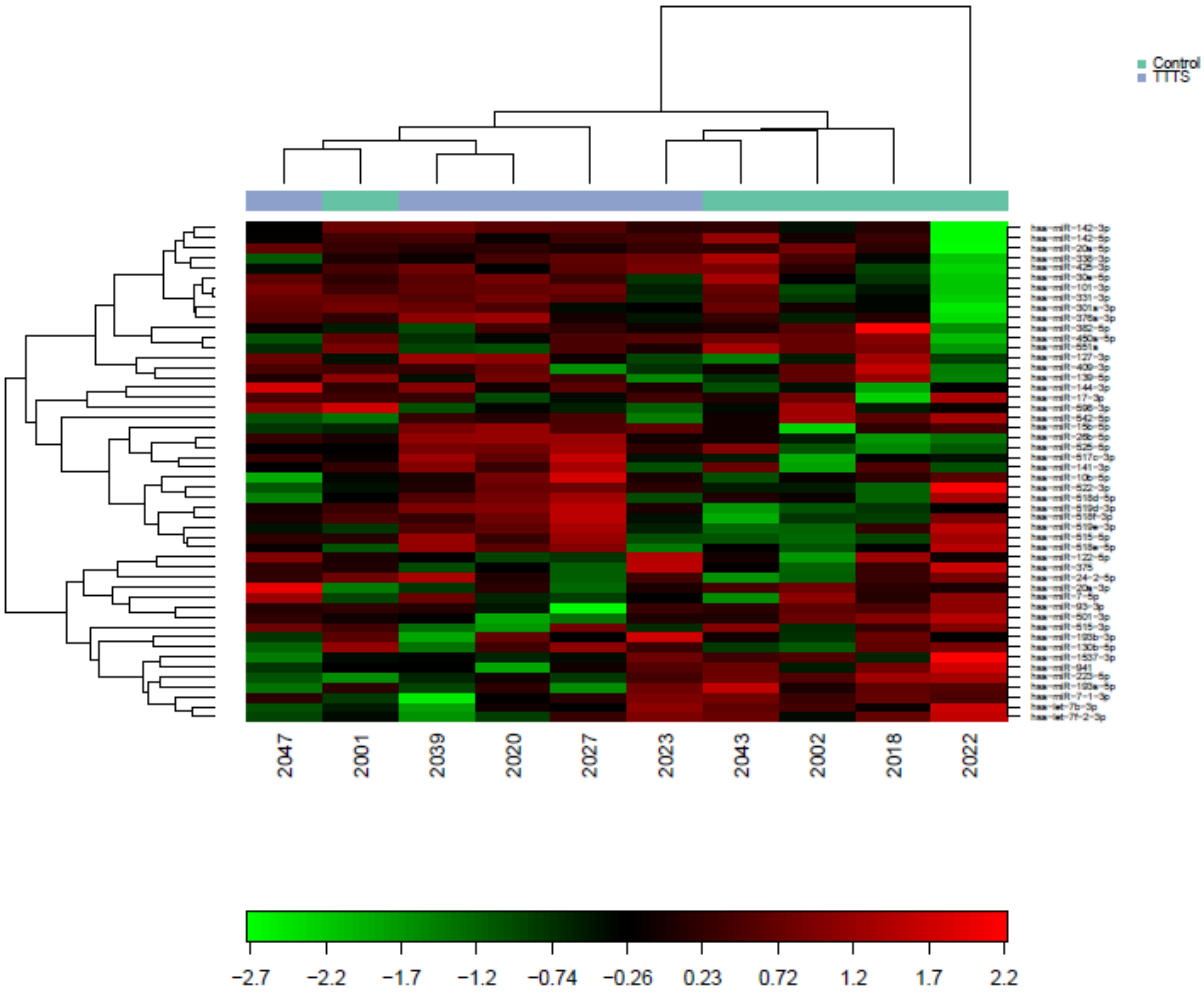
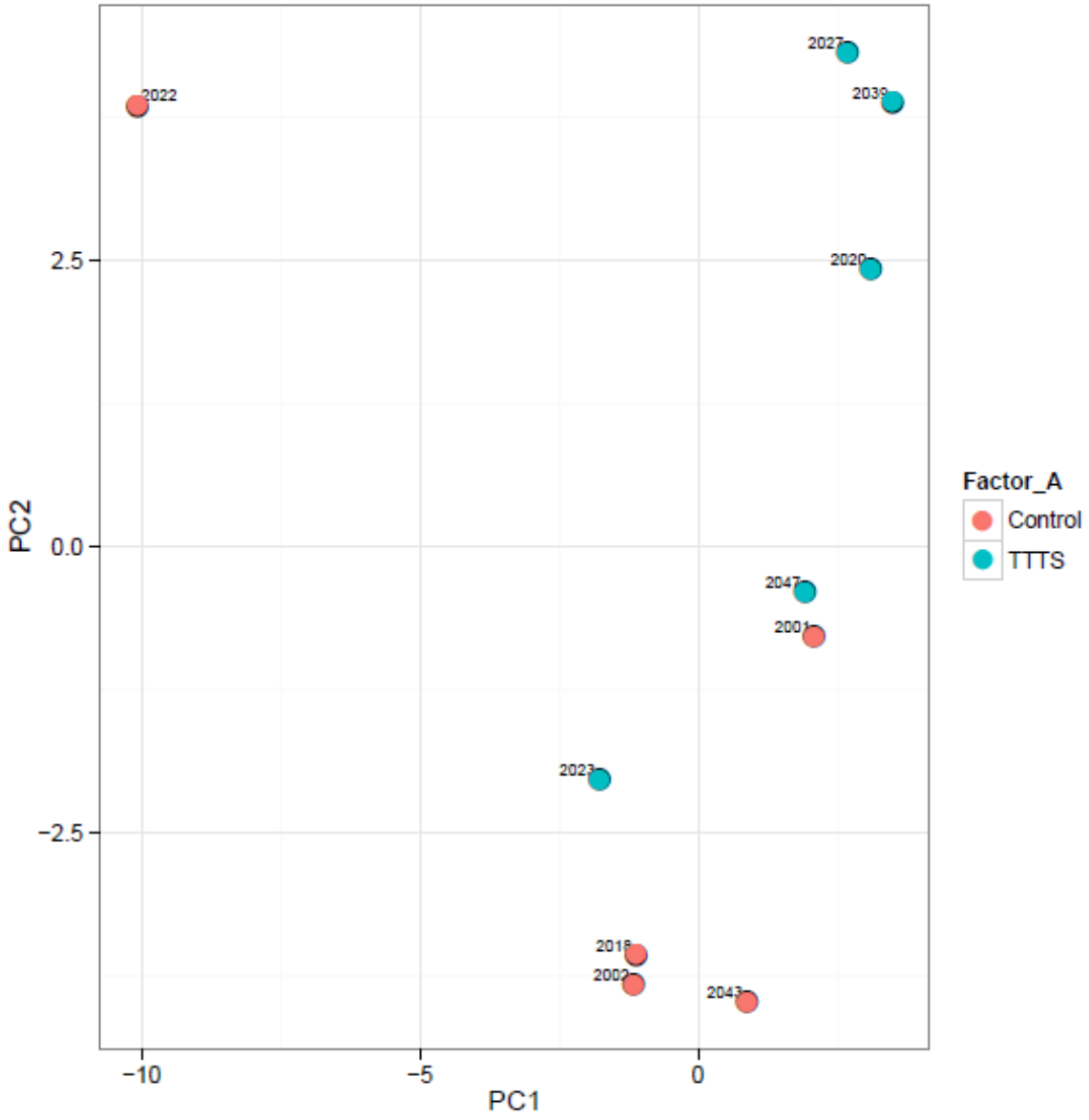


Figure 6.4 Principal Component Analysis (PCA) plot of top 50 miRNA with largest variation in expression across all maternal serum samples from pregnancies with twin-twin transfusion syndrome (TTTS) compared to matched controls with uncomplicated monochorionic diamniotic twin pregnancies

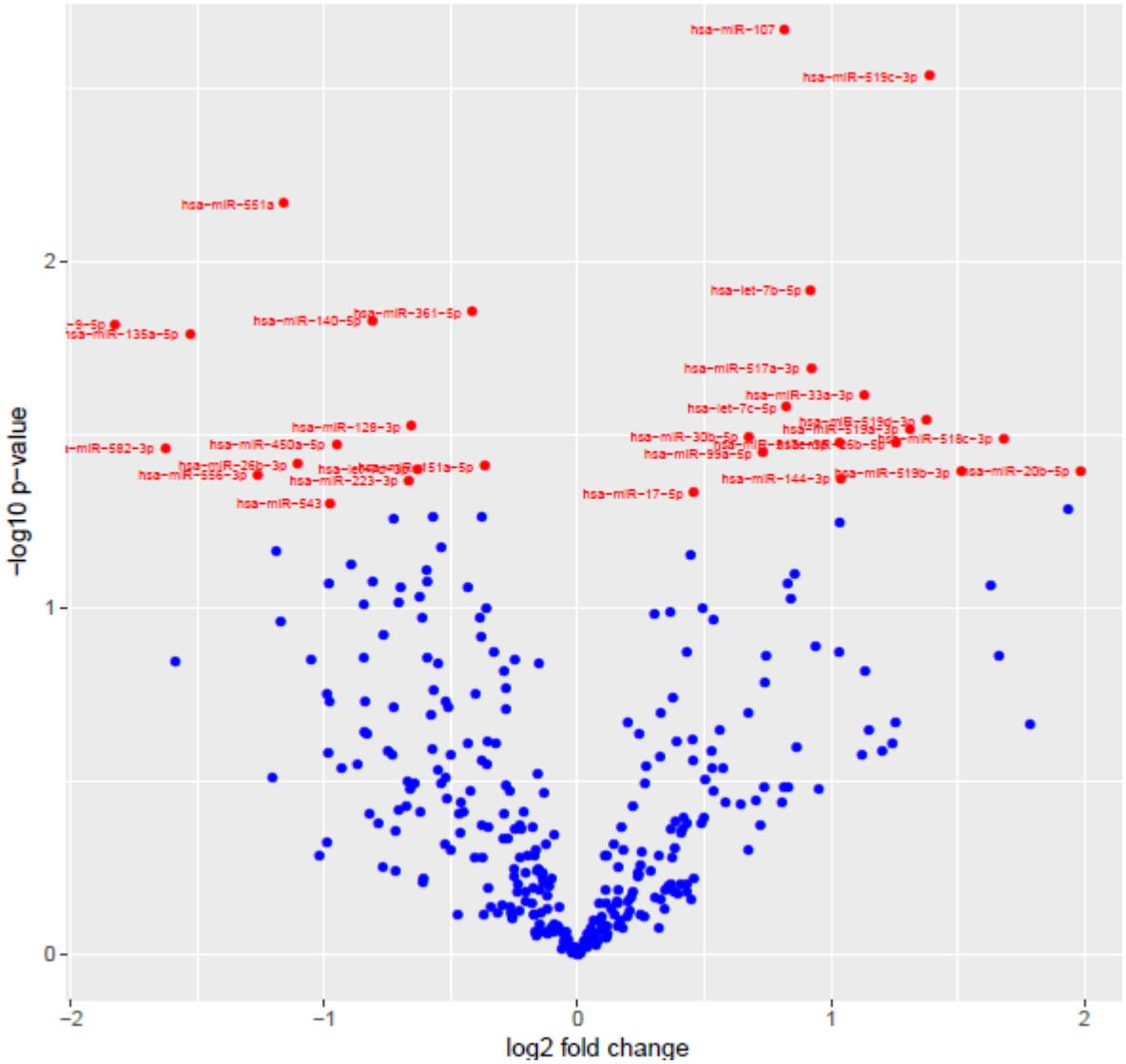


6.4.3 miRNA profiling array differences between TTTS and uncomplicated monochorionic twin pregnancies

555/752 miRNAs were detected. 185 miRNAs were identified in all samples, with a mean of 313 miRNAs detectable per sample. In the PCA there was a trend towards bimodal clustering of the 9 samples (excluding 2022), depending on whether they were TTTS or control samples which suggests there may be a difference between the groups, although there was still dispersion. The volcano plot demonstrated a number of significantly different miRNA, some were upregulated and some downregulated (Figure 6.5)**.**

Figure 6.5 Volcano plot comparing fold change of miRNA expression in maternal serum samples from pregnancies with twin-twin transfusion syndrome (TTTS) compared to matched controls with uncomplicated monochorionic diamniotic twin pregnancies

1 biological outlier excluded. Red denote miRNAs with statistically significant fold change difference in TTTS, blue denotes miRNAs with no significant difference



31 miRNAs were significantly different in the TTTS compared to control group (see Appendix 10.9 for all miRNAs, and Table 6.3 for ‘Top 5’). 17 miRNAs were upregulated in the TTTS samples, in order of statistical significance: hsa-miR-107, hsa-miR-519c-3p, hsa-let-7b-5p, hsa-miR-517a-3p, hsa-miR-33a-3p, hsa-let-7c-5p, hsa-miR-519d-3p, hsa-miR-519a-3p, hsa-miR-30b-5p, hsa-miR-518c-3p, hsa-miR-26b-5p, hsa-miR-517c-3p, hsa-miR-99a-5p, hsa-miR-20b-5p, hsa-miR-519b-3p, hsa-miR-144-3p, hsa-miR-17-5p; and 14 were downregulated: hsa-miR-551a, hsa-miR-361-5p, hsa-miR-140-5p, hsa-miR-9-5p, hsa-miR-135a-5p, hsa-miR-128-3p, hsa-miR-450a-5p, hsa-miR-582-3p, hsa-miR-26b-3p, hsa-miR-151a-5p, hsa-let-7d-3p, hsa-miR-556-3p, hsa-miR-223-3p, hsa-miR-543. They did not remain significant following the Benjamini-Hochberg correction for multiple testing.

A Bayesian model technique was used to examine the differential expression between TTTS and control (Table 6.4), both unadjusted and with adjusted for maternal age, BMI, ethnicity, parity and gestational age at blood sampling. Neither adjusted nor non-adjusted models demonstrated any significant differential expression between TTTS and control groups by Bayes factor.

Table 6.3 'Top 5' significantly upregulated miRNAs, and downregulated miRNAs in profiling array

Prior to Benjamini-Hochberg correction, in maternal serum samples from pregnancies complicated by twin-twin transfusion syndrome (TTTS), compared to matched controls with uncomplicated monochorionic diamniotic twin pregnancies

Assay	Average dcq TTTS (SD)	Average dcq Control (SD)	ddcq TTTS - Control	Fold change TTTS / Control	t-test p-value
hsa-miR-107	2.581 (-0.305)	1.765 (0.209)	0.816	1.761	0.002
hsa-miR-519c-3p	-3.212 (0.551)	-4.600 (0.373)	1.388	2.618	0.003
hsa-miR-551a	-2.259 (0.560)	-1.098 (0.219)	-1.160	-2.235	0.007
hsa-let-7b-5p	3.219 (0.366)	2.299 (0.409)	0.919	1.891	0.012
hsa-miR-361-5p	1.196 (0.175)	1.611 (0.191)	-0.416	-1.334	0.014
hsa-miR-140-5p	0.493 (0.145)	1.303 (0.359)	-0.810	-1.753	0.015
hsa-miR-9-5p	-6.594 (0.567)	-4.768 (0.529)	-1.826	-3.545	0.015
hsa-miR-135a-5p	-6.030 (0.900)	-4.501 (0.331)	-1.529	-2.886	0.016
hsa-miR-517a-3p	0.695 (0.522)	-0.228 (0.404)	0.923	1.896	0.020
hsa-miR-33a-3p	-4.193 (0.483)	-5.326 (0.608)	1.133	2.193	0.024

Table 6.4 Bayesian Model of significant miRNAs in profiling array in pregnancies complicated by twin-twin transfusion syndrome, compared to uncomplicated monochorionic diamniotic twin pregnancies

*Adjusted for maternal age, BMI, ethnicity, parity and gestational age at blood sampling

(a) Unadjusted

	Log Fold Count	Average Expression	t	P value	Adjusted P Value	B
hsa-miR-494-3p	10.78839	7.202803	5.912049	0.000116	0.01834	-0.02452
hsa-miR-204-5p	9.935406	6.635394	5.731394	0.00015	0.01834	-0.15169
hsa-miR-520c-3p	8.480225	4.711236	5.070715	0.0004	0.01834	-0.66378
hsa-miR-411-5p	9.925229	7.538905	4.817742	0.000592	0.01834	-0.88045
hsa-miR-181a-2-3p	10.45718	8.027257	4.742297	0.000667	0.01834	-0.94735
hsa-miR-381-3p	10.75888	8.367614	4.601182	0.000834	0.01834	-1.07533
hsa-miR-518d-3p	10.82702	8.443192	4.579189	0.000864	0.01834	-1.09561
hsa-miR-369-5p	10.26154	7.965421	4.576583	0.000868	0.01834	-1.09802
hsa-miR-517-5p	10.31679	8.030072	4.552294	0.000902	0.01834	-1.12054
hsa-miR-10a-5p	10.44629	8.173513	4.503198	0.000976	0.01834	-1.16638

(b) Adjusted*

	Log Fold Count	Average Expression	t	P value	Adjusted P Value	B
hsa-miR-379-5p	-186.379	6.396119	-9.89661	0.002952	0.574946	-4.59509
hsa-miR-9-5p	-197.243	7.054207	-9.77597	0.003053	0.574946	-4.59509
hsa-let-7c-5p	-81.7966	7.004665	-7.37649	0.006547	0.574946	-4.59509
hsa-miR-577	205.4049	3.385849	6.465976	0.009306	0.574946	-4.59509
hsa-miR-503-5p	-190.476	6.641287	-5.89464	0.011881	0.574946	-4.59509
hsa-miR-589-3p	210.6642	7.959102	5.791917	0.012443	0.574946	-4.59509
hsa-miR-365b-5p	-79.0397	2.389523	-5.55616	0.013871	0.574946	-4.59509
hsa-miR-877-3p	-81.4977	2.40734	-5.14132	0.016964	0.574946	-4.59509
hsa-miR-219a-2-3p	83.26375	1.219888	5.016209	0.018075	0.574946	-4.59509
hsa-miR-548d-5p	82.48287	1.208447	4.997538	0.018249	0.574946	-4.59509

6.4.4 Identification of candidate miRNAs for validation

Eight miRNAs were identified for validation (Table 6.5). All miRNAs were detected in all samples in the investigation cohort. No MTI studies were found in maternal serum in pregnancy. See Appendix 10.11 for more information on the biological plausibility of the candidate miRNAs.

Table 6.5 Candidate miRNAs for validation

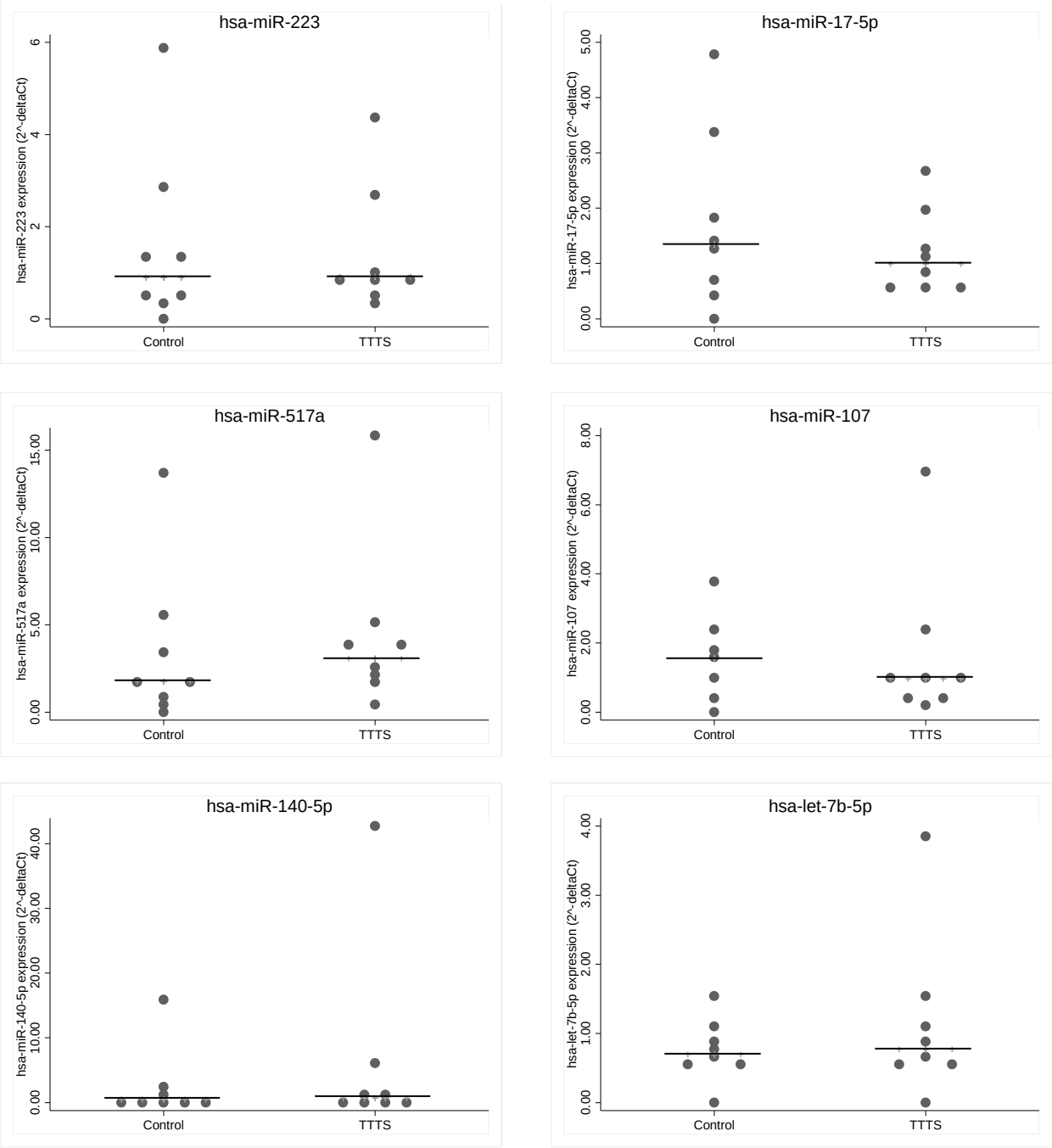
MTI: microRNA-Target Interaction. *according to miRTarBase (Chou 2018)

miRNA	Fold change in investigation cohort	Change in TTTS maternal serum compared to control	Number of papers with strong evidence of functional MTI*	Functional MTI target genes*	TTTS biological plausibility
hsa-let-7b-5p	1.89	Upregulated	42	34	Upregulated in congestive heart failure
hsa-miR-17-5p	1.37	Upregulated	120	80	Associated with angiogenesis
hsa-miR-107	1.76	Upregulated	49	38	Upregulated in congestive heart failure, same gene targets as those involved in pre-eclampsia and spiral artery remodelling
hsa-miR-140-5p	-1.75	Downregulated	37	30	Downregulated in congestive heart failure
hsa-miR-223-3p	-1.59	Downregulated	64	48	Downregulated in Stage 3 and 4 chronic kidney disease
hsa-miR-517a-3p	1.90	Upregulated	2	2	Placenta-specific miRNA
hsa-miR-519a-3p	2.48	Upregulated	12	9	Placenta-specific miRNA
hsa-miR-519c-3p	2.62	Upregulated	8	6	Placenta-specific miRNA

6.4.5 Validation of candidate miRNAs

Figure 6.6 Validation of candidate miRNAs

No significant difference between groups (n=8 controls, n=8 TTTS pregnancies) (Wilcoxon signed-rank test) Line represents median. miR-451a used as reference.



There was no significant difference in miRNA expression between the TTTS group and the control group (Figure 6.6). It was not possible to perform RT-PCR of miR519a-3p or miR519c-3p and the validation results should be interpreted with caution as the concentration of the RNA, as quantified by the RNA integrity number (RIN) was low.

6.5 Discussion

6.5.1 miRNA profiling array differences between TTTS and uncomplicated monochorionic twin pregnancies

The initial miRNA profiling array suggested that there was a difference in the miRNA profiles of maternal serum taken from TTTS and uncomplicated MC twin pregnancies. Although the results were not significant following statistical correction for multiple testing, the thresholds for these corrections are naturally conservative and it was possible the small sample size for the profiling array was associated with a type II statistical error, thus candidate miRNAs were investigated and validated.

6.5.2 Validation of candidate miRNAs

None of the candidate miRNAs were confirmed as being significantly different in the validation cohort. There are no other studies on maternal serum miRNA in TTTS to compare the findings. However, the quality control of the validation work was low and although some of the assays were able to be performed, the two placenta-specific miRNA were unable to be assessed.

One reason why the results may not have been significant is that maternal circulating miRNA may come from different sources (fetus, placenta or maternal endothelium) and the pathophysiology of TTTS may affect miRNAs from each source differently. Even if it was possible to identify the source of the miRNA and which were maternal and which were fetal, an added complication with exploring miRNA in maternal serum in MCDA twin pregnancies is that although they are monozygotic with the same germline and same environmental influences in-utero, discordant conditions still occur antenatally (Machin 2009, Saffery 2012). In the case of TTTS, there may be different epigenetic mechanisms at play in each fetus, due to the different problems the donor and recipient fetuses develop. It is possible that the epigenetic changes in one twin could “mask” a difference in the other twin, and therefore changes will not be seen in maternal serum. In-utero inter-twin miRNA differences in fetal circulation have not been described; practically and ethically it would be difficult to obtain individual fetal blood samples, as an invasive procedure would be required, which would increase the risk of miscarriage in a clinical scenario that is already highly morbid.

Inter-twin miRNA differences have been explored in adults; in adults discordant for non-alcoholic fatty liver disease (4 monozygotic twin pairs, 2 dizygotic twin pairs), a difference in miRNAs was seen (Zarrinpar 2016), which persisted when sub-group analysis was performed on the monozygotic twins. Another study looking at monozygotic twins with discordant life expectancies, defined as a difference in age of death of at least 5 years, found a difference in miRNA in plasma samples taken at age 44-51 years in the twin pairs (Wu 2016), but this difference did not remain

significant following Bonferroni correction for multiple testing. Therefore there remains a lot to be elucidated regarding miRNAs in monozygotic twin pairs, particularly antenatally.

It may be that as we were unable to recruit pregnancies with TTTS all at the same Quintero stage, that miRNA expression is affected by different stages, but unfortunately it was not possible to investigate this further. Another reason why there may not be a significant difference in miRNA in maternal serum in TTTS pregnancies is that TTTS is a disease which affects the placenta and fetuses, but rarely causes additional extra-uterine symptoms, such as maternal proteinuria and hypertension which characterise pre-eclampsia, therefore miRNA changes in TTTS may not be seen in the maternal circulation. There is a rare condition called ‘maternal mirror syndrome’ which describes maternal oedema in the presence of fetal oedema (Hayashi 2006, Chai 2014, Chang 2014) and is associated with changes in the maternal serum (Prefumo 2010), thus one could hypothesise that there may be changes in miRNA in maternal serum in TTTS. However mirror syndrome is only associated with Quintero Stage IV and V and none of the participants had Stage IV or V TTTS prior to FLA when blood sampling was performed. It is interesting to note that whilst miRNA changes are seen in both maternal plasma and placental tissue in conditions which clinically affect both the mother and the fetus, such as GDM (Zhao 2011, Zhao 2014), and pre-eclampsia (Akehurst 2015); in conditions which only affect the fetus, such as fetal growth restriction, it is not as clear. Studies by Hromadnikova et al. have found conflicting results in changes in miRNA in maternal plasma, with miRNA differences apparent at 12-16 weeks gestation (Hromadnikova

2012), but not at 10-13 weeks (Hromadnikova 2017). In two studies from the same group in which the gestation at blood sampling was not stated but is assumed to be at a later gestation, no difference in miRNA in maternal plasma samples was seen in placenta-specific miRNA i.e. those only produced by placental tissue but able to be transferred to maternal blood (Hromadnikova 2013), but a significant difference was seen in miRNAs associated with cardiovascular and cerebrovascular disease (Hromadnikova 2016).

Changes have been demonstrated in miRNA in placental tissue from fetal growth restricted pregnancies (Maccani 2011), but again the picture is not clear. Higasijima et al. (Higashijima 2013) looked at placental tissue and maternal plasma in the same study and demonstrated changes in placental tissue, but not maternal plasma, despite miRNAs being able to cross into the maternal circulation, whereas Mouillet et al. found no difference in maternal plasma, or placental tissue, although they only looked at miRNAs associated with hypoxia (Mouillet 2010). Therefore, the significant miRNAs for TTTS may not be placenta-specific, despite the condition being placental in origin. The panels used in this work did include the cardiovascular and cerebrovascular miRNAs which Hromadnikova found to be significantly different, as one may hypothesise they would also be different in TTTS, given the cardiovascular sequelae in the fetuses, however no significant difference in these miRNAs were seen.

6.5.3 Strengths and limitations

This is the first study to compare human maternal serum miRNAs in pregnancies complicated with TTTS, with uncomplicated MCDA twin pregnancies and it has demonstrated that it is possible to measure miRNA in maternal serum taken prior to FLA for TTTS. An attempt was made to match the samples as closely as possible as demographic factors including maternal age, BMI and ethnicity affect miRNA expression (Morales-Prieto 2012, Enquobahrie 2017, Timofeeva 2018), and this is vital in miRNA research (Barchitta 2017). The sample size did not allow the examination of the impact of other factors such as stage of TTTS, pregnancy outcome following TTTS, maternal age, BMI, parity, fetal sex on miRNA in TTTS which may have provided further information, although a Bayesian model was produced in the investigation cohort to adjust for these factors, and the samples were matched as closely as possible. This does mean that one limitation of this work is that only white European women were examined because of the patient population, and thus the results may not be generalisable to other ethnicities.

Another strength is that there was good technical performance in the profiling experiment as demonstrated by the RNA spike-in and no template control. Due to the high risk nature of MCDA twins, it was not possible to find a group of control MCDA patients with no comorbidities, therefore a pragmatic approach was taken and included 2 patients with comorbidities (isolated polyhydramnios in 1 twin at 34 weeks, pregnancy-induced hypertension at 34 weeks) in the control group in the investigation cohort which is not believed to have affected the study results as they are unrelated to TTTS. In the control group in the validation cohort 3 patients had

comorbidities (1 patient developed mild pre-eclampsia at 33 weeks, 1 patient developed moderate pre-eclampsia at 35 weeks, and 1 patient developed chorioamnionitis at 30+6 weeks following spontaneous rupture of membranes at 30+3 weeks) but they are unlikely to have affected the study results.

RT-PCR was used for validation as opposed to Next Generation Sequencing as PCR is the only platform able to generate absolute quantification, and this is the most frequently used method of validation, and is considered an acceptable approach (Barchitta 2017, Moldovan 2017). Both methods are comparable, but when detecting pre-selected miRNAs, RT-PCR provides a higher sensitivity and requires a smaller amount of biofluid (Blondal 2017).

6.5.4 Clinical implications and future research

These findings cannot be used clinically yet as this was initial discovery work and more research is required. Due to poor quality control in the validation work, this work should be repeated in a different cohort. Although no difference was seen in the 752 miRNAs compared, it may be that the differences in TTTS occur in miRNAs transferred in exosomes as exosome-specific RNA extraction was not performed, and exosomal miRNAs have recently been shown to be important in the regulation of angiogenesis in the maintenance of normal pregnancy (Jia 2018), or that changes may occur in miRNAs which have not yet been discovered. miRNAs were first discovered in 1993, and since then PubMed cites over 75,000 publications on miRNAs, and more than 1880 human miRNAs have been reported (Chiofalo 2017) therefore there are more miRNAs to explore in future research. However, to do this,

and investigate those which have not yet been discovered would be costly and require Next Generation Sequencing (Ge 2011).

Another area to focus on would be to examine miRNA expression in placentas of pregnancies complicated by TTTS, particularly as it was not possible to get the RT-PCR of maternal serum to work to validate miR519a-3p or miR519c-3p which are placenta-specific miRNAs, however, there are a couple of issues with using placental tissue. The collection of TTTS placentas is logistically difficult as most patients whom are treated with FLA return to their referring unit to deliver, and as placentas need to be collected and sampled within an hour of delivery, given the large geographical area from which referrals are received, this will not be possible. There is also the question regarding if the TTTS resolves following FLA, whether collecting placentas at delivery will demonstrate any changes, as miRNAs are transient and the miRNA profile may have returned to normal, or if changes are present this may be a cause of TTTS, a consequence of TTTS, or an effect of FLA. Alternatively, placentas could be collected from pregnancies from the extreme end of the TTTS spectrum: which have ended in a dIUFD, however even if it was possible to collect the placenta, there is the issue of whether the miRNA changes were due to TTTS, FLA or IUFD. Cord blood at delivery may offer an alternative solution, but again presents the same problems as with placental collection and sampling. Alternatively, analysing amniotic fluid taken prior to FLA may provide a way to assess fetal miRNA changes. Ideally one would sample the recipient, and the donor twin, but it would be technically very difficult, and potentially dangerous to sample amniotic fluid from the donor twin sac due to the oligohydramnios.

6.6 Conclusion

This is the first study to look at miRNAs in maternal serum from pregnancies complicated by TTTS. Although the initial profiling array demonstrated a difference in miRNAs in pregnancies complicated by TTTS compared to control MCDA pregnancies, these finding were not confirmed in the validation cohort. Further investigation using alternative biofluids, particularly placental tissue samples, would be interesting, but pragmatically difficult to obtain.

CHAPTER 7 PROGNOSIS OF THE CO-TWIN FOLLOWING SPONTANEOUS SINGLE INTRAUTERINE FETAL DEATH IN TWIN PREGNANCIES: SYSTEMATIC REVIEW AND META-ANALYSIS

- These findings were presented as a poster presentation at the British Maternal and Fetal Medicine Society 19th Annual Conference, April 2018, Brighton.
- These findings have published in full article form [Mackie FL, Rigby A, Morris RK and Kilby MD (2018). "Prognosis of the co-twin following spontaneous single intrauterine fetal death in twin pregnancies: a systematic review and meta-analysis." BJOG; 126:569-78.]

7.1 Introduction

Twin pregnancies are associated with increased perinatal morbidity and mortality compared to singletons. The prevalence of sIUFD is up to 6% in twin pregnancies, making it a common adverse event (Pharoah 2000). Monochorionic (MC) twins with placental inter-twin anastomoses conjoining the fetal circulations are associated with an increased risk of sIUFD and consequential fetal morbidity (Bajoria 1999b, D'Antonio 2017b). Many are first trimester fetal losses, but sIUFD after 14 weeks gestation is associated with greatest adverse effect on the surviving fetus (Malinowski 2001). Sequelae of sIUFD in twin pregnancy include: co-twin IUFD, PTB (spontaneous or iatrogenic), and long term comorbidity; most commonly ante- or postnatal brain injury. A critical appraisal and interpretation of the literature is

complicated by significant heterogeneity in the incidence and management in reported studies (Ong 2006). In 2011, our group completed a systematic review and meta-analysis of co-twin prognosis following sIUFD, with outcomes stratified by chorionicity. In the 22 included manuscripts there were 343 cases of sIUFD reported in 6225 twin pregnancies (Hillman 2011). A meta-analysis of event rates was not undertaken as there was a high risk of heterogeneity and low number of events within each study. A summary point estimate was produced with a simple binomial confidence interval, thus not allowing for the non-independence of the different studies. This manuscript demonstrated an increased OR of co-twin death and neurodevelopmental morbidity after sIUFD in MC compared to DC twin pregnancies. The management of multiple pregnancies in general, and MC pregnancies in particular, has received considerable attention since 2011 with national and international guidelines being published by international professional bodies (Morin 2011, NICE 2011, ACOG 2016, Khalil 2016b, Kilby 2016, RANZCOG 2017). Importantly the 2011 review included twin pregnancies that had undergone intervention for TTTS and IUGR, thus confounding factors will have affected the prognosis (Morris 2010). This review will focus on spontaneous sIUFD only and will not include pregnancies that have undergone treatment for TTTS or IUGR.

7.1.1 Objectives

To determine the prognosis of the surviving co-twin following spontaneous sIUFD. The outcomes explored will be: co-twin IUFD, PTB, abnormal postnatal brain imaging and neurodevelopmental comorbidity as analysed in the previous systematic review and meta-analysis, and the additional outcomes of abnormal antenatal brain imaging

and NND will also be examined. This review will allow inclusion of the recent literature informing clinical practice to aid counselling patients and highlight areas of future research.

7.2 Materials and methods

The systematic review was performed according to an *a priori* protocol and complied with recommended guidance including MOOSE and PRISMA guidelines (Stroup 2000, Moher 2009). Ethical approval was not required.

7.2.1 Eligibility criteria

The gestation of the initial sIUFD must have been after 14 weeks. Twin chorionicity had to be defined but studies did not have to include both MC and DC twin pregnancies in the same study. Studies were excluded if the following conditions could not be removed for analysis i.e. if the following cases were not identifiable in analysis: selective termination, higher order multiple pregnancies, twin reversed arterial perfusion (TRAP) sequence, structural or chromosomal anomalies, conjoined twins, monoamniotic twins, or first-trimester miscarriages associated with twins. As the objective of the study was to assess spontaneous IUFD, IUFDs which occurred following an intervention for TTTS or sIUGR, including FLA or BCO, were not included in the analysis as there are confounding factors that may affect the outcome of the pregnancy, including surgeon experience, which make this group heterogeneous (Morris 2010). As FLA dichorionises the placenta and this was considered to have more of an effect on outcome, whereas amniodrainage was not

considered an intervention which would affect the prognosis in the co-twin as the main reason for IUFD following amniodrainage is likely due to TTTS itself, rather than a complication of the procedure, thus these pregnancies remained in the analysis. All study designs were included, although case series needed to include at least 5 cases of sIUFD in twin pregnancies.

7.2.2 Outcomes

There is no core outcome set for multiple pregnancy, particularly sIUFD co-twin survivors, thus the outcomes assessed were the outcomes in the previous review, with the addition of antenatal brain imaging and NND. The outcomes were defined *a priori* as:

- Co-twin IUFD >14 weeks gestation but prior to delivery.
- PTB, defined as a live birth of the surviving co-twin, irrespective of whether the birth was spontaneous or iatrogenic which will be explored as a sub-group analysis, between 24^{+0}-34^{+0} weeks gestation as some MCDA twins are routinely delivered at <36 weeks, and with little long-term consequence.
- Abnormal antenatal brain imaging. There was no limit on timing of imaging post-IUFD or type of imaging due to no consensus guidance existing at the time of this review.
- Abnormal postnatal brain imaging. There was no limit on imaging modality.
- Neurodevelopmental comorbidity, defined as per study, as there is no standard test to assess this in sIUFD.

- NND, defined as death within 28 days of live birth.

7.2.3 Information sources

The search was performed according to previously published methods (Hillman 2011). In brief, Medline, Embase, Web of Science, Cochrane Library and British Nursing Index were searched. Due to including the new outcomes of abnormal antenatal brain imaging, and NND, the searches were run from 1980 in keeping with the introduction of ultrasound into clinical practice, to 9th June 2017.

7.2.4 Search strategy

Keywords and variants of “intrauterine” “death” and “twin” were used (see Appendix 10.14 for search strategy). Bibliographies were manually checked and there was no restriction on language.

7.2.5 Study selection and data extraction

Three investigators (FLM, AR and RKM) independently extracted the data needed to assess the quality of the studies and form a 2x2 contingency table, using piloted data collection forms (Appendix 10.15). Data from the previous systematic review by Hillman (Hillman 2011) was re-extracted by FLM and RKM. Any discrepancies were resolved by MDK. If clarification was required authors were contacted.

7.2.6 Quality assessment of included studies

The quality of the studies was assessed according to the STROBE checklist (von Elm 2007).

7.2.7 Assessment of heterogeneity

Heterogeneity between the studies was assessed visually using forest plots and statistically using the I^2 statistic. An I^2 statistic ≥50% indicated a high-risk of heterogeneity. Heterogeneity was investigated via sub-group and sensitivity analysis.

7.2.8 Assessment of reporting bias

If >10 studies were included in a meta-analysis, a funnel plot was generated using the *metafunnel* command (Sterne 2003) in Stata (Stata, 2015 Release 13.1, StataCorp. Texas, USA) and Egger's test was performed using the *metabias* command (Harbord 2009), with $p<0.05$ considered a significant risk of small-study effects publication bias.

7.2.9 Data synthesis

With the additional 20 studies, a summary event rate statistic was produced which has allowed for the non-independence of different studies when the data is pooled, as is appropriate in a meta-analysis. This was calculated using the *metan* command (Harris 2009). ORs with random effects were calculated to compare the risk in MC twin pregnancies with DC twin pregnancies using the *metan* command. 0.5 was added to 0 cells in all analyses to allow inclusion of more studies (Sankey 1996). If a

study only included MC twin pregnancies, the study was used to calculate the summary event rate for MC twins only, and was not included in the DC summary event rate or OR calculation of MC vs. DC twins, and vice versa if a study only included DC twin pregnancies. Sub-group analysis, in analyses of ≥3 studies, was planned to evaluate the effect of factors identified as potential causes of heterogeneity prior to commencing analysis: gestational age of sIUFD <28 weeks, TTTS (managed conservatively meaning no intervention but continued surveillance), IUGR (managed conservatively), year of publication pre-and post-2011. Twenty-eight weeks was chosen as a cut-off to distinguish between trimesters as there is no research to determine an evidence-based cut-off. PTB as an outcome was also divided by iatrogenic and spontaneous where possible. Antenatal and postnatal brain imaging were divided by imaging modality, and the postnatal outcomes were also divided by PTB where possible, the latter irrespective of whether the PTB was iatrogenic or spontaneous. The sub-group summary event rate was reported as the rate of the outcome (e.g. co-twin IUFD) in women with or without that factor (e.g. sIUFD at <28 weeks, TTTS, IUGR) to enable maximum clinical utility for counselling women in each scenario. ORs were calculated to compare the summary event rate for each factor in MC and DC twin pregnancies.

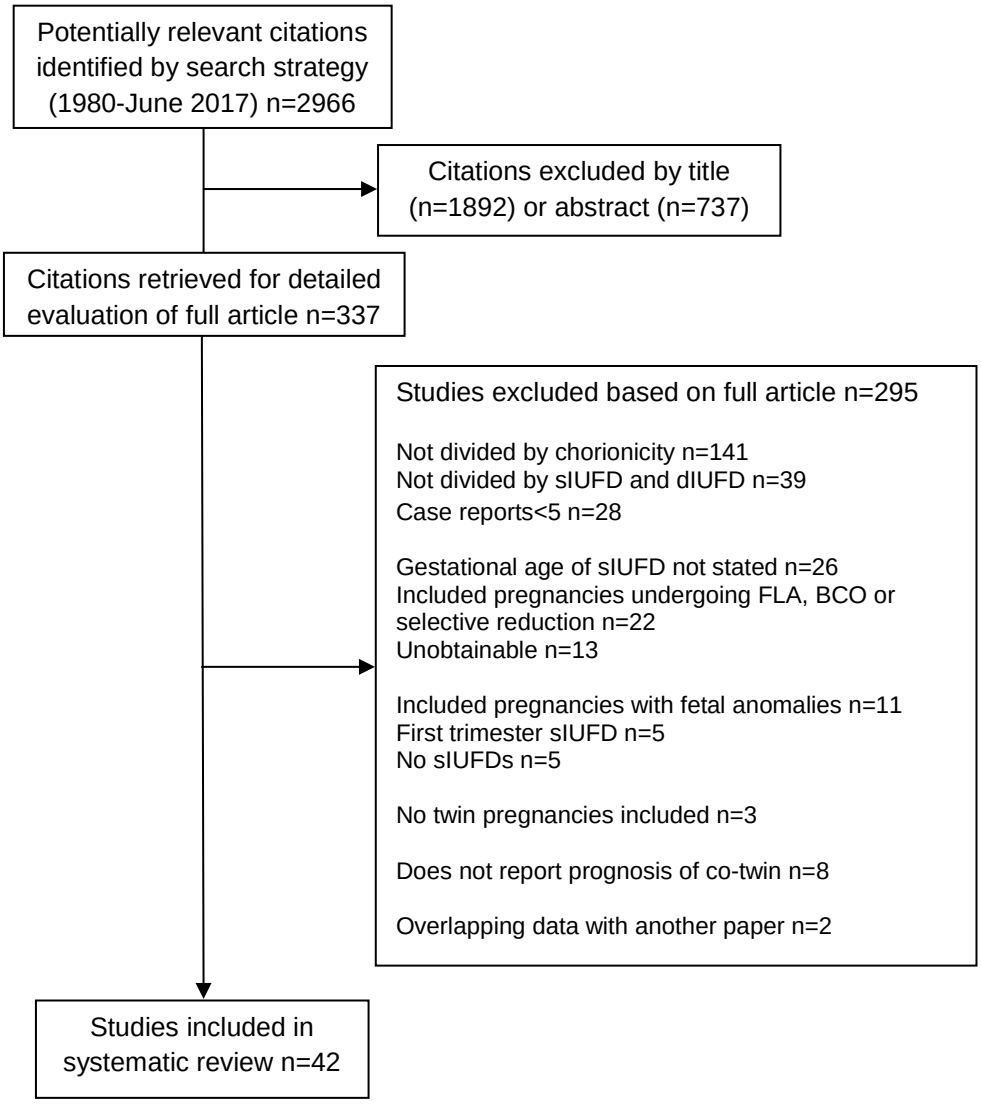
7.3 Results

7.3.1 Study selection

The search revealed 2966 citations potentially eligible for inclusion, of which 2629 were excluded on the title or abstract, 337 full papers were assessed, and 42 full papers were eligible for inclusion (Hagay 1986, Szymonowicz 1986, Cherouny 1989,

Fusi 1990, Gaucherand 1994, Ishimatsu 1994, Kilby 1994, Santema 1995, Jou 1996, Tordjeman 1996, Sebire 1997, Krayenbuhl 1998, van Heteren 1998, Axt 1999, Bajoria 1999b, Lin 1999, Petersen 1999, Saito 1999, Malinowski 2000, Wang 2000, Woo 2000, Baghdadi 2003, Malinowski 2003, Gratacós 2004, Barigye 2005, Gratacós 2008, Jelin 2008, Lewi 2008, Chelli 2009, Fichera 2009, Dias 2011, Mahony 2011, Farah 2012, McPherson 2012, Deveer 2013, Hoffmann 2013, Griffiths 2015, van Klink 2015, Wang 2016, D'Antonio 2017b, Robinson 2017, Rustico 2017) (Figure 7.1).

Figure 7.1 Study selection from initial search



7.3.2 Studies not included in meta-analyses

Although Woo 2000, Wang 2000 and Wang 2016 were eligible for inclusion in the systematic review, it was not possible to include them in meta-analysis (Wang 2000, Woo 2000, Wang 2016). In Woo, once the cases with insufficient information were removed, the number of sIUFD cases was reduced to 4 therefore this study was not

included in analysis, unlike in the previous review. Wang 2000 was not able to be included as all 9 sIUFD pregnancies had TTTS, 8/9 women were delivered prematurely to avoid dIUFD, they were unable to report on the presence of congenital anomalies and the authors limited their cohort to third trimester sIUFDs. In Wang 2016 6/16 (38%) cases of sIUFD who had TTTS had undergone FLA and could not be removed for analysis. This proportion of heterogeneous sIUFDs was felt to be too high to include the study in meta-analysis. The 10 DC pregnancies in Fichera 2009 were not included as a high proportion 3/10 (30%) had chromosomal anomalies, although the MC pregnancies are included in analysis (Fichera 2009).

Six studies were not included in the co-twin IUFD outcome: 2 studies did not report this outcome (Jelin 2008, D'Antonio 2017b) and 4 studies focused on neonatal outcomes thus dIUFDs were excluded by the authors (Szymonowicz 1986, van Heteren 1998, Lin 1999, Saito 1999). It was not possible to include Chelli 2009 in the PTB outcome as the authors defined PTB as 26-33 weeks which was not in-keeping with the definition used in this review (Chelli 2009).

