TWIN-TWIN TRANSFUSION SYNDROME – INVESTIGATION OF THE EFFECT OF FETOSCOPIC LASER ABLATION AND REVIEWS OF DIAGNOSIS AND TREATMENT

By

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A thesis submitted to

the University of Birmingham

for the degree of

DOCTOR OF MEDICINE

School of Clinical and Experimental Medicine

University of Birmingham

AUGUST 2013

ABSTRACT

The first aim of this thesis was to systematically review the literature to determine the diagnostic accuracy of ultrasound in the first trimester to screen for twin-twin transfusion syndrome (TTTS) and predict its outcome after diagnosis, as well as the effectiveness of its two main treatments. Quantitative analysis revealed that a crown rump discordance or abnormal nuchal translucency in the first trimester was useful to screen for TTTS, but after diagnosis no single test could reliably predict outcome. In both situations a negative test was not reliable for excluding TTTS or a poor outcome. Fetoscopic laser ablation (FLA) is likely to confer a benefit both in terms of survival and morbidity in survivors.

The second aim was to determine what happened to biological markers in TTTS and how FLA affected them. It appeared that TTTS may be associated with abnormal placentation as maternal serum α-fetoprotein (MSAFP) approximately doubled and free β-human chorionic gonadotrophin (f-βhCG) tripled. The balance of angiogenic factors i.e. 2-3 fold increased angiogenin 2 and 1.5 fold increased soluble vascular endothelial growth factor receptor 1 appeared to favour angiogenesis in response to hypoxia or ischaemia in TTTS. Maternal cell-free messenger RNA was reliably detected and showed similar alteration in angiogenic markers. Interestingly, TTTS was associated with minimal changes in cytokine levels. In response to FLA there was an increase in transplacental haemorrhage (MSAFP increased 445%) rather than trophoblast destruction (f-βhCG unchanged) as well as a transient increase in some anti-angiogenic markers. Although, in general angiogenic factors and cytokines are altered little by this therapy. If the biomarker changes detected precede the onset of clinically apparent disease, they may be useful to improve the performance of first trimester ultrasound screening. Composite tests may be more useful to predict outcome and FLA should continue to be utilised to reduce morbidity.

DEDICATION

To my friends and family for all the help and encouragement they have given me. With particularly special mention to my husband Rob for his ever tolerant ways and my daughter Annabel for her ability to provide perspective and a healthy disregard for my work.

ACKNOWLEDGMENTS

I would like to acknowledge and offer my sincere thanks to

Wellbeing of Women and Birmingham Women's Foundations Trust Research and Development grant for giving me the chance to develop my interest in research and make the research which culminated in this thesis possible

Jenny Tamblyn for her help as a second reviewer for the diagnostic accuracy reviews and for reminding me how young and enthusiastic we all start out and can be again, enthusiastic at least

Katie Morris for her help as a second reviewer, independent arbitrator, source of much wisdom and general encouragement

The girls in the office for cheering me up and keeping me in coffee and chocolate when times were tough

Sam Pretlove, Ben Chan and Dave Lissauer for their help collecting the TTTS samples and never letting a small thing like a their own in-patient status get in the way of this

Gendie Lash for her assistance and guidance in learning to perform ELISA and FASTQUANT analysis and work out what it all meant afterwards

Akihiko Sekizawa for his help and advice with the RNA analysis

Dr. Francoise Muller for her help and advice with the free βHCG multiples of the median

Ian Mills for his help and advice with the AFP multiples of the median and analysis

Roger Holder for his patience and statistical serum rhubarb wizardry

Richard Riley for his down to earth statistics advice

Christine Macarthur for being a brilliant supervisor, mentor and friend

Mark Kilby for inspiring me to do Obstetrics as a student and for keeping me on the straight and narrow ever since.

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LIST OF ABBREVIATIONS

AA	Arterioarterial
AFP	α-fetoprotein
AGF	Angiogenic growth factor
Ang	Angiopoietin
AUC	Area under curve
AV	Arteriovenous
βhCG	β-human chorionic gonadotrophin
cDNA	Complementary DNA
Cf-mRNA	Cell free messenger RNA
CI	Confidence interval
CHOP	Children's Hospital of Philadelphia
COP	Colloid osmotic pressure
CRL	Crown rump length
CVS	Chorionic villous sampling
DC	Dichorionic
df	Degrees of freedom
DNA	Deoxyribonucleic acid
DV	Ductus venosus
DZ	Dizygous
EFW	Estimated fetal weight
ELISA	Enzyme-linked immunosorbent assay
Eng	Endoglin

f-βhCG	free β-human chorionic gonadotrophin
FLA	Fetoscopic laser ablation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IFN	Interferon
IL	Interleukin
IQR	Inter-quartile range
IUD	Intrauterine death
KGF	Keratinocyte growth factor
l	Litre
LR	Likelihood ratio
ml	Millilitre
MC	Monochorionic
MCDA	Monochorionic diamniotic
MCMA	Monochorionic monoamniotic
MeSH	Medical subject heading
MHC	Major histocompatilbilty complex
MSAFP	Maternal serum α-fetoprotein
MVP	Maximal vertical pool
MZ	Monozygous
NND	Neonatal death
NNtest	Number needed to test
NNtreat	Number needed to treat
NT	Nuchal translucency
PCR	Polymerase chain reaction

PlGF	Placental growth factor
RCT	Randomised controlled trial
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
RR	Relative risk
TGF- β1	Transforming growth factor-β1
TNF-α	Tumour necrosis factor-α
TOP	Termination of pregnancy
TTTS	Twin-twin transfusion syndrome
VEGF	Vascular endothelial growth factor
VV	Venovenous
Δ	Difference

Publications arising from this work

1. Fox CE, Lash GE, Pretlove SJ, Chan BC, Holder R, Kilby MD. Maternal plasma and amniotic fluid angiogenic factors and their receptors in monochorionic twin pregnancies complicated by twin-to-twin transfusion syndrome. Ultrasound Obstet Gynecol. 2010 Jun;35(6):695-701.
2. Fox CE, Sekizawa A, Pretlove SJ, Chan BC, Okai T, Kilby MD. Maternal cell-free messenger RNA in twin pregnancies: the effects of chorionicity and severe twin to twin transfusion syndrome (TTTS). Acta Obstet Gynecol Scand. 2012 Oct;91(10):1206-11.
3. Fox CE, Pretlove SJ, Chan BC, Mahony RT, Holder R, Kilby MD. Maternal serum markers of placental damage in uncomplicated dichorionic and monochorionic pregnancies in comparison with monochorionic pregnancies complicated by severe twin-to-twin transfusion syndrome and the response to fetoscopic laser ablation. Eur J Obstet Gynecol Reprod Biol. 2009 Jun;144(2):124-9.
4. Fox CE, Lash GE, Pretlove SJ, Chan BC, Holder R, Kilby MD. Maternal plasma and amniotic fluid cytokines in monochorionic, diamniotic twin pregnancies complicated by twin-to-twin transfusion syndrome. Fetal Diagn Ther. Epub Apr 2014.

CHAPTER 1

INTRODUCTION

1.1 The epidemiology and aetiology of twin-twin transfusion syndrome

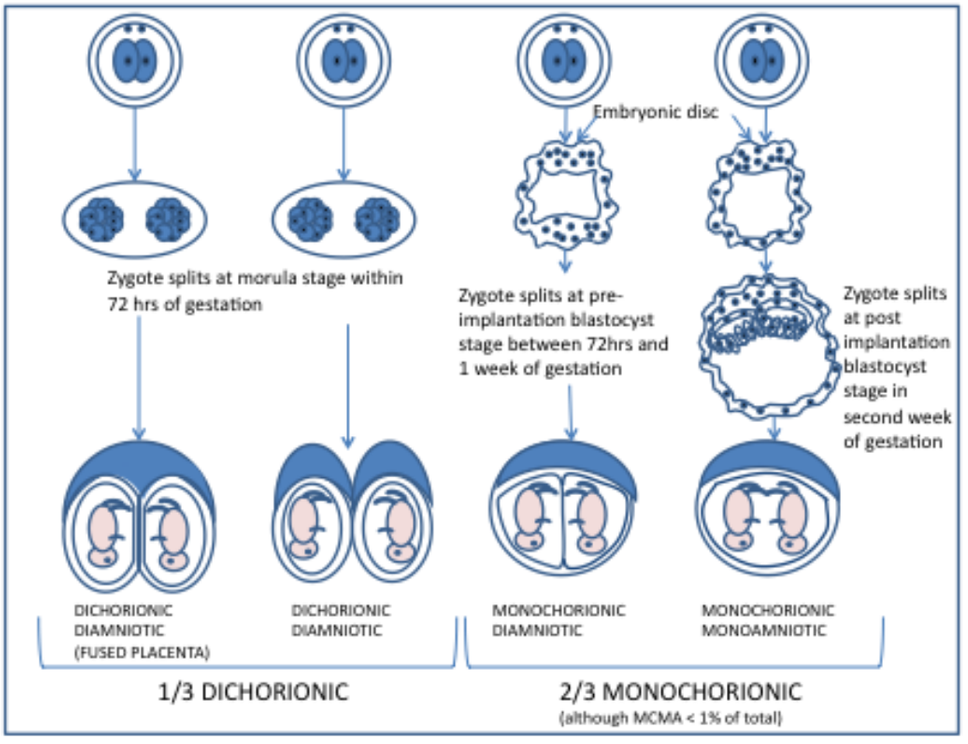
The incidence of multiple pregnancy is increasing, with a rise in the use of assisted conception techniques and delayed child bearing[1]. More specifically, in 2010, 11,053 women gave birth to twins, equating to 1 in 32 babies being born a twin (this figure includes both live and stillbirths)[2]. This is a considerable rise from 30 years ago, when the figure was 1 in 52[2], and of great significance to modern obstetric care as twin pregnancy, as with any multiple pregnancy, carries increased risks of maternal and perinatal complications[1 3].

1.1.1 The Relationship between zygosity and chorionicity

Twin pregnancies can either result from fertilisation of a single ovum by a single sperm, monozygous (MZ) twins, or from fertilization of two ova by two sperm, dizygous (DZ) twins. Approximately one-third of twins are MZ and two-thirds are DZ. Dizygous twins are non-identical and will always have separate placentae (dichorionic twins). However, in MZ or identical twins the number of placentae that develop is dependent on the timing of the single fertilised egg (zygote) splitting (see figure 1-1). In one-third the zygote

splits before 72 hours and two placental discs develop producing dichorionic (DC) twins[4]. In the remaining two-thirds splitting of the zygote occurs after 72 hours, when the inner cell mass has formed and then a single placenta develops (monochorionic (MC) twins), most commonly with two amnions, monochorionic diamniotic (MCDA) twins[4]. However, if the cellular mass splits after one week then the result is twins that share not only the same placenta but also the same amniotic sac, monochrionic monoamniotic (MCMA) twins (<1%)[2]. Overall, approximately one-fifth of twins are MC and four-fifths are DC[5].

Figure 1-1 Diagrammatic representation of the development and placentation of monozygotic twins depending on the timing of the zygote splitting.



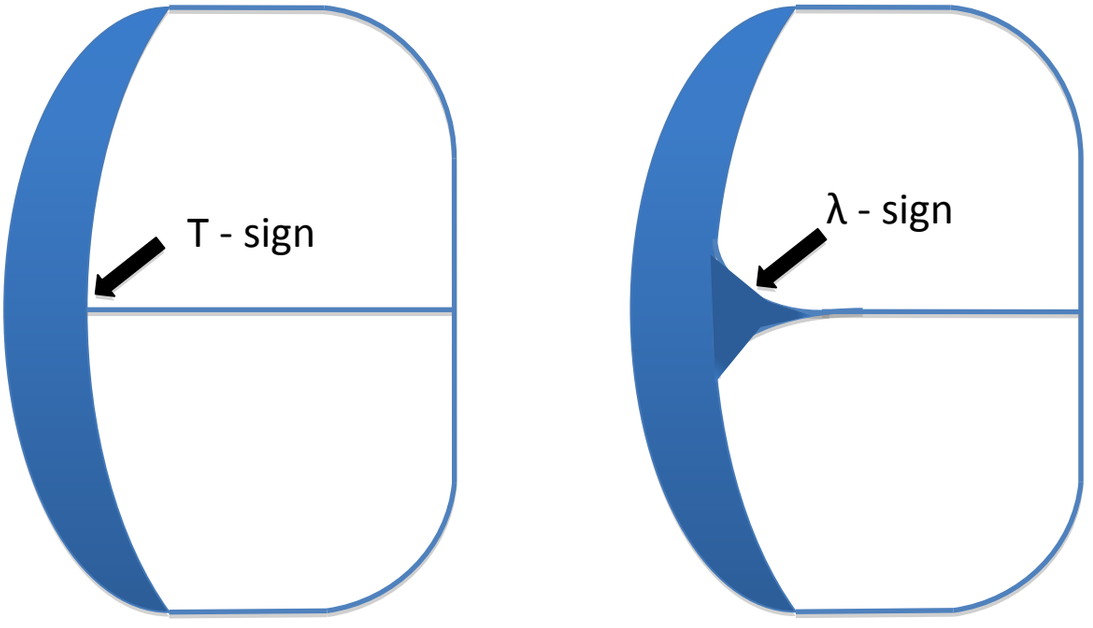
Adapted from Wigglesworth JS. The Placenta in twins. In: Ward RH, Whittle M, editors. Multiple Pregnancy. London: RCOG Press. 1995. Pages 48 - 55[4].

It is however, chorionicity rather than zygosity that determines perinatal risk in a twin pregnancy, such that perinatal mortality in MC twin pregnancies is up to five times higher than in DC twin pregnancies[6 7]. This is important because chorionicity can be reliably diagnosed by first trimester ultrasound scan[8-10] by noting the following features:

a) The number of placental masses

b) The presence of a triangular tissue projection extending from the base of the inter-twin membrane, referred to as a λ-sign, or the absence of this, referred to as a T-sign. The pregnancy is diagnosed as MC in the presence of a single placental mass and evidence of a T-sign, and DC if a λ-sign or two separate placental masses are present.

Figure 1-2 showing the difference between the T-sign in monochorionic twins and the λ-sign in dichorionic twins



It is the case that all MC twins are monozygous, although the same cannot be assumed with respect to DC twins, as these can be either mono- or dizygous depending on the timing of the zygote splitting as described above.

1.1.2 Definition of TTTS

Twin-twin transfusion syndrome (TTTS) is a relatively unusual condition as genetically concordant (i.e. monozygous) twins develop phenotypically discordant features. It is in MCDA twins that TTTS commonly develops, complicating 10-15% overall, of whom 50% are severely affected with the clinical features being apparent by 22 weeks of gestation[11]. TTTS is a clinical condition characterised by the development of discordant amniotic fluid volumes (usually in the diamniotic sacs). The criteria for diagnosis are ultrasound based and require demonstration of a significant discrepancy in amniotic fluid volumes with the presence of oligohydramnios (defined as a maximal vertical pool (MVP) of $\leq$2 cm) in one sac, and of polyhydramnios (a MVP of $\geq$8 cm, or $\geq$10cm after 20 weeks) in the other sac in a MC twin pair. TTTS is thought to be secondary to a chronic imbalance of inter-twin blood flow and haemodynamic pressures within the twin fetal circulations[12] and the twins are described in terms of being either the 'donor' or the 'recipient' in this imbalance. The 'donor' is relatively hypoperfused and hypotensive with associated poor renal perfusion and subsequently urine output. This makes ultrasound visualisation of a fetal bladder difficult and leads to significant oligohydramnios. In the 'recipient' twin, there is a hyperdynamic circulation, with associated increased renal perfusion, polyuria and polyhydramnios[13].

1.1.3 Placental angioarchitecture in TTTS

It was the German Obstetrician Friedrich Schatz who first described TTTS in 1875 based on his work on monochorionic placentas[14]. He discovered the presence of connections between twins, for which he coined the term the "third circulation". He recognised the vascular connections within the placenta that are now considered an anatomical prerequisite for the development of TTTS. Work since then has largely focused on the inter-twin angioarchitecture, with numerous complex variations having been described[15]. In the second week of embryological development trophoblast invasion of maternal blood vessels occurs to form lucanae or intervillous spaces, between which villi begin to appear in branches[16]. From the third week the intravillous vascular network develops and connects with the allantoic vessels to establish the feto-placental circulation[17]. All monochorionic placentae have vascular connections and the majority of connections will form appropriately from the vein to artery of the same embryo[17]. However, where the expanding chorionic vessels from individual embryos overlap there is the potential for inter-twin connections to form[17]. Attrition of chorionic villi is a normal process in placental development[18], however, if an intertwin connected villous area is lost the overall pattern of the connections may change[19]. TTTS has been postulated to develop where the random disruption of anastomoses, and regression of their associated villous districts, results in unequal loss of these placental anastomoses[17]. It is therefore known that all monochorionic placentae have vascular anastomoses in two forms: either direct connections – arterioarterial (AA)/ venovenous (VV), or via a shared cotyledon where an artery from one twin drains into the vein of the other, so-called arterio-venous (AV) connections[20]. However, flow between the twins is usually roughly balanced as there are AV anastomoses in both directions, and any

imbalance can usually be compensated for by AA/VV anastomoses[21]. In particular AA anastomoses appear to have a 'protective role' given that where an AA anastomoses is detected the incidence of TTTS is 15% compared to 61% if no AA anastomoses are present[22]. In summary, TTTS occurs when the pattern of anastomoses present facilitates an overall preponderance of flow from the donor to the recipient[21].

TTTS is uncommon, which, coupled with the lack of a suitable animal model have made studying the angioarchitecture of the placenta more difficult. Specialists have traditionally had to limit themselves to in-utero assessment by ultrasound, with subsequent pathological examinations of the placenta. More recently, Van Gemert's group has performed work using mathematical modelling[23-25]. They have suggested models incorporating different patterns of vascular connections, which seek to explain why only some MCDA placentae develop TTTS and why the severity varies[23]. In the first model, MC twins with unidirectional AV anastomoses, the "net transfusion" from donor to recipient reduces the donor's blood volume, blood pressure, urine production, blood osmolality and therefore colloid osmotic pressure (COP)[25]. It follows that the donor becomes progressively growth restricted. In addition, donor swallowing and intramembranous absorption of amniotic fluid declines in line with the blood osmolality/COP. The reduction in COP will therefore eventually cause a decline in net flow to the recipient. The size of the AV anastomoses will dictate the severity of the discrepancy between the donor and the recipient, such that large calibre AV anastomoses result in a more pronounced discordance in fetal size and amniotic fluid volumes.

In the proposed second model, compensating superficial anastomoses exist but the original net flow from the donor twin causes the same decrease in blood pressure as previously described. However, this facilitates flow in the compensating AA anastomoses in the opposite direction[25] and mitigates the decrease in the donor's haemodynamic (blood) pressure so that urine production will be maintained, albeit at a lower rate (than in uncomplicated monochorionic twins). In the third model, bi-directional AV anastomoses are present such that the flow from the donor to the recipient is compensated by oppositely directed anastomoses, as is suggested to be the most common scenario in TTTS[12]. The result is similar to that seen in AV anastomoses with compensating AA anastomoses, i.e. a steady haemodynamic state of relatively small net transfusion[25]. Finally, the fourth model includes "unequal placental sharing" so the twins develop discordance in size and amniotic fluid volume. However, if a compensating AV anastomosis exists from the larger to the smaller twin the onset of TTTS will be at a later gestational age and the earlier discordance in growth/amniotic fluid accounted for by the differences in placental mass will be reversed, with the larger twin becoming the donor and the smaller the recipient[25].

These models help to explain the differing clinical presentations and perinatal outcomes observed in twin pairs affected by TTTS. In summary, they propose that unidirectional transfusion from donor to recipient causes progressive and irreversible fetal discordance because anastomotic blood flow increases faster than the natural growth rate expected for each fetus[24]. In MC placentae with only unidirectional AV anastomoses this fetal discordance will continue until the driving anastomotic pressure difference becomes small, whereas in the presence of compensating anastomoses the

discordance will develop but the net twin-to-twin transfusion will then trend to zero as compensation occurs (and discordance will therefore persist but not increase)[24]. Conversely, where there is “unequal sharing of the placental disc” and compensating anastomoses, anastomotic blood flow will grow faster than natural fetal growth and this will reverse the discordant growth expected by the unequal placental masses[24].

Such theoretical, mathematical work has offered comprehensive and logical models, which describe and help to explain the varying timing and severity of clinical presentation in TTTS. The authors of this work acknowledge that by necessity various simplifications and assumptions were necessary to account for gaps in our current knowledge and make the equations from which they worked feasible. One of these simplifications relates to overlooking potential fetal compensatory mechanisms, as well as other humoural and endocrine components, which have been implicated in the pathophysiology of TTTS[26].

1.1.4 Ultrasound findings in TTTS

The severity of TTTS, in terms of reflecting prognosis and outcome, has been outlined in a clinical staging classification first described by Dr. Ruben Quintero[27].

Stage I: The fetal bladder of the donor twin remains visible ultrasonographically.

Stage II: The bladder of the donor twin is collapsed and not visible by ultrasound.

Stage III: Critically abnormal fetal Doppler studies noted. This may include absent or reversed end-diastolic velocity in the umbilical artery, reverse flow in the ductus venosus and/ or pulsatile flow in the umbilical vein.

Stage IV: Fetal hydrops in either twin (most commonly the recipient).

Stage V: Demise of either twin.

Quintero staging[27] describes the prerequisite inter-twin discrepancy in amniotic fluid volumes as well as inter-twin haemodynamic disturbance observed ultrasonographically in the MC pregnancy[27]. Donor and recipient twin haemodynamic disturbance is therefore reflected in the amniotic fluid volumes and the presence or absence of a donor fetal bladder but ultrasound also allows measurement of blood velocities within the peripheral and intracardiac arterial and venous fetal circulations by Doppler insonation. In-utero, the right ventricle contributes 60-70% of cardiac output and this is achieved by flow being distributed through the ductus arteriosus, to bypass the lungs, and the foramen ovale, to shunt blood directly from the right atrium to the left atrium[28]. The other fetal circulatory adaptation involves the ductus venosus, which is the shunt from the umbilical vein to the inferior vena cava that allows blood to bypass the liver[28]. Objective ultrasound measurements of fetal cardiac function (or dysfunction) include: cardiothoracic ratio; ventricular chamber dimensions and freewall thickness; valvular regurgitation (atrioventricular (mitral/tricuspid), aortic and pulmonary); and assessment of systolic and diastolic function[29-31]. Systolic function describes ventricular contraction and ejection, whereas diastolic function refers to the ability of the ventricle to fill[29]. Abnormalities such as reversed flow in the ductus venosus and/or pulsatile flow umbilical vein are indirect measures of fetal diastolic dysfunction[29]. In TTTS, the donor twin has been demonstrated to have a higher heart rate than the recipient, which is thought to be a compensatory mechanism for the decreased circulating volume[32]. However, it is in the recipient where abnormal cardiovascular changes correspond to

the severity of TTTS[29]. In the recipient, there is an increase in cardiothoracic ratio, which is thought to be due to cardiac hypertrophy rather than dilatation[29 32]. It has also been shown that ventricular hypertrophy and diastolic dysfunction precedes systolic dysfunction, and that there is greater compromise of the right ventricular rather than left ventricular function[29]. This is exemplified by the higher incidence of tricuspid valve (the atrioventricular valve on the right side of the heart) than mitral valve regurgitation (the atrioventricular valve on the left side of the heart) (60% vs. 38%)[29]. Right ventricular dysfunction, encompassed by hypertrophy and reduced RV ejection fraction, in combination with tricuspid regurgitation can contribute to anatomical right ventricular outflow obstruction and eventually hydrops[29].

Doppler assessment can also assess the feto-placental circulation. Umbilical artery Doppler velocimetry reflects placental function and any abnormality can identify a placental lesion[33]. Although it cannot predict the extent of fetal compromise abnormality does indicate a pregnancy at higher risk of perinatal mortality[33] and it is therefore useful for monitoring in the setting of twin pregnancy, where perinatal mortality is known to be higher[1]. Cardiovascular scores, which can include umbilical artery Doppler assessment, have been suggested to standardise the assessment of the changes seen in TTTS, and several have been described[34-36]. The Children's Hospital of Philadelphia (CHOP) score is the most widely used and encompasses 12 parameters, 11 in the recipient and one in donor, which are graded according to severity[36].

This thesis assesses the diagnostic accuracy of conventional ultrasound and Doppler insonation measurements. Firstly, from measurements taken in the first trimester for

the prediction of which MC twins will develop TTTS and secondly, after TTTS has been diagnosed, which measurements can help to predict outcome.

1.1.5 Treatment for TTTS

In 1875 Freidrich Shatz made observations not only about the placental circulation in TTTS but also that it was condition not amenable to treatment and with very high perinatal loss rates. Since that time, this condition has offered many academic challenges. This is not only in terms of reaching a consensus on diagnosis and understanding the underlying pathophysiology, but also in attempting to alter the clinical course of the condition. Work of vital importance, as TTTS is a condition that affects 10-15% of MC twins but whose natural outcome would be at least 90% perinatal loss of both twins and very high (>50%) neuromorbidity in any surviving babies[11 37]. Crucially, TTTS usually present prior to viability, most commonly less than 22 weeks of gestation, and results in miscarriage or extreme preterm birth of both twins[38]. Where this is not the case in-utero demise of one twin can still lead to jeopardy for the surviving co-twin[39 40]. Treatment strategies have therefore focused on prolonging the pregnancy and have included interrupting the “third circulation” to minimise the harm to the surviving co -twin. Treatment to ameliorate the discrepancy in amniotic fluid between the two amniotic sacs has taken on two forms: intentional septostomy of the dividing membrane[41] or removal of amniotic fluid from the recipient sac[42 43]. Other options have been to perform selective termination of one twin[44], or to interrupt the connecting vessels on the placental surface by fetoscopic laser ablation (FLA) [45-47]. However, the two main approaches that have been utilised have been serial, aggressive amnioreduction and FLA. Briefly, these procedures are performed as follows:

a) *Serial, aggressive amnioreduction*[42 43] - involves the repetitive removal of large volumes of amniotic fluid (usually in excess of 1-2 litres)[43 48]. Under continual ultrasound assessment, an 18-G needle is inserted percutaneously into the recipient amniotic sac. Amniodrainage is performed until the MVP is normal (usually 5-6cms) and repeated whenever polyhydramnios recurs.

b) *Fetoscopic laser ablation*[45-47] – is performed percutaneously under local or regional anaesthesia. A 3.3-mm cannula with trocar is inserted under continuous ultrasound guidance and a 2mm fetoscope (Stortz, Germany) introduced. Primarily, coagulation selectively targets superficial and deep anastomoses on the chorionic plate using a Diode laser system (30-50 W). The total numbers of vascular anastomoses on the chorionic plate crossing the inter-twin membrane are counted prior to the coagulation. At the end of the procedure, excess amniotic fluid is drained to a MVP of 5-6cm. Although not widely performed septostomy involves intentional puncture of the intertwin membrane, usually at the junction of the donor and recipient twins' sacs close to its attachment to the chorionic plate[49]. Again this is performed percutaneously and under continual ultrasound guidance[49].

Serial, aggressive amnioreduction is a relatively simple and widely available treatment. The rationale of this technique is to prevent pre-term labour related to polyhydramnios and to improve fetal haemodynamics by decreasing pressure on the placental surface[42]. Until recently amnioreduction was the mainstay of treatment with overall survival rates of 60%[50] but uncontrolled case series have indicated associated neurodevelopmental morbidity of 40%[51]. Alternatively, FLA of the vascular anastomoses appears to have the potential to modify the underlying disease. A Cochrane review[52] of three studies:

two comparing FLA with serial amniodrainage (one randomised controlled trial, one observational); and one (observational) comparing serial amniodrainage with septostomy, showed there is evidence that FLA, to selectively coagulate all unidirectional vascular anastomoses, may be superior in terms of survival with up to 80% of treated cases having at least one survivor and neurodevelopmental morbidity being as low as 5%. This thesis assesses whether in light of further randomised controlled trial evidence this is still the case.

1.2 Systematic reviews

Systematic reviews are widely regarded as a robust form of evidence on which to base practice, as they aim to reduce bias by use of explicit and systematic methodology [53-55]. The biases that can be reduced include: language bias (selective inclusion of studies published in English); availability bias (selective inclusion of studies that are easily accessible to the researcher); cost bias (selective inclusion of studies that are available free or at low cost); familiarity bias (selective inclusion of studies only from one's own discipline), and outcome bias (selective reporting by the author of a primary study of some outcomes but not others, depending on the direction and statistical significance of the results)[5].

Systematic reviews are based on clearly formulated questions, from which all relevant studies can be identified and their quality appraised[56]. They can summarise and interpret large quantities of information in an efficient and objective manner[57]. They establish the generalisabilty of the findings and examine consistency in the data. In

doing so they can explain any inconsistency and conflicts within the data. Where they include meta-analysis this can increase the precision and power of results[57]. Overall these techniques need to be explicitly described to ensure that the systematic review is robust to allow the results to be interpreted in a clinical meaningful way and therefore inform practice. It is also possible if there is a paucity of evidence that systematic reviews can highlight areas for subsequent research[57].

1.2.1 Existing evidence on accuracy of diagnostic tools

Literature was identified by searching for existing evidence addressing the accuracy of ultrasound in investigation of TTTS. This showed that in the last two decades there have been a number of publications indicating that ultrasound measurements firstly, from the first trimester may have a role in predicting which MC twins will develop TTTS, and secondly, once TTTS develops may have a role in predicting its outcome. Tests in the first trimester include crown rump length discordance, nuchal translucency discordance, nuchal translucency > 95th percentile and absent/reversed flow in the ductus venosus. Tests to predict outcome after diagnosis include abnormalities in flow in the umbilical artery, middle cerebral artery, ductus venosus and umbilical vein as well as single e.g. valve dysfunction and composite measures of fetal cardiac dysfunction. However, individual studies addressing the accuracy of these widely available ultrasound tools vary greatly in size and have produced heterogeneous and imprecise estimates of accuracy. The reporting of conflicting data has led to confusion and therefore made clinical interpretation difficult. The absence of a uniform strategy for the investigation of pregnancies at risk of TTTS, or risks once it has been diagnosed, reflects the lack of a rigorous assessment of these ultrasound tests.

No systematic reviews of the diagnostic accuracy of ultrasound tools in TTTS were available at the commencement of the research leading to this thesis. Therefore the need to conduct high quality comprehensive reviews on this aspect of TTTS was clear.

1.2.2 Existing evidence on effectiveness of interventions

Literature was identified by searching for existing published evidence addressing the effectiveness of interventions for TTTS. A previous systematic review[58] found that of the four possible intervention options (serial amnioreduction, FLA, septostomy and selective feticide) only serial amnioreduction and FLA had been robustly evaluated. A subsequent Cochrane review in 2008[52] has evaluated three interventions (serial amnioreduction, FLA and septostomy), but also concluded that serial amnioreduction and FLA were the only two treatment modalities that have been rigorously tested. This review included one randomised controlled trial (RCT)[59] and stated that FLA should be considered in treatment of TTTS to improve perinatal outcome. Further study of interventions has focused on FLA and serial amnioreduction, including a second RCT[60] that was reported after the Cochrane review was produced. As this is only the second RCT comparing FLA and serial amnioreduction it provides a substantial contribution to the total number of cases studied, and as systematic reviews of RCTs are considered the most robust form of evidence[53] the need to include this in an up-to date and again comprehensive review is clear.

1.3 Existing evidence on biological markers in the pathophysiology of TTTS

1.3.1 Markers of placental destruction (αFP/βhCG)

α-fetoprotein (AFP) is a glycoprotein produced by the fetal liver and gastrointestinal tract in the second trimester of pregnancy[61]. It has a half life of approximately five days[62]. It enters the maternal circulation by crossing the placenta, mainly by paracellular diffusion in a unidirectional manner [63 64], but can also diffuse across the fetal membranes[65]. Elevated maternal serum AFP (MSAFP) is accepted to be associated with a number of complications including growth restriction, preterm birth, placenta praevia, placental abruption, fetal loss, gestational hypertension and pre-eclampsia[66]. Similarly low levels are also associated with complications such as miscarriage, stillbirth, preterm birth and macrosomia[66]. Theoretical mechanisms for these abnormal maternal serum levels, particularly high levels, relate to placental vascular damage, feto-placental ischaemia or feto-maternal-placental barrier disruption[66]. Human chorionic gonadotrophin (hCG) has an α and a β subunit and has a half life of 24 to 36 hours[67]. The biologically intact ₊β–heterodimer, the "free β -subunit" is secreted by the trophoblast as syncytial differentiation occurs[68]. Elevations of free βhCG (f- βhCG) have been associated with miscarriage, pre-eclampsia, fetal growth restriction and fetal loss[69]. With a higher level associated with a greater risk of complications. In common with elevated MSAFP, placental dysfunction appears to be the underlying cause and therefore MSAFP and f- βhCG are considered markers of placental function[66]. As described previously some of the morphological changes evident in conditions such as pre-eclampsia and growth restriction are shared by TTTS;

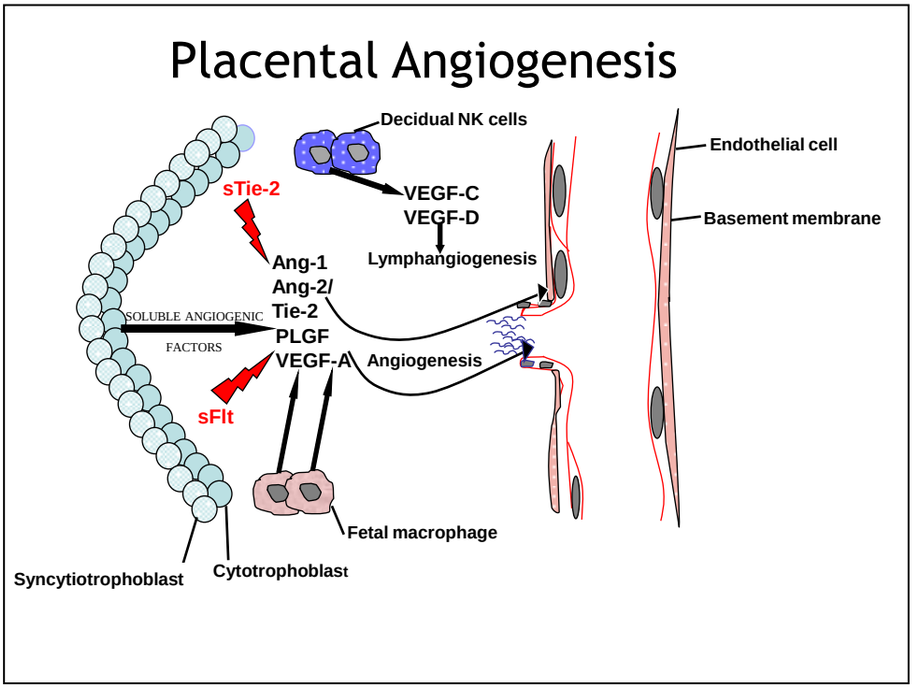
therefore it may be that these placental markers may also be raised in TTTS. Similar elevations of MSAFP and free β-hCG have also been described after multifetal reduction procedures[70]. Additionally, placental microtrauma[71] e.g. chorionic villous sampling (CVS) causes a transplacental haemorrhage releasing AFP normally stored for controlled transport between the fetal and maternal circulations[64]. It has therefore been inferred that elevations in MSAFP after fetal blood sampling and CVS represent placental damage [72 73]. It is hypothesised that FLA may be associated with similar changes in MSAFP and free β-hCG.

1.3.2 Angiogenic growth factors

The vascular endothelial growth factor (VEGF)-family of proteins, comprising VEGF isoforms A-E and placental growth factor (PlGF), interact with various receptors including VEGFR-1 (formally known as Flt-1)[74], see figure 1-3. VEGF-A is a macrophage-derived angiogenic growth factor which, along with PlGF from the trophoblast, has been shown to be potent angiogenic factor, being critical for placental angiogenesis and also as a permeability factor leading to vascular leakage[74 75]. VEGF-C and VEGF-D arise from natural killer (NK) cells and are primarily involved in lymphangiogenesis, the process of creating new lymphatic vessels from previous ones, although they also possess some angiogenic properties[75]. The Tie-2 receptor, a member of the receptor tyrosine kinase family[76], and its ligands angiopoietins, Angiopoietin- 1 and -2 (Ang-1, Ang-2) produced by decidual endothelial cells[77 78], play fundamental roles in angiogenesis and remodelling of vessel structure[75]. Ang-1 does not induce endothelial cell proliferation *in-vitro*, but is chemotactic for human endothelial cells and promotes angiogenesis *in-vivo*[79]. It acts to stabilise blood vessels as opposed

to the antagonist ligand for Tie-2, Ang-2 which plays a role in vessel remodelling through dilatation of vessels and disruption of vessel integrity[79 80]. Transforming growth factor-β1 (TGF- β1) is a further angiogenic factor that binds to Endoglin (Eng) and plays an important role in trophoblast differentiation[81]. In contrast to the angiogenic role these factors and their receptors play, soluble VEGFR-1 and Eng act as natural inhibitors of VEGF-A and TGF- β1 respectively, and are therefore considered anti-angiogenic[74 82]. These factors are likely to have their predominant effects locally as the tissues that synthesise these proteins are immediately adjacent.

Figure 1-3 Diagrammatic representation of angiogenesis in the human placenta.



Adapted from Dunk C, Ahmed A. Growth Factor Regulators of Placental Angiogenesis. In: Kingdom JCP & Baker PN, editors. Intrauterine Growth restriction: aetiology & management. Springer. 1999. Pages 149-162.

Human haemochorial placentation involves vasculogenesis, the formation of blood vessels from non-vascular (haemangioblastic) precursors, in combination with

angiogenesis, the remodelling of existing blood vessels to create new ones[75]. Both of these processes are vital to the fetus and the placenta to facilitate provision of nutrients and oxygen alongside removal of waste products. In addition, optimal growth depends not only on the initial formation of maternal and fetal vasculatures but also on increased blood flow through these as gestation advances[75]. The successful development of the human placenta and the necessary paracrine and autocrine "signals" that regulate the migration and organisation of the various cell types within the placenta (i.e. trophoblast, endothelial cells) are numerous and unsurprisingly complex. The fetal vasculature of the placenta is thought to be dictated by the actions of angiogenic growth factors and their receptors, rather than the action of the trophoblast,[83] and involves members of the vascular endothelial growth factor (VEGF) family as well as transforming growth factor-β 1 (TGF-β 1)[75]. Fetal vascularisation of placental villi begins three weeks after conception by the formation of haemangioblastic cords[84]. This is followed by a period of branching angiogenesis from weeks four to 25, which involves high levels of VEGF in relation to more modest PlGF levels[83], after which angiogenesis switches from branching to non-branching form[75], associated with increased expression of PlGF and the soluble form of VEGF receptor 1 (sVEGFR-1)[84]. Thus, in an uncomplicated pregnancy PlGF levels usually increase in the first two trimesters, peaking at 29 to 32 weeks, before declining to term. sVEGFR-1 levels are usually constant until 33 to 36 weeks, and then increase to term[85]. It is thought that the balance of high PlGF and low sVEGFR-1 in the second trimester of uncomplicated pregnancy creates a pro-angiogenic state[85]. Conversely, at term the balance favours high sVEGFR-1 and lower PlGF, thought to be a reflection of a more anti-angiogenic state[85]. Pre-eclampsia is associated with an earlier increase in sVEGFR-1and fall in PlGF, which has been

considered to represent early onset of the anti-angiogenic state seen in an uncomplicated term pregnancy[85]. Furthermore, It has been shown that if an embryo lacks the VEGF receptor-1, there is a relative excess of its soluble form, the known VEGF antagonist, and this combination is lethal[84].

Angiogenic factors can all be regulated by local oxygen concentration and the normal partial pressure of oxygen in the intervillous space is known to vary with gestation; being low in the first trimester and then peaking at approximately 16 weeks, before declining gradually to term[75 86]. However, there is a time lag between changes in oxygen tension and placental morphological changes and changes in the level of angiogenic factors[75]. The overall environment for placental development is one of relative hypoxia compared to maternal tissues, although as gestation progressed intervillous oxygen tension increases[87]. Peculiar to the placenta, when compared to most other developing organs, is the fact that advancing vascularisation i.e. increased numerical density of fetal capillaries, instead of improving oxygenation appears to be associated with a decrease in tissue oxygenation in placental villi[88]. In conditions such as intrauterine growth restriction and pre-eclampsia there is an increase in the amount of branching angiogenesis resulting in malformation of terminal villi, and a reduction in the growth of capillaries, so that capillary volumes and surfaces may be limited[89].

Twin placentae have been shown to not only display abnormal villus maturation but also other pathological lesions such as subchiorial fibrin deposition, retroplacental haematoma and infarction[90]. These pathological lesions are all more common in monochorionic than dichorionic placentae, with infarction and abnormal villus maturation

being twice as common[90]. In addition, there is an increased incidence of non-central cord insertion in twin pregnancies, which is found almost twice as commonly in MC twins when compared with DC twins (31.5% vs. 17.1%)[91]. Non-central cord insertion is thought to represent competition for suitable implantation sites because although the cord maintains its initial implantation site there is simultaneous proliferation of villi in regions of favourable endometrial blood supply and atrophy of villi in poorly perfused areas[92]. This is significant as this is another feature of the placenta associated with pregnancy complications such as prematurity and fetal growth restriction[93 94]. Specific to TTTS, it has been shown that the placental villi of the recipient are smaller and there is also advanced syncytiovascular membrane formation, the only physical barrier between the fetal ad maternal blood[95], a feature also seen in pre-eclampsia[96]. There is also evidence that where discordant growth exists in TTTS there is evidence of increased resistance in the spiral artery of the donor twin, a feature suggestive of impaired trophoblast invasion, which has been shown in singleton pregnancies with fetal growth restriction and/or pre-eclampsia to equate to abnormal placental development[97]. These placental findings help to explain why twin pregnancies, including those complicated by TTTS, are predisposed to maternal and fetal complications such as pre-eclampsia and fetal growth restriction[98].

Pre-eclampsia and fetal growth restriction have been described as having abnormal placentation and are associated with uteroplacental hypoxia[89]. It is suggested that it may be the relative constancy of oxygen at the different gestations, rather than the absolute value, which may be needed for normal placental development[86]. There is controversy as to whether relative ischaemia and/or hypoxaemia is the causative

mechanism predisposing to abnormal angiogenic orchestration both within the placenta and at the site of trophoblast vascular endothelial cell interaction[82 99]. However, placental expression, circulating levels of angiogenic growth factors and the circulating receptor sVEGFR-1 have been implicated in this process[98]. Indeed, there is growing evidence that the homeostasis between angiogenic and anti-angiogenic growth factors released from the human fetoplacental unit is involved in the pathogenesis of pre-eclampsia[99-105], intrauterine growth restriction[100-102 105-107] and conditions associated with fetal hydrops[108]. A descriptive case study has also reported increased expression of mRNA encoding VEGFR-1 in the villous syncytiotrophoblast of the donor portion of the placenta in TTTS, as compared to that of the recipient[109]. In addition, a cross sectional study has demonstrated an increased circulating maternal plasma sVEGFR-1 concentration and reduced plasma PlGF concentration in TTTS as compared to uncomplicated, gestationally matched monochorionic pregnancies[110]. These data raise the possibility that TTTS may be a relatively anti-angiogenic condition and therefore this thesis will address this question as well as determine the impact of FLA.

1.3.3 Maternal cell-free messenger RNA for angiogenic growth factors

Since 1997, when it was shown that fetal nucleic acids are present in maternal serum and plasma[111], much work has been done to explore new avenues for non-invasive prenatal diagnosis and monitoring. Cell-free fetal DNA is thought to originate from the trophoblast cells of the placenta[112]. The majority results from turnover of villous trophoblasts, as occurs in normal pregnancy, and a smaller amount arises due to apoptosis of fetal nucleated erythrocytes in the maternal circulation[113]. Cell free DNA is detectable in maternal plasma from the first trimester (from approximately seven

weeks)[114]. However, it is cleared very rapidly and undetectable 24 hours after delivery, [115] so can be considered as a biomaker for the current pregnancy, in contrast with intact fetal cells that circulate in the maternal blood for decades[116]. It is known that cell-free DNA is fragmented, and that the maternal DNA fragments are markedly bigger than the corresponding fetal DNA fragments, and is easily extracted and quantified using real-time polymerase chain reaction amplification techniques[117]. Higher levels of cell free DNA have been noted in the plasma of women with conditions associated with abnormalities of the placenta such as placenta accreta[118], as well as in pre-eclampsia, in which there is delayed clearance of fetal cell-free nucleic acids[117] It has been shown that both monochorionic and dichorionic pregnancies have significantly higher free fetal DNA concentrations than singleton pregnancies, with a trend towards a higher concentration in MC than DC twins[119].

Analysis of plasma fetal DNA can indicate the presence and concentration of fetal genetic material in the maternal circulation[111]. However, it is also possible to detect fetal messenger RNA (mRNA), as this is stable in maternal blood due to the fact that it circulates in apoptotic bodies[117] or in association with subcellular particles[120]. Studies have shown that mRNA of placental origin is detectable in maternal plasma by use of real-time quantitative polymerase chain reaction and that it is also rapidly cleared hours after delivery[120 121]. Longitundinal studies have demonstrated that mRNA is present at varying levels during pregnancy but absent within hours of delivery allowing authors to conclude that the mRNA they are measuring is fetal rather than maternal in origin[120 122]. There is also evidence that there is unidirectional transfer of mRNA across the placenta into the maternal blood[120]. It is thought analysis of plasma fetal mRNA can impart

valuable clues as to the gene expression patterns of fetal tissues[123], and plasma levels have been suggested to reflect recent pathological or physiological events due to the rapid kinetics of circulating placental mRNA[124]. It is therefore considered by leaders in this field that measuring fetal mRNA levels offers the possibility of providing non-invasive real-time "fetal functional development information"[117]. For example, changes in oxygen supply or demand, and therefore available concentration, lead to acute and chronic cellular responses[75]. It is thought that chronic responses can be seen in mRNA transcription level changes and so measuring mRNA levels can give complementary information to measuring protein levels alone[75] and that for most genes changes in mRNA are related to changes in protein levels[125]. AGFs have received particular interest due to their link with conditions associated with abnormal placentation, and it was recently suggested that a panel of cell free mRNA (cf-mRNA) markers could be used in the prediction of pre-eclampsia[126 127]. Given the association of MC twin pregnancies with abnormal placentation[90], and that TTTS may be a relatively anti-angiogenic condition[110] it is possible that cf-mRNA for AGFs may be aberrant in TTTS. In addition, cf-mRNA has been demonstrated to be sensitive in defining prospective risk of malplacentation syndromes[126] and it is possible that if cf-mRNA changes are demonstrated they could perform a similar role in TTTS as well.

1.3.4 Cytokines

Cytokines are soluble glycoprotein messengers of the immune system, released predominantly by T lymphocytes. More specifically, it is the T lymphocytes expressing $CD4^+$, which may be further classified into TH1 and TH2 subtypes, that are the richest source of cytokines[128]. TH1 cytokines are involved in pro-inflammatory responses that include release of interferon-gamma (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin (IL) subtypes: IL-2, IL-6, IL-12, IL-1β[128]. TH2 cytokines are released by lymphocytes involved in anti-inflammatory and allergic immune responses and include interleukin sub-types: IL-4, IL-5, IL-10 and IL-13[128]. Cytokines are known to be expressed in the placenta, decidua, and fetal membranes during normal pregnancy and are considered integral to the establishment and function of the placental/maternal interface[129]. The trophoblast does not express classical major histocompatibility complex (MHC) molecules but instead a non-classical MHC to suppress maternal immune cells, and the immunological balance of pregnancy favours TH2 promoted humoural immunity in preference to TH1 cell-mediated immunity to facilitate this[129]. Disruption to cytokine balance has been implicated in conditions associated with failure of trophoblast invasion as well as placental hypoxia and vascular changes within the placenta, such as those seen in recurrent miscarriage, intrauterine growth restriction and pre-eclampsia[129 130]. The TH1 inflammatory cytokines, particularly IL-1β and IL-6, are thought to stimulate the invasive and proliferative phenotypes of the placenta in the first trimester; however, in later pregnancy TH2 cytokines are thought to suppress the production of TH1 cytokines[129]. Increased placental production of some TH1 cytokines, in conjunction with their interaction with growth factors, has been suggested to represent failure of trophoblast invasion in recurrent miscarriage[129] and the continued

placental hypoxia seen in pre-eclampsia[130]. Specifically increased amniotic fluid levels of IL-6 and IL-8 in the mid-trimester have been associated with the subsequent development of pre-eclampsia[131]

In contrast parturition is associated with an increase in production of the TH1 pro-inflammatory cytokines and the chemokine IL-8, which increase with gestation to a peak in labour[129 130]. This is particularly relevant to amniotic and cervico-vaginal fluid levels of IL-1β, TNF-α, IL-6, which stimulate prostaglandin production[130]. There is also evidence that increasing levels of TH2 cytokines IL-4 and IL-10 at term, usually associated with decreased prostaglandin production, may reflect a reversal in function of these usually anti-inflammatory agents to facilitate the cascade of events that precipitates labour[130]. As well as stimulating prostaglandin production from the amnion cytokines also have a role in membrane remodelling and function[130] Rupture of membranes occurs when there is digestion of the extracellular membrane by matrix metalloproteinases and trophoblast apoptosis, both of which are stimulated by inflammatory cytokines (IL-1β, TNF-α, IL-6) and IL-8[130]. There is evidence that elevated levels of these same inflammatory cytokines in the extraplacental membranes and consequently amniotic fluid are also associated with spontaneous preterm labour and the associated reaction when infection is also implicated in this process[130]. This is particularly important as levels of IL-1β, IL-6 and IL-8 in amniotic fluid are also considered risk factors for the development of white matter damage and cerebral palsy in the infant and child[132 133].

There is little specific study of cytokines in multiple pregnancies, particularly reporting the effects of chorionicity. However, a study looking at mid-trimester amniotic fluid cytokines in singleton and twin pregnancies (including four MC and 25 DC twins) reported higher levels of IL-1β, TNF-α and IL-4 in twins than in singletons and supposed that this represented evidence of increased fetal immune system activation in twins[134]. A further study, including two sets of twins, of 18 fetal blood samples showed evidence of expression of fetal mRNA for TNF-α and IL-1β from as early as 21 weeks although, as this was a small sample it did not try to show any quantitative differences between singletons and twins[135]. Another study suggested a link between increased IL-8 in cervico-vaginal secretions and preterm birth in twins[136], and this has since been supported by analysis of 523 blood spot specimens, which also reported a significant increase in IL-8 levels in twins who delivered prematurely[137]. IL-6 is another cytokine that has received particular attention as a possible marker of spontaneous preterm birth and intrauterine infection[130] and this has also been investigated in relation to TTTS[138] [139]. It was measured in amniotic fluid before FLA and found to be higher than in singleton pregnancies[138]. In another study it was measured after FLA but was not found to correlate with the risk of premature delivery[139]. However, other cytokines in maternal plasma or amniotic fluid in relation to TTTS have not been described.

It is known that in multiple pregnancies, there is an increased risk of abnormal placentation, predisposing to complications such as pre-eclampsia and fetal growth restriction[98]. In addition, in TTTS there are numerous, asymmetric anastomoses[17] within the placenta, which account for the characteristic haemodynamic imbalance in the fetal circulations. It is therefore possible that cytokine balance may be aberrant in

the condition itself. FLA causes occlusion by coagulation of the anastomoses[140] and there is some evidence of minimal increases in feto-maternal haemorrhage and trophoblast destruction[141]. It is possible that such a 'reaction' leads to the local release of cytokines and these may have an effect both on decidual-myometrial interaction (affecting the risks of preterm delivery)[130] and potentially also the risk of cerebrovascular morbidity. This is particularly important as neurodevelopmental morbidity occurs in between 11-19% of survivors of TTTS, in spite of treatment[142], and cytokines have been implicated in neonatal white matter lesions, and by extension cerebral palsy[132]. The need to study a range of both pro- and anti-inflammatory cytokines in relation to TTTS and FLA is therefore clear.

1.4 Summary

In summary there is an absence of a uniform strategy for the investigation of pregnancies at risk of TTTS, or risks once it has been diagnosed, and this reflects the lack of a rigorous assessment of the relevant ultrasound tests. This thesis assesses the diagnostic accuracy of conventional ultrasound and Doppler insonation measurements to provide such an assessment. Firstly, from measurements taken in the first trimester for the prediction of which MC twins will develop TTTS and secondly, after TTTS has been diagnosed, which measurements can help to predict outcome.
Furthermore, a third systematic review will update the existing evidence, including the only two RCTs, to compare FLA and serial amnioreduction in the treatment of TTTS. As the existing contemporaneous Cochrane review predated the second of these RCTs the need to include both trials in an up-to date and again comprehensive review is clear. It was for this reason that the Cochrane review has also subsequently been updated and published in January 2014.

With respect to the pathophysiology of TTTS and the effect that FLA has on biological markers several aspects need to be considered. MSAFP and levels of free β-hCG have been associated with conditions associated with abnormal placentation such as pre-eclampsia, fetal growth restriction and miscarriage[69]. Elevations of MSAFP have also been described after CVS[71], and elevations of both MSAFP and free β-hCG after multifetal reduction procedures[70], suggesting FLA, as a further invasive procedure, may also cause changes in their levels. In addition, placental expression and circulating levels of AGFs and their receptors have been implicated in the pathogenesis of conditions associated with abnormal placentation such as pre-eclampsia[99-105],

intrauterine growth restriction[100-102 105-107] and conditions associated with fetal hydrops[108]. TTTS is also associated with abnormal placentation, and has been suggested to be a relatively anti-angiogenic condition[110]. Information regarding AGF at the protein level can be supplemented by fetal nucleic acid study, as plasma fetal RNA can impart valuable clues as to the gene expression patterns of fetal tissues[123]. Cytokines are known to be expressed in the placenta, decidua, and fetal membranes during normal pregnancy and are considered integral to the establishment and function of the placental/maternal interface[129]. Disruption to cytokine balance has been implicated in conditions associated with failure of trophoblast invasion as well as placental hypoxia and vascular changes within the placenta, such as those seen in recurrent miscarriage, intrauterine growth restriction and pre-eclampsia[129 130]. This thesis will assess how these biological factors may contribute to the development of TTTS, as well as investigate what effect treatment by FLA has.

The research presented in this thesis was undertaken with two overall aims:

1. To systematically review and critical appraise the current evidence relating to the diagnostic accuracy of ultrasound in the prediction of TTTS and its outcome, as well as the effectiveness of the two principal treatments for TTTS.
2. To investigate markers of placental destruction, angiogenic growth factor and cytokine levels in uncomplicated twins and twin pregnancies complicated by TTTS. In addition, in the TTTS cohort to investigate the effect of FLA on these same markers.

CHAPTER 2

METHODS

2.1 Systematic review methods

To determine the accuracy of ultrasound tests in the prediction of TTTS and its outcome, and the effectiveness of its two principal treatments three quantitative systematic reviews were performed. Each of the three systematic reviews followed a prospectively developed protocol and used widely recommended and comprehensive methodology[143-145]. Broadly this involves five steps[56]:

i) Framing the question – in terms of the population, the index test or intervention to be studied, the reference standard or outcome and the type of study needed to evaluate these elements.

ii) Identifying relevant studies – a comprehensive search to identify all possibly relevant citations, from which the final studies were selected and the review based.

iii) Assessing the quality of the literature – the confidence that measures were taken to minimize bias in the study design, conduct, analysis and reporting.

iv) Summarising the evidence – collating the findings, including meta-analysis if appropriate.

v) Interpreting the findings – to explore how the findings impact on decision making.

2.1.1 Framing questions

a) What is the diagnostic accuracy of ultrasound in the first trimester for the prediction of which MC twin pregnancies will be affected by TTTS?

b) What is the diagnostic accuracy of ultrasound in the prediction of outcome of TTTS after diagnosis?

c) What is the effectiveness of FLA versus serial amnioreduction in the treatment of TTTS?

These questions were formed a priori in terms of the population, index test/intervention and outcomes and these components are illustrated below, where they were integral to study selection.

2.1.2 Identification of relevant studies

The systematic reviews were designed and performed with careful attention to the need to avoid bias (as described in the introduction). The studies relevant to each review were identified by searching: general bibliographic databases including MEDLINE, EMBASE, and Web of Science; as well as specialist electronic databases (the Cochrane Library - Cochrane Database of Systematic Reviews (2013: Issue 1), Centre for Reviews and Dissemination (2013) and Medion (2013). Hand searching of specialist journals was also performed and reference lists of all known reviews and primary studies were searched. Language restrictions were not applied during search or selection, and unpublished studies were sought. This search informed the planned series of three reviews looking at several aspects of TTTS from screening, to outcome prediction after diagnosis, as well comparing its two main treatments. The search strategy used is detailed in Appendix 1 and encapsulated term combinations with the

relevant population using relevant MeSH terms, text words, and their word variants. Methodological filters were also applied for identification of diagnostic, therapeutic and prognostic studies. All databases were searched from inception until January 2013. A comprehensive database of the literature was constructed (Endnote X5). The electronic searches were examined, to enable initial study selection based on the title and/or abstract. Full manuscripts of all potentially relevant citations were obtained. The final inclusion/exclusion decisions were made after evaluation of the full papers using piloted proformas. The proformas used for each of the three reviews are shown in Appendices 2-4. For multiple/duplicate publication of the same data set only the most recent and/or complete study was included. Two reviewers performed the study selection process independently (see acknowledgments). If there was ambiguity about duplicate publication, studies were initially included for close examination of the reports during data extraction and synthesis.

For the diagnostic accuracy of ultrasound in the first trimester for the prediction of which MC twin pregnancies will be affected by TTTS study selection criteria included:

a) Population	MC twins (single placental mass and presence of T sign)
b) Index tests	Test performed in the first trimester (prior to 14 weeks of gestation) - crown rump length discordance, nuchal translucency discordance, nuchal translucency > 95th percentile and absent/reversed flow in the ductus venosus.
c) Reference standard	Development of TTTS (polyhydramnios surrounding the recipient (MVP > 8cm, or ≥10cm after 20 weeks) and oligohydramnios (MVP < 2cm) surrounding the donor).

d) Study design	Trials or cohort studies were considered ideal but case-control studies were included.

For the diagnostic accuracy of ultrasound in the prediction of outcome of TTTS after diagnosis study selection criteria included:

a) Population	MC twins (single placental mass and presence of T sign) with TTTS (polyhydramnios surrounding the recipient (MVP > 8cm, or ≥10cm after 20 weeks) and oligohydramnios (MVP < 2cm) surrounding the donor).
b) Index tests	Test performed after diagnosis of TTTS – abnormalities in flow in the umbilical artery, middle cerebral artery, ductus venosus, umbilical vein or measures of fetal cardiac dysfunction.
c) Reference standard	Intrauterine or neonatal death
d) Study design	Trials or cohort studies were considered ideal but case-control studies were included.

For the diagnostic accuracy reviews studies that reported a series of less than five cases or failed to correlate the test with the outcome specified were excluded. Although both of the diagnostic accuracy reviews aimed to assess ultrasound the search was not limited to exclude other diagnostic tests. This was to ensure that if there were any additional tests that had been evaluated in robust studies these could be retrieved for detailed examination of their applicability to the prediction of TTTS or its outcome.

For the review of effectiveness of FLA and serial amnioreduction in the treatment of TTTS study selection criteria included:

a) Population	MC twins (single placental mass and presence of T sign) with TTTS (polyhydramnios surrounding the recipient (MVP > 8cm, or ≥10cm after 20 weeks) and oligohydramnios (MVP < 2cm) surrounding the donor).
b) Interventions	Fetoscopic laser ablation and amnioreduction
c) Primary outcome	Survival of at least one fetus.
d) Study design	Both cohort and randomised designs that compared the two interventions in a primary study were considered. Studies that only reported a series of less than five cases or one intervention were excluded.

2.1.3 Quality assessment

All included studies were assessed for their methodological quality based on validated tools[4 55 146-149]. For the diagnostic accuracy reviews the items considered important for a good quality study included prospective design with consecutive recruitment (to avoid selection bias), verification of the index test with reference standard (>90%) (to avoid verification bias), adequate description of the index test and, both appropriate and timely application of the reference standard.

For the review of effectiveness items considered important for a good quality study included randomisation with concealment of allocation (to avoid selection bias), complete description of the intervention (to allow replication by others), blinding of participants, personnel and outcome assessors (to avoid performance and detection

bias), avoidance of deviations from protocol and complete (>90%) follow up (to avoid attrition bias) and examination of any possibility of selective outcome reporting (to avoid reporting bias).

A detailed explanation of how quality was assessed in each review can be found in tables 2.1 and 2.2.

Table 2-1 Quality assessment and definitions for diagnostic accuracy reviews.

Feature	QUADAS 2 Domain	Applicability and criteria fulfilled when
Patient selection Risk of bias Concerns re applicability	1	For the study to be classed as having a low risk of bias it should: have a consecutive or random sample, avoid a case control design and inappropriate exclusions. The patients included should match the review question (RV 1 – unselected MC twins in the first trimester, RV 2 – MC twins with TTTS)
Index test Risk of bias Concerns re applicability	2	For the study to be classed as having a low risk of bias the index test should be interpreted without knowledge of the reference standard and have a pre-specified threshold. The index test should match the review question (RV 1 – test performed in the first trimester to predict TTTS, RV 2 – test after TTTS diagnosed to predict outcome).
Reference standard Risk of bias Concerns re applicability	3	For the study to be classed as having a low risk of bias the reference test should correctly identify the target condition and be interpreted without the knowledge of the index test. The target condition as defined by the reference standard should match the review question (RV 1 - development of TTTS, RV 2 – in utero/neonatal death)
Flow and timing Risk of bias	4	For the study to be classed as having a low risk of bias there should be an appropriate time interval between the index test and reference standard, all

		patients should receive the same reference standard and be included in the analysis.

Table 2.2 Quality assessment and definitions for review of effectiveness.

Feature	Quality assessment
Study design	Randomisation with concealment of allocation was considered ideal. However, it is acknowledged that in observational studies use of adjustment for confounding in analysis is ideal for this type of study design, therefore was also accepted for assessment of quality.
Data collection	Prospective collection of data was considered ideal, retrospective collection was considered second best
Patient selection	Consecutive recruitment with the use of specific eligibility criteria was considered ideal.
Definition of TTTS	MC twins (single placental mass and presence of T sign) with TTTS (polyhydramnios surrounding the recipient (maximum vertical pool (MVP) > 8cm) and oligohydramnios (MVP< 2cm) surrounding the donor) was considered adequate.
Description of intervention	Complete and transparent description to allow replication by others was considered adequate. To include gestational age at intervention and number of procedures performed.
Outcome ascertainment	Complete (>90%) follow up of the original study population with adequate description of the outcome measures were considered ideal. Blinding of carers and patients was not considered essential for quality, however, blinding of outcome assessors was considered ideal.

Piloted checklists (based on QUADAS 2[148] for the diagnostic accuracy reviews and the CASP checklists[150 151] for the effectiveness review) were used to identify and record items relating to study quality. This assessment was performed independently, in duplicate for each review. Any disagreements were resolved by consensus. Quality assessment was displayed on a bar chart. An assessment of individual components of study quality was made to classify studies as high or low quality, and this was utilised in sub-group analysis, including meta-regression if the number of studies allowed. This approach was utilised in preference to assigning a quality score as these can obscure the strengths and weaknesses of the study and have been shown to have little validity[4 152]. Diagnostic accuracy studies were considered high quality if they reported an independent, prospective comparison of the index test with the reference standard in an appropriate population of consecutive patients and moderate quality if they fulfilled all except the criterion for consecutive recruitment. All other studies were considered low quality.

2.1.4 Summarising the evidence

Data extraction forms were devised and piloted and can be found in Appendices 2-4. All data were extracted independently and in duplicate. For the diagnostic accuracy reviews this allowed construction of two-by-two tables detailing the result of the index test under scrutiny and the results of the reference standard, namely crown rump length (CRL) discordance, nuchal translucency (NT) discordance, NT > 95th percentile or absent/reversed flow in the ductus venosus (DV) against the development of TTTS for the prediction review, and abnormalities of flow in the umbilical artery (UA), middle cerebral artery (MCA), DV, umbilical vein (UV), estimated fetal weight discordance

(EFW) or fetal cardiac dysfunction against in-utero/neonatal death for the review of outcome. If the study reported different cut-off levels for an abnormal test result then separate two-by-two tables were constructed to reflect this. For the effectiveness review two-by-two tables detailed survival after FLA and amnioreduction.

For the diagnostic reviews the following measures of test accuracy, with their 95% confidence intervals (CI), were calculated from the two-by-two tables: sensitivity (true positive rate), specificity (true negative rate), and likelihood ratios (LR, summarising how many times more (or less likely) patients with the disease are to have a particular result than patients without the disease)[153]. Where any of the cells in the two-by-two tables contained zero cells, a half (0.5) was added to each cell to enable calculations[154]. The results from studies with similar characteristics and the same threshold for the index test, as well as the reference standard, were pooled. Where bivariate meta-analysis was not possible (if there were less than four studies) analysis also included exploration of threshold effect, using Receiver Operating Characteristic (ROC) analysis and calculation of Spearman correlation coefficients[155]. LRs were favoured for meta-analysis as these are widely regarded as of most relevance clinically[156] as they can be directly applied to give a probability that an individual has a disease[157]. This was done using Bayes' theorem and the formula:

Post test probability = LR x pre-test probability / [(1-pre-test probability) x (1-LR)][158]

An estimate for pre-test probability was calculated from the prevalence of the pathology in the population studied. If a study reported a prevalence that was markedly different from the other studies and inconsistent with the general literature this estimate was not

included. To deal with the uncertainty in estimation 95% confidence intervals (CI) for each point estimate (LR) were calculated.

For the effectiveness review the included studies had either randomised or cohort designs therefore effect estimates were computed using Relative risk (RR) as an effect measure, along with 95% confidence intervals[159]. Intervention failure and complication rates were compared using Fisher's exact test.

The extracted data were tabulated to allow qualitative inspection for clinical and methodological heterogeneity. Heterogeneity was also explored graphically and statistically. Graphical assessment for the diagnostic accuracy reviews involved examining the distribution of sensitivities and specificities as points on a ROC plot[157] and LRs as a measurement of accuracy size using a Forest plot. For the effectiveness review the distribution of effect estimates was explored graphically using Forest plots. All reviews also examined heterogeneity statistically using the χ^2 test and I^2 [160]. Where there was evidence of heterogeneity e.g. χ^2 p value < 0.05 then a random effects pooling method was used, conversely if the studies were homogeneous then a fixed effects pooling method was used. The reasons for heterogeneity were explored using meta-regression and sub-group analysis. Where only a single study existed single estimates are reported.

Sub-groups were planned a priori based on clinical criteria known to influence prognosis and study quality and were performed if there were at least two studies with those characteristics. These included:

- study design – trials or cohort studies only

- study quality

- variations in index test e.g. type of test parameter, cut off used

- variations in reference standard – e.g. in-utero or neonatal death

Statistical analyses were performed using Meta-DiSc[97] and STATA 11 (StataCorp., 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp. LP) for meta-analysis (bivariate with the "metandi" command[120] where sufficient (4) studies were available), meta-regression and plotting ROC curves, and Statsdirect (Statsdirect Ltd) for calculations and producing Forest plots. In the diagnostic accuracy reviews sensitivity analyses were performed to check the robustness of all results. Statistical significance was accepted at a P value of < 0.05 for all reviews.

2.1.5 Interpreting the findings

The interpretation of the review findings takes into account the credibility of the findings and their relevance to clinical practice. This will involve exploration of publication bias both graphically, through the production of funnel plots, and statistically, using Egger's regression method[161]. Funnel plots are scatter plots of a measure of individual study size (i.e. precision, overall sample size or standard error) against accuracy or effect size[161 162]. The smaller studies are distributed towards the bottom of the graph and, due to their estimates of accuracy/effect being more heavily influenced by random variation, across a range of values[161]. If the funnel plot is asymmetric this implies publication bias is present, as the missing plots are likely to reflect the fact that smaller or non-significant studies are less likely to be published[162]. Conversely, a funnel shape would be

expected if publication bias is absent. Egger's regression method is intended to quantify the bias depicted by a funnel plot by use of the studies' accuracy/effect sizes and their precision[87 161]. Using this method the presence of publication bias is evident when $p<0.1$[87]. However, both funnel plots and Egger's regression method are more reliable if a large number of studies are being analysed as smaller studies are more likely to be of lower methodological quality and show larger treatment effects or accuracy, known as small-study effects[87 161]. For this reason, when there was evidence of small-study effects this was considered in the reporting of any meta-analysis to aid interpretation of the review findings. The use of sub-group analysis based on study quality was also performed to ensure this process was explicit. This allowed recommendations for practice to reflect the strengths and weakness of the assimilated evidence[56].

2.2 Investigation of biological markers in the pathophysiology of TTTS methods

2.2.1 Study design

A cohort of monochorionic (MC) twins complicated by TTTS (n=23) was prospectively studied at a single Fetal Medicine Unit, between October 2006 and December 2007[141] [163-165]. Twins were eligible for inclusion if they were confirmed by ultrasound scan (in the first trimester) to be MC (there was a single placental mass and only a thin dividing membrane (T-sign)) and subsequently presenting with a diagnosis of TTTS but no other fetal or maternal condition. The diagnosis was made according to internationally accepted ultrasound criteria: a MC twin pregnancy with polyhydramnios (maximum vertical pool (MVP) $\geq$8cm) in the recipient (or $\geq$10cm from 20 weeks onwards) and oligohydramnios (MVP $\leq$2cm) in the donor[27]. Recruitment was consecutive and all those recruited were available for follow up to birth.

The stage of TTTS was reported based on: the presence of the donor twin's bladder (stage I); the absence of the donor twin's bladder but no Doppler abnormalities (stage II); Doppler abnormalities present (stage III); the presence of hydrops (stage IV); demise of one or both twins (stage V)[27]. In addition the inter-twin and amniotic fluid volume difference were reported as these give useful clinical information regarding the severity of the condition[166 167]. Severe polyhydramnios was defined as a MVP > 12cm, as this is the level above which a reduction in utero-placental blood flow and increased local hypoxemia has been described[167]. The percentage estimated fetal weight difference (ΔEFW) was calculated as the difference in weight, divided by the larger twin's weight,

multiplied by 100, with growth defined as concordant if the ΔEFW was less than 25%[166]. Fetal ultrasound and echocardiography were performed with curvilinear array transducers with variable frequencies (7-3.5MHz) on a Siemens Antares (Siemens Ltd, Erlangan, Germany). These were performed by a single operator (MDK).

A relatively small comparison group of uncomplicated dichorionic (n=12) and monochorionic (n=7) twin pregnancies was also studied. All of these twin pregnancies were appropriately grown for gestation with normal amniotic fluid volumes in each of the diamniotic sacs. All uncomplicated twins were designated as DC or MC at ultrasound between 11-14 weeks. Monochorionicity was based on the whether there was a single placental mass and only a thin dividing membrane (T-sign). They were recruited in the second trimester and used for basal plasma comparison only. They did not undergo any form of treatment and all delivered > 34 weeks.

This study had Ethical approval from Birmingham Black Country Local Research Ethics Committee (No: 06/Q2702/71) and all participants gave informed consent.

2.2.2 Sample collection and assay

Maternal peripheral venepuncture was performed under basal conditions (several hours prior to any fetal therapy in the TTTS cohort, and at an antenatal visit in the uncomplicated twins) and then intervals of 6, 24 hours and one week post-treatment in the TTTS cohort. Venous blood was collected either into a Sarstedt Lithium heparin 7.5ml tube and placed on ice (for plasma samples) or into a Sarstedt Serum Gel S 7.5ml tube and allowed to clot for at least one hour at room temperature (for serum

samples). Plasma samples were centrifuged within one hour, and serum samples were centrifuged after they had been allowed to clot. Centrifugation occurred at 3000 rpm for 10 minutes at 4°C (Heraeus Labofuge 400). A sample of amniotic fluid was collected from the recipient twin sac just prior to fetoscopic laser ablation or amniodrainage and again at the end of the procedure.

Briefly, the procedures were performed as follows:

i). Fetoscopic laser ablation. This was performed percutaneously under regional anaesthesia by a single operator (MDK)[46]. A 3.3-mm cannula with trocar was inserted under continuous ultrasound guidance and a 2mm fetoscope (Stortz, Germany) introduced. Primarily, coagulation selectively targeted superficial and deep anastomoses on the chorionic plate using a Diode laser system (30-50 W)[46]. The total numbers of vascular anastomoses on the chorionic plate crossing the inter-twin membrane were counted prior to coagulation. At the end of the procedure, excess amniotic fluid was drained to a MVP of 5-6cm (median 1500mL; range 900-4300mL). This was the primary treatment if stage II-IV TTTS was diagnosed.

ii) Amniodrainage. Under continual ultrasound assessment, an 18-G needle was inserted percutaneously into the recipient amniotic sac. The initial 5mls sample was discarded and then 5mls of amniotic fluid taken for assay. An amniodrainage was performed until the MVP was measured as 5-6cms (median 2500 mL; range 1700 – 5100 mL). This was the primary treatment if stage I TTTS was diagnosed.

All treatment was performed within 24 hours of diagnosis.

Both maternal plasma/serum and amniotic fluid samples were divided into 1000μl aliquots and stored at -80°C until assay (all within 16 months of collection [range 1.2 – 15.3 months]). Where appropriate, the optimal concentration for plasma and amniotic

fluid for each analyte was determined by assay of a range of concentrations against the appropriate standard and if necessary diluted in accordance with manufacturer's instructions. All samples for any given analyte were assayed in duplicate at the same time using the same standard curve to minimize inter-assay variation. The methodology has previously been described previously[168-170]. All angiogenesis and cytokine assays were performed by the author and Dr Gendie Lash (see acknowledgements)[164].