7.3.3 Study characteristics

The characteristics of the included studies are described in Table 7.1 which summarises the study design, study population, and details of abnormal brain imaging and neurodevelopmental comorbidity. 10/42 studies were published in the 5 years prior to June 2017 when the search was performed. The oldest study was from 1986. 40 studies were cohort studies, 2 studies were case-controls. The majority of studies performed retrospective data collection (n=32), 7 performed prospective data collection, and it was not clear in 3 studies. Enrolment was consecutive in 17 studies,

not stated in 24 studies, and random in 1 study. 28 groups of patients were from 11 European countries, the UK being the commonest (n=9), 3 from the USA, 3 from Taiwan, 2 from Japan, 2 from Australia, 2 from Israel, 1 from Hong Kong and 1 from China. The longest study ran for 16 years, with the modal time period being 4 years and shortest being 21 months. The smallest study analysed 9 sIUFDs, and the largest analysed 62 sIUFDs. The previous review included 22 studies (Hagay 1986, Szymonowicz 1986, Fusi 1990, Jou 1993, Gaucherand 1994, Ishimatsu 1994, Kilby 1994, Santema 1995, Krayenbuhl 1998, van Heteren 1998, Axt 1999, Bajoria 1999b, Lin 1999, Petersen 1999, Saito 1999, Malinowski 2000, Wang 2000, Woo 2000, Baghdadi 2003, Malinowski 2003, Chelli 2009, Fichera 2009). Of the 42 studies, 39 were included in the meta-analysis (for details of excluded studies see section 7.3.2). The additional outcomes of antenatal brain imaging and NND were reported by 6 studies, and 19 studies respectively. The imaging modalities used were ultrasound and fetal magnetic resonance imaging (fMRI) antenatally, CT scan was also used postnatally.

Table 7.1 Characteristics of studies eligible for inclusion

Reproduced from Hillman et al. (Hillman 2011)
Abbreviation: FLA: fetoscopic laser ablation, FU: follow-up, IUGR: intrauterine growth restriction, IVH: intraventricular haemorrhage, MCDA: monochorionic diamniotic, MA: monoamniotic, MRI: magnetic resonance imaging, NA: not applicable, PTB: preterm birth, PVH: periventricular haemorrhage, sIUFD: spontaneous intrauterine fetal death, sIUGR: selective intrauterine growth restriction, TRAP: twin reversed arterial perfusion sequence, TTTS: twin-twin transfusion syndrome, USS: ultrasound scan
†: new study added

Author (year)	Study design, data collection, enrolment	Study population (location, years, inclusion description, PTB information, additional information)	Details of abnormal brain imaging	Details of neurodevelop-mental comorbidity	Follow-up
Axt (1999) (Axt 1999)	Cohort, retrospective, not reported	*Location:* Germany *Year:* 1992-1998 *Description*: 185 twin pregnancies. 7 twin pregnancies with sIUFD but we excluded 1 due to structural anomalies to fit our own criteria. Death of twin confirmed by USS. Placenta examined histologically to confirm chorionicity. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic stated. *Additional*: Confounders of TTTS although no mention of intervention, fetal anomaly, IUGR and maternal illness recorded	*Mode of imaging*: postnatal USS and MRI *When imaged*: "after birth" *Definition of abnormal imaging*: Periventricular leukomalacia, multiple small cystic lesions in white matter	Mild delay in gross motor development and muscle rigidity. No details of method of assessment given.	Different for individual infants. From hospital discharge – 1.5 years of age.
Baghdadi (2003) (Baghdadi 2003)	Cohort, prospective, consecutive	*Location:* UK *Year:* 1996-1998 *Description*: 252 twin pregnancies, authors excluded 14 cases as either; fetal anomaly on USS, loss to FU or mother's medical condition, therefore 238 in cohort. 13 cases of sIUFD included. Placenta examined histologically to confirm chorionicity. *PTB information:* gestational age at delivery reported	NA	NA	11 cases lost to follow up out of 252 (4.4%)

		individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*. Kaplan –Meir analysis of survival. Confounders of TTTS although no mention of intervention, fetal anomaly and maternal illness recorded. All 4 NNDs were in fetuses delivered <24 weeks			
Bajoria (1999) (Bajoria 1999b)	Cohort, retrospective, not reported	*Location:* UK *Year:* 1980-1998 *Description*: 3 hospitals included. 101 cases of twin pregnancy, 9 cases excluded as type of anastomoses/neonatal data not present therefore 92 pregnancies analysed. Also excluded if had abortion, acardiac twin, FLA for TTTS, or delivered before 20 weeks. Histological confirmation of chorionicity recorded. *PTB information:* gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Information on confounders TTTS including 4 who underwent amniodrainage, IUGR and maternal illness were recorded but not congenital anomaly.	*Mode of imaging*: postnatal USS *When imaged*: neonatal *Definition of abnormal imaging*: IVH, periventricular leukomalacia, subependymal haemorrhage. Excludes transient echo-dense areas.	Obtained from the neonatal notes. Only information on major neurological handicaps such as cerebral palsy, blindness and paralysis was obtained.	"Long-term follow-up" duration not specified.
† **Barigye (2005) (Barigye 2005)**	Case-control, retrospective, not reported	*Location:* UK *Year:1992-2004* *Description:* 151 uncomplicated MCDA pregnancies, 7 with sIUFD after 24 weeks. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional:* excluded TTTS, IUGR, structural anomalies, TRAP, HOM, MA, conjoined twins. Maternal illness not recorded.	NA	NA	Not reported
Chelli (2009) (Chelli 2009)	Cohort, retrospective, not reported	*Location:* France *Year:2000-2008* *Description:* 1107 twin pregnancies. 33 with sIUFD after 26 weeks, but only 28 with chorionicity determined.	*Mode of imaging*: postnatal USS *When imaged*: day 7 of life	NA	USS at day 7 post-delivery in 100% survivors

		Histological confirmation of chorionicity recorded. *PTB information*: could only extract 26-33 weeks due to way grouped therefore unable to include in analysis, spontaneous or iatrogenic not clear. One monoamniotic twin pregnancy included as unable to remove for analysis. *Additional:* Information on confounders TTTS including 1 who had amniodrainage, IUGR, maternal illness and congenital anomaly recorded.	*Definition of abnormal imaging*: IVH, periventricular hyperechogenicity, Subependymal haemorrhage		
† **Cherouny (1989) (Cherouny 1989)**	Cohort, retrospective, consecutive	*Location:* USA *Year:* 1980-1987 *Description*: 20 multiple pregnancies with sIUFD >20 weeks or birthweight >350g. We excluded 1 which was a triplet pregnancy, 4 which were MA, and 3 which chorionicity was not determined therefore 12 pregnancies were analysed. Histological confirmation of chorionicity recorded. *PTB information*: NA *Additional*: Confounders of TTTS and IUGR recorded, fetal anomaly and maternal illness not recorded	NA	NA	Not reported
† **D'Antonio (2017) (D'Antonio 2017b)**	Cohort, retrospective, consecutive	*Location:* UK *Year:* 2000-2010 *Description*: 3013 twin pregnancies, of which 65 had a sIUFD and the authors excluded 3 with dIUFD but provided insufficient details to include in co-twin IUFD outcome. The authors excluded TTTS, TOP, chromosomal anomalies, MA, HOM, miscarriage <24 weeks, dIUFD and sIUFD >34 weeks therefore we only included the study in PTB analysis. Histological confirmation of chorionicity recorded. *PTB information*: grouped to <34, <32 and <28 weeks therefore first group used for analysis. Spontaneous or iatrogenic not clear. *Additional:* Information on confounder maternal illness not recorded.	NA	NA	Not reported

† **Deveer (2013) (Deveer 2013)**	Cohort, prospective, not reported	*Location:* Turkey *Year:* Not reported, but over 21 months *Description:* 47 IUFDs but 9 women discontinued follow-up after diagnosis of IUFD therefore not included, 13 IUFDs diagnosed in the first trimester and therefore not included, 25 cases 2nd and 3rd trimester IUFDs included. Chorionicity determined on USS. *PTB information*: no definition given therefore could not include *Additional:* Information on confounders TTTS but no mention of intervention, maternal illness and congenital anomaly recorded. 1 fetus with a congenital anomaly included, but unable to identify and remove from analysis, therefore included.	NA	"neurological impairment" no further details given, did not report on whole cohort, just TTTS patients therefore could not include	Not reported
† **Dias (2011) (Dias 2011)**	Cohort, retrospective, consecutive	*Location:* UK *Year:* 1997-2008 *Description*: 147 MCDA pregnancies. 19 with sIUFD but we excluded 1 TRAP, 1 who underwent FLA for TTTS, and 1 with a congenital malformation, therefore 16 included in analysis. Placenta examined histologically to confirm chorionicity. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Confounders of TTTS and IUGR recorded, but maternal illness not.	NA	NA	Not reported
† **Farah (2012) (Farah 2012)**	Cohort, retrospective, not reported	*Location:* Southern Ireland *Year:* 1999-2007 *Description*: 208 uncomplicated MCDA twin pregnancies, 9 twin pregnancies included with sIUFD. Placenta examined histologically to confirm chorionicity. Authors excluded TTTS, IUGR, growth discordance, TRAP and structural anomalies. *PTB information*: NA *Additional*: Confounder of maternal illness not recorded	NA	NA	Not reported

Fichera (2009) (Fichera 2009)	Cohort, retrospective, not reported	*Location:* Italy *Year:* 2001-2006 *Description*: 23 twin pregnancies with sIUFD, we excluded 1 who underwent termination of pregnancy, and did not include the DC twin pregnancies as a high proportion (3/10) had chromosomal anomalies. Study also excluded MCMA twins and sIUFD in first trimester. Chorionicity determined by USS. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Confounder of TTTS with 1 patient undergoing FLA and 4 amniodrainage was recorded although it was not possible to remove the pregnancy which underwent FLA. sIUGR and maternal illness also recorded.	*Mode of imaging*: antenatal and postnatal USS *When imaged*: 'prenatal' or 'neonatal' *Definition of abnormal imaging*: severe IVH, bilateral ventriculomegaly, PVH, periventricular hyperechogenicities, calcifications and multilocular cysts on pavements of normal ventricles	Assessed by neuropediatric specialist according to Dubowitz method, Prechtl evaluation, or Griffith's Mental Developmental Scales as appropriate.	Follow up of surviving twin in 18/20 cases (85%). Follow up ranged from 12-56 months
Fusi (1990) (Fusi 1990)	Cohort, retrospective, not reported	*Location:* UK *Year:* 1977-1988 *Description*: 485 twin pregnancies. 11 cases of sIUFD included from first hospital. An additional 5 cases from a second hospital, however 1 MCMA therefore excluded from analysis. Authors excluded if death occurred during labour or within a week of delivery. Death of twin confirmed by USS. Placenta examined histologically to confirm chorionicity *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Maternal illness and IUGR as confounders were recorded but TTTS not mentioned.	NA	Cerebral atrophy, spastic quadriplegia	Not reported
Gaucherand (1994) (Gaucherand 1994)	Cohort, retrospective, not reported	*Location:* France *Year:* 1985-1992 *Description*: 248 multiple pregnancies. The authors excluded 3 cases of dIUFD because they were affected by TTTS thus 10 cases of sIUFD but we excluded the 1	*Mode of imaging*: postnatal USS *When imaged*: not stated but postnatal *Definition of*	Tetraplegia and convulsions, no further information given.	Not reported

		selective termination of pregnancy for Down's syndrome and 1 triplet pregnancy. 8 cases included. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Data on maternal illness, IUGR and TTTS as potential confounders included but definition of latter not clear.	*abnormal imaging*: porencephaly		
† **Gratacós (2004) (Gratacós 2004)**	Cohort, prospective, consecutive	*Location:* not stated *Year:* not stated *Description*: 50 MC twin pregnancies. Authors excluded 8 as underwent cord occlusion, 6 sIUFDs all of which had sIUGR. Chorionicity confirmed on US. Pregnancies with TTTS were excluded. *PTB information*: NA *Additional*: Confounders of fetal anomaly and maternal illness not recorded.	*Mode of imaging*: postnatal USS *When imaged*: all twins at or before 4 days and 28 +/- 7 days of life *Definition of abnormal imaging*: suggestive of parenchymal brain damage, particularly periventricular leukomalacia. "The latter was classified as follows: (I) transient periventricular echodensities for ≥7 days; (II) periventricular echodensities evolving into small localised frontoparietal cystic lesions; (III) periventricular densities evolving	NA	Not reported

			into extensive periventricular cystic lesions; (IV) densities extending into the deep white matter evolving into extensive cystic lesions"		
† **Gratacós (2008) (Gratacós 2008)**	Cohort, retrospective, consecutive	*Location:* Spain and Belgium *Year:* 2003-2006 *Description*: 49 MC twin pregnancies with sIUGR Type III. 31 had expectant management of which 9 had a sIUFD. We did not include the 18 who underwent laser treatment. *PTB information*: NA *Additional*: Confounders of fetal anomaly and maternal illness not recorded	NA	NA	Not reported
† **Griffiths (2015) (Griffiths 2015)**	Cohort, retrospective, not reported	*Location:* UK *Year:* 2004-2013 *Description:* 68 monochorionic IUFDs who underwent fetal MRI. Birth outcome data available for 64. Cohort divided into spontaneous IUFD (n=41) and IUFD post-FLA for TTTS (n=27). We only included spontaneous IUFD group, of which we excluded 2 first-trimester losses and 10 with no pregnancy outcome data. 29 sIUFDs included. Chorionicity determined by USS. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional:* Data on congenital anomaly as confounder included, but not maternal illness.	*Mode of imaging*: antenatal USS and MRI in all pregnancies *When imaged*: 1-12 weeks post-IUFD *Definition of abnormal imaging*: encephalomalacia, ventriculomegaly	NA	Not reported
Hagay (1986) (Hagay 1986)	Cohort, not reported, not reported	*Location:* Israel *Year:* 1969-1983 *Description*: 1192 multiple pregnancies. 21 cases of	NA	NA	Not reported

		sIUFD recorded 1 set triplets and 20 twins. We excluded the 1 case of triplets, 2 MCMA pregnancies, and 7 further cases (2 MC, 5 DC) where the gestational age of sIUFD was not recorded therefore we analysed 11 cases. Placenta examined histologically to confirm chorionicity. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Data on maternal illness and congenital anomaly was recorded but not TTTS.			
† **Hoffmann (2013) (Hoffmann 2013)**	Cohort, prospective, not reported	*Location:* Israel *Year:* 2007-2010 *Description:* 34 monochorionic IUFDs who underwent fetal MRI. 18 were a consequence of selective reduction and 10 underwent FLA for TTTS and were excluded from our analysis. In the 6 with a spontaneous IUFD, we excluded 2 from the co-twin death and PTB outcome as they decided to terminate the pregnancy following antenatal scan. Chorionicity determined by USS. Authors included only included those with sIUFD after 1st trimester. In-utero transfusion was performed in 1 twin included in analysis *PTB information*: NA *Additional:* Information on maternal illness and congenital anomalies not reported.	*Mode of imaging*: antenatal USS and MRI *When imaged*: within 6 days of sIUFD *Definition of abnormal imaging*: acute ischemic lesions	Information obtained from hospital charts and telephone interviews with parents. Sequelae of prematurity and asphyxia. No further information given.	Not reported
Ishimatsu (1994) (Ishimatsu 1994)	Cohort, not reported, not reported	*Location:* Japan *Year:* 1986-1992 *Description*: 100 twin pregnancies. 15 cases of sIUFD included. Death of twin confirmed by USS. Histological assessment placental chorionicity in all cases. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Information on congenital anomaly recorded	*Mode of imaging*: postnatal USS and CT *When imaged*: neonatal *Definition of abnormal imaging*: periventricular leukomalacia,	Cerebral palsy or mental retardation. Assessment methods not described.	Not reported

		but not TTTS or maternal illness.	intracranial haemorrhage, microcephaly, brain atrophy		
† **Jelin (2008) (Jelin 2008)**	Cohort, retrospective, consecutive	*Location:* USA *Year:* 1997-2007 *Description*: 47 MC twin pregnancies with sIUFD undergoing fMRI, The authors excluded 25 who underwent radiofrequency ablation or placental ablation, and the 1 case with no antenatal USS with which to compare the fMRI, therefore 21 sIUFDs in the study. We excluded 2 TRAP pregnancies, and 1 1st trimester loss, thus 18 in our analysis. Due to not reporting the outcome of the pregnancy, this study was only included in the antenatal brain imaging outcome. *PTB information*: NA *Additional*: Confounders of TTTS, fetal anomaly and maternal illness recorded	*Mode of imaging*: antenatal USS and MRI in all pregnancies *When imaged*: 0-12 weeks post sIUFD *Definition of abnormal imaging*: ventriculomegaly, abnormal sylvian fissures, evidence of cerebral injury, cystic changes, haemorrhage	NA	Doesn't report on outcome of pregnancy after antenatal MRI.
Jou (1996) (Jou 1996)	Cohort prospective, consecutive	*Location:* Taiwan *Year:* 1991-1995 *Description*: Monochorionic pregnancies with sIUFD after 20 weeks, 14 cases in cohort. sIUFD confirmed by USS. Authors excluded 2 cases where 2nd twin was terminated following sIUFD, therefore 12 cases included in analysis. Placenta chorionicity determined histologically. 6 pregnancies with TTTS underwent amniodrainage but were included in our analysis. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional*: Presence of TTTS was noted but congenital anomaly and maternal illness was not.	NA	Spastic cerebral palsy	Not reported
Kilby (1994) (Kilby 1994)	Cohort, retrospective, not reported	*Location:* UK *Year:* 1988-1993 *Description*: 342 twin pregnancies 20 cases of sIUFD	NA, results not divided by chorionicity	NA, results not divided by chorionicity	Not reported

		with surviving twin born after 20 weeks; we excluded 5 with congenital anomalies. sIUFD confirmed by USS. Placentas examined histologically for chorionicity. *PTB information*: NA *Additional*: Details on congenital anomaly, karyotyping and maternal illness recorded. 1 case of mild TTTS reported but included in our analysis.			
Krayenbuhl (1998) (Krayenbuhl 1998)	Cohort, retrospective, not reported Cohort	*Location:* Switzerland *Year:* 1984-1994 *Description*: 541 twin pregnancies. 19 cases of sIUFD included. Chorionicity determined histologically. sIUFD in first trimester excluded. *PTB information*: NA *Additional*: Data on TTTS (n=5 cases) but no mention of treatment, and congenital anomaly but not maternal illness. Two pregnancies with congenital anomalies included, but unable to remove as doesn't report the chorionicity of the 2 cases.	NA	NA	Not reported
† **Lewi (2008) (Lewi 2008)**	Cohort, retrospective, consecutive	*Location:* Belgium, Germany *Year:* 2002-2007 *Description*: 202 MCDA twin pregnancies, excluded HOM, 1 TRAP sequence, 1 1st trimester dIUFD, 1 with sirenomelia. 45 had sIUFD but we excluded those who underwent FLA, cord occlusion or termination of pregnancy for TTTS, therefore 13 included in analysis. Placenta examined histologically to confirm chorionicity. *PTB information*: NA *Additional*: Confounders of TTTS, IUGR and fetal anomaly and recorded, but not maternal illness.	*Mode of imaging*: not stated *When imaged*: postnatally *Definition of abnormal imaging*: not stated	NA	Not reported
Lin (1999) (Lin 1999)	Cohort, retrospective, not reported	*Location:* Taiwan *Year:* 1988-1997 *Description:* 302 twin pregnancies. 17infant survivors of sIUFD included. We excluded 5 cases as chorionicity was not known, 2 did not undergo neurological assessment. The study did not include dIUFD therefore	*Mode of imaging*: not stated *When imaged*: postnatally *Definition of abnormal imaging*:	Cerebral palsy	Not reported

		not included in co-twin IUFD outcome. *PTB information*: NA *Additional:* No data on confounders TTTS, congenital anomaly and maternal illness.	IVH, periventricular echogenicity, encephalomalacia		
† **Mahony (2011) (Mahony 2011)**	Cohort, retrospective, consecutive	*Location:* Ireland *Year:*1997-2006 *Description:* 1178 twin pregnancies. 22 sIUFD pregnancies included. Only included IUFDs after 23+6 weeks. Placenta examined histologically to confirm chorionicity. Authors excluded pregnancies with fetal anomalies. *PTB information*: NA *Additional:* data on TTTS reported with several who underwent amniodrainage but were kept in for analysis, IUGR also reported, but not maternal illness.	NA	NA	Not reported
Malinowski (2000) (Malinowski 2000)	Cohort, prospective, consecutive	*Location:* Poland *Year:* 1989-1999 *Description:* 11 cases of sIUFD recorded, we excluded 3 as 1st trimester sIUFD and 2 with congenital malformations. Placenta examined histologically to confirm chorionicity. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear. *Additional:* Data on TTTS but no mention of intervention, congenital anomaly recorded but not maternal illness.	NA	NA	Not reported
Malinowski (2003) (Malinowski 2003)	Cohort Multi-centre, retrospective, not reported	*Location:* Poland *Year:* 1995-1999 *Description*: 295 twin pregnancies. 12 cases of sIUFD *PTB information*: NA *Additional*: Pregnancies all in the third trimester. Data on congenital anomaly, TTTS but no mention of intervention, and maternal illness recorded.	NA	NA	Not reported

† **McPherson (2012) (McPherson 2012)**	Cohort, retrospective, consecutive	*Location:* USA *Year:* 1990-2008 *Description*: 2454 twin pregnancies. 77 twin pregnancies with sIUFD included. Authors excluded MA, TTTS, higher order multiples and IUFD <20 weeks. Placenta examined histologically to confirm chorionicity. *PTB information:* NA *Additional*: Confounders of TTTS, fetal anomaly and maternal illness recorded	NA	NA	Not reported
Petersen (1999) (Petersen 1999)	Cohort, retrospective, not reported	*Location:* Denmark *Year:* 1991-1995 *Description*: 310 twin pregnancies. sIUFD occurred in 28 multiple pregnancies. We excluded 6 as they were a 1st trimester loss, 9 because they were from selective fetal reduction, 1 because the chorionicity was not determined,1 with Fallot's tetralogy, and 1 set of triplets, therefore 10 cases were analysed. Histopathological examination of chorionicity for all cases. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic was reported. *Additional*: Information on TTTS, IUGR, congenital anomaly and maternal illness as potential confounders recorded.	NA	Psychomotor development commented on, but no further details given.	4 out of 8 surviving twins (50%) at mean of 19 months, range 8-51 months.
† **Robinson (2017) (Robinson 2017)**	Cohort, retrospective, not reported	*Location:* Australia *Year:* 2007-2016 *Description*: 33 complicated MC twin pregnancies that had fetal MRIs, 10 of which were spontaneous sIUFDs. Pregnancies with structural anomalies were excluded. We excluded 2 from the co-twin death and PTB outcome as they decided to terminate the pregnancy following antenatal scan. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear *Additional*: Confounders of TTTS,	*Mode of imaging*: antenatal USS and MRI in all pregnancies *When imaged*: 2 weeks post IUFD, usually after 24 weeks gestation *Definition of abnormal imaging*: IVH,	NA	Not reported

		IUGR, fetal anomaly and maternal illness recorded	ventriculomegaly, infarction, abnormal cranial biometry		
† **Rustico (2017) (Rustico 2017)**	Cohort, retrospective, consecutive	*Location:* Italy *Year:* 2004-2012 *Description*: 140 MC twin pregnancies with sIUGR. 18 twin pregnancies included with sIUFD. TTTS and TAPS excluded. We did not include the 20 who had cord occlusion or 5 who had a termination of pregnancy *PTB information*: NA. *Additional*: Confounders of fetal anomaly and maternal illness not recorded	NA	Detailed assessment outlined in article	All surviving infants were followed up as per Italian protocol by paediatric neurologist-psychiatrists. The range of follow up in the study was 1-7 years (median 2 years)
Saito (1999) (Saito 1999)	Cohort, retrospective, not reported	*Location:* Japan *Year:* 1971-1997 *Description*: 481 cases of twins. 30 cases of sIUFD of which we have excluded 3 MCMA and 2 with fetal anomalies. Method of determining chorionicity and gestational age of twin demise not recorded. Amniodrainage performed for TTTS in some cases but not clear which. As the aim of the study was to assess the neonatal outcome of sIUFD twins, the study was not included in the co-twin IUFD outcome as dIUFD were not included. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear *Additional:* Confounders such as TTTS congenital anomaly and maternal illness recorded.	NA	Cerebral palsy, assessment method not stated.	Not reported
Santema (1995) (Santema 1995)	Case-control, prospective, consecutive	*Location:* The Netherlands *Year:* 1973-1993 *Description:* 531 twin pregnancies. 29 cases of sIUFD, confirmed by USS with sIUFD >20 weeks, and >12h	NA	NA	3/22 (16%) lost to follow-up. Median age of FU 4years and 2

		before delivery. Control group was normal twin pregnancies, used to compare outcomes but not include in our analysis. Placentas were examined histologically to determine chorionicity. *PTB information*: NA *Additional*: Data on congenital anomaly, maternal illness recorded but not TTTS.			months range 2 months to 18 years
† **Sebire (1997) (Sebire 1997)**	Cohort, retrospective, not reported	*Location:* UK *Year:* 1992-1996 *Description*: 486 twin pregnancies. 27 twin pregnancies included with sIUFD. Placenta examined histologically to confirm chorionicity. The authors excluded chromosomal anomalies but analysis included 1 MC twin with gastroschisis, and 1 DC twin with arthrogryposis *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear *Additional*: Confounders of TTTS recorded although intervention not mentioned.	NA	NA	Not reported
Szymonowicz (1986) (Szymonowicz 1986)	Cohort, retrospective, not reported	*Location:* Australia *Year*: not stated *Description*: 6 cases of MCDA sIUFDs. How chorionicity determined is not recorded. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear *Additional:* Data on TTTS, congenital anomaly and maternal illness not recorded	*Mode of imaging*: USS and CT *When imaged*: Not stated whether antenatal or postnatal therefore not included in analysis *Definition of abnormal imaging*: parietal / occipital / cerebellar / cortical / parietal-occipital infarcts, hydranencephaly, hydrocephalus.	Cerebral palsy and mental retardation, no other information given.	2 out of 2 cases that survived (100%) at 3 and 5 years of age

† **Tordjeman (1996) (Tordjeman 1996)**	Cohort, retrospective, not reported	*Location:* France *Year:* 1984-1994 *Description*: 21 pregnancies with sIUFD, we excluded 3 triplets, 1 quadruplet, 2 MCMA twins, 3 with congenital anomalies, 2 termination of pregnancy for haemophilia A and TTTS. 10 twin pregnancies included with sIUFD. Placenta examined histologically to confirm chorionicity. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear *Additional*: Confounders of TTTS, IUGR, fetal anomaly and maternal illness recorded	*Mode of imaging*: postnatal USS *When imaged*: not stated but know is postnatal *Definition of abnormal imaging*: severe multicystic encephalopathy	NA	Not reported
van Heteren (1998) (van Heteren 1998)	Cohort, retrospective, consecutive	*Location:* The Netherlands *Year:* 1990-1996 *Description:* Only included sIUFD in MC twin pregnancies with TTTS but did not undergo intervention. 11 cases included. Chorionicity was determined by placental histological. Not included in co-twin IUFD as aim of paper to look at outcomes for child, therefore not dIUFDs included. *PTB information*: gestational age at delivery reported individually thus sub-group possible. Spontaneous or iatrogenic not clear *Additional:* Data on TTTS, congenital anomaly were recorded but not concurrent maternal illness. Did change management for last 2/9 pregnancies due to high comorbidity rate: delivered as soon as possible after sIUFD when compatible with neonatal survival.	*Mode of imaging*: postnatal USS *When imaged*: neonatal but does not state specifically *Definition of abnormal imaging*: periventricular leukomalacia, (transient) periventricular echodensities, multicystic encephaloleucomalacia	Taken from neonatal notes. Psychomotor retardation and spastic tetraplegia. Spastic diplegia. Further information re mode of assessment not stated.	FU of the eight survivors (100%) at 1.5-3 years of age.
† **van Klink (2015) (van Klink 2015)**	Cohort, retrospective, consecutive	*Location:* The Netherlands *Year:* 2002-2013 *Description:* 49 MC pregnancies with sIUFD. Authors excluded sIUFD post-FLA, selective feticide, dIUFD on same day. There was 1 case of triplets that we excluded from all 4 outcomes (co-twin death, antenatal brain imaging, postnatal brain imaging, NND), 5 MCMA twins	*Mode of imaging*: antenatal and postnatal USS and MRI *When imaged*: antenatal diagnosis made at median 26.5	NA	28/47 had postnatal US, of whom 11/28 had MRI as well. All cerebral imaging performed up to 1 year of age

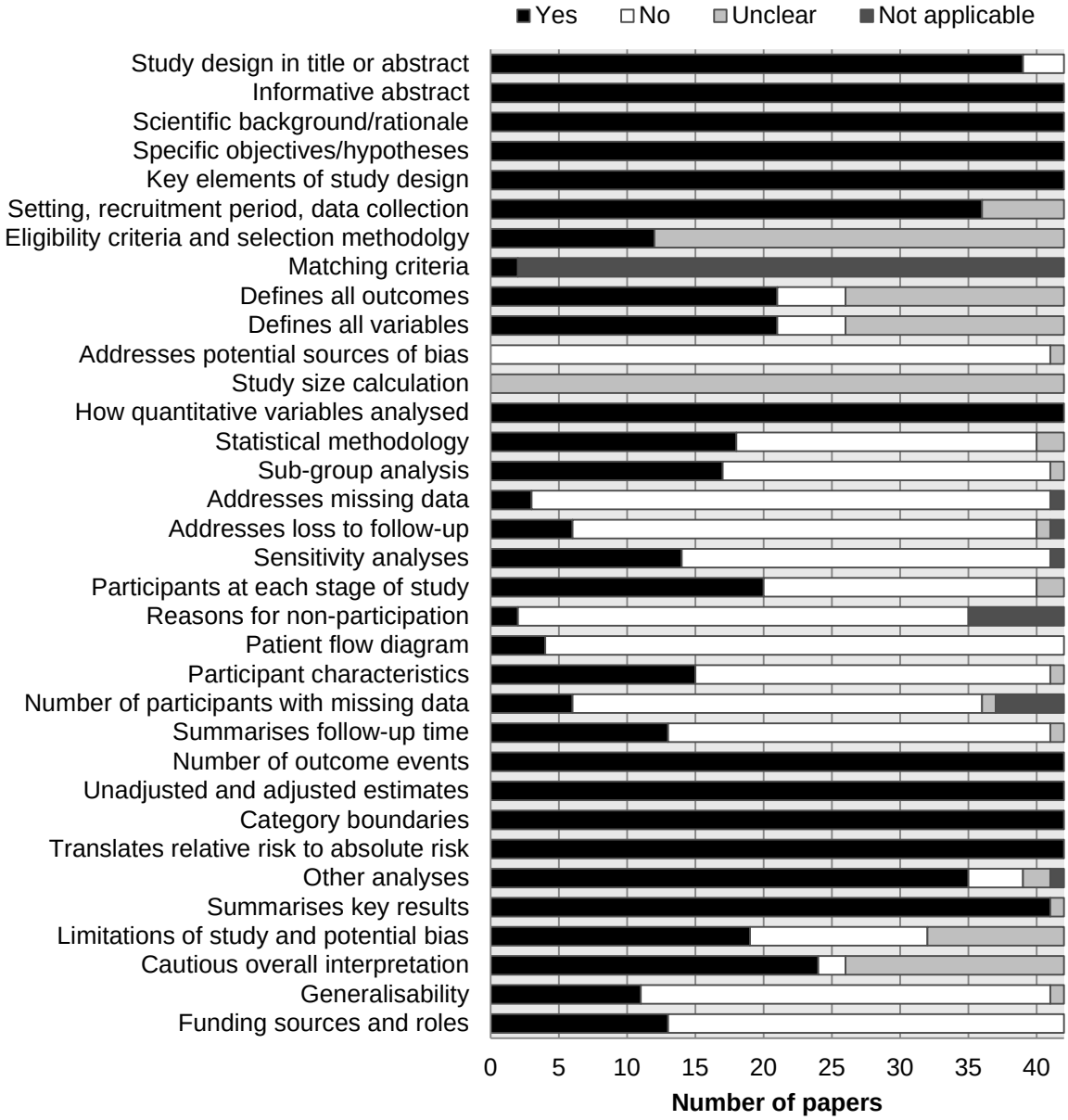
		that we were only able to exclude from co-twin death, 1 with congenital anomalies that we were only able to exclude from co-twin death, and 2 with a termination of pregnancy that were excluded from co-twin death, postnatal brain imaging and NND. 6 underwent in-utero transfusions, but included in analysis as it was not possible to remove them. *PTB information*: NA *Additional:* Data on TTTS and congenital anomalies reported, but no record of maternal illness.	weeks (IQR 22.3-30.8), postnatal imaging performed up to 1 year of age, not all pregnancies scanned *Definition of abnormal imaging*: IVH, parenchymal haemorrhage, cystic periventricular leukomalacia, porencephalic cyst, ventricular dilatation, infarction and hypoxic-ischemic injury of basal ganglia, thalamus and/or cortex. Severe cerebral injury was defined as at least one of: IVH≥Grade III, cPVL≥Grade II, ventricular dilatation≥97th percentile, porencephalic cyst, arterial or venous infarction, basal ganglia, thalamic and/or cortical injury or other severe cerebral lesions		reported.

			associated with an adverse neurological outcome.		
Wang (2000) (Wang 2000)	Cohort, retrospective, consecutive	*Location:* Taiwan *Year:* 1993-1998 *Description*: 9 cases of MC twin with sIUFD. All had a sIUFD associated with TTTS but intervention was delivery therefore the study could not be included in analysis. Not recorded how chorionicity or gestation of sIUFD was derived. *PTB information*: NA *Additional:* No record of potential confounders, maternal illness and congenital anomaly	NA	NA	Not reported
† **Wang (2016) (Wang 2016)**	Cohort, retrospective, random enrolment	*Location*: China *Year:* not stated *Description:* 47 cases of sIUFD, although the study excluded 1st trimester miscarriages and selective reductions, the study did include 2 MCMA pregnancies, 2 terminations of pregnancies and 7/16 cases of TTTS who underwent FLA therefore the study could not be included in analysis. *PTB information*: NA *Additional:* Did report potential confounders of maternal illness, but not congenital anomaly	*Mode of imaging*: postnatal USS *When imaged*: neonatal but does not state specifically *Definition of abnormal imaging:* IVH, ventriculomegaly	NA	FU average of 32 months (range 12-37 months)
Woo (2000) (Woo 2000)	Cohort, not reported, not reported	*Location:* Hong Kong *Year:* 1993-1997 *Description*: 182 twin pregnancies. 7 cases of sIUFD included. We excluded 3 as the chorionicity was not recorded in one case and the gestation of sIUFD was not recorded in 2 cases, therefore the study could not be included in analysis. Chorionicity was confirmed histologically. *PTB information*: NA *Additional:* Confounders; TTTS, maternal illness and congenital anomaly were recorded	NA	NA	All 6 surviving twins were FU (100%) but timings of FU were not recorded.

7.3.4 Risk of bias of included studies

The quality of the included studies is displayed in Figure 7.2. All the studies reported study design and the number of outcome events. None of the studies explained how their sample size was determined and it is likely that a sample of convenience was used. The poor reporting of enrolment methods mean that selection bias is not clear.. The number of participants at each stage of the study was reported in 20/42 (47.6%) studies which may be that selective reporting and attrition bias occurred in some studies and results should be interpreted with caution as it is possible that adverse outcomes were over reported. Only 15/42 (35.7%) studies reported which data were missing, and 19/42 (45.2%) adequately reported the limitations of their study. The funnel plots and Egger's test results are reported in Appendix 10.17; some analyses did suggest small-study effects publication bias which is important when interpreting the results as caution should be heeded.

Figure 7.2 Quality assessment of included studies according to 'Strengthening The Reporting of Observational studies in Epidemiology' (STROBE) checklist



7.3.5 Synthesis of results

As some studies only included MC, or DC twin pregnancies, these studies could only be included in the summary event rate calculation and not in the comparison of MC with DC twin pregnancies. The extracted 2x2 data used to calculate the summary

event rates, and the forest plots not included in the main text comparing MC with DC twin pregnancies for each adverse outcome are in Appendix 10.16. Reasons why data have not been included in analysis are described in Table 7.1.

7.3.6 Summary event rates

Table 7.2 Summary event rates and odds ratio of adverse outcome in surviving co-twin following sIUFD in monochorionic and dichorionic twin pregnancies

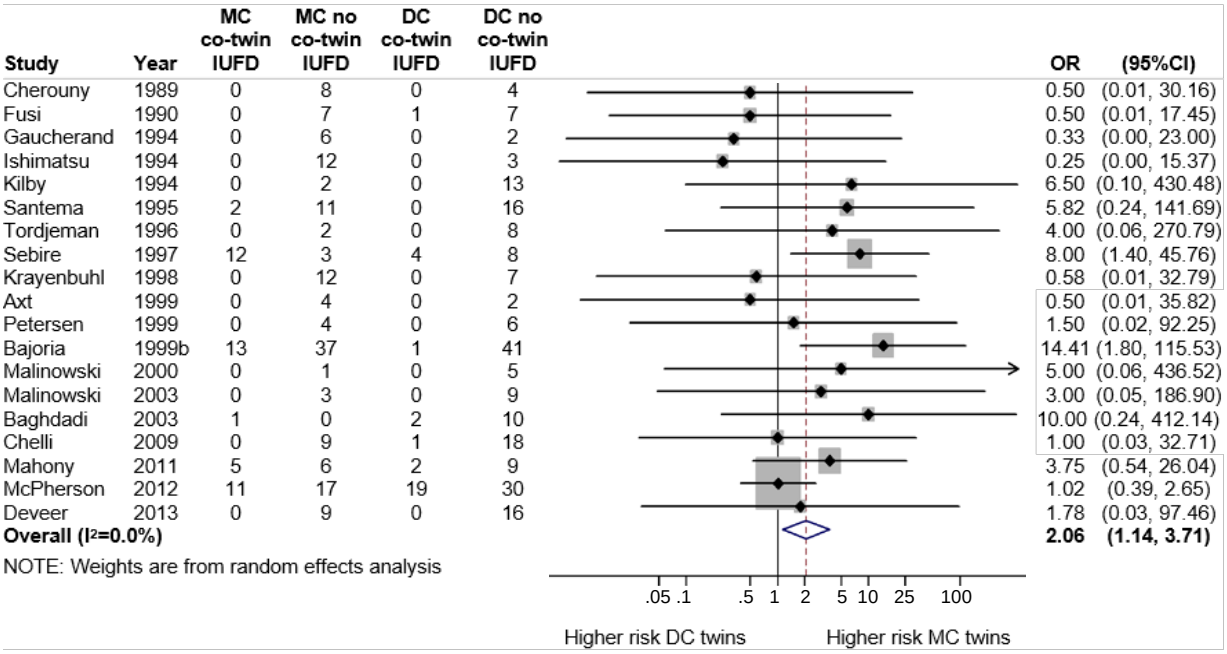
fMRI: fetal magnetic resonance imaging, NP: not possible to calculate odds ratios. p value in the OR column denotes the significance of OR=1. Statistically significant results are in bold.

Adverse outcome	Monochorionic event rate	Dichorionic event rate	Odds ratio [95%CI] comparing MC v DC
Co-twin intra-uterine fetal death	41.0% [95%CI 33.7, 49.9] I^2=44.2%, 32 studies, 379 pregnancies	22.4% [95%CI 16.2, 30.9] I^2=21.7%, 20 studies, 255 pregnancies	**2.06 [95%CI 1.14, 3.71] p=0.016, I^2=0.0%, 19 studies, 441 pregnancies**
Preterm birth	58.5% [95%CI 48.2, 70.9] I2=11.7%, 20 studies, 202 pregnancies	53.7% [95%CI 40.8, 70.6] I^2=0.0%, 12 studies, 107 pregnancies	1.42 [95%CI 0.67, 2.99] p=0.356, I^2=1.5%, 10 studies, 167 pregnancies
Abnormal antenatal brain fMRI	20.0% [95%CI 12.8, 31.1] I^2=21.9%, 6 studies, 116 pregnancies	NP	NP
Abnormal postnatal brain imaging	43.0% [95%CI 32.8, 56.3] I^2=12.4%, 12 studies, 140 pregnancies	21.2% [95%CI 10.6, 42.4] I^2=0.7%, 7 studies, 75 pregnancies	**5.41 [95%CI 1.03, 28.58] p=0.047, I^2=45.8%, 7 studies, 142 pregnancies**

Adverse outcome	Monochorionic event rate	Dichorionic event rate	Odds ratio [95%CI] comparing MC v DC
Neuro-developmental comorbidity	28.5% [95%CI 19.0, 42.7] I^2=0.0%, 13 studies, 103 pregnancies	10% [95%CI 3.9, 27.7] I^2=0.0%, 8 studies, 62 pregnancies	3.06 [95%CI 0.88, 10.61] p=0.08, I^2=0.0%, 8 studies, 129 pregnancies
Neonatal death	27.9% [95%CI 21.1, 36.9] I^2=0.0%, 18 studies, 206 pregnancies	21.2% [95%CI 14.5, 31.2] I^2=0.0%, 12 studies, 130 pregnancies	1.95 [95%CI 1.00, 3.79] p=0.051, I^2=0.0%, 11 studies, 232 pregnancies

The co-twin survivor in MC twin pregnancies was at significantly higher risk of co-twin IUFD (Table 7.2, Figure 7.3) and abnormal postnatal brain imaging than co-twin survivors in DC twin pregnancies. No significant difference was found between MC and DC twin pregnancies in the rate of PTB, neurodevelopmental comorbidity or NND, although the latter outcome was borderline significant. The rate of abnormal antenatal brain imaging in MC twin pregnancies was 20%, but as no studies were found reporting this outcome in DC twin pregnancies, the OR was not calculated. Additional forest plots and extracted 2x2 data are shown in Appendix 10.16.

Figure 7.3 Forest plot comparing the risk of co-twin intrauterine fetal death (co-twin IUFD) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies



7.3.7 Sub-group analysis

Sub-group analysis demonstrated that in MC twin pregnancies, those with an sIUFD <28 weeks were significantly more likely to have a co-twin IUFD than those with an sIUFD ≥28 weeks. The pathologies of TTTS and IUGR were not associated with an increased risk of co-twin IUFD (Table 7.3). Pregnancies complicated by TTTS were significantly more likely to have a PTB than twin pregnancies without TTTS. It was not possible to calculate ORs to examine if there was a significant difference in the proportion of PTB which were spontaneous or iatrogenic as the proportions were not known for the births which were not preterm, however the summary event rates may suggest a difference (Appendix 10.16). When PTB was divided according to whether it was iatrogenic or spontaneous, in MC twins the summary event rate of iatrogenic PTB was 60.4% ([95%CI 33.5, 109.1] I^2=0.0%, 3 studies, 7 pregnancies) compared

to a spontaneous PTB rate of 37.1% % ([95%CI 20.5, 66.9] I^2=24.1%, 3 studies, 4 pregnancies). In DC twins the summary event rate of iatrogenic PTB was 32.4% ([95%CI 14.6, 72.1] I^2=32.7%, 3 studies, 6 pregnancies) compared to a spontaneous PTB rate of 70.7% ([95%CI 31.8, 157.4] I^2=0.0%, 3 studies, 6 pregnancies), although the wide 95% CIs and low numbers of included pregnancies should be noted. Other sub-group analysis in DC twins was limited due to small numbers, but the following analyses were possible, none of which found a significant difference: sIUFD <28 weeks did not affect co-twin IUFD, PTB, abnormal postnatal brain imaging, neurodevelopmental comorbidity or NND; IUGR did not affect co-twin IUFD or PTB, neurodevelopmental comorbidity or NND; PTB did not affect abnormal postnatal brain imaging, neurodevelopmental comorbidity or NND.

Table 7.3 Significant results for sub-group analysis of adverse outcomes in surviving co-twin following single intrauterine fetal death in monochorionic twin pregnancies

Summary event rates for each sub-group are presented, and the significant odds ratio (OR) comparing the two sub-groups fMRI: fetal magnetic resonance imaging, GA: gestational age, IUGR: intrauterine growth restriction, NA: not applicable as a sub-group for outcome, NP: not possible to calculate odds ratio, NS: not statistically significant, PTB: preterm birth, TTTS: twin-twin transfusion syndrome, USS: ultrasound scan. p value in the OR column denotes the significance of OR=1. Note TTTS and IUGR were conservatively managed. Statistically significant results are in bold.

Adverse outcome	GA of sIUFD <28 weeks	TTTS	IUGR	PTB versus no PTB
Co-twin intra-uterine fetal death	60.6% ([95%CI 45.8, 80.2] I^2=30.4%, 14 studies, 114 pregnancies) 29.6% ([95%CI 19.2, 45.6] I^2=0.0%, 15 studies, 85 pregnancies) **OR 2.31 ([95%CI 1.02, 5.25] p=0.046, I^2=0.0%, 12 studies, 184 pregnancies)**	NS	NS	NA

Adverse outcome	GA of sIUFD <28 weeks	TTTS	IUGR	PTB versus no PTB
Preterm birth	NS	74.9% ([95%CI 54.0, 103.8] I^2=0.0%, 6 studies, 36 pregnancies) 43.3% ([95%CI 32.5, 57.6] I^2=76.0%, 7 studies, 47 pregnancies) **OR 3.48 ([95%CI 1.17, 10.84] p=0.03, I^2=0.0%, 6 studies, 80 pregnancies)**	NS	NA
Abnormal antenatal brain imaging	NP	NP	NP	NA
Abnormal postnatal brain imaging	NS	NP	NS	NS
Neurodevelopmental comorbidity	NS	NS	NS	NS
Neonatal death	55.0% ([95%CI 36.4, 83.1] I^2=0.0%, 10 studies, 47 pregnancies) 25.2% ([95%CI 15.9, 40.0] I^2=0.0%, 12 studies, 76 pregnancies) **OR 2.84 ([95%CI 1.18, 6.77] p=0.019, I^2=0.0%, 10 studies, 117 pregnancies)**	NS	34.5% ([95%CI 23.5, 50.6] I^2=68.5%, 7 studies, 26 pregnancies) 25.3% ([95%CI 19.2, 33.4] I^2=0.0%, 7 studies, 50 pregnancies) **OR 4.83 ([95%CI 1.14, 20.47] p=0.03, I^2=0.0%, 6 studies, 60 pregnancies)**	41.9% (95%CI 33.6, 52.3] I^2=19.4%, 12 studies, 79 pregnancies) 11.3% (95%CI 8.6, 15.0] I^2=24.1%, 11 studies, 49 pregnancies) **OR 4.95 ([95%CI 1.71, 14.30] p=0.003, I^2=0.0%, 11 studies, 124 pregnancies)**

All six MC twin pregnancy studies which reported antenatal brain imaging compared fMRI with fetal ultrasound in the same pregnancy (Jelin 2008, Fichera 2009, Hoffmann 2013, Griffiths 2015, van Klink 2015, Robinson 2017). Ultrasound "missed" 6/19 (31.5%) lesions detected on fMRI in 3 studies (Jelin 2008, Hoffmann 2013, Robinson 2017) although this difference was not statistically significant. The other 3 studies demonstrated concordance between the two imaging modalities (Fichera 2009, Griffiths 2015, van Klink 2015). In abnormal postnatal brain imaging, it was not possible to perform sub-group analysis based on the imaging modalities of MRI or CT scan as 2 studies used ultrasound and MRI (Gaucherand 1994, van Klink 2015), 1 study used ultrasound and CT (Lin 1999), and 2 studies did not state the mode of imaging (Gratacós 2004, Lewi 2008). The rate of NND was higher in MC twin pregnancies where the initial sIUFD occurred <28 weeks gestation, in those with IUGR, and those with a PTB. No factors affected the risk of adverse outcome in DC twin survivors. It was not possible to calculate ORs for the year of publication sub-group analysis.