AFP and free β-HCG

Maternal serum and amniotic fluid samples were analysed for AFP and free β-HCG using solid phase, two site fluoroimmunometric assays (Delfia free β -HCG, Perkin Elmer Ltd, Finland). Intra-assay and inter-assay coefficient of variation was 1% and 1.9%[141]. All values for AFP and free β-HCG are given as multiples of the median (MoM) to correct for the fact that they are known to change with gestational age[171]. The reference ranges utilised to calculate these MoM were supplied by Dr Francoise Muller at the Service de Biochimie-Hormonologie Hôpital Robert Debré (Paris, France) for free β-HCG and Ian Mills at the Clinical Chemistry laboratory, Birmingham Women's Hospital (England) for MSAFP. However, where the analysis is limited to the TTTS cohort undergoing FLA AFP and free β-HCG are analysed as absolute values to allow a percentage change to be seen.

Angiogenic growth factors

Maternal plasma and amniotic fluid samples were analysed for Ang-1 (sensitivity 312.5pg/ml), PlGF (sensitivity 31.25pg/ml), VEGF-C (sensitivity 93.75pg/ml), VEGF-D (sensitivity 31.25pg/ml), sTie-2 (sensitivity 31.25pg/ml) and sVEGFR-1 (sensitivity

31.25pg/ml) by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). In addition, samples were analysed for VEGF-A (sensitivity 27.4pg/ml) and Ang-2 (sensitivity 137pg/ml) using a FAST Quant human angiogenesis array (Whatman Schleicher & Schuell, Dassel, Germany). The intra-assay coefficients of variation using these methods ranged from 4.2% to 10.1%.

Multiplex analysis and cytokines

Plasma and amniotic fluid samples were analysed for the TH1/TH2 cytokines: IFN-γ, TNF-α, IL-2, IL-6, IL-1β, IL-4, IL-5, IL-10, and IL-13; as well as keratinocyte growth factor (KGF), platelet derived growth factor-BB (PDGF-BB), fibroblast growth factor-basic (FGF-basic), tissue inhibitor of matalloproteinases-1 (TIMP-1) and intercellular adhesion molecule-1 (ICAM-1) by Human TH1/TH2 or Human Angiogenesis FASTQuant kits (Kerafast, NC, USA). IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). The sensitivity of all assays ranged between 3.04pg/ml and 15.2pg/ml, and these values were utilised if the assay result was outside the reference range. The intra-assay coefficients of variation using these methods ranged from 2.7% - 16.5%.

Cell free mRNA

Molecular analysis was performed in the Department of Obstetrics and Gynaecology at Showa University School of Medicine in Tokyo, Japan (see acknowledgements). Processing of blood samples has been described previously[165 172]. Total RNA was extracted from 1ml of maternal plasma using the following technique[165]. The plasma was mixed with 3ml of Trizol LS reagent (Invitrogen, Carlsbad, CA) and 0.25ml of

chloroform to disrupt the plasma cells and allow isolation of high quality RNA. This mixture was centrifuged at 12,000 times gravity for 15 min at 4 °C, and then the aqueous layer was extracted as this contains the RNA. After 1 volume of 700ml/l ethanol was added to 1 volume of aqueous layer and the RNA precipitated out, the mixture was applied to a QIAamp MinElute Virus column (Qiagen, Hilden, Germany), causing the RNA to bind, and processed according to the manufacturer's instructions. Total RNA was then eluted with 20µL of RNase-free water and directly reverse-transcripted using an Omniscript RT kit (Qiagen) according to the manufacturer's instructions. The resulting complementary DNA (cDNA) products were amplified by real-time quantitative polymerase chain reaction (PCR) according to the manufacturer's instructions (QuantiTect Probe PCR kit; Qiagen) using a 2µL aliquot of cDNA and the kit components in a reaction volume of 20µL. TaqMan PCR analyses for VEGF-A, VEGFR-1 (Flt1), endoglin (Eng), PlGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using pre-developed and commercially available primers and probe sets (Cat # Hs00167155_m1 for PAI-1, Cat # Hs00263492_m1 for tPA, Cat # Hs00900054_m1 for VEGFA, Cat # Hs01052936_m1 for FLT1, Cat # Hs00923997_g1 for ENG, Cat # Hs00182176_m1 for PlGF and Cat # 4310884E for GAPDH; Applied Biosystems, Foster City, CA, USA). TaqMan polymerases are thermostable 5'3' exonuclease assays which cleave 5' terminal nucleotides to release mono and oligonucleotides which can be used as probes[173]. The PCR reaction utilises the 5' nuclease activity of the TaqMan DNA polymerase to cleave a Taqman probe[174]. Attached to the probe are a reporter dye at the 5'-end and a quencher dye at the 3'-end. The reporter will not fluoresce while both are attached however, during the reaction cleavage of the probe separates the reporter and quencher dyes allowing the reporter

dye to fluoresce[174]. The increase in fluorescence signal is only detected if the target sequence is complementary to the probe and is amplified during the PCR. To verify that each PCR assay was specific to mRNA and not to genomic DNA the primers used all spanned an exon junction and would therefore only amplify cDNA from mRNA transcripts and not genomic DNA. Amplification data were collected and analysed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analysed in duplicate, and multiple negative water blanks were included in every analysis. The thermal profile used was as follows: 15 min of denaturation at 95 °C, 15 seconds of annealing at 94 °C and 1 min of extension at 60 °C. Quantification of gene expression was performed by investigators blinded to the outcome of pregnancy.
Amounts of mRNA were calculated using both absolute value (copies/mL) and relative value (by comparison of the target nucleic acid threshold cycle with a calibrator nucleic acid threshold cycle), amounts of mRNA samples were expressed in term of copies per ml. It is known that there is variation in amounts of starting material across samples[175], therefore several genes were investigated for use as internal controls and the gene with the least variation selected for expressing relative value as previously utilised[176-178]. However, in line with previous studies of fetal mRNA[178-181] and as the more consistent result in this study gene expression values are given in copies per ml.
To quantify mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described[182].

2.2.3 Statistical analysis

Statistical analysis was performed using Graphpad Instat® and graphs generated using Graphpad Prism version4 (Graphpad Software Inc, CA, 2004). As data did not fit a

normal distribution, values are expressed as median and inter-quartile ranges (IQR) and non-parametric statistical tests used. The unpaired Mann Whitney U test was used to compare numerical variables where there were two groups and for comparison of three or more groups the Kruskal-Wallis test with Dunn's post-hoc testing. For analysis of 'paired' plasma and amniotic fluid samples the Wilcoxon Signed rank test was used. Correlations were performed using Spearman's rank correlation coefficient. A multiple regression model was used to look at associations between biomarkers and duration after FLA, with particular reference to the influence of gestational age, operation time, amount of amniotic fluid drained at the end of the procedure, and the number of chorionic vessels coagulated. A P value of < 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Systematic review results

3.1.1 Diagnostic accuracy review of ultrasound in the first trimester to predict TTTS in uncomplicated MCDA twins

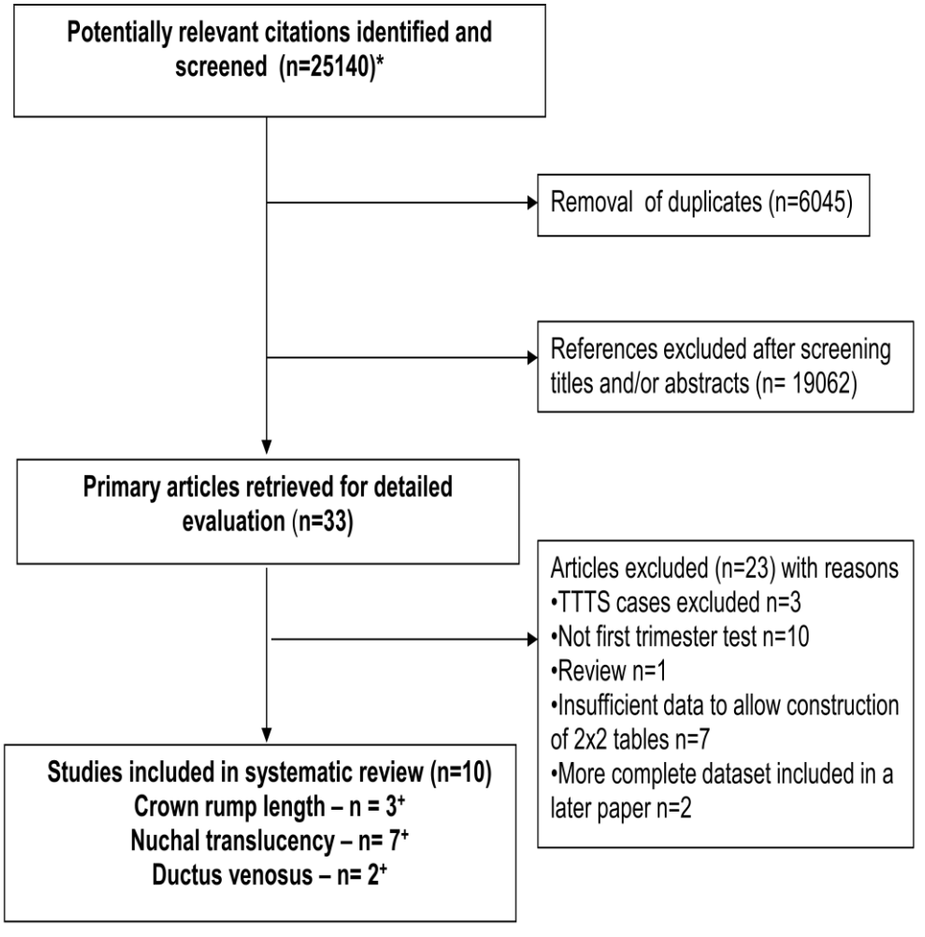
3.1.1.1 Question

What is the accuracy of ultrasound in the first trimester for the prediction of TTTS in uncomplicated MCDA twins?

3.1.1.2 Identification of relevant studies

The electronic and hand search relevant to all three planned reviews generated 25140 citations, of which 33[22 183-214] were thought to be relevant: 31 were published in English, one in Chinese and one in German. After independent review of the full manuscript of these 33 articles, 10[204-213] were selected for inclusion in this review (figure 3-1). The characteristics of the included studies are shown in table 3-1. The list of excluded studies is included in Appendix 5**.**

Figure 3-1 Study selection process for a diagnostic accuracy review of ultrasound in the first trimester to predict development of TTTS in uncomplicated MCDA twins.



*Overarching search encompassing three TTTS reviews

+Some articles included more than one test

Table 3-1 Study Characteristics for a diagnostic accuracy review of ultrasound in the first trimester to predict development of TTTS in uncomplicated MCDA twins.

Study	**Population** **(country/study design)**	Reference standard	**Index test/s**
Casabuenas et al (2008)	Inclusion – 27 MCDA twin pregnancies and 3 triplet pregnancies with 1 set of MC fetuses (2 diamniotic, 1 monoamniotic) evaluated in the first trimester. Time period unspecified. No exclusions. Method of confirmation of chorionicity – USS. (Chile) (retrospective cohort)	Development of second trimester TTTS – defined as the polyhydramnios/ oligohydramnios sequence (as described by Quintero).	∆NT ≥ 20% ∆CRL ≥ 10% NT and CRL measurements followed the UK Fetal Medicine Foundation recommendations. NT and CRL discrepancies were calculated as a percentage of the larger twin. All scans were performed transabdominally by maternal-fetal medicine specialists.

El Kateb et al (2007)	Inclusion – 103 consecutive MCDA twin pregnancies followed up from 11-14 weeks between June 2002 and February 2006. Exclusions – cases with chromosomal or congenital malformation. Method of confirmation of chorionicity – NS. (France) (Prospective cohort)	Development of TTTS- defined by the association of polyuric polyhydramnios in one sac with a DVP of at least 8 or 10 cms before and after 20 weeks respectively together with oliguric oligohydramnios in the other sac with a DVP of at most 2 cm.	ΔCRL $\geq$ 10% NT > 95th percentile. CRL and NT were measured to the nearest millimeter in a sagittal section of the fetus with the head in a neutral position.
Fratelli et al (2011)	Inclusion – 136 consecutive MCDA twin pregnancies in the first trimester assessed between February 2001 and April 2009. Exclusion – 1 case with trisomy 21 in both fetuses. Method of confirmation of chorionicity – USS. (Italy) (retrospective cohort)	Development of severe TTTS – defined by the presence of DVP < 2cm in the donor and $\geq$ 8 cm (or 10cm if after 20 weeks) in the recipient	NT > 95th percentile in the pregnancy. NT was measured in a sagittal section of the fetus with the head in a neutral position. Reference values for NT measurement Percentiles were those provided by the Fetal Medicine Foundation. The ultrasound examinations were performed by experienced sonographers accredited by the Fetal medicine Foundation.

Linskens et al (2009)	Inclusion – 61 consecutive MCDA twin pregnancies that were referred for Down's syndrome screening and had serial follow up ultrasonography. Taken from the fetal database at a tertiary centre between 2004 and 2008. Exclusion – 3 cases IUD, 3 cases born immaturely. Method of confirmation of chorionicity – USS. (Netherlands) (retrospective cohort)	Development of TTTS according to the Quintero staging system.	∆NT ≥ 20% NT was measured on an early first trimester scan according to the standards defined by the Fetal Medicine Foundation.
Maiz et al (2009)	Inclusion – 733 diamniotic twin pregnancies undergoing USS at 11 0/7 to 13 6/7 weeks gestation assessed between January 2006 and January 2008. Exclusion – 5 unable to get DV waveforms, 33 with a missing outcome 516 DC, 179 MC. Only MC included in this review. Method of confirmation of chorionicity – USS. (United Kingdom) (prospective cohort)	Development of TTTS.	Reversed a wave in the DV The Doppler studies were undertaken during fetal quiescence. The image was magnified and a right ventral mid-sagittal view of the fetal trunk was obtained, and color flow mapping was used to demonstrate the umbilical vein, ductus venosus, and fetal heart. The insonation angle was less than 30°, the filter was set at allow frequency (50–70 Hz) to allow visualization of the whole waveform, and the sweep speed was high (2–3 cm/s) so that the waveforms were spread widely, allowing better assessment of the a-wave. Transabdominal USS by sonographers with the appropriate Fetal medicine Foundation Certificate of Competence.

Matias et al (2010)	Inclusion – 103 MCDA twin pregnancies assessed at 11-14 weeks gestation between December 1997 and October 2008 Exclusions – 2 cases with congenital malformations, 2 cases with single IUD prior to development of TTTS Method of confirmation of chorionicity – USS and placental histology. (Portugal) (prospective cohort)	Development of TTTS - defined by the presence of oligohydramnios and non-visible bladder in the donor in combination with polyhydramnios and dilated bladder in the recipient, along with different stages of Doppler deterioration.	Abnormal flow in the DV DV measurements were measured during fetal quiescence, the image was magnified and a right ventral mid-sagittal view of the fetal trunk was obtained. The insonation angle was systematically kept below 30° the high-pass filter was set at 50–60 Hz and the sweep speed was high (2–3 cm/s) to better visualize the A-wave. An average of five consecutive high-quality waveforms was used to qualitatively assess the flow in the DV, which was considered to be abnormal if the A-wave was absent or reversed. Transabdominal and transvaginal approaches as required. Experienced sonographers accredited by the Fetal medicine Foundation performed the ultrasound examinations.
Matias et al (2005)	Inclusion – 50 MCDA pregnancies referred to a single centre between 11-14 weeks. No exclusions. Method of confirmation of chorionicity – USS. (Portugal) (prospective cohort)	Development of TTTS - was defined sonographically based on the presence of anhydramnios and non-visible bladder in the donor in combination with polyhydramnios and dilated bladder in the recipient.	$\Delta NT \geq 20\%$ NT thickness was measured at a maximum magnification of the fetus and plotted against crown-rump length.

O'Kagan et al (2007)	Inclusion – 560 MCDA twin pregnancies at 11- 13+6 weeks between January 2001 and April 2006. Exclusions – 28 chromosomal or major structural defects, 20 no pregnancy outcome data, 42 with IUD one or both twins. Method of confirmation of chorionicity – USS. (United Kingdom) (prospective cohort)	Development of severe TTTS treated with laser coagulation. The indication for laser being ultrasound diagnosis of polyhydramnios/anhydramnios and absent or reversed flow in either the umbilical artery or ductus venosus in one or both fetuses.	ΔCRL $\geq$ 10% ΔNT $\geq$ 20% or ΔNT $\geq$ 30% Inter-twin discordance in NT and CRL was calculated as the difference in each measurement between the two fetuses (NT1−NT2 and CRL1−CRL2, respectively) expressed as a percentage of the larger measurement.
Sebire et al (2000)	Inclusion – 303 consecutive MC twin pregnancies examined at 10-14 weeks identified from September 1992 and estimated to deliver by June 1999. Exclusions – 16 pregnancies with structural or chromosomal abnormalities. Method of confirmation of chorionicity – USS. (United Kingdom) (prospective cohort)	Development of severe TTTS - was defined by the ultrasound features of anhydramnios and non-visible bladder in the donor in combination with polyhydramnios and dilated bladder in the recipient, which resulted in either miscarriage or fetal death or required intrauterine treatment or post-mortem evidence that the cause of death was TTTS.	NT > 95th percentile in the pregnancy.

Tai et al (2007)	Inclusions – 178 twin pregnancies scanned between 7 and 14 weeks of gestation and subsequently delivered between June 2000 and March 2006 at a single centre. Exclusions - Fetuses with known chromosomal or major congenital anomalies, women who underwent first- or second-trimester pregnancy termination, those pregnancies that were monoamniotic. Only 43 MC twin pregnancies included in this review. Method of confirmation of chorionicity – USS. (USA) (retrospective cohort)	Development of TTTS	∆CRL >11% The CRL was calculated as the difference in the twin CRLs divided by the CRL of the larger twin and expressed as a percentage.

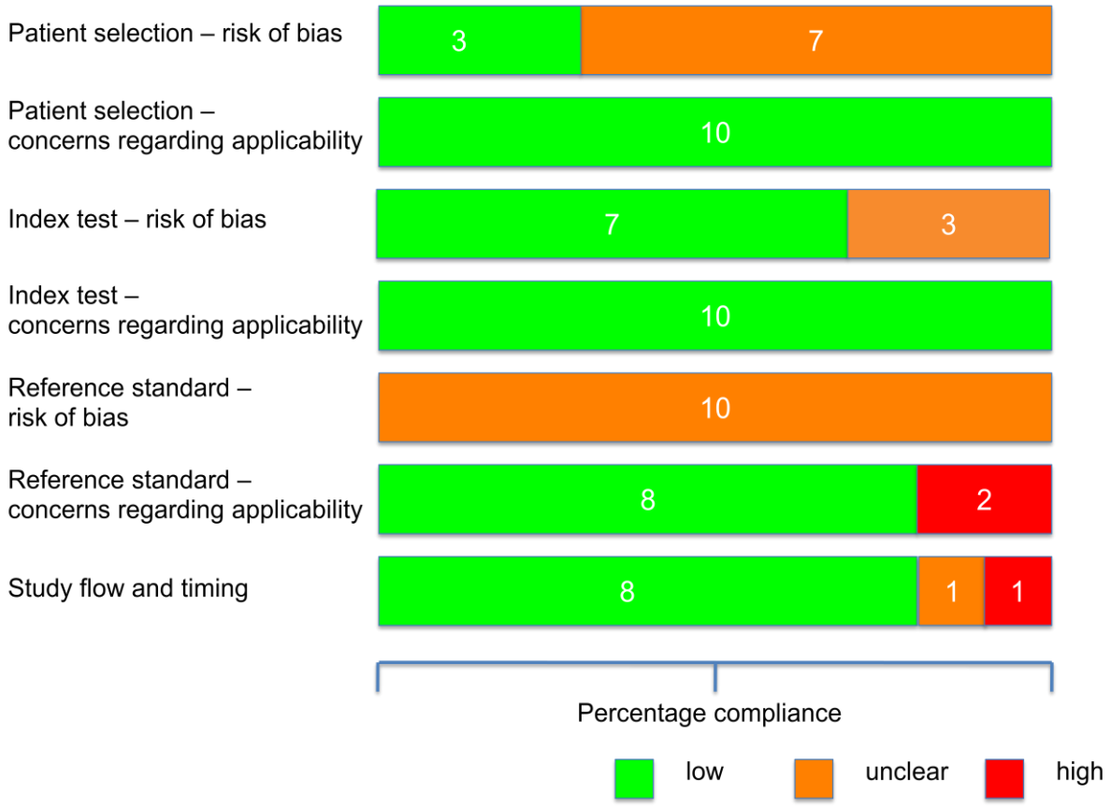
CRL = Crown-rump length, DC = dichorionic, DV = ductus venosus, DVP = deepest vertical pool, GA = gestational age, MC = monochorionic, MCDA = monochorionic diamniotic, NS= not specified, NT = nuchal translucency, USS = ultrasound scan.

Data relating to 1451 pregnancies were reported in the ten included studies, and included evaluation of 4 diagnostic tests – crown rump length discordance (646 pregnancies), NT discordance (605 pregnancies), NT > 95th percentile (555 pregnancies) and abnormality in the ductus venosus (278 pregnancies).

3.1.1.3 Quality assessment

Quality assessment for the included studies is summarised in Figure 3-2. Recruitment was prospective in six (60%) of the studies, but only two of these studies (20% overall) also employed consecutive patient selection, and thus were considered to have avoided selection bias. All ten studies (100%) avoided verification bias by ensuring that verification of the index test with the reference standard took place in >90% of cases. The index test was adequately described in eight (80%) of studies. Appropriate and timely application of the reference standard was also present in eight (80%) studies, with two studies (20%) failing to provide an adequate description of what they meant by development of TTTS. In considering the assessment made using the QUADAS 2 checklist[148], the risk of bias with regard to the applicability of patient selection to the review question was the only domain that was universally low, conversely blinding of the reference standard was not reported by any study. Two studies[207 210] were found to be of high quality, three studies[204 208 213] of moderate quality and five studies[205 206 209 211 212] of low quality.

Figure 3-2 Bar chart showing quality of evidence in relation to the diagnostic accuracy of ultrasound in the first trimester to predict development of TTTS in uncomplicated MCDA twins.



Data presented as 100% stacked bar chart with numbers in bars representing numbers of studies. (see methods for details of quality items)

3.1.1.4 Summarising the evidence

The results are summarised in figure 3-3 – 3-7 and table 3-2.

A CRL discordance of ≥ 10% predicted the development of TTTS with a pooled positive likelihood ratio (LR+) of 2.82 (95% CI 1.75 – 4.55) and a negative likelihood ratio (LR-) of 0.89 (95% CI 0.76 – 1.05), with no significant heterogeneity (χ^2 < degrees of freedom (df) p= 0.53, I^2 =0%). The area under curve (AUC) for the summary ROC analysis

suggested excellent accuracy for this test (0.92). A NT > 95th percentile predicted the development of TTTS with a pooled LR+ of 2.45 (95% CI 1.15 – 5.19) and a LR- of 0.87 (95% CI 0.73 – 1.02), although some heterogeneity existed, this was not significant (χ^2 > df p= 0.275, I^2 =22.6%) and the AUC was fair/good at 0.80. In the O'Kagan study[212] it was possible to extract data for NT discordances of 20 and 30%. Sub-group analysis was possible for discordance of 20%, as this was the same threshold utilised by the other three studies. A NT discordance of ≥ 20% predicted the development of TTTS with a pooled LR+ of 1.98 (95% CI 1.23 – 3.19) and a LR- of 0.64 (95% CI 0.43 – 0.94), although some heterogeneity existed, this was not significant (χ^2 > df p= 0.234, I^2 = 29.7%) and the AUC was good at 0.83. In the Matias study[204] it was possible to extract data for abnormalities in the DV for one fetus, both fetuses and at least one fetus. As the only other study reporting DV measurement only reported data on abnormal DV flow in at least one fetus, this was the threshold that was utilised for meta-analysis, although there was still noted to be significant heterogeneity (χ^2 > df p= 0.01, I^2=84.8%) and the AUC revealed the test to be no better than chance. An absent or reversed a wave in the DV in at least one twin predicted the development of TTTS with a pooled LR+ of 4.80 (95% CI -1.44 – 11.03) and a LR- of 0.50 (95% CI 0.06 – 0.95). Sensitivity analysis utilising the different thresholds for the index test e.g. NT discordance of 30% did not affect these estimates of diagnostic accuracy.

When considering the possibility of publication bias it was noted that there were only a few studies in each analysis however, where assessment was possible funnel plot asymmetry was not detected (CRL discordance p=0.88, NT > 95th percentile p= 0.40, NT discordance p= 0.29).

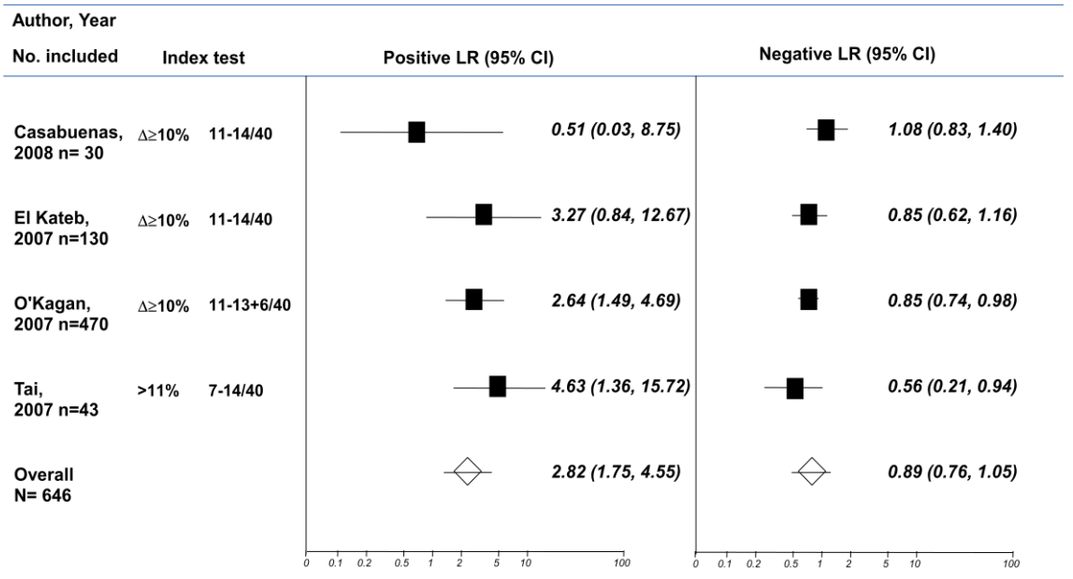
All tests showed poor sensitivity but better specificity. This is reflected in the LRs and subsequent post-test probabilities, as those for a negative test show little change from the underlying prevalence, whereas those for a positive test were more discriminating.

Table 3-2 Pooled analysis of ultrasound in the first trimester to predict development of TTTS in uncomplicated MCDA twins.

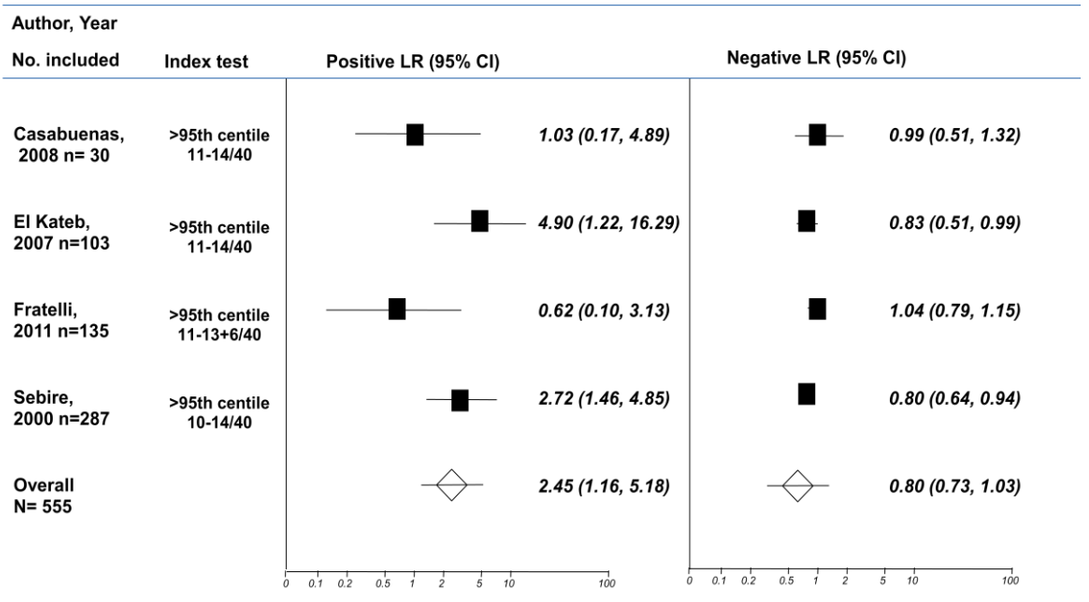
	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95%CI)	LR- (95% CI)	Pre-test probability %	Post-test probability % (range)	
						Test +	Test -
CRL discordance ≥ 10%	0.23 (0.14 – 0.33)	0.92 (0.90 – 0.94)	2.82 (1.75 – 4.55)	0.89 (0.76 – 1.05)	13.8 (9.69 – 17.9)	31.1 (21.9 – 42.2)	12.5 (10.9 – 14.4)
NT > 95th percentile	0.21 (0.01 – 0.37)	0.92 (0.87 – 0.95)	2.45 (1.15 – 5.19)	0.87 (0.73 – 1.02	13.8 (9.69 – 17.9)	28.2 (15.6 – 45.4)	12.2 (10.5 – 14.0)
NT discordance ≥ 20%	0.53 (0.38 – 0.68)	0.73 (0.65 – 0.80)	1.98 (1.23 – 3.19)	0.64 (0.43 – 0.94)	13.8 (9.69 – 17.9)	24.1 (16.5 – 33.8)	9.30 (6.44 – 13.1
DV a wave absent or reversed	0.5 (0.33 – 0.67)	0.88 (0.83 – 0.91)	4.77 (1.34 – 17.04)	0.49 (0.17 – 1.41)	13.8 (9.69 – 17.9)	43.3 (17.7 – 73.2)	7.28 (2.65 – 18.4)

Figure 3-3 Forest plots of positive and negative likelihood ratios for ultrasound in the first trimester to predict development of TTTS in uncomplicated MCDA twins.

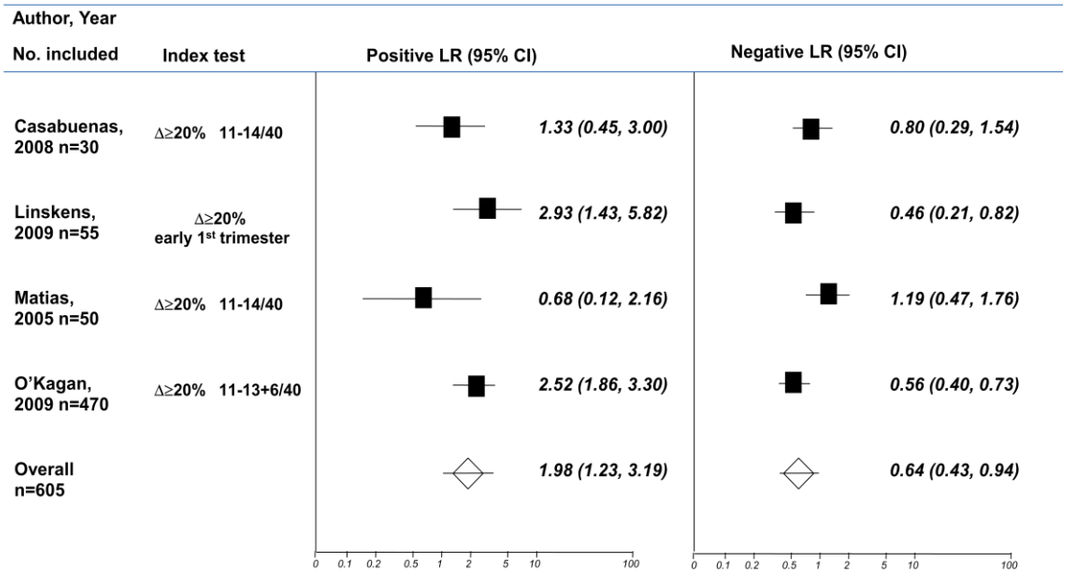
a) Crown rump length discordance



b) Nuchal translucency >95th percentile



c) Nuchal translucency discordance



d) Absent or reversed flow in the ductus venosus in ≥ 1 fetus

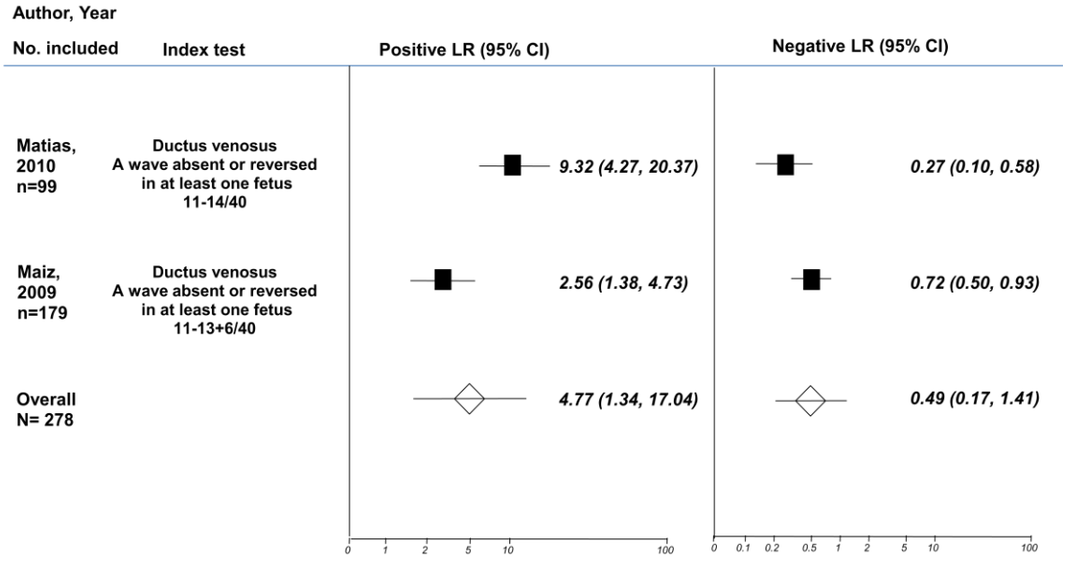


Figure 3-4 Summary ROC curve for CRL discordance of ≥ 10% to predict development of TTTS in uncomplicated MCDA twins.

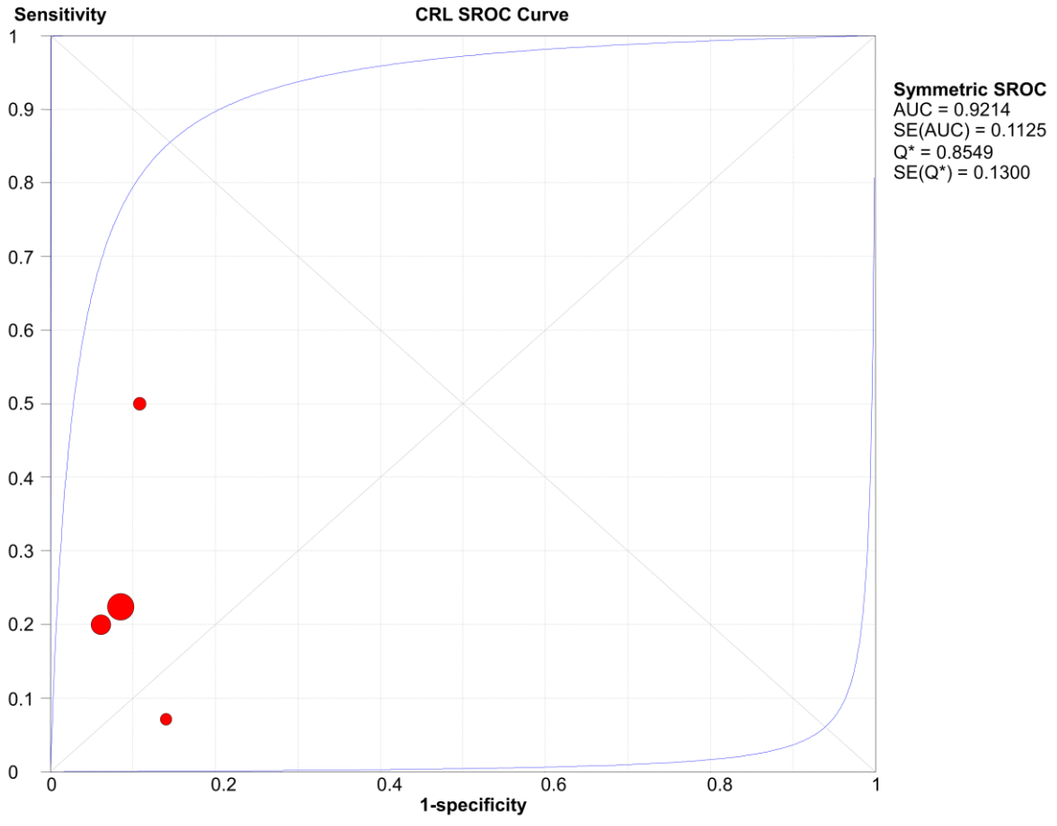


Figure 3-5 Summary ROC curve for NT >95th to predict development of TTTS in uncomplicated MCDA twins.