7.3.8 Publication bias

The funnel plots for co-twin IUFD, PTB, abnormal postnatal brain imaging and neurodevelopmental comorbidity appear asymmetrical and Egger's test suggests small-study effects publication bias may exist in MC and the DC twins (Appendix 10.17).

7.4 Discussion

7.4.1 Main findings

Abnormal antenatal brain imaging following sIUFD has not previously been meta-analysed; this review reports a rate of 1 in 5 surviving MC co-twins demonstrating abnormal antenatal brain imaging, which doubled on postnatal brain imaging. NND was another novel outcome in this review with a rate of almost 3 in 10 surviving MC co-twins resulting in a NND, and 2 in 10 DC co-twins being reported. In MC twins, if the initial sIUFD occurred at <28 weeks gestation, this significantly increased the rate of co-twin IUFD and NND compared to pregnancies in which the initial sIUFD occurred >28 weeks. The presence of TTTS was associated with a significant increase in the rate of PTB, but no other adverse outcome.

7.4.2 Strength and limitations

This rigorous and robust systematic review provides clinicians and parents with the most up to date rates of complications in the surviving twin following spontaneous sIUFD as reported by the literature. It also allows more tailored counselling, for example, depending on the gestation of the initial sIUFD. According to international guidance (Morin 2011, NICE 2011, ACOG 2016, Khalil 2016b, Kilby 2016, RANZCOG 2017), MC twins should be scanned at a minimum frequency of every 2 weeks, and DC twins every 4 weeks, therefore it is possible that some cases of co-twin IUFD have been missed by studies as there may appear to be a double IUFD at the subsequent ultrasound scan, although the surviving co-twin may have been alive

for a substantial period following the initial sIUFD. Some of the sub-group analysis was limited because these data were not reported by the included studies. For example it was not possible to perform the sub-group analysis based on year of publication, thus the inclusion of older studies with different antenatal care guidance and neonatal care provision may increase the risk of heterogeneity. Ideally for the PTB outcome, further analysis would have been performed using cut-offs of 24-28, 28-32 weeks etc. as the review definition of <34 weeks was somewhat crude, however there were insufficient numbers of pregnancies to do this. It would also be more clinically useful if the gestation of sIUFD could be more specific than before or after 28 weeks, but this would require individual patient data. There was a myriad of differences between studies reporting brain imaging findings, including different referral criteria, different timing of antenatal imaging which varied from 0-12 weeks post IUFD, different imaging modalities, antenatal imaging findings were rarely linked to postnatal imaging findings and neurodevelopmental comorbidity, follow-up was poor and no studies were found reporting antenatal brain imaging in DC twins. Different methods of assessing neurodevelopment were used, making interpretation difficult. The results of this meta-analysis are not applicable to women in low-income countries as most studies include populations from developed countries.

7.4.3 Interpretation

When co-twin IUFD is viewed in the context of the summary event rates, the rate appears higher in both MC and DC twins compared to the previous review from 2011 (Hillman 2011). Caution is advised when interpreting this result as it is possibly an

overestimate. This may be because of the existence of small-study effects publication bias in this outcome, and it is likely that there is selective bias as authors are more likely to report adverse outcomes than normal outcomes. Nevertheless, these event rates are the most recent data available and 10 additional studies have been published since the previous review. The smaller 95%CI when comparing co-twin IUFD between chorionicities suggests that the most recent results are more realistic, and the increased rate seen in MC twins compared to DC twins is to be expected given the presence of vascular anastomoses in the former. The significant difference may also be a consequence of an improved ability to determine chorionicity, better knowledge, and changes in monitoring over time. The lack of difference in adverse outcome, including co-twin IUFD, in TTTS pregnancies may be because of excluding TTTS pregnancies undergoing FLA or BCO, thus there was a higher proportion of milder cases of TTTS. This was different to the previous review but as the treatment for TTTS has advanced dramatically, its use is more widespread since 2011, and there are different confounding factors compared to in spontaneous sIUFD, it was important to include this restriction. TTTS was associated with an increased PTB rate, although it was not possible to determine if they were spontaneous or iatrogenic. No difference was found in PTB between MC and DC surviving co-twins, suggesting that the mechanism of PTB in these cases is not inherent to chorionicity or vascular anastomoses, but to factors common to all twin pregnancies. With regards to abnormal antenatal and postnatal brain imaging, these results are difficult to interpret for reasons previously outlined. The higher rate of abnormal postnatal brain imaging in MC twins compared to DC twins was expected as it is believed that

when one MC twin dies, acute transfusional events through inter-twin placental anastomoses occur (as reviewed by Mackie 2016) resulting in cerebral injury detectable on postnatal brain imaging in the surviving co-twin. Whereas in DC twins the cause of the cerebral pathology is more likely a result of the pathological condition which killed the other twin, rather than a consequence of the sIUFD. The similarity between chorionicities and sub-group analysis in the neurodevelopmental comorbidity outcome may be due to small study size or a reflection of there being no difference in PTB between the chorionicities. The borderline-significantly higher rate of NND in MC twins compared to DC twins was to be expected, particularly as if the initial sIUFD was <28 weeks, or IUGR or PTB was involved, the rate of NND was significantly higher in MC twins. It would have been interesting to explore the relationship between these factors further, but it was not possible.

7.5 Conclusion

These results will help clinicians counsel parents with a sIUFD and give information based upon chorionicity. The high rate of adverse outcomes highlights the importance of close antenatal surveillance, particularly in MC surviving co-twins, and those in which the sIUFD has occurred at <28 weeks. PTB was the commonest adverse outcome and clinicians and parents should be aware of the high risk of PTB in these pregnancies, and the potential NNU admission. Outcomes regarding brain imaging and neurodevelopmental comorbidity are an important area for future research as this outcome is important to parents and will affect the quality of life of

not only the surviving twin, but also other family members. The high rate of 20% of co-twins with an abnormal antenatal fMRI highlights that parents should always be offered antenatal brain imaging. In line with these findings, and those of the MERIDIAN study, the imaging modality should be fMRI not ultrasound (Griffiths 2017). A study is needed examining antenatal and postnatal brain imaging and neurodevelopmental comorbidity in the same surviving co-twins, in a standardised manner, with adequate follow-up. The studies included in this meta-analysis were small and small study effects were shown to exist, consequently the authors have recognised the need to perform a large population-based study and are in the process of conducting a study using data from UKOSS. This will be the largest study of complications in the surviving co-twin in a population cared for using the same national guidance (for further details see (UKOSS 2016)).

CHAPTER 8 PARENTAL ATTACHMENT AND DEPRESSIVE SYMPTOMS IN PREGNANCIES COMPLICATED BY TWIN-TWIN TRANSFUSION SYNDROME

- These findings have been accepted for publication in full article form [Mackie FL, Pattison H, Jankovic J, Morris RK, Kilby MD (2019). “Parental attachment and depressive symptoms in pregnancies complicated by twin-twin transfusion syndrome.” BMC Pregnancy Childbirth]

8.1 Overview

There has been little research on the psychological impact of TTTS on mothers and fathers antenatally and postnatally. As the disease process is so morbid, it is probable that there are emotional effects for both parents, antenatally and postnatally but these have been poorly described. This chapter will investigate maternal and paternal antenatal fetal attachment and postnatal infant attachment in pregnancies complicated by TTTS. Parental depression will also be explored in this setting, and an association between parental attachment and depression will be evaluated.

8.1.1 Psychological and emotional issues in TTTS pregnancies

Twin pregnancies are known to generate complex emotional and practical demands on parents compared to singleton pregnancies even in the absence of complications (Nys 1998, Beretta 2007, Wenze 2015). However, TTTS is a highly morbid condition

that carries a risk of neurological comorbidity, and dIUFD or sIUFD, even with antenatal treatment (see section 1.2.6). These risks continue throughout pregnancy which is why MC twin pregnancies are closely monitored. Regular sequential ultrasound monitoring may reassure parents by allowing timely detection of abnormality, but conversely can cause concern that a complication is going to be detected (Laxton-Kane 2002, Righetti 2005, Yarcheski 2009, Atluru 2012). The unusual scenario where both twins are at risk, often to varying degrees, and one twin may die and one twin may survive, or one or both may be handicapped even after FLA means that parents may face difficult paradoxical situations. Studies performed on parents of MC twin pregnancies affected by sIUFD have reported that as MC twins are identical they find it difficult to cope with grieving for one twin whilst caring for the other, with the reminder of the deceased twin in the surviving twin (Swanson 2009, Richards 2015, Jordan 2018). Consequently women with pregnancies affected by TTTS are at risk of altered materno-fetal attachment and symptoms of depression and anxiety, both antenatally and postnatally, compared to women with uncomplicated MC twins and DC twins (Beauquier-Maccotta 2016, Falletta 2018). There have been no studies examining these potential emotional impacts of TTTS on fathers.

8.1.2 Maternal and paternal antenatal and postnatal attachment

As Mercer states: “The purpose of studying the developing mother-infant relationship is to identify determinants of sensitive, competent mothering that foster healthy child development, and vulnerable populations for intervention” (Mercer 1993a). Laxton-

Kane describes that: “Pre- and postnatal attachment may require different conceptual frameworks” as there is reciprocation with postnatal attachment, which may affect both maternal and paternal attachment to the fetus/infant (Laxton-Kane 2002). Fetal attachment is known to occur antenatally in mothers and fathers through fantasy before the infant is born, and to increase with gestational age and continue into the postnatal period (Leifer 1977, Cranley 1981, Heidrich 1989, Raphael-Leff 2001, Condon 2013), with a positive correlation reported between maternal antenatal attachment and subsequent maternal postnatal attachment (Müller 1996, van Bussel 2010, Dubber 2015). In fathers, antenatal attachment has been demonstrated to be the strongest predictor of postnatal attachment, with paternal depression thought to be the second strongest predictor of decreased attachment (Ferketich 1995b, Hjelmstedt 2008). Conflicting results have been reported when comparing levels of attachment in mothers and fathers. Some studies report higher levels of attachment in mothers compared to fathers (Mercer 1988, Lorensen 2004, Ustunsoz 2010, Kaur 2017), particularly antenatally, with speculation that the difference may be due to the mother being able to feel physical cues from the fetus, whereas for the father the absence of the ’real’ child may affect his ability to form a bond with the fetus (Colpin 1998). Other studies have reported higher attachment in fathers (Schodt 1989, White 1999) and one study reported no difference in antenatal or postnatal maternal and paternal attachment (Wilson 2000).

8.1.3 Importance of parental attachment

Materno-fetal attachment positively influences maternal health choices antenatally including dietary, smoking, alcohol and drug-taking decisions, and thus may affect neonatal outcome (Alhusen 2008). Parento-fetal and parento-infant attachment is important as it shapes parental postnatal behaviour (Siddiqui 2000, Condon 2013); in the short term improving early infant development (Ard 2000, Damato 2000, Alhusen 2008). In the long term parental attachment is associated with the child's feeling of self-worth, ability to explore and deal with their environment, and their future capability of forming relationships (Ainsworth 1979, Bowlby 1982, Egeland 1984, Damato 2000).

8.1.4 Parental attachment in TTTS pregnancies

A high-risk pregnancy may decrease the mother's ability to make psychologically adaptive changes to the pregnancy, thus preventing antenatal attachment for fear the fetus may not survive (Moore 1983, Kemp 1987, Stainton 1992). This protective mechanism may exist in pregnancies affected by TTTS. Only one study has explored materno-fetal attachment in TTTS pregnancies (Beauquier-Maccotta 2016). Beauquier-Maccotta et al. compared pregnancies with TTTS to uncomplicated MC twins, and DC twins in France. They found that materno-fetal antenatal attachment increased during pregnancy in mothers with uncomplicated MC and DC twins, but not in mothers with pregnancies complicated by TTTS. Postnatal attachment was not examined, and the investigators did not distinguish between the 22 women with

TTTS pregnancies with 2 survivors, and 9 women with TTTS pregnancies with 1 survivor.

8.1.5 Maternal and paternal antenatal and postnatal depression

Parents of twins are generally reported to have higher rates of postnatal depressive symptoms compared to parents of singletons (Wenze 2015). Fewer studies have explored parental antenatal depression in twin pregnancy, and results are conflicting when mothers and fathers of twins are compared to singletons (Wenze 2015). When mothers and fathers of twins were compared to each other, mothers appear to experience higher postnatal distress, and lower well-being levels than fathers (Zanardo 1998, Baor 2004) although little research has been conducted in the antenatal period, and in comparing parents of MC and DC twins. It is interesting to note that fathers whose partners scored highly on the Edinburgh Postnatal Depression Scale (EPDS) i.e. reported greater depressive symptoms, scored more highly than fathers whose partners scored lower on the EPDS (Lovestone 1993, Morgan 1997, Matthey 2001, Paulson 2010, Underwood 2017) suggesting there is a link between maternal and paternal postnatal depression in couples (see section 8.2.2 for more information on the EPDS). The most important risk factor for postnatal depression, in mothers and fathers, is believed to be antenatal depression (Becker 2016).

8.1.6 Importance of parental depression

Maternal postnatal depression affects 10-15% of the general obstetric population, and can lead to self-harm, suicide and infanticide (Palumbo 2017). Antenatally, maternal depression and anxiety can have effects on the fetus as well as the mother as they are associated with LBW, PTB, and pre-eclampsia (Bonari 2004, Grote 2010, Grigoriadis 2013) thus compounding the increased background risk MC twin pregnancies have of these complications. In the longer term maternal antenatal and postnatal depression is negatively associated with child development, and an increased number of behavioural problems (Murray 1992, Cummings 1994, Bonari 2004, Grigoriadis 2013). Paternal postnatal depression is associated with behavioural problems, with children of fathers with depression displaying significantly more hyperactivity and conduct disorders at 3.5 years of age compared to children whose fathers were not depressed, including when maternal depression was adjusted for (Ramchandani 2005). Paternal postnatal depression has an important impact on the whole family, and there is a tendency of high attrition rates of fathers in postnatal depression studies meaning evidence is lacking (Ramchandani 2008, Oladosu 2012).

8.1.7 Parental depression in TTTS pregnancies

There is a dearth of research on parents of TTTS pregnancies and depression, particularly fathers. Beauquier-Maccotta et al. reported at 20 weeks gestation when TTTS was diagnosed, the total mean EPDS score of the mothers in the TTTS group (mean 12.06 +/- 5.58) was significantly higher than the score of mothers in

gestationally-matched uncomplicated MC (5.68 +/- 5.00) and DC twin (5.40 +/- 4.64) pregnancy groups, with 72% of the TTTS group scoring above the cut-off of 11 for major depressive symptoms in French speaking women (Beauquier-Maccotta 2016) (Table 8.1). At 30 weeks gestation there was no significant difference between the groups, and the percentage of mothers with TTTS pregnancies who scored above the cut-off decreased to 9%. At 3 months postnatally the TTTS group reported the highest rate of depression again, with an increase to 33%. Sub-group analysis was not performed according to pregnancy outcome.

Table 8.1 Percentage of women with Edinburgh Postnatal Depression Scale scores >11 at each time point

Comparing TTTS pregnancies with uncomplicated monochorionic (MC) and dichorionic (DC) twin pregnancies (taken from (Beauquier-Maccotta 2016)). The number of women in each group at each time point is not clear, hence only able to provide percentages, but there were 77 women in the study in total.

	At diagnosis of TTTS	**20 weeks gestation**	**26 weeks gestation**	**30 weeks gestation**	**3 months postnatal**
TTTS	68	72	25	9	33
Uncomplicated MC	NA	20	22	33	25
Uncomplicated DC	NA	5	11	11	9

Another study reported high rates of depressive symptoms in 350 women in the USA with a pregnancy complicated by TTTS: 11.4% of women reported ‘some’ or ‘serious’ depressive symptoms before pregnancy, 66.0% during pregnancy, and 67.7% after

pregnancy (Falletta 2018). However, one limitation of this study was the retrospective design as participants were recruited following completion of their TTTS pregnancy, as the authors highlighted, a prospective study is warranted. A recent study from Belgium which surveyed 92 women at a median of 7 years post-FLA for TTTS self-reported a higher rate of “psychological and emotional” problems compared to mothers of uncomplicated MCDA twin pregnancies (n=107) (Vergote 2018). These problems were defined as “anxiety concerning health of the baby, fear for repetition; grieving the loss of a child, feelings of guilt”; and there was another group described as “others”. They did not demonstrate a significant difference in “depressed mood” between the 2 groups, although as the authors state, one limitation of their study was the self-reported nature of their survey and lack of standardised tools for assessing depressive symptoms.

8.1.8 Parental depression in TTTS pregnancies according to pregnancy outcome

The American TTTS cohort was divided by outcome and they found that the highest percentage of women with depressive symptoms during pregnancy were those with a sIUFD (46/57, 80.7%) (Falletta 2018). The rate of depression in the sIUFD women increased postnatally (52/57, 92.1%), however the highest percentage of women with depressive symptoms postnatally were those with a dIUFD (48/49, 98.0%). Interestingly, even in the double survivor group there were high rates of mothers who experienced depressive symptoms during pregnancy following diagnosis of TTTS (132/199, 66.2%), which remained high (107/199, 53.8%) postnatally, much higher than before pregnancy, indicating that TTTS is associated with depression,

irrespective of pregnancy outcome. These rates are substantially higher that those reported in uncomplicated twin pregnancies (Wenze 2015). There have been no studies looking at this in fathers.

8.1.9 Association between maternal and paternal attachment and depression

Depression has a negative impact on antenatal materno-fetal attachment (Condon 1997, Martins 2000, Alhusen 2008) and postnatal materno-infant attachment (Goecke 2012, Dubber 2015) in low-risk singleton pregnancies. Colpin et al. demonstrated that a higher quality of antenatal materno-fetal attachment was associated with a higher “psychosocial wellbeing” as measured by the General Health Questionnaire (GHQ-30) in women with twin pregnancies (Colpin 1998), though this was not the case in the fathers. Other studies have demonstrated a relationship between paternal postnatal depression and paternal postnatal attachment, although there has been little research exploring this (Ferketich 1995a). In TTTS pregnancies, there was no difference in materno-fetal attachment between mothers with and without depressive symptoms at diagnosis of TTTS (Beauquier-Maccotta 2016). In mothers with depressive symptoms there was no correlation between antenatal attachment and EPDS scores in any group at any gestation. Postnatal attachment in fathers was not investigated.

8.1.10 Aims

The aims of this study are to investigate in a cohort of parents with pregnancies complicated by TTTS if:

1) There is a difference in parental attachment, and depressive symptoms, in mothers compared to fathers.
2) Parental attachment, and maternal and paternal depressive symptoms change at diagnosis of TTTS, following FLA, and postnatally.
3) There is a difference in parental attachment, and depressive symptoms, in mothers and fathers who have a past/current history of mental health problems.
4) There is an association between parental attachment and depressive symptoms.

Hypotheses

In the context of pregnancies complicated by TTTS:

1) Mothers will report higher levels of parental attachment, and more depressive symptoms, than fathers, particularly antenatally.
2) Parental attachment will increase, and depressive symptoms will decrease, antenatally to postnatally.
3) Mothers and fathers who have a past/current history of mental health problems will report lower parental attachment and greater depressive symptoms than those with no past/current history of mental health problems.
4) There will be an inverse relationship between parental attachment and depressive symptoms in mothers and fathers.

8.2 Methods

8.2.1 Participants

Women with MCDA twin pregnancies, and their partners, who were referred to the West Midlands Fetal Medicine Centre for FLA for TTTS at <24 weeks gestation were prospectively and consecutively recruited between 28/1/2016 and 5/9/2017, and follow-up continued to 1/2/2018. Both the woman and her partner had to provide individual written informed consent to participate. If a woman attended the initial consultation or came for FLA without her partner, they were not eligible for recruitment as the couple were recruited as a pair. Participants had to be able to read English due to needing to understand the follow-up postal questionnaires. Women with higher order pregnancies, or whose pregnancies were affected by chromosomal/structural anomalies were not eligible for inclusion. If a couple suffered a dIUFD or sIUFD prior to FLA, meaning that FLA would not be performed, they were not eligible for recruitment. Recruits received no compensation for participation.

8.2.2 Measures

In this work, parental attachment refers to either maternal or paternal attachment to the fetus(es) or infant(s) depending on whether the child is in-utero or has been born. The maternal and paternal scores were never combined.

1. Parental Attachment Scale questionnaires

Parental attachment was assessed using four self-reported Attachment Scales created by Condon et al. (Condon 1998, Condon 2013, Condon 2015b, Condon 2015a):

- Maternal Antenatal Attachment Scale (MAAS) 19 items, range of scores 19-95, sub-groups of ‘quality’ and ‘intensity’ of attachment.
- Paternal Antenatal Attachment Scale (PAAS) 16 items, range of scores 16-80, sub-groups of ‘quality’ and ‘intensity’ of attachment.
- Maternal Postnatal Attachment Scale (MPAS) 19 items, range of scores 19-95, sub-groups of ‘quality of attachment’, ‘absence of hostility’ and ‘pleasure in interaction’.
- Paternal Postnatal Attachment Scale (PPAS) 19 items, range of scores 19-95, sub-groups of ‘patience and tolerance’, ‘pleasure in interaction’ and ‘affection and pride’.

A higher score denotes greater attachment. These attachment tools have acceptable internal consistency, test-retest reliability, construct validity and have been demonstrated to be a valid measure of parento-fetal attachment and parento-infant attachment in numerous countries, including English-speaking countries as in this study (Condon 1998, Condon 2013). No studies were found using these questionnaires in twin pregnancies or TTTS due to little research having been conducted in this area, but it was favoured over the Prenatal Attachment Inventory (PAI) (Muller 1993) which although it has been used in twin pregnancies, the PAI has been used less in fathers than the PAAS and PPAS which was believed to be more

important than being validated in twin pregnancy. The PAI is also believed to focus more on attitudes towards pregnancy and motherhood, whereas the scales created by Condon et al. are more of a reflection of attitudes towards the fetus/infant (Alhusen 2008).

No cut-offs were used as low parental attachment is not recognised as a pathological condition, and there are no validated cut-offs. As the antenatal and postnatal sub-groups were different, longitudinal comparison could not be performed between sub-groups. Participants were asked to complete the Attachment Scales per pregnancy, not per fetus/infant, and in the pre-FLA and post-FLA questionnaires the time point of "since the diagnosis of TTTS" was specified, as opposed to the last two weeks as stated in the original questionnaires, thus the wording was amended to reflect this. The reworded questionnaires were piloted on couples with twin pregnancies for sense prior to use in the actual study (see Appendix 10.19 for questionnaires).

2. Edinburgh Depression Scale (EDS) and Edinburgh Postnatal Depression Scale (EPDS)

Depressive symptoms were assessed antenatally and postnatally using the EDS and EPDS respectively, which will be referred to as 'EPDS' irrespective of whether it was used antenatally or postnatally. The EDS and EPDS both consist of the same 10 questions and a 4-point self-rated scale of depressive symptoms. The response to each question corresponds to a score of 0-3, with a total lowest score possible of 0, and maximum score of 30 (Cox 1987). A higher score denotes greater depressive

symptoms. A cut-off of 15 or more was used for maternal antenatal depression to give a sensitivity of 91% and specificity of 95% for indicating major depressive disorders in English speaking women, and 13 or more for maternal postnatal depression (Matthey 2006). Although the questionnaire is validated for use in fathers antenatally a cut-off for major depressive disorders has not been validated (Matthey 2006) so a cut-off of 12 or more was used for paternal antenatal depression as in Ramchandani et al. and Buist et al. (Buist 2003, Ramchandani 2008). A cut-off of 10 or more was used for paternal postnatal depression to give a sensitivity of 71.4% and specificity of 93.8% (Matthey 2001). The EPDS has been validated for use in mothers and fathers antenatally and postnatally including English-speaking parents as in this study (Cox 1987, Murray 1990, Cox 1996, Matthey 2001, Matthey 2006). Due to the rapid disease progression of TTTS, patients are referred to the Fetal Medicine Centre for treatment the following day, thus the pre-FLA and post-FLA EPDS questionnaires were amended to ask about time “since the diagnosis of TTTS” as opposed to in the last 7 days. The postnatal questionnaire asked about the last 7 days.

3. Mental health history questionnaire

Participants were asked to complete several questions on current and past mental health problems, including questions regarding diagnoses, medication and therapy (see Appendix 10.19). At the postnatal time point, participants were asked if they had received any new psychiatric diagnoses, and if they were taking any additional medication or undergoing therapy for any mental health problems since the diagnosis of TTTS.

8.2.3 Procedure

Women and their partners were approached by a trained researcher after they had been consented for FLA by the Fetal Medicine Consultant. This was the day before FLA. The woman and her partner were asked to complete the questionnaire on parento-fetal attachment and depressive symptoms separately. The Attachment and EPDS questionnaires were completed at three time points:

- Pre-FLA: the day prior to FLA (MAAS, PAAS, maternal and paternal EPDS, mental health history)
- Post-FLA: one month following FLA (MAAS, PAAS, maternal and paternal EPDS)
- Postnatal: 6–10 weeks following delivery (MPAS, PPAS, maternal and paternal EPDS, mental health history).

As the West Midlands Fetal Medicine Centre treats patients from a large geographical area, the follow-up questionnaires were posted to participants, with a stamped addressed envelope enclosed. If follow-up questionnaires were not received, a reminder was sent in the post, and contact with the couple was attempted by telephone. The timing of the questionnaires was related to medical care as 4 weeks post-FLA is when a fMRI is advised to assess for brain injury, and 6-10 weeks postnatal allows time for NNU admission if required. If FLA was planned but was not possible intra-operatively, the initial pre-FLA questionnaires were included in analysis, but the post-FLA and postnatal questionnaires were not sent out. If following FLA the couple suffered a dIUFD, double NND, or terminated the whole pregnancy, the post-FLA and/or postnatal questionnaires were not sent out according

to the timing of the double IUFD/NND or termination. This was because the Attachment Scales would no longer be relevant. Those with a sIUFD or single NND were still eligible and were asked to complete the follow-up questionnaires. If a participant was considered at high-risk of suicide based on the answers of the questionnaire, the participant and general practitioner (GP) were contacted. Completed questionnaires were stored in a secure place, and data were anonymised prior to data entry onto the statistical package.

8.2.4 Missing data

If a participant only completed the Attachment Scale, or EPDS portion of the questionnaire, their answers were included for the completed portion. Where one person in the couple did not complete the questionnaire, the answers of the other person were included in the analysis examining either mothers or fathers individually, but the couple was not included in the comparison between mothers and fathers. For single missing answers, median substitution was performed as indicated in Table 8.3, no other imputations were performed.

8.2.5 Statistical analysis

All statistical analysis was performed in Stata (Stata, 2015 Release 13.1 StataCorp, Texas, USA). As this was a sample of convenience, no power calculation was performed.

The skewing and kurtosis of the data were assessed using the *sktest* command (StataCorp. 2013i), and the Shapiro-Wilk test of normality was performed using the

swilk command (StataCorp. 2013k). Descriptive data are reported as medians and IQRs. As the maximum total score of the PAAS was different to the MAAS, MPAS and PPAS, the scores were converted into percentages of the maximum total score to allow comparison.

Box plots were generated using the *graphbox* command (StataCorp. 2013c). The Wilcoxon rank-sum test was performed to compare the maternal and paternal scores at each time point, and those with and without mental health problems, using the *ranksum* command (StataCorp. 2013f). Fisher's exact test was used to compare the proportion of mothers and fathers above the EPDS cut-off at each time point, using the *exact* command (StataCorp. 2013f).

A line plot was generated using the *twoway connected* command (StataCorp. 2013d) to visually display the data longitudinally. To assess any difference between the three time points within the mothers or fathers who completed questionnaires at all 3 time points, the Shapiro-Wilk test of normality was performed, and the presence of outliers was examined using box plots. All data were normally distributed except the paternal pre-FLA EPDS score, and the only outliers were 2 paternal attachment scores pre-FLA. Consequently one-way repeated-measures analysis of variance (ANOVA) was performed using the *anova* command (StataCorp. 2013a) to assess maternal attachment and EPDS scores over time. If a significant difference was found, this was investigated by linear regression using the *regress* command (StataCorp. 2013g). Paternal attachment and EPDS scores were examined by the Kruskall-Wallis test using the *kwallis* command (StataCorp. 2013e).

To evaluate if there was an association between attachment and EPDS scores Kendall's rank correlation coefficients were calculated using the *ktau* command (StataCorp. 2013j). Sub-group analysis was planned to assess the effect of those with past/current mental health problems, and those with one survivor compared to two survivors. A post-hoc sub-group analysis was performed to assess the effect of participants who did not complete the pre-FLA questionnaires before FLA and completed it immediately after FLA instead.

8.2.6 Ethical approval

This study received ethical approval from East Midlands Research Ethics Committee (15/EM/0244) and all participants gave written informed consent (see Appendix 10.12 for patient information sheet and Appendices 10.13 and 10.18 for the consent forms).

8.3 Results

8.3.1 Participant characteristics

Fifty-four women, under 24 weeks gestation, were booked for FLA for TTTS at the West Midlands Fetal Medicine Centre during the 19 month recruitment period, of which 27 couples were approached, and 27 couples (100%) consented and agreed to participate. One couple was missed in the screening process. The other 26/54 women were ineligible to participate for the following reasons: no partner present at appointment (n=16), seen by researcher the morning of FLA therefore insufficient time to complete questionnaire (n=7), dIUFD the following morning/prior to

commencing FLA (n=1), unable to read English (n=1), declined to talk to researcher (n=1). Two couples did not complete the questionnaire prior to FLA and when asked about the questionnaires immediately following FLA stated that they had “forgotten” and were unable to complete them, thus the data presented here are for 25 couples (25 mothers, and 25 fathers). The demographic information is given in Table 8.2. There were no same-sex couples. All mothers and fathers who participated were couples at the time of recruitment, but not all were married or co-habiting and biological parental status was not confirmed. One couple was local and was booked at Birmingham Women’s and Children’s NHS Foundation Trust thus was already undergoing routine antenatal care at the same hospital as the West Midlands Fetal Medicine Centre. The other 24 couples were booked at other hospitals and had to travel for FLA.

Table 8.2 Participant demographic information

Maternal characteristics (n=25)	
Maternal age median (IQR) years	28.76 (26-32)
Parity n (%) Nulliparous Multiparous	 16 (64) 9 (36)
Maternal ethnicity n (%) White European	 26 (100)
Quintero staging at FLA median (IQR)	3 (2-3)
Gestation at FLA median (IQR) weeks	19+3 (18+2-20+6)
Gestation 1 month following FLA median (IQR) weeks	23+5 (22+4-25+1)

23/25 (92%) couples completed all sections of the pre-FLA questionnaire (Table 8.3), although 6/25 (24%) of couples completed the pre-FLA questionnaires immediately following FLA. In one couple, neither the mother nor father completed the EPDS: the mother had current mental health problems, and the father had a history of mental health problems. In another couple the father did not complete the pre-FLA questionnaires, and in another couple the father did not complete the post-FLA questionnaire although his partner did, therefore these couples were not included in the mother to father comparisons. All pregnancy outcomes for those who underwent FLA were collected: 9/23 (39.1%) pregnancies resulted in a double survivor, 8/23 (34.8%) resulted in a single survivor and 6/23 (26.1%) in no survivors. FLA was unable to be performed in 2 pregnancies therefore these couples were not eligible for follow-up. The questionnaire return rate from eligible couples post-FLA was 8/18

(44.4%), and postnatal was 5/17 (29.4%) with one reminder due to the tight time frame of the follow-up and progressing pregnancy. The postnatal questionnaires were completed when baby(ies) had been discharged from the NNU as they were posted out 6 weeks after their planned delivery date of 36 weeks.

8.3.2 Mental health history of participants

At diagnosis of TTTS, one mother reported a current mental health problem (anxiety), and 6 mothers reported a combination of previous mental health problems (5 anxiety, 2 with concurrent depression, 1 postnatal depression, 1 post-traumatic stress disorder, 1 anorexia nervosa). Five fathers reported a current mental health problem at diagnosis of TTTS (1 depression, 4 anxiety, 2 with concurrent depression, 1 obsessive compulsive disorder), and 2 fathers reported a past mental health problem (1 anxiety, 1 depression). There were no new mental health problems diagnosed during the study.

Table 8.3 Questionnaires received at each time point and pregnancy outcome

Study ID	Pre-FLA	Post-FLA	Post-natal	Pregnancy outcome	Issues with questionnaire completion
2033	y	y	y	2 survivors born at 32w	None
2036	y	y		1 survivor born at 35w, sIUFD at 20w	Father did not complete post-FLA
2039	y			0 survivors, dIUFD at 23w	None
2040	y			1 survivor born at 26w, 1 NND	None
2041	y			1 survivor	None
2042	y	y		2 survivors born at 30w	Maternal and paternal pre-FLA completed just after FLA
2047	y	y	y	2 survivors born at 30w	None
2054	y			2 survivors born at 32w	None
2055	y	y	y	1 survivor born at 36w, sIUFD 17w	None
2066	y			1 survivor born at 28w, sIUFD at 18w	Answer MAAS 1.3 missing (median substitution)
2069	y			2 survivors born at 30w	None
2071	y	y	y	2 survivors	None
2078	y			0 survivors, dIUFD at 21w	None
2095	y			1 survivor born at 31w	None
2097	y	y	y	2 survivors	Maternal and paternal pre-FLA completed just after FLA
2098	y			1 survivor born at 34w	None
2101	y			Unable to perform FLA	Maternal and paternal pre-FLA completed just after FLA. No maternal or paternal EPDS completed
2102	y			0 survivors, sIUFD at 19w then TOP	None
2105	y			Unable to perform FLA	Answer MAAS 1.14 missing (median substitution)

Study ID	Pre-FLA	Post-FLA	Post-natal	Pregnancy outcome	Issues with questionnaire completion
2107	y			0 survivors, dIUFD	None
2116	y			0 survivors, PPROM at 18w then TOP	Father did not complete pre-FLA. Answer MAAS 1.16 missing (median substitution)
2118	y			1 survivor born at 30w, sIUFD at 30w	Maternal and paternal pre-FLA completed just after FLA
2119	y			2 survivors born at 31w	Maternal and paternal pre-FLA completed just after FLA
2123	y	y		2 survivors born at 29w	Maternal and paternal pre-FLA completed just after FLA
2125	y			0 survivors, dIUFD at 21w	Answer MAAS 1.15 missing (median substitution)

8.3.3 Maternal and paternal attachment

Table 8.4 Maternal and paternal attachment pre-fetoscopic laser ablation (FLA), post-FLA and postnatally

NA: not applicable as sub-group does not exist in questionnaire. Median and IQR presented.

	Maternal pre-FLA (n=25)	**Maternal post-FLA (n=8)**	**Maternal postnatal (n=5)**	**Paternal pre-FLA (n=24)**	**Paternal post-FLA (n=7)**	**Paternal postnatal (n=5)**
Total Attachment Scale score	79 (74-82)	82 (70.5-86.5)	86.8 (74.4-88.2)	63.5 (55-67)	65 (58-68)	82.1 (77.4-84.8)
Total Attachment Scale score as percentage of maximum possible	83.2 (77.9-86.3)	86.3 (74.2-91.1)	91.4 (78.3-92.8)	79.4 (68.8-83.8)	81.3 (72.5-85)	86.4 (81.5-89.3)
Sub-groups of Attachment Scales						
Quality of attachment	43 (40-45)	44 (40-46.5)	42.2 (42.2-42.2)	35 (32-36.5)	34 (32-37)	NA
Intensity of attachment	30 (29-34)	33 (26-34)	NA	20 (15-21.5)	21 (16-22)	NA
Pleasure in interaction	NA	NA	24 (21-25)	NA	NA	27.2 (26.9-27.3)
Absence of hostility	NA	NA	19.6 (17.6-21)	NA	NA	NA
Patience and tolerance	NA	NA	NA	NA	NA	36.2 (30.1-37.2)
Affection and pride	NA	NA	NA	NA	NA	20 (19-20)

There was no significant difference between maternal and paternal attachment scores at each time point (Table 8.4, Figure 8.1). When maternal and paternal attachment in the 5 couples who completed questionnaires at all 3 time points was

examined over time, ANOVA demonstrated a significant difference in maternal attachment, $F(2, 4)=7.86$, $p=0.0258$ with Greenhouse and Geisser correction of sphericity ($\varepsilon=0.7359$) (Greenhouse 1959). Post hoc linear regression revealed a significant increase in maternal attachment from pre-FLA to postnatal ($p=0.004$) (Figure 8.2 (a)). This result should be interpreted with caution as only 5 couples were able to be included in this analysis and the antenatal score at diagnosis of TTTS may be more of a reflection of adjustment to the diagnosis of TTTS than of antenatal attachment. There was no significant change in paternal attachment over time using the Kruskal Wallis test, $\chi^2(2)=2.414$, $p=0.30$ (Figure 8.2(b)). It was not possible to compare couples with 1 survivor to those with 2 survivors due to insufficient numbers. However, it was interesting to note that the one couple with a sIUFD who completed the questionnaires at all 3 time points reported the lowest attachment scores pre-FLA and postnatally (Figure 8.2(a) and Figure 8.2(b)).

Figure 8.1 Box and whisker plots of maternal and paternal Attachment Scale scores as percentages of total maximum score pre-fetoscopic laser ablation (FLA), post-FLA and postnatally

(n=25 mothers, n=25 fathers) No significant difference was found between mothers and fathers. Median and IQR presented.

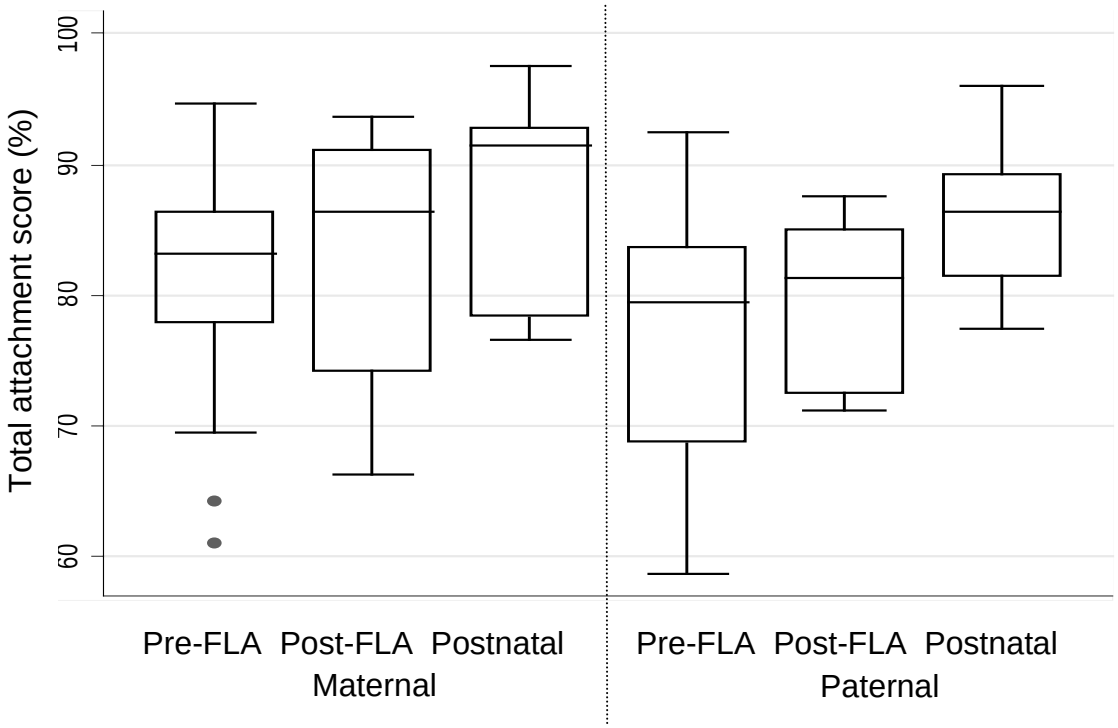
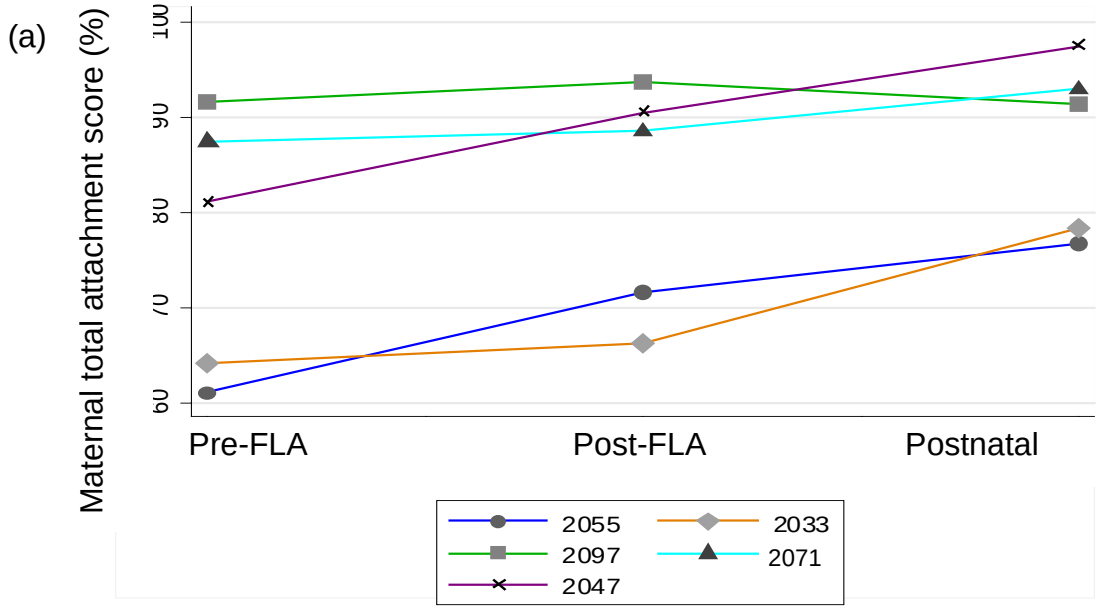
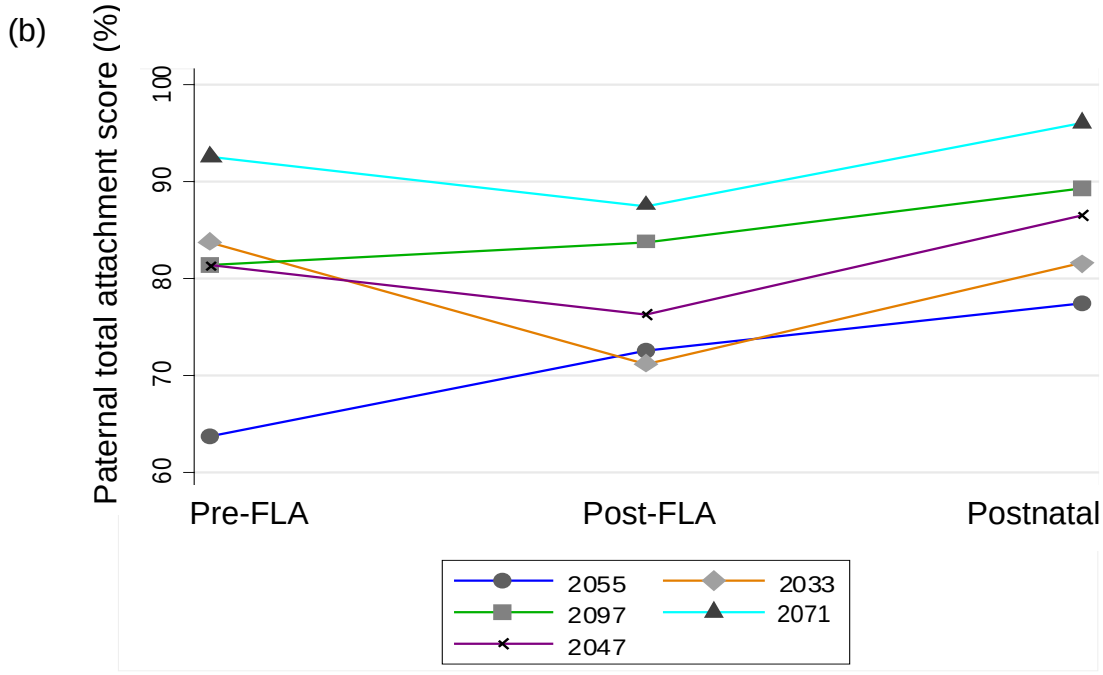


Figure 8.2 Line plot of individual (a) maternal (b) paternal Attachment Scale scores as a percentage of the maximum possible score pre-fetoscopic laser ablation (FLA), post-FLA and postnatally

(n=5 couples included at all 3 time points) The bottom dark blue line with circle markers is the couple with 1 survivor, the other 4 couples had 2 survivors. p<0.05 maternal attachment pre-FLA to postnatal





When the cohort was divided based on mental health problems, there were no statistically significant differences between those with and without mental health problems (Table 8.5). Mothers with a history of mental health problems reported a trend of lower attachment scores at all time points than mothers with no history of mental health problems, but there was only one mother in the postnatal group with a history of mental health problems. The opposite was true in fathers: fathers with current or a past mental health problems reported a trend of higher attachment scores than fathers with no history of mental health problems, but there was only one father with current mental health problems, and one father with past mental health problems who completed the pre-FLA and post-FLA questionnaires.

Table 8.5 Total parental attachment scores as a percentage of the maximum possible score pre-fetoscopic laser ablation (FLA), post-FLA and postnatally according to presence of mental health problems

NA: not applicable as no participants in the sub-group. Median and IQR presented.

	Maternal pre-FLA	**Maternal post-FLA**	**Maternal postnatal**	**Paternal pre-FLA**	**Paternal post-FLA**	**Paternal postnatal**
Current mental health problems	85.26 n=1	NA n=0	NA n=0	81.25 (71.25-83.75) n=5	87.50 n=1	96.00 n=1
Past mental health problems	78.95 (76.32-82.37) n=6	71.58 (68.95-74.21) n=2	78.32 n=1	85.00 (83.13-86.88) n=2	83.75 n=1	89.26 n=1
No history of mental health problems	83.68 (79.21-87.11) n=18	89.47 (85.26-91.32) n=6	92.11 (87.68-94.00) n=4	77.50 (67.50-82.50) n=17	76.25 (72.50-81.25) n=5	81.47 (79.42-83.95) n=3

There was no significant difference between those who completed the pre-FLA attachment questionnaire before FLA, and those who completed it immediately after FLA (data not shown).

8.3.4 Maternal and paternal depressive symptoms

There was a significant difference between the maternal and paternal EPDS scores pre-FLA and post-FLA, but not postnatally (Table 8.6, Figure 8.3). When the scores were translated into the number of participants above the cut-off for major depressive disorders there was no significant difference between the mothers and fathers at each time point. The time point with the highest proportion of mothers above the cut-off was post-FLA (4/8, 50.0%). Postnatally no mothers had an EPDS score above the cut-off, irrespective of pregnancy outcome. The time point with the highest proportion of fathers above the cut-off was pre-FLA (6/23, 26.1%). Postnatally 1/5 (20%) fathers had an EPDS score above the cut-off, which interestingly was the pregnancy with 1 survivor whereas the other 4/5 pregnancies had 2 survivors. When EPDS scores in the 5 couples who completed questionnaires at all 3 time points were examined over time, ANOVA demonstrated a significant difference in maternal depressive symptoms $F(2, 4)=8.03$, $p=0.0308$ with Greenhouse and Geisser correction of sphericity ($\varepsilon=0.6552$) (Greenhouse 1959). Post hoc linear regression revealed a significant decrease in maternal EPDS score from pre-FLA to postnatal ($p=0.006$) Figure 8.4(a). This result should be interpreted with caution as only 5 couples were able to be included in this analysis and as previously highlighted this may be more of an acute adjustment reaction to the diagnosis of TTTS rather than antenatal depression. There was no significant change in paternal depressive symptoms over time using the

Kruskal Wallis test, $\chi^2(2)=2.738$, p=0.25 Figure 8.4(b). It was not possible to formally compare couples with 1 survivor to those with 2 survivors due to insufficient numbers. However, the couple with a sIUFD who completed the questionnaires at all 3 time points reported the highest EPDS scores postnatally (Figure 8.4(a) and Figure 8.4(b)).