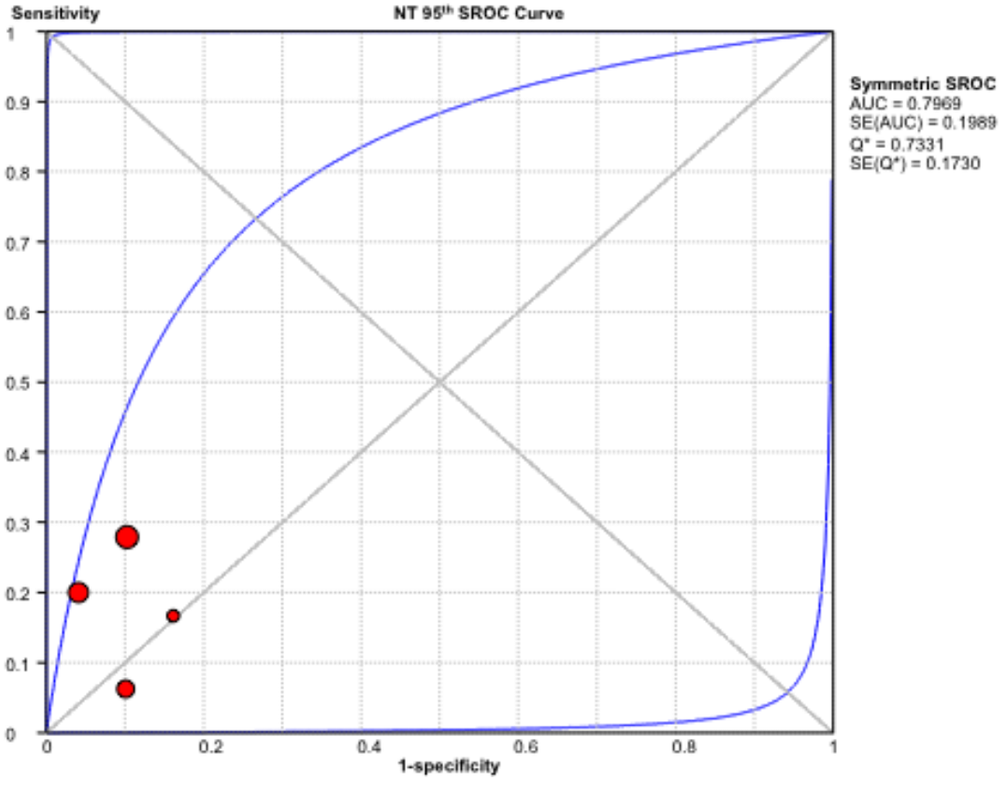


Figure 3-6 Summary ROC curve for NT discordance of ≥ 20% to predict development of TTTS in uncomplicated MCDA twins.

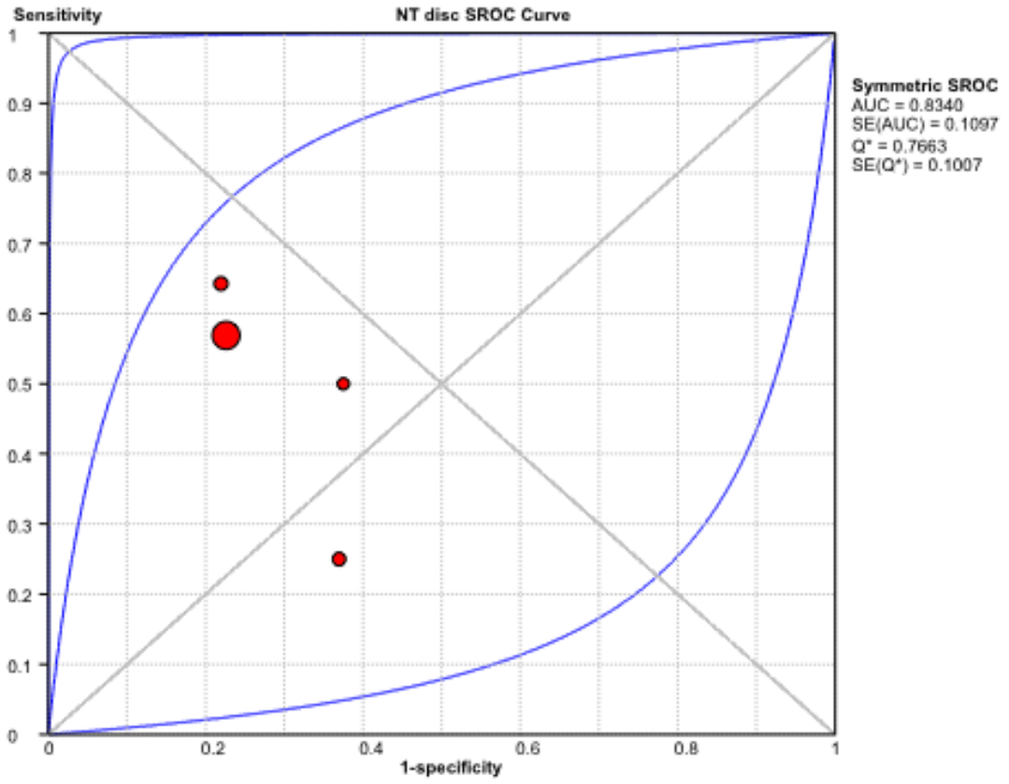
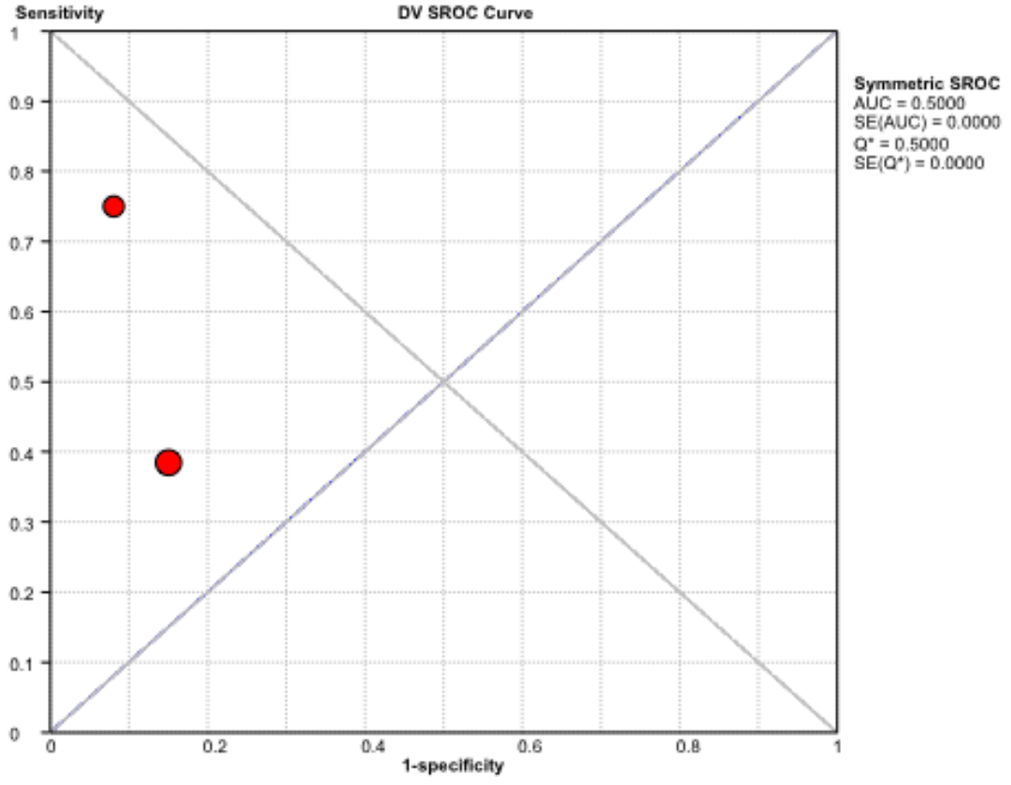


Figure 3-7 Summary ROC curve for absent/reversed a wave in the DV in ≥ 1 fetus to predict development of TTTS in uncomplicated MCDA twins.



3.1.2 Diagnostic accuracy of tests after diagnosis to predict outcome of TTTS

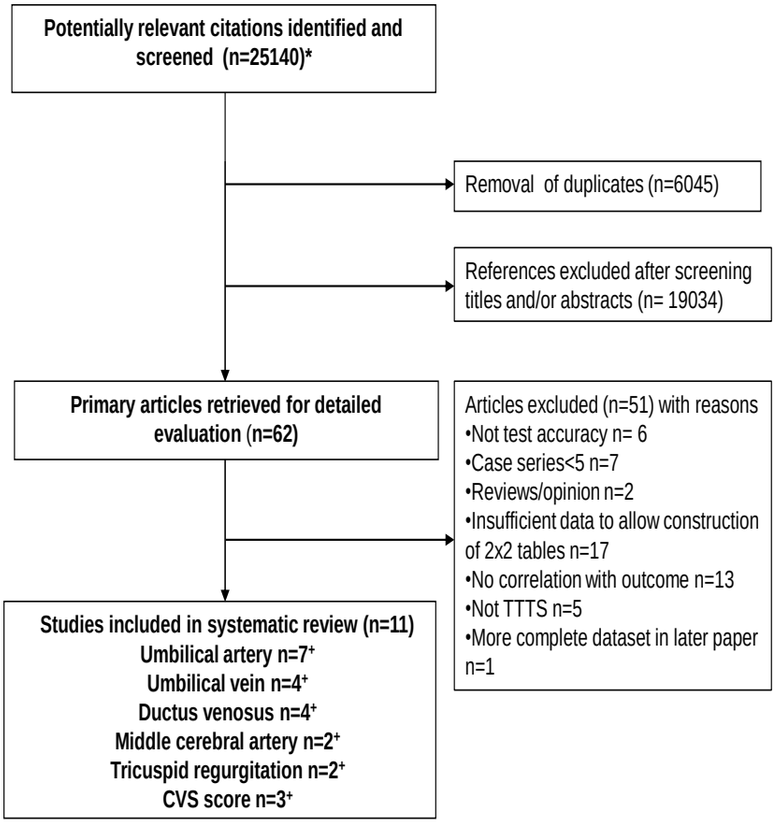
3.1.2.1 Question

What is the diagnostic accuracy of ultrasound in the prediction of outcome of TTTS after diagnosis?

3.1.2.2 Study selection

The electronic and hand search relevant to all three planned reviews generated 25140 citations, of which 62[23 27 34 36 38 185 197 202 215-268] were thought to be relevant: 57 were published in English, one in Serbian, one in Italian, one in German, one in Japanese and one in Chinese. After independent review of the full manuscript of these 61 articles, 11 were selected for inclusion in this review (figure 3-8). The characteristics of the included studies are shown in table 3-3. The list of excluded studies is included in Appendix 6**.**

Figure 3-8 Study selection process for a diagnostic accuracy review of ultrasound after diagnosis of TTTS to predict outcome.



*Overarching search encompassing three TTTS reviews

+Some articles included more than one test

Table 3-3 Study characteristics for a diagnostic accuracy review of ultrasound after diagnosis of TTTS to predict outcome.

Study	**Population** **(country/study design)**	Reference standard **IUD/NND**	**Index test/s**
Bruner et al (1997)	Inclusion – 9 pregnancies with second trimester twin gestations with identical twins, MC placenta and co-existent polyhydramnios of larger twin and oligohydramnios of smaller twin. Single centre. Treatment was by serial amnioreduction. Method of confirmation of chorionicity – USS and placental histology. (USA) (cohort)	**IUD** Test and outcome data only available for 6 pregnancies	UA abnormal Duplex pulsed-wave Doppler measurements of UA from a free-floating loop of cord. Calculations of the flow velocity waveform systolic-diastolic (S-D) ratio were performed on three to five sequential normally shaped waveforms. Results were reported as means and plotted against a normogram of age-corrected S-D values. All measurements by a single examiner. Other parameters recorded - ΔEFW
Ishii et al (2007)	Inclusion – 55 pregnancies diagnosed with TTTS and underwent SLPCV at four centres between July 2002 and February 2005. Exclusion - 2 Donor fetuses (one miscarriage and one IUD due to cord entanglement) 3 Recipient fetuses (one miscarriage and two IUD due to cord entanglement) All underwent SLPCV Median GA 21 (range from 16 - 25). Method of confirmation of chorionicity – USS.	**IUD** Test and outcome data only available for 53 donors and 52 recipients.	Critically abnormal Doppler studies: UA – AREDV UV – pulsatile flow DV – absent or reversed flow during atrial contraction Colour and pulsed Doppler studies within 24hrs of surgery. 3.5-MHz or 5-MHz curved array transducer with spatial peak temporal average intensities of <100mW/cm^2. The high-pass filter was set at the lowest level. Doppler measurements were obtained in the absence

	(Japan) (cohort)		of fetal breathing or movements. UA/UV measurements were recorded from a free loop of cord or at the placental insertion site. The sample volume for the DV was determined from its inlet portion at the UV.
Kontopoulos et al (2007)	Inclusion – 401 pregnancies referred to a single centre between August 2001 and August 2005 Exclusion – triplets and higher order pregnancies, monoamniotic twin pregnancies All patients underwent SLPCV. Median GA donor survived 20.3; donor died 19.7 (range for both 16 – 26) Method of confirmation of chorionicity – placental histology. (USA) (retrospective cohort)	**Donor IUD**	UA – AEDV Pre-operative ultrasound examination included Doppler examination of the UA in a free loop of cord. The angle of insonation was kept at <30°. The wall motion filter was kept at <100MHz and Doppler energy output levels were <50 mW/cm^2. Doppler measurements were obtained in the absence of fetal breathing. If intermittently absent EDV considered to have positive EDV. None with reversed EDV
Martinez et al (2003)	Inclusion – 110 consecutive pregnancies undergoing SLPCV for TTTS at a single centre between October 1997 and February 2001. All underwent SLPCV. Median GA 20.7 (range, 16.1 – 25.7) Method of confirmation of chorionicity –	**IUD** ***Outcomes reported separately for donor and recipient*** Not all measurements and outcomes available pre/post op (indicates numbers of fetuses with data available)	Critically abnormal Doppler studies: UA – AREDV UV – pulsatile flow at the placental cord insertion or umbilical loop MCA – PI < 5th percentile DV – reversed flow during atrial contraction

	NS (USA) (cohort)	UA – D=110, R=110/ D=90, R= 94 UV – D=106, R=107/ D=89, R=95 MCA – D=102, R=105/ D=81, R=91 DV – D=108, R=109/ D=90, R=95 TR – D= 81, R = 102/ D=67, R=71	TR Colour and pulsed Doppler studies. Energy output levels were lower than 50 mW/cm^2 spatial peak temporal average, and the high pass filter was set at the lowest level. Doppler measurements were obtained in the absence of fetal breathing or movements. Measurements taken 6-24hrs pre-op and within 24hrs after surgery
Murakoshi et al (2008)	Inclusion – 138 pregnancies with severe TTTS treated with fetoscopic laser surgery at five centres between July 2002 and August 2006. Exclusion – 7 pregnancies that miscarried, 49 twins with stage I, II or IV TTTS. All underwent fetoscopic laser ablation of all communicating vessels. Method of confirmation of chorionicity – NS (Japan) (prospective cohort)	**Donor IUD** Test and outcome data only available for 82 pregnancies with stage III TTTS	UA Doppler studies – absent or reversed EDV

Quintero et al (1999)	Inclusion – 80 patients referred with a diagnosis of TTTS between August 1993 and January 1999. 48 patients underwent LPCV or SLPCV. Other treatments included umbilical cord occlusion (n=17), serial amniocentesis (n=10) and 1 termination of pregnancy. 4 patients were lost to follow up. (USA) (prospective cohort)	**Donor IUD** Test and outcome data only available for 45 pregnancies treated with LPCV	∆EFW
Shah et al 2008	Inclusion – 62 consecutive pregnancies from a single centre between January 2004 and April 2006. Exclusions – pregnancies where either twin was known to have congenital heart disease, severe non-cardiac fetal anomalies and cases of selective feticide. 30 underwent SLPCV, 32 underwent AR (of whom 8 also needed SLPCV). Method of confirmation of chorionicity – NS (USA) (retrospective cohort)	**Recipient NND** (within 30 days of birth)	Doppler studies were performed immediately pre-procedure (is SLPCV or AR) and considered to be abnormal if: UA – AREDV UV – pulsatile flow in a free loop of cord DV – atrial systolic flow reversal Other parameter correlated with outcome AVVR (mild/moderate/severe), abnormal RV MPI, abnormal LV MPI, hydrops, overall Cardiovascular profile score All measurements by a single investigator blinded to outcome status

Skupski et al (2010)	Inclusion - 466 consecutive pregnancies from eight centres (part of North American Fetal Therapy Network) January 2002 – June 2009. All underwent SLPCV. Method of confirmation of chorionicity – USS or pathology of placenta (USA) (cohort - retrospective analysis of prospectively collected data)	**IUD and NND** (within 30 days of birth) ***Outcomes reported separately for donor and recipient*** Not all measurements and outcomes available for all fetuses. The figures below indicate the number of fetuses with data available. Letter in brackets refers to twin in whom the measurement is taken. UA – AEDV – (D) – IUD D=R=462 / NND D=321, R=355 UA – AEDV – (R) – IUD D=R=462 / NND D=322, R=356 UA – REDV – (D) – IUD D=R=464 / NND D=323, R=357 UA – REDV – (R) – IUD D=R=464 / NND D=319, R=351 UV – pulsatile flow – (D) – IUD D=R=435 / NND D=306, R=336 UV – pulsatile flow – (R) – IUD D=R=433 / NND D=305, R=334 DV – absent a wave – (D) – IUD D=392, R=374 / NND D=281, R=311 DV – absent a wave – (R) – IUD D=R=392 / NND D=280, R=310 DV – reversed a wave – (D) – IUD D=R=400 / NND D= 286, R=316 DV – reversed a wave – (R) – IUD D=R=398 / NND D=284, R=314 TR or MR – (D) – IUD D=R=464 / NND D=324, R=358	Doppler studies UA – AEDV* UA – REDV* UV – pulsatile flow DV – absent a wave* DV – reversed a wave* TR or MR *intermittent or persistent All tests were reported separately for donor and recipient fetuses Other parameter correlated with outcome Absence of fetal bladder, EFW discrepancy, ascites, pericardial/pleural effusion, skin/cord oedema, hydrops fetalis, global cardiac dysfunction [(abnormal tei index, ventricular dyskinesia, abnormal EF (either ventricle), abnormal cardiac "score" or other measure of cardiac dysfunction)]

		TR or MR – (R) – IUD D=R=464 / NND D=322, R=355 Global cardiac dysfunction – (D) – IUD D= R=463/ NND D=323, R=357 Global cardiac dysfunction – (R) – IUD D=R=458/ NND D=320, R=352	
Stirnemann et al (2010a)	Inclusion – 107 consecutive MC pregnancies complicated by severe TTTS and referred for prenatal care over a one-year period. All underwent SLPCV. Cardiac profile and outcome data only available for 58 pregnancies (44 incomplete data and 5 lost to follow up) Method of confirmation of chorionicity NS. (France/Canada) (prospective cohort)	**NND** (within 28 days of birth)	A six variable cardiac profile was defined for each recipient twin including: RV – SF, LV – SF, LV – MPI, RV – MPI, DV – PI and Cardiac Index. Profile 1 - considered close to normal, although DV-PI and RV-MPI slightly increased. Profile 2 - significantly increased RV and LV MPI with moderately increased DV-PI. Demonstrating diastolic and global myocardial impairment. Profile 3 - significant depression of LV+RV shortening fraction, very high DV-PI and increased biventricular MPI. Demonstrating a severe systolic, diastolic and overt global cardiac failure. Fetal echocardiography performed pre-operatively by one highly trained sonologist blinded to outcome. The mean of three measurements was taken for each variable. For pulsed Doppler measurements, the angle of insonation was kept under 20°.

Stirnemann et al (2010b)	Inclusion – 215 consecutive patients with TTTS treated with selective laser coagulation of placental vessels at a single centre between 2006 and 2008. Median gestational age 20 weeks (18+2 – 22+3). Quintero stage I – 46, 2 – 31, 3 – 134, IV – 2. Exclusion – fetal malformations, premature rupture of membranes prior to surgery and amniocentesis prior to surgery. 25 patients were lost to follow up. All underwent SLPCV within 24hrs of echocardiography. Method of confirmation of chorionicity – pathology of the placenta (France) (prospective cohort)	**IUD/NND** Pregnancy outcome was not available in the 25 patients lost to follow up and CHOP evaluation was incomplete in 34 pregnancies. There were 158 pregnancies available for prognostic evaluation of the CHOP scoring system. IUD (per pregnancy) CHOP 1 25/116, CHOP 2 3/36, CHOP 3 0/6 NND (per fetus) CHOP 1 70/232 CHOP 2 16/72 CHOP 3 1/12 (though reporting of NND figures was inconsistent)	Echocardiograms were performed within 24hrs prior to surgery and followed a predefined protocol based on the recommendations of the American College of Echocardiography. All measurements were performed using an Aloka ProSound Alpha 10, a GE Voluson 730 or a GE Voluson E8. Pulsed Doppler measurements were performed with an angle of insonation of <20° and the mean of three consecutive measurements used for all quantitative parameters. The CHOP score was computed as the sum of 12 cardiovascular parameters (11 in the recipient and 1 in the donor) scored according to severity. It included measurements in the recipient relating to: Ventricular hypertrophy, cardiomegaly, ventricular systolic function, MR, TR, MV EA, TV EA, DV, pulsatile UV, PR, RV outflow tract obstruction; and in the donor: UA. Score – stage 1=0-5, 2=6-10, 3=11-15, 4=16-20.

Trieu et al (2012)	Inclusion – 86 consecutive pregnancies with TTTS treated with laser coagulation of the placental anastomoses at a single centre between 2004 and 2009. Exclusion – any patient in whom it was not possible to measure MCA (Pre-op – 11 D and 15 R, Post-op – 3 D and 5 R) All underwent laser coagulation GA 20.5 ±2.9 Method of confirmation of chorionicity – USS (France) (retrospective cohort)	**IUD within 7 days of laser** Not all measurements and outcomes available pre/post op 5 D and 2 R IUD prior to 48hrs so no post-op measurement possible Overall numbers Pre-op – D=75, R=71 Post-op – D=67, R=66	MCA-PSV by experienced operators before (within 24hrs) and 48hrs after laser treatment. The circle of Willis and the MCA were identified using B-mode and colour Doppler. The window was placed at the proximal segment of the MCA, close to the origin of the internal carotid artery with an angle as close to 0° as possible. An angle >30° was a criteria for exclusion. The values were then converted to MoM. The PSV was considered to be elevated if it was ≥ 1.5 MoM.

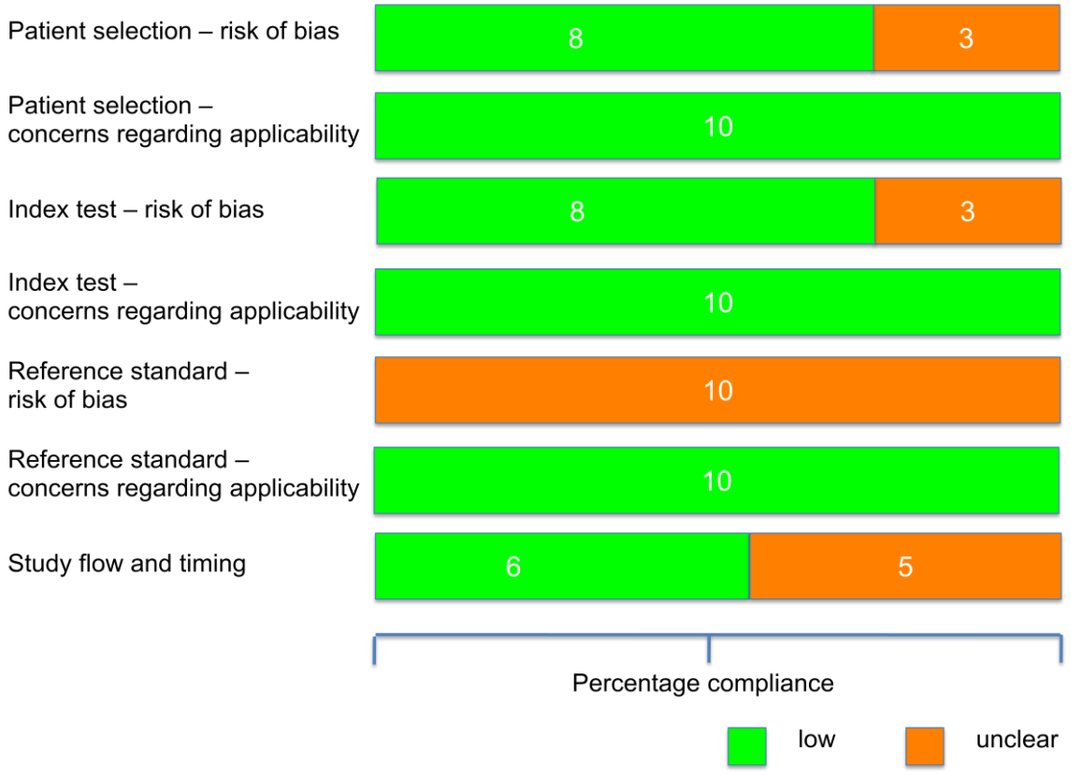
AREDV = absent or reversed end-diastolic velocity, AR = amnioreduction, AVVR = atrioventricular valve regurgitation, D=donor twin, DV = ductus venosus, EDV = end-diastolic velocity, ∆EFW = estimated fetal weight difference, GA = gestational age, IUD= intrauterine death, LPCV = laser photocoagulation of communicating vessels, LV = left ventricle, MC = monochorionic, MCA-PSV = middle cerebral artery peak systolic velocity, MoM = multiples of the median, MPI = myocardial performance index, NND= neonatal death, NS= not specified, PI = pulsatility index, R=recipient twin, RV = right ventricle, SF = shortening fraction, SLPCV = selective laser photocoagulation of communicating vessels, TOP= termination of pregnancy, TR = tricuspid valve regurgitation, UA = umbilical artery, UAD = umbilical artery Doppler, USS = ultrasound scan, UV = umbilical vein.

Data relating to 1635 pregnancies were reported in the eleven included studies, and included evaluation of seven diagnostic tests – umbilical artery abnormality (absent or reversed EDV) (1182 pregnancies), MCA abnormality (PI<5th percentile or PSV>1.5 MoM) (196 pregnancies), DV abnormality (absent or reversed a wave) (693 pregnancies), umbilical vein (UV) abnormality (pulsatile flow) (693 pregnancies), tricuspid regurgitation (576 pregnancies), abnormal cardiac profile (384 pregnancies) and ΔEFW (45 pregnancies).

3.1.2.3 Quality assessment

Quality assessment for the included studies is summarised in Figure 3-9. Recruitment was prospective in five (45%) of the studies, but only three of these studies (27% overall) also employed consecutive patient selection, and thus were considered to have avoided selection bias. Ten studies (91%) avoided verification bias by ensuring that verification of the index test with the reference standard took place in >90% of cases. The index test was adequately described in eight (73%) of studies. Appropriate and timely application of the reference standard was present in all 11 (100%) studies; with seven studies utilising IUD (64%), one (9%) NND, and three (27%) both IUD and NND as outcomes. In considering the assessment made using the QUADAS 2 checklist[148], the risk of bias with regard to applicability of patient selection, index test and reference standard to the review question were the domains that was universally low, conversely blinding of the reference standard was not reported by any study. Two studies[34 221] were found to be of high quality, six studies[27 202 216 219 222 268] of moderate quality and three studies[217 218 220] of low quality.

Figure 3-9 Bar chart showing quality of evidence in relation to the diagnostic accuracy of ultrasound after diagnosis of TTTS to predict outcome.



Data presented as 100% stacked bar chart with numbers in bars representing numbers of studies. (see methods for details of quality items). No studies were judged to be at high risk of bias.

3.1.2.4 Summarising the evidence

The results are summarised in Figure 3-10 – 3-13 and Table 3-4

With respect to prediction of intrauterine death (IUD), six tests were evaluated (see table 3-4 a). No test performed well in terms of both sensitivity and specificity, although the trend was for much better specificity than sensitivity, with the exception of EFW difference. An abnormal UA Doppler predicted donor IUD with a pooled positive LR+ of

2.29 and a negative LR- of 0.44, although there was significant heterogeneity noted (χ^2 > df, p=0.013 and I^2 = 65.4%). An abnormal UA Doppler predicted recipient IUD with a pooled positive LR+ of 1.07, a LR- of 1.00 and there was no significant heterogeneity (χ^2 < df, p=0.91 and I^2 = 0%). An abnormal MCA Doppler predicted donor IUD with a pooled LR+ of 1.08, a LR- of 0.97 and there was no significant heterogeneity (χ^2 < df, p=0.51 and I^2 = 0%). An abnormal MCA Doppler predicted recipient IUD with a pooled LR+ of 1.88 and a LR- of 0.90, and although there was some heterogeneity this was not significant (χ^2 > df, p=0.11 and I^2 = 61.1%). An abnormal DV in the donor predicted donor IUD with a pooled LR+ of 2.12 and a LR- of 0.97, some heterogeneity was noted, although again this was not significant (χ^2 > df, p=0.14 and I^2 = 49.8%). An abnormal DV in the recipient predicted recipient IUD with a pooled LR+ of 1.53, a LR- of 0.86 and there was no significant heterogeneity (χ^2 < df, p=0.86 and I^2 = 0%). A pulsatile UV predicted donor IUD with a pooled LR+ of 1.02, a LR- of 1.00 and there was no significant heterogeneity (χ^2 < df, p=0.89 and I^2 = 0%). A pulsatile UV predicted recipient IUD with a pooled LR+ of 1.29 and a LR- of 0.89, however some heterogeneity was noted but was not significant (χ^2 > df, p=0.07 and I^2 = 61.6%). The presence of mitral or tricuspid regurgitation (TR/MR) predicted donor IUD with a pooled LR+ of 1.04, a LR- of 1.00 and no significant heterogeneity was noted (χ^2 < df, p=0.93 and I^2 = 0%). The presence of TR/MR predicted recipient IUD with a pooled LR+ of 1.12, a LR- of 0.93 and no significant heterogeneity was noted (χ^2 < df, p=0.44 and I^2 = 0%). An EFW difference of >20% predicted donor IUD with a LR+ of 0.75 and a LR- of 1.71. As all classifications of CHOP score include some abnormality it was not possible to dichotomise this test into a positive or negative result, however increasing CHOP score was associated with decreasing IUD CHOP 1 21%, CHOP 2 8% and CHOP 3 0%. The

majority of tests performed poorly when the sROC curve was considered. However, with respect to donor IUD DV abnormality (AUC 0.99) and pulsatile UV (AUC 0.94) showed excellent accuracy, with UAD abnormality showing fair accuracy (AUC 0.78).

In terms of prediction of neonatal death (NND) this outcome was not as commonly reported for the donor as the recipient and five tests were evaluated (see table 3-4b). Again these tests proved to have higher specificities than sensitivities, with the exception of cardiac profile and overall NND. An abnormal UA Doppler predicted donor NND with a positive LR+ of 1.78 and a negative LR- of 0.80. An abnormal UA Doppler predicted recipient NND with a pooled positive LR+ of 0.90 and a negative LR- of 1.00, however significant heterogeneity was noted ($\chi^2 >$ df, p=0.06 and I^2 = 72.4%). Reversed flow in the DV predicted recipient NND with a pooled LR+ of 1.18 and a LR- of 0.96, although some heterogeneity was noted it was not significant ($\chi^2 >$ df, p=0.21 and I^2 = 37.9%). A pulsatile UV predicted recipient NND with a pooled LR+ of 1.18 and a LR- of 0.96, although some heterogeneity was noted it was not significant ($\chi^2 >$ df, p=0.20 and I^2 = 38.2%). The presence of TR/MR predicted donor NND with a LR+ of 0.68 and a LR- of 1.01. The presence of TR/MR predicted recipient NND with a LR+ of 0.76 and a LR- of 1.19. The presence of global cardiovascular dysfunction predicted donor NND with a LR+ of 0.71 and a LR- of 1.19. The presence of global cardiovascular dysfunction predicted recipient NND with a LR+ of 1.03 and a LR- of 0.99. The presence of global cardiovascular dysfunction predicted overall NND (including donor and recipient deaths) with a LR+ of 0.95 and a LR- of 1.08 . When considering the use of the CHOP cardiovascular score and its relation to prediction of

NND the inverse relationship shown with IUD was again apparent: CHOP 1 30%, CHOP 2 22%, CHOP 3 8%.

When considering the possibility of publication bias it was noted that none of the meta-analyses contained more than six studies and many contained only two or three. It was only possible to make a graphically and statistically assessment in relation to UA Doppler, where funnel plot asymmetry was not significant ($p>0.1$).