Table 8.6 Maternal and paternal Edinburgh Postnatal Depression Scale (EPDS) scores pre-fetoscopic laser ablation (FLA), post-FLA and postnatally

*p=0.01 pre-FLA: maternal vs paternal, †p=0.02 post-FLA: maternal vs paternal

	Maternal pre-FLA (n=24)	**Maternal post-FLA (n=8)**	**Maternal postnatal (n=5)**	**Paternal pre-FLA (n=23)**	**Paternal post-FLA (n=7)**	**Paternal postnatal (n=5)**
Total EPDS score median (IQR)	12.5* (7-17)	12.5† (9.5-17.5)	4 (3-7)	8* (5-11.5)	6† (4.5-9.5)	3 (2-9)
Number of participants above cut-off n/N (%)	10/24 (41.7)	4/8 (50.0)	0/5 (0.0)	6/23 (26.1)	1/7 (14.2)	1/5 (20.0)

Figure 8.3 Box and whisker plots of maternal and paternal Edinburgh Postnatal Depression Scale (EPDS) scores pre-fetoscopic laser ablation (FLA), post-FLA and postnatally

(n=24 mothers, n=24 fathers) *p<0.05 pre-FLA, and post-FLA, when mothers were compared to fathers. Median and IQR presented.

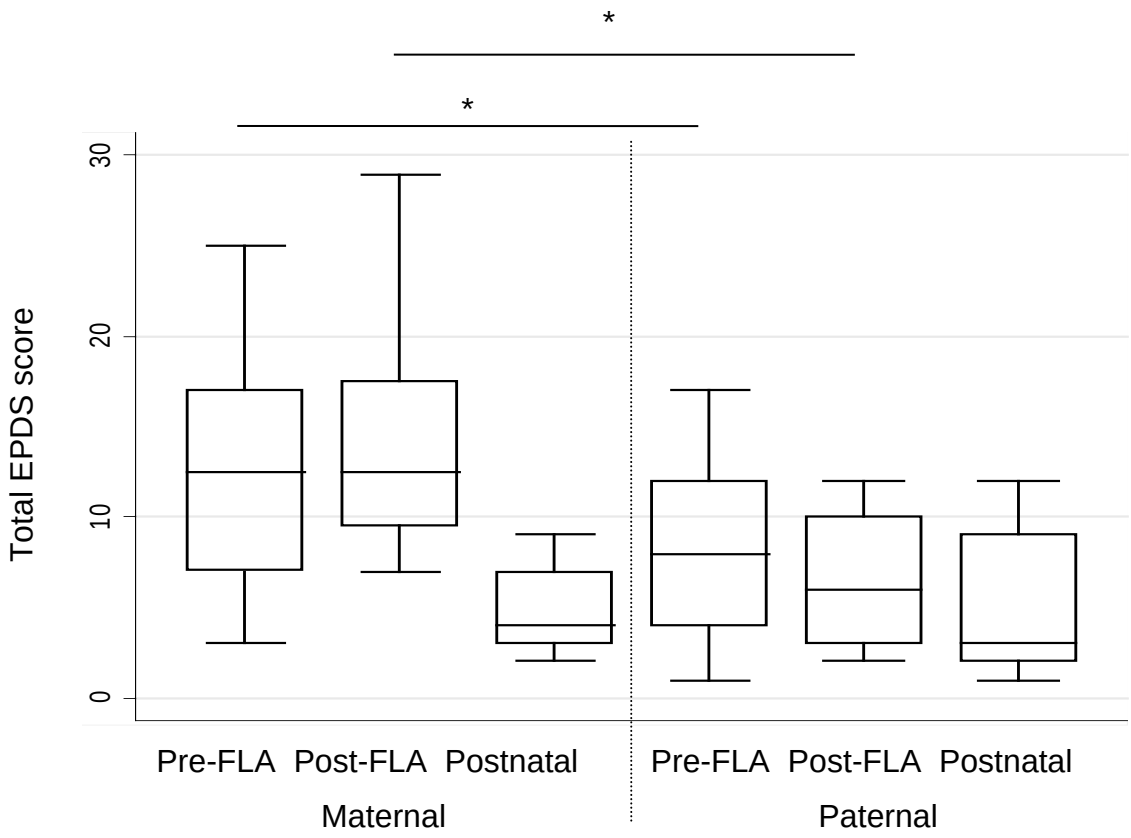
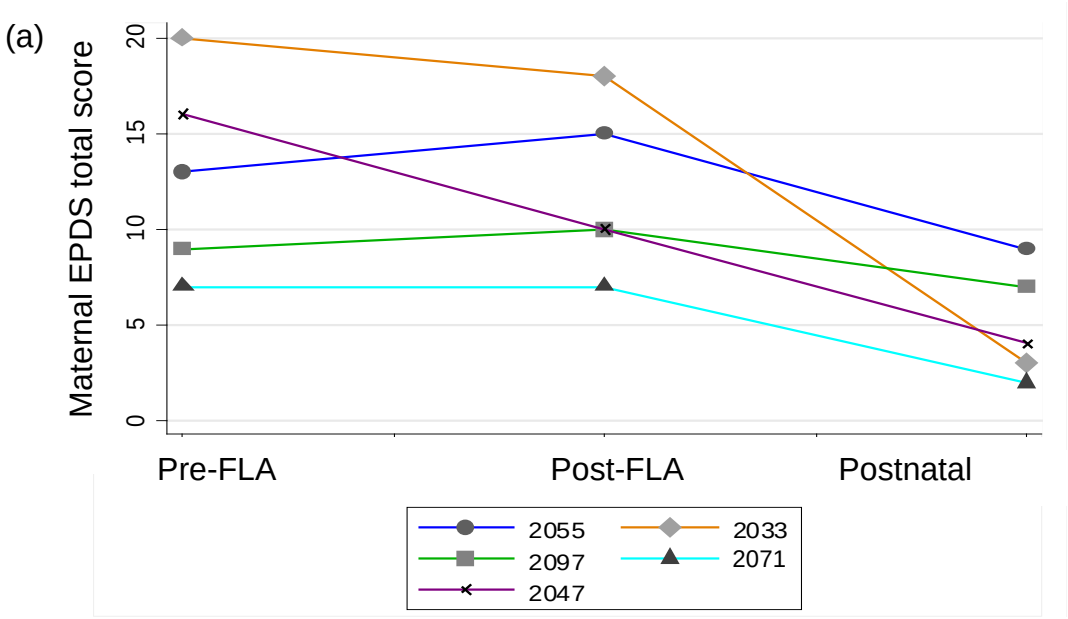
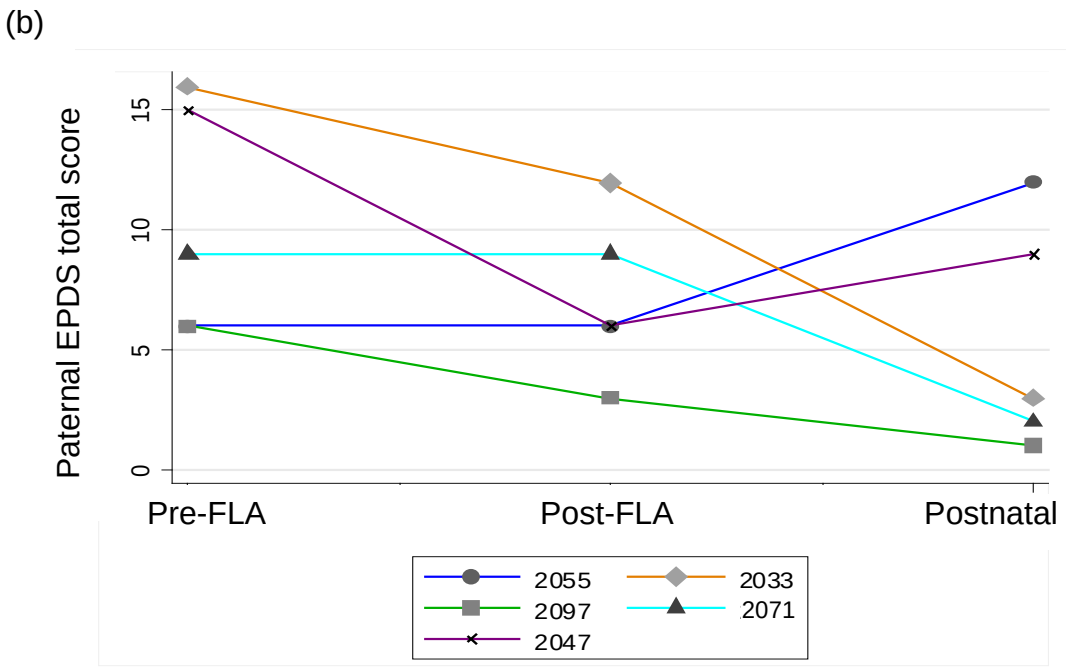


Figure 8.4 Line plot of individual (a) maternal (b) paternal Edinburgh Postnatal Depression Scale (EPDS) scores pre-fetoscopic laser ablation (FLA), post-FLA and postnatally

(n=5 couples included at all 3 time points) The bottom dark blue line with circle markers (2055) is the couple with 1 survivor, the other 4 couples had 2 survivors. p<0.05 maternal EPDS total pre-FLA to postnatal





When the cohort was divided based on mental health problems, mothers with a history of mental health problems reported significantly greater depressive symptoms post-FLA than mothers with no mental health problems (Table 8.7).
Fathers with current mental health problems reported significantly greater depressive symptoms pre-FLA than fathers with no history of mental health problems. However, these increases in EPDS score did not translate into a significant difference in the proportion of mothers and fathers above the cut-offs. There was a trend of greater depressive symptoms pre-FLA in mothers with a history of mental health problems compared to mothers with no mental health problems, and in fathers with current mental health problems compared to fathers with no mental health problems. It is not possible to draw conclusions regarding postnatal depression because of insufficient numbers.

There was a significant difference (p=0.03) in the median maternal EPDS scores between those who completed the pre-FLA questionnaire before the FLA (10.5 [IQR: 7-16.75] 18 mothers) and those who completed it immediately after FLA (19 [IQR: 4.75-22.5] 6 mothers). This did not translate to a difference in the proportion of mothers who scored above the cut-off. There was no difference in the fathers (data not shown).

Table 8.7 Total maternal and paternal Edinburgh Postnatal Depression Scale (EPDS) scores pre-fetoscopic laser ablation (FLA), post-FLA and postnatally according to presence of mental health problems

*p<0.05 maternal post-FLA: past mental health problems vs no mental health problems †p<0.05 paternal pre-FLA: current mental health problems vs no mental health problems. NA: not applicable as no participants in the sub-group. Median (IQR) presented.

	Maternal pre-FLA	**Maternal post-FLA**	**Maternal postnatal**	**Paternal pre-FLA**	**Paternal post-FLA**	**Paternal postnatal**
Current mental health problems	NA	NA	NA	12† (10-15)	9	2
Above cut-off n/N (%)	0/0	0/0	0/0	3/5 (60)	0/1 (0)	0/1 (0)
Past mental health problems	17 (11-20)	23.5* (20.75-26.25)	3	6	3	1
Number of participants above cut-off n/N (%)	4/6 (66.6)	2/2 (100)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
No history of mental health problems	10 (7-16.75)	10* (9.25-13.75)	5.5 (3.5-7.5)	7† (4-9)	6 (6-10)	9 (6-10.5)
Number of participants above cut-off n/N (%)	6/18 (33.33)	2/6 (33.33)	0/4 (0)	3/17 (17.65)	1/5 (20)	1/3 (33.33)

8.3.5 Association between parental attachment and depressive symptoms

There was no association between parental attachment and depressive symptoms in mothers or fathers, at any time point (Table 8.8, scatter plots not shown).

Table 8.8 Correlation between maternal and paternal attachment scores and Edinburgh Postnatal Depression Scale (EPDS) scores pre-fetoscopic laser ablation (pre-FLA), post-FLA and postnatally.

	Maternal pre-FLA (n=24)	**Maternal post-FLA (n=8)**	**Maternal postnatal (n=5)**	**Paternal pre-FLA (n=24)**	**Paternal post-FLA (n=8)**	**Paternal postnatal (n=5)**
Kendall's tau-b correlation coefficient	-0.28	-0.47	-0.40	-0.15	-0.39	-0.60
p value	0.07	0.13	0.46	0.35	0.29	0.22

8.4 Discussion

8.4.1 Maternal and paternal attachment

There was no significant difference between maternal and paternal attachment scores at each time point. This may be because the fathers who were more attached were more likely to attend the Fetal Medicine Centre, and agree to participate in the study, or may be reflective of small sample size. Although results are conflicting in other studies regarding whether mothers or fathers report higher levels of attachment there is a dearth of recent studies (Ustunsoz 2010), and very few in the UK, thus it could be that as fathers have become more involved in family life, paternal

attachment levels have increased. Another explanation is that mothers with TTTS pregnancies decrease their attachment to the fetuses in the antenatal period as a protective mechanism (Damato 1998), consequently mothers are no longer more attached than fathers. The employment of a protective mechanism in mothers of high-risk pregnancies has been reported by other studies (Penticuff 1982, Moore 1983, Stainton 1992, Feldman 1999), and is supported in this study by the level of maternal attachment increasing from at time of diagnosis of TTTS to postnatally, but there being no significant increase at the post-FLA time point when the fetuses are still in danger. This is supported by Beauquier-Maccotta et al. who also reported no increase in antenatal maternal attachment in TTTS pregnancies (Beauquier-Maccotta 2016). Alternatively, the increase in maternal attachment over time may be a reflection of the general increase in maternal attachment in relation to gestational age, as in other studies (Leifer 1977, Cranley 1981, Heidrich 1989, Raphael-Leff 2001, Condon 2013). No change over time was seen in the fathers which may reflect that fathers vary in the way they cope with stressful situations, with some fathers employing protective mechanisms and others not. It is important to highlight that the time period for the MAAS and PAAS was changed to “since the diagnosis of TTTS” as opposed to “over the past 2 weeks” thus the pre-FLA MAAS and PAAS should be interpreted with caution as the score may be more reflective of an acute adjustment reaction. Unfortunately it was not possible to assess the effect that time from diagnosis/suspicion of TTTS had on attachment as 24/25 couples underwent antenatal care in other hospitals and may have had discordant liquor volumes prior to referral to the West Midlands Fetal Medicine Centre.

8.4.2 Maternal and paternal depression

Mothers reported significantly greater depressive symptoms than fathers at diagnosis of TTTS, and 1 month post-FLA, but there was no difference in postnatal depressive symptoms between mothers and fathers. This may link to the guilt and responsibility that mothers feel as it is them who are growing the fetuses inside them, and who may blame themselves for the pregnancy being complicated by TTTS. Another interesting finding was from the post-hoc analysis of mothers who forgot to complete the pre-FLA questionnaire before the FLA and completed it immediately after FLA who reported significantly greater depressive symptoms than mothers who completed the questionnaire pre-FLA. This may be because the mothers were too nervous or depressed to remember to complete the questionnaire prior to FLA. No difference was seen in the fathers, but mothers had the additional stress of being the ones physically going through the FLA.

Maternal depressive symptoms decreased from diagnosis of TTTS to postnatally. In-keeping with the results of this study, Beauquier-Maccotta et al. also reported a lower rate of maternal depressive symptoms postnatally than at the diagnosis of TTTS which fits with the pregnancy continuing to be at risk throughout the antenatal period and mothers experiencing relief at the delivery of the survivor(s) (Beauquier-Maccotta 2016). The mean EPDS score reported by Beauquier-Maccotta et al. at the diagnosis of TTTS was 12.06 (SD 5.58) which is comparable to the median score of 12.5 (IQR: 7-17) in this study, however they used a cut-off of 11.5 for their French cohort (compared to a validated cut-off of 15 and above in English speaking cohort) and

therefore they reported a higher proportion of mothers scoring above the cut-off at the diagnosis of TTTS compared to in this study (72% vs 41.7% respectively). Although in the current study there were significant changes in the EPDS scores, this did not translate into changes in the proportion of participants above the cut-offs. The rate of 0/5 (0%) women in the postnatal period with an EPDS score above the cut-off, is likely to be an underestimate due to insufficient numbers. There was no change over time in fathers which may reflect that fathers experience potentially high-risk situations in a more variable way than mothers. Multiple studies have reported that fathers believe their role is to provide support to the mother and to stay in control of the situation, and consequently do not feel able to express their emotions (Due 2017). Alternatively it could be that mental health history played a part as fathers with current mental health problems reported significantly greater depressive symptoms at diagnosis of TTTS than fathers with no history of mental health problems. Another explanation is that other confounding factors including quality of relationship with their partner and low social support, that are known to affect paternal depressive symptoms (Wee 2011), were not accounted for in this study due to the small numbers, and were beyond the scope of the current study. It is important to highlight that the time period for the maternal and paternal pre-FLA EPDS was changed to "since the diagnosis of TTTS" as opposed to "in the past seven days" thus the pre-FLA EPDS should be interpreted with caution as the score may be more reflective of an acute adjustment reaction. Unfortunately it was not possible to assess the effect that time from diagnosis/suspicion of TTTS had on depressive symptoms as 24/25 couples underwent antenatal care in other hospitals and may have had discordant liquor volumes prior to referral to the West Midlands Fetal Medicine Centre.

8.4.3 Effect of number of survivors on attachment and depression

It was not possible to formally assess if the number of survivors affected parental attachment, but the one couple with one survivor who completed the questionnaires at all three time points did report the lowest attachment score at diagnosis of TTTS and postnatally, and the highest postnatal depression scores. As the attachment scores were lowest at diagnosis of TTTS prior to the sIUFD, it may be that the mother and father in this couple naturally have a lower attachment tendency, or they may have already employed a protective mechanism prior to assessment at the Fetal Medicine Centre.

It was interesting that postnatally they both had the lowest attachment scores and highest depression scores. This suggestion that parents of twins with sIUFD report greater depressive symptoms than those with 2 survivors is supported by Falletta 2018 (Falletta 2018), and may reflect the difficulty these parents have in caring for a new-born, whilst grieving the loss of the other twin, with the surviving twin acting as a reminder of the deceased identical twin (Swanson 2009, Richards 2015, Jordan 2018).

8.4.4 Effect of mental health problems on attachment and depression

When participants with mental health problems were examined as a sub-group, although no statistically significant difference was found with parental attachment, interestingly a trend did appear of mental health history having opposing effects on maternal and paternal attachment: fathers with past and current mental health problems reported higher attachment scores than fathers with no mental health problems at all three time points, whereas mothers with past mental health problems,

including previous postnatal depression, reported lower attachment scores than mothers with no history of mental health problems. As mentioned previously, this may be a reflection of protective mechanisms being employed by mothers, but not fathers. When perinatal depressive symptoms were examined in the context of mental health problems, mothers with a history of mental health problems reported significantly greater depressive symptoms 1 month post-FLA than mothers with no mental health problems, and fathers with current mental health problems reported significantly greater depressive symptoms at diagnosis of TTTS than fathers with no history of mental health problems. This is in-keeping with what is reported in the literature with antenatal depression being linked to existing mental health problems (Cox 2005, Becker 2016). It was difficult to draw conclusions regarding postnatal depression because of insufficient numbers.

8.4.5 Association between parental attachment and depression

Parental attachment was not associated with depressive symptoms in mothers or fathers. In the context of TTTS pregnancies, the maternal findings are supported by Beauquier-Maccotta et al., but the relationship between paternal attachment and depressive symptoms has not been explored before (Beauquier-Maccotta 2016). Studies in various countries have shown that decreased maternal attachment is associated with increased maternal depression in singleton pregnancies (Taylor 2005, Goecke 2012), although paternal attachment does not appear to be associated with paternal depression (Kunkle 2003). Findings in twin pregnancies, particularly those complicated by TTTS may differ from singletons as attachment in twin pregnancies is different to in singleton pregnancies (Abbink 1982, Van der Zalm

1995, Damato 2004). The diagnosis of a twin pregnancy can cause conflicting feelings as not all mothers and fathers are happy with the diagnosis of a twin pregnancy (Nys 1998, Beretta 2007) as the diagnosis carries with it not only implications for pregnancy risk, but there are physical implications in terms of potentially needing to move house, and other additional financial implications such as buying a new car. Consequently when the fetuses are in danger, this can cause contradictory feelings. In a Danish study of 61 women with twin pregnancies, half of women (54%, 33/61) reported happiness at the diagnosis of a twin pregnancy, 34% (21/61) reported shock/negative feelings, and 33% (20/61) were uncertain whether they were capable of parenting two infants at the same time (Nys 1998). At 27 weeks gestation, the percentage of women who reported happiness increased to 70% (43/61), and those with shock/negative feelings decreased to 2% (1/61), but those uncertain of their parenting ability remained high at 31% (19/61). The chorionicity of the twins in the study was not reported, but one would imagine that there would be a difference between parents of MC and DC twins due to MC twins being considered higher-risk than DC twins. Anxiety also seems to play a role in parental attachment and depression (Condon 1997, Kunkle 2003, van Bussel 2010, Dubber 2015) and therefore warrants further investigation in TTTS pregnancies, particularly as fathers are more likely to report anxiety symptoms than depressive symptoms (Matthey 2001), and Beauquier-Maccotta et al. reported significantly higher anxiety symptoms in mothers with TTTS pregnancies than those with uncomplicated MC or DC twin pregnancies (Beauquier-Maccotta 2016).

8.4.6 Strengths and limitations

This is the first time attachment and depression has been explored in a UK cohort of mothers and fathers whose pregnancies have been affected by TTTS. All participants approached were willing to take part, and there was a good return of pre-FLA questionnaires (92.6%), but the proportion of returned post-FLA questionnaires (44.4%) and postnatal questionnaires (29.4%) was lower than the generally agreed acceptable survey response rate of 60% (Livingston 2012). Consequently, this work is at risk of sampling bias as substantial proportions of the population have not been represented. The amount of missing data in the returned questionnaires was acceptable and is a problem common to postal questionnaire studies. Importantly sub-group analysis was performed according to past/current mental health problems which have been shown to be one of the biggest predictors of lower parental attachment and greater depression symptoms. By only including parents with twin pregnancies affected by TTTS, it meant that the confounding factor of including DC twins was ameliorated as mothers of DC twins have demonstrated thinking differently about their twins compared to mothers of MC twins. Mothers of DC twins defined each baby with different characteristics, whereas MC twin mothers think of each baby as being in a pair (Van der Zalm 1995). As maternal scores were compared with paternal scores, this also decreased the role of other potential confounding founders such as household income, due to the couple acting as an internal control in some analyses. Although ethnicity was not an exclusion criteria, only one ethnicity was able to be included in this cohort which may be seen as a limitation on one hand because the results are not generalisable to other ethnicities and cultures, but it does mean that ethnicity was not a confounding factor either, and there was no effect of cultural

bias (Matthey 2001). Another strength of this study was the use of a validated depression screening tool specific to pregnancy that has been used in twin pregnancy before. Although the EPDS does not provide a definitive diagnosis of a depressive disorder according to the DSM-IV criteria, it does have a high sensitivity and specificity and is therefore considered an acceptable screening tool, and is used in routine clinical care. It was decided to explore attachment per total surviving number of fetuses/infants, and not per fetus which is believed to be a unique characteristic of this study. Although mothers in a study of 214 low- and high-risk twins reported a significantly higher attachment score in the second born twin, the actual difference in the score was not believed to have a practical impact on attachment, and no reference was made to chorionicity which is known to affect attachment (Damato 2000). When Beauquier-Maccotta et al. asked mothers to complete a Prenatal Attachment Inventory (PAI) for each twin fetus from TTTS pregnancies there was no significant difference between the twins at any time point (20, 26, 32 weeks gestation) including no difference depending on whether the twin was the donor or recipient (Beauquier-Maccotta 2016).

There were several limitations to this study. One was the geographical spread of patients who are treated at the West Midlands Fetal Medicine Centre, which may have meant that fewer participants completed the follow-up questionnaires than if they had had their antenatal follow-up care at the Fetal Medicine Centre. Only one couple was local whereas the other 24 couples had to travel for FLA, which one could hypothesise may affect how parents cope psychologically with TTTS. How couples are counselled at the start of a MC twin pregnancy may also differ between

units, with some parents reporting not having heard of the condition until they were diagnosed with it, and others being aware of the risk from their booking appointment. The issue of geographical patient spread is a problem with all studies of FLA for TTTS as FLA needs to be performed by experienced operators, and thus treatment has to be centralised (Morris 2010). This also meant that interviews to delineate the reasons behind the questionnaire responses were not possible within this study. However there is a benefit to using questionnaires as respondents are believed to be more truthful when answering questionnaires than in face to face interviews (Leedy 2001) as 'acceptability influence' is a known issue of self-report questionnaires (Edwards 1957). This could have been a potential issue with this study as Condon et al. explains that participants may answer attachment questions in the way that they believe reflects well on them, and in the way that they perceive society expects them to answer (Condon 1998). However, one way to view it is that if the 'average person' embellishes their answers to appear more socially desirable, the whole distribution of the cohort's responses will shift, which has a minimal effect when the scores are converted to standardised scores (Nunnally 1970). As the study was not concerned with absolute attachment scores, but the comparison between mothers and fathers, and the change in scores over time, the ability to discriminate between the low- and high-attachment groups is more important than the absolute scores, and the MAAS, PAAS, MPAS, PPAS do discriminate between the groups (Condon 1998, Condon 2013).

By the nature of the study the results are not generalisable to all parents with a pregnancy complicated by TTTS. It was not possible to include couples who did not

read English who may have a different cultural background and thus may be psychologically affected by TTTS differently. It was also not possible to include couples in which the father did not attend the Fetal Medicine Centre (16/54, 29.6%) which would be an interesting group to assess as it may be that not being at the appointment affects paternal attachment and depression, particularly in the antenatal period. Additionally, it may be that not having the paternal support during the procedure impacts maternal psychological well-being. Another group who are important to evaluate are those with a dIUFD, but as the attachment questionnaires would not be relevant they were not included in this study. There is a risk of selection bias as those very distressed may be less willing to participate, leading to an underestimate of the negative reactions in these circumstances, and they may also be less likely to return follow-up questionnaires. Performing interviews may be a way to explore this further. The missing outcomes for some pregnancies may mean that confounding factors such as an abnormal fMRI, or neonatal comorbidity were unable to be accounted for.

No studies in twin pregnancies were found that have used the Attachment Scales by Condon et al. which is a limitation of the study. The majority of questions on the scale seemed appropriate, although 1 question in the MAAS and PAAS was difficult to interpret in the context of TTTS: “Since the diagnosis of TTTS when I think about the babies inside me I get feelings which are:” the responses vary from “Very sad” to “Very happy”. As the pregnancy is in danger at this point the question may not be able to discriminate between parents with high and low fetal attachment. The scoring of this question was not altered, in line with other studies which have used the MAAS

in high-risk pregnancies (White 2008, Pisoni 2015). Some wording of the questionnaires was amended to reflect twin pregnancies, and the time over which parents were asked to think back over, as due to the rapid disease progression of TTTS and the necessity for treatment, the pre-FLA and post-FLA questionnaires were changed to “since diagnosis of TTTS” from “over the last 7 days”. The postnatal questionnaires were not changed. There is also no validated cut-off for the paternal antenatal EPDS score (Matthey 2001) but 12 and above was used as a cut-off, akin to 2 other studies that investigated antenatal paternal depression using the EPDS. This cut-off was 2 points higher than the validated postnatal cut-off, which is the difference between the maternal antenatal and postnatal cut-offs (Matthey 2006), but it is important to highlight that the paternal antenatal depressive symptoms cut-off may not be reflective of pathology.

Demographic data on mothers were collected but not fathers, and results were not adjusted for demographic factors. The main limitation is the sample size, particularly in the longitudinal analyses as only 5 couples completed questionnaires at all 3 time points thus the results should be interpreted with caution.

Research into the relationship between attachment and demographic variables/potential confounding factors such as: IVF, whether pregnancy was planned or not, parental educational level, and family income has been conflicting (Damato 2004). The majority of evidence does not support any association with these factors (Kemp 1987, Laxton-Kane 2002, Lahann 2008). Lahann et al. (Lahann 2008) reported a significant relationship between maternal antenatal attachment in twin pregnancy and maternal age, parity and known gender of fetuses, but these

variables were not adjusted for as these findings conflict with other studies which did not find a significant difference (Mercer 1993b, Siddiqui 2000). The only factors that have been consistently reported to affect attachment are gestational age (Damato 2004, Beauquier-Maccotta 2016), and the ability to feel fetal movements ('quickening') which have demonstrated an association with increased maternal attachment (Lerum 1989, Bloom 1995, Damato 2004). These factors were incorporated in this study as attachment over time was explored, and the pre-FLA and post-FLA time points represent pre- and post-'quickening'.

8.4.7 Clinical implications and future research

This work demonstrates that a high proportion of mothers with TTTS pregnancies score above the EPDS cut-off, particularly 1 month after FLA (50%). Therefore referral centres providing on-going antenatal care for these women after FLA should be aware of this risk and screen and refer for additional psychological support as necessary. The clinical team in France routinely offer all women referred to their centre for FLA psychological follow-up, although the effect of this follow-up has not been evaluated. In light of the findings of this study, psychological follow-up should be considered in the UK patient population, (Beauquier-Maccotta 2016). Patients and health care professionals can be reassured that generally maternal depressive symptoms decrease in the postnatal period although it has been highlighted that the maternal postpartum depression rate may be an underestimate. With regards fathers, health care practitioners should be aware of the variable way fathers experience TTTS, and although the EPDS has been validated as a screening tool for fathers, there is not a validated antenatal cut-off for depressive disorders, and fathers may

not always feel able to express their emotions. Health care professionals should be particularly aware of mothers and fathers who have a history of mental health problems, and those whose pregnancy has resulted in 1 survivor. A recent study found that psychological issues thought to be a result of a TTTS pregnancy persist for at least 7 years following FLA (Vergote 2018), and that the loss of a child in a previous pregnancy impacts subsequent pregnancies (O'Leary 2004) thus health care professionals should be aware of this when caring for women in pregnancies subsequent to their TTTS pregnancy.

Continued research in this area, with larger cohorts in different countries and ethnicities using translated questionnaires with appropriate validation is required. Future research should include the exploration of anxiety symptoms, and their relationship with parental attachment and depression, particularly in fathers. Qualitative interviews are important to ascertain reasons behind the scores on the questionnaires, and confirm the diagnosis of depression. Parental attachment scores in TTTS pregnancies should be validated with another measure of attachment, and a cut-off for paternal antenatal depression using the EPDS is required. Future studies should ensure that pregnancy outcome and current and past mental health problems are considered. Other potential confounding factors should be investigated in a TTTS pregnancy context, including paternal demographic data, and partner relationship satisfaction (Condon 1997, Colpin 1998, van Bussel 2010, Goecke 2012). It would also be interesting to assess fathers who were unable to attend the Fetal Medicine Centre, but due to the rapid progression of TTTS and the large geographical referral area, it would be difficult to consent these fathers. Different methods of improving survey response rate should be explored.

8.5 Conclusion

In this small preliminary study of parental antenatal and postnatal attachment and depression in TTTS pregnancies, maternal attachment increases in the postnatal period, and maternal depressive symptoms decrease in the postnatal period, whereas paternal scores do not appear to change over time. The study has also highlighted the importance of health care professionals in referral centres monitoring mothers and fathers following FLA for depressive symptoms, particularly those with a history of mental health problems, and in TTTS pregnancies which involve fetal loss and the possible requirement of additional psychological support for high-risk pregnancies undergoing invasive procedures. Further work is needed in this area in larger cohorts.

CHAPTER 9 DISCUSSION

9.1 Overview

This thesis has explored the prediction, diagnosis and management of complications in MC twin pregnancies. MC twin pregnancies are high risk and adverse outcomes can have devastating effects on families. They are closely monitored antenatally as stipulated in international guidance (NICE 2011, Khalil 2016b, Kilby 2016) because it is not possible to predict which pregnancies will develop complications. Following this guidance necessitates health care professionals' time and resources, in addition to the effects it has on parents' time and anxiety. The ability to predict which pregnancies will develop complications may allow more tailored antenatal care for women with MC twin pregnancies, and allow clinicians to practice stratified medicine. Additionally, it may enable the development of new treatments for complications and inform future research studies (Riley 2013).

Although this thesis did not find any maternal serum biomarkers with sufficient predictive ability to be used clinically, this thesis has progressed knowledge in the field of MC twin pregnancies and the findings do have clinical implications.

9.1.1 How this thesis has progressed knowledge of MC twin pregnancies

So-called 'negative' findings are still valid and important findings. In CHAPTER 3 the OMMIT study found that first trimester inter-twin NT discordance, CRL discordance, and first trimester maternal serum β-hCG, PAPP-A, AFP, PlGF and sFlt-1 were unable to predict adverse outcomes as individual prognostic factors. However, the

results are exciting from a pathophysiological perspective as some associations with adverse outcomes were found, and they suggest that pathophysiological changes may occur in the first trimester, prior to the appearance of the ultrasound signs of polyhydramnios and oligohydramnios, and IUFD. This supports that first trimester prognostic factors may exist; warranting further investigation. Interestingly no potential prognostic factors affected both growth restriction and TTTS supporting that they have different pathological mechanisms.

As changes in these markers were demonstrated in the second trimester at 20 weeks gestation by previous work, but not at 12 weeks gestation in this work, the concentrations in maternal serum were measured longitudinally over the first and second trimester in women with uncomplicated MC twin pregnancies in CHAPTER 4. The patterns of these markers have not been measured before in MC twin pregnancies and may improve understanding of the pathophysiology of TTTS.

The discovery of changes in the amniotic fluid metabolome from the recipient twin pre- and post-FLA; and a relationship between cardiac function in the recipient twin and a switch from fatty acid to carbohydrate metabolism was demonstrated in CHAPTER 5. These are thrilling new findings, suggesting that metabolomics may play a role in the pathophysiology of TTTS and provide other potential prognostic factors and therapeutic targets to explore in future work.

The work on miRNA in second trimester maternal serum from pregnancies complicated by TTTS in CHAPTER 6 is the first time that miRNA has been investigated in this context. The finding of no significant difference in the 6 candidate miRNAs which were validated increases knowledge surrounding TTTS and suggests that maternal serum may not reflect possible miRNA changes in TTTS.

The systematic review in CHAPTER 7 reported updated and more detailed prognoses of the surviving co-twin after spontaneous sIUFD, demonstrating that pregnancies in which the sIUFD occurred at 14-28 weeks are at higher risk. The rate of abnormal antenatal brain imaging and NND were calculated for the first time in this context which also adds to existing knowledge.

The preliminary study of parento-fetal antenatal and postnatal attachment and depression in TTTS pregnancies in CHAPTER 8 is the first time these outcomes have been explored in mothers in a UK cohort, and in fathers in the World. This thesis found that maternal attachment increased postnatally and depressive symptoms decreased, whereas paternal scores did not change, and a higher-risk group for developing adverse psychological outcome was identified.

9.1.2 Strengths and limitations

A major strength of this thesis is that it is the first time many factors have been explored in MC twin pregnancies, and especially in those with TTTS. The lack of animal models makes it difficult to investigate the pathophysiology, thus impeding advances in this area. However, collaborations were formed, and pragmatic choices were made, to maximise what could be learned in this area. The methodologies and statistical analyses used were robust and as five of the seven chapters have been accepted for publication/are published to date this indicates that the scientific community believes the work is relevant and important.

One of the main limitations of the work was the sample size, which was unsurprising given the relative scarcity of MCDA twin pregnancies in the general obstetric population. As discussed in CHAPTER 3, the general rule of thumb for powering investigation of individual prognostic factors is at least ten events for every candidate prognostic factor of interest, consequently a fetal adverse outcome composite was used. If the study was to be powered to demonstrate a difference in TTTS alone, the number of cases of TTTS would have to be 70. Given that TTTS affects 10-15% of MCDA twin pregnancies, this would equate to needing to recruit approximately 700 MCDA twin pregnancies. If a recruitment rate of 75% was used, as was found in CHAPTER 4, this would mean approximately 935 women with MC twin pregnancies would need to be approached, which would take roughly 43 years if women were recruited from BWH alone (a tertiary referral unit with 8,000 deliveries/year), therefore collaboration is essential, and biobanking for rare conditions is important. The sample size was adequate for the hypothesis generating untargeted metabolomics work, and according to the power calculation performed in the miRNA work. Although the initial recruitment rate to the psychological work was good, the return rate of follow-up questionnaires was sub-optimal.

Recruiting appropriate controls was also a limitation, and is particularly hindered when invasive techniques are needed to obtain tissue samples. There are many options for future research and research resources and time could be, in my opinion, better spent in other ways (see Section 9.4).

9.1.3 Clinical implications for clinicians and patients

The main clinical implication from the first trimester potential prognostic factors work is that, in line with the RCOG MC twin pregnancy guidance, NT % discordance, and individual NT measurements alone should not be used to predict TTTS (Kilby 2016).

New knowledge has been synthesised to produce a more personalised risk prediction for parents with a sIUFD, which has the clinical implications of improving patient counselling and decision making. The high risk of preterm birth in DC and MC twins should prompt clinicians to discuss with patients the possibility of NNU admission, and to have a low threshold to seek medical advice if they have concerns that they are going into preterm labour. The importance of offering antenatal brain imaging to parents follow sIUFD in MC twin pregnancies has also been highlighted and the rate of 20% may help parents decide whether to undergo imaging.

Additional knowledge has been obtained to help referring centres identify mothers and fathers who may need additional psychological support following FLA. Patients and health care professionals can be reassured that generally maternal depressive symptoms decrease in the postnatal period although it has been highlighted that the maternal postpartum depression rate may be an underestimate. With regards fathers, health care practitioners should be aware of the variable ways fathers experience TTTS, and although the EPDS has been validated as a screening tool for fathers, there is not a validated antenatal cut-off for depressive disorders, and fathers may not always feel able to express their emotions. Health care professionals should be particularly aware of mothers and fathers who have a history of mental health problems, and those whose pregnancy has resulted in 1 survivor. The findings in this

thesis should prompt them to ask them to complete an EPDS, and advise either referral to a specialist perinatal mental health team, or to seek advice from their GP.

9.1.4 Future research

A wealth of research ideas has been generated from this thesis. An overriding issue highlighted in this thesis was the lack of a core outcome set for twin pregnancy research, thus pragmatic decisions were made. The importance of a core outcome set has been recognised by other groups, and this work is currently being performed. As previously stated, sample size was an issue, but in order to power the work to look at specific adverse outcomes, such as TTTS, it would take a long time to recruit an adequately sized cohort, thus collaboration would be better. What would be more helpful would be to explore other biomarkers, but to continue examining outcomes as a composite. Careful consideration would be needed of what the action would be following the output from the eventual prognostic model. For example if a high risk outcome from the prognostic model is calculated, the action may be to increase ultrasound surveillance, and it would be appropriate to combine sIUGR, TTTS and sIUFD together, but if it was to identify which pregnancies to give a new prophylactic treatment for TTTS, the use of a composite would not be appropriate. Ideally new biomarkers to be explored in maternal blood would be identifiable as fetal material rather than maternal, such as cffDNA, as TTTS and sIUGR are considered placental/fetal diseases.

The longitudinal work in CHAPTER 4 exploring maternal serum biomarkers in the first and second trimester should be repeated in a larger cohort of MC twins, and with the inclusion of complicated MC twin pregnancies, to allow comparison between the two

groups, and provide gestational cut-offs for when each biomarker can be accurately measured. It may also improve knowledge surrounding the pathogenesis of MC twin complications as by recruiting complicated MC twin pregnancies this will enable investigation as to whether the biomarkers are low only in the first trimester, or remain low throughout the first and second trimester when signs and symptoms begin to appear. It would be interesting to compare these levels in DC twin pregnancies, although this may have less clinical utility as DC twins develop fewer, and different complications compared to MC twins.

The results of the metabolomics work in CHAPTER 5 need to be validated, ideally by another metabolomics centre, with amniotic fluid samples from different MC twin pregnancies. Following this, the biological function of the significantly different metabolites should be investigated by performing targeted assays to assess the phenotype and identify candidate biomarkers worthy of future prognosis research. The biomarkers could be used to predict outcome following FLA, and could also be explored prior to the appearance of clinical signs of TTTS. It would be interesting to collect amniotic fluid samples and placental samples at delivery and to correlate these samples with fetal cardiac function prior to delivery.

As this is the first time miRNA has been explored in TTTS there is a lot of future work. The work should be repeated in a different cohort to ensure that the negative findings of the validation work are true and that miRNA is not incorrectly discounted. Exosomal miRNAs should be explored, and other new miRNAs could be examined using Next Generation Sequencing. Another captivating area to focus on is miRNA expression in placentas of pregnancies complicated by TTTS, particularly as it was not possible to get the RT-PCR of maternal serum to work to validate miR519a-3p or

miR519c-3p which are placenta-specific miRNAs. This could be extended to placentas from uncomplicated MC and DC twin pregnancies, and twins with sIUGR and sIUFD. The miRNA work could be repeated in amniotic fluid samples, and cord blood to investigate possible inter-twin differences.

The systematic review on sIUFD prognosis in CHAPTER 7 revealed a dearth of research regarding antenatal brain imaging in DC twins, and very little research linking antenatal brain imaging with postnatal brain imaging. As it can be difficult to counsel patients on their baby's/babies' quality of life based on antenatal brain imaging, investigating this in the context of follow-up postnatal brain imaging and standardised long-term neurodevelopmental follow-up assessment is vital. The studies included in this meta-analysis were small and small study effects were shown to exist, thus a large population-based study has been performed using data from UKOSS. This will be the largest study of complications in the surviving co-twin in a population cared for using the same national guidance (for further details see (UKOSS 2016)).

The psychological work should be continued in a larger cohort with additional strategies to improve questionnaire return rates. It would be fascinating to perform qualitative interviews to confirm the diagnosis of depression, and delineate the reasons behind the answers on the questionnaires. Interviews would also enable further investigation as to how couples can be best supported, and allow health care professionals to provide holistic care. It is important to repeat this research in different countries and ethnicities using translated questionnaires with appropriate validation. Future research should also include the exploration of anxiety symptoms, and their relationship with parental attachment and depression, particularly in fathers.

Parental attachment scores in TTTS pregnancies should be validated with another measure of attachment, and a cut-off for paternal antenatal depression using the EPDS is required. Future studies should ensure that pregnancy outcome and current and past mental health problems are considered. Other potential confounding factors should be investigated, including paternal demographic data, and partner relationship satisfaction (Condon 1997, Colpin 1998, van Bussel 2010, Goecke 2012). It would also be interesting to assess fathers who were unable to attend the Fetal Medicine Centre, but due to the rapid progression of TTTS and the large geographical referral area, it would be difficult to consent these fathers. Different methods of improving survey response rate should be explored.

9.2 Conclusion

This thesis has explored the prediction, diagnosis and management of complications in MC twin pregnancies. Stimulating findings have been reported, some of which have direct clinical implications, others excitingly advance knowledge regarding complications of MC twin pregnancies. Conducting further research in this group is essential as complications are associated with high rates of morbidity, however there are various obstacles that make research in this field difficult. Pragmatic solutions must be found and collaborations formed.

CHAPTER 10 APPENDICES

10.1 Early prognostic factors of outcomes in monochorionic twin pregnancy systematic review search strategy

1. fetofetal blood transfusion or fetofetal transfusion or twin twin transfusion syndrome or twin to twin transfusion syndrome or twin-twin transfusion syndrome or twin-to-twin transfusion.mp.
2. twin anemia* polycythemia* sequence or TAPS.mp.
3. twin oligohydramnios-polyhydramnios sequence or TOPS.mp.
4. fetal death or intrauterine death or intrauterine demise or single twin demise or perinatal mortality or perinatal outcome* or neonatal mortality.mp.
5. small-for-gestational age or lbw or small for gestational age or sgr or small for date* or small for gestation* or fgr or iugr or intrauterine growth retard* or intrauterine growth restrict* or fetal growth retard* or fetal growth restrict* or growth restrict* or growth retard* or “Fetal growth retardation” or “Infant, Low Birthweight” or low birthweight.mp.
6. Diseases in Twins/
7. amniotic fluid or amniotic fluid metabolism.mp.
8. placenta* or placental circulation or placental metabolism or placental blood supply or amnion or chorion.mp.

9. alpha-fetoprotein* or angiogenesis inducing agents or biological markers or chorionic gonadotropin, beta subunit or angiogenesis inducing agents or metabolomics* or vascular endothelial growth factor* or placental growth factor.mp.

10. neck/ultrasonography or nuchal translucency.mp. crown-rump length.mp. or biometry.mp or blood flow velocity.mp. or regional blood flow.mp. or umbilical arteries*.mp. or umbilical veins*.mp. or Doppler.mp. or ductus venosus.mp.

11. predictive value or tests* or risk assessment or risk factors* or prognostic* factors* or predictive* factors* or prognostic model or prognosis* or prediction* or predictor or formula or algorithm.mp.

12. twins*.mp.

13. monochorionic*.mp.

14. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11

15. 12 and 13 and 14

10.2 Early prognostic factors of outcomes in monochorionic twin pregnancy systematic review data collection form

Section A: Study Information

1)Ref ID:		**4)Publication year:**	
2)Rev name:		**5)First Author:**	
3)Country:		**6)Language:**	
7) Authors contacted?	Yes ☐$_1$ No ☐$_2$ Not required ☐$_3$		

Section B: Population

8) Number of participating centres: _____

9) Setting: tertiary referral centre □$_1$ DGH □$_2$ Other _____

13) What was the chorionicity / amnionicity of the (case) group?

Monochorionic/diamniotic □$_1$ Monochorionic/monoamniotic □$_2$ Dichorionic/diamniotic □$_3$

Unreported □$_4$

13.ii) Was chorionicity / amnionicity appropriately assessed? Yes □$_1$ No □$_2$ Unreported □$_3$

13.iii) How was chorionicity assessed?

USS?

Placental postnatal assessment?

14) Were all patients primigravid?

Yes □$_1$ No □$_2$ Unreported □$_3$

15) List other eligibility/ in-/exclusion criteria e.g. chromosomal abnorm, Quintero staging

14.i) Were eligibility/in-/exclusion criteria appropriate? Yes □$_1$ No □$_2$ Unclear □$_3$

15) Study population: (describe age (mean +/- SD or median/range), ethnicity, smoking, BMI etc.)

16) Start of patient inclusion (year) : ☐ Unreported □1

17) End of patient inclusion (year) : ☐ Unreported □1

18) Study Design:

cohort □1 case control □2 RCT/CCT □3 cross sectional □4 before and after □5 case series □6 (n=___)

other _____□7

19) Data collection: prospective □1 retrospective □2 unreported □3 other □4

20) Enrolment: consecutive □1 arbitary (random) □2 unreported □3 other □4

21) Blinding: single □1 double □2 none □3 not reported □4

1st Predictive factor (use separate section for each factor)

10.i) Which predictive factor evaluated?

NT □1 CRL □2 Ductus venosus □3 Cord insertion □4 Plasma biochemical markers □5 Amniotic biochemical markers □6 Other _____

10.ii) More detail (eg. if USS how calculated, if biomarker which and normal values)

11.i) Gestation at time of predictive factor measured

<11 weeks □1 11-13^{+6} weeks □2 14-24 weeks □3 24^{+1}-37 weeks □4 Unreported □5 Other _____

11.i) Mean (range)_____Unreported □1

11.iii) Median (range) _____Unreported □1

12) Indication for predictive factor:

Routine clinical care □1 Down's screening □2 No clinical reason □3 Unreported □4

Other _____

2nd Predictive factor (use separate section for each factor)

10.i) Which predictive factor evaluated?