Table 3-4 Pooled and single estimates for ultrasound tests after diagnosis of TTTS to predict outcome.

a) IUD

	No. of studies	No. of women	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95%CI)	LR- (95% CI)	Pre-test probability % (95% CI)	Post-test probability % (range)	
								Test +	Test -
UA abnormal and donor IUD	6	1118	0.69 (0.36 – 0.89)	0.70 (0.51 – 0.83)	2.29 (1.61 – 3.25)	0.44 (0.22 – 0.88)	23.3 (15.3 – 31.4)	41.1 (32.9 – 49.7)	11.8 (6.28 – 21.1)
UA abnormal and recipient IUD	4	634	0.08 (0.02 – 0.25)	0.93 (0.73 – 0.98)	1.07(0.10 – 12.1)	1.00 (0.81 – 1.22)	23.3 (15.3 – 31.4)	24.6 (2.95 – 78.7)	23.3 (19.8 – 27.1)
MCA abnormal and donor IUD	2	196	0.22 (0.12 – 0.37)	0.81 (0.73 – 0.87)	1.08 (0.58 – 2.02)	0.97 (0.82 – 1.14)	23.3 (15.3 – 31.4)	24.7 (15.0 – 38.1)	22.8 (20.0 – 25.8)
MCA abnormal and recipient IUD	2	196	0.33 (0.18 – 0.52)	0.67 (0.59 – 0.75)	1.88 (0.31 – 11.2)	0.90 (0.75 – 1.08)	23.3 (15.3 – 31.4)	36.4 (8.62 – 77.3)	21.5 (18.6 – 24.7)
DV abnormal and donor IUD	3	629	0.08 (0.04 – 0.14)	0.95 (0.93 – 0.97)	2.12 (0.37 – 12.2)	0.97 (0.87 – 1.08)	23.3 (15.3 – 31.4)	39.2 (10.1 – 78.8)	22.8 (20.9 – 24.7)
DV abnormal and recipient IUD	3	628	0.33 (0.16 – 0.55)	0.79 (0.63 – 0.89)	1.53 (1.12 – 2.08)	0.86 (0.72 – 1.02)	23.3 (15.3 – 31.4)	31.8 (25.4 – 38.8)	20.8 (18.0 – 23.7)
UV-P and donor IUD	3	629	0.04 (0.02 – 0.09)	0.96 (0.94 – 0.98)	1.02 (0.42 – 2.52)	1.00 (0.96 – 1.04)	23.3 (15.3 – 31.4)	23.7 (11.3 – 43.4)	23.3 (22.6 – 24.1)
UV-P and recipient IUD	3	629	0.34 (0.25 – 0.45)	0.70 (0.65 – 0.74)	1.29 (0.73 – 2.27)	0.89 (0.62 – 1.27)	23.3 (15.3 – 31.4)	28.2 (18.2 – 40.9)	21.3 (15.9 – 27.9)
TR/MR and donor IUD	2	576	0.02 (0.002 – 0.05)	0.98 (0.97 – 0.99)	1.04 (0.25 – 4.30)	1.00 (0.97 – 1.03)	23.3 (15.3 – 31.4)	24.1 (7.07 – 56.7)	23.3 (22.8 – 23.9)
TR/MR and recipient IUD	2	576	0.44 (0.33 – 0.54)	0.61 (0.56 – 0.65)	1.12 (0.87 – 1.45)	0.93 (0.77 – 1.12)	23.3 (15.3 – 31.4)	25.4 (20.9 – 30.6)	22.1 (19.0 – 25.4)
EFW	1	45	0.56 (0.31 –	0.26 (0.11 –	0.75 (0.47 –	1.71 (0.76 -	23.3 (15.3 –	18.6 (12.5 –	34.2 (18.8 –

difference>20% and donor IUD			0.79)	0.46)	1.20)	3.89)	31.4)	26.8	54.2)

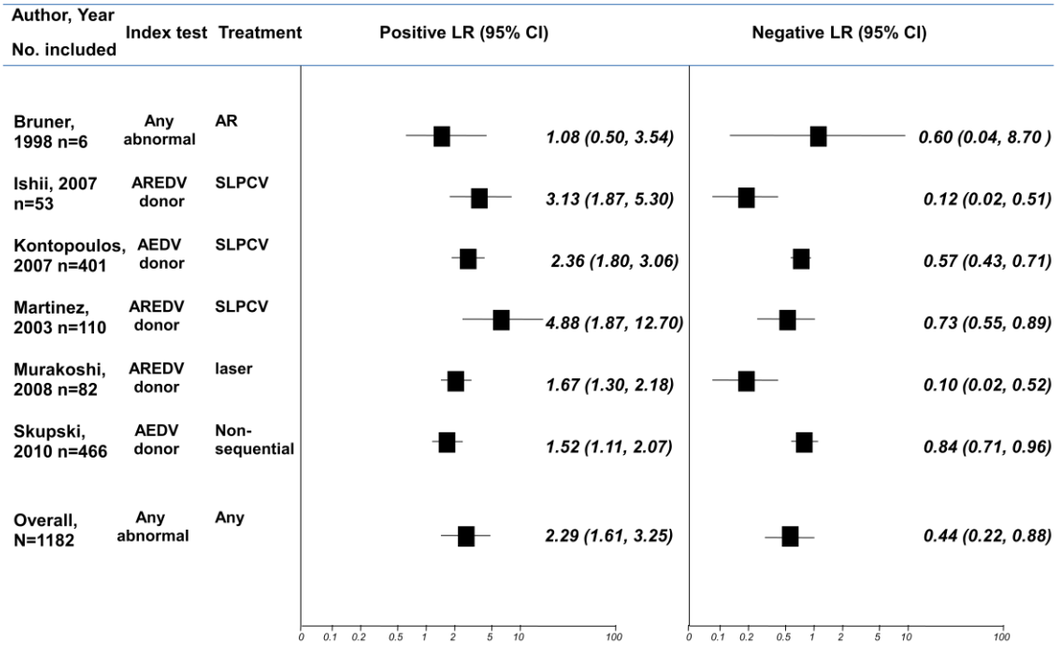
b) NND

	No. of studies	No. of women	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95%CI)	LR- (95% CI)	Pre-test probability % (95% CI)	Post-test probability % (range)	
								Test +	Test -
UA abnormal and donor NND	1	466	0.36 (0.22 – 0.51)	0.80 (0.75 – 0.85)	1.78 (1.10 – 2.74)	0.80 (0.62 – 0.97)	14.5 (9.70 – 19.3)	23.2 (15.7 – 31.8)	12.0 (9.52 – 14.1)
UA abnormal and recipient NND	2	528	0.04 (0.01 – 0.12)	0.93 (0.90 – 0.96)	0.90 (0.03 – 26.30)	1.00 (0.83 – 1.20)	14.5 (9.70 – 19.3)	13.3 (0.51 – 81.7)	14.5 (12.0 – 16.9)
DVRF and recipient NND	2	498	0.25 (0.15 – 0.37)	0.71 (0.66 – 0.76)	1.18 (0.52 – 2.69)	0.96 (0.80 – 1.14)	14.5 (9.70 – 19.3)	16.7 (8.11 – 31.4)	14.0 (12.0 – 16.2)
UV-P and recipient NND	2	498	0.23 (0.14 – 0.35)	0.74 (0.69 – 0.78)	1.18 (0.51 – 2.71)	0.96 (0.81 – 1.14)	14.5 (9.70 – 19.3)	10.4 (7.97 – 31.5)	14.0 (12.1 – 16.2)
TR/MR and donor NND	1	436	0 (0 – 0.08)	0.99 (0.96 – 1.00)	0.68 (0.04 – 12.35)	1.01 (0.97 - 1.04)	14.5 (9.70 – 19.3)	11.4 (0.67 – 67.7)	14.6 (14.1 – 15.0)
TR/MR and recipient NND	1	436	0.33 (0.20 – 0.50)	0.56 (0.50 – 0.62)	0.76 (0.48 – 1.18)	1.19 (0.94 – 1.51)	14.5 (9.70 – 19.3)	10.8 (7.53 – 16.7)	16.8 (13.8 – 20.4)
CVS dysfunction and donor NND	1	436	0.26 (0.14 – 0.41)	0.64 (0.58 – 0.70)	0.71 (0.42 – 1.21)	1.17 (0.96 – 1.42)	14.5 (9.70 – 19.3)	14.9 (6.65 – 17.0)	16.6 (14.0 – 19.4)
CVS dysfunction and recipient NND	1	436	0.37 (0.22 – 0.531)	0.64 (0.59 – 0.70)	1.03 (0.67 – 1.58)	0.99 (0.77 – 1.26)	14.5 (9.70 – 19.3)	13.9 (10.2 – 21.2)	14.4 (11.6 – 17.6)
CVS dysfunction and NND overall	1	58	0.58 (0.41 – 0.75)	0.39 (0.28 – 0.50)	0.95 (0.69 – 1.32)	1.08 (0.67 – 1.73)	14.5 (9.70 – 19.3)	13.9 (10.5 – 18.3)	15.5 (10.2 – 22.7)

CHOP score not included in the above table as it was not possible to dichotomise the results into a positive or negative test.

Figure 3-10 Forest plots of positive and negative likelihood ratios for ultrasound after diagnosis of TTTS to predict outcome

a) UA Doppler and donor IUD



b) UA Doppler and recipient IUD

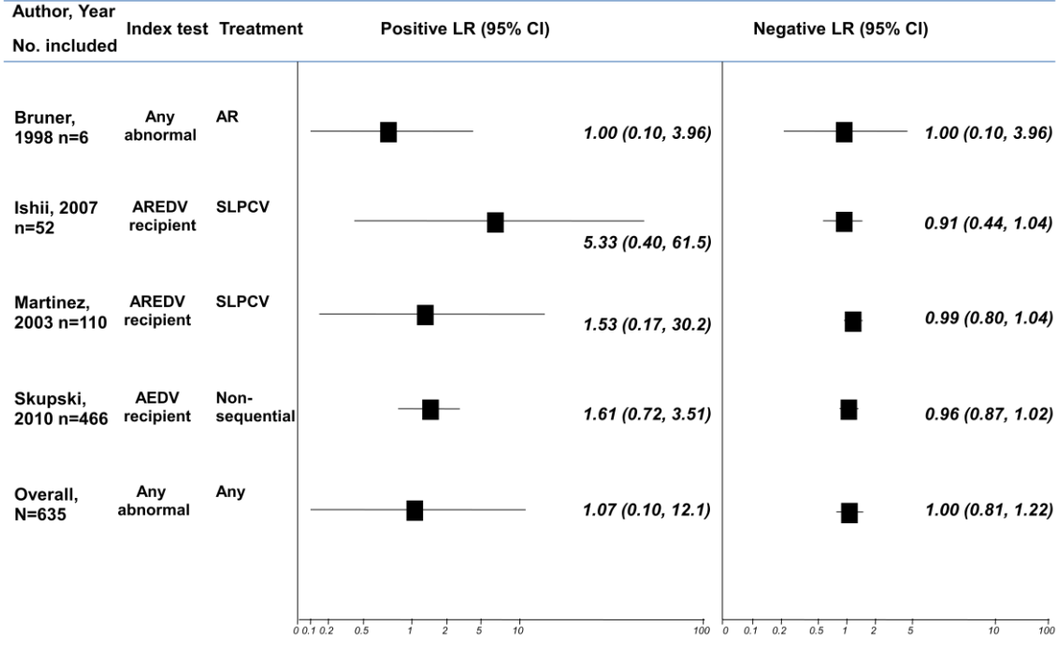


Figure 3-11 Summary ROC curve for UA abnormality to predict donor IUD a diagnosis of TTTS.

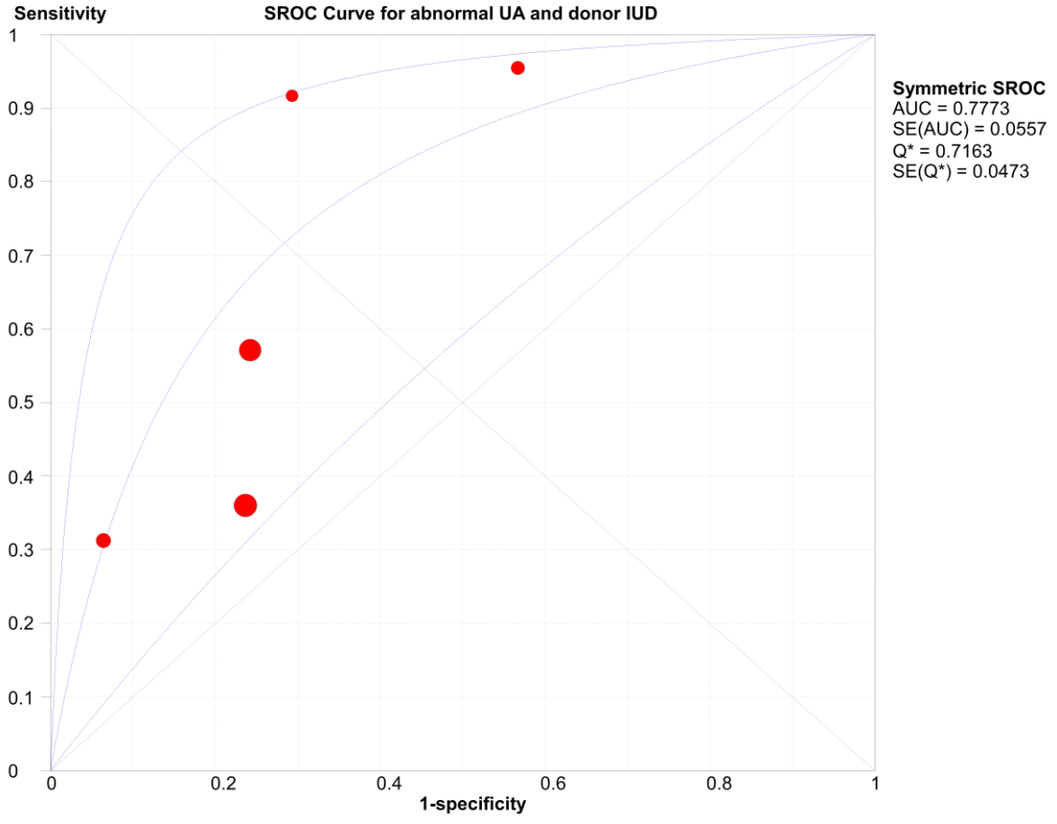


Figure 3-12 Summary ROC curve for donor DV abnormality to predict donor IUD after diagnosis of TTTS.

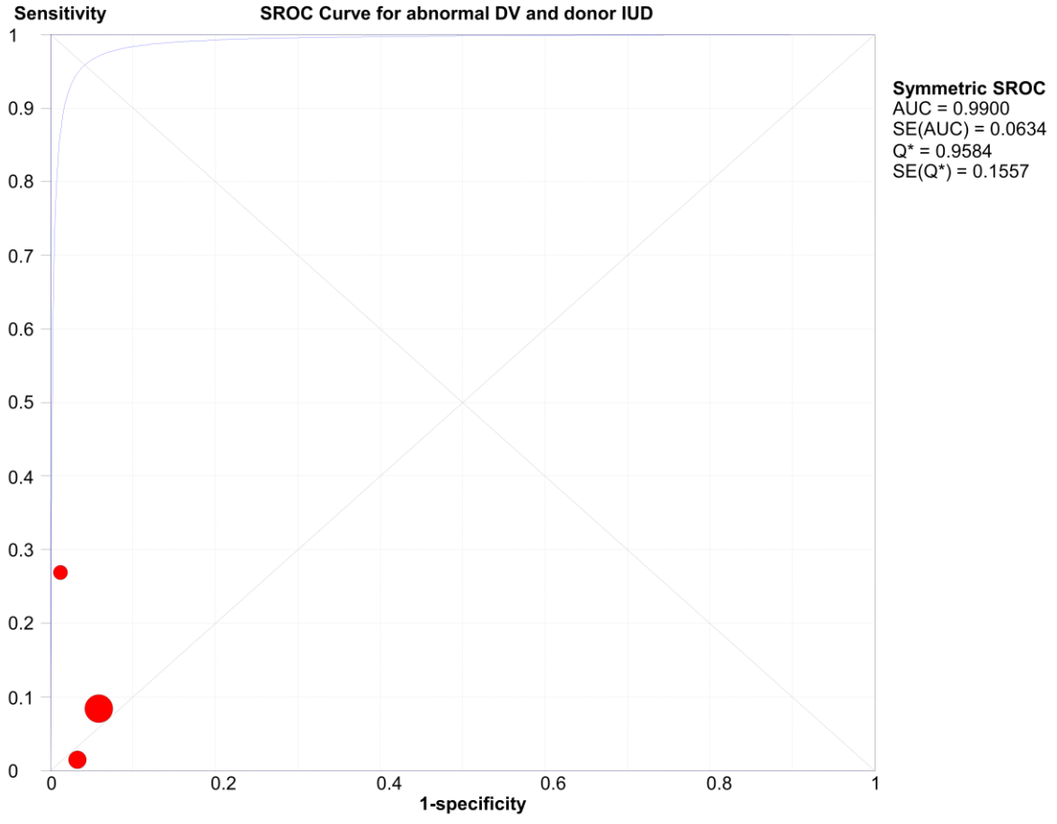
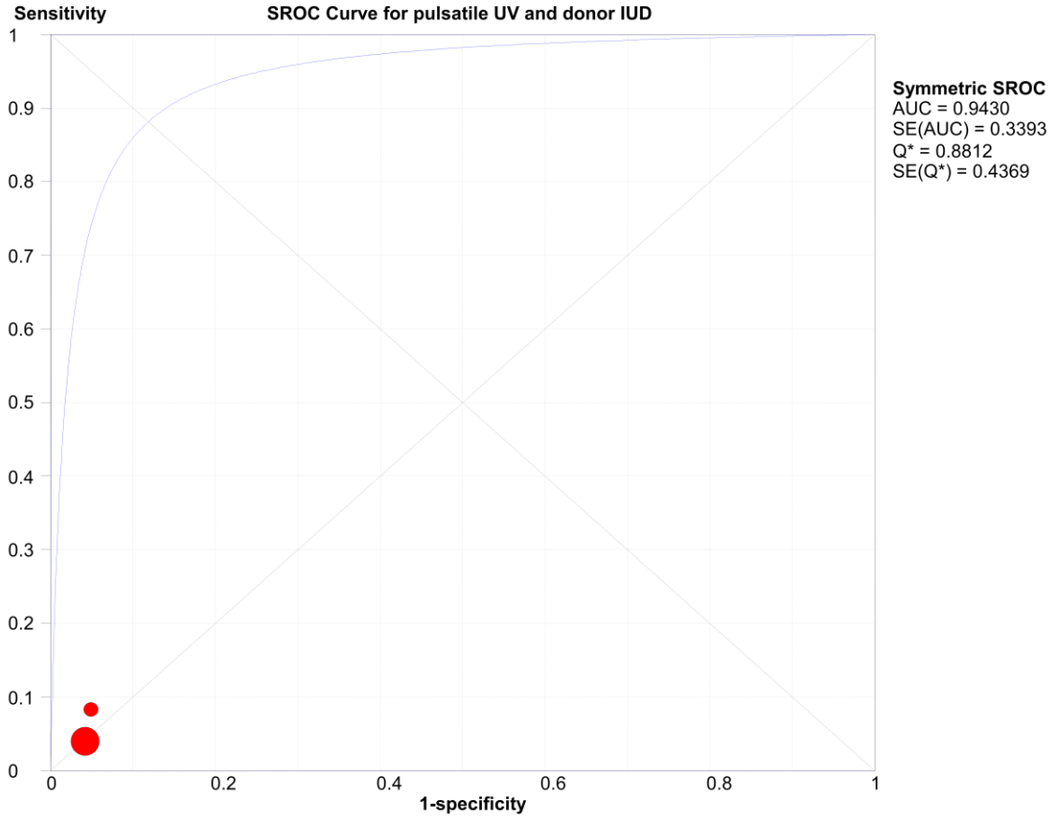


Figure 3-13 Summary ROC curve for pulsatile UV to predict donor IUD after diagnosis of TTTS.



3.1.3 Effectiveness review of FLA and serial amnioreduction for the treatment of TTTS.

3.1.3.1 Question

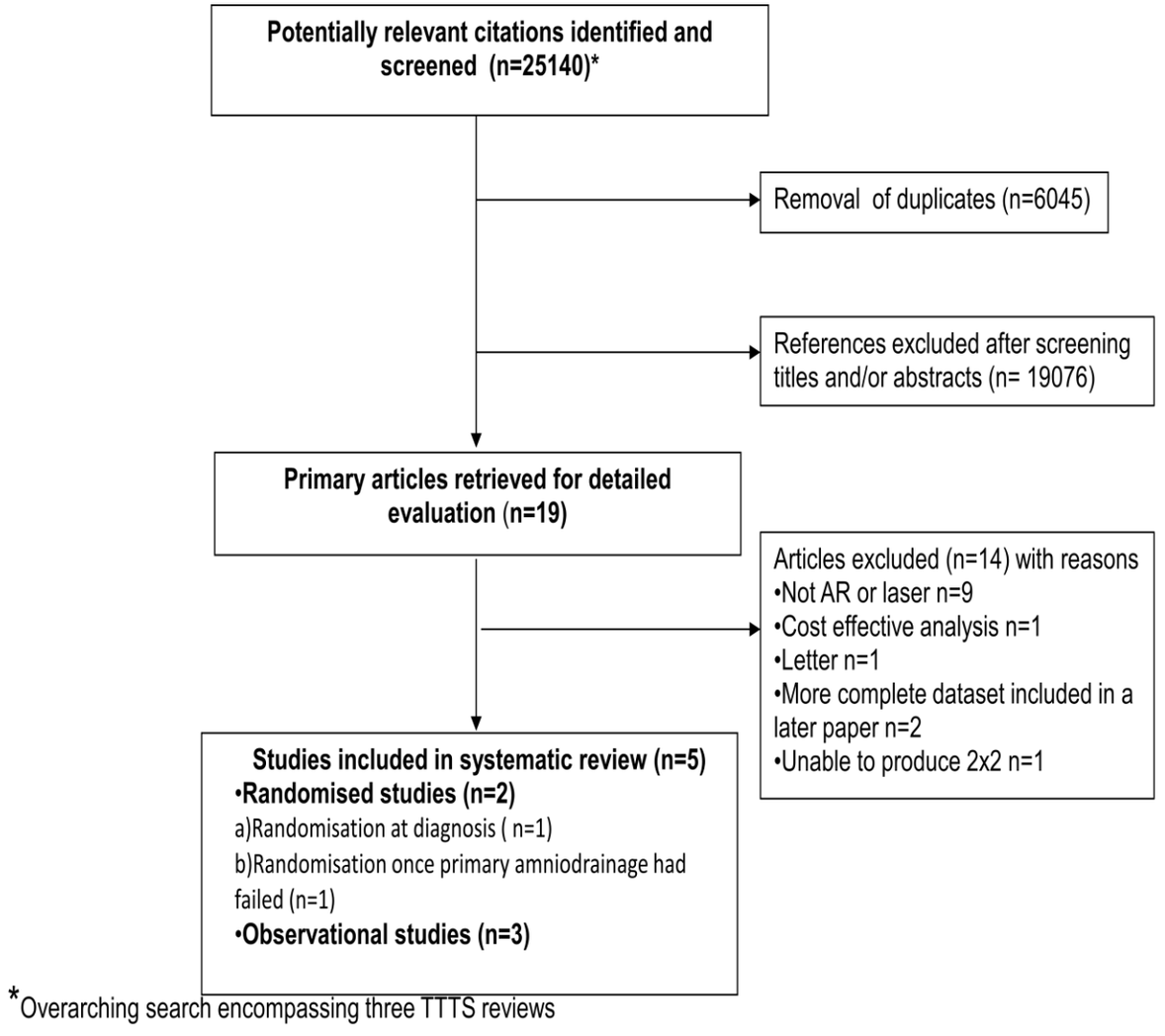
What is the effectiveness of FLA versus serial amnioreduction for the treatment of TTTS?

3.1.3.2 Study selection

The electronic and hand search relevant to all three planned reviews generated 25140 citations, of which 19 articles[59 60 269-284] were thought to be relevant: 18 were published in English and one in German. After independent review of the full manuscript of these 19 full articles, five were selected for inclusion in this review (figure 3-14). Thus, a total of five primary studies (all in English language), including 495 twin pregnancies, were selected for review (Table 3-5). Two randomised trials (185 twin pregnancies) and three observational studies (310 twin pregnancies) were identified.

The list of excluded studies is included in Appendix 7**.**

Figure 3-14 Study selection process for a systematic review of effectiveness of FLA and serial amnioreduction for the treatment of TTTS.



*Overarching search encompassing three TTTS reviews

Table 3-5 Study Characteristics for a systematic review of the effectiveness of FLA and serial amnioreduction for the treatment of TTTS.

(unless otherwise stated all interventions are carried out with continuous US guidance, and all twins undergoing laser also underwent a single amnioreduction as part of the procedure) Where survival differences are known to be significant p values are stated.

	Study	**Population**	**Interventions**	Outcomes (survival)	Follow up	
					Length (Months)	**%**
Randomised controlled trials	***Crombleholme et al (2007)***	43 monochorionic twin pregnancies complicated by TTTS (prior to 22 weeks) who had failed to respond to initial qualifying amnioreduction. 1 withdrew prior to randomisation and 2 dropped out after randomisation but prior to treatment (one from each arm). Ultrasound was used to determine chorionicity.	**Selective laser (n=20)** performed in 3 units Under epidural anaesthesia with intravenous sedation. *Technique:* most through a single percutaneous port, however some with an anterior placenta required a mini-laparotomy. 3.3 mm 3 port fetoscope, one for the 600µ laser endostat and a second for rapid infusion of physiologic saline if needed to clear the amniotic cavity. Selective coagulation using 60 Watt power with Endostat 1cm from the surface of the vessel, after the chorionic plate of the placenta was mapped 3 times. All procedures digitally recorded and independently reviewed.	***Overall at 30/7*** Laser 18/40 (45%) AR 24/40 (60%) ***At least one twin at 30/7*** Laser 13/20 (65%) AR 15/20 (75%) ***Donor* at 30/7** Laser 11/20 (55%) AR 11/20 (55%) **Recipient at 30/7** Laser 6/20 (30%) AR 9/20 (25%)	NS	>90%

	All treatments were performed prior to 24 weeks but mean gestation for laser and amnioreduction not specified. *Initial recruitment planned to 146 but trial stopped early at investigators request and the Trial Oversight Committee's recommendation based on interim analyses.*	Amnioreduction performed to a deepest vertical pocket (DVP) of <5cm. Antibiotics were instilled into the amniotic cavity prior to trocar removal. **Vs** **Aggressive Amnioreduction (AR) (n=20)** Ultrasound performed thrice weekly and AR performed whenever the DVP in the recipient twin sac was ≥8cm. *Technique:* 20 gauge needle and vacuum suction bottle to a DVP of ≤5cm. USS to assess for inadvertent septostomy 1hr post-procedure. No. of procedures NS Treatment failures Laser (n=0), AR (n=7).	None of these differences were statistically significant		
Senat et al (2004)	142 monochorionic twin pregnancies complicated by TTTS (15-26 weeks) Gestational age at treatment not specified but all randomised prior to 24 weeks. Postnatal examination of the placenta confirmed monochorionicity	**Selective Laser (n=72)** performed in 3 units Under local/regional anaesthesia *Technique*: through recipient sac, 3.3mm cannula with trocar, 2mm fetoscope, 400 or 600 µm diameter Nd:YAG or diode laser fibre. Selective coagulation by non-touch technique using an output 30-60 Watts. Post-laser amniotic fluid drained until	***Overall at 6/12*** Laser 81/144 (56%), SA 54/140 (39%) ***At least one twin at 6/12*** Laser 55/72 (76%), SA 36/70 (51%) p=0.002 ***Donor* at 6/12** Laser 39/72 (54%), SA 28/70 (40%)	Median 20 Range 6-42	>90%

	in all but 1 case (in SA group). *Initial recruitment planned to 172 but trial stopped early after second interim analysis.*	maximum pool depth 5-6cm. n=3 didn't undergo procedure (n=2 didn't meet selection criteria; n=1 IUD both fetuses) Primary treatment failures within 2 weeks n=3 (n=2 had repeat laser, n=1 underwent additional amnioreduction) **Vs** **Serial amnioreduction (SA) (n=70)** performed in 17 units *Technique:* 18 gauge needle syringe aspiration or wall suction, until maximum pool depth 5-6cm. Mean no. of procedures 2.6 n=2 didn't undergo the procedure (n=1 withdrew consent, had laser instead; n=1 IUD both fetuses) Primary treatment failures n=6 so also underwent laser. Prophylactic tocolytics and antibiotics were given.	***Recipient* at 6/12** Laser 42/72 (58%), SA 26/70 (37%) ***Without major neurological complications at 6/12*** ***Donor*** Laser 36/72 (50%), SA 25/70 (35%) p=0.09 ***Recipient*** Laser 39/72 (54%), SA 19/70 (27%) p= 0.003 **TOP** Laser 0/72, SA 11/70 **Delivery<24wks** Laser 24/144, 16/140 **IUD≥ 24 wks** Laser 27/144, SA 29/140 **NND/infant death** Laser 12/144, SA 41/140		
Observational studies	***Middeldorp et al (2007)*** 21 consecutive monochorionic pregnancies complicated by TTTS (after 26 weeks) Ultrasound was used to determine chorionicity.	**Laser (n=10)** Median gestational age at treatment 27 weeks. *Technique:* maximum power used 70 watts and post-procedure amnioreduction median 1800ml amniotic fluid removed.	***Overall (liveborn)*** Laser 20/20 (100%) SA 22/22 (100%) ***At least one (liveborn)*** Laser 20/20 (100%), SA 22/22 (100%)	NS	>90%

	All cases were seen at a single tertiary referral centre.	n=1 had amniodrainage 3/7 prior to laser. n=1 laser abandoned due to poor visualisation. **Vs** **SA (n=11)** *Technique:* not described Median gestational age at treatment 27 weeks. Mean no. of procedures 1.6.	***NND*** Laser 0/20 (0%), SA 3/22 (14%) ***Any adverse outcome*** Laser 3/20 (15%), SA 8/22 36%)		
Quintero et al (2003)	173 consecutive monochorionic pregnancies complicated by TTTS. All treated prior to 27 weeks.	**Selective Laser (95)** 1 centre Median gestational age at treatment 21.6 weeks. Under General anaesthesia *Technique:* 2.7-3.3mm fetoscope with 3.5mm trocar, 600 µm diameter non-contact Nd:YAG using 10-40 Watts. Prophylactic antibiotics and tocolysis were given. **Vs** **SA (78)** 3 centres Median gestational age at treatment 20.7 weeks.	***Overall*** (to NN period) Laser 122/190 (62.4%), SA 90/156 (57.7%) ***At least one twin*** Laser 79/95 (83.2%), SA 52/78 (67.7%) ***Donor*** Laser 60/95 (63.2%), SA 45/78 (57.7%) ***Recipient*** Laser 63/95 (66.3%), SA 45/78 (57.7%) **Miscarriage<24wks** Laser 8/95, SA 8/78 **IUD** Laser 43/190 (includes 1 pregnancy with IUD of D and 2	NS	>90%

		Technique: sterile aspiration to MVP 6-7cm. Weekly scanning and repeat procedures if MVP ≥8cm No of procedures NS	pregnancies with IUD of D+R before miscarriage), SA 12/156 (includes 1 pregnancy with IUD of D and 1 pregnancies with IUD of R before miscarriage) **NND** Laser 14/190 (6=D, 8=R), SA 40/156 (21=D,19=R)		
Hecher et al (1999)	116 consecutive monochorionic pregnancies complicated by TTTS. (17-25 weeks gestation)	**Laser (73)** 1 centre *Technique*: through recipient sac, 1.9mm fetoscope in 9.8F sheath, 400 µm diameter Nd:YAG. Selective coagulation using an output 50-60 Watts from 1cm. n=1 also underwent SA **Vs** **Serial Amnioreduction (43)** 1 centre *Technique:* 18 gauge spinal needle, until normal amniotic fluid volume in recipient sac Median no of procedures 3 (range 1-15) Amniotic fluid drained after laser was significantly more than that removed at 1st amniocentesis in SA group (median 2500ml vs 1990ml)	***Overall*** Laser 89/146 (61%), SA 44/86 (51%) ***At least one twin*** Laser 58/73 (79%), SA 26/43 (60%) ***Donor*** Laser 38/73 (52%), SA 21/43 (49%) ***Recipient*** Laser 51/73 (70%), *SA 23/43 (53%)* **TOP** Laser 2/73, SA 1/43 **SF** (of donor) Laser 1/73, SA 4/43 (all resulted in death of co-twin as well) **IUD of 1 fetus** Laser	NS	>90%

		24/73, SA 3/43 **NND** Laser 6/95, SA 7/51

D=donor twin, DVP = deepest vertical pocket, IUD= intrauterine death, MVP = maximum vertical pool, NND= neonatal death, NS= not specified, R=recipient twin, SA= serial amnioreduction, SF = selective feticide, TOP= termination of pregnancy

See Appendix 8 for a summary of morbidity, gestational age and birth weight data for review of effectiveness of FLA and serial amniodrainage for treatment of TTTS

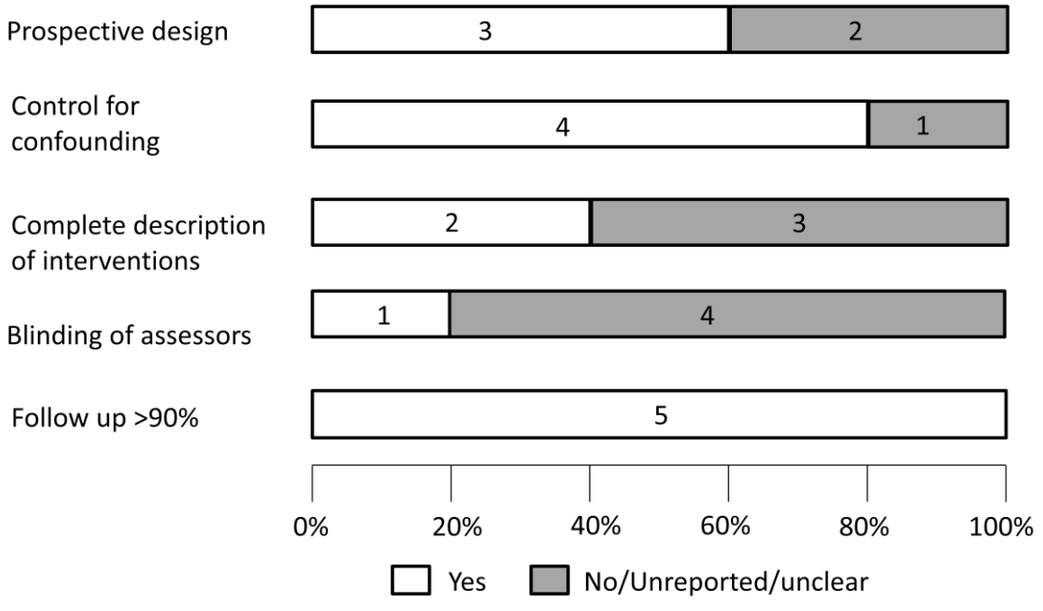
3.1.3.3 Quality assessment

Details of the study characteristics, methods, populations, interventions (including failed procedures) and outcomes (including complications) are shown in Table 3-5 and quality assessment in Figure 3-15. All included pregnancies had ultrasonic evidence of monochorionic twins and oligo-polyhydramnios, however, only one of the RCTs [59] also made postnatal examinations of the placenta, which revealed one placenta to be dichorionic. In the majority of studies [59 269-271] recruitment was based on the presence of TTTS, however in one of the RCTs[60] all patients were required to undergo a "qualifying" amnioreduction and it was only if this did not resolve the features of TTTS that patients were eligible for randomisation. As the rationale of a randomised controlled trial is to eliminate bias it is considered that the two randomised controlled trials [59 60] provided ideal control for confounding factors, however in an attempt to minimise bias two of the observational, comparative studies [270 271] used statistical adjustment for differences between the groups (TTTS stage was recorded for both treatment groups and was not found to be significantly different- Quintero; gestational age and abdominal biometry was not significantly different between treatment groups - Hecher). Method of data collection was prospective in 3/5 studies (60%) and in 2/5 (40%) it was either retrospective or unreported. All of the studies employed consecutive patient enrolment. The majority of studies [59 60 270 271] reported intervention for TTTS prior to 26 weeks gestation, whereas Middeldorp et al [269] reported intervention after 26 weeks. Description of intervention was considered incomplete in 3/5 (60%) of the studies. Although all studies provided detailed descriptions of the technique and equipment used, this detail was undermined in several instances: two studies [59 60] did not report the gestation at which the first intervention occurred, and another [270] failed to

provide numerical data for the mean or median number of amnioreductions performed in its serial amnioreduction cohort. The remaining 2/5 (40%)[269 271] provided adequate detail of both interventions.

It was possible to extract data on overall survival, survival of at least 1 twin and differential survival of donor and recipient twins in all trials (5/5). However, the timing of the definition of survival varied from birth [269 271], to the perinatal/neonatal period (28/30 days respectively)[60 270] and 7-12 months [59]. Data on neurological abnormality or morbidity in survivors were available in 4/5 (80%) [59 269-271]. Data relating to maternal morbidity were available in 3/5 (60%)[59 60 269]

Figure 3-15 Bar chart showing quality of evidence in relation to the effectiveness of FLA and serial amnioreduction in the treatment of TTTS.



Data presented as 100% stacked bar chart with numbers in bars representing numbers of studies. (see methods for details of quality items)

3.1.3.4 Summarising the evidence

Data from all studies are grouped, unless otherwise specified, to allow overall descriptive comparisons to be made.

Survival outcomes (see figure 3-16 for relative risk calculations)

All twins survived in both the FLA and the AR arms of the Middeldorp study[269], so relative risk calculation was not performed and these figures have been excluded from the following ranges to avoiding masking the results from the other studies.

When considering the primary outcome of this review, FLA conferred better survival of at least one twin in all except one trial[60], with values ranging between 53.2-83.2%. This trend was also repeated in relation to the secondary outcome of overall survival, range 45.0% -62.4%. However, with respect to differential survival of donor and recipient twins all studies showed equivalent or improved survival with FLA, ranges 52%-63.2% and 30-70% respectively. This is in comparison with amnioreduction, which resulted in survival ranges: 51%-75% for survival of at least one twin, 39%-60% for overall survival, with differential survival of 40%-57.7% and 25%-57.7% for donor and recipient twins respectively. Meta-analysis was not performed on all the studies as clear differences noted in inclusion criteria meant the populations included were not comparable. The reasons for not including a meta-analysis in this evidence summary are explained in further detail in the discussion.

Intervention Failures and Complications

Of the 270 patients scheduled to receive FLA nine (3.3%) either did not get FLA or had treatment failures. Of these four did not undergo the planned treatment (1.5%), one (0.4%) underwent amnioreduction prior to FLA, and failure occurred in a further four

(1.5%); necessitating two patients to undergo repeat laser coagulation, and two additional amnioreduction. Of the 222 patients who were scheduled to receive amnioreduction 15 (6.8%) either did not get the treatment or had treatment failures. Of these two (0.9%) did not undergo the planned treatment (one requested laser instead and one had a double in-utero death prior to treatment), six (2.7%) required FLA as well and there were seven (3.2%) treatment failures. There was no statistically significant difference between FLA and AR in terms of treatment failures (p=0.09).

Four studies [59 269-271] also reported more specific data regarding complications including fetal and neonatal deaths, as well as fetal morbidity. There were statistically fewer TOPs and NNDs in twins treated by FLA than amnioreduction, 2/155 (1.3%) vs 12/ 124 (9.7%) (p=0.002), and 32/449 (7.1%) vs 91/369 (24.7%) (p<0.0001) respectively. There were similar numbers of miscarriages and IUDs in both the FLA and amnioreduction groups, 25/250 (10.0%) vs 19/202 (9.4%) (p=0.87) and 74/500 (14.8%) vs 57/404 (14.1%) (p=0.78) respectively. All four studies reported fewer central nervous system (CNS) complications in relation to FLA. Two studies reported survival with no CNS complications and showed a significant benefit with FLA: FLA 75/144 (52.0%) vs amnioreduction 44/140 (30.5%) p=0.005[59]; FLA 75/95 (78.9%) vs amnioreduction 40/78 (51.3%) p=0.002[270].

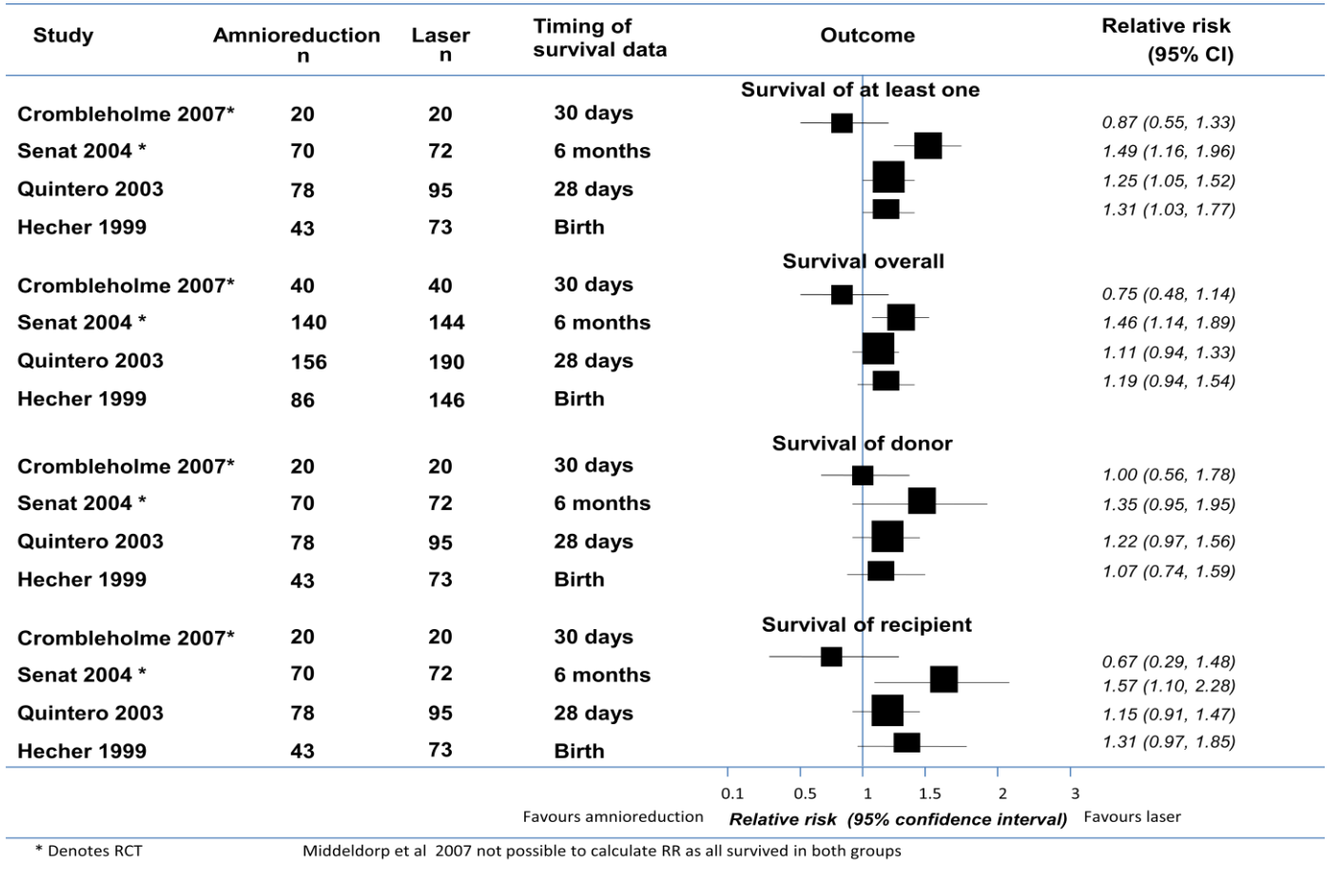
Serious maternal complications were reported in three studies and were infrequent. Placental abruption necessitating delivery occurred in 1/72 (1.4%) who underwent FLA, as opposed to 2/70 (2.9%) who underwent amnioreduction [59] and this difference was not statistically significant (p=0.67). The two other studies reported no serious maternal

complications in either group [60 271]. Minor maternal complications were more frequent with FLA and included a spinal headache (1/20; 5%) and a mini laparotomy (required in seven women (7/20; 35%) to expose the surface of the uterus) in the Crombleholme study[60].

Individual patient data analysis

Despite contacting all authors it was only possible to obtain individual patient data for two of the observational studies[269 270]. Repeated attempts to contact the authors yielded replies from all other authors, except the Eurofetus group, who stated that original ethics or logistically constraints prevented them from being able to share their data. It was therefore not possible to perform this analysis.

Figure 3-16 Forest plot showing the relative risks of FLA and serial amnioreduction for the treatment of TTTS.



* Denotes RCT

Middeldorp et al 2007 not possible to calculate RR as all survived in both groups

3.2 Results of investigation of biomarkers in the pathophysiology of TTTS

3.2.1 Markers of placental destruction (AFP/βhCG)

a) **Demographics of the twin pregnancy cohorts**

There were 31 potentially eligible MC twin pregnancies complicated by TTTS in the study period; however eight of these declined participation. Anonymised demographic data regarding these eight were however available and were not statistically different. All 23 recruited women were available for follow up. The demographics and outcomes of the uncomplicated dichorionic and monochorionic twin pregnancies and the twins complicated by TTTS have been previously described[141 164 165] and are shown in Table 3-6. The majority of cases had severe TTTS (21/23: 91.3%: with stage III 18/21 or IV disease 3/21), whereas only two cases (2/23, 8.7%) had stage I TTTS[285].

Overall, in 16/23 cases (69.6%), the recipient amniotic sac had a maximum pool depth that was less than 12cm and in 7/23 (30.4%) the maximum pool depth was ≥12cm. In 14/23 (60.8%) of cases of TTTS, the inter-twin EFW difference (ΔEFW) was <25%, whilst 9/23 (39.1%) had an ΔEFW of ≥25%. When considering only the severe cases (n=21): 18 (85.7%) had stage III disease and three (14.3%) had stage IV disease; 8/21 (38.1%) of recipient twins had ultrasound signs of cardiac dysfunction alone; in 5/21 (23.8%) the donors demonstrated AEDFV only and in 8/21 (38.1%) both recipient cardiac dysfunction and donor AEDFV were present. One case was excluded from all but basal analyses as fetoscopic laser ablation was not performed due to major inter-twin membrane disruption following a previous amniodrainage (at another centre).

Table 3-6 Demographics of uncomplicated di- and monochorionic twins and monochorionic twin pregnancies complicated by TTTS.

	DC twins (n=12)	MC twins (n=7)	MC twins with TTTS (n=23)
Maternal age (y)	33.5 (29.5 – 37)	35 (26 – 40)	32 (25 – 34)
Parity	0 (0 – 1)	1 (0 – 2)	1 (0 – 1)
Blood pressure at diagnosis (MAP; mmHg).	82 (80 – 84)	83 (79.5 – 87.5)	82.5 (79.5 – 87)
Gestational age (wk) at recruitment	20.5 (20.22 – 21.5)	22.14 (20 – 22.43)	21 (19 – 22.14)
Gestational age (wk) at delivery of survivors	37 (34.5 – 37.5)	35 (35 – 36)	33.93 (31.29 – 35.36)
Birthweight (survivors) (g)	Twin 1 2480 (1743 – 2680) Twin 2 2180 (1674 – 2580)	Twin 1 2497 (2165 – 2600) Twin 2 1900 (1830 – 2497)	Recipient 2370 (1972 – 2693) Donor 1880 (1355 – 1996)

Data analysed by Kruskal Wallis test ($p < 0.05$), represented as medians (IQR).

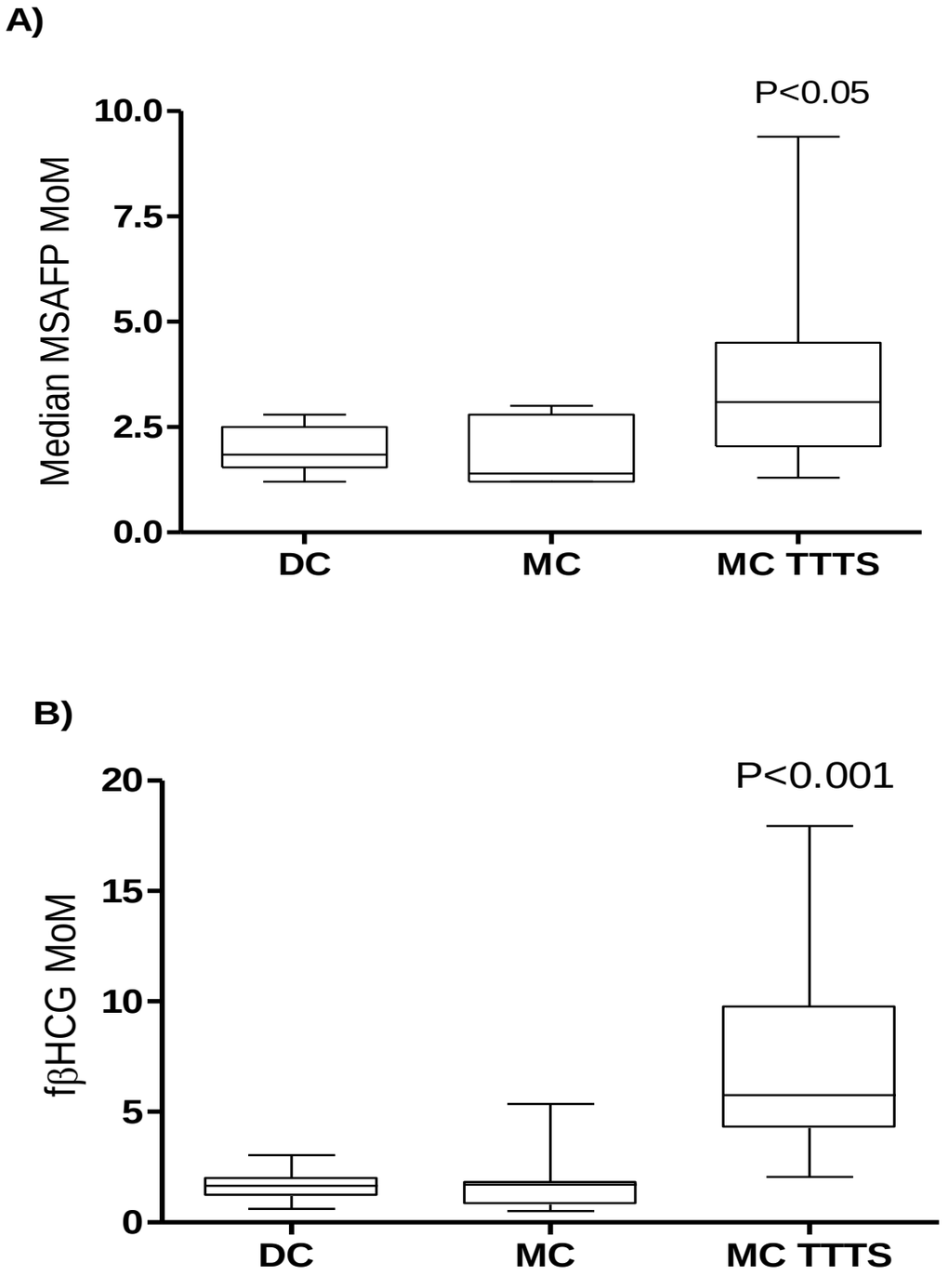
Of the TTTS cohort who were treated by either fetoscopic laser ablation (n=20; 90.1%) or amniodrainage (n=2) (cases with stage I TTTS only; 9.9%), the overall survival was 68.2% live births (30/44) and an outcome of at least one twin survivor in 86.4% (19/22) of cases.

b) Circulating MSAFP and f-βhCG concentrations under basal conditions in uncomplicated multiple pregnancies and those complicated by TTTS

The median MoM were not significantly different in uncomplicated DC twin pregnancies for MSAFP 1.85 (IQR 1.62 – 2.34) or f-βhCG 1.66 (95%CI 1.21 – 2.04) as compared to uncomplicated MC twin pregnancies (MSAFP 1.40 (IQR 1.16 – 2.58 and f-βhCG 1.70

(IQR 0.32 – 3.35)). The median MSAFP MoM and f-βhCG MoM in MC twin pregnancies complicated by severe TTTS were significantly increased (MSAFP 3.10 (IQR 2.67 – 4.43); $p<0.05$ and f-βhCG MoM 5.75 (IQR 5.22 – 9.12); $p<0.001$) compared to uncomplicated twin pregnancies (Figure 3-17A and B). These significant differences were also found on post hoc analysis of uncomplicated MC twins vs those affected by TTTS and uncomplicated DC twins vs those affected by TTTS.

Figure 3-17 Box and whisker plot showing basal comparisons of MSAFP and f-βhCG and the effects of chorionicity and TTTS.



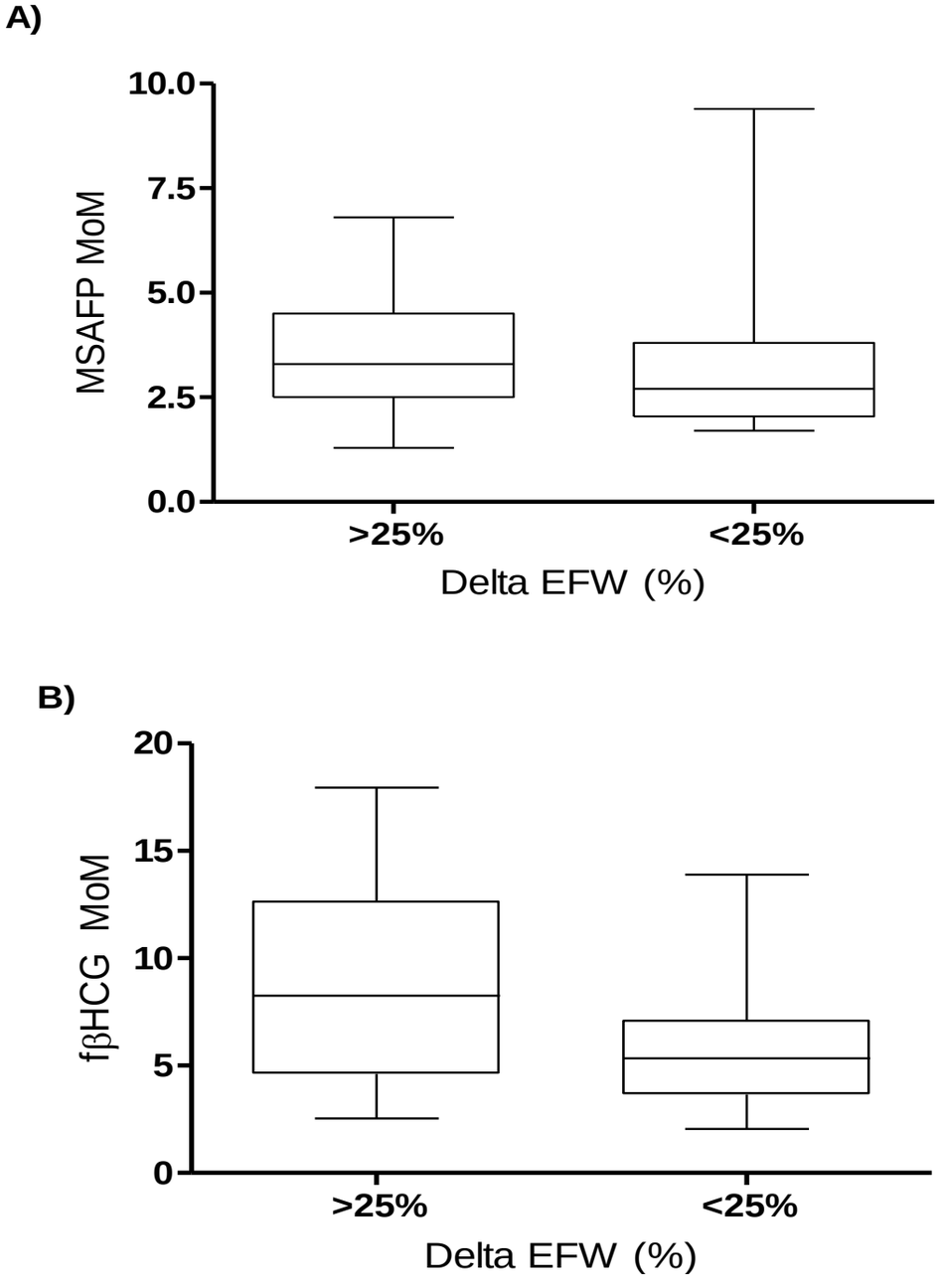
(Representing the median value, the IQR and the overall range. Analysed by Kruskal-Wallis test.)

c) Sub-group analysis of circulating MSAFP and f-βhCG in the cohort of MC twins complicated by TTTS

In the TTTS cohort, there was no significant difference, including on post hoc analysis across all three groups, in median MSAFP MoM or f-βhCG MoM between sub-groups where the recipient had signs of cardiac dysfunction only (MSAFP 3.15 (IQR 1.64 – 6.19) and f-βhCG 5.50 (IQR 3.91 – 9.18)), where the donor had AEDFV only (MSAFP 2.70 (IQR 2.04 – 3.19) and f-βhCG 3.21 (IQR 1.09 – 7.34)) or both recipient and donor had both abnormal cardiac and feto-placental velocity (MSAFP 3.20 (IQR 2.15 - 4.80) and f-βhCG 6.80 (IQR 5.19 – 14.12)).

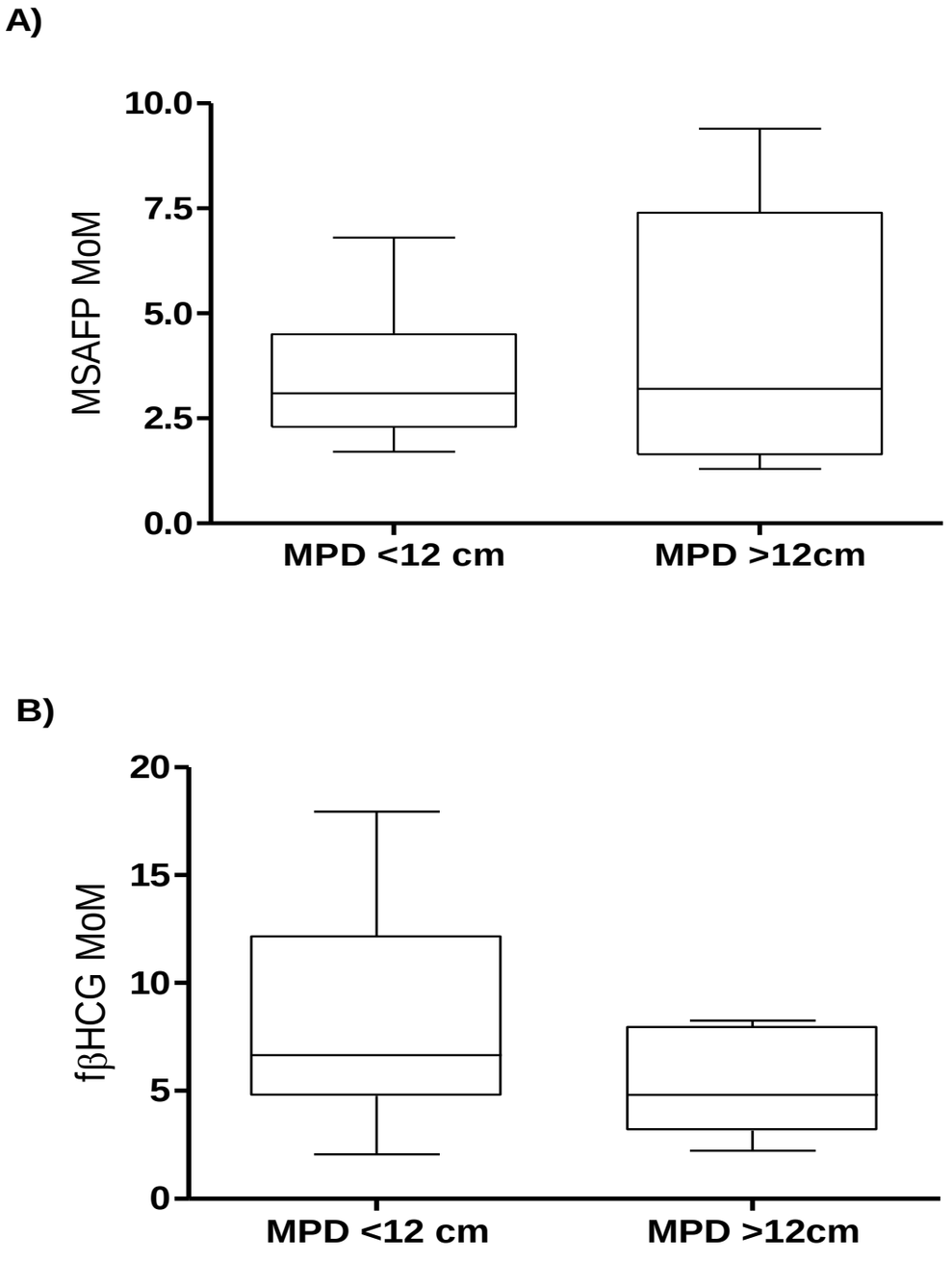
No significant difference in median MSAFP MoM or f-βhCG MoM was noted when subdivided by severity of inter-twin EFW difference (Figure 3-18) or severity of hydramnios in the recipient amniotic sac (Figure 3-19).

Figure 3-18 Box and whisker plot showing the effect of EFW difference on the levels of MSAFP and f-βHCG for the TTTS cohort.



(Representing the median value, the IQR and the overall range. Analysed by unpaired Mann Whitney U test.)

Figure 3-19 Box and whisker plot showing the effect of severe hydramnios on the levels of MSAFP and f-βhCG for TTTS cohort.



(Representing the median value, the IQR and the overall range. (Analysed by unpaired Mann Whitney U test)

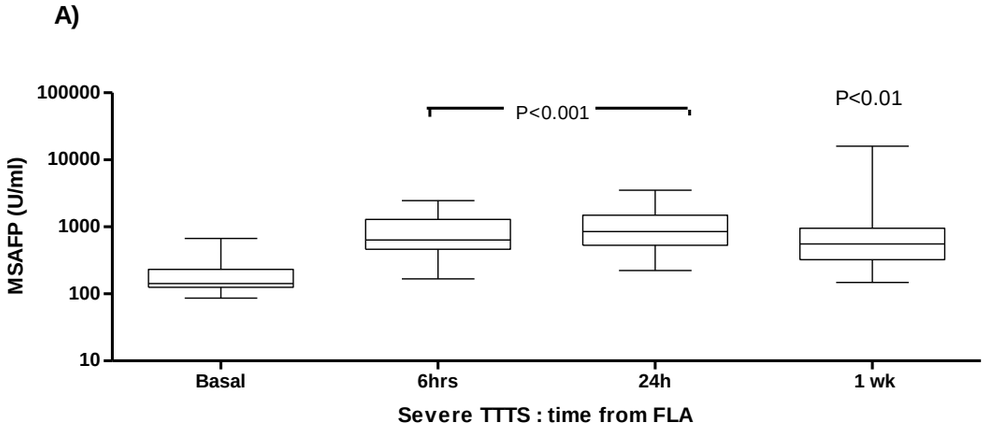
d) Changes in circulating MSAFP and f-βhCG concentrations after FLA

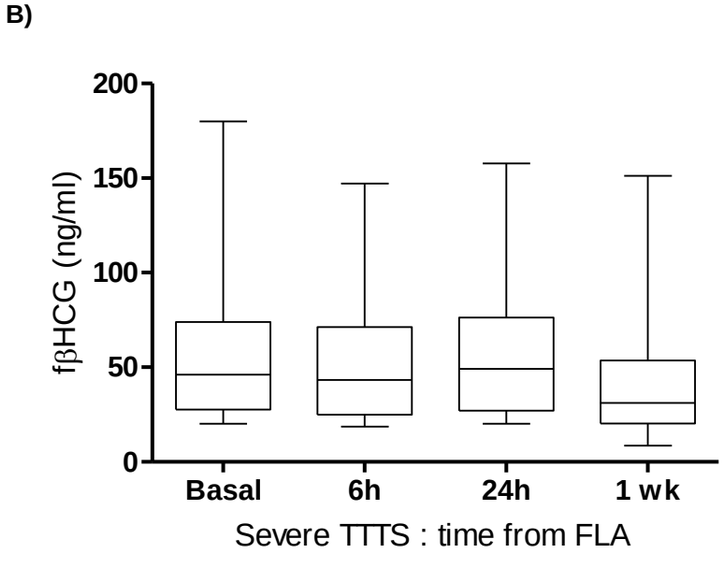
The median MSAFP increased by 445% (636.65 (IQR 616 – 1216.9 U/ml)) by six hours post-procedure and remained elevated at one week (553.4 (IQR 203.7 –3020.8U/ml); p=0.001) (Figure 3-20a). No significant difference in median f-βhCG was noted post-FLA (p=0.36) (Figure 3-20b). There was no difference in the increase in MSAFP concentration (ΔMSAFP), whether there were two survivors or one or more deaths post-FLA (Figure 3-21). There was no significant correlation between the Δ MSAFP between basal and 6 hours post-FLA and total energy used (Spearman r=0.251 (95%CI -0.216 – 0.62), the total number of AVA coagulated (Spearman r=0.27 (95%CI -0.19 – 0.64), the duration of the procedure (r= -0.24 (95%CI -0.62 – 0.22) or the amniotic fluid drained/gestational age ratio) (r= -0.29 (95%CI -0.65 – 0.17). There was no significant correlation between f-βhCG and total energy used (Spearman r=-0.032 (95%CI -0.468 – 0.417) or the total number of AVA coagulated (Spearman r=0.148 (95%CI -0.316 – 0.554). Multiple linear regression models demonstrated that the rise in MSAFP was independent of the effect of gestational age, operation time, amount of amniotic fluid drained at the end of the procedure, the amniotic fluid drained/gestational age ratio, and the number of chorionic vessels coagulated.

There was no significant difference in basal MSAFP or f-βhCG (prior to or after treatment) noted in those twins who both died post-FLA (MSAFP 3.0 (IQR 0.23 - 5.96) and f-βhCG 7.60 (IQR 5.87 – 21.18)), compared to those with one survivor (MSAFP 3.10 (IQR 2.34 – 5.08) and f-βhCG 6.80 (IQR 4.66 – 9.02)) or two survivors (MSAFP 2.70 (IQR 1.39 – 5.09; p=0.66) and f-βhCG 4.45 (IQR 1.97 – 10.7); MSAFP p=0.6652, f-βhCG p=0.28).

In the small number (n=2) of TTTS cases (stage I) treated by amnioreduction there were no significant changes in MSAFP and f-βhCG after treatment (p= 0.6652, p= 0.5083 respectively).

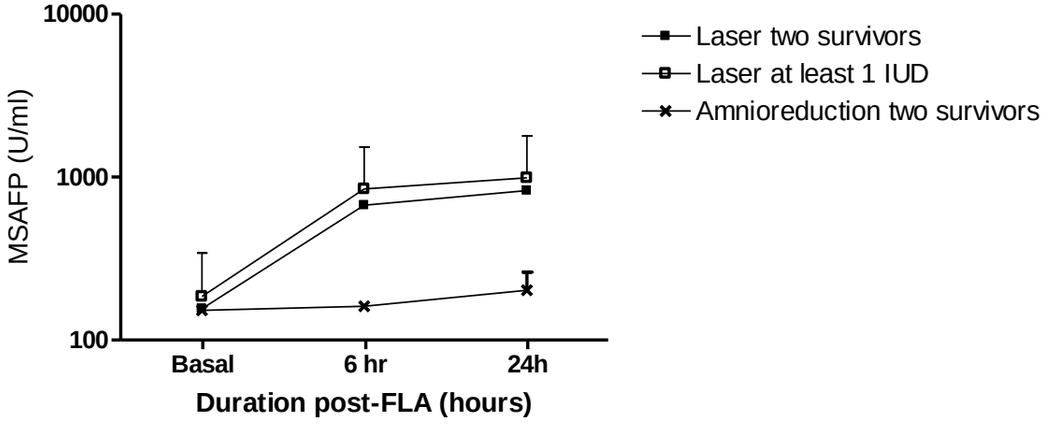
Figure 3-20 Box and whisker plot showing the changes in A) MSAFP and B) f-βhCG over time after FLA.





(Representing the median value, the IQR and the overall range. (Analysed by Kruskal-Wallis test)

Figure 3-21 showing the effect of the numbers of survivors on levels of MSAFP in those treated by amnioreduction and by FLA.



Graph demonstrating the change of geometric mean MSAFP (+/-75th geometric centile) after fetoscopic laser ablation in a cohort where there are two fetal survivors (filled square) as compared to those pregnancies where there is at least one IUD (black square). No significant difference in MSAFP increase was noted over time in those treated with amnioreduction or with two survivors vs at least one IUD after FLA (black cross).

3.2.2 Angiogenic growth factors

a) Demographics of the twin pregnancy cohorts.

See 3.2.1 section a) for demographics

b) Circulating plasma angiogenic factors and receptors, sVEGFR1 and sTie-2, concentrations in uncomplicated twin pregnancies and TTTS.

The median (IQR) plasma concentrations for all the measured angiogenic factors and the receptors sVEGFR-1 and sTie-2 are shown in Table 3-7. There was a significant difference in the plasma VEGF-C, Ang-2 and sVEGFR-1 concentrations between uncomplicated MC and MC twin pregnancies with TTTS. Maternal plasma VEGF-C was significantly lower in TTTS cases ($P< 0.0001$) and this significance was confirmed on post-hoc analysis of uncomplicated DC vs TTTS and uncomplicated MC vs TTTS. Plasma Ang-2 was significantly higher in the TTTS cohort ($P=0.0012$), with post-hoc analysis showing a significant difference between uncomplicated DC twins and the TTTS cohort. In terms of angiogenic growth factor receptors, only plasma sVEGFR-1 was significantly higher in the MC twins with TTTS ($P=0.042$), but this significance was not seen on post-hoc analysis. The sVEGFR-1/PIGF ratio was significantly higher in the TTTS cohort compared to MC and DC cohorts ($P=0.0071$), with post-hoc analysis showing a significant difference between uncomplicated DC twins and the TTTS cohort.

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VEGF-C was higher in uncomplicated MC twins than uncomplicated DC twins, and lowest in TTTS ($P<0.0001$), while Ang-2 and sVEGFR-1 was highest in TTTS ($P=0.0012$ and $P=0.042$ respectively).

Table 3-7 Plasma concentrations (pg/ml) of angiogenic growth factors and their receptors in uncomplicated di- and monochorionic twins and twins complicated by TTTS.

	DC twins (n=12)	MC twins (n=7)	MC twins with TTTS (n=23)	P value
VEGF-C	1500, 1300 – 1800	1700, 1500 – 1800	190, 190 – 1300 (↓)	**<0.0001**
VEGF-D	98, 81 – 140	240, 63 – 3900	79, 63 – 140	0.1277
VEGF-A	1100, 480 – 1700	1400, 450 – 25000	1600, 700 – 5100	0.2499
Ang-1	5038, 4736 – 6126	5314, 4779 – 5811	4478, 2519 – 5273	0.0576
PlGF	280, 230 – 420	310, 150 – 560	170, 100 – 320	0.1181
Ang-2	7100, 3500 – 12000	9300, 4300 – 20000	22000, 16000 – 31000 (↑)	**0.0012**
sVEGFR-1	3700, 3100 – 6300	3900, 3000 – 4700	5700, 4200 – 11000 (↑)	**0.042**
sTie-2	8100, 7100 – 8400	9000, 7800 – 9200	7200, 6000 – 8900	0.1440
sVEGFR-1/PlGF	12.63, 9.52 – 19.42	14.92, 7.33 – 20.73	26.10, 18.07 – 92.24 (↑)	**0.0071**

Data analysed by Kruskal Wallis test, represented as medians (IQR).

c) Basal concentrations of angiogenic factors and sVEGFR-1 and sTie-2 receptors in plasma and amniotic fluid in MC twins complicated by TTTS.

There was a significant difference between plasma and amniotic fluid concentrations of all angiogenic factors, with the exception of VEGF-D. VEGF-C (*P*< 0.0001) and Ang-1 (*P*=0.0004) were significantly elevated in amniotic fluid samples compared to 'paired' maternal plasma samples shown in table 3-8. In contrast, PlGF (*P*=0.0003) and Ang-2 (*P*<0.0001) were significantly higher in maternal plasma compared to amniotic fluid samples in TTTS. Concentrations of the angiogenic growth factor receptor sTie-2 were higher in plasma than amniotic fluid (*P*<0.0001), whereas sVEGFR-1 was higher in amniotic fluid compared to maternal plasma in TTTS (*P*=0.0002). These significant

differences were also noted on multivariate analysis correcting for stage of TTTS (*P*< 0.001). Again, we compared sVEGFR-1/PlGF in plasma and amniotic fluid and found this to be significantly higher (*P*=0.0018) in amniotic fluid compared to maternal plasma.

No significant association (using Spearman's correlation) was noted between any maternal plasma and amniotic fluid angiogenic factors.

Table 3-8 Concentrations (pg/ml) of angiogenic growth factors and their receptors VEGFR-1 and sTie-2 receptors in plasma and amniotic fluid in MC twins complicated by TTTS.

	Plasma	Amniotic fluid	P value
VEGF-C	187.5 (187.5 – 1170)	7209 (6043 – 8429)	**<0.0001**
VEGF-D	71 (63 - 130)	76 (66 – 85)	0.716
VEGF-A	1600 (870 – 5300)	15000 (8500 – 25000)	**0.0038**
Ang-1	4300 (2500 – 5200)	7400 (5400 – 11000)	**0.0004**
PlGF	210 (100 – 330)	65 (60 – 69)	**0.0003**
Ang-2	25000 (18000 – 32000)	11000 (7000 – 14000)	**<0.0001**
sVEGFR-1	5400 (4300 – 10000)	21000 (11000 – 34000)	**0.0002**
sTie-2	7300 (6000 – 9000)	1600 (1500 – 2000)	**<0.0001**
sVEGFR-1/PlGF	25.5 (18.0 – 34.1)	319.0 (173.8 – 536.4)	**0.0018**

Data analysed by Wilcoxon signed rank test, represented as medians (IQR).

d) Sub-group analysis of plasma, amniotic fluid angiogenic factors, sVEGFR-1 and sTie-2 receptor concentrations in MC twin pregnancies complicated by TTTS.

There was no significant difference in median plasma or amniotic fluid angiogenic factors or receptors when the TTTS cohort was subdivided by Quintero stage of TTTS. There were significant differences in the following angiogenic factors and their receptors when the TTTS cohort was analysed by sub-group:

(i) Severity of polyhydramnios in recipient sac *(median values)***.**

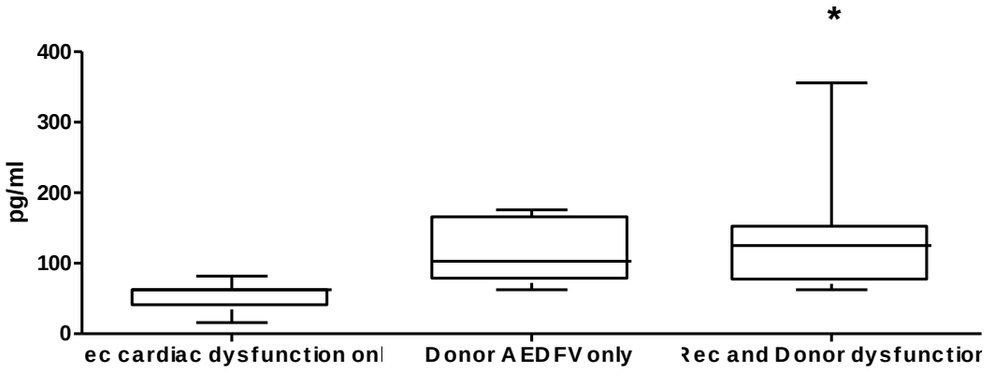
In maternal plasma VEGF-C (MPD< 12cm 1000pg/ml vs. MPD≥ 12cm 190pg/ml; *P*< 0.01) and Ang-1 (MPD< 12cm 4700pg/ml, vs. MPD≥ 12cm 2300pg/ml; *P*< 0.05) were lower in the sub-group of TTTS complicated by severe hydramnios.

In amniotic fluid, concentrations of VEGF-A (MPD< 12cm 17000pg/ml, vs. MPD≥ 12cm 9100pg/ml, *P*= 0.0299), Ang-2 (MPD< 12cm 13000pg/ml vs. MPD≥ 12cm 6400pg/ml; *P*= 0.0083) and sTie-2 (MPD< 12cm 1800pg/ml vs. MPD≥ 12cm 1400pg/ml; *P*= 0.0148) were significantly lower in the sub-group of TTTS complicated by severe hydramnios (MPD ≥ 12cm).