NT □1 CRL □2 Ductus venosus □3 Cord insertion □4 Plasma biochemical markers □5 Amniotic biochemical markers □6 Other _____

10.ii) More detail (eg. if USS how calculated, if biomarker which and normal values)

11.i) Gestation at time of predictive factor measured

<11 weeks □1 11-13+6 weeks □2 14-24 weeks □3 24+1-37 weeks □4 Unreported □5 Other _____

11.i) Mean (range)_____Unreported □1

11.iii) Median (range) _____Unreported □1

12) Indication for predictive factor:

Routine clinical care □1 Down's screening □2 No clinical reason □3 Unreported □4

Other _____

3rd Predictive factor (use separate section for each factor)

10.i) Which predictive factor evaluated?

NT □1 CRL □2 Ductus Venosus □3 Cord insertion □4 Plasma biochemical markers □5 Amniotic biochemical markers □6 Other _____

10.ii) More detail (eg. if USS how calculated, if biomarker which and normal values)

11.i) Gestation at time of predictive factor measured

<11 weeks □$_1$ 11-13^{+6} weeks □$_2$ 14-24 weeks □$_3$ 24^{+1}-37 weeks □$_4$ Unreported □$_5$ Other _____

11.i) Mean (range)_____Unreported □$_1$

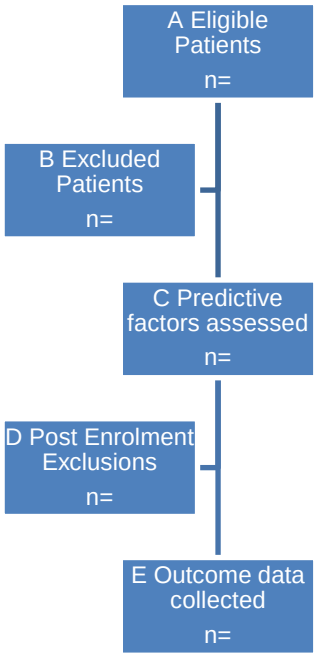
11.iii) Median (range) _____Unreported □$_1$

12) Indication for predictive factor:

Routine clinical care □$_1$ Down's screening □$_2$ No clinical reason □$_3$ Unreported □$_4$

Other _____

22) Numbers:



B Reasons _____

23) Completeness of Verification:

(= E / C x 100 = %)

> 90% □$_1$ 81-90% □$_2$ < 81% □$_3$

Outcome(s)

11.i) Outcome(s) assessed by predictive factor:

TTTS □$_1$ sFGR □$_2$ Fetal death □$_3$ TAPS □$_4$ TOPS □$_5$ Discordant fetal anomalies □$_6$ Pre-term labour □$_7$ Neurological comorbidity □$_8$ Birth weight □$_9$ Other □$_{10}$

Other _____

11.ii) Definition of outcome:

24.i) Adequate □1 Inadequate □2 Unclear □3

25) Follow-up details (e.g. mode, frequency, length)

24.i) Adequate □1 Inadequate □2 Unclear □3

Results

Predictive factor:

Predictive: Yes □1 No □2

Reasons given for being predictive:

10.3 OMMIT data collection form

Woman's Demographic Details

Study number (OMMIT1 ____ ____ ____) (see patient list for study number)

Date of birth _____

Marital status single / married or CP / Partner / Separated / Divorced / Widowed

Was the woman in paid employment at booking? Yes / No

If Yes, what is her occupation? _____

What is her partner's (if any) occupation _____

Smoking status never smoked / current smoker / ex-smoker

If ex-smoker, date gave up _____

Ethnicity of mother of baby _____

Ethnicity of father of baby _____

Height at booking (cm) _____

Weight at booking (kg) _____

Previous Obstetric History

Number of completed pregnancies beyond 24 weeks _____

Number of pregnancies less than 24 weeks _____

2.2.a) If pregnancy <24 weeks, please specify gestation of each pregnancy

_____

2.3 Any history of previous preterm birth Yes / No

2.3a) If yes, please specify number of pregnancies and gestation at delivery of each._____

2.4 Any history of neonatal death: Yes / No

If yes, please specify gestation at delivery and age at death if known

_____

Did the women have a previous history of multiple pregnancy? Yes / No

2.5a If yes, please give details

_____

Did the woman have any other <u>previous</u> pregnancy problems Yes / No

If Yes, please specify details

Problem	**Yes**	**Details e.g. medication**
Ovarian hyperstimulation syndrome		
Hyperemesis requiring admission		
Gestational diabetes		
Severe infection e.g. pyelonephritis		
Pregnancy induced hypertension		
Pre-eclampsia		
Eclampsia		
Placenta praevia		
Placental abruption		
Post-partum haemorrhage requiring transfusion		
Surgical procedure in pregnancy		
Puerperal psychosis		
Thrombotic event		
Amniotic fluid embolism		

Problem	Yes	Details e.g. medication
Stillbirth		
Baby with a major congenital abnormality		
Small for gestational age (SGA) infant		
Large for gestational age (LGA) infant		
Other (please specify:		

Previous Medical History

Did the woman have any pre-existing medical problems Yes / No

If Yes, please specify details

Problem	Yes	Details
Cardiac disease (congenital or acquired)		
Renal disease		
Endocrine disorders e.g. hypo or hyperthyroidism		
Psychiatric disorders		
Haematological disorders e.g. sickle cell disease, diagnosed thrombophilia		
Inflammatory disorders e.g. inflammatory bowel disease		

Problem	Yes	Details e.g. medication
Autoimmune diseases		
Cancer		
HIV		
Other (please specify):		

This Pregnancy

Estimated date of delivery (EDD) (Use the best estimate (ultrasound scan or date of last menstrual period [based on a 40 week gestation]) _____

Was this an assisted conception pregnancy? Yes / No

4.2a) If yes specify type of artificial reproductive technique e.g. IVF, ICSI, clomiphene etc.

Was there evidence of any fetal complications of monochorionic pregnancy?

Yes / No

4.3a) If yes, please complete the table below

Growth discordance between twins (>20% difference)	**Diagnosis present?**	**Description of severity of complication**	**Antenatal management instituted?**	**Did the woman have an antenatal ultrasound or MRI to look for neurological damage?**
(If yes, please provide details indicated in boxes to the right)	Yes / No Date of diagnosis: Gestation at **first** diagnosis:	**Greatest** estimated fetal weight discordance (%): Gestation of **greatest** discordance: Which twin smallest: Twin 1 / 2 Did the growth plot below the 10th centile: twin 1 / twin 2 / neither Umbilical artery Doppler end-diastolic flow in twin 1: positive / **constant** absent or reversed / **intermittent** absent or reversed Umbilical artery Doppler end-diastolic flow in twin 2: positive / **constant** absent or reversed / **intermittent** absent or reversed Any other details:	Yes / No Type of intervention: Date of procedure:	Yes / No Type of imaging: Date of imaging: Findings (please continue on separate sheet):

Twin to twin transfusion syndrome (TTTS)	**Diagnosis present?**	**Description of severity of complication by Quintero stage*.**	**Antenatal management instituted?**	**Did the woman have an antenatal ultrasound or MRI to look for neurological damage?**
(If yes, please provide details indicated in boxes to the right)	Yes / No Date of diagnosis:	Donor twin: Twin 1 / 2 Recipient twin: Twin 1 / 2 Quintero staging* (see end of data collection form for classification):	Yes / No Type of intervention: Date of procedure:	Yes / No Type of imaging: Date of imaging: Findings (please continue on separate sheet):
Intrauterine death	**Diagnosis present?**	**Description of complication**	**Antenatal management instituted?**	**Did the woman have an antenatal ultrasound or MRI to look for neurological damage?**
(If yes, please provide details indicated in boxes to the right)	Twin 1: Yes / No Date of diagnosis: Twin 2: Yes / No Date of diagnosis:	Cause (if known?): Any other details:	Yes / No Type of intervention: Date of procedure:	Yes / No Type of imaging: Date of imaging: Findings (please continue on separate sheet):

Chromosomal anomaly	**Diagnosis present?**	**Description of chromosomal anomaly**	**Antenatal management instituted?**	**Did the woman have an antenatal ultrasound or MRI to look for neurological damage*?***
(If yes, please provide details indicated in boxes to the right)	Twin 1: Yes / No Twin 2: Yes / No Date of diagnosis:	Twin 1: Twin 2:	Yes / No Type of intervention: Date of procedure:	Yes / No Type of imaging: Date of imaging: Findings (please continue on separate sheet):
Structural anomaly	**Diagnosis present?**	**Description of structural anomaly**	**Antenatal management instituted?**	**Did the woman have an antenatal ultrasound or MRI to look for neurological damage?**
(If yes, please provide details indicated in boxes to the right)	Twin 1: Yes / No Twin 2: Yes / No Date of diagnosis:	Twin 1: Twin 2:	Yes / No Type of intervention: Date of procedure:	Yes / No Type of imaging: Date of imaging: Findings (please continue on separate sheet):

Other problem e.g. TOPS, TAPS	**Diagnosis present?**	**Description of complication**	**Antenatal management instituted?**	**Did the woman have an antenatal ultrasound or MRI to look for neurological damage?**
(If yes, please provide details indicated in boxes to the right)	Twin 1: Yes / No Twin 2: Yes / No Date of diagnosis:	Twin 1: Twin 2:	Yes / No Type of intervention: Date of procedure:	Yes / No Type of imaging: Date of imaging: Findings (please continue on separate sheet):

Were there any other problems in <u>this</u> pregnancy? Yes / No

If Yes, please specify details

Problem	**Yes**	**Details**
Ovarian hyperstimulation syndrome		
Hyperemesis requiring admission		
Dehydration requiring admission		
Gestational diabetes		
Severe infection e.g. pyelonephritis		
Pregnancy induced hypertension		
Pre-eclampsia		
Eclampsia		
HELLP		
Placenta praevia		
Significant placental abruption		
Post-partum haemorrhage requiring intevention		
Surgical procedure in pregnancy		

Problem	Yes	Details
Puerperal psychosis		
Thrombotic event		
Cerebrovascular accident		
Amniotic fluid embolism		
Adult respiratory distress syndrome		
Disseminated intravascular coagulopathy		
Pulmonary oedema		
Mendelson's syndrome		
Renal failure		
Septicaemia		
Maternal death (if yes, please state date of death, and primary cause of death as stated on the death certificate)		
Other (please specify:		

Did she receive any of the following treatments? Yes / No

Medication	**Yes**	**Gestation given**	**Reason given**
Steroids for fetal lung maturation			Routine elective caesarean section At risk of spontaneous preterm delivery At risk of iatrogenic preterm delivery Other (please specify):
Magnesium Sulphate			Prevention of maternal fitting Fetal neuroprotection Other (please specify):
Tocolysis (please list agents):			
Other preterm labour prevention agents (may be part of a research study) e.g. Arabin pessary, Cervical cerclage, Progesterone pessary			

Delivery (this pregnancy)

Did this woman have a miscarriage? Yes / No

If Yes, please specify date _____

Did this woman have a termination of pregnancy? Yes / No

If Yes, please specify date _____

Has this women delivered? Yes / No

If she has not delivered, will she receive the rest of her antenatal care from your hospital? Yes / No

If No, please indicate name of hospital providing future care

_____

Will she be delivered at your hospital? Yes / No

If No, please indicate name of hospital where she will be delivered

_____

Did this woman have a routine Category 4 Elective Caesarean Section?

Yes / No

If Yes, please specify date _____

If Yes, please specify indication _____

What was the onset of labour? Spontaneous/ Induced / Did not labour

5.7a) If induced, please state indication _____

Did the woman reach full dilatation? Yes / No

What was mode of delivery? (please tick all that apply)

Mode of delivery	**Twin 1**	**Twin 2**
Caesarean section cat 1		
Caesarean section cat 2		
Caesarean section cat 3		
Instrumental delivery		
Normal vaginal delivery		

RCA/RCOG/CEMACH/CNST Classification for urgency of caesarean section:

1 Immediate threat to life of woman or fetus
2 Maternal or fetal compromise which is not immediately life-threatening
3 Needing early delivery but no maternal or fetal compromise
4 At a time to suit the woman and maternity team

If delivery was by Emergency Caesarean section (category 1, 2 or 3), please state the indication:

_____

Which anaesthesia was used? Regional / General / Both

5.11a) If general anaesthesia, indication: _____

Outcomes: Maternal

Was the woman admitted to HDU or ITU? Yes / No

6.1a) If Yes, please state indication for admission

_____

6.1b) specify duration of stay (days) _____

6.1c) Or Tick if woman is still in HDU or ITU ___

6.1d) Or Tick if woman was transferred to another hospital ___

Outcomes: Infants

Outcome	Twin 1	Twin 2
Date of delivery		
Time of delivery		
Gestation at delivery		
Birthweight (g)		
Sex of infant	M / F / Indeterminate	M / F / Indeterminate
Condition at birth	Live / Stillborn	Live / Stillborn
If stillborn, date of death		
Timing of stillbirth	Antenatal / Intrapartum	Antenatal / Intrapartum
Presumed cause of death		
Apgar @ 5 mins		
Cord gas (venous)		
Cord gas (arterial)		
NNU admission?	Y / N	Y / N
Date of NNU admission		
Reason for NNU admission		
Were the babies transferred to a different NNU?	Y / N	Y / N
If transferred, which NNU were they transferred to?		
Discharge date from hospital to home		

Did any major infant complications occur? Yes / No

If Yes, please tick

Problem	**Twin 1 - Yes**	**Twin 2 - yes**
Respiratory distress syndrome		
Intraventricular haemorrhage or other brain injury?		
Necrotising enterocolitis		
Neonatal encephalopathy		
Chronic lung disease		
Severe jaundice requiring phototherapy		
Major congenital anomaly		
Severe infection e.g. septicaemia, meningitis		
Exchange transfusion		
Did this infant die?	Y / N / still in NNU	Y / N / still in NNU
If yes, what was the primary cause of death as stated on the death certificate? (please state if not known)		
Other complications (please specify):		

Are there any signs of potential long-term damage? Yes / No

If yes, please tick and provide details

Problem	**Twin 1**	**Twin 2**
Ultrasound evidence of neurological damage postnatally?	Y / N / test not performed	Y / N / test not performed
If yes, date of imaging		
Type of abnormality		
MRI evidence of neurological damage postnatally?	Y / N / test not performed	Y / N / test not performed
If yes, date of imaging		
Type of abnormality (please continue on separate page if required)		
Abnormal neurological signs noted in the neonatal period prior to discharge?	Y / N / Not known	Y / N / Not known
If yes, please detail		
Evidence of cardiac impairment?	Y / N / Not known	Y / N / Not known
If yes, please detail		

Section 8: The end

Name of person completing the form:

Designation:

Today's date:

You may find it useful in the case of queries to keep a copy of this form.

If posting forms back, please send to:
Dr Fiona Mackie
3rd Floor O&G Academic Department
Birmingham Women's Hospital
Mindelsohn Way
Edgbaston
Birmingham
B15 2TG

Email: [redacted] (if patient sensitive information)

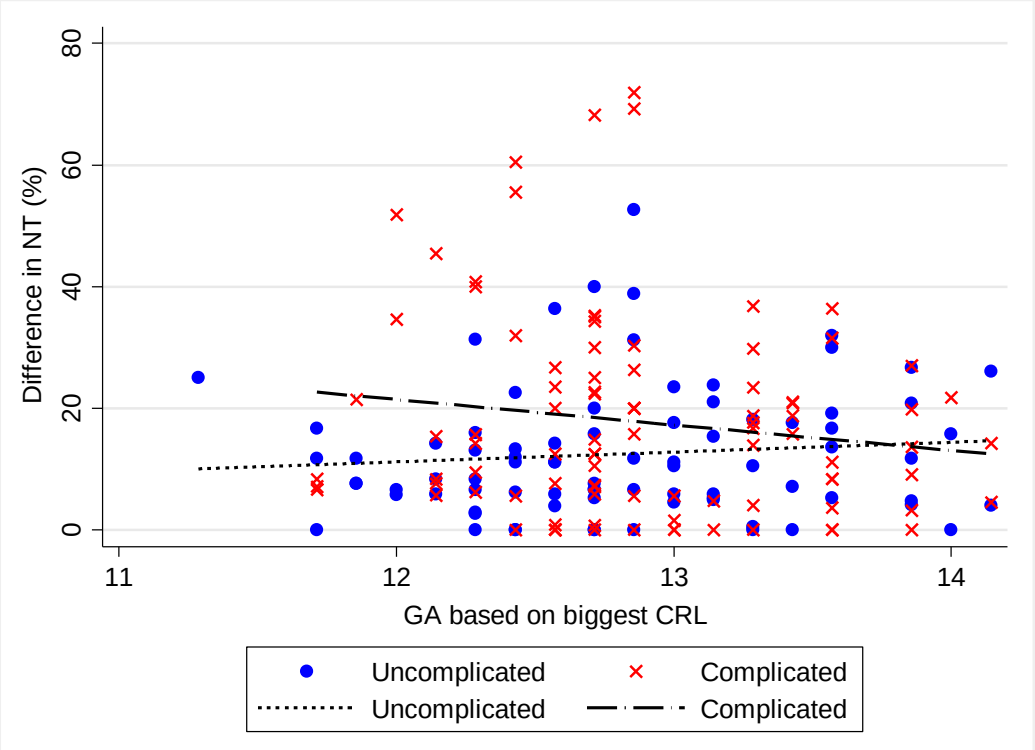
Twin to twin transfusion syndrome, please state which twin donor and which twin recipient and then state Quintero stage:

Stage I.	Poly/Oligohydramnios with bladder of the Donor still visible
Stage II.	Bladder of the Donor not visible
Stage III.	Presence of Either AEDFV in the UA, reverse flow in the DV, or pulsatile UV in either twin
Stage IV.	Hydrops in either twin
Stage V.	Demise of one or both twins

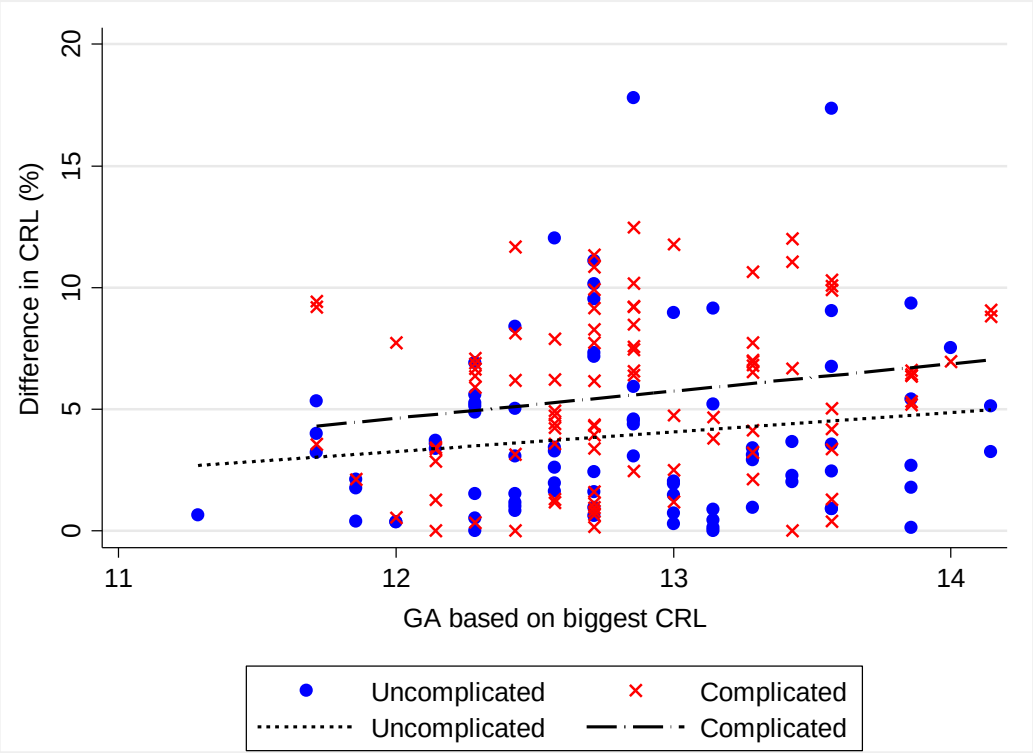
10.4 OMMIT scatter plots

The scatterplots and lines of best fit demonstrate the spread of data points for each potential prognostic factor. An uncomplicated pregnancy was defined as no adverse outcome and the delivery of 2 healthy twins after 34 weeks gestation. A complicated pregnancy was defined as being affected by at least one outcome in the fetal adverse outcome composite (TTTS, TAPS, IUFD, growth restriction).

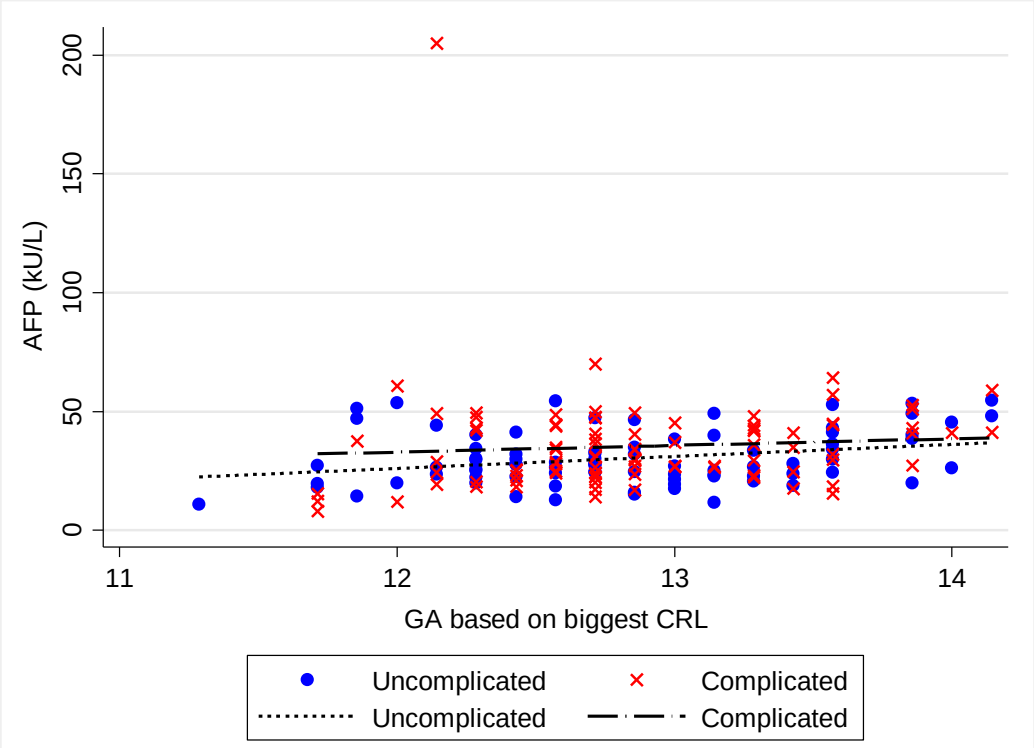
NT % discordance



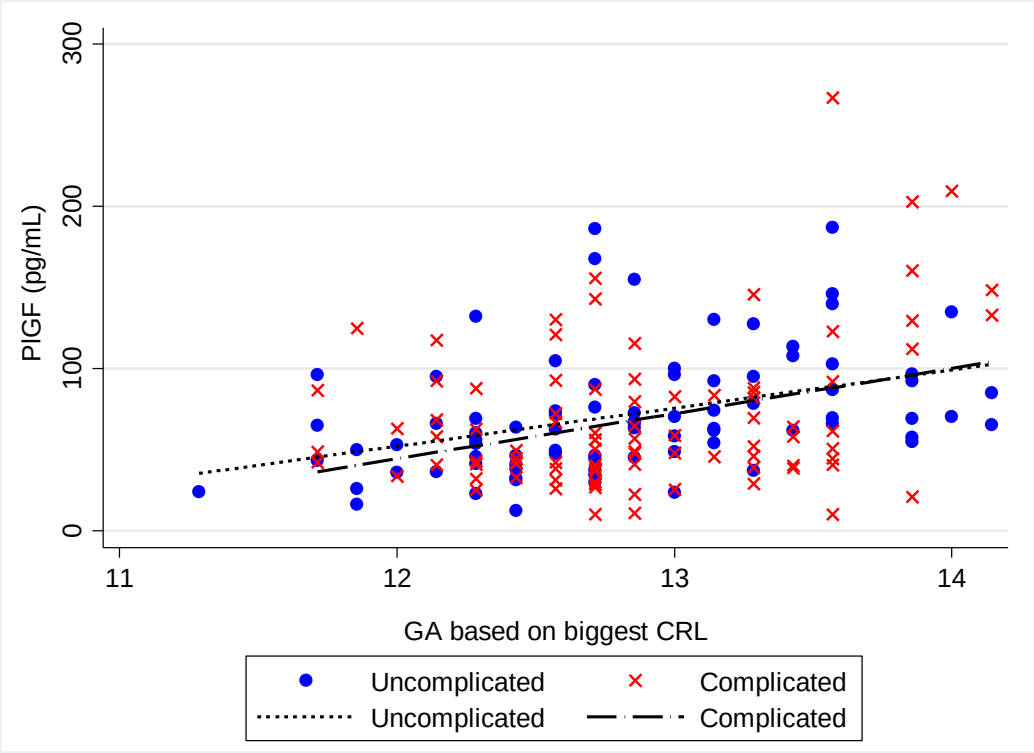
CRL % discordance



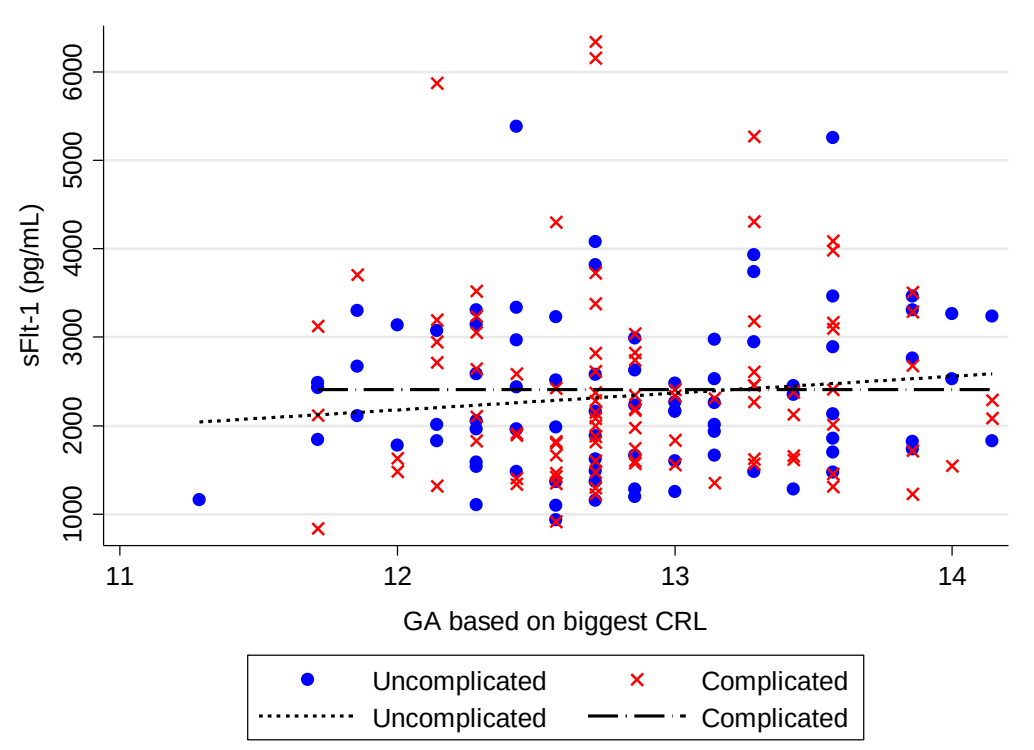
AFP



PlGF


PlGF (pg/mL)
0
100
200
300
11
12
13
14
GA based on biggest CRL
Uncomplicated
Complicated
Uncomplicated
Complicated

sFlt-1


sFlt-1 (pg/mL)
1000
2000
3000
4000
5000
6000
11
12
13
14
GA based on biggest CRL
Uncomplicated
Complicated
Uncomplicated
Complicated

10.5 Post-FLA compared to pre-FLA metabolites

All metabolites are grouped into classes of chemical structure or metabolic pathway. Fold change is calculated as the median (pre-treatment)/median (post-treatment) and the 95% confidence intervals are included in brackets.

Mass/ charge ratio	Retention time	Ion Mode	Metabolites	Metabolite class	P value	Fold change (pre/post)
129.9757	820	Negative	Nitrate and/or Peroxynitrite	Inorganic ionic metabolites	0.00270	0.74 (0.55,0.98)
186.1134	242	Negative	8-Amino-7-oxononanoate	Biotin metabolism	0.00162	0.39 (0.21,0.89)
202.0337	215	Negative	8-Hydroxy-7-methylguanine and/or Adenine	Nucleosides	0.00270	0.69 (0.52,0.89)
259.0137	230	Negative	D-Galactose 6-sulfate and/or D-Glucose 6-sulfate and/or O3-Sulfonylgalactose and/or O4-Sulfonylgalactose	Carbohydrates	0.00079	0.81 (0.58,1.10)
410.1620	816	Negative	(4E,8E,10E-d18:3) Sphingosine	Ceramides and sphingolipids	0.00468	0.61 (0.50,0.73)
424.0174	36	Negative	D-4'-Phosphopantothenate	CoA metabolism	0.00134	0.74 (0.60,0.91)
523.1454	38	Negative	Fucosyllactose	Carbohydrates	0.00389	0.80 (0.44,1.39)
532.2874	484	Negative	Glycocholic acid	Sterols and steroids	0.00270	0.70 (0.55,0.88)

539.1405	38	Negative	(1->3)-beta-D-Galactopyranans and/or (2,6-beta-D-Fructosyl)n and/or 1,3-beta-D-Oligoglucan and/or 1,6-beta-D-Glucan and/or 1-alpha-D-(1,4)-alpha-D-Glucosyl(n-1)-alpha-D-glucopyranoside and/or 1F-beta-D-Fructosylsucrose and/or 1-kestotriose and/or 3-Galactosyllactose and/or 6-alpha-D-(1,4-alpha-D-Glucano)-glucan and/or 6-alpha-Maltosylglucose and/or 6F-alpha-D-Galactosylsucrose and/or 6G-kestotriose and/or 6-kestotriose and/or beta-D-Fructofuranosyl O-beta-D-glucopyranosyl-(1-6)-alpha-D-glucopyranoside and/or beta-D-Glucan and/or Cellotriose and/or Dextrin and/or D-Gal alpha 1->6D-Gal alpha 1->6D-Glucose and/or fagopyritol B2 and/or Galactomannan and/or Isomaltotriose and/or Laminarin and/or Levan and/or Maltotriose and/or manninotriose and/or Melezitose and/or Panose and/or Polysaccharide and/or Raffinose and/or Umbelliferose	Carbohydrates	0.00058	0.79 (0.63,0.98)
565.9458	35	Negative	8-hydroxydeoxyguanosine 5'-triphosphate and/or GTP and/or Guanosine-3'-Monophosphate-5'-Diphosphate	Nucleosides	0.00451	0.73 (0.57,0.92)
573.3282	506	Negative	PC(20:4)	Glycero-phospholipids	0.00468	0.67 (0.35,1.07)
604.0563	37	Negative	CDP-ribitol	Carbohydrates	0.00134	0.78 (0.52,1.14)

612.5577	531	Negative	DG(34:0)	Acyl glycerides	0.00228	1.53 (1.04,2.39)
666.6060	567	Negative	DG(38:1)	Acyl glycerides	0.00047	2.11 (1.34,3.71)
668.6194	573	Negative	DG(38:0)	Acyl glycerides	0.00228	1.59 (1.07,2.57)
677.1417	453	Negative	2-Acetyl-Protoporphyrin I	Heme metabolism	0.00468	0.85 (0.65,1.09)
680.6215	580	Negative	DG(39:1)	Acyl glycerides	0.00389	1.64 (1.10,2.63)
680.8807	30	Negative	3'-Phosphoadenylylselenate	Other class	0.00286	0.71 (0.51,0.96)
692.1633	37	Negative	3'-Sialyllactose and/or 6'-Sialyllactose and/or Lactose Sialic Acid	Carbohydrates	0.00270	0.74 (0.61,0.89)
692.6215	570	Negative	DG(40:2)	Acyl glycerides	0.00047	2.27 (1.38,4.37)
694.6371	593	Negative	DG(40:1)	Acyl glycerides	0.00047	2.05 (1.32,3.57)
700.5307	533	Negative	PE(16:0/dm18:1) and/or PE(16:1/dm18:0) and/or PE(18:1/dm16:0)	Glycero-phospholipids	0.00031	1.47 (1.03,2.21)

701.1941	40	Negative	1,3-alpha-D-Mannosyl-1,2-alpha-D-mannosyl-1,2-alpha-D-mannosyl-D-mannose and/or 1,6-kestotetraose and/or 1F-alpha-D-Galactosylraffinose and/or 3F-alpha-D-Galactosylraffinose and/or 6G,6-kestotetraose and/or alpha-D-Galactosyl-(1-6)-alpha-D-galactosyl-(1-6)-beta-D-fructosyl-(2-1)-alpha-D-glucoside and/or Cellotetraose and/or fagopyritol B3 and/or Glycogen and/or Lychnose and/or Maltotetraose and/or Stachyose	Carbohydrates	0.00010	0.67 (0.45,0.99)
719.4780	464	Negative	DG(38:3)	Acyl glycerides	0.00286	0.86 (0.60,1.14)
722.5140	516	Negative	PE(18:3/dm18:1) and/or PE(18:4/dm18:0) and/or PE(20:4/dm16:0) and/or PE(16:0/dm18:1) and/or PE(16:1/dm18:0) and/or PE(18:1/dm16:0)	Glycero-phospholipids	0.00228	1.32 (0.96,1.90)
732.1963	40	Negative	3-Sialyl-N-acetyllactosamine and/or 6-Sialyl-N-acetyllactosamine and/or alpha-N-Acetylneuraminyl-2,6-beta-D-galactosyl-1,4-N-acetyl-beta-D- glucosamine	Carbohydrates	0.00006	0.66 (0.53,0.81)
744.4982	33	Negative	PE(18:3/dm18:1) and/or PE(18:4/dm18:0) and/or PE(20:4/dm16:0)	Glycero-phospholipids	0.00162	0.52 (0.38,0.68)
750.5382	510	Negative	PC(15:0/dm18:1) and/or PE(18:0/dm18:1) and/or PE(18:1/dm18:0) and/or PE(20:1/dm16:0)	Glycero-phospholipids	0.00389	1.27 (0.96,1.75)
776.5459	513	Negative	PC(32:1) and/or PE(35:1)	Glycero-phospholipids	0.00047	1.86 (1.26,3.12)

778.5610	526	Negative	PC(32:0) and/or PE(35:0)	Glycero-phospholipids	0.00016	1.44 (0.94,2.02)
779.5651	526	Negative	PC(32:0) and/or PE(35:0)	Glycero-phospholipids	0.00004	1.60 (1.08,2.67)
780.5686	526	Negative	PC(32:0) and/or PE(35:0)	Glycero-phospholipids	0.00091	1.16 (0.83,1.71)
788.5466	580	Negative	PS(36:1) and/or PC(33:2) and/or PE(36:2)	Glycero-phospholipids	0.00058	1.85 (1.16,3.36)
803.5655	517	Negative	PT(36:1)	Glycero-phospholipids	0.00027	1.93 (1.27,3.37)
804.5769	529	Negative	PS(37:0)	Glycero-phospholipids	0.00010	1.74 (1.15,2.92)
812.5823	524	Negative	PC(18:2/dm18:1) and/or PC(18:3/dm18:0) and/or PC(20:3/dm16:0)	Glycero-phospholipids	0.00389	1.39 (0.94,2.26)
826.5633	513	Negative	PC(36:4)	Glycero-phospholipids	0.00027	2.30 (1.49,4.17)
828.5775	519	Negative	PC(36:3)	Glycero-phospholipids	0.00047	2.00 (1.26,3.71)
832.6086	548	Negative	PC(36:1)	Glycero-phospholipids	0.00027	1.73 (1.12,3.00)
838.6000	529	Negative	PC(20:3/dm18:1) and/or PC(20:4/dm18:0) and/or PC(22:4/dm16:0)	Glycero-phospholipids	0.00389	1.51 (0.99,2.61)
852.5793	516	Negative	PC(38:5)	Glycero-phospholipids	0.00079	1.94 (1.22,3.60)
854.5957	528	Negative	PC(38:4)	Glycero-phospholipids	0.00047	1.96 (1.28,3.52)
859.6930	596	Negative	SM(d18:0/24:1) and/or SM(d18:1/24:0)	Ceramides and sphingolipids	0.00270	1.74 (1.17,2.99)

878.5944	524	Negative	PC(40:6)	Glycero-phospholipids	0.00134	1.63 (0.98,3.12)
884.5509	510	Negative	PC(O-17:0/18:1) and/or PE(20:0/dm18:0) and/or PE(22:0/dm16:0)	Glycero-phospholipids	0.00018	1.48 (1.05,2.31)
888.6808	539	Negative	PC(O-19:0/22:0)	Glycero-phospholipids	0.00228	1.74 (1.23,2.57)
951.5361	510	Negative	PI(40:4)	Glycero-phospholipids	0.00134	1.38 (1.03,1.99)
971.5852	482	Negative	PE(44:2)	Glycero-phospholipids	0.00270	0.62 (0.46,0.82)
982.6173	482	Negative	PE(44:5)	Glycero-phospholipids	0.00468	0.73 (0.50,1.00)
127.0359	66	Positive	Hydroxybutyric acid	Oxidised fatty acids	0.00451	1.13 (0.89,1.44)
132.1018	69	Positive	Isoleucine and/or L-Leucine	Amino acid metabolism	0.00001	1.31 (0.98,1.78)
138.0524	69	Positive	Proline	Amino acid metabolism	0.00001	1.41 (1.17,1.72)
142.9387	92	Positive	Sulfate	Inorganic ionic metabolites	0.00468	1.19 (0.98,1.44)
147.1128	48	Positive	Lysine	Amino acid metabolism	0.00001	1.36 (1.08,1.73)
155.0681	362	Positive	Hydroxyhexanoic acid	Oxidised fatty acids	0.00058	1.27 (0.98,1.66)
156.0765	55	Positive	Histidine	Amino acid metabolism	0.00097	1.45 (1.14,1.86)
186.0732	60	Positive	Norvaline and/or Valine	Amino acid metabolism	0.00016	1.45 (1.20,1.78)

203.1502	67	Positive	N,N-dimethylarginine	Acyl amino acids and related metabolites	0.00058	1.19 (0.93,1.54)
204.1230	68	Positive	Acetylcarnitine	Acyl carnitines	0.00010	1.22 (1.01,1.48)
228.0636	365	Positive	Methoxyindoleacetate	Other class	0.00010	1.35 (1.14,1.62)
230.0783	394	Positive	N-Acetyl-phenylalanine	Acyl amino acids and related metabolites	0.00228	1.11 (0.76,1.61)
241.1418	399	Positive	Decanoate	Fatty acid metabolism	0.00010	1.30 (1.04,1.66)
243.1338	414	Positive	Dodecenoic acid	Fatty acid metabolism	0.00016	1.46 (1.10,1.98)
249.0593	379	Positive	Dimethylxanthine and/or Theobromine and/or Theophylline	Other class	0.00286	1.17 (0.96,1.43)
260.1857	396	Positive	Hexanoylcarnitine	Acyl carnitines	0.00001	1.37 (1.06,1.78)
267.0587	68	Positive	Pseudouridine and/or Uridine	Nucleosides	0.00058	1.20 (0.93,1.56)
267.1191	394	Positive	S-Acetyldihydrolipoamide	Oxidative phosphory-lation	0.00053	1.23 (0.87,1.85)
272.2586	575	Positive	Hexadecenoic acid	Fatty acid metabolism	0.00058	0.92 (0.85,0.99)
282.1198	64	Positive	Methyladenosine and/or Methyl-deoxyguanosine	Nucleosides	0.00004	1.27 (1.11,1.46)

287.2442	57	Positive	N,N-Diacetylspermine	Acyl amino acids and related metabolites	0.00001	1.36 (0.98,1.93)
289.2204	453	Positive	Octanoylcarnitine	Acyl carnitines	0.00010	1.36 (0.97,1.95)
290.2473	603	Positive	5a-Androst-3-en-17-one	Thyroid and steroid hormones	0.00286	0.91 (0.72,1.12)
301.1411	585	Positive	Hydroxydodecanoic acid	Oxidised fatty acids	0.00001	0.50 (0.43,0.60)
305.0841	62	Positive	Methylinosine	Nucleosides	0.00027	1.27 (1.09,1.49)
313.2740	643	Positive	Oxo-nonadecanoic acid	Oxidised fatty acids	0.00270	1.62 (1.09,2.33)
316.2484	495	Positive	Decanoylcarnitine	Acyl carnitines	0.00010	1.32 (0.95,1.86)
318.3004	509	Positive	4-hydroxysphinganine and/or Phytosphingosine	Ceramides and sphingolipids	0.00286	0.91 (0.85,0.98)
325.2732	610	Positive	11(R)-HEDE and/or 11S-HEDE and/or 15(R)-HEDE	Oxidised fatty acids	0.00286	0.86 (0.70,1.06)
357.1956	560	Positive	Octadecenoic acid	Fatty acid metabolism	0.00058	1.63 (0.64,2.65)
368.1528	70	Positive	Estrone sulfate	Thyroid and steroid hormones	0.00058	1.36 (1.13,1.64)

370.2571	685	Positive	(13E)-11alpha-Hydroxy-9,15-dioxoprost-13-enoate and/or (5e,13e)-9,15-Dihydroxy-11-Oxoprosta-5,13-Dien-1-Oicacid and/or (5Z)-(15S)-11alpha-Hydroxy-9,15-dioxoprostanoate and/or (5Z,13E)-(15S)-11-alpha,15-dihydroxy-9-oxoprosta-5,13-dienoate and/or (5z,13e)-(15s)-6,9-alpha-epoxy-11-alpha,15-dihydroxyprosta-5,13-dienoate and/or (5Z,13E)-(15S)-9alpha,11alpha-epoxy-15-hydroxythromboxa-5,13-dienoate and/or (5Z,13E)-(15S)-9alpha,15-Dihydroxy-11-oxoprosta-5,13-dienoate and/or (6Z,8E,10E,14Z)-(5S,12R)-5,12,20-Trihydroxyicosa-6,8,10,14- tetraenoate and/or 11beta-PGE2 and/or 13,14-dihydro-15-keto-PGD2 and/or 13,14-dihydro-15-oxo-lipoxin A4 and/or 15-epi-lipoxin A4 and/or 15-epi-lipoxin B4 and/or 15-Keto-prostaglandin F2alpha and/or 15-oxo-PGE1 and/or 15R-PGD2 and/or 15R-PGE2 and/or 20-OH-hepoxilin A3 and/or 20-OH-Leukotriene B4 and/or 20-OH-Leukotriene B4 and/or 5,12,20-TriHETE and/or 5,14,15-trihydroxy-6,8,10,12-Eicosatetraenoic acid and/or 5S,6S-Lipoxin A4 and/or 5-trans-PGE2 and/or 8-iso-15-keto-PGF2a and/or 8-iso-PGF3a and/or 8-isoprostaglandin E2 and/or D17 PGE1 and/or delta-12-PGD2 and/or Dinoprostone (JAN/USP/INN) and/or	Oxidised fatty acids	0.00010	1.66 (1.14,2.52)

			Epoprostenol (USAN/INN) and/or Levuglandin D2 and/or Levuglandin E2 and/or Lipoxin A4 and/or Lipoxin B4 and/or Narbonolide and/or PGE2 and/or PGK1 and/or Prostaglandin E2 and/or Prostaglandin F3alpha and/or Prostaglandin H2 and/or Prostaglandin I2 and/or prostaglandin-H2 and/or Thromboxane A2 and/or w-hydroxyl leukotriene B4			
386.3996	607	Positive	dimethyl-docosanoic acid and/or methyl-tricosanoic acid and/or tetracosanoate	Fatty acid metabolism	0.00027	1.30 (0.61,2.95)
395.1381	536	Positive	QH2	Oxidative phosphory-lation	0.00010	0.23 (0.17,0.33)
400.4154	612	Positive	Pentacosanoic acid	Fatty acid metabolism	0.00157	1.24 (0.58,2.85)
401.2579	655	Positive	Methyl-eicosanoic acid and/or Heneicosanoic acid	Fatty acid metabolism	0.00286	0.74 (0.55,0.93)
405.2759	513	Positive	Tetracosatetraenoic acid	Fatty acid metabolism	0.00004	0.38 (0.31,0.47)
406.1446	539	Positive	Poly-L-glutamate	Amino acid metabolism	0.00270	0.23 (0.17,0.36)
414.4314	617	Positive	Hexacosanoic acid	Fatty acid metabolism	0.00004	1.22 (0.56,2.88)
427.2670	536	Positive	1(3)-glyceryl-PGD2 and/or 1(3)-glyceryl-PGE2 and/or 1(3)-glyceryl-PGH2 and/or 2-glyceryl-PGD2 and/or 2-glyceryl-PGE2 and/or 2-glyceryl-PGH2	Oxidised fatty acids	0.00011	0.02 (0.01,0.03)

459.1296	383	Positive	FMNH	Oxidative phosphory-lation	0.00010	1.37 (1.12,1.70)
496.3403	596	Positive	LysoPC(16:0)	Glycero-phospholipids	0.00010	1.76 (1.25,2.49)
504.4365	655	Positive	Ceramide (d18:1/12:0)	Ceramides and sphingolipids	0.00468	1.89 (1.26,2.62)
509.1768	439	Positive	Estriol-glucuroinide	Thyroid and steroid hormones	0.00058	1.23 (0.94,1.62)
510.4886	683	Positive	N-(Tetradecanoyl)-sphing-4-enine	Ceramides and sphingolipids	0.00011	1.48 (1.01,2.17)
522.2863	526	Positive	Chenodeoxycholoyltaurine and/or Taurochenodeoxycholate and/or Taurodeoxycholic acid and/or Tauroursodeoxycholic acid	Bile acid metabolism	0.00058	1.3 (0.83,2.09)
522.3579	598	Positive	LysoPC(18:1)	Glycero-phospholipids	0.00097	1.82 (1.27,2.65)
524.3708	617	Positive	LysoPC(18:0)	Glycero-phospholipids	0.00010	1.68 (1.24,2.25)
529.2423	521	Positive	11-hydroxyprogesterone 11-glucuronide	Thyroid and steroid hormones	0.00058	1.20 (0.63,2.11)
535.4343	693	Positive	DG(28:0)	Acyl glycerides	0.00286	1.33 (1.09,1.61)

539.5236	700	Positive	N-Palmitoylsphingosine	Ceramides and sphingolipids	0.00001	1.57 (1.14,2.20)
541.2332	695	Positive	Leukotriene D4	Oxidised fatty acids	0.00010	1.54 (1.18,2.10)
542.2777	543	Positive	Octadecenoyl carnitine	Acyl carnitines	0.00286	1.26 (0.96,1.67)
542.3224	581	Positive	LysoPC(20:5) and/or LysoPC(18:2)	Glycero-phospholipids	0.00451	1.47 (1.05,2.04)
545.2362	416	Positive	13-cis-retinoyl-beta-D-glucuronide and/or all-trans-Retinoyl-beta-glucuronide	Other class	0.00134	1.23 (0.79,1.98)
562.5149	700	Positive	Cer(d18:0/16:0)	Ceramides and sphingolipids	0.00001	1.37 (1.03,1.84)
563.2452	460	Positive	Tetrahydroaldosterone-3-glucuronide	Thyroid and steroid hormones	0.00010	1.37 (1.04,1.81)
564.2495	510	Positive	LysoPE(22:6)	Glycero-phospholipids	0.00010	1.42 (1.08,1.87)
565.4355	612	Positive	PC(O-3:1/O-18:1)	Glycero-phospholipids	0.00004	1.78 (1.11,2.89)
568.5664	727	Positive	Cer(d18:0/18:0)	Ceramides and sphingolipids	0.00091	1.29 (0.91,1.85)
575.4666	670	Positive	DG(33:4) and/or DG(31:1)	Acyl glycerides	0.00006	2.11 (1.19,3.74)
583.4003	618	Positive	Hentriacontanoic acid	Fatty acid metabolism	0.00468	1.44 (1.09,1.88)