(ii) Severity of haemodynamic changes in Donor and Recipient twins.

In stage III or IV TTTS maternal plasma VEGF-D concentrations were significantly different when the severity of hemodynamic changes was considered. VEGF-D was significantly elevated where there was evidence of either absent umbilical artery Doppler velocimetry in the donor alone or absent umbilical artery Doppler velocimetry in the donor and signs of cardiac dysfunction in the recipient. (*P*= 0.0061), and post-hoc analysis showed a significant difference if there was recipient cardiac dysfunction only vs recipient and donor dysfunction- figure 3-22.

Figure 3-22 Box and whisker plot showing that the severity of haemodynamic changes in TTTS causes a significant difference in basal plasma VEGF-D levels.



*** *P* = 0.0061**

(Representing the median value, the IQR and the overall range. Analysed by Kruskal-Wallis test.)

(iii) Inter-twin estimated fetal weight difference (at diagnosis) of ≥ 25%.

Multivariate analysis correcting for stage of TTTS, gestational and maternal age noted that VEGF-A and sVEGFR-1 concentrations were significantly higher in maternal plasma when the cohort was subdivided by ΔEFW at diagnosis (≥ 25%) (*P*= 0.045).

e) Changes in plasma and amniotic fluid angiogenic factors and sVEGFR-1 and sTie-2 receptor concentrations after FLA in MC twin pregnancies complicated by TTTS.

Plasma PlGF was the only factor that was significantly different after fetoscopic laser ablation. Maternal plasma PlGF was transiently decreased after this therapy returning to baseline by one week (*P*= 0.0314) (basal 210- IQR 100-330, 6hr 130 IQR 65-210, 24hr 120 IQR 40-230, 1 week 210 IQR 170-590). The plasma sVEGFR-1/PlGF ratio

was also affected by FLA, with a transient increase after therapy, followed by a significant reduction to below basal concentrations by one week (*P*= 0.0102). Only VEGF-D was significantly different (+8.3%; *P*=0.0155) in amniotic fluid immediately after completion of FLA. Multiple logistic regression analysis was also performed. These observed changes in both maternal plasma and amniotic fluid, in response to fetoscopic laser ablation appeared to be independent of the number of AVA ablated, the volume of amniotic fluid removed at amniodrainage, the duration of surgery, the gestation at which surgery was performed or the outcome of the surgery in terms of fetal survival.

3.2.3 Maternal cell-free mRNA for angiogenic growth factors

a) Demographics of the twin pregnancy cohorts.

See 3.2.1 section a) for demographics

b) Percentage of maternal plasma samples that express cf-mRNA

We found that cf-mRNA was detectable for a number of angiogenic factors and receptors. In uncomplicated DC, MC and TTTS pregnancies respectively the proportion of samples with detectable cf- mRNA were as follows: GAPDH - 80%, 100% and 96%; VEGFR-1 - 10%, 0% and 26%; VEGF-A- 80%, 71% and 96%; Endoglin – 60%, 71% and 91%; PlGF – 70%, 57%, 22%; Tie-1 0%, 43%, 0%; Ang-1 71%, 50% and 60% and Ang-2 83%, 50% and 89%. The median (IQR) plasma concentrations for all the measured angiogenic factors and the receptors VEGFR-1, Endoglin and Tie-1 are shown in Table 3-9. There is no IQR for Ang-2 as insufficient samples had detectable levels to enable this calculation, so the overall range is shown.

Table 3-9 Maternal cell-free mRNA levels (copies/ml plasma) in uncomplicated twin pregnancies and those complicated by TTTS.

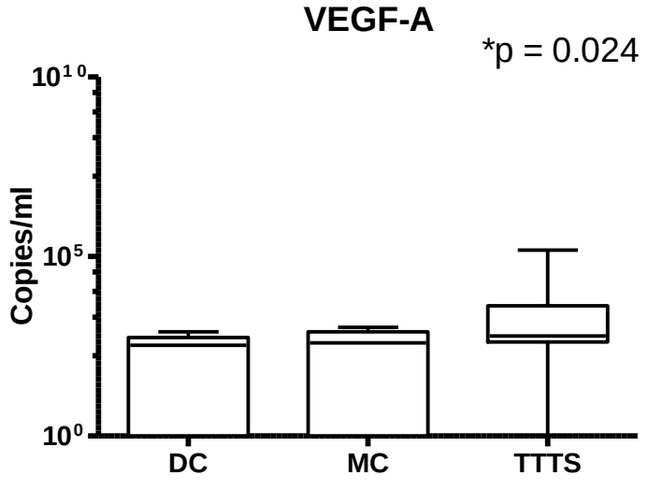
	DC twins (n=12)	MC twins (n=7)	MC twins with TTTS (n=23)	P value
GAPDH	44.7 (1.9 – 163.4)	176.1 (37.7 – 240.4)	140.4 (7.9 – 283.9)	NS
VEGFR-1	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)	0.0 (0.0 – 2.5)	NS
VEGF-A	337.7 (0.0 – 595.1)	390.8 (0.0 – 795.8)	618.6 (418.3 – 4279)	**0.024**
Endoglin	14.5 (0.0 – 3457)	1171 (0.0 – 1517)	2896 (1365 – 6187)	**0.027**
Tie-1	0.0 (0.0 – 0.0)	0.0 (0.0 – 17.9)	0.0 (0.0 – 0.0)	NS
PlGF	106.7 (0.0 – 126.0)	114.6 (0.0 – 119.6)	0.0 (0.0 – 71.47)	NS
Ang-1	3.1 (0.0 – 8.3)	1.6 (0.0 – 8.5)	5.1 (0.0 - 17.2)	NS
Ang-2	13.7 (2.3 – 23.4)	8.5 (0.0 – 23.2)	44.8 (26.8 – 76.7)	**0.007**

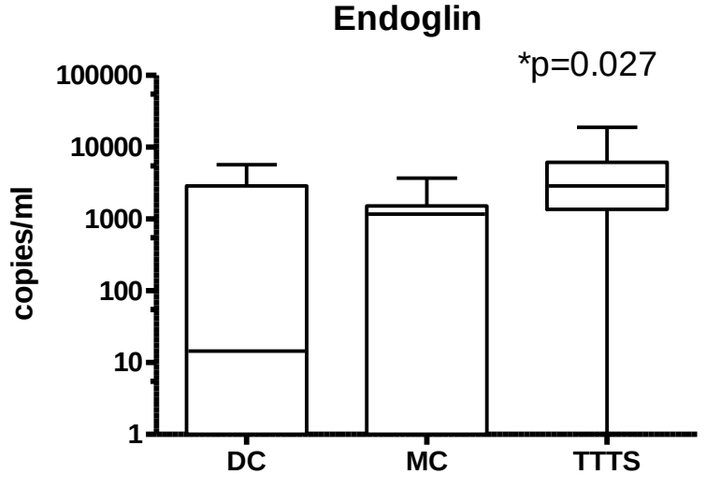
Data analysed by Kruskal-Wallis test, represented as medians (IQR), except Ang – 2 shown as median and overall range.

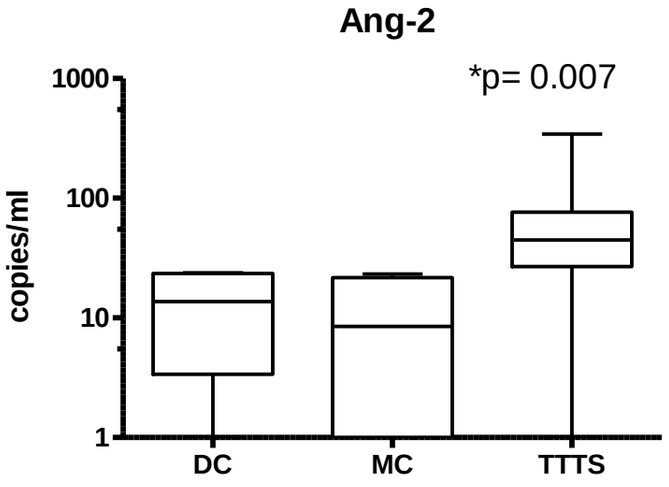
c) Changes and differences in expression with chorionicity and in TTTS

There was a significant difference in VEGF-A (medians DC -337.3, MC - 390.8, TTTS - 618.6 copies/ml plasma p=0.024), Endoglin (medians DC-14.49, MC-1171, TTTS - 2896 copies/ml plasma p=0.027) and Ang-2 (medians DC-13.66, MC-8.49, TTTS 44.80 copies/ml plasma p=0.007) (figure 3-23). On post-hoc analysis significant differences were found in VEGF-A between uncomplicated DC twins and the TTTS cohort and in Ang-2 between uncomplicated DC or MC twins and the TTTS cohort.

Figure 3-23 Box and whisker plot showing significant differences in VEGF-A, Endoglin and Ang-2 between uncomplicated twin pregnancies and pregnancies complicated by TTTS.







(Representing the median value, the IQR and the overall range. Analysed by Kruskal-Wallis test.)

3.2.4 Cytokines

a) Demographics of the twin pregnancy cohorts.

See 3.2.1 section a) for demographics

b) Circulating plasma cytokine concentrations in uncomplicated twin pregnancies and those complicated by TTTS.

The median (IQR) plasma levels for all the measured cytokines are shown in Table 3-10. There was a significant difference in the plasma PDGF-BB and TIMP-1 concentrations noted between uncomplicated MC and MC twin pregnancies complicated by TTTS (Figure 3-24). Median maternal plasma PDGF-BB was lower in uncomplicated MC twins (479.6 pg/ml) than in TTTS pregnancies (1071 pg/ml) and DC twin pregnancies (1368 pg/ml) *(P*=0.049) and no groups were significantly different on

post-hoc analysis. However, TIMP-1 was higher in TTTS pregnancies than in uncomplicated twins (*P*=0.003), with uncomplicated DC twins vs TTTS significant on post-hoc analysis.

Table 3-10 Basal circulating plasma cytokine concentrations (pg/ml) in uncomplicated twin pregnancies and in the cohort of MC complicated by TTTS.

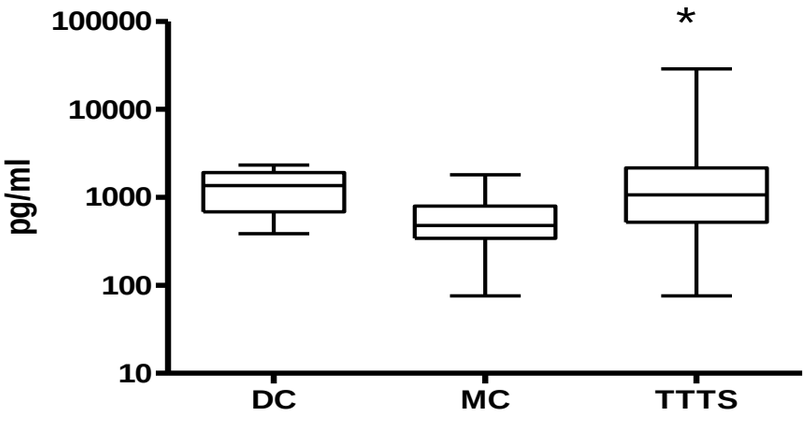
	DC twins *(n=12)*	**MC twins *(n=7)***	**MC twins with TTTS *(n=23)***	**P value**
IL-6	6.1, 6.1 – 6.1	6.1, 6.1 – 50.5	6.1, 6.1 – 43.2	0.079
IL-1β	6.1, 6.1 – 14.9	38.5, 6.1 – 215.0	6.1, 6.1 – 85.5	0.297
IL-10	30.4, 30.4 – 669.6	1179, 30.40 – 10870	30.4, 30.4 – 3484	0.178
IL-2	10.25, 6.6 – 23.6	37.70, 6.10 – 213.8	9.9, 6.1 – 188.2	0.600
IL-13	69.15, 37.8 – 208.9	220.9, 111.1 – 2392	40.2, 30.4 – 5528	0.415
IL-4	6.1, 6.1 – 6.1	6.1, 6.1 – 71.0	6.1, 6.1 – 267.6	0.308
IFN-γ	45.25, 30.40 – 170.4	392.8, 30.4 – 1057	62.2, 30.4 – 3337	0.510
IL-5	23.7, 9.35 – 63.0	218.6, 11.6 – 690.3	17.5, 6.1 – 529.8	0.521
TNF-α	7.2, 6.1 – 39.3	40.2, 6.1 – 144.6	8.2, 6.1 – 141.2	0.539
KGF	133.9, 78.68 – 200.5	272.9, 210.8 – 3670	212.1, 106.5 – 1962	0.090
PDGF-BB	***1368, 684.3 – 1906***	***479.6, 342.5 – 799.3***	***1071, 523.1 – 2158***	**0.049**
FGF-basic	552.7, 304.9 - 2247	2448, 1337 – 22530	1115, 389.0 – 6512	0.106
TIMP-1	***1911, 1860 – 2085***	***2110, 1561 – 3683***	***2615, 2196 – 3118***	**0.003**
ICAM-1	82700, 71010 – 86190	85610, 83810 – 88080	74510, 68050 – 80800	0.071

Data analysed by the Kruskal-Wallis test and represented as medians (IQR)

No IL-8 detectable in plasma

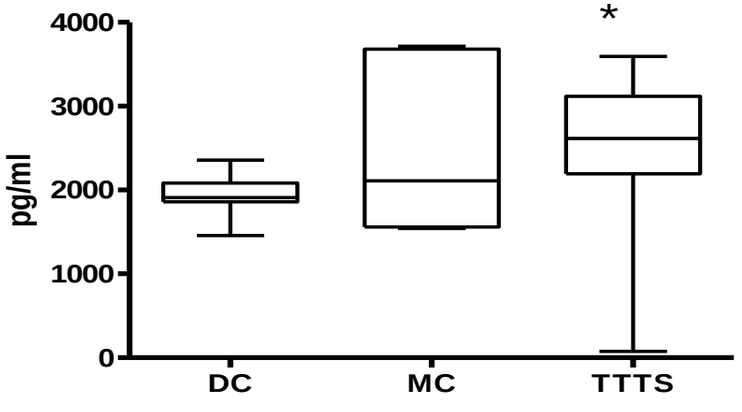
Figure 3-24 Box and whisker plot showing significant differences in basal plasma cytokines between uncomplicated di- and monochorionic pregnancies and monochorionic pregnancies complicated by TTTS.

A) PDGF-BB



* *P*= 0.0486

B) TIMP-1



P= 0.0031

(Representing the median value, the IQR and the overall range. Analysed by Kruskal-Wallis test.)

c) Basal concentrations of cytokines in plasma and amniotic fluid in MC twins complicated by TTTS.

There was a significant difference between basal plasma and amniotic fluid concentrations of IL-6, IL-10, IFN-γ, TIMP-1 and ICAM-1 (Table 3-11). These cytokines were elevated in amniotic fluid samples as compared to 'paired' maternal plasma samples. There were no significant differences noted between plasma and amniotic fluid concentrations of IL-2, IL-1β, TNF-α, IL-4, IL-5, IL-13, KGF, PGF-BB and FGF-basic

No significant association was noted between maternal plasma and amniotic fluid cytokines, with the exception of the trend noted for TIMP-1. There was a significant correlation showing when plasma TIMP-1 increases, amniotic fluid TIMP-1 decreases (Spearman r= -0.464; 95%CI – 0.7417 to -0.05157; *P*=0.03).

Table 3-11 Basal concentrations of cytokines (pg/ml) in plasma and amniotic fluid in the cohort of MC twins complicated by TTTS (n=23).

	Plasma	**Amniotic fluid**	**P value**
IL-6	***6.1, 6.1 – 6.1***	***446.8, 148.3 - 1020***	***<0.001***
IL-1b	6.1, 6.1 – 85.5	16, 15.2 – 31.3	0.994
IL-10	***30.4, 30.4 - 3484***	***3119, 935.1 – 6260***	***0.032***
IL-2	9.9, 6.1- 188.2	46.8, 15.2 – 88.2	0.982
IL-13	40.2, 30.4 – 5528	986.9, 512.2 – 1352	0.958
IL-4	6.1, 6.1 – 267.6	151.1, 46.5 – 318.9	0.277
IFN- γ	***62.2, 30.4 – 3337***	***7764, 1810 – 16380***	***0.010***
IL-5	17.5, 6.1 – 529.8	82.1, 32.2 – 106.8	0.589
TNF-a	8.2, 6.1 – 141.2	61.9, 23.6 – 96	0.434
KGF	212.1, 106.5 – 1962	282.5, 76 – 505.4	0.090
PDGF-bb	1071, 523.1 – 2158	628.2, 76 – 1182	0.079
FGF-basic	1115, 389 – 6512	2240, 1403 – 2895	0.131
TIMP-1	***2615, 2196 – 3118***	***124800, 109800 – 139000***	***<0.001***
ICAM-1	***74510, 68050 – 80800***	***40560, 21300 – 55510***	***<0.001***

Data analysed by the paired Wilcoxon signed rank test and represented as medians (IQR)

d) Sub-group analysis of plasma and amniotic fluid cytokine concentrations in MC twin pregnancies complicated by TTTS.

There was no significant difference in median plasma or amniotic fluid cytokines when the TTTS cohort was subdivided by Quintero stage. There were significant differences in the following cytokines when the TTTS cohort was analysed by sub-group:

(ii) Severity of polyhydramnios in recipient sac.

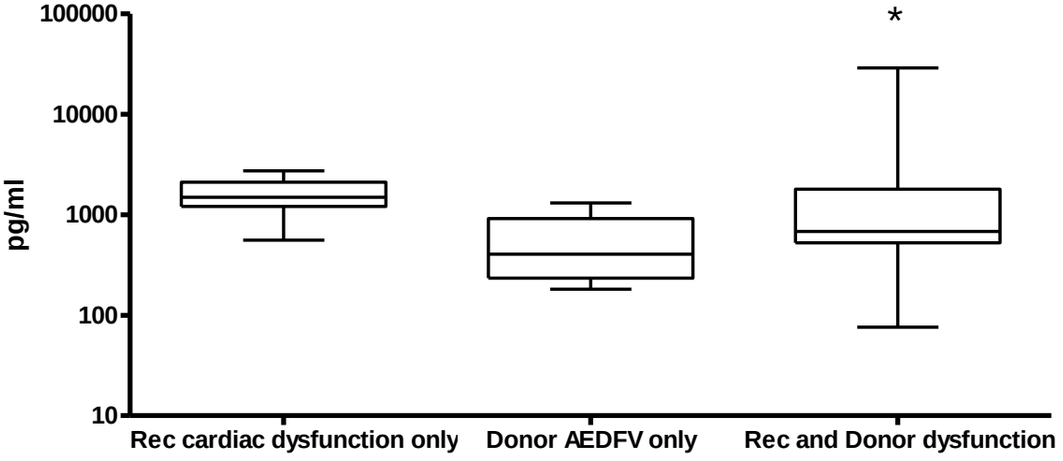
In maternal plasma only ICAM-1 was significantly different (MPD <12cm median 75850 pg/ml, 75% CI 70290-83210 pg/ml vs. MPD ≥12cm median 68241 pg/ml, 75% CI 55520-74510 pg/ml; *P*=0.03). ICAM-1 was lower in the sub-group of TTTS complicated by severe polyhydramnios.

In amniotic fluid, concentrations of most cytokines were significantly different, except IL-6, IL-1β, PDGF-BB and ICAM-1. All were significantly lower in the sub-group of TTTS complicated by severe polyhydramnios (MPD ≥12cm).

(ii) Severity of haemodynamic changes in Donor and Recipient twins.

In severe TTTS (Quintero stage III or IV) we noted only maternal plasma PDGF-BB concentrations to be significantly different when the severity of hemodynamic changes was considered. PDGF-BB was significantly elevated where there was evidence of cardiac dysfunction in the recipient, either in combination with absent umbilical artery Doppler velocimetry in the donor or alone. (*P*= 0.03; Figure 3-25). Post-hoc analysis showed significance for twins with recipient dysfunction only vs donor dysfunction only.

Figure 3-25 Box and whisker plot showing that the severity of haemodynamic changes in TTTS causes a significant difference in basal plasma PDGF-BB levels.



**P*=0.0344

(Representing the median value, the IQR and the overall range. Analysed by Kruskal-Wallis test.)

There were no significant changes in cytokine concentrations in amniotic fluid on sub-group analysis with respect to the severity of haemodynamic changes.

(iii) Inter-twin estimated fetal weight difference (at diagnosis) of ≥25%. There were no significant differences in any cytokine concentrations in maternal plasma with respect to sub-group analysis for inter-twin EFW difference. There was however, a significant difference in ICAM-1 in amniotic fluid. ICAM-1 was significantly lower with EFW difference ≥ 25% (<25% 52090 pg/ml, 38330- 57320 pg/ml vs. ≥25% 21530 pg/ml, 16700 – 28010 pg/ml *P*=0.0033).

e) Changes in plasma and amniotic fluid cytokine concentrations after FLA in MC twin pregnancies complicated by TTTS.

There was no significant change in cytokines in maternal plasma before and at six hours, 24 hours and one week after fetoscopic laser ablation. However, KGF was significantly lower after FLA in amniotic fluid (pre laser median 349.9 (IQR 110.2 – 554.7), post laser median 183.2 (IQR 76.0 – 322.8) p= 0.046). There was also a significant difference in IL-8, although the median values were the same the IQR showed a trend towards higher levels pre-laser. (Table 3-12)

Table 3-12 C*ytokine concentrations (*pg/ml) *in the cohort of MC twins complicated by severe TTTS before and at specified intervals after treatment by FLA (n=20).*

a) Maternal plasma

	Basal	6hrs	24hrs	1 week	P value
IL-6	6.4, 6.1 – 52.5	12.8, 9.3 – 24.7	10.5, 6.1 – 56.8	6.1, 6.1 - 31.9	0.192
IL-1β	6.1, 6.1 – 107.6	14.2, 6.1 – 109.4	20.5, 6.1 – 131.9	6.1, 6.1 – 181.1	0.828
IL-10	30.4, 30.4 – 4071	30.4, 30.4 – 2279	31.2, 30.4 – 3008	30.4, 30.4 – 4407	0.991
IL-2	8, 6.1 – 207.9	8.2, 6.1 – 140.3	17.1, 6.1 – 195.6	6.1, 6.1 – 173.1	0.851
IL-13	35.3, 30.4 – 36100	227.2, 30.4 – 8663	220.6, 30.4 – 34340	79.8, 30.4 – 2760	0.833
IL-4	6.1, 6.1 – 335	6.1, 6.1 – 64	7.350, 6.1 – 222.2	6.1, 6.1 – 165.7	0.844
IFN-γ	46.3, 30.4 – 4873	30.4, 30.4 – 676.9	138.4, 30.4 – 2665	33.4, 30.4 – 1692	0.809
IL-5	17, 6.1 – 623.8	28.2, 6.1 – 343.9	42.3, 6.1 – 476.9	13.8, 6.1 – 503.4	0.976
TNF-α	9.9, 6.1 – 147.4	11.7, 6.1 – 68.1	26.7, 6.1 – 120.2	11.9, 6.1 – 115.9	0.960
KGF	212.8, 109.5 – 2063	226.5, 94.7 – 2007	298.1, 109.8 – 2856	271.2, 108.9 – 2324	0.971
PDGF-BB	945.4, 540.5 – 1587	970.5, 550.6 – 2060	1271, 249.5 – 2213	1238, 384.1 – 1535	0.963
FGF-basic	2168, 450.5 – 14220	1894, 478.0 – 12780	1774, 492.8 – 25320	2908, 266.9 – 13630	0.939
TIMP-1	2805 2440 – 3176	2923, 2076.0 – 3274	2846, 2206 – 3484	2488, 1916 – 3042	0.487
ICAM-1	74440, 68040 – 81950	70130, 59800 – 77790	74470, 66930 – 85310	72660, 38700 – 79890	0.670

Data analysed by the unpaired Mann Whitney U test and represented as medians (IQR).

No IL-8 detected in plasma

b) Amniotic fluid

	Pre - laser	Post - laser	P value
IL-6	363.9, 157.7 – 806.2	312.2, 192.5 – 1027	0.318
IL-1β	15.59, 15.2 – 30.22	15.2, 15.2 – 22.9	0.465
IL-10	2858, 705.7 – 6095	2669.0, 1241 – 4208	0.661
IL-2	44.2, 15.2 – 83.9	42.1, 16.4 – 67.9	0.561
IL-13	907.9, 530.3 – 1295	938.8, 558 – 1120	0.688
IL-4	135.3, 42.4 – 296.6	113.7, 34.4 – 213	0.417
IFN-γ	7715, 1505 – 15090	7680, 1685 – 11350	0.801
IL-5	76.0, 35.2 – 106.1	51.5, 23.5 – 88.9	0.216
TNF-α	58.5, 23.3 – 91.1	52.3, 29.2 – 81.0	0.333
KGF	***349.9, 110.2 – 554.7***	***183.2, 76.0 – 322.8***	***0.046***
PDGF-BB	613.4, 76.0 – 1073.0	640.0, 76.0 – 1580.0	0.712
FGF-basic	2300.0, 1518.0 – 2898.0	1911.0, 1605.0 – 2430.0	0.439
TIMP-1	123500.0, 109200.0 – 137300.0	127900.0, 109700.0 – 141100.0	0.439
ICAM-1	36670.0, 20970.0 – 51230.0	31890.0, 17780.0 – 48850.0	0.376
IL-8	1563.0, 1563.0 – 2822.0	1563.0, 1169.0 – 1563.0	0.130

Data analysed by the Wilcoxon signed rank test and represented as medians (IQR).

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4.1 Systematic reviews

4.1.1 Diagnostic accuracy reviews of tests in the first trimester to predict TTTS and tests after diagnosis to predict outcome of TTTS

The first diagnostic accuracy review examined the role of ultrasound in screening in the first trimester to predict the development of TTTS. It found that tests already commonly utilised for dating twin pregnancies and screening for Down's syndrome such as crown rump length and nuchal translucency, could be applied for screening for TTTS if discordancy ≥ 10% or 20% respectively were calculated or nuchal translucency > 95th percentile noted. The second review assessed the accuracy of ultrasound tests after diagnosis of TTTS to predict outcome in terms of IUD or NND. The test that performed the best was donor DV abnormality in prediction of donor IUD. With respect to IUD there was an association between an abnormal test result and worse rates of survival, however interestingly this was not the case with prediction of NND. Here, several abnormal tests, including cardiac predictors such as TR/MR or worsening CVS score, were associated with improved outcome.

The first review evaluated the accuracy of four first trimester ultrasound tests in a total of 1451 pregnancies. For all four screening tests a positive test result had a high reliability for predicting the development of TTTS but a negative test result did not completely exclude TTTS, as the sensitivity of all tests was poor (ranging from 0.21 – 0.53). This was particularly true for CRL discordance and NT > 95th percentile as they both had specificities of 92% and gave post test probabilities for a positive test of 31.1% and 28.2% respectively (more than doubling the pre-test probability). Although no screening test demonstrated an ideal overall predictive accuracy, CRL discordance greater than 10% (based on analysis of four studies, including 646 pregnancies) appeared to be the best. This is of clinical relevance as all pregnancies, including multiple pregnancies, are recommended to have CRL performed as a routine test in the first trimester to date the pregnancy[1 286]. So this test is already being performed but if the CRL discordance in the MC twin pair were routinely calculated (the difference in the CRLs of the twins divided by the CRL of the larger twin), where discordance was greater than 10% this pregnancy could be discussed with or referred on to a centre with expertise in treating TTTS. This would have the advantage that the TTTS may be treated at an earlier stage, and stage at treatment has been shown to be an independent predictor of neurodevelopmental outcome[287]. In addition, all women are offered screening for Down's syndrome including measurement of nuchal translucency[1 286], and NT > 95th percentile was the other test with the highest specificity and could be utilised for risk stratification of MC twin pregnancies. So these are both routinely available tests but currently not widely utilised for the purpose of screening for risk of TTTS. This review suggests that these tests could be utilised for screening for TTTS, with the caveat that if the test is negative it does not exclude the condition. However, as

centres should monitor MC twins with scans every week or fortnight from 16 weeks[1], so even if the screening test e.g. CRL discordance was negative, these pregnancies would still receive regular follow up. However, as treatment for TTTS is only offered at regional and sometimes supra-regional centres, that are often a considerable distance from the referring unit, early referral could be reserved for women whose pregnancies screen positive on first trimester test as there is evidence that twins referred early (prior to 20 weeks) to a specialist centre have better outcomes than those referred later[288].

The clinical utility of the review findings has been explored further through consideration of the combination of these first trimester tests with the currently accepted treatment for TTTS, fetoscopic laser ablation[289]. This is done by calculating the numbers needed to test (NNTest) with CRL/NT or DV scanning, and treat (NNTreat) with FLA to avoid death of both infants as shown in table 4-1. The NNTest are 56, 52, 22 and 11 for CRL discordance, NT >95th percentile, NT discordance and DV abnormality in $\geq$ 1 fetus respectively and the NNTreat 5, 7, 8 and 5 respectively. To describe these more fully, it would be necessary to test 56 pregnancies using CRL discordance of 10% to prevent death of both infants if all screen positive cases are treated by FLA. The numbers are similar for NT > 95th percentile but lower at 22 needed to be tested and screen positve cases treated by FLA to avoid death of both twins. This review considers that as CRL and NT are already being performed as routine practice, these figures add further weight to this review's findings that they should now be utilised as predictors for development as TTTS as well to allow risk stratification and onward referral for MC twin pregnancies found to be at higher risk.

Table 4-1 Ultrasound screening in uncomplicated MCDA pregnancies and number of women needed to be tested and treated with FLA to prevent death of both infants.

Test result	Sensitivity %	Specificity %	Prevalence of TTTS (%)	Probability of TTTS after testing positive (%)	Risk of death of both infants after treatment*	NNTest[1]	NNTreat[2]
CRL discordance >10%	22.5	91.9	13.8	31.1	0.49	56	5
NT>95th	20.8	91.5	13.8	28.2	0.49	52	7
NT discordance >20%	53.2	73.1	13.8	24.1	0.49	22	8
DV absent/reversed flow in ≥ 1 fetus	50.0	87.5	13.8	43.3	0.49	11	5

*RR 0.49 (95% CI 0.30 – 0.79) Roberts et al[289].

[1]NNTest is the number needed to test and treat with FLA to prevent death of both infants calculated by 1/ (proportion of true positives(TP) – (proportion TPxRR)).

[2]NNTreat is the number needed to treat if only test positives are treated with FLA calculated by 1/ (probability of testing positive – probability after treatment).

The second diagnostic review assessed the accuracy of ultrasound tests after diagnosis of TTTS to predict outcome in terms of IUD or NND. The test that performed the best was DV abnormality in prediction of donor IUD. With respect to IUD all tests reported a trend between an abnormal test result and worse rates of survival, however interestingly this was not the case with prediction of NND. Here, several abnormal tests, including cardiac predictors such as TR/MR or worsening CVS score, were associated with improved outcome. This may be due to the fact that these cardiovascular abnormalities are common in recipient twins and can be transient[290], and that cardiovascular abnormalities can be amenable to treatment postnatally[291]. It is acknowledged that this finding is inconsistent with a previous study in TTTS[215], and information from singleton pregnancies [292] although singletons usually differ from recipient twins in that they are growth-restricted, however, this is an area that warrants further study of the physiological mechanisms at play.

This review evaluated the accuracy of seven tests performed after diagnosis of TTTS to determine its outcome, including 1635 pregnancies. Overall these tests showed poor predictive ability for either IUD (six tests) or NND (five tests). No test showed high sensitivity for the prediction of outcome, so a normal test is not useful for excluding IUD or NND. It is perhaps surprising that cardiovascular assessment denoting deteriorating cardiodynamic function, as represented by a higher CHOP score or cardiac profile, was associated with an improvement in survival in terms of NND. In keeping with the findings of the review of tests in the first trimester some tests showed high specificities such that a positive test makes IUD or NND likely. With respect to donor IUD an abnormal DV, a pulsatile UV or the presence of TR/MR showed high specificity (DV –

93% - from 3 studies, UV – 96% - from 3 studies, TR/MR – 98% - 2 studies), whereas only TR/MR was useful for NND (specificity 99% - 1 study). For recipient outcomes: an abnormal UA Doppler showed high specificity for IUD (93% - 4 studies) and NND (93% - 2 studies). As the likelihood of IUD and NND is very important for counselling in TTTS these tests with high specificities could be useful. Unfortunately, it is only for an abnormal DV and its prediction of donor IUD that this is likely to be true. This was the only test that had both high specificity and a significant LR+ giving a clinically meaningful post-test probability for a positive test of 39.2% (compared with a pre-test probability of 23.3%). This test also showed the best accuracy with an AUC of 0.99. This is logical as DV abnormality has been shown to be associated with fetal acidaemia[293] and has been considered a late change in fetal Doppler assessments, therefore showing a higher association with perinatal death than for example umbilical artery Doppler abnormalities[294]. Ultrasound assessment of both fetuses prior to treatment is ubiquitous and the likely accuracy of DV in predicting IUD, as shown in this review, leads to the recommendation that this should be a part of this routine examination and utilised in counselling regarding the possible outcome of treatment.

The strengths and the validity of the findings of the diagnostic accuracy reviews of tests used to predict the development and outcome of TTTS are derived from the use of rigorous methodology, in accordance with accepted guidelines for performing systematic reviews[56 145 157 295]. They were based on clear research questions from which prospective protocols were designed. The search strategy was thorough and was performed without language restrictions. The resulting articles underwent a detailed assessment of the quality of study design and reporting based on validated

tools[4 146-149]. The evidence was summarised both descriptively and quantitatively, including reporting summary likelihood ratios on the basis of the recommendation of the Evidence Based Medicine Working Group[156]. These likelihood ratios were utilised with Bayes' theorem to calculate clinically meaningful post-test probabilities. It is acknowledged that research has suggested that independently pooled likelihood ratios should be interpreted with caution, as positive and negative likelihood ratios, like sensitivity and specificity, are related statistics[296]. However, sensitivity analysis with pooled sensitivity and specificity, as well as bivariate analysis,[116] which not only preserves the two dimensional nature of the data but can acknowledge any possible correlation between these two measures, were also performed. These additional analyses confirmed the interpretation of the results therefore confidence in the inferences made is likewise confirmed.

The limitations of these reviews are predominantly related to the absence of clear reporting by the primary studies. Initiatives to standardise the design and reporting of studies have become widely recognised and supported over the last decade[149 286 295 297 298] and this transparency is particularly important in relation to screening or diagnostic tests, as these are more prone to bias[299]. However, it is known that study design can affect estimates of diagnostic accuracy and in particular that diagnostic performance can be overestimated if interpretation of the reference standard is not blinded[300]. This is pertinent to these two reviews as none of the included studies (21 in total, 10 in the first review and 11 in the second) reported whether the reference standard was blinded. Ideally, meta-regression using items of study quality can assess the impact of study design[300], however due to the small number of studies relating to each test this was not

possible[297] although, where possible sub-group analysis using only high quality studies was performed, and this confirmed the diagnostic accuracy estimates.

The review of first trimester ultrasound tests for the prediction of TTTS reported four tests, three of which (CRL discordance, NT >95th percentile and NT discordance) included analysis of four studies and one of which included review of only two (DV abnormality). There was only evidence of heterogeneity in analysis of DV abnormality and this may be explained by the inclusion of studies reporting absent a wave in one and absent or reversed a wave in the other. However, no significant heterogeneity was found in any of the other analysis, and these tests were the ones shown to be of greater value clinically. It is acknowledged that the numbers of studies for each test are small but, due to the thorough literature search and the absence of evidence of publication bias, confidence can be had that the findings provide the best evaluation of the currently available evidence.

The review of prediction of outcome (in terms of IUD or NND) of TTTS after diagnosis reported a range of different tests and although some studies reported more than one test not all tests included all of the participants. This was apparent in all of the studies and although acknowledged no explanation was given as to whether the pregnancies that were unable to be included differed from those included. So although several of the studies had been designed to avoid selection bias, by using prospective and consecutive recruitment[34 221 268], the omission of analysis to confirm that the patients who had missing data were not statistically different from those included means that this bias may still exist. These studies could therefore be considered equivalent to the

other studies in this review and in the review of prediction of development of TTTS, with non-consecutive and/or retrospective recruitment, but reassuringly these aspects of study design have not been shown to give different results in reviews of this type[300]. It is therefore reasonable to conclude that despite these limitations the results of these diagnostic accuracy reviews remain valid. As the populations included reflect those encountered in everyday practice and ultrasound is a safe modality to employ clinicians can be reassured that the recommendations to incorporate routinely available first trimester tests as screening for TTTS, as well as DV assessment in prediction of its outcome can be implemented immediately.

4.1.2 Effectiveness review of FLA and serial amniodrainage in the treatment of TTTS

This systematic review examined the effectiveness of the two principal treatments for TTTS, FLA and serial amnioreduction. It found that there was a lack of consistency between the studies as to the relative effects of these two treatments but in a previously untreated population it appears that FLA offers improved survival chances.