585.4814	696	Positive	Tritriacontanoic acid	Fatty acid metabolism	0.00468	1.57 (0.98,2.48)
594.5827	745	Positive	Ceramide (d18:1/20:0)	Ceramides and sphingolipids	0.00006	1.41 (0.99,2.01)
595.3468	441	Positive	Urobilin	Heme metabolism	0.00031	1.37 (0.90,2.18)
601.4813	662	Positive	DG(35:5) and/or DG(33:2)	Acyl glycerides	0.00010	2.05 (1.22,3.34)
609.2601	614	Positive	2-hydroxyestrone-1-S-glutathione	Thyroid and steroid hormones	0.00286	1.26 (0.99,1.62)
614.2843	572	Positive	LysoPE(22:4)	Glycero-phospholipids	0.00270	1.27 (0.90,1.80)
628.3592	682	Positive	LysoPC(19:0) and/or PC(3:0/O-16:0) and/or PC(O-16:0/3:0) and/or PC(O-17:0/2:0) and/or PC(O-18:0/1:0)	Glycero-phospholipids	0.00286	0.79 (0.65,0.96)
633.4487	633	Positive	PA(31:1)	Glycero-phospholipids	0.00058	1.41 (1.07,1.88)
634.5399	612	Positive	DG(36:4)	Acyl glycerides	0.00097	1.37 (1.15,1.63)
636.4600	633	Positive	PC(25:0) and/or PE(28:0)	Glycero-phospholipids	0.00001	2.04 (1.17,3.57)
641.5126	733	Positive	DG(38:6) and/or DG(36:3)	Acyl glycerides	0.00058	1.54 (1.15,2.03)
643.5279	753	Positive	DG(38:5) and/or DG(36:2)	Acyl glycerides	0.00058	1.63 (1.22,2.17)

644.5958	777	Positive	Ceramide (d18:1/22:0)	Ceramides and sphingolipids	0.00058	1.34 (0.90,1.97)
647.4595	763	Positive	DG(35:4)	Acyl glycerides	0.00058	0.82 (0.72,0.93)
651.6487	803	Positive	Cer(d18:0/24:1)	Ceramides and sphingolipids	0.00468	1.06 (0.67,1.94)
651.7983	483	Positive	Triiodothyronine	Thyroid and steroid hormones	0.00058	1.24 (0.97,1.59)
652.6011	761	Positive	Episteryl palmitoleate and/or fecosteryl palmitoleate	Fatty acid metabolism	0.00053	1.26 (0.82,1.99)
658.6116	797	Positive	Tricosanamide	Fatty acid metabolism	0.00010	1.51 (1.06,2.19)
667.5279	752	Positive	DG(40:7) and/or DG(38:4) and/or DG(36:1)	Acyl glycerides	0.00010	1.78 (1.22,2.72)
670.6115	770	Positive	Ceramide (d18:1/24:1)	Ceramides and sphingolipids	0.00001	1.82 (1.19,2.97)
675.5445	688	Positive	SM(d18:1/14:0)	Ceramides and sphingolipids	0.00001	1.63 (1.18,2.34)
689.5604	709	Positive	CE(18:1)	Cholesterol esters	0.00001	1.62 (1.15,2.40)
690.5638	719	Positive	N-(2-hydroxyhenicosanoyl)-4,8-sphingadienine	Ceramides and sphingolipids	0.00010	1.75 (1.22,2.70)

690.6378	801	Positive	N-(24-hydroxytetracosanyl)sphinganine and/or N-tetracosanylphytosphingosine	Ceramides and sphingolipids	0.00270	1.27 (0.92,1.79)
695.5107	672	Positive	SM(d18:0/12:0)	Ceramides and sphingolipids	0.00027	1.53 (1.05,2.29)
700.4900	694	Positive	PE(33:3) and/or PC(28:0) and/or PE(31:0)	Glycero-phospholipids	0.00011	1.54 (1.07,2.34)
700.5603	55	Positive	PC(O-14:0/O-16:0)	Glycero-phospholipids	0.00047	1.35 (1.00,1.82)
701.5604	693	Positive	Episteryl oleate and/or fecosteryl oleate and/or lanosteryl palmitoleate	Fatty acid metabolism	0.00001	1.61 (1.14,2.37)
704.5794	712	Positive	N-(2-hydroxydocosanoyl)-4,8-sphingadienine	Ceramides and sphingolipids	0.00001	1.43 (1.07,1.96)
707.5434	697	Positive	TG(36:0)	Acyl glycerides	0.00058	1.46 (1.05,2.11)
713.5494	701	Positive	3-demethylubiquinone-8	Oxidative phosphory-lation	0.00016	1.81 (1.23,2.80)
717.5915	715	Positive	CE(20:1)	Cholesterol esters	0.00001	1.48 (1.04,2.21)
718.5754	701	Positive	PC(14:0/dm18:0) and/or PC(16:0/dm16:0) and/or PC(O-14:0/18:1)	Glycero-phospholipids	0.00058	1.49 (1.09,2.13)
720.5549	726	Positive	PC(31:0) and/or PE(34:0)	Glycero-phospholipids	0.00002	1.44 (1.01,2.16)
726.5614	702	Positive	SM(d18:1/16:0)	Ceramides and sphingolipids	0.00001	1.46 (1.07,2.05)

727.1948	487	Positive	Dehydroisocoproporphyrinogen	Heme metabolism	0.00468	1.36 (0.72,2.53)
727.5681	713	Positive	Coenzyme Q8 and/or ubiquinone-8	Oxidative phosphory-lation	0.00001	1.42 (1.06,1.95)
729.2107	517	Positive	Coproporphyrin and/or Uroporphyrin	Heme metabolism	0.00468	1.09 (0.85,1.41)
731.6074	728	Positive	SM(d18:0/18:1)	Ceramides and sphingolipids	0.00001	1.53 (1.13,2.14)
733.5858	775	Positive	PC(14:0/dm18:1) and/or PC(14:1/dm18:0) and/or PC(16:1/dm16:0)	Glycero-phospholipids	0.00468	1.28 (0.86,1.84)
738.5995	781	Positive	DG(44:8)	Acyl glycerides	0.00468	1.26 (0.87,1.82)
739.5508	677	Positive	DG(40:5)	Acyl glycerides	0.00001	1.60 (1.18,2.19)
742.5731	734	Positive	PC(16:1/dm18:1) and/or PC(18:2/dm16:0)	Glycero-phospholipids	0.00010	1.39 (0.97,2.06)
748.4906	61	Positive	PC(32:4) and/or PE(35:4)	Glycero-phospholipids	0.00058	1.66 (1.15,2.53)
748.5861	755	Positive	PC(33:0) and/or PE(36:0)	Glycero-phospholipids	0.00270	1.18 (0.77,1.98)
752.5602	729	Positive	PE(20:3/dm18:1) and/or PE(20:4/dm18:0) and/or PE(22:4/dm16:0) and/or PE(O-16:0/22:5) and/or PE(O-18:0/20:5) and/or PE(O-18:1/20:4)	Glycero-phospholipids	0.00286	1.58 (1.13,2.31)
753.5817	727	Positive	Ubiquinol 8	Oxidative phosphory-lation	0.00010	1.46 (1.07,2.06)

755.5405	710	Positive	CE(20:5)	Cholesterol esters	0.00010	1.44 (1.08,1.98)
759.4971	687	Positive	DG(44:11)	Acyl glycerides	0.00016	1.54 (1.13,2.17)
759.5735	717	Positive	PC(34:2)	Glycero-phospholipids	0.00010	1.57 (1.14,2.22)
760.5863	738	Positive	PC(34:1) and/or PE(37:1)	Glycero-phospholipids	0.00010	1.45 (1.10,1.97)
766.4087	597	Positive	PS(32:4) and/or PE(32:5)	Glycero-phospholipids	0.00010	0.86 (0.76,0.97)
766.5732	750	Positive	PC(18:3/dm18:1) and/or PC(18:4/dm18:0) and/or PC(20:4/dm16:0)	Glycero-phospholipids	0.00001	1.59 (1.11,2.43)
768.5534	726	Positive	PC(35:4) and/or PE(38:4)	Glycero-phospholipids	0.00058	1.46 (1.12,1.97)
770.5698	670	Positive	PC(35:3) and/or PE(36:0)	Glycero-phospholipids	0.00010	1.70 (1.23,2.38)
772.5887	747	Positive	PC(35:2)	Glycero-phospholipids	0.00079	1.35 (1.08,1.78)
774.5422	724	Positive	PE(22:6/dm18:1)	Glycero-phospholipids	0.00286	1.51 (1.10,2.15)
774.6011	698	Positive	PC(35:1) and/or PE(38:1)	Glycero-phospholipids	0.00468	1.59 (1.07,2.49)
777.6960	485	Positive	O-(4-Hydroxy-3,5-diidophenyl)-3,5-diiodo-L-tyrosine and/or thyroxine	Thyroid and steroid hormones	0.00162	1.21 (0.96,1.53)
781.6198	734	Positive	SM(d18:1/20:0)	Ceramides and sphingolipids	0.00001	1.52 (1.11,2.14)

785.6545	764	Positive	SM(d18:1/22:1)	Ceramides and sphingolipids	0.00010	1.60 (1.13,2.35)
787.6053	740	Positive	PC(36:2)	Glycero-phospholipids	0.00010	1.55 (1.12,2.22)
787.6698	793	Positive	SM(d18:1/22:0)	Ceramides and sphingolipids	0.00001	1.59 (1.10,2.39)
796.5841	739	Positive	PC(37:4) and/or PE(40:4) and/or PE(38:1)	Glycero-phospholipids	0.00001	1.56 (1.11,2.32)
813.5459	734	Positive	PS(36:1) and/or PG(38:6) and/or PC(33:2) and/or PE(36:2)	Glycero-phospholipids	0.00286	1.46 (1.06,1.97)
813.6855	798	Positive	SM(d18:1/24:1)	Ceramides and sphingolipids	0.00016	1.63 (1.08,2.60)
816.5653	56	Positive	PE(20:0/dm18:1) and/or PE(20:1/dm18:0) and/or PE(22:1/dm16:0)	Glycero-phospholipids	0.00097	1.51 (1.05,2.31)
818.6050	731	Positive	PC(22:5/dm18:1) and/or PC(22:6/dm18:0) and/or PC(20:2/dm18:1) and/or PC(20:3/dm18:0)	Glycero-phospholipids	0.00001	1.62 (1.13,2.45)
821.5239	689	Positive	PG(36:1)	Glycero-phospholipids	0.00270	1.52 (1.06,2.39)
825.0334	703	Positive	Diguanosine diphosphate	Nucleosides	0.00157	1.54 (1.03,2.33)
828.5073	733	Positive	PC(O-15:0/20:4) and/or PE(20:2/dm18:1) and/or PE(20:3/dm18:0)	Glycero-phospholipids	0.00058	1.26 (0.98,1.59)

833.6522	768	Positive	SM(d18:0/22:0)	Ceramides and sphingolipids	0.00010	1.62 (1.16,2.36)
836.5374	721	Positive	PS(36:0) and/or PE(38:4) and/or PE(36:1)	Glycero-phospholipids	0.00010	1.52 (1.15,2.06)
837.5403	726	Positive	PG(36:0)	Glycero-phospholipids	0.00027	1.45 (1.11,1.94)
842.6039	744	Positive	PC(22:4/dm18:1) and/or PC(22:5/dm18:0) and/or PC(37:0 and/or PE(40:0)	Glycero-phospholipids	0.00053	1.30 (0.95,1.83)
844.5390	712	Positive	PC(18:1/dm18:1) and/or PC(18:2/dm18:0) and/or PC(20:2/dm16:0)	Glycero-phospholipids	0.00010	1.68 (1.21,2.42)
854.6703	745	Positive	GlcCer(d18:0/22:0)	Ceramides and sphingolipids	0.00053	1.41 (1.07,1.88)
868.6010	411	Positive	PC(37:2) and/or PE(40:2)	Glycero-phospholipids	0.00286	1.23 (0.91,1.68)
872.5699	756	Positive	PC(20:1/dm18:1) and/or PC(20:2/dm18:0) and/or PC(22:2/dm16:0)	Glycero-phospholipids	0.00006	1.53 (1.09,2.24)
875.6235	742	Positive	PC(42:8)	Glycero-phospholipids	0.00011	1.45 (1.05,2.06)
901.6392	750	Positive	PC(44:9)	Glycero-phospholipids	0.00016	1.56 (1.09,2.34)
910.5107	704	Positive	PC(42:11)	Glycero-phospholipids	0.00006	1.56 (1.07,2.34)
912.5263	735	Positive	PC(42:10)	Glycero-phospholipids	0.00010	1.68 (1.21,2.43)
916.5281	717	Positive	PC(44:12)	Glycero-phospholipids	0.00011	1.54 (1.14,2.11)

918.5439	735	Positive	PC(44:11)	Glycero-phospholipids	0.00016	1.59 (1.16,2.26)
923.5036	716	Positive	PI(38:5)	Glycero-phospholipids	0.00004	1.46 (1.09,1.99)
924.5716	733	Positive	PC(42:9)	Glycero-phospholipids	0.00389	1.36 (0.97,1.92)
938.6262	407	Positive	PE(44:3)	Glycero-phospholipids	0.00451	1.24 (0.92,1.71)
944.2437	664	Positive	Decanoyl-CoA	CoA metabolism	0.00468	0.89 (0.78,1.01)

10.6 Metabolites correlated with RV MPI pre-FLA

All metabolites listed demonstrated a Spearman Rank correlation between -1.0 to -0.3 or +0.3 to +1.0 and are grouped into classes of chemical structure or metabolic pathway.

Mass/ charge ratio	Retention time	Ion mode	Metabolite	Metabolite Class	Correlation Coefficient
203.1502	67	Positive	N,N-dimethylarginine	Acyl amino acids	0.30
287.2442	57	Positive	N,N-Diacetylspermine	Acyl amino acids	0.37
542.2777	543	Positive	Octadecenoyl carnitine	Acyl carnitine	-0.43
204.1230	68	Positive	Acetylcarnitine	Acyl carnitine	-0.40
184.0943	56	Positive	Carnitine	Acyl carnitine	-0.36
232.1543	75	Positive	Butanoylcarnitine	Acyl carnitine	-0.33
289.2204	453	Positive	Octanoylcarnitine	Acyl carnitine	-0.32
260.1857	396	Positive	Hexanoylcarnitine	Acyl carnitine	-0.31
547.4343	635	Positive	DG(29:1)	Acyl glycerides	-0.49
739.5508	677	Positive	DG(40:5)	Acyl glycerides	-0.40
707.5434	697	Positive	TG(36:0)	Acyl glycerides	-0.37
713.5494	701	Positive	DG(40:3)	Acyl glycerides	-0.34

633.4487	633	Positive	DG(36:7);DG(34:4)	Acyl glycerides	-0.33
745.5988	751	Positive	DG(40:2)	Acyl glycerides	-0.31
957.6695	391	Positive	TG(56:10)	Acyl glycerides	0.32
429.1987	580	Positive	MG(14:0)	Acyl glycerides	0.41
953.6869	399	Positive	TG(55:10)	Acyl glycerides	0.60
339.0221	346	Positive	Vanillactic acid	Aromatic metabolites	-0.41
532.2909	338	Negative	Glycocholic acid	Bile acid metabolism	0.31
651.2658	335	Negative	Taurallocholic acid;Tauroursocholic acid	Bile acid metabolism	0.31
570.2418	531	Positive	Deoxycholic acid disulfate	Bile acid metabolism	0.36
187.0577	59	Positive	1,5-Anhydro-D-glucitol;Deoxy-galactose;Deoxy-glucose;Fucose;Rhamnose; Rhamnulose	Carbohydrates	-0.33
275.0655	36	Negative	1-Amino-1-deoxy-scyllo-inositol 4-phosphate;Glucosamine 1-phosphate;Aminofructose 6-phosphate;Galactosamine 1-phosphate;Galactosamine 6-phosphate;Glucosamine-4-Phosphate;Glucosamine-1-Phosphate	Carbohydrates	0.30

673.2322	41	Negative	3-Sialyl-N-acetyllactosamine;6-Sialyl-N-acetyllactosamine	Carbohydrates	0.35
634.2114	37	Negative	3'-Sialyllactose;6'-Sialyllactose	Carbohydrates	0.45
867.6750	750	Positive	SM(d18:1/25:0)	Ceramides and sphingolipids	-0.49
792.5794	736	Positive	Cer(d18:1/26:1)	Ceramides and sphingolipids	-0.41
660.5699	766	Positive	Cer(d18:1/22:0)	Ceramides and sphingolipids	-0.38
510.4886	683	Positive	N-(Tetradecanoyl)-sphing-4-enine	Ceramides and sphingolipids	-0.37
654.4016	733	Positive	N-(2-hydroxypentadecanoyl)-4,8-sphingadienine	Ceramides and sphingolipids	-0.36
340.2821	519	Positive	4-hydroxysphinganine; Phytosphingosine	Ceramides and sphingolipids	-0.33
539.5236	700	Positive	N-Palmitoylsphingosine	Ceramides and sphingolipids	-0.30
458.2247	606	Positive	Sphinganine-1-phosphate	Ceramides and sphingolipids	0.40
738.6497	693	Positive	Cer(d18:0/26:0)	Ceramides and sphingolipids	0.42
912.0391	378	Positive	Formyl-CoA	CoA metabolism	-0.40
242.0998	82	Positive	Pantothenic acid	CoA metabolism	-0.30
201.1111	433	Positive	Decenedioic acid	Fatty acid metabolism	-0.52
213.1101	407	Positive	Octanoic acid	Fatty acid metabolism	-0.45
374.3033	592	Positive	Anandamide (20:2, n-6)	Fatty acid metabolism	-0.43
357.1956	560	Positive	Octadecenoic acid	Fatty acid metabolism	-0.41

583.4003	618	Positive	Hentriacontanoic acid	Fatty acid metabolism	-0.40
451.3777	625	Positive	Pentacosanoic acid	Fatty acid metabolism	-0.35
377.3224	594	Positive	Anandamide (20:l, n-9)	Fatty acid metabolism	-0.32
160.1335	317	Positive	Aminooctanoic acid;Octenoic acid	Fatty acid metabolism	-0.30
467.2860	596	Positive	Docosanoic acid	Fatty acid metabolism	0.32
401.1641	270	Negative	Docosahexaenoic acid	Fatty acid metabolism	0.32
289.2932	515	Positive	Methyl-hexadecanoic acid;Heptadecanoic acid	Fatty acid metabolism	0.33
266.2085	551	Positive	Amino-tetradecanoic acid	Fatty acid metabolism	0.38
608.6364	33	Negative	Docosanyl octadecanoate;eicosanyl icosanoate;hexadecyl tetracosanoate;octadecyl docosanoate;tetracosanyl hexadecanoate;tetradecyl hexacosanoate;;	Fatty acid metabolism	0.46
880.5985	450	Positive	PC(36:0)	Glycerophospholipids	-0.64
720.5549	726	Positive	PE(34:0)	Glycerophospholipids	-0.45
872.6479	801	Positive	PC(22:0/dm18:1);PC(22:1/dm18:0);PC(24:1/dm16:0)	Glycerophospholipids	-0.45

614.2843	572	Positive	LysoPE(22:6)	Glycerophospholipids	-0.44
748.5861	755	Positive	PE(36:0)	Glycerophospholipids	-0.42
742.5731	734	Positive	PC(16:1/dm18:1);PC(18:2/dm16:0);PC(O-16:0/18:3);PC(P-16:0/18:2)	Glycerophospholipids	-0.42
791.5685	731	Positive	PE(22:6/dm18:1)	Glycerophospholipids	-0.42
718.5394	715	Positive	PE(34:1)	Glycerophospholipids	-0.41
888.5360	736	Positive	PC(18:0/dm18:1);PC(18:2/O-18:0);PC(20:1/dm16:0);PC(P-18:0/18:1)	Glycerophospholipids	-0.40
842.6039	744	Positive	PE(40:0)	Glycerophospholipids	-0.39
649.3451	516	Positive	PG(24:0)	Glycerophospholipids	-0.38
958.7256	440	Positive	PE(48:1)	Glycerophospholipids	-0.38
837.5403	726	Positive	PG(36:0)	Glycerophospholipids	-0.36
700.4900	694	Positive	PC(28:0)	Glycerophospholipids	-0.36
932.6470	405	Positive	PC(44:4)	Glycerophospholipids	-0.35
747.5136	717	Positive	PG(34:2)	Glycerophospholipids	-0.35
767.5788	750	Positive	PC(18:3/dm18:1);PC(18:4/dm18:0);PC(20:4/dm16:0);PC(O-16:0/20:5);PC(P-16:0/20:4)	Glycerophospholipids	-0.35
923.5036	716	Positive	PI(38:5)	Glycerophospholipids	-0.32

752.5602	729	Positive	PE(20:3/dm18:1);PE(20:4/dm18:0);PE(22:4/dm16:0)	Glycerophospholipids	-0.32
590.3300	492	Positive	LysoPI(16:0)	Glycerophospholipids	-0.32
818.6050	731	Positive	PC(22:5/dm18:1);PC/dm18:0);PC(P-18:0/22:6);PC(20:2/dm18:1;PC(20:3/dm18:0);PC(O-16:0/22:4);PC(O-18:0/20:4)	Glycerophospholipids	-0.32
844.6192	713	Positive	PC(22:4/dm18:0);PC(O-18:0/22:5);PC(20:0/dm18:1);PC(20:1/dm18:0);PC(22:1/dm16:0)	Glycerophospholipids	-0.31
718.5754	701	Positive	PC(14:0/dm18:0);PC(16:0/dm16:0);PC(O-14:0/18:1)	Glycerophospholipids	-0.31
912.5263	735	Positive	PC(42:10)	Glycerophospholipids	-0.30
982.6173	482	Negative	PE(44:2)	Glycerophospholipids	0.31
573.3282	506	Negative	PC(20:4)	Glycerophospholipids	0.31
834.5431	33	Negative	PE(40:3)	Glycerophospholipids	0.36
511.1917	415	Positive	PG(12:0)	Glycerophospholipids	0.51
695.2615	775	Positive	Protoporphyrinogen IX	Heme metabolism	-0.34
355.2245	479	Positive	Dihydroxy-stearic acid;methyl-hexadecanoic acid	Mixed class	-0.59

331.0182	57	Positive	2-deoxyglucose-6-phosphate;Glycerophosphoglycerol	Mixed class	0.46
306.0926	64	Positive	Orotidine	Nucleoside	-0.36
667.1006	632	Positive	2'-Deoxycytidine-2'-Deoxyadenosine-3',5'-Monophosphate	Nucleosides	-0.50
385.0209	363	Positive	2',3'-Dehydro-2',3'-Deoxy-Thymidine 5'-Diphosphate	Nucleosides	-0.34
565.9458	35	Negative	8-hydroxydeoxyguanosine 5'-triphosphate;Guanosine triphosphate	Nucleosides	0.54
743.5405	695	Positive	Cholesteryl 11-hydroperoxy-eicosatetraenoate	Other class	-0.43
263.0796	419	Positive	Hydroxynicotine;Nicotine-N-oxide	Other class	-0.41
450.0877	209	Negative	Succinyladenosine	Other class	0.32
420.2155	604	Positive	S-Octyl GSH	Other class	0.38
511.0943	506	Positive	CDP-choline	Other class	0.57
604.0563	37	Negative	CDP-ribitol	Other class	0.71
579.2165	486	Positive	Leukotriene D5	Oxidised fatty acids	-0.48
187.1328	401	Positive	Hydroxydecenoic acid;Oxodecanoic acid	Oxidised fatty acids	-0.40

398.2885	731	Positive	PGF2alpha-EA;;16,16-dimethyl-PGD2;16,16-dimethyl-PGE2;1a,1b-dihomo-PGD2;1a,1b-dihomo-PGE2;20-ethyl-PGE2	Oxidised fatty acids	-0.31
346.3319	535	Positive	Hydroxyeicosanoic acid	Oxidised fatty acids	0.32
329.1605	496	Positive	Prostaglandin M	Oxidised fatty acids	0.36
159.1029	283	Negative	Hydroxyoctanoic acid	Oxidised fatty acids	0.48
361.2354	603	Positive	10,11-dihydro-12-epi-leukotriene B4;11,12-DHET;11-deoxy-PGE1;11-deoxy-PGF2a;11-deoxy-PGF2beta;12-keto-tetrahydro-Leukotriene B4;14,15-DHET;15-hydroperoxyeicosa-8Z,11Z,13E-trienoate;5,6-DHET;5,6-dihydroxy-8,11,14-eicosatrienoic acid;6,7-dihydro-12-epi-leukotriene B4;8,9-DHET;8,9-dihydroxy-5,11,14-eicosatrienoic acid	Oxidised fatty acids	0.57
259.0926	38	Negative	L-alpha-glutamyl-L-hydroxyproline	Peptides	0.51
696.9590	60	Positive	UDP-D-galacturonate;UDP-D-glucuronate	Sugar nucleotide metabolism	-0.65

602.0526	50	Negative	Thymidine-5'-Diphospho-Beta-D-Xylose	Sugar nucleotide metabolism	0.36
267.1191	394	Positive	S-Acetyldihydrolipoamide	Oxidative phosphorylation	0.32
651.7983	483	Positive	Triiodothyronine	Thyroid and steroid metabolism	-0.37

10.7 Metabolites correlated with LV MPI pre-FLA

All metabolites listed demonstrated a Spearman Rank correlation between -1.0 to -0.3 or +0.3 to +1.0 and are grouped into classes of chemical structure or metabolic pathway.

Mass/ charge ratio	Retention time	Ion mode	Metabolite	Metabolite Class	Correlation Coefficient
203.1502	67	Positive	N,N-dimethylarginine	Acyl amino acids	0.34
287.2442	57	Positive	N,N-Diacetylspermine	Acyl amino acids	0.39
270.1092	72	Positive	Butanoylcarnitine	Acyl carnitine	-0.46
444.3049	636	Positive	Palmitoylcarnitine	Acyl carnitine	-0.42
406.2173	447	Positive	Decanoylcarnitine	Acyl carnitine	-0.34
202.1093	252	Negative	Acetylcarnitine	Acyl carnitine	-0.31
548.2890	606	Positive	Docosapentaenoyl carnitine	Acyl carnitine	0.41
781.6437	650	Positive	DG(43:0)	Acyl glycerides	-0.52
855.7422	797	Positive	TG(52:4) and/or TG(50:1)	Acyl glycerides	-0.47
634.5399	612	Positive	DG(36:4)	Acyl glycerides	-0.41
535.4343	693	Positive	DG(28:0)	Acyl glycerides	-0.40
707.5434	697	Positive	TG(36:0)	Acyl glycerides	-0.39

547.4343	635	Positive	DG(29:1)	Acyl glycerides	-0.38
759.4971	687	Positive	DG(44:11)	Acyl glycerides	-0.34
698.6628	650	Positive	DG(40:0)	Acyl glycerides	-0.32
961.6651	384	Positive	TG(58:12)	Acyl glycerides	-0.31
843.6096	437	Positive	DG(46:6)	Acyl glycerides	-0.31
647.4595	763	Positive	DG(35:4)	Acyl glycerides	0.40
909.7193	435	Positive	TG(51:4)	Acyl glycerides	0.54
281.1008	231	Negative	S-aminomethyldihydrolipoamide	Amino acid metabolism	-0.48
154.0836	68	Positive	Isoleucine and/or Leucine	Amino acid metabolism	-0.47
172.0397	60	Positive	Methionine	Amino acid metabolism	-0.41
314.0174	349	Positive	Indoleacrylic acid	Aromatic metabolites	-0.37
228.0636	365	Positive	Methoxyindoleacetate and/or Indolelactate	Aromatic metabolites	-0.31
495.2383	512	Positive	Chenodeoxycholic acid sulfate and/or Ursodeoxycholic acid 3-sulfate	Bile acid metabolism	-0.38
532.2874	484	Negative	Glycocholic acid	Bile acid metabolism	0.32
478.2889	549	Positive	Glycolithocholic acid	Bile acid metabolism	0.44
259.0137	230	Negative	Galactose 6-sulfate and/or Glucose 6-sulfate	Carbohydrates	-0.57

692.1633	37	Negative	3'-Sialyllactose and/or 6'-Sialyllactose	Carbohydrates	0.33
732.1963	40	Negative	3-Sialyl-N-acetyllactosamine and/or 6-Sialyl-N-acetyllactosamine	Carbohydrates	0.65
792.5794	736	Positive	Cer(d18:1/26:1)	Ceramides and sphingolipids	-0.43
873.6764	438	Positive	SM(d18:1/25:0)	Ceramides and sphingolipids	-0.41
854.6703	745	Positive	GlcCer(d18:0/22:0)	Ceramides and sphingolipids	-0.34
729.5918	715	Positive	SM(d18:1/18:1)	Ceramides and sphingolipids	-0.34
814.6887	800	Positive	SM(d18:1/24:1)	Ceramides and sphingolipids	-0.33
608.4995	640	Positive	Cer(d18:1/18:1)	Ceramides and sphingolipids	-0.32
675.5445	688	Positive	SM(d18:1/14:0)	Ceramides and sphingolipids	-0.31
759.6381	703	Positive	SM(d18:1/20:0)	Ceramides and sphingolipids	-0.30
952.1884	414	Positive	Octanoyl-CoA	CoA metabolism	-0.34
242.0998	82	Positive	Pantothenic acid	CoA metabolism	0.36
285.1799	77	Positive	Pentadecenoic acid	Fatty acid metabolism	-0.67
194.1525	526	Positive	Decanamide	Fatty acid metabolism	-0.46
399.2425	678	Positive	Heneicosenoic acid	Fatty acid metabolism	-0.43
369.2408	529	Positive	Octadecanoic acid	Fatty acid metabolism	-0.39
413.1948	585	Positive	Nonadecenoic acid	Fatty acid metabolism	-0.37
374.3033	592	Positive	Anandamide (20:2, n-6)	Fatty acid metabolism	-0.37

272.2586	575	Positive	Amino-hexadecanoic acid and/or hexadecenoic acid	Fatty acid metabolism	-0.34
241.1418	399	Positive	Decanoic acid	Fatty acid metabolism	-0.34
224.1623	499	Positive	Amino-undecanoic acid	Fatty acid metabolism	0.31
551.4441	709	Positive	Hentriacontanoic acid	Fatty acid metabolism	0.54
720.4816	464	Negative	PC(28:1)	Glycerophospholipids	-0.53
786.6395	389	Positive	PE(22:0/dm18:1) and/or PE(22:1/dm18:0) and/or PE(24:1/dm16:0)	Glycerophospholipids	-0.49
580.2962	572	Positive	PC(18:1)	Glycerophospholipids	-0.48
946.5888	423	Positive	PC(42:6)	Glycerophospholipids	-0.46
636.3620	636	Positive	PC(22:1)	Glycerophospholipids	-0.45
802.5731	746	Positive	PE(22:4/dm18:0) and/or PE(20:0/dm18:1) and/or PE(20:1/dm18:0) and/or PE(22:1/dm16:0)	Glycerophospholipids	-0.44
608.2570	618	Positive	LysoPS(18:1)	Glycerophospholipids	-0.43
615.1862	515	Positive	PG(16:0)	Glycerophospholipids	-0.43
923.5036	716	Positive	PI(38:5)	Glycerophospholipids	-0.43

778.6338	646	Positive	PA(40:0)	Glycerophospholipids	-0.42
496.3403	596	Positive	LysoPC(16:0)	Glycerophospholipids	-0.41
890.5874	399	Positive	PE(42:5) and/or PE(40:2)	Glycerophospholipids	-0.40
626.3007	466	Positive	LysoPC(22:6)	Glycerophospholipids	-0.40
692.5241	684	Positive	PE(32:0)	Glycerophospholipids	-0.40
896.6722	437	Positive	PC(22:0/dm18:1) and/or PC(22:1/dm18:0) and/or PC(24:1/dm16:0)	Glycerophospholipids	-0.40
760.5012	643	Positive	PE(16:0/dm18:1) and/or PE(16:1/dm18:0) and/or PE(18:1/dm16:0)	Glycerophospholipids	-0.39
932.6470	405	Positive	PC(44:4)	Glycerophospholipids	-0.39
912.5263	735	Positive	PC(42:10)	Glycerophospholipids	-0.38
623.3181	598	Positive	LysoPI(18:0)	Glycerophospholipids	-0.38
700.4900	694	Positive	PC(28:0)	Glycerophospholipids	-0.37
759.5735	717	Positive	PC(34:2)	Glycerophospholipids	-0.37
836.5374	721	Positive	PC(34:0) and/or PE(38:4) and/or PE(36:1)	Glycerophospholipids	-0.36
910.5107	704	Positive	PC(42:11)	Glycerophospholipids	-0.36

522.3579	598	Positive	LysoPC(18:1)	Glycerophospholipids	-0.35
918.5439	735	Positive	PC(44:11)	Glycerophospholipids	-0.35
837.5403	726	Positive	PG(36:0)	Glycerophospholipids	-0.34
546.3537	614	Positive	LysoPC(20:3)	Glycerophospholipids	-0.34
923.5885	528	Negative	PC(38:4)	Glycerophospholipids	-0.34
524.3708	617	Positive	LysoPC(18:0)	Glycerophospholipids	-0.34
768.5534	726	Positive	PE(38:4) and/or PE(36:1)	Glycerophospholipids	-0.31
720.5549	726	Positive	PE(34:0)	Glycerophospholipids	-0.31
544.3379	585	Positive	LysoPC(20:4)	Glycerophospholipids	-0.31
791.5685	731	Positive	PE(22:6/dm18:1)	Glycerophospholipids	-0.31
916.5281	717	Positive	PC(44:12)	Glycerophospholipids	-0.30
740.5576	748	Positive	PC(18:3/dm16:0) and/or PC(O-14:0/20:4) and/or PC(16:0/dm16:0) and/or PC(O-14:0/18:1)	Glycerophospholipids	0.34
922.5065	443	Positive	PS(37:0)	Glycerophospholipids	0.35
554.2765	469	Positive	LysoPC(dm16:0)	Glycerophospholipids	0.41
642.2114	605	Positive	LysoPE(22:4)	Glycerophospholipids	0.44
619.3017	280	Negative	PC(20:2)	Glycerophospholipids	0.46
922.7293	446	Positive	PC(42:4)	Glycerophospholipids	0.48

830.6646	444	Positive	PE(42:1)	Glycerophospholipids	0.50
629.2355	592	Positive	Phytochromobilin and/or Dihydrobiliverdin and/or Bilirubin	Heme metabolism	-0.61
605.2399	643	Positive	Biliverdin	Heme metabolism	-0.41
595.3468	441	Positive	Urobilin	Heme metabolism	0.38
729.2107	517	Positive	Coproporphyrin and/or Uroporphyrin	Heme metabolism	0.42
331.0182	57	Positive	2-deoxyglucose-6-phosphate and/or Glycerophosphoglycerol	Mixed class	0.44
310.0095	96	Positive	N-Acetylgalactosaminate and/or Choline sulfate	Mixed class	0.61
168.0631	64	Positive	Isobutyrylglycine and/or Aminoadipate 6-semialdehyde and/or Methyl-pyrrolidinone	Mixed class	0.66
320.0972	58	Positive	Methylguanosine and/or Deoxyadenosine	Nucleoside	-0.48
338.0624	38	Negative	Methyladenosine and/or deoxyguanosine	Nucleoside	-0.43
306.0926	64	Positive	Orotidine	Nucleoside	0.34
210.1101	382	Positive	8-Amino-7-oxononanoate	Other class	-0.39
743.5405	695	Positive	Cholesteryl 11-hydroperoxy-eicosatetraenoate	Other class	-0.32

511.0943	506	Positive	CDP-choline	Other class	0.31
933.6153	378	Positive	all trans Decaprenyl diphosphate	Oxidative phosphorylation	-0.56
833.5874	732	Positive	Ubiquinone-9	Oxidative phosphorylation	-0.39
459.1296	383	Positive	Oxidised flavin mononucleotide	Oxidative phosphorylation	0.42
744.5905	482	Positive	Coenzyme Q8 and/or ubiquinone-8	Oxidative phosphorylation	0.42
209.1154	444	Positive	Oxo-decanoic acid and/or hydroxy-decenoic acid	Oxidised fatty acids	-0.31
325.1607	490	Positive	Oxo-hydroxy-tetradecenoate	Oxidised fatty acids	-0.31
442.2607	665	Positive	Leukotriene E3	Oxidised fatty acids	-0.30
435.3804	646	Positive	Hydroxyhexacosanoic acid	Oxidised fatty acids	-0.30
259.0926	38	Negative	L-alpha-glutamyl-L-hydroxyproline	Peptides	0.32
463.0587	374	Positive	5'-Butyrylphosphoinosine	Purine metabolism	-0.36
255.9818	81	Positive	Se-Methylselenomethionine	Selenocompound metabolism	-0.48
448.9128	53	Positive	Selenohomocystine	Selenocompound metabolism	-0.30
651.7983	483	Positive	Triiodothyronine	Thyroid and steroid metabolism	-0.45
369.1749	324	Negative	Dihydrotestosterone sulfate and/or Androsterone sulfate	Thyroid and steroid metabolism	-0.41

10.8 All miRNAs tested by Exiqon profiling array

hsa-miR-107	hsa-miR-543	hsa-miR-30e-3p
hsa-let-7b-5p	hsa-miR-625-3p	hsa-miR-99a-5p
hsa-miR-30b-5p	hsa-miR-335-3p	hsa-miR-181a-5p
hsa-miR-26b-5p	hsa-miR-194-5p	hsa-miR-146b-5p
hsa-let-7c-5p	hsa-miR-432-5p	hsa-miR-362-3p
hsa-miR-582-3p	hsa-miR-525-5p	hsa-miR-941
hsa-miR-9-5p	hsa-miR-193a-5p	hsa-miR-126-5p
hsa-miR-135a-5p	hsa-miR-146a-5p	hsa-miR-93-3p
hsa-miR-519d-3p	hsa-miR-26b-3p	hsa-miR-101-3p
hsa-miR-556-3p	hsa-miR-340-5p	hsa-miR-378a-5p
hsa-miR-517c-3p	hsa-miR-130a-3p	hsa-miR-101-5p
hsa-miR-197-3p	hsa-miR-15a-3p	hsa-miR-532-3p
hsa-miR-33a-3p	hsa-miR-1296-5p	hsa-miR-139-3p
hsa-miR-361-5p	hsa-miR-502-3p	hsa-miR-628-3p
hsa-miR-517a-3p	hsa-miR-491-5p	hsa-miR-15b-5p
hsa-miR-17-5p	hsa-miR-345-5p	hsa-miR-376a-3p
hsa-miR-223-3p	hsa-miR-501-5p	hsa-miR-519c-3p
hsa-miR-144-3p	hsa-miR-1249	hsa-miR-505-5p
hsa-let-7d-3p	hsa-miR-339-5p	hsa-miR-126-3p
hsa-miR-519a-3p	hsa-let-7i-5p	hsa-miR-15b-3p
hsa-miR-140-5p	hsa-miR-191-5p	hsa-let-7f-2-3p
hsa-miR-140-3p	hsa-miR-769-5p	hsa-miR-33a-5p
hsa-miR-518c-3p	hsa-miR-484	hsa-miR-342-3p
hsa-miR-501-3p	hsa-miR-1271-5p	hsa-miR-337-5p
hsa-miR-519b-3p	hsa-miR-21-5p	hsa-miR-518f-3p
hsa-miR-629-5p	hsa-miR-582-5p	hsa-miR-223-5p
hsa-miR-20b-5p	hsa-miR-493-3p	hsa-miR-339-3p
hsa-miR-28-3p	hsa-miR-431-5p	hsa-miR-152-3p

hsa-miR-940
hsa-miR-148b-5p
hsa-miR-660-5p
hsa-miR-424-5p
hsa-miR-186-5p
hsa-miR-103a-3p
hsa-miR-125a-5p
hsa-miR-155-5p
hsa-miR-218-5p
hsa-miR-331-3p
hsa-miR-329-3p
hsa-let-7b-3p
hsa-miR-1
hsa-miR-335-5p
hsa-miR-127-3p
hsa-let-7g-3p
hsa-miR-509-3p
hsa-miR-518b
hsa-miR-551a
hsa-miR-515-3p
hsa-miR-598-3p
hsa-miR-196b-5p
hsa-miR-1537-3p
hsa-miR-24-3p
hsa-miR-214-3p
hsa-miR-454-3p
hsa-miR-525-3p
hsa-miR-365a-3p
hsa-miR-148a-3p
hsa-miR-324-3p
hsa-miR-320b
hsa-miR-520h
hsa-miR-2110
hsa-miR-320d
hsa-miR-142-3p
hsa-miR-377-3p
hsa-miR-515-5p
hsa-miR-379-5p
hsa-miR-96-5p
hsa-miR-32-5p
hsa-miR-627-5p
hsa-miR-27b-3p
hsa-miR-153-3p
hsa-miR-145-3p
hsa-miR-16-2-3p
hsa-miR-664a-3p
hsa-miR-542-5p
hsa-miR-618
hsa-miR-26a-5p
hsa-miR-550a-3p
hsa-miR-16-5p
hsa-miR-99b-5p
hsa-miR-520a-5p
hsa-miR-450b-5p
hsa-miR-520g-3p
hsa-miR-122-3p
hsa-miR-143-3p
hsa-miR-425-3p
hsa-miR-25-3p
hsa-let-7f-5p
hsa-miR-376a-5p
hsa-miR-30e-5p
hsa-miR-326
hsa-miR-376b-3p
hsa-miR-328-3p
hsa-miR-106b-5p
hsa-miR-744-5p
hsa-miR-624-5p
hsa-miR-518c-5p
hsa-miR-370-3p
hsa-miR-524-5p
hsa-miR-106b-3p
hsa-let-7g-5p
hsa-miR-10a-5p
hsa-miR-19b-1-5p
hsa-miR-106a-5p
hsa-miR-574-3p
hsa-miR-144-5p
hsa-miR-889-3p
hsa-miR-203a
hsa-miR-1260a
hsa-miR-320a
hsa-miR-7-1-3p
hsa-miR-136-3p
hsa-miR-519e-3p
hsa-miR-30a-3p
hsa-miR-128-3p

hsa-miR-425-5p
hsa-miR-19b-3p
hsa-miR-337-3p
hsa-miR-125b-5p
hsa-miR-221-5p
hsa-miR-132-3p
hsa-miR-502-5p
hsa-miR-654-5p
hsa-miR-188-3p
hsa-miR-30a-5p
hsa-miR-141-3p
hsa-miR-496
hsa-miR-20a-5p
hsa-miR-1972
hsa-miR-423-3p
hsa-miR-526b-5p
hsa-let-7d-5p
hsa-miR-584-5p
hsa-miR-579-3p
hsa-miR-518e-5p
hsa-miR-301a-3p
hsa-miR-182-5p
hsa-miR-151a-5p
hsa-miR-29a-5p
hsa-miR-224-5p
hsa-miR-143-5p
hsa-miR-23b-3p
hsa-miR-185-5p
hsa-miR-548j-5p
hsa-miR-215-5p
hsa-miR-450a-5p
hsa-miR-22-3p
hsa-miR-100-5p
hsa-miR-29b-3p
hsa-miR-512-3p
hsa-miR-874-3p
hsa-miR-518f-5p
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hsa-miR-142-5p
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hsa-miR-330-3p
hsa-miR-495-3p
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hsa-miR-148b-3p
hsa-miR-320c
hsa-miR-369-3p
hsa-miR-181b-5p
hsa-miR-545-3p
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hsa-miR-628-5p
hsa-miR-27a-5p
hsa-miR-133b
hsa-miR-181c-3p
hsa-miR-375
hsa-miR-522-3p

hsa-miR-188-5p	hsa-miR-150-5p	hsa-miR-200c-3p
hsa-miR-93-5p	hsa-miR-34a-5p	hsa-miR-130b-3p
hsa-miR-18a-3p	hsa-miR-27a-3p	hsa-miR-877-5p
hsa-miR-766-3p	hsa-miR-323a-3p	hsa-miR-485-3p
hsa-miR-210-3p	hsa-miR-219a-5p	hsa-miR-30d-3p
hsa-miR-376c-3p	hsa-miR-193b-3p	hsa-miR-20a-3p
hsa-let-7i-3p	hsa-miR-151a-3p	hsa-miR-199a-5p
hsa-miR-19a-3p	hsa-miR-29c-5p	hsa-miR-548a-3p
hsa-miR-122-5p	hsa-miR-181c-5p	hsa-miR-340-3p
hsa-miR-29c-3p	hsa-miR-421	hsa-miR-651-5p
hsa-miR-652-3p	hsa-miR-29a-3p	hsa-miR-92a-3p
hsa-miR-382-5p	hsa-miR-483-5p	hsa-miR-519e-5p
hsa-miR-33b-5p	hsa-miR-363-3p	hsa-miR-374a-5p
hsa-miR-548c-5p	mmu-miR-378a-3p	hsa-miR-28-5p
hsa-miR-338-3p	hsa-miR-134-5p	hsa-miR-590-5p
hsa-miR-885-5p	hsa-miR-409-5p	hsa-miR-29b-2-5p
hsa-miR-493-5p	hsa-miR-17-3p	hsa-miR-497-5p
hsa-miR-136-5p	hsa-miR-98-5p	hsa-miR-221-3p
hsa-miR-222-3p	hsa-miR-518a-3p	hsa-miR-30c-5p
hsa-miR-200b-3p	hsa-miR-190a-5p	hsa-miR-410-3p
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hsa-miR-1911-5p
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hsa-miR-193b-5p
hsa-miR-194-3p
hsa-miR-195-3p
hsa-miR-196a-5p
hsa-miR-196b-3p
hsa-miR-198
hsa-miR-19a-5p
hsa-miR-200a-3p
hsa-miR-200b-5p
hsa-miR-204-5p
hsa-miR-206
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hsa-miR-20b-3p
hsa-miR-2113
hsa-miR-211-5p
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hsa-miR-214-5p
hsa-miR-216a-5p
hsa-miR-219a-2-3p
hsa-miR-224-3p
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hsa-miR-23b-5p
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hsa-miR-373-5p
hsa-miR-374b-3p
hsa-miR-379-3p
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hsa-miR-512-5p
hsa-miR-514a-3p
hsa-miR-516a-3p
hsa-miR-516a-5p
hsa-miR-517-5p
hsa-miR-518d-3p
hsa-miR-520a-3p
hsa-miR-520c-3p
hsa-miR-520d-3p
hsa-miR-520d-5p
hsa-miR-520e
hsa-miR-521
hsa-miR-523-3p
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hsa-miR-548a-5p
hsa-miR-548b-3p
hsa-miR-548d-5p
hsa-miR-548e-3p
hsa-miR-548i
hsa-miR-548l
hsa-miR-548n
hsa-miR-550a-5p
hsa-miR-552-3p
hsa-miR-566
hsa-miR-567
hsa-miR-570-3p
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hsa-miR-576-3p
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hsa-miR-610
hsa-miR-615-3p
hsa-miR-616-3p
hsa-miR-616-5p
hsa-miR-619-3p
hsa-miR-623
hsa-miR-629-3p
hsa-miR-631
hsa-miR-635
hsa-miR-640
hsa-miR-642a-5p
hsa-miR-643
hsa-miR-645

hsa-miR-646	hsa-miR-708-5p	hsa-miR-887-3p
hsa-miR-649	hsa-miR-758-3p	hsa-miR-922
hsa-miR-650	hsa-miR-760	hsa-miR-92a-1-5p
hsa-miR-655-3p	hsa-miR-761	hsa-miR-92b-3p
hsa-miR-661	hsa-miR-765	hsa-miR-92b-5p
hsa-miR-662	hsa-miR-769-3p	hsa-miR-933
hsa-miR-663a	hsa-miR-770-5p	hsa-miR-934
hsa-miR-663b	hsa-miR-873-5p	hsa-miR-936
hsa-miR-668-3p	hsa-miR-875-5p	hsa-miR-937-3p
hsa-miR-671-3p	hsa-miR-876-5p	hsa-miR-9-3p
hsa-miR-671-5p	hsa-miR-877-3p	hsa-miR-99a-3p
hsa-miR-708-3p	hsa-miR-885-3p	hsa-miR-99b-3p

10.9 Additional validation of candidate miRNAs methods

The validation of the candidate miRNAs was performed by Dr Andrew Beggs and Miss Agata Stodolna at the University of Birmingham. miRNA was extracted from serum with miRNeasy Serum/Plasma Kit (Qiagen, Manchester, UK) according to manufacturer's instructions. The quality and the concentration of the samples was investigated with High Sensitivity RNA TapeStation (Agilent, Stockport, UK). Two endogenous controls were used: miR-361-5p and miR-451a. Samples were converted to cDNA and miRNA was amplified using TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher Scientific, Warrington UK). To create the cDNA templates, samples, and reagents were thawed on ice and vortexed to ensure adequate mixing. The samples were then centrifuged to remove air bubbles. 3 µL of Poly(A) Reaction Mix was added to each well of the reaction plate with 2 µL of serum sample. The reaction plates were sealed, vortexed and centrifuged. The plate was placed in the thermal cycles at 37°C for 45 minutes, 65°C for 10 minutes, and then held at 4°C before performing the adaptor ligation reaction. 10 µL of Ligation Reaction Mix was added to each well and the reaction plate was sealed, vortexed and centrifuged. The plate was placed in the thermal cycler and incubated at 16°C for 60 minutes, and then held at 4°C.

RT-PCR was performed with TaqMan Advanced miRNA Assays (Applied Biosystems). The RT Reaction Mix was prepared and 15 µL were added to each well of the reaction plate, the plate was sealed, vortexed and centrifuged. The plate was

placed in the thermal cycler and incubated at 42°C for 15 minutes, 85°C for 5 minutes, and then held at 4°C.

miR-Amp Reaction Mix was prepared and 45 µL was added to a new reaction plate. 5 µL of the RT reaction product was added to each reaction well, the plate was sealed, vortexed and centrifuged. The plate was placed in the thermal cycler and incubated at 95°C for 5 minutes for 1 cycle, 95°C for 3 seconds then 60°C for 30 seconds for 14 cycles, 99°C for 60 minutes and then held at 4°C.

5µl of 1:10 dilution of cDNA template was used for the RT-PCR reaction with 10µl of 2X TaqMan Fast Advanced Master Mix (Applied Biosystems), 1µl of 20X TaqMan Advanced miRNA Assay and 4µl of RNase-free water. The reaction was run on the QuantStudio 5 instrument (Applied Biosystems) under conditions: 1 cycle for 20 seconds at 95ºC, then 40 cycles of 1 second at 95ºC and 20 seconds at 60ºC.

10.10 Significantly different miRNAs in investigation cohort

Significantly different miRNAs, prior to Benjamini-Hochberg correction, in pregnancies complicated by twin-twin transfusion syndrome, compared to uncomplicated MCDA pregnancies

Assay	Average dcq TTTS (SD)	Average dcq Control (SD)	ddcq TTTS – Control	Fold change TTTS / Control	t-test p-value	Benjamini-Hochberg FDR
hsa-miR-107	2.581 (-0.305)	1.765 (0.209)	0.816	1.761	0.002	0.486
hsa-miR-519c-3p	-3.212 (0.551)	-4.600 (0.373)	1.388	2.618	0.003	0.486
hsa-miR-551a	-2.259 (0.560)	-1.098 (0.219)	-1.160	-2.235	0.007	0.494
hsa-let-7b-5p	3.219 (0.366)	2.299 (0.409)	0.919	1.891	0.012	0.494
hsa-miR-361-5p	1.196 (0.175)	1.611 (0.191)	-0.416	-1.334	0.014	0.494
hsa-miR-140-5p	0.493 (0.145)	1.303 (0.359)	-0.810	-1.753	0.015	0.494
hsa-miR-9-5p	-6.594 (0.567)	-4.768 (0.529)	-1.826	-3.545	0.015	0.494
hsa-miR-135a-5p	-6.030 (0.900)	-4.501 (0.331)	-1.529	-2.886	0.016	0.494
hsa-miR-517a-3p	0.695 (0.522)	-0.228 (0.404)	0.923	1.896	0.020	0.494
hsa-miR-33a-3p	-4.193 (0.483)	-5.326 (0.608)	1.133	2.193	0.024	0.494
hsa-let-7c-5p	-0.032 (0.516)	-0.856 (0.361)	0.823	1.769	0.026	0.494
hsa-miR-519d-3p	-0.868 (0.635)	-2.246 (0.770)	1.377	2.598	0.029	0.494
hsa-miR-128-3p	-1.117 (0.250)	-0.460 (0.376)	-0.656	-1.576	0.030	0.494
hsa-miR-519a-3p	-2.319 (0.743)	-3.632 (0.697)	1.313	2.484	0.031	0.494

hsa-miR-30b-5p	2.585 (0.147)	1.908 (0.383)	0.677	1.599	0.032	0.494
hsa-miR-518c-3p	-2.275 (1.176)	-3.958 (0.499)	1.683	3.211	0.033	0.494
hsa-miR-26b-5p	0.995 (0.578)	-0.262 (0.736)	1.257	2.390	0.033	0.494
hsa-miR-517c-3p	-0.793 (0.602)	-1.828 (0.562)	1.035	2.048	0.034	0.494
hsa-miR-450a-5p	-1.175 (0.684)	-0.224 (0.160)	-0.950	-1.932	0.034	0.494
hsa-miR-582-3p	-3.722 (0.977)	-2.096 (0.877)	-1.627	-3.088	0.035	0.494
hsa-miR-99a-5p	-0.643 (0.421)	-1.375 (0.411)	0.732	1.661	0.036	0.494
hsa-miR-26b-3p	-4.091 (0.689)	-2.986 (0.609)	-1.105	-2.151	0.039	0.494
hsa-miR-151a-5p	1.935 (0.193)	2.300 (0.219)	-0.365	-1.288	0.039	0.494
hsa-let-7d-3p	2.093 (0.480)	2.724 (0.169)	-0.630	-1.548	0.040	0.494
hsa-miR-20b-5p	-4.324 (0.561)	-6.309 (1.228)	1.985	3.958	0.040	0.494
hsa-miR-519b-3p	-4.841 (0.552)	-6.357 (0.707)	1.516	2.859	0.041	0.494
hsa-miR-556-3p	-5.746 (0.547)	-4.483 (0.489)	-1.262	-2.399	0.041	0.494
hsa-miR-144-3p	6.066 (0.566)	5.028 (0.637)	1.039	2.054	0.042	0.494
hsa-miR-223-3p	9.903 (0.437)	10.570 (0.373)	-0.667	-1.588	0.043	0.494
hsa-miR-17-5p	-1.374 (0.353)	-1.832 (0.196)	0.457	1.373	0.047	0.515
hsa-miR-543	-2.876 (0.797)	-1.899 (0.293)	-0.976	-1.967	0.050	0.526

10.11 Biological plausibility of candidate miRNAs for validation identified in the investigation cohort

The miRNAs in which a difference was revealed in TTTS pregnancies in the investigation cohort may reflect overlapping clinical features often seen in the disease process. Up to 70% of recipient twins have associated cardiac right ventricular dysfunction (Van Mieghem 2013); interestingly, in adults with congestive heart failure, circulating hsa-miR-140-5p is reported as being downregulated, and hsa-miR-107 and hsa-let-7b-5p appears upregulated (Marques 2016), which is what was found in TTTS patients in the investigation cohort. TTTS is also associated with significant changes in renal physiology in both the donor and recipient (Kilby 2001, Mahieu-Caputo 2001, Verbeek 2017). Circulating hsa-miR-223-3p has been found to be downregulated in adults with chronic kidney disease (stages 4–5) (Ulbing 2017), again echoing the findings in the TTTS investigation cohort.

Approximately 60% of TTTS pregnancies are also affected by fetal growth restriction (FGR) (Lewi 2014) and changes have been demonstrated in miRNA in placental tissue from FGR singleton pregnancies, although there was no overlap with the miRNA changes demonstrated in the maternal serum samples from the investigation cohort (Maccani 2011). However, another study compared placental tissue and maternal plasma from FGR pregnancies and demonstrated changes in placental tissue, but not maternal plasma, despite miRNAs being able to cross into the maternal circulation (Higashijima 2013). A recent study compared miRNA expression in placental samples taken from each twin's umbilical cord root insertion, within monochorionic twinsets in which one twin had FGR and the other twin was normally

grown (Wen 2017). This was performed as although monochorionic placentation involves a single placenta, there is differing ‘vascular territories’ between the two fetuses and this may vary considerably depending upon the severity of the FGR (Chang 2009). Wen et al. identified 14 differentially expressed miRNAs (7 upregulated, 7 downregulated) in the placental samples of the larger twin compared with smaller twin. These 14 miRNAs were not significantly different between the two normally grown twins in the one monochorionic twin pregnancy which acted as their control for the miRNA profiling. All 14 miRNAs were successfully validated in a separate unmatched cohort (15 monochorionic twin pregnancies with FGR, 15 monochorionic twin pregnancies with appropriately grown fetuses). Unfortunately Wen et al. did not collect the respective maternal blood samples to compare with the miRNA expression in the placental samples, although there was an overlap with four miRNAs that were significantly different in TTTS pregnancies in the investigation cohort: hsa-let-7c, hsa-miR-518c-3p, hsa-miR-144-3p, and hsa-miR-543. hsa-let-7c, was upregulated in TTTS pregnancies in the investigation cohort and upregulated in the larger twin in Wen’s study, however the changes in the other three miRNAs were in line with the smaller twin, not the larger twin: hsa-miR-518c-3p and hsa-miR-144-3p were upregulated in TTTS and upregulated in the smaller twin, and hsa-miR-543 was downregulated in TTTS, and downregulated in the smaller twin, indicating that an association may exist between miRNA expression in placental samples and maternal serum, and TTTS and FGR.

Changes were also demonstrated in the investigation cohort in hsa-miR-517a-3p, hsa-miR-519a-3p and hsa-miR-519c-3p which are members of the C19MC group of placenta-specific miRNAs (Morales-Prieto 2012, Zhang 2016), and are detectable

from the first trimester in chorionic villi stromal cells (Flor 2012). hsa-miR-17-5p is known to be associated with angiogenesis (Hromadnikova 2015), and increased hsa-miR-17 and hsa-miR-20b expression with reduced spiral artery remodelling (Chen 2013a) and pre-eclampsia (Wang 2012b). A recent study has found that downregulation of hsa-miR-17-5p improves cardiac function after myocardial infarction, thought to be due to aiding repair of vascular injury, and decreasing the rate of apoptosis (Yang 2018). Other miRNAs that were significant prior to correction for multiple testing have also been demonstrated to be significantly different in maternal serum from singleton pregnancies complicated by pre-eclampsia: hsa-miR-107, hsa-miR-223, hsa-miR-26b, hsa-miR-30b, hsa-miR-517c, hsa-miR518c, hsa-miR-519d, hsa-miR-99a (Li 2013, Ura 2014). This again fits with issues of placentation and abnormal placental vasculogenesis seen in TTTS, although the majority of this work has been performed in singleton pregnancies.

With regards to potential gene targets of the differentially expressed miRNA in the investigation cohort identified from miRTarBase, although there is a paucity of gene target validation studies in pregnancy, the potential gene targets do have biological plausibility. One example is miR-107 whose targets include *NOTCH2* and *DICER1* which have been shown to be involved in pre-eclampsia and spiral artery remodelling (Hunkapiller 2011, Doridot 2013). This supports further investigation of these miRNAs in pregnancy to both to improve knowledge of the pathophysiology of TTTS, and also to enable further study of miRNAs as potential biomarkers of TTTS.

10.12 OMMIT 2 Patient information sheet

Birmingham Women's NHS
NHS Foundation Trust

Monochorionic Twins Screening Study - Patient Information Sheet

What is the purpose of the study?

Approximately 15% of monochorionic (identical) twins develop a complication, such as twin-twin transfusion syndrome (TTTS) or selective in-uterine growth restriction (sIUGR) where there is unbalanced blood flow between the twins because they share the placenta. However, we don't know which twins will develop these complications. The aim of this study is to develop a formula which we can use to predict which twins will develop complications, from measurements we do in the first trimester (up to 14 weeks). We will do this using results from your routine ultrasound scan and a blood test. If you are unfortunate enough to develop a complication such as TTTS or sIUGR and require fetoscopic laser ablation (FLA) we will ask to perform blood tests and take extra amniotic fluid during FLA which is part of normal clinical care to try to help us discover more about why women develop these complications.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information to keep and asked to sign a consent form. If you decide to take part you are free to withdraw at any time without giving a reason. If you do not feel able to take part it will not in any way affect the care you or your family receives.

What will happen to me if I take part?

If you agree to take part we will ask your permission to access your hospital records to obtain the results of your ultrasound scan, basic information about you such as your age, and the outcome of your pregnancy. We will also ask you to give us a maximum of 2 extra blood samples during your pregnancy. In addition (with your permission) we will store your blood sample for use in future, ethically approved research. You would not get results from this.

If there are complications at this time we may ask you to consent to further tests: an extra 4 small blood tests after FLA. We will also take an extra small bottle of blood as part of the routine blood tests we perform prior to FLA. During FLA it is routine to take a small sample of amniotic fluid, we would also take a small sample before we start the FLA, and another sample after we have finished the FLA. If you deliver at Birmingham Women's Hospital, we will ask your permission to collect your placenta after delivery. In addition (with your permission) we will store your blood samples and amniotic fluid samples for use in future, ethically approved research. With your permission, we may contact you in the future regarding further research on your stored samples. You would not get results from this as it will not change your medical care.

We are also interested to see how having FLA can affect how you feel about your babies, and your emotions, so we will ask you and your partner to complete a questionnaire before FLA, after FLA, and after you have the babies.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information will be handled in confidence. Any information you give us will only be used in the course of the research to develop these new tests. Any samples and data stored will be stored securely in a swipecard access area. They will be coded, and no personal data (name and address) will be available to the researchers. We need to keep this information to match up your test results with the outcome of your pregnancy. We will inform your GP that you are participating in the study, unless you would prefer your GP not to know. If your answers to the questionnaire reveal that you are at high-risk of postnatal depression then we will tell you and advise you to see your GP for further assessment so that they are able to provide additional support if necessary.

What are the possible benefits of taking part?

There are no direct benefits to you and your twins, but you will be helping future women with monochorionic (identical) twin pregnancies.

What are the possible disadvantages and risks of taking part?

The main disadvantage will be collecting extra blood samples, but this will not require any extra appointments to what you have as part of your routine care. This will be carried out by someone who is skilled in venepuncture (taking blood). Some people may experience bruising at the site which will resolve over a few days. Taking the amniotic fluid samples will not put you or your babies at any higher risk, as it is all performed as part of the FLA, and doesn't require any extra needles into your womb.

What will happen if I don't want to continue in the study?

You are free to withdraw at anytime. If you withdraw from the study we will not access any further samples and will destroy any of your samples that were collected for the study. We will also remove any data held on our own computers.

What will happen to any samples I give?

Blood samples, amniotic fluid samples and placental samples will be collected for this study. The samples will be stored and analysed in Birmingham Women's Hospital, the University of Birmingham and collaborative Universities both in the UK and overseas.

What will happen to the results of the research study?

The results from our project will be published as research papers in medical journals. No data will be published that will allow individuals to be identified.

Where can I get further information or discuss any problems?

Please contact a member of the research team on 0121-623-6652/627-2778 to discuss any questions or worries about the study, or if you have any complaints. If the problems are not resolved, or if you wish to speak to someone independent of the study please contact the Patient Advisory Liaison Services (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint. Please phone contact PALS on 0121 627 2747 or email pals@bwnft.nhs.uk , you can also visit PALS by asking at any hospital reception.

Who is organising and funding the research?

This research is organised by Birmingham Women's Hospital, and funded by the Wiseman Trust (a medical research charity into twin pregnancy).

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the East Midlands Committee.

****Thank you for taking the time to read this information leaflet.****

Detail of researchers

1. Dr Fiona Mackie,
   Clinical Research Fellow,
   Department of Maternal & Fetal Medicine, Floor 3,
   Birmingham Women's Hospital,
   Edgbaston, Birmingham, B15 2TG.
   Telephone No: 0121-623-6652

2. Professor M.D. Kilby,
   Department of Maternal & Fetal Medicine, Floor 3,
   Birmingham Women's Hospital,
   Edgbaston, Birmingham, B15 2TG.
   Telephone No: 0121-627-2778

   Patient Advice Liaison Service
   Tel: 0121 627 2747
   Email: pals@bwnft.nhs.uk

Fetal Medicine Centre, Birmingham Women's Foundation Trust,
Metchley Park Road, Edgbaston, Birmingham. B15 2TG
Tel.No: 0121 627 2683

10.13 OMMIT 2 consent forms

local care:
global impact

Birmingham Women's NHS
NHS Foundation Trust

Centre Number: Study Number:
Patient Identification Number for this trial:

CONSENT FORM
Monochorionic Twin Pregnancy Outcomes – a prospective screening study
Name of Researcher: Professor Mark Kilby, Miss Katie Morris, Dr Fiona Mackie

SECTION A – AT BOOKING APPOINTMENT

Please initial box

1. I confirm that I have read and understand the information sheet version 3.0 dated 04/11/15 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities, the University of Birmingham or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. (a) I agree to allow medical information about my pregnancy to be entered on a confidential computer database. ☐

   (b) If further medical information is requested by members of the project team, I agree to be contacted again for this purpose and agree to be contacted for any future ethically agreed studies. ☐

   (c) I agree to extra blood samples being taken, by a trained professional. ☐

5. I agree to take part in the above **Monochorionic Twin Screening and Pregnancy Outcomes study**. ☐

6. I agree that some of my blood (the plasma) can be stored by the laboratory for use in future ethically approved research. (OPTIONAL) ☐

7. I understand that I will not be told the results of the extra blood samples as it will not change the medical care that I receive. ☐

8. I agree to my GP being informed of my participation in the study. ☐

_____	_____	_____
Name of Patient	Signature	Date
_____	_____	_____
Name of Person (taking consent)	Signature	Date

local care: global impact Birmingham Women's NHS Foundation Trust

Centre Number: Study Number:

CONSENT FORM

SECTION B – When a complication of monochorionic twin pregnancy is diagnosed or if an intervention in pregnancy is being considered

Please initial box

1. I confirm that I have read and understand the information sheet version 3.0 dated 04/11/15 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities, the University of Birmingham or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. (a) I agree to allow medical information about my pregnancy to be entered on a confidential computer database. ☐

   (b) If further medical information is requested by members of the project team, I agree to be contacted again for this purpose and agree to be contacted for any future ethically agreed studies. (OPTIONAL) ☐

5. I agree to have an extra blood test (maximum 10mls) taken at the time of ultrasound diagnosis of a complication and prior to any intervention and then at time points after any intervention (6 hours, 24 hours and at 1 week). ☐

6. If undergoing a fetoscopic laser ablation procedure I agree to have a small sample (5mls) of my amniotic fluid being taken just before and after fetoscopic laser ablation procedure. ☐

7. If undergoing amniocentesis for another indication as part of the standard investigation, I agree to a small extra sample (5mls) being collected. ☐

8. I agree that some of my blood sample (the plasma) and/or amniotic fluid sample can be stored by the laboratory for future research which gains ethical approval. (OPTIONAL) ☐

9. I agree that my placenta can be collected after delivery for testing, and stored (OPTIONAL) ☐

10. I agree that researchers may use stored blood samples from my first trimester screening, if these samples were analysed and stored at Birmingham Women's Hospital. ☐

11. I agree to complete the questionnaires before laser, after laser, and after delivery. I understand that if my answers to these questions show that I am at high risk of depression that the researcher will contact me and advise me to see my GP. ☐

12. I agree to being contacted again in the future by the researchers for any follow up information. ☐

13. I understand that I will not be told the results of the extra blood or amniotic fluid samples or placental analysis as they will not change the medical care that I receive. ☐

14. I agree to my GP being informed of my participation in the study. ☐

_____	_____	_____
Name of Patient	Signature	Date
_____	_____	_____
Name of Person (taking consent)	Signature	Date

10.14 Prognosis of the co-twin following spontaneous single intrauterine fetal death systematic review search strategy

1. exp Fetal Death/
2. exp Diseases in Twins/
3. exp pregnancy, multiple/ or exp pregnancy outcome/
4. single twin death.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, ui, sy, tc, id, tm]
5. co-twin demise.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, ui, sy, tc, id, tm]
6. single twin demise.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, ui, sy, tc, id, tm]
7. co-twin death.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, ui, sy, tc, id, tm]
8. surviving twin.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, ui, sy, tc, id, tm]
9. intrauterine death.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, ui, sy, tc, id, tm]
10. intrauterine demise.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, an, eu, pm, ui, sy, tc, id, tm]
11. perinatal death.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, an, eu, pm, ui, sy, tc, id, tm]
12. single fetal death.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, an, eu, pm, ui, sy, tc, id, tm]

13. single fetal demise.mp. [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, an, eu, pm, ui, sy, tc, id, tm]

14. fetal loss . [mp=ti, ab, hw, tn, ot, dm, mf, dv, kw, fs, nm, kf, px, rx, an, eu, pm, ui, sy, tc, id, tm]

15. 1 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14

16. 2 or 3

17. 15 and 16

10.15 Prognosis of the co-twin following spontaneous single intrauterine fetal death systematic review data collection form

Reviewer ID:……. paper:…….

Selection or rejection (must have all 5 as yes)

a) Population- pregnant with twins with ≥1 twin which died >14 weeks Y / N

b) Outcome- co-twin IUFD/NND, AN/PN brain imaging, neurodevelopment, gestation at delivery Y / N

c) Population ≥ 5 sIUFDs Y / N

d) Chorionicity defined Y / N

e) Excluded twins affected by structural/chromosomal anomalies Y / N/ not stated

Data Collection

Population

Total number of patients recruited (n) …….

Country of study: ……..

Data collection: retrospective / prospective / unreported / other

Patient enrolment: consecutive / arbitrary / unreported / other

Method of confirming chorionicity: Scan / placental pathology / not stated…….

Gestational age at single twin demise (i) average (……..)

(ii) range ( ) to ( )wks

Inclusion criteria:

……

……...

……

……

Exclusion criteria:

……

……

……

……

Interventions

Description of management following sIUFD e.g. frequency of monitoring, delivery, not stated etc.

……

……...

……

……

Outcomes

Percentage of follow up: (……..…..)%

Number of patients with data available…….

Study design

Well defined sample at uniform early stage

antenatal care protocolised yes/ no/ can't tell

Length of antenatal follow up ……

Length of neonatal follow up ……

“Surviving” co-twin intrauterine demise (IUFD)

1st Twin demise ≥14 weeks; 2nd (co-twin) intrauterine death any GA

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) intrauterine death any GA

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) intrauterine death any GA

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

Any further sub-group analysis:

“Surviving” co-twin early neonatal death (<7 days)

1st Twin demise ≥14 weeks; 2nd (co-twin) early NND

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) early NND

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) early NND

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

Any further sub-group analysis:

“Surviving” co-twin late neonatal death (7-28 days)

1st Twin demise ≥14 weeks; 2nd (co-twin) late NND

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) late NND

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) late NND

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

Any further sub-group analysis:

Surviving co-twin preterm delivery (24-34 weeks)

What was the definition of preterm delivery?

Did the paper divide preterm delivery into spontaneous and iatrogenic? Yes / No

(If yes, further sub-group analysis)

1st Twin demise ≥14 weeks; 2nd (co-twin) preterm delivery (spont. and iatrogenic)

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) preterm delivery (spont. and iatrogenic)

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) preterm delivery (spont. and iatrogenic)

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

Any further sub-group analysis:

Abnormal antenatal brain imaging in surviving co-twin

Mode of scanning: ultrasound / fetal MRI / other

Timing of scan (e.g. 4 weeks post-sIUFD):

……..

Definition of abnormal head scan:

……

……

……

1st Twin demise ≥14 weeks; 2nd (co-twin) abnormal antenatal brain imaging (state mode) ……

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) abnormal antenatal brain imaging (state mode) ……

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) abnormal antenatal brain imaging (state mode) ……

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

Any further sub-group analysis (e.g. TTTS vs no TTTS, antenatal USS vs antenatal MRI):

Abnormal postnatal brain imaging in surviving co-twin

Mode of scanning: ultrasound / fetal MRI / other

Timing of scan (e.g. 4 weeks of age): …….

Definition of abnormal head scan:

……

……

……

1st Twin demise ≥14 weeks; 2nd (co-twin) abnormal postnatal brain imaging (state mode) ……

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) abnormal postnatal brain imaging (state mode) ……

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) abnormal postnatal brain imaging (state mode) ……

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

Any further sub-group analysis (e.g. TTTS vs no TTTS, postnatal USS vs postnatal MRI):

Abnormal postnatal neurological signs in surviving co-twin

Mode of assessment: ……

Age of assessment: ……

Definition of abnormal neurological signs:

……

……

……

Length of follow-up: ……

1st Twin demise ≥14 weeks; 2nd (co-twin) abnormal neurological signs

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) abnormal neurological signs

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) abnormal neurological signs

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

Any further sub-group analysis (e.g. TTTS vs no TTTS, postnatal USS vs postnatal MRI):

TTTS

Definition of TTTS:

……

……

1st Twin demise ≥14 weeks; 2nd (co-twin) intrauterine death any GA

	TTTS	No TTTS	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) intrauterine death any GA

	TTTS	No TTTS	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) intrauterine death any GA

	TTTS	No TTTS	Total
Present			
Absent			
Total			

Any further sub-group analysis (e.g. intervention vs. no intervention, stage of TTTS):

selective IUGR (or other fetal complication)

Definition of sIUGR:

……

……

1st Twin demise ≥14 weeks; 2nd (co-twin) intrauterine death any GA

	TTTS	No TTTS	Total
Present			
Absent			
Total			

1st Twin demise 14-27+6 weeks; 2nd (co-twin) intrauterine death any GA

	TTTS	No TTTS	Total
Present			
Absent			
Total			

1st Twin demise ≥28 weeks; 2nd (co-twin) intrauterine death any GA

	TTTS	No TTTS	Total
Present			
Absent			
Total			

Any further sub-group analysis (e.g. intervention vs. no intervention, stage of TTTS):

Coagulopathy and pre-eclampsia

1st Twin demise ≥14 weeks; maternal coagulopathy

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥14 weeks; pre-eclampsia

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

1st Twin demise ≥14 weeks; other maternal complication …….

	Monochorionic	Dichorionic	Total
Present			
Absent			
Total			

10.16 Additional forest plots and extracted 2x2 data for prognosis of surviving co-twin systematic review

MC vs DC twins risk of co-twin intrauterine fetal death

Barigye, Dias, Farah, Fichera, Gratacós 2004 and 2008, Griffiths, Hoffmann, Jou, Lewi, Robinson, Rustico and van Klink did not include DC twin pregnancies in their studies (Jou 1996, Gratacós 2004, Barigye 2005, Gratacós 2008, Lewi 2008, Fichera 2009, Dias 2011, Farah 2012, Hoffmann 2013, Griffiths 2015, van Klink 2015, Robinson 2017, Rustico 2017), Hagay (Hagay 1986) did not include MC twins, therefore these studies were only included in the summary event rate calculation for MC and DC twins respectively.

Studies and extracted data used to calculate the summary event rate and perform sub-group analysis of co-twin intrauterine fetal death (IUFD) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies

GA: gestational age at sIUFD, IUGR: intrauterine growth restriction, TTTS: twin-twin transfusion syndrome

Study	Year	MC co-twin IUFD	MC twins total	DC co-twin IUFD	DC twins total	Sub-group
Hagay	1986	0	0	0	11	GA
Cherouny	1989	0	8	0	4	-
Fusi	1990	0	7	1	8	IUGR
Gaucherand	1994	0	6	0	2	GA, IUGR
Ishimatsu	1994	0	12	0	3	GA
Kilby	1994	0	2	0	13	-
Santema	1995	2	13	0	16	-
Jou	1996	1	12	0	0	GA, TTTS

Study	Year	MC co-twin IUFD	MC twins total	DC co-twin IUFD	DC twins total	Sub-group
Tordjeman	1996	0	2	0	8	GA, IUGR
Sebire	1997	12	15	4	12	GA, TTTS
Krayenbuhl	1998	0	12	0	7	TTTS
Axt	1999	0	4	0	2	GA, TTTS, IUGR
Bajoria	1999b	13	50	1	42	GA, TTTS, IUGR (concurrent)
Petersen	1999	0	4	0	6	TTTS, IUGR, only GA for MC twins
Malinowski	2000	0	1	0	5	GA
Baghdadi	2003	1	1	2	12	-
Malinowski	2003	0	3	0	9	GA, TTTS
Gratacós	2004	3	6	0	0	IUGR cohort only
Barigye	2005	3	7	0	0	GA
Gratacós	2008	3	9	0	0	IUGR cohort only
Lewi	2008	11	13	0	0	GA, TTTS, IUGR
Chelli	2009	0	9	1	19	-
Fichera	2009	1	12	0	0	TTTS, IUGR (MC only)
Dias	2011	9	16	0	0	GA, IUGR, TTTS
Mahony	2011	5	11	2	11	GA, TTTS, IUGR
Farah	2012	6	9	0	0	GA, TTTS (acute only thus not in sub-group)
McPherson	2012	11	28	19	49	-
Deveer	2013	0	9	0	16	-
Hoffmann	2013	0	4	0	0	-
Griffiths	2015	1	28	0	0	GA
van Klink	2015	0	40	0	0	-
Robinson	2017	0	8	0	0	GA, IUGR
Rustico	2017	10	18	0	0	IUGR cohort only

MC vs DC twins risk of preterm birth

Bajoria, Barigye, Dias, Fichera, Griffiths, Hoffmann, Jou, Robinson, Szymonowicz and van Heteren (Szymonowicz 1986, Jou 1996, van Heteren 1998, Bajoria 1999b, Barigye 2005, Fichera 2009, Dias 2011, Hoffmann 2013, Griffiths 2015, Robinson

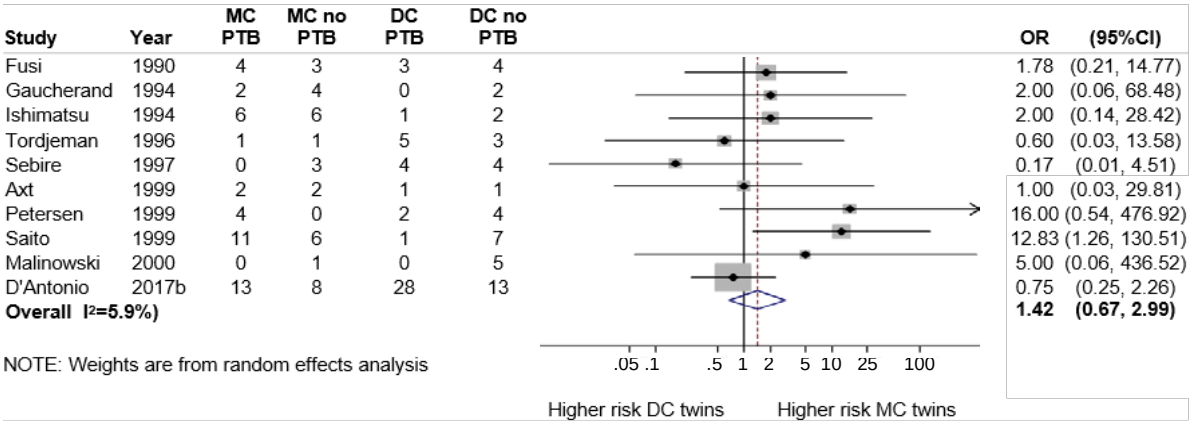
2017) did not include DC twins in their studies, Baghdadi and Hagay (Hagay 1986, Baghdadi 2003) did not include MC twins, therefore these studies were only included in the summary event rate calculations for MC and DC twins respectively.

Studies and extracted data used to calculate the summary event rate and perform sub-group analysis of preterm birth (PTB) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies

GA: gestational age at sIUFD, IUGR: intrauterine growth restriction, spont: spontaneous delivery PTB, TTTS: twin-twin transfusion syndrome

Study	Year	MC PTB	MC twins total	DC PTB	DC twins total	Sub-group
Hagay	1986	0	0	2	11	GA
Szymonowicz	1986	5	6	0	0	GA
Fusi	1990	4	7	3	7	iatrogenic/spont, IUGR
Gaucherand	1994	2	6	0	2	GA, IUGR
Ishimatsu	1994	6	12	1	3	GA
Jou	1996	2	11	0	0	GA, TTTS
Tordjeman	1996	1	2	5	8	GA, IUGR
Sebire	1997	0	3	4	8	GA, TTTS
van Heteren	1998	9	11	0	0	GA, TTTS
Axt	1999	2	4	1	2	GA, iatrogenic/spont, TTTS, IUGR
Bajoria	1999b	29	37	0	0	GA, TTTS, IUGR (MC only)
Petersen	1999	4	4	2	6	GA, TTTS, iatrogenic/spont, IUGR
Saito	1999	11	17	1	8	GA, TTTS
Malinowski	2000	0	1	0	5	GA
Baghdadi	2003	0	0	3	6	GA
Barigye	2005	0	4	0	0	GA
Fichera	2009	4	11	0	0	GA
Dias	2011	3	7	0	0	GA, TTTS, IUGR
Hoffmann	2013	1	4	0	0	-
Griffiths	2015	5	27	0	0	GA
D'Antonio	2017b	13	21	28	41	-
Robinson	2017	1	7	0	0	GA, IUGR

Forest plot comparing the risk of preterm birth (PTB) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies



MC twins risk of abnormal antenatal brain imaging

None of the six studies in the antenatal brain imaging analysis included DC twin pregnancies therefore a forest plot could not be drawn (Jelin 2008, Fichera 2009, Hoffmann 2013, Griffiths 2015, van Klink 2015, Robinson 2017). Each study performed USS and MRI in the same pregnancies.

Studies and extracted data used to calculate the summary event rate and perform sub-group analysis of abnormal antenatal brain imaging following single intrauterine fetal death in monochorionic (MC) twin pregnancies

fMRI: fetal magnetic resonance imaging, fUSS: fetal ultrasound scan, GA: gestational age at sIUFD, IUGR: intrauterine growth restriction, TTTS: twin-twin transfusion syndrome

Study	Year	Abnormal antenatal brain fUSS	MC twins total	Abnormal antenatal brain fMRI	MC twins total	Sub-group
Jelin	2008	3	18	3	18	GA, TTTS, imaging modality
Fichera	2009	0	11	0	11	Imaging modality
Hoffmann	2013	0	6	0	6	Imaging modality
Griffiths	2015	5	39	5	39	GA, imaging modality
van Klink	2015	3	35	3	35	GA, TTTS, IUGR (abnormal scan group only), imaging modality
Robinson	2017	2	10	2	10	GA, IUGR, imaging modality

MC vs DC risk of abnormal postnatal brain imaging

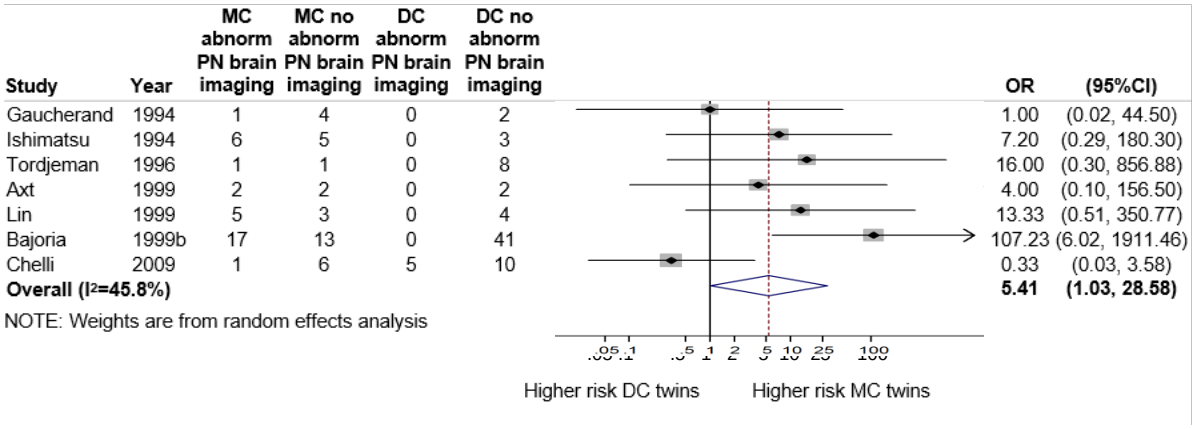
Fichera, Gratacós, Lewi, van Heteren and van Klink did not include DC twins in their studies therefore these studies were only included in the summary event rate calculation for MC twins (van Heteren 1998, Gratacós 2004, Lewi 2008, Fichera 2009, van Klink 2015).

Studies and extracted data used to calculate the summary event rate and perform sub-group analysis of abnormal postnatal brain imaging following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies

GA: gestational age at sIUFD, IUGR: intrauterine growth restriction, TTTS: twin-twin transfusion syndrome

Study	Year	MC abnormal postnatal brain	MC twins total	DC abnormal postnatal brain	DC twins total	Sub-group
Gaucherand	1994	1	5	0	2	GA, IUGR
Ishimatsu	1994	6	11	0	3	GA
Tordjeman	1996	1	2	0	8	GA, IUGR
van Heteren	1998	7	11	0	0	GA, TTTS
Axt	1999	2	4	0	2	GA, TTTS, IUGR
Bajoria	1999b	17	30	0	41	GA, TTTS, IUGR (MC only)
Lin	1999	5	8	0	4	-
Gratacós	2004	1	3	0	0	IUGR only in cohort
Lewi	2008	0	2	0	0	GA
Chelli	2009	1	7	5	15	-
Fichera	2009	2	11	0	0	GA
van Klink	2015	10	28	0	0	GA, TTTS, IUGR (abnormal scan group only)

Forest plot comparing the risk of abnormal postnatal brain imaging (abnorm brain PN) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies



MC vs DC risk of neurodevelopmental comorbidity

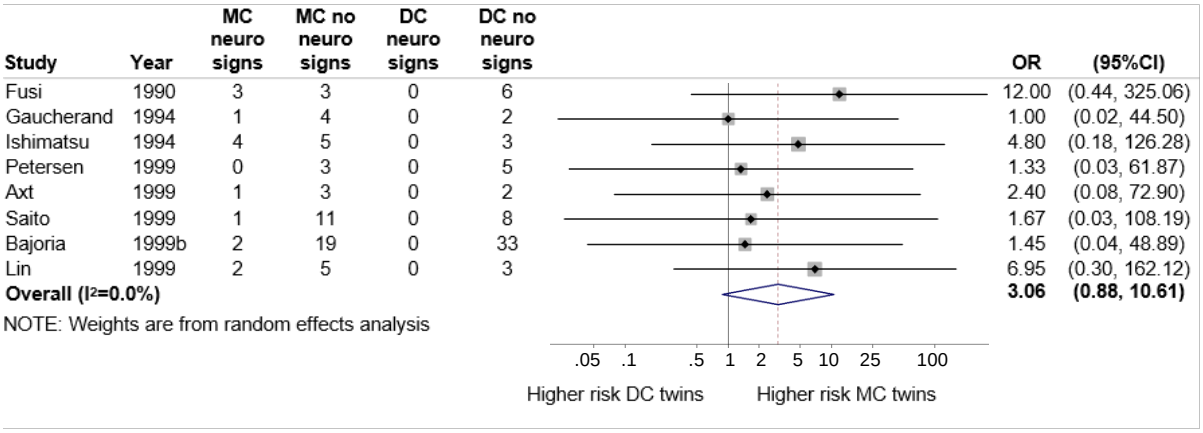
Fichera, Jou, Rustico, Szymonowicz and van Heteren did not include DC twins in their studies therefore these studies were only included in the summary event rate calculation for MC twins (Szymonowicz 1986, Jou 1996, van Heteren 1998, Fichera 2009, Rustico 2017).

Studies and extracted data used to calculate the summary event rate and perform sub-group analysis of neurodevelopmental comorbidity (neuro signs) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies

GA: gestational age at sIUFD, IUGR: intrauterine growth restriction, TTTS: twin-twin transfusion syndrome

Study	Year	MC neuro signs	MC twins total	DC neuro signs	DC twins total	Sub-group
Szymonowicz	1986	2	2	0	0	GA
Fusi	1990	3	6	0	6	IUGR
Gaucherand	1994	1	5	0	2	GA, IUGR
Ishimatsu	1994	4	9	0	3	GA
Jou	1996	1	8	0	0	GA, TTTS
van Heteren	1998	3	9	0	0	GA, TTTS
Axt	1999	1	4	0	2	GA, IUGR, TTTS
Lin	1999	2	7	0	3	-
Petersen	1999	0	3	0	5	GA, TTTS
Saito	1999	1	12	0	8	GA, TTTS, IUGR
Bajoria	1999b	2	21	0	33	GA, IUGR, TTTS (MC only)
Fichera	2009	2	11	0	0	GA
Rustico	2017	1	6	0	0	GA, IUGR in cohort only

Forest plot comparing the risk of neurodevelopmental comorbidity (neuro signs) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies



MC vs DC risk of neonatal death

Fichera, Jou, Lewi, Rustico, van Heteren and van Klink did not include DC twins in their studies (Jou 1996, van Heteren 1998, Lewi 2008, Fichera 2009, van Klink 2015, Rustico 2017), Baghdadi did not include MC twins (Baghdadi 2003), therefore these studies were only included in the summary event rate calculation for MC, and DC twins respectively.

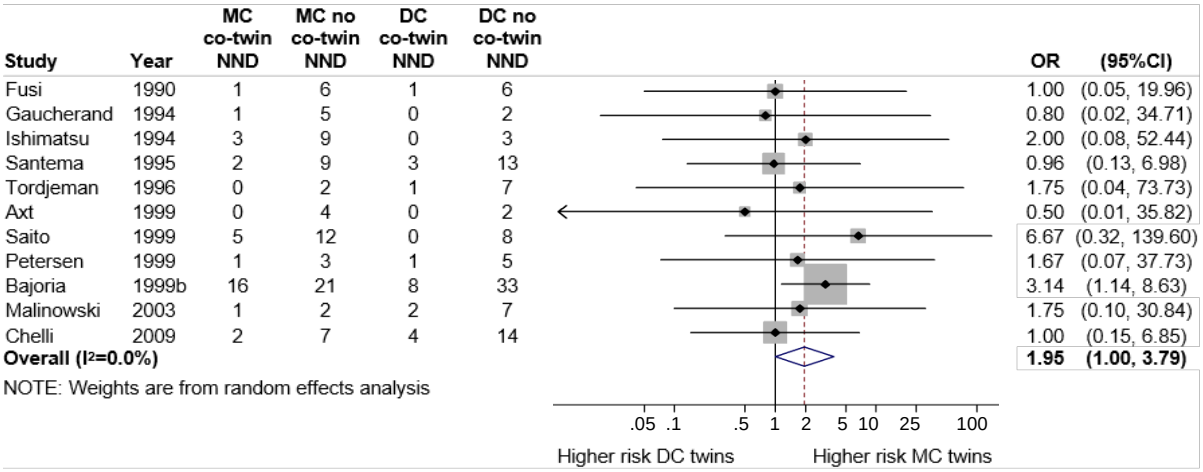
Studies and extracted data used to calculate the summary event rate and perform sub-group analysis of neonatal death (NND) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies

GA: gestational age at sIUFD, IUGR: intrauterine growth restriction, TTTS: twin-twin transfusion syndrome

Study	Year	MC NND	MC twins total	DC NND	DC twins total	Sub-group
Szymonowicz	1986	4	6	0	0	GA
Fusi	1990	1	7	1	7	IUGR
Gaucherand	1994	1	6	0	2	GA, IUGR

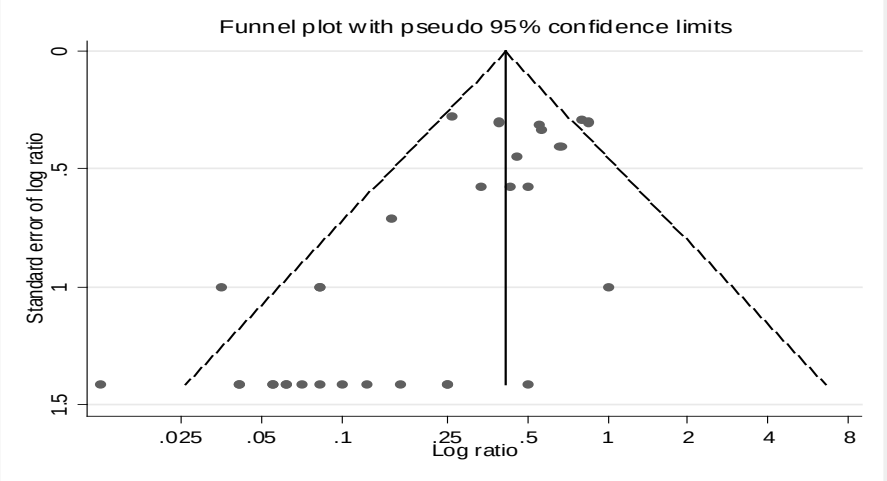
Study	Year	MC NND	MC twins total	DC NND	DC twins total	Sub-group
Ishimatsu	1994	3	12	0	3	GA
Santema	1995	2	11	3	16	-
Jou	1996	3	11	0	0	GA, TTTS
Tordjeman	1996	0	2	1	8	
van Heteren	1998	2	11	0	0	GA, IUGR
Axt	1999	0	4	0	2	GA, IUGR, TTTS
Petersen	1999	1	4	1	6	GA, TTTS, IUGR
Saito	1999	5	17	0	8	GA, TTTS
Bajoria	1999b	16	37	8	41	GA, IUGR, TTTS
Malinowski	2003	1	3	2	9	-
Baghdadi	2003	0	0	4	10	GA
Lewi	2008	0	2	0	0	GA
Fichera	2009	1	11	0	0	GA
Chelli	2009	2	9	4	18	TTTS, IUGR
van Klink	2015	5	46	0	0	-
Rustico	2017	1	7	0	0	GA, IUGR in cohort only

Forest plot comparing the risk of neonatal death (NND) following single intrauterine fetal death in monochorionic (MC) and dichorionic (DC) twin pregnancies

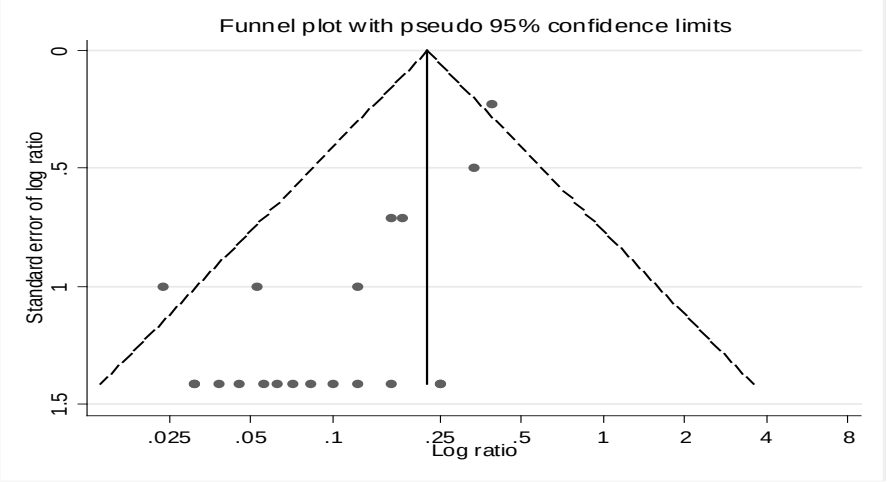


10.17 Funnel plots for prognosis of surviving co-twin systematic review

Studies included in outcome: summary event rate of co-twin IUFD in MC twin pregnancies. Egger's test p=0.00.

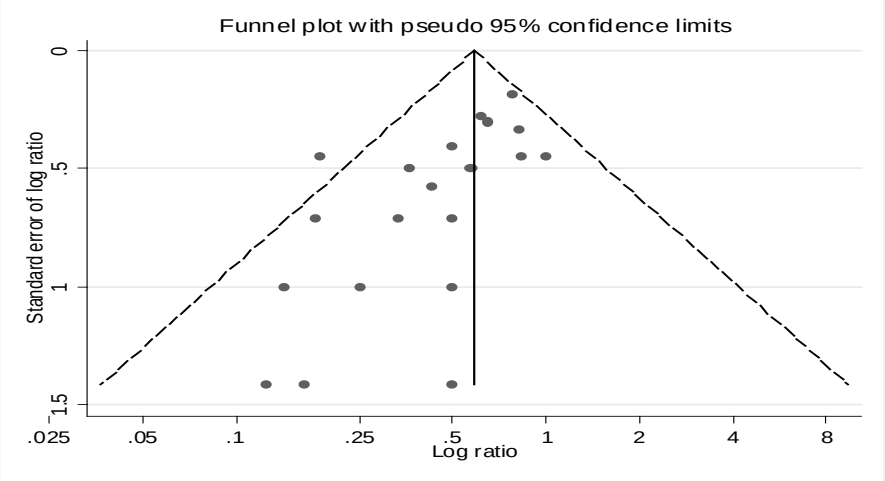


Studies included in outcome: summary event rate of co-twin IUFD in DC twin pregnancies. Egger's test p=0.00.



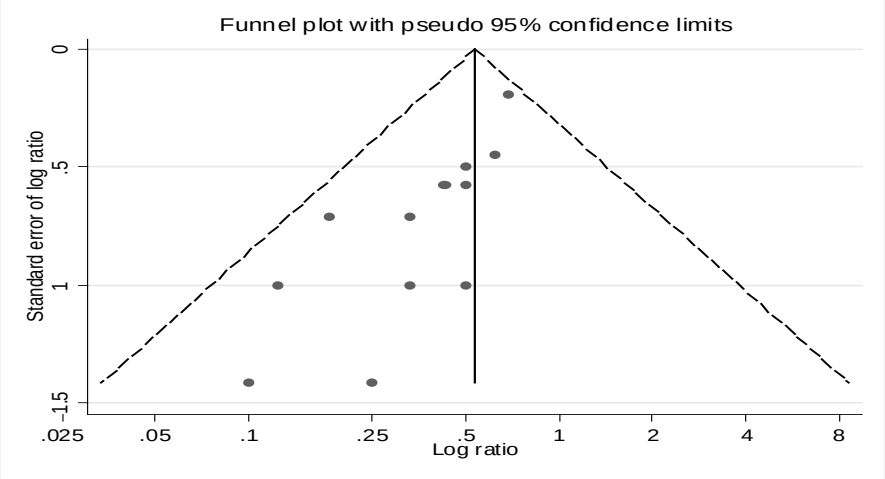
Studies included in outcome: summary event rate of PTB in MC twin pregnancies.

Egger's test p=0.005.

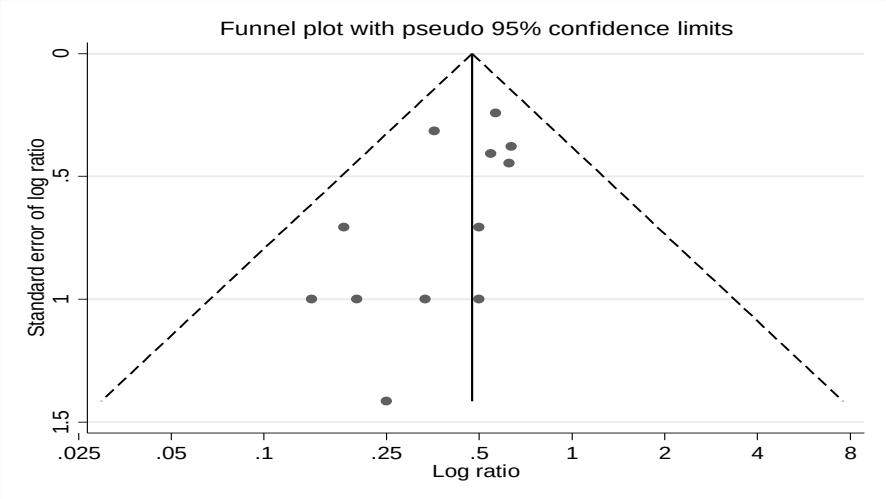


Studies included in outcome: summary event rate of PTB in DC twin pregnancies.

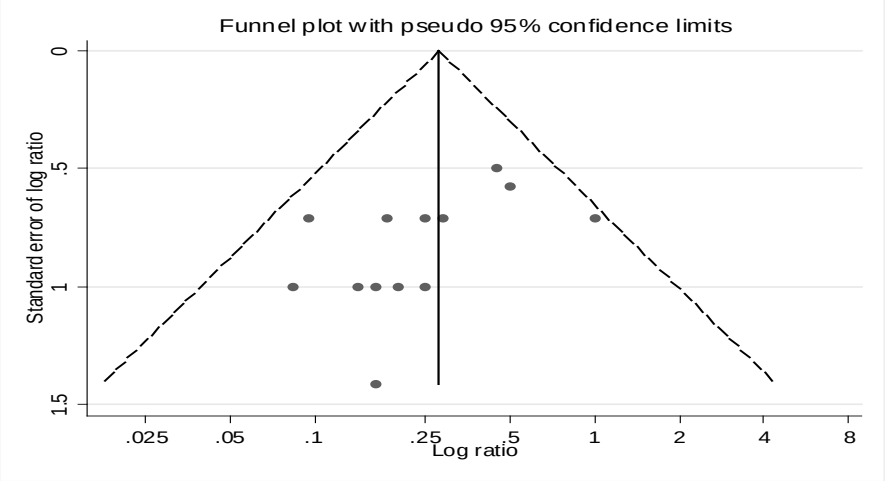
Egger's test p=0.001.



Studies included in outcome: summary event rate of abnormal postnatal brain imaging in MC twin pregnancies. Egger's test p=0.40.

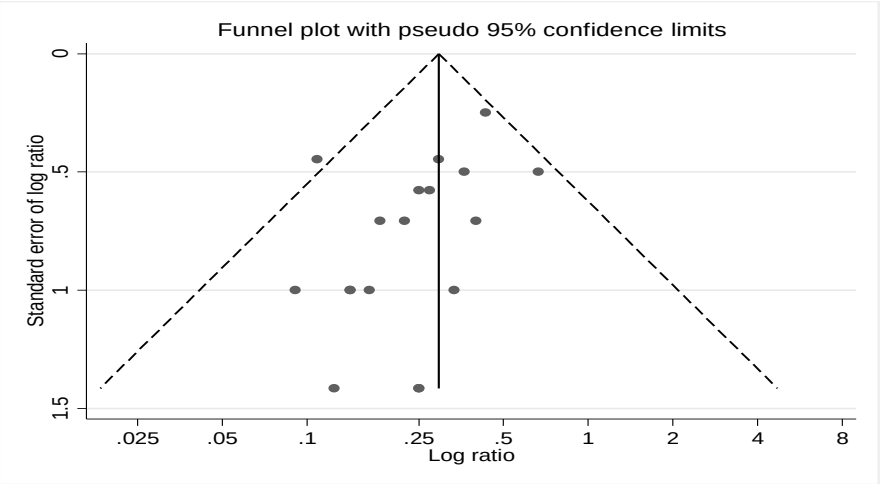


Studies included in outcome: summary event rate of neurodevelopmental comorbidity in MC twin pregnancies. Egger's test p=0.058.



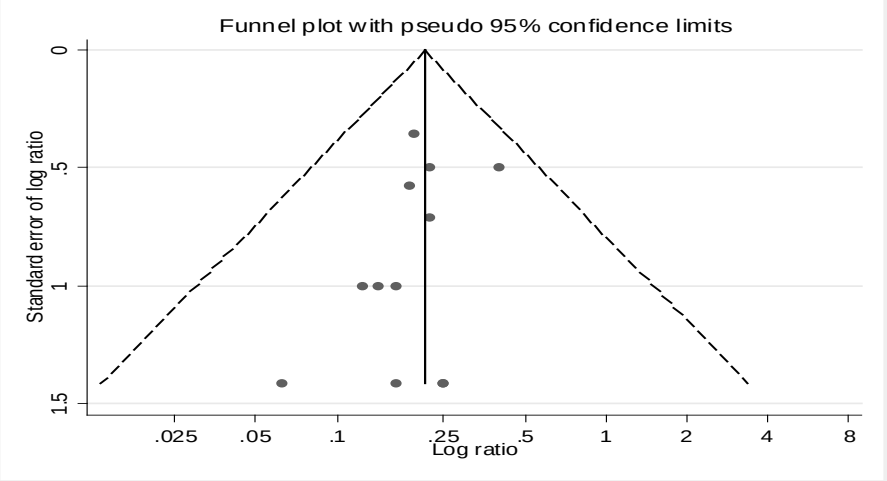
Studies included in outcome: summary event rate of NND in MC twin pregnancies.

Egger's test p=0.04.



Studies included in outcome: summary event rate of NND in DC twin pregnancies.

Egger's test p=0.225



10.18 Paternal consent form for parental attachment and depressive symptoms work

Birmingham Women's NHS
NHS Foundation Trust

Centre Number: Study Number:

Patient Identification Number for this trial:
CONSENT FORM - PATERNAL

SECTION B – When a complication of monochorionic twin pregnancy is diagnosed or if an intervention in pregnancy is being considered

Please initial box

1. I confirm that I have read and understand the information sheet version 3.0 dated 4.11.15 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without the medical care or my legal rights being affected. ☐
3. I agree to complete the questionnaires before laser, after laser, and after delivery. I understand that if my answers to these questions show that I am at high risk of depression then the researcher will contact me and advise me to see my GP. ☐
4. I agree to being contacted again in the future by the researchers for any follow up information ☐

_____	_____	_____
Name of Patient	Signature	Date
_____	_____	_____
Name of Person (taking consent)	Signature	Date

10.19 Questionnaires for parental attachment and depressive symptoms

Birmingham Women's NHS
NHS Foundation Trust

OMMIT (Optimal Management of Monochorionic Twins) Patient Questionnaire
Pre laser Maternal

Twin to twin transfusion syndrome is a very nasty complication of pregnancy that if untreated carries a very high risk of mortality for babies. The treatment of fetoscopic laser ablation significantly improves fetal outcomes in many pregnancies, but outcomes may be unpredictable. We realise that having laser surgery is a difficult time for most parents, so we would be very grateful if you could answer the following questions so that we can help other people in the same position as you in the future.

We will ask you to complete the questionnaire at different time points (before laser, 1 month after laser, and 6 weeks after you deliver). The aim of our questionnaire is to:

1) investigate if undergoing laser surgery affects parental bonding/attachment during pregnancy and after delivery
2) learn more about how parents feel who are having laser surgery, so that we are able to provide better support to parents in the future.

These questionnaires will be slightly different, but some of the questions are the same so that we can look at how your feelings change during pregnancy, and after you've delivered. Please read each question carefully. There are no right or wrong answers. If you would like to discuss any issues raised by this questionnaire with a member of the research team, the contact information is at the bottom.

Background Information

OMMIT study number _____

1. **Do you currently have a mental health illness?** Yes / No
   a. If yes, please specify which condition(s)
      _____
      _____
      _____
2. **Do you currently take any medication for a mental health illness?** Yes / No
   a. If yes, please list any medication
      _____
      _____
3. **Do you currently attend any counselling for a mental health illness?** Yes / No
   a. If yes, please list any counselling / types of therapy
      _____
      _____

4. **Have you ever had a mental health illness in the past?** Yes / No
    a. If yes, please specify which condition(s)

       _____
       _____
       _____

5. **Did you take any medication for a previous mental health illness?** Yes / No
    a. If yes, please list any medication

       _____
       _____

6. **Did you attend any counselling for a previous mental health illness?** Yes / No
    a. If yes, please list any counselling / types of therapy

       _____
       _____

Condon Fetal Attachment Questions

These questions are about your thoughts and feelings about the developing babies.
Please tick one box only in answer to each question.

MA1: Since the diagnosis of TTTS I have thought about, or been preoccupied with the babies inside me:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

MA2: Since the diagnosis of TTTS when I have spoken about, or thought about the babies inside me I got emotional feelings which were:	Please tick
Very weak or non-existent	
Fairly weak	
In between strong and weak	
Fairly strong	
Very strong	

MA3: Since the diagnosis of TTTS my feelings about the babies inside me have been:	Please tick
Very positive	
Mainly positive	
Mixed positive and negative	
Mainly negative	
Very negative	

MA4: Since the diagnosis of TTTS I have the desire to read about or get information about the developing babies. This desire is:	Please tick
Very weak or non-existent	
Fairly weak	
Neither strong nor weak	
Moderately strong	
Very strong	

MA5: Since the diagnosis of TTTS I have been trying to picture in my mind what the developing babies actually look like in my womb:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

MA6: Since the diagnosis of TTTS I think of the developing babies mostly as:	Please tick
Real little people with special characteristics	
Babies like any other babies	
Human beings	
Living things	
Things not really yet alive	

MA7: Since the diagnosis of TTTS I have felt that the babies inside me is dependent on me for its well-being:	Please tick
Totally	
A great deal	
Moderately	
Slightly	
Not at all	

MA8: Since the diagnosis of TTTS I have found myself talking to my babies when I am alone:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost all the time I am alone	

MA9: Since the diagnosis of TTTS when I think about (or talk to) my babies inside me, my thoughts:	Please tick
Are always tender and loving	
Are mostly tender and loving	
Are a mixture of both tenderness and irritation	
Contain a fair bit of irritation	
Contain a lot of irritation	

MA10: The picture in my mind of what the babies at this stage actually looks like inside the womb is:	Please tick
Very clear	
Fairly clear	
Fairly vague	
Very vague	
I have no idea at all	

MA11: Since the diagnosis of TTTS when I think about the babies inside me I get feelings which are:	Please tick
Very sad	
Moderately sad	
A mixture of happiness and sadness	
Moderately happy	
Very happy	

MA12: Some pregnant women sometimes get so irritated by the babies inside them that they feel like they want to hurt them or punish them:	Please tick
I couldn't imagine I would ever feel like this	
I could imagine I might sometimes feel like this, but I never actually have	
I have felt like this once or twice myself	
I have occasionally felt like this myself	
I have often felt like this myself	

MA13: Since the diagnosis of TTTS I have felt:	Please tick
Very emotionally distant from my babies	
Moderately emotionally distant from my babies	
Not particularly emotionally close to my babies	
Moderately close emotionally to my babies	
Very close emotionally to my babies	

MA14: Since the diagnosis of TTTS I have taken care with what I eat to make sure the babies get a good diet:	Please tick
Not at all	
Once or twice when I ate	
Occasionally when I ate	
Quite often when I ate	
Every time I ate	

MA15: When I first see my babies after the birth I expect I will feel:	Please tick
Intense affection	
Mostly affection	
Dislike about one or 2 aspects of the babies	
Dislike about quite a few aspects of the baby	
Mostly dislike	

MA16: When my babies are born I would like to hold the babies:	Please tick
Immediately	
After they have been wrapped in a blanket	
After they have been washed	
After a few hours for things to settle down	
The next day	

MA17: Since the diagnosis of TTTS I have had dreams about the pregnancy or babies:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost every night	

MA18: Since the diagnosis of TTTS I have found myself feeling, or rubbing with my hand, the outside of my stomach where the babies are:	Please tick
A lot of times each day	
At least once per day	
Occasionally	
Once only	
Not at all	

MA19: If the pregnancy was lost at this time (due to miscarriage or other accidental event) without any pain or injury to myself, I expect I would feel:	Please tick
Very pleased	
Moderately pleased	
Neutral (i.e. neither sad nor pleased; or mixed feelings)	
Moderately sad	
Very sad	

Edinburgh Postnatal Depression Scale

These questions assess your risk for depression. Although it is called the Postnatal Depression Scale, it is also used antenatally before you have your babies. If you score highly, we will inform you and advise you to see your GP for further assessment and support if required.

Please tick one box only in answer to each question which comes closest to how you have felt **since the diagnosis of TTTS.**

EPDSM1: I have been able to laugh and see the funny side of things:	Please tick
As much as I always could	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSM2: I have looked forward with enjoyment to things:	Please tick
As much as I ever did	
Rather less than I used to	
Definitely less than I used to	
Hardly at all	

EPDSM3: I have blamed myself unnecessarily when things went wrong:	Please tick
Yes, most of the time	
Yes, some of the time	
Not very often	
No, never	

EPDSM4: I have been anxious or worried for no good reason:	Please tick
No not at all	
Hardly ever	
Yes, sometimes	
Yes, very often	

EPDSM5: I have felt scared or panicky for no very good reason:	Please tick
Yes, quite a lot	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSM6: Things have been getting on top of me:	Please tick
Yes, most of the time I haven't been able to cope at all	
Yes, sometimes I haven't been coping as well as usual	
No, most of the time I have coped quite well	
No, I have been coping as well as ever	

EPDSM7: I have been so unhappy that I have had difficulty sleeping:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSM8: I have felt sad or miserable:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSM9: I have been so unhappy that I have been crying:	Please tick
Yes, most of the time	
Yes, quite often	
Only occasionally	
No, never	

EPDSM10: The thought of harming myself has occurred to me:	Please tick
Yes, quite often	
Sometimes	
Hardly ever	
Never	

Thank-you very much for taking the time to complete this questionnaire

Detail of researchers

1. Dr Fiona Mackie,
   Clinical Research Fellow,
   Department of Maternal & Fetal Medicine, Floor 3,
   Birmingham Women's Hospital,
   Edgbaston, Birmingham, B15 2TG.
   Telephone No: 0121-626-4535

2. Professor M.D. Kilby,
   Department of Maternal & Fetal Medicine, Floor 3,
   Birmingham Women's Hospital,
   Edgbaston, Birmingham, B15 2TG.
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Email: pals@bwnft.nhs.uk

Fetal Medicine Centre, Birmingham Women's Foundation Trust,
Metchley Park Road, Edgbaston, Birmingham. B15 2TG,
Tel.No: 0121 627 2683

Birmingham Women's NHS
NHS Foundation Trust

OMMIT (Optimal Management of Monochorionic Twins) Patient Questionnaire
Pre laser Paternal

Twin to twin transfusion syndrome is a very nasty complication of pregnancy that if untreated carries a very high risk of mortality for babies. The treatment of fetoscopic laser ablation significantly improves fetal outcomes in many pregnancies, but outcomes may be unpredictable. We realise that having laser surgery is a difficult time for most parents, so we would be very grateful if you could answer the following questions so that we can help other people in the same position as you in the future.

We will ask you to complete the questionnaire at different time points (before laser, 1 month after laser, and 6 weeks after your partner delivers). The aim of our questionnaire is to:

1) investigate if undergoing laser surgery affects parental bonding/attachment during pregnancy and after delivery
2) learn more about how parents feel who are having laser surgery, so that we are able to provide better support to parents in the future.

These questionnaires will be slightly different, but some of the questions are the same so that we can look at how your feelings change during pregnancy, and after your partner has delivered. Please read each question carefully. There are no right or wrong answers. If you would like to discuss any issues raised by this questionnaire with a member of the research team, the contact information is at the bottom.

Background Information

OMMIT study number _____P

1. **Do you currently have a mental health illness?** Yes / No
    a. If yes, please specify which condition(s)
    _____
    _____
    _____
2. **Do you currently take any medication for a mental health illness?** Yes / No
    a. If yes, please list any medication
    _____
    _____
3. **Do you currently attend any counselling for a mental health illness?** Yes / No
    a. If yes, please list any counselling / types of therapy
    _____
    _____
4. **Have you ever had a mental health illness in the past?** Yes / No
    a. If yes, please specify which condition(s)
    _____
    _____
    _____
5. **Did you take any medication for a previous mental health illness?** Yes / No
    a. If yes, please list any medication
    _____
    _____
6. **Did you attend any counselling for a previous mental health illness?** Yes / No
    a. If yes, please list any counselling / types of therapy
    _____
    _____

Condon Fetal Attachment Questions

These questions are about your thoughts and feelings about the developing babies.
Please tick one box only in answer to each question.

PA1: Since the diagnosis of TTTS I have thought about, or been preoccupied with the babies:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

PA2: Since the diagnosis of TTTS when I have spoken about, or thought about the developing babies I got emotional feelings which were:	Please tick
Very weak or non-existent	
Fairly weak	
In between strong and weak	
Fairly strong	
Very strong	

PA3: Since the diagnosis of TTTS my feelings about the developing babies have been:	Please tick
Very positive	
Mainly positive	
Mixed positive and negative	
Mainly negative	
Very negative	

PA4: Since the diagnosis of TTTS I have the desire to read about or get information about the developing babies. This desire is:	Please tick
Very weak or non-existent	
Fairly weak	
Neither strong nor weak	
Moderately strong	
Very strong	

PA5: Since the diagnosis of TTTS I have been trying to picture in my mind what the developing babies actually look like in my partner's womb:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

PA6: Since the diagnosis of TTTS I think of the developing babies mostly as:	Please tick
Real little people with special characteristics	
Babies like any other babies	
Human beings	
Living things	
Things not really yet alive	

PA7: Since the diagnosis of TTTS when I think about the developing babies my thoughts:	Please tick
Are always tender and loving	
Are mostly tender and loving	
Are a mixture of both tenderness and irritation	
Contain a fair bit of irritation	
Contain a lot of irritation	

PA8: Since the diagnosis of TTTS my ideas and possible names for the babies have been:	Please tick
Very clear	
Fairly clear	
Fairly vague	
Very vague	
I have no idea at all	

PA9: Since the diagnosis of TTTS when I think about the developing babies I get feelings which are:	Please tick
Very sad	
Moderately sad	
A mixture of happiness and sadness	
Moderately happy	
Very happy	

PA10: Since the diagnosis of TTTS I have been thinking about what kind of child the baby will grow into:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost all the time	

PA11: Since the diagnosis of TTTS I have felt:	Please tick
Very emotionally distant from the babies	
Moderately emotionally distant from the babies	
Not particularly emotionally close to the babies	
Moderately close emotionally to the babies	
Very close emotionally to the babies	

PA12: When I first see my babies after the birth I expect I will feel:	Please tick
Intense affection	
Mostly affection	
Affection, but I expect there may be a few aspects of the babies I will dislike	
I expect there may be quite a few aspects of the babies I will dislike	
I expect I might feel mostly dislike	

PA13: When the babies are born I would like to hold the babies:	Please tick
Immediately	
After they have been wrapped in a blanket	
After they have been washed	
After a few hours for things to settle down	
The next day	

PA14: Since the diagnosis of TTTS I have had dreams about the pregnancy or babies:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost every night	

PA15: Since the diagnosis of TTTS I have found myself feeling, or rubbing with my hand, the outside of my partner's stomach where the babies are:	Please tick
A lot of times each day	
At least once per day	
Occasionally	
Once only	
Not at all	

PA16: If the pregnancy was lost at this time (due to miscarriage or other accidental event) without any pain or injury to my partner, I expect I would feel:	Please tick
Very pleased	
Moderately pleased	
Neutral (i.e. neither sad nor pleased; or mixed feelings)	
Moderately sad	
Very sad	

Edinburgh Postnatal Depression Scale

These questions assess your risk for depression. Although it is called the Postnatal Depression Scale, it is also used antenatally before you have your babies. If you score highly, we will inform you and advise you to see your GP for further assessment and support if required.

Please tick one box only in answer to each question which comes closest to how you have felt **since the diagnosis of TTTS.**

EPDSP1: I have been able to laugh and see the funny side of things:	Please tick
As much as I always could	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSP2: I have looked forward with enjoyment to things:	Please tick
As much as I ever did	
Rather less than I used to	
Definitely less than I used to	
Hardly at all	

EPDSP3: I have blamed myself unnecessarily when things went wrong:	Please tick
Yes, most of the time	
Yes, some of the time	
Not very often	
No, never	

EPDSP4: I have been anxious or worried for no good reason:	Please tick
No not at all	
Hardly ever	
Yes, sometimes	
Yes, very often	

EPDSP5: I have felt scared or panicky for no very good reason:	Please tick
Yes, quite a lot	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSP6: Things have been getting on top of me:	Please tick
Yes, most of the time I haven't been able to cope at all	
Yes, sometimes I haven't been coping as well as usual	
No, most of the time I have coped quite well	
No, I have been coping as well as ever	

EPDSP7: I have been so unhappy that I have had difficulty sleeping:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSP8: I have felt sad or miserable:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSP9: I have been so unhappy that I have been crying:	Please tick
Yes, most of the time	
Yes, quite often	
Only occasionally	
No, never	

EPDSP10: The thought of harming myself has occurred to me:	Please tick
Yes, quite often	
Sometimes	
Hardly ever	
Never	

Thank-you very much for taking the time to complete this questionnaire

Detail of researchers

1. Dr Fiona Mackie,
   Clinical Research Fellow,
   Department of Maternal & Fetal Medicine, Floor 3,
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Birmingham Women's NHS
NHS Foundation Trust

OMMIT (Optimal Management of Monochorionic Twins) Patient Questionnaire
Post laser Maternal

We would be grateful if you could complete this questionnaire so that we can help people who need laser surgery in the future. As you know twin pregnancies are high-risk and people that have twins can have a wide variety of outcomes. Whilst we have tried our best to send you the correct questionnaire depending on your outcome, we may sometimes get this wrong for which we apologise.
There are no right or wrong answers. If you would like to discuss any issues raised by this questionnaire with a member of the research team, the contact information is at the bottom.

Condon Fetal Attachment Questions

These questions are about your thoughts and feelings about the developing baby/babies.
Please tick one box only in answer to each question.

MA1: Since the diagnosis of TTTS I have thought about, or been preoccupied with the baby/babies inside me:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

MA2: Since the diagnosis of TTTS when I have spoken about, or thought about the baby/babies inside me I got emotional feelings which were:	Please tick
Very weak or non-existent	
Fairly weak	
In between strong and weak	
Fairly strong	
Very strong	

MA3: Since the diagnosis of TTTS my feelings about the baby/babies inside me have been:	Please tick
Very positive	
Mainly positive	
Mixed positive and negative	
Mainly negative	
Very negative	

MA4: Since the diagnosis of TTTS I have the desire to read about or get information about the developing baby/babies. This desire is:	Please tick
Very weak or non-existent	
Fairly weak	
Neither strong nor weak	
Moderately strong	
Very strong	

MA5: Since the diagnosis of TTTS I have been trying to picture in my mind what the developing baby/babies actually look like in my womb:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

MA6: Since the diagnosis of TTTS I think of the developing baby/babies mostly as:	Please tick
Real little people with special characteristics	
Baby/babies like any other baby/babies	
Human beings	
Living things	
Things not really yet alive	

MA7: Since the diagnosis of TTTS I have felt that the baby/babies inside me is dependent on me for its well-being:	Please tick
Totally	
A great deal	
Moderately	
Slightly	
Not at all	

MA8: Since the diagnosis of TTTS I have found myself talking to my baby/babies when I am alone:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost all the time I am alone	

MA9: Since the diagnosis of TTTS when I think about (or talk to) my baby/babies inside me, my thoughts:	Please tick
Are always tender and loving	
Are mostly tender and loving	
Are a mixture of both tenderness and irritation	
Contain a fair bit of irritation	
Contain a lot of irritation	

MA10: The picture in my mind of what the baby/babies at this stage actually looks like inside the womb is:	Please tick
Very clear	
Fairly clear	
Fairly vague	
Very vague	
I have no idea at all	

MA11: Since the diagnosis of TTTS when I think about the baby/babies inside me I get feelings which are:	Please tick
Very sad	
Moderately sad	
A mixture of happiness and sadness	
Moderately happy	
Very happy	

MA12: Some pregnant women sometimes get so irritated by the baby/babies inside them that they feel like they want to hurt them or punish them:	Please tick
I couldn't imagine I would ever feel like this	
I could imagine I might sometimes feel like this, but I never actually have	
I have felt like this once or twice myself	
I have occasionally felt like this myself	
I have often felt like this myself	

MA13: Since the diagnosis of TTTS I have felt:	Please tick
Very emotionally distant from my baby/babies	
Moderately emotionally distant from my baby/babies	
Not particularly emotionally close to my baby/babies	
Moderately close emotionally to my baby/babies	
Very close emotionally to my baby/babies	

MA14: Since the diagnosis of TTTS I have taken care with what I eat to make sure the baby/babies get a good diet:	Please tick
Not at all	
Once or twice when I ate	
Occasionally when I ate	
Quite often when I ate	
Every time I ate	

MA15: When I first see my baby/babies after the birth I expect I will feel:	Please tick
Intense affection	
Mostly affection	
Dislike about one or 2 aspects of the baby/babies	
Dislike about quite a few aspects of the baby	
Mostly dislike	

MA16: When my baby/babies are born I would like to hold the baby/babies:	Please tick
Immediately	
After they have been wrapped in a blanket	
After they have been washed	
After a few hours for things to settle down	
The next day	

MA17: Since the diagnosis of TTTS I have had dreams about the pregnancy or baby/babies:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost every night	

MA18: Since the diagnosis of TTTS I have found myself feeling, or rubbing with my hand, the outside of my stomach where the baby/babies are:	Please tick
A lot of times each day	
At least once per day	
Occasionally	
Once only	
Not at all	

MA19: If the pregnancy was lost at this time (due to miscarriage or other accidental event) without any pain or injury to myself, I expect I would feel:	Please tick
Very pleased	
Moderately pleased	
Neutral (i.e. neither sad nor pleased; or mixed feelings)	
Moderately sad	
Very sad	

Edinburgh Postnatal Depression Scale

These questions assess your risk for depression. Although it is called the Postnatal Depression Scale, it is also used antenatally before you have your baby/babies. If you score highly, we will inform you and advise you to see your GP for further assessment and support if required.

Please tick one box only in answer to each question which comes closest to how you have felt **since the diagnosis of TTTS.**

EPDSM1: I have been able to laugh and see the funny side of things:	Please tick
As much as I always could	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSM2: I have looked forward with enjoyment to things:	Please tick
As much as I ever did	
Rather less than I used to	
Definitely less than I used to	
Hardly at all	

EPDSM3: I have blamed myself unnecessarily when things went wrong:	Please tick
Yes, most of the time	
Yes, some of the time	
Not very often	
No, never	

EPDSM4: I have been anxious or worried for no good reason:	Please tick
No not at all	
Hardly ever	
Yes, sometimes	
Yes, very often	

EPDSM5: I have felt scared or panicky for no very good reason:	Please tick
Yes, quite a lot	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSM6: Things have been getting on top of me:	Please tick
Yes, most of the time I haven't been able to cope at all	
Yes, sometimes I haven't been coping as well as usual	
No, most of the time I have coped quite well	
No, I have been coping as well as ever	

EPDSM7: I have been so unhappy that I have had difficulty sleeping:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSM8: I have felt sad or miserable:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSM9: I have been so unhappy that I have been crying:	Please tick
Yes, most of the time	
Yes, quite often	
Only occasionally	
No, never	

EPDSM10: The thought of harming myself has occurred to me:	Please tick
Yes, quite often	
Sometimes	
Hardly ever	
Never	

Thank-you very much for taking the time to complete this questionnaire

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OMMIT (Optimal Management of Monochorionic Twins) Patient Questionnaire

Post laser Paternal

We would be grateful if you could complete this questionnaire so that we can help people who need laser surgery in the future. As you know twin pregnancies are high-risk and people that have twins can have a wide variety of outcomes. Whilst we have tried our best to send you the correct questionnaire depending on your outcome, we may sometimes get this wrong for which we apologise.

There are no right or wrong answers. If you would like to discuss any issues raised by this questionnaire with a member of the research team, the contact information is at the bottom.

Condon Fetal Attachment Questions

These questions are about your thoughts and feelings about the developing baby/babies. Please tick one box only in answer to each question.

PA1: Since the diagnosis of TTTS I have thought about, or been preoccupied with the baby/babies:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

PA2: Since the diagnosis of TTTS when I have spoken about, or thought about the developing baby/babies I got emotional feelings which were:	Please tick
Very weak or non-existent	
Fairly weak	
In between strong and weak	
Fairly strong	
Very strong	

PA3: Since the diagnosis of TTTS my feelings about the developing baby/babies have been:	Please tick
Very positive	
Mainly positive	
Mixed positive and negative	
Mainly negative	
Very negative	

PA4: Since the diagnosis of TTTS I have the desire to read about or get information about the developing baby/babies. This desire is:	Please tick
Very weak or non-existent	
Fairly weak	
Neither strong nor weak	
Moderately strong	
Very strong	

PA5: Since the diagnosis of TTTS I have been trying to picture in my mind what the developing baby/babies actually look like in my partner's womb:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

PA6: Since the diagnosis of TTTS I think of the developing baby/babies mostly as:	Please tick
Real little people with special characteristics	
Baby/babies like any other baby/babies	
Human beings	
Living things	
Things not really yet alive	

PA7: Since the diagnosis of TTTS when I think about the developing baby/babies my thoughts:	Please tick
Are always tender and loving	
Are mostly tender and loving	
Are a mixture of both tenderness and irritation	
Contain a fair bit of irritation	
Contain a lot of irritation	

PA8: Since the diagnosis of TTTS my ideas and possible names for the baby/babies have been:	Please tick
Very clear	
Fairly clear	
Fairly vague	
Very vague	
I have no idea at all	

PA9: Since the diagnosis of TTTS when I think about the developing baby/babies I get feelings which are:	Please tick
Very sad	
Moderately sad	
A mixture of happiness and sadness	
Moderately happy	
Very happy	

PA10: Since the diagnosis of TTTS I have been thinking about what kind of child the baby will grow into:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost all the time	

PA11: Since the diagnosis of TTTS I have felt:	Please tick
Very emotionally distant from the baby/babies	
Moderately emotionally distant from the baby/babies	
Not particularly emotionally close to the baby/babies	
Moderately close emotionally to the baby/babies	
Very close emotionally to the baby/babies	

PA12: When I first see my baby/babies after the birth I expect I will feel:	Please tick
Intense affection	
Mostly affection	
Affection, but I expect there may be a few aspects of the baby/babies I will dislike	
I expect there may be quite a few aspects of the baby/babies I will dislike	
I expect I might feel mostly dislike	

PA13: When the baby/babies are born I would like to hold the baby/babies:	Please tick
Immediately	
After they have been wrapped in a blanket	
After they have been washed	
After a few hours for things to settle down	
The next day	

PA14: Since the diagnosis of TTTS I have had dreams about the pregnancy or baby/babies:	Please tick
Not at all	
Occasionally	
Frequently	
Very frequently	
Almost every night	

PA15: Since the diagnosis of TTTS I have found myself feeling, or rubbing with my hand, the outside of my partner's stomach where the baby/babies are:	Please tick
A lot of times each day	
At least once per day	
Occasionally	
Once only	
Not at all	

PA16: If the pregnancy was lost at this time (due to miscarriage or other accidental event) without any pain or injury to my partner, I expect I would feel:	Please tick
Very pleased	
Moderately pleased	
Neutral (i.e. neither sad nor pleased; or mixed feelings)	
Moderately sad	
Very sad	

Edinburgh Postnatal Depression Scale

These questions assess your risk for depression. Although it is called the Postnatal Depression Scale, it is also used antenatally before you have your baby/babies. If you score highly, we will inform you and advise you to see your GP for further assessment and support if required.

Please tick one box only in answer to each question which comes closest to how you have felt **since the diagnosis of TTTS.**

EPDSP1: I have been able to laugh and see the funny side of things:	Please tick
As much as I always could	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSP2: I have looked forward with enjoyment to things:	Please tick
As much as I ever did	
Rather less than I used to	
Definitely less than I used to	
Hardly at all	

EPDSP3: I have blamed myself unnecessarily when things went wrong:	Please tick
Yes, most of the time	
Yes, some of the time	
Not very often	
No, never	

EPDSP4: I have been anxious or worried for no good reason:	Please tick
No not at all	
Hardly ever	
Yes, sometimes	
Yes, very often	

EPDSP5: I have felt scared or panicky for no very good reason:	Please tick
Yes, quite a lot	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSP6: Things have been getting on top of me:	Please tick
Yes, most of the time I haven't been able to cope at all	
Yes, sometimes I haven't been coping as well as usual	
No, most of the time I have coped quite well	
No, I have been coping as well as ever	

EPDSP7: I have been so unhappy that I have had difficulty sleeping:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSP8: I have felt sad or miserable:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSP9: I have been so unhappy that I have been crying:	Please tick
Yes, most of the time	
Yes, quite often	
Only occasionally	
No, never	

EPDSP10: The thought of harming myself has occurred to me:	Please tick
Yes, quite often	
Sometimes	
Hardly ever	
Never	

Thank-you very much for taking the time to complete this questionnaire

Detail of researchers

1. Dr Fiona Mackie,
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OMMIT (Optimal Management of Monochorionic Twins) Patient Questionnaire
Postnatal Maternal

As you know twin pregnancies are high-risk and people that have twins can have a wide variety of outcomes. Whilst we have tried our best to send you the correct questionnaire depending on your outcome, we may sometimes get this wrong for which we apologise. Thank-you very much for being part of our study, which we hope will improve care for people in your position in the future.
Please read each question carefully. There are no right or wrong answers. If you would like to discuss any issues raised by this questionnaire with a member of the research team, the contact information is at the bottom.

Background Information

OMMIT study number _____P

7. **Since the first time we asked you this question before your laser surgery, have you been diagnosed with a mental health illness?** Yes / No
    a. If yes, please specify which condition(s)
    _____
    _____
    _____
8. **Do you currently take any medication for a mental health illness?** Yes / No
    a. If yes, please list any medication
    _____
    _____
9. **Do you currently attend any counselling for a mental health illness?** Yes / No
    a. If yes, please list any counselling / types of therapy
    _____
    _____

Condon Fetal Attachment Questions

These questions are about your thoughts and feelings about your baby/babies. Please tick one box only in answer to each question.

MP1: When I am caring for the baby/babies, I get feelings of annoyance or irritation:	Please tick
Very frequently	
Frequently	
Occasionally	
Very rarely	
Never	

MP2: When I am caring for the baby/babies I get feelings that they are deliberately being difficult or trying to upset me:	Please tick
Very frequently	
Frequently	
Occasionally	
Very rarely	
Never	

MP3: Over the last 2 weeks I would describe my feelings for the baby/babies as:	Please tick
Dislike	
No strong feelings towards the baby/babies	
Slight affection	
Moderate affection	
Intense affection	

MP4: Regarding my overall level of interaction with the baby/babies I:	Please tick
Feel very guilty that I am not more involved	
Feel moderately guilty that I am not more involved	
Feel slightly guilty that I am not more involved	
I don't have any guilty feelings regarding this	

MP5: When I interact with the baby/babies I feel:	Please tick
Very incompetent and lacking in confidence	
Moderately incompetent and lacking in confidence	
Moderately competent and confident	
Very competent and confident	

MP6: When I am with the baby/babies I feel tense and anxious:	Please tick
Very frequently	
Frequently	
Occasionally	
Almost never	

MP7: When I am with the baby/babies and other people are present, I feel proud of the baby/babies:	Please tick
Very frequently	
Frequently	
Occasionally	
Almost never	

MP8: I try to involve myself as much as I possibly can PLAYING with the baby/babies:	Please tick
This is true	
This is untrue	

MP9: When I have to leave the baby/babies:	Please tick
I usually feel rather sad (or it's difficult to leave)	
I often feel rather sad (or it's difficult to leave)	
I have mixed feelings of both sadness and relief	
I often feel rather relieved (and it's easy to leave)	
I usually feel rather relieved (and it's easy to leave)	

MP10: When I am with the baby/babies:	Please tick
I always get a lot of enjoyment/satisfaction	
I frequently get a lot of enjoyment/satisfaction	
I occasionally get a lot of enjoyment/satisfaction	
I very rarely get a lot of enjoyment/satisfaction	

MP11: When I am not with the baby/babies, I find myself thinking about the baby/babies:	Please tick
Almost all the time	
Very frequently	
Frequently	
Occasionally	
Not at all	

MP12: When I am with the baby/babies:	Please tick
I usually try to prolong the time I spend with him/her/them	
I usually try to shorten the time I spend with him/her/them	

MP13: When I have been away from the baby/babies for a while and I am about to be with him/her/them again, I usually feel:	Please tick
Intense pleasure at the idea	
Moderate pleasure at the idea	
Mild pleasure at the idea	
No feelings at all about the idea	
Negative feelings about the idea	

MP14: I now think of the baby/babies as:	Please tick
Very much as my own baby/babies	
A bit like my own baby/babies	
Not yet really my own baby/babies	

MP15: Regarding the things that we have had to give up because of the baby/babies:	Please tick
I find that I resent it quite a lot	
I find that I resent it a moderate amount	
I find that I resent it a bit	
I don’t resent it at all	

MP16: Over the past three months, I have felt that I do not have enough time for myself or to pursue my own interests:	Please tick
Almost all the time	
Very frequently	
Occasionally	
Not at all	

MP17: Taking care of these baby/babies is a heavy burden of responsibility. I believe this is:	Please tick
Very much so	
Somewhat so	
Slightly so	
Not at all	

MP18: I trust my own judgement in deciding what the baby/babies needs:	Please tick
Almost never	
Occasionally	
Most of the time	
Almost all the time	

MP19: Usually when I am with the baby/babies:	Please tick
I am very impatient	
I am a bit impatient	
I am moderately patient	
I am extremely patient	

Edinburgh Postnatal Depression Scale

These questions assess your risk for depression. If you score highly, we will inform you and advise you to see your GP for further assessment and support if required.

Please tick one box only in answer to each question which comes closest to how you have felt **in the last 7 days.**

EPDSM1: I have been able to laugh and see the funny side of things:	Please tick
As much as I always could	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSM2: I have looked forward with enjoyment to things:	Please tick
As much as I ever did	
Rather less than I used to	
Definitely less than I used to	
Hardly at all	

EPDSM3: I have blamed myself unnecessarily when things went wrong:	Please tick
Yes, most of the time	
Yes, some of the time	
Not very often	
No, never	

EPDSM4: I have been anxious or worried for no good reason:	Please tick
No not at all	
Hardly ever	
Yes, sometimes	
Yes, very often	

EPDSM5: I have felt scared or panicky for no very good reason:	Please tick
Yes, quite a lot	
Not quite so much now	
Definitely not so much now	
Not at all	

EPDSM6: Things have been getting on top of me:	Please tick
Yes, most of the time I haven't been able to cope at all	
Yes, sometimes I haven't been coping as well as usual	
No, most of the time I have coped quite well	
No, I have been coping as well as ever	

EPDSM7: I have been so unhappy that I have had difficulty sleeping:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSM8: I have felt sad or miserable:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSM9: I have been so unhappy that I have been crying:	Please tick
Yes, most of the time	
Yes, quite often	
Only occasionally	
No, never	

EPDSM10: The thought of harming myself has occurred to me:	Please tick
Yes, quite often	
Sometimes	
Hardly ever	
Never	

Thank-you very much for taking the time to complete this questionnaire and being part of this study

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11. **Do you currently take any medication for a mental health illness?** Yes / No
    a. If yes, please list any medication
    _____
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    _____

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Very frequently	
Frequently	
Occasionally	
Very rarely	
Never	

PP2: When I am caring for the baby/babies I get feelings that they are deliberately being difficult or trying to upset me:	Please tick
Very frequently	
Frequently	
Occasionally	
Very rarely	
Never	

PP3: Over the last 2 weeks I would describe my feelings for the baby/babies as:	Please tick
Dislike	
No strong feelings towards the baby/babies	
Slight affection	
Moderate affection	
Intense affection	

PP4: I can understand what my baby/babies needs or wants:	Please tick
Almost always	
Usually	
Sometimes	
Rarely	
Almost never	

PP5: Regarding my overall level of interaction with the baby/babies I believe I am:	Please tick
Much more involved than most fathers in my position	
Somewhat more involved than most fathers in my position	
Involved to the same extent as most fathers in my position	
Somewhat less involved than most fathers in my position	
Much less involved than most fathers in my position	

PP6: When I am with the baby/babies I feel bored:	Please tick
Very frequently	
Frequently	
Occasionally	
Almost never	

PP7: When I am with the baby/babies and other people are present, I feel proud of the baby/babies:	Please tick
Very frequently	
Frequently	
Occasionally	
Almost never	

PP8: I try to involve myself as much as possible in child care and looking after the baby/babies:	Please tick
This is true	
This is untrue	

PP9: I find myself talking to people (other than my partner) about the baby/babies:	Please tick
Many times each day	
A few times each day	
Once or twice a day	
Rarely on any one day	

PP10: When I have to leave the baby/babies:	Please tick
I usually feel rather sad (or it's difficult to leave)	
I often feel rather sad (or it's difficult to leave)	
I have mixed feelings of both sadness and relief	
I often feel rather relieved (and it's easy to leave)	
I usually feel rather relieved (and it's easy to leave)	

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Frequently	
Occasionally	
Not at all	

MP13: When I am with the baby/babies:	Please tick
I usually try to prolong the time I spend with him/her/them	
Neither	
I usually try to shorten the time I spend with him/her/them	

PP14: When I have been away from the baby/babies for a while and I am about to be with him/her/them again, I usually feel:	Please tick
Intense pleasure at the idea	
Moderate pleasure at the idea	
Mild pleasure at the idea	
No feelings at all about the idea	
Negative feelings about the idea	

PP15: Over the past 3 months I have found myself just sitting looking at the sleeping baby/babies for periods of five minutes or more:	Please tick
Very frequently	
Frequently	
A few times	
Not at all	

PP16: I now think of the baby/babies as:	Please tick
Very much my own baby/babies	
A bit like my own baby/babies	
Not really my own baby/babies	

PP17: Regarding the things that we have had to give up because of the baby/babies:	Please tick
I find that I resent it quite a lot	
I find that I resent it a moderate amount	
I find that I resent it a bit	
I don't resent it at all	

PP18: Over the past 3 months, I have felt that I do not have enough time for myself or to pursue my own interests:	Please tick
Almost all the time	
Very frequently	
Occasionally	
Not at all	

PP19: Usually when I am with the baby/babies:	Please tick
I am very impatient	
I am a bit impatient	
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Definitely less than I used to	
Hardly at all	

EPDSP3: I have blamed myself unnecessarily when things went wrong:	Please tick
Yes, most of the time	
Yes, some of the time	
Not very often	
No, never	

EPDSP4: I have been anxious or worried for no good reason:	Please tick
No not at all	
Hardly ever	
Yes, sometimes	
Yes, very often	

EPDSP5: I have felt scared or panicky for no very good reason:	Please tick
Yes, quite a lot	
Not quite so much now	
Definitely not so much now	
Not at all	

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Yes, sometimes I haven't been coping as well as usual	
No, most of the time I have coped quite well	
No, I have been coping as well as ever	

EPDSP7: I have been so unhappy that I have had difficulty sleeping:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSP8: I have felt sad or miserable:	Please tick
Yes, most of the time	
Yes, sometimes	
Not very often	
No, not at all	

EPDSP9: I have been so unhappy that I have been crying:	Please tick
Yes, most of the time	
Yes, quite often	
Only occasionally	
No, never	

EPDSP10: The thought of harming myself has occurred to me:	Please tick
Yes, quite often	
Sometimes	
Hardly ever	
Never	

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