It is considered that this review satisfied criteria for performing a rigorous systematic review[144 301]. It examines both randomised controlled trials and observational studies and demonstrated that FLA and serial amnioreduction have been studied in 495 pregnancies affected by TTTS. However, the number of studies considered worthy of inclusion was small. These included four studies (2 randomised controlled and 2

observational) comparing FLA with serial amnioreduction in pregnancies complicated by twin–twin transfusion syndrome presenting before 26 weeks gestation[59 60 270 271] and one observational study comparing the same treatments but after 26 weeks[269]. The quality of reporting of the studies included is a limitation of this review. Of the five studies included in this systematic review, no study fulfilled all five aspects of quality assessment. The only aspect universally well described and conducted was follow-up of >90% of subjects enrolled. In considering other sources of bias three studies have a potential bias in that the laser procedure was performed in relatively few centres, whereas amnioreduction was performed by multiple operators in many centres[59 60 270]. It should also be recognised that the technique used in FLA differs between centres. Some using a non-selective technique with all vessels running across the intertwin membrane being ablated, whereas other groups are more selective in ablating communicating vessels[270].

Despite only a small number of studies reporting the effect of FLA versus amnioreduction there was considerable heterogeneity noted. The most stark example of this is in the study inclusion criteria and relates to the differences noted between the two RCTs. As the Crombleholme study[60] required patients to fail to respond to a qualifying amnioreduction prior to randomisation to either FLA or AR, the generalisability of this study to an untreated TTTS population as included in the other RCT[59] and two of the observational studies [270 271] is likely to be affected. This may explain why the treatment effects reported by the two RCTs were diametrically opposed. The other major difference noted was in the timing of the measurement of the primary outcome of survival, which varied from birth to 6 months. It is noted that four

of the studies described how their planned analyses accounted for the impact of clustering within the twin pair, and that the study that did not all twins survived. Unfortunately, the planned individual patient data (IPD) analysis, which would have enabled this review to perform analyses accounting for these differences was not possible as the majority of studies, including the two RCTs, were unable to provide this data. This was however considered in the design of this review so the primary outcome of survival of a least one fetus, a calculation possible from the extracted two-by-two data, could still be performed and interpreted.

The two RCTs[59 60] were both stopped early quoting the O'Brien-Fleming multiple testing procedure. This involves fixing the number of observations between tests and the number of tests in advance to allow a trial to be stopped early, with little impact on trial accuracy or power, if one treatment is markedly better than the other[299]. However, this methodology is not accepted by all as it has been shown that even large effects seen early in a trial can be reversed or disappear over its duration[302]. In the Senat trial the second interim analysis showed FLA to offer better survival for at least one fetus, hence the trial was stopped. However, the Crombleholme trial was stopped at the investigators' request as clinicians were increasingly unwilling to only offer patients FLA on a randomised basis. Interestingly, this trial's interim analysis showed better survival with amnioreduction, but the reliability of this analysis may well be affected by the uncertainty as to whether many eligible women were not referred because of their clinicians' preconceptions that FLA was the better treatment. Therefore despite reference to the O'Brien-Fleming procedure the fact that trials were both stopped early leads to less certainty regarding their results.

Systematic reviews and meta-analyses of data from observational studies are sometimes conducted to obtain evidence for practice, reflecting the relative paucity of experimental designs[303]. Reviews of observational studies, therefore, have a role in evaluating medical effectiveness[144] especially strengthened by being comparative, as in this series. There is however controversy about the differences in effects observed in randomised and observational studies[304]. Although meta-analysis of observational data can sometimes provide more precise estimates, there is the potential for producing spurious results as a consequence of confounding and selection biases[299]. It is for this reason, in combination with the heterogeneity noted particularly in relation to inclusion criteria and timing of outcome measure in the RCTs, that this review does not provide an overall meta-analysis, as it was not felt this would provide clinically useful information and may even be misleading. It is acknowledged that both a previous review[289] and an update to the Cochrane review (in press)[286] have performed meta-analysis of the two RCTs, however these papers recognise that significant heterogeneity exists and that the two trials appear to have opposite directions of effect. In view of this, comprehensive summaries of the characteristics and methodological quality of all included studies are reported. To supplement this information sub-group analysis by inclusion criteria was performed so a pooled effectiveness estimate was obtained that could be generalised to the most common TTTS presentation encountered in clinical practice, i.e. prior to 26 weeks and without prior treatment. This showed that the relative risk of survival of at least one twin was 1.33 (95% CI 1.17 – 1.52), in favour of treatment with FLA and that these studies were statistically homogeneous. With respect to differential survival of the donor and recipient this trend

was repeated, as was the evidence of homogeneity, although the confidence intervals for the donor crossed the line of no effect.

In summary the evidence on which to base decisions regarding treatment for TTTS is not robust, although it is acknowledged that FLA is likely to confer improved survival chances for the pregnancy. TTTS therefore remains a morbid condition and neurological morbidity remains a problem despite treatment[305-307]. However, as both RCTs were stopped early it seems unlikely that a further RCT will be funded but it should be acknowledged that trials stopped early provide less certainty with respect to treatment effect[302 308].

4.2 Biological markers for the investigation of the pathophysiology of TTTS

4.2.1 Markers of placental destruction (AFP/βhCG)

Elevations in MSAFP and levels of free β-hCG have been associated with conditions associated with abnormal placentation such as pre-eclampsia, fetal growth restriction and miscarriage[69]. Elevations of MSAFP have also been described after CVS[71], and elevations of both MSAFP and free β-hCG after multifetal reduction procedures[70]. This study is the first to investigate both the effect of TTTS and FLA on these same markers. It found there was no significant difference between median MSAFP and f-βhCG MoM in uncomplicated DC and MC twin pregnancies in the mid-gestational period. However,

in TTTS there was a significant increase in median MSAFP and f-βhCG MoM compared to uncomplicated twin pregnancies. These data have also demonstrated a significant increase in MSAFP concentration post- FLA that persists until at least one week post-procedure, with no associated increase in maternal f-βhCG.

A large cohort study[309] has previously investigated the median MSAFP MoM in DC and MC twin pregnancies and is in agreement with these results. However, this group noted a significant increase in median f-βhCG MoM in monochorionic twin pregnancies, which this study did not find. However, the previous study was focused mainly upon the role of second trimester analytes in screening for Down's syndrome and there was limited information on the outcomes of these pregnancies. It is possible that some of the cohort of twins may have suffered significant complications, such as TTTS and this may explain the inconsistency in Muller's data[309] and these data with respect to f-βhCG.

This study found that in TTTS, there is a significant increase in median MSAFP and f-βhCG MoM compared to uncomplicated twin pregnancies. Several hypotheses may be proposed to explain the difference in circulating maternal f-βHCG in pregnancies with severe TTTS. The polyhydramnios surrounding the recipient fetus may be associated with relatively impaired uteroplacental blood flow increasing the risks of hypoxaemia[167]. Several factors influence the placental production of HCG, including the total number of trophoblast cells, the degree of syncytialisation of cytotrophoblast[302] and oxygenation within the placenta[121]. The increase in maternal f-βHCG may therefore reflect the large placental size in TTTS and changes in placental oxygen tension secondary to uteroplacental hypoperfusion.

Increased placental transfer of AFP may occur across fibrinoid deposits at discontinuities in the syncytiotrophoblast[64] or by release from the decidua entering the maternal circulation[300]. In syndromes with abnormal placentation, a prospective increase in MSAFP and hCG levels has been described[297]. The increase in median MSAFP MoM in this cohort of pregnancies with severe TTTS may reflect the abnormal placentation in this condition. It is not possible from this study to know whether the observed elevation in MSAFP and f-βhCG in subjects with TTTS precedes the onset of the disease process. If so, their measurement may be of use to aid prediction of TTTS, increasing the sensitivity of first trimester ultrasound screening[212].

Laser ablation of arterio-venous anastomoses ideally discontinues the inter-twin transfusion process. These data have demonstrated a significant increase in MSAFP concentration post- FLA that persists until at least one week post-procedure, with no associated increase in maternal f-βhCG. Immediate effects from laser have been described with coagulation necrosis from thermal injury observed around the coagulation site and complete cotyledon infarction caused by the arrest of blood flow[310]. The damaged cotyledon may continuously release trophoblast debris including DNA, RNA and proteins into the maternal circulation[310]. The number of chorionic vessels coagulated may reflect the number of ischaemic placental cotyledons but in this study, no association was noted between the rise in MSAFP and either the number of chorionic vessels coagulated or the total energy used during FLA. MSAFP is selectively elevated compared to f-βhCG levels suggesting a transplacental leak of fetal haematopoietic cells[300]. Histological examination of the MC placentae post-FLA shows evidence of microvascular collapse with associated focal subchorionic haemorrhage[310].

Feto-maternal transfusion from this surgery may increase placental permeability to AFP and lead to the observed rapid rise in MSAFP within six hours post-surgery. This may indicate a continuing abnormality of transplacental AFP transport or clearance[300 310]. Despite abnormal fetoplacental blood flow being associated with at least one fetal death post-FLA, this study did not note any significant increase in MSAFP or f-βHCG above twin pregnancies with dual survivors. A rise in MSAFP has also been described where there is 'microtrauma' to the placenta, such as in chorionic villous sampling[71] or in procedures where there is single fetal demise in multiple pregnancies[311]. Deprest described a persistent elevation of plasma fetal DNA levels (but not RNA) after laser for up to 48 hours[312 313] associated with longer operation time, number of chorionic vessels ablated and IUD of at least one twin after FLA for TTTS. This indicates that the passage of proteins, RNA and DNA from the fetal to maternal circulations post-FLA may require a more complex explanation than simple ablative trophoblast damage or increased transplacental permeability.

We have also investigated a small number of pregnancies complicated by mild TTTS where only amnioreduction was performed. It is interesting to note that neither MSAFP nor f-βhCG increase when this therapy was utilised, making it unlikely that amnioreduction is the stimulus to the observed rise in MSAFP after FLA (a procedure where amnioreduction is also performed).

These data suggest that the basal rise in MSAFP and f-βhCG associated with TTTS may be secondary to abnormal placentation, a process that precedes the onset of clinical disease. Post-FLA there is a rise in MSAFP, with no corresponding rise in f-

βhCG, indicating an association with increased transplacental haemorrhage rather than trophoblast destruction. This study therefore aids our understanding of the pathophysiology of TTTS as well as the effect of FLA, although it is acknowledged that these changes need to be confirmed to precede the onset of clinical disease as has been shown in relation to the pathophysiology of pre-eclampsia. Of clinical relevance, if confirmed to precede disease onset, measurement of MSAFP and f-βhCG may be useful adjuncts to ultrasound predictors of the development of disease.

4.2.2 Angiogenic growth factors

Placental expression and circulating levels of AGFs and their receptors have been implicated in the pathogenesis of conditions associated with abnormal placentation such as pre-eclampsia[99-105], intrauterine growth restriction[100-102 105-107] and conditions associated with fetal hydrops[108]. In addition TTTS, which is also associated with abnormal placentation, has been suggested to be a relatively anti-angiogenic condition[110]. This study supports these findings by demonstrating a significant increase in median plasma sVEGFR-1 (and indeed the circulating sVEGFR-1/PlGF ratio) in those pregnancies complicated by severe TTTS as compared to uncomplicated di- and monochorionic twin pregnancies. In addition, the amniotic fluid sVEGFR-1 concentration was significantly higher than the maternal plasma concentration. More comprehensively, it is the first study to also examined the circulating plasma and amniotic fluid concentrations of the VEGF isoforms (VEGF-A, VEGF-C and VEGF-D), PlGF and the angiopoietin/Tie complex in relation to the severity of TTTS and in response to FLA. This showed that with more severe hydramnios angiogenesis in

response to hypoxic or inflammatory stimuli may be favoured. Interestingly it did not show a marked change in AGFs and receptors in response to FLA.

Angiogenesis and trophoblast/vascular interaction play an important role in human placentation[75]. There is accumulating evidence that angiogenic factors, including those of the VEGF family, PlGF and the angiopoietins orchestrate materno-fetal circulatory interaction within the haemochorial placenta[83 314]. Although there was no significant difference in the concentration of maternal plasma VEGF isoforms/sVEGFR-1 and angiopoietin/Tie-2 complex with increasing severity of TTTS (as denoted by Quintero staging alone), plasma VEGF-D was significantly increased in those pregnancies complicated by Stage III/IV TTTS when there was evidence of haemodynamic compromise in both the recipient and the donor, particularly in comparison with the recipient alone. This is in keeping with the findings of other studies; in that maternal circulating angiogenic factors appear to be most altered when both utero- and fetoplacental blood flow are abnormal[105].

Interestingly, the expression of VEGF-A within the stromal core of the villi and syncytiotrophoblast is significantly increased when there is hydrops fetalis[315], a situation mirrored within the fetal myocardium[316]. Such an up regulation in these fetal organs is thought to be secondary to hypoxaemia. There is some evidence that VEGF-A, expressed in amnion and chorionic, may control the intra-membranous absorption of fluid across fetal membranes and be involved in amniotic fluid regulation, a process that is abnormal in TTTS[286 317]. However, measured plasma VEGF-A did not appear to be significantly different in uncomplicated monochorionic twin pregnancies as compared to

those complicated by TTTS. Conversely, the plasma level of VEGF-C (a factor believed to orchestrate lymphangiogenesis) was significantly lower in twin pregnancies complicated by TTTS, as compared to uncomplicated MC twin pregnancies.

These data demonstrate that maternal plasma concentrations of Ang-2 are increased in pregnancies complicated by TTTS compared to uncomplicated twin pregnancies, particularly uncomplicated DC twin pregnancies. Physiologically, Ang-2 has the potential to favour angiogenesis in response to hypoxaemic or inflammatory stimuli, if concentrations are high relative to concentrations of Ang-1 and the VEGF isoforms[318]. Of interest is the observation from this study that in those MC twin pregnancies with TTTS in which there was severe hydramnios (as defined by the maximum pool depth $\geq$ 12cm), the amniotic fluid concentrations of VEGF-C and Ang-1 are significantly reduced compared to those with a maximum pool depth between 8-12cms, an observation independent of the effect of gestational age. This is not thought to represent simply a dilutional effect as this would have been expected to affect all angiogenic factor comparisons. A previous study comparing amniotic fluid protein levels between non-TTTS controls and TTTS recipients (by definition affected by polyhydramnios) showed higher overall protein levels in controls implying some dilutional effect but showed changes in markers of cardiac dysfunction despite this effect[319], as is believed to have occurred here. When gross polyhydramnios is present, a reduction in uteroplacental blood flow and increased local hypoxaemia has been reported[167]. The observed changes in amniotic fluid VEGF-C and Ang-1 may be in response to this.

Abnormal placentation is associated with the development of excess rates of fetal loss, intrauterine growth restriction and maternal pre-eclampsia[320], with associated uteroplacental ischaemia and endothelial cell dysfunction[321]. The human placenta (and its underlying vasculature) appears to be a rich source of angiogenic factors, both the VEGF family[314 322], the angiopoietins[323 324] and their respective receptors. Circulating plasma concentrations of angiogenic factors and the soluble VEGFR-1 are increased from early gestation, in at risk pregnancies, preceding the development of pre-eclampsia[85 105 325], intrauterine growth restriction[101 105 326 327] and even fetal demise[108 328]. In addition, decreased plasma concentrations of sTie-2 have been reported in pregnancies complicated by pre-eclampsia and intrauterine growth restriction[102], also implicating the angiopoietin/Tie system in the processes related to abnormal placentation. The association of an anti-angiogenic process in severe IUGR appears strongest when abnormal blood flows, in both the uteroplacental and fetoplacental circulation, are noted[105].

Multiple pregnancies, and in particular monochorionic twin pregnancies, have significantly increased rates of pregnancy complications, especially pre-eclampsia and intrauterine growth restriction[6 98]. In uncomplicated twin pregnancies, an elevation in maternal serum sVEGFR-1/PlGF ratio and sVEGFR-1 concentrations has been noted above singleton pregnancies[98 329]. Expression studies have indicated that it is the relative size of the placenta (compared to singletons) rather than an angiogenic response to relative hypoxaemia, which was responsible for this finding[98]. However, expression of placental sVEGFR-1 is further increased if the twin pregnancy is complicated by intrauterine growth restriction[330]. The effects of chorionicity are

unreported however, in TTTS, the placental size is significantly larger than in an uncomplicated monochorionic twin pregnancy[331]. In this study there was no difference demonstrated between any of the circulating angiogenic factors or their receptors between uncomplicated dichorionic and monochorionic twin pregnancies at mid-gestation, although it is conceded that the sample sizes are small, making a type 2 statistical error possible.

Kusanovic and colleagues[110] have investigated maternal plasma circulating angiogenic factors PlGF and sVEGFR-1 in a cohort of monochorionic twin pregnancies complicated by TTTS (n=16). Of this cohort 50% (8/16) had relatively mild TTTS with stage I/II disease. Furthermore, in one of the other eight twin pregnancies, a co-twin death had already occurred (a process observed to increase sVEGFR-1 concentrations, irrespective of the underlying pathology)[108]. It was noted that there was a higher median plasma concentration of sVEGFR-1 and a significantly lower PlGF concentration in the monochorionic twin cohort complicated by TTTS and the authors postulated that TTTS is associated with an 'anti-angiogenic state'. This study has replicated their findings in relation to sVEGFR-1 and PlGF so could be taken to support this assertion. More specifically, this study has shown that the balance of angiogenic factors i.e. higher angiogenin 2 and soluble vascular endothelial growth factor receptor 1 appears to favour angiogenesis in response to hypoxia or ischaemia in TTTS.

This study also investigated changes in maternal (and amniotic fluid) angiogenic factors in response to fetoscopic laser ablation, the treatment that appears to most consistently modify outcome in TTTS[52]. This process allows the prospective identification of

pathological arteriovenous anastomoses linking the cotyledonary circulations of both twins and the subsequent ablation of this placental abnormal angioarchitecture. The process inevitably causes some secondary trophoblast destruction and increased permeability between the maternal/fetal circulations[141]. However, these data indicate that changes in maternal (and amniotic fluid) angiogenic growth factor concentrations in response to fetoscopic laser ablation are modest, if they occur at all. They also appear to be independent of the number of AVA ablated, the amniotic fluid volume removed at the end of the procedure and as to the outcome of the pregnancy, in terms of fetal survival.

Such data further aids understanding of the pathogenesis of the condition of TTTS, the responses to treatment and the relation to outcome. These data support that anti-angiogenic activity is increased in severe TTTS.

4.2.3 Maternal cell-free mRNA for angiogenic growth factors

Analysis of plasma fetal DNA can indicate the presence and concentration of fetal genetic material in the circulation[111]. In addition, plasma fetal RNA can impart valuable clues as to the gene expression patterns of fetal tissues[123]. These data demonstrate that maternal cf- mRNA could be reliably detected for VEGF-A, Endoglin, PlGF, Ang-1 and Ang-2 in twin pregnancies. This represents a broader range of cf-mRNA encoding genes than previously reported in twin pregnancies. Furthermore, a significant difference in VEGF-A, Endoglin and Ang-2 was demonstrated between uncomplicated twins and MC twin pregnancies complicated by TTTS.

Angiogenesis and trophoblast/vascular interaction play an important role in human placentation[75]. There is accumulating evidence that angiogenic factors, including those of the VEGF family, PlGF, TGF- β1 and the angiopoietins orchestrate materno-fetal circulatory interaction within the haemochorial placenta[81 83 314]. There is some evidence that VEGF-A, expressed in amnion and chorion, may control the intra-membranous absorption of fluid across fetal membranes and be involved in amniotic fluid regulation, a process that is abnormal in TTTS[286 317]. The finding of elevated maternal cf-mRNA VEGF-A in TTTS, in comparison with uncomplicated twin pregnancies (particularly in comparison with uncomplicated DC twin pregnancies), supports this conclusion. As an antagonist ligand for Tie-2, Ang-2 plays a role in dilatation of vessels and disruption of vessel integrity[79 80]. Physiologically, Ang-2 has the potential to favour angiogenesis in response to hypoxemic or inflammatory stimuli, if concentrations are high relative to concentrations of Ang-1 and the VEGF isoforms[318]. As TTTS is a condition associated with abnormal placentation, and there may be link with relative ischemia and/or hypoxemia, this could explain our finding of increased levels of cf-mRNA Ang-2. The finding of increased Eng in TTTS is in keeping with previous work that has suggested that TTTS may be a relatively anti-angiogenic state[110], as it is associated with endothelial dysfunction[332] and its soluble form is known to inhibit the important role of TGF- β1 in trophoblast differentiation[81].

The number of twin pregnancies, complicated and uncomplicated, was relatively small but for certain cf-mRNAs significant differences were noted. However, the possibility that this represents a type 1 statistical error cannot be excluded. It is also acknowledged that there are inherent difficulties in measuring fetal nucleic acids in the

maternal circulation and this is a field of ongoing advancement. It is however, widely accepted that the placenta is the predominant source of fetal nucleic acid in the maternal plasma but that fetal nucleic acids only accounts for on average 3-6% of the cell-free nucleic acid present[333]. For this reason the method used to detect this subpopulation of cell free nucleic acid must be able to differentiate this from the background maternal nucleic acid[333]. Initially this was done by targeting genetic markers on the Y chromosome, but this was therefore only applicable in male fetuses. More recently several techniques have been developed to make prenatal nucleic acid evaluation relevant to all pregnancies. As the size of fetal nucleic acid fragments is known to be smaller than maternal[334], this size difference has been used to allow selectively enrichment of fetal nucleic acid[335]. The main limitations of this approach are that the degree of enrichment possible is only moderate, and that the manipulations involved are liable to external contamination[333]. Fetal epigenetic markers have also been used where methylation status differs between the fetus and the mother, but one marker tried[336] involved techniques that can lead to destruction of a large proportion of the treated DNA[337]. Due to these limitations the use of plasma RNA markers has also been explored after the discovery of placental specific mRNA[120 338]. Reverse transcriptase PCR also has potential problems in quantitatively measuring RNA. These include the need for a specific, sensitive and reproducible assay[339]. Firstly, the use of mRNA-specific primers, as in this study, seeks to restrict the likelihood of background genomic DNA amplification[339]. Secondly, there is a need to correct for any inter-sample variation by the use of an internal standard against which RNA values can be normalised[339]. To find a reliable internal standard for this study experience from previous studies[176-178] was utilised and after investigating a number of genes the one

with the least variation was chosen. With a view to needing a method that was consistent Taqman was chosen as this has been shown to have a very low coefficient of variation[340]. It was for these reasons that the analysis of fetal mRNA was conducted at a laboratory with proven expertise and reputation for previous work in this area. A large prospective study measuring angiogenic factors in the late first trimester in all monochorionic twins, who could then be followed up to assess which were complicated by TTTS, would help to clarify the diagnostic possibilities of these plasma tests.

Overall these findings aid the further understanding of the pathophysiology of the morbid condition of TTTS. In addition, if the alterations in maternal cf-mRNA precede the onset of clinically apparent disease, they may be useful as an adjuvant blood test to complement first trimester ultrasound screening.

4.2.4 Cytokines

Cytokines are known to be expressed in the placenta, decidua, and fetal membranes during normal pregnancy and are considered integral to the establishment and function of the placental/maternal interface[129]. Disruption to cytokine balance has been implicated in conditions associated with failure of trophoblast invasion as well as placental hypoxia and vascular changes within the placenta, such as those seen in recurrent miscarriage, intrauterine growth restriction and pre-eclampsia[129 130]. This study is the first to report a comprehensive range of cytokines in relation to severe TTTS, as well as the effect of treatment by FLA. In addition, by the use of comparison groups of uncomplicated DC and MC twins it was found that TTTS is associated with minimal differences in cytokine levels. Treatment by FLA did not cause a significant

change in maternal plasma cytokine concentrations. However, the amniotic fluid concentration of cytokines was significantly higher than those in maternal plasma in TTTS, for the TH1 inflammatory cytokines IFN-γ, IL-1β, IL-6, TNF-α; the TH2 anti-inflammatory cytokines IL-4, IL-10; the chemokine IL-8; as well as TIMP-1 and ICAM-1.

The inclusion of both uncomplicated DC and MC twins in the basal comparison allowed examination of whether changes seen in TTTS were specific to this complication. TH1/TH2 cytokines were not significantly different in maternal plasma between the cases of uncomplicated MC and DC twins and those affected by severe TTTS. This is in keeping with Huber's study, which looked at AF IL-6, comparing TTTS pregnancies treated with laser with singleton pregnancies undergoing amniocentesis for karyotyping[138], and did not find a significant difference either. Only two cytokine related markers showed significant differences when uncomplicated MC and DC twins were compared with MC twins complicated by TTTS: these were PDGF-BB and TIMP-1. PDGF-BB is known to have a role in regulating cell growth and division[341] as well as vessel stabilisation[168] and was higher in twins with a DC placenta than those with a MC placenta (DC>TTTS>uncomplicated MC). An increase in PDGF-BB has been observed in pregnancy-induced hypertension in association with diminished vascular remodelling[168]. It is possible that there may be less vascular modelling in the DC than MC placenta, although given the association of vascular connections within the TTTS placenta[17] one may have expected this level to be lowest, and as this finding only just reached significance further work is needed to evaluate the role of PDGF-BB. TIMP-1 is a member of the tissue inhibitors of metalloproteinase family known to modulate matrix metalloproteinase activity and suppress extracellular matrix turnover[342]. It also

acts directly to inhibit cell growth and possibly induce apoptosis[342]. The finding that TIMP-1 is higher in maternal plasma in TTTS than in uncomplicated twin pregnancies suggests usual cell proliferation and turnover may be disordered in TTTS. Further work to evaluate the relevance of TIMP-1 to a causative mechanism would require a larger cohort, and possibly a broader range of members of the TIMP and MMP family, to be studied in MC twins prior to the onset of TTTS.

In terms of sub-group analysis, there were modest changes. When considering the cytokines found to be significantly different when severity of polyhydramnios was considered it was evident that a more severe presentation was associated with lower concentrations of cytokines .e.g. recipient twin with polyhydramnios ≥12cm: maternal plasma ICAM-1 lower; AF IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, IL-13, KGF, FGF-basic, and TIMP-1 lower. As this level of polyhydramnios is associated with a reduction in uteroplacental blood flow and increased local hypoxemia[167] it is possible that placental expression of cytokines is also reduced as a result. With respect to EFW difference the trend for severity being associated with lower levels was continued but only AF ICAM-1 was significantly different. Increased AF ICAM-1 has been suggested to be associated with preterm birth[343] but has not been studied in TTTS before. With respect to the severity of haemodynamic changes, i.e. whether recipient cardiac dysfunction was absent or present, only maternal plasma PDGF-BB showed a significant difference, and this was higher. Higher PDGF-BB has been associated with a reduction in vascular modelling[168] and so we suppose that this may be contributing to worsening haemodynamics in TTTS. However, we consider these relatively isolated findings are currently of uncertain significance.

All cytokines, with the exception of PDGF-BB, were found in higher concentrations in amniotic fluid prior to FLA than maternal plasma. This difference was significant for the TH1 inflammatory cytokines IFN-γ, IL-1β, IL-6, TNF-α; the TH2 anti-inflammatory cytokines IL-4, IL-10; the chemokine IL-8; as well as TIMP-1 and ICAM-1. This is in keeping with a previous study describing the use of multiplex assays of inflammatory markers for analysis of different types of sample[344]. It is known that the human placenta is a rich source of cytokines, as are the decidua and fetal membranes[129]. This may explain the higher concentrations in amniotic fluid compared to maternal plasma. The lack of IL-8 in plasma is also of interest. As IL-8 is thought to promote angiogenesis[121], given the likelihood that TTTS is associated with anti-angiogenic activity[110 163] its absence in TTTS is perhaps not surprising; however it absence, despite ELISA testing, in uncomplicated twins in this study and previous reports of higher levels in conditions associated with abnormal placentation, such as pre-eclampsia[300], suggests this is an area that merits further study.

There were no significant differences found in plasma cytokine levels before and after FLA. KGF was the only amniotic fluid marker to change and this was lower after FLA. KGF is an epithelial growth factor that is known to be expressed by endometrial epithelium and is thought to modulate PLAC1 (a trophoblast-specific gene) to promote trophoblast growth or differentiation[345]. It may also be involved in regeneration of the endometrium[346], as well as the interaction between the decidua and chorion in early pregnancy[347]. It has also been studied in relation to preterm birth but no significant difference found between those delivering preterm or term[343]. Animal studies have investigated its role in lung abnormalities in relation to prematurity[348 349]. There is some

evidence that KGF may protect against the development of pulmonary hypertension[349]. Given the possibility that TTTS may be associated with abnormal placentation, a process known to be associated with abnormal trophoblast invasion, it would be interesting to study the role of KGF in TTTS further, including the significance of the change in amniotic fluid levels noted.

FLA does not appear to be associated with significant trophoblast destruction[141] and this may explain why cytokines levels in maternal plasma and amniotic fluid are not affected by such treatment. The only previous study investigating cytokines in TTTS treated by fetoscopy measured IL-6 levels and their relation to very preterm birth but did not find any association in this cohort of 166 MC pregnancies complicated by TTTS[139]. As TTTS is associated with neurological morbidity in surviving infants, whether treated or not, it is important to investigate whether cytokine levels increased post treatment. It was particularly reassuring that amniotic fluid levels of inflammatory cytokines previously shown to be associated with cerebral palsy (IL-1β, IL-6 and TNF-α)[132] did not change. However, it cannot be assumed that such an association is causal. It is acknowledged that amniotic fluid samples were collected immediately after FLA and there may be a greater time lag before changes in cytokine levels are apparent. However, ethical constraints prevented us from obtaining later amniotic fluid samples and we have obtained maternal plasma samples up to one week to provide as detailed information as possible with regard to the effect of FLA.

This study reported the use of predominantly multiplex assays, which have the advantage of giving a broad picture of the immune response. It is noteworthy that this

study found TTTS is associated with minimal changes in cytokine levels when compared to uncomplicated twins, although the majority of cytokine levels were higher in amniotic fluid than maternal blood. Through the use of assays providing sensitivities for cytokines akin to levels reported by previous studies[138 139 350 351] it does not appear that FLA provokes a significant cytokine response. It is acknowledged that where differences were not found with multiplex assays it may be worthwhile conducting ELISA testing to confirm these findings and that the numbers of twin pregnancies studied was small so a type 2 error is possible.

This study provides a novel insight into cytokines in relation to TTTS and some reassurance regarding treatment with FLA. To shed further light on the pathophysiology of TTTS it would be useful to measure cytokines in a large cohort of MC twins prior to the onset of TTTS.

4.3 Conclusions

CRL and NT measurement in the first trimester is routine practice in twin pregnancies. Calculation of CRL discordance of ≥ 10% is the best screening tool for TTTS, with nuchal translucency > 95th percentile and calculation of nuchal translucency discordance of ≥ 20% also of value. These tests are useful for showing those at high risk of TTTS but all MCDA twins still need regular follow up as a negative test does not exclude TTTS. Donor DV abnormality showed moderate predictive accuracy for predicting donor IUD after diagnosis of TTTS and should be incorporated into routine assessment. Again a negative test does not ensure a good outcome and all TTTS pregnancies require regular follow up regardless of whether treated. FLA appears to offer both survival and morbidity advantages over serial amniodrainage in the treatment of TTTS. Unfortunately, TTTS remains a high-risk condition with considerable morbidity despite treatment.

In terms of the pathophysiology of TTTS the changes seen in relation to markers of placental destruction and angiogenesis support the understanding that TTTS is one of a number of conditions associated with abnormal placentation. It seems that in TTTS the balance of angiogenic factors appears to favour angiogenesis in response to hypoxia or ischaemia however, it is associated with minimal changes in cytokine levels. FLA appears to be associated with transplacental haemorrhage rather than trophoblast destruction. Interestingly, FLA does not seem to provoke much, if any, response in angiogenic growth factors or cytokines.

4.4 Recommendations for future research in TTTS

Ultrasound tests in the first trimester seem to be of value in screening for TTTS. However, further research to evaluate the use of a combination of tests, such as the both CRL and NT discordance, may be able to improve on the current sensitivity of individual tests. This supposition could also relate to ultrasound tests to predict outcome of TTTS after diagnosis. There are currently no tests that can reliably predict either a good or poor outcome however, any combination test utilised in further research should include DV assessment as this was the test that showed the most promise.

In terms of treatment for TTTS it seems unlikely future randomised trials to evaluate FLA and serial amnioreduction would be funded, given that both RCTs were stopped early. It appears that FLA does offer survival and morbidity advantages but individual patient data analysis could increase clinicians' certainty. Although some authors were not able to provide data for this review it has been indicated that with further time and the possibly the establishment of a collaborative group of experts in this area the difficulties encountered could be overcome.

With respect to the work on biomarkers in TTTS several exciting prospects in terms of angiogenic growth factors and markers of placental function have been highlighted. Further research should focus on conducting a large prospective cohort study in monochorionic twins in the first trimester. This would allow biomarkers to be measured prior to the onset of TTTS and noting any differences evident in twins that went on to develop TTTS in comparison with those that did not. This would test the hypothesis

that the changes noted in this thesis are part of the pathogenesis of the condition. This study could employ both individual ELISA and multiplex assay testing. If biomarker changes are confirmed to pre-date the onset of clinical disease, as this is diagnosed on ultrasound, these biomakers could be incorporated into first or early second trimester testing utilising ultrasound, more specifically CRL and NT, to produce a combination test with greater sensitivity and specificity than ultrasound alone.

APPENDICES

Appendix 1

Search strategy for reviews

1. Twins/ or Diseases in Twins/
2. Fetofetal Transfusion/
3. fetofetal transfusion.mp. or Fetofetal Transfusion/
4. Twin Oligohydramnios-Polyhydramnios Sequence.mp.
5. twin to twin.mp.
6. feto fetal.mp.
7. twin twin.mp.
8. feto feto.mp
9. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8
10. limit 9 to "therapy (sensitivity)"
11. limit 9 to "diagnosis (sensitivity)"
12. limit 9 to "prognosis (sensitivity)"
13. 10 or 11 or 12
14. limit 13 to animals
15. 13 not 14

Appendix 2

Study selection and data extraction form for diagnostic accuracy of ultrasound in the first trimester for the prediction of which MC twin pregnancies will be affected by TTTS.

Reviewer ID …… **Date** …… **Paper no** ……

Year of publication ……... **Language** ……. **Region** ……...

Selection or rejection (must have all Y)

a) Population – (pregnant with MC twins) Y/N
b) Index test: Y/N
c) Reference test/ outcome measure: Y/N
d) 2x2 table possible: Y/N

Select this study? Y/N If this is Y – complete this form

If N must describe why…….……

Data extraction

<u>Population</u>

Total number of patients/fetuses recruited (n)/(n) (……)/(…….)

Confirmation of chorionicity Y/N

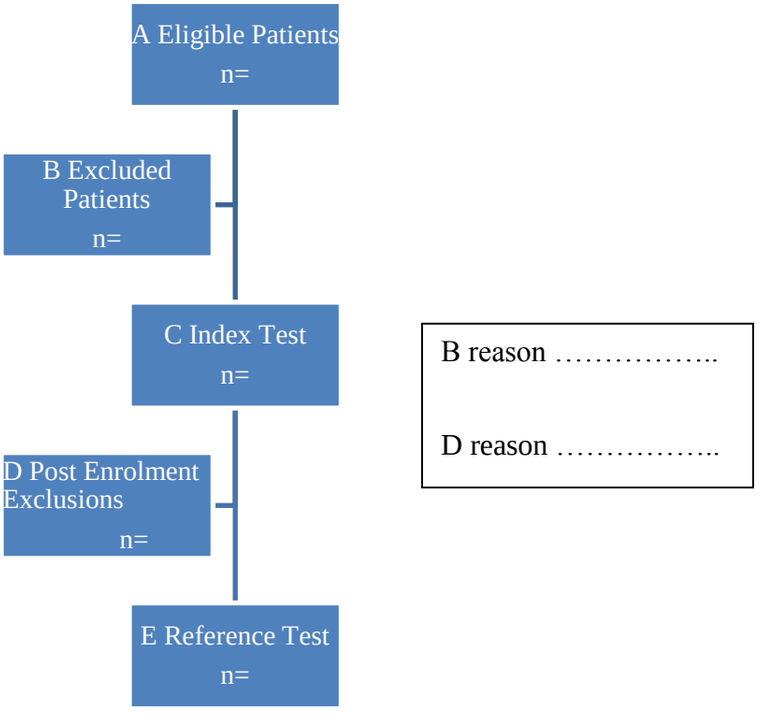
Method described Y/ N ……

<u>Study Design</u>

RCT/ Controlled observational study/ Uncontrolled study

Data collection retrospective/prospective/unreported/other

Patient enrolment consecutive/arbitrary/unreported/other



Outcomes

Complete follow up: yes/ no/ can't tell

Percentage of follow up **(>90%; 81-91%; <81%)** n (…….)/ % …….

Comments on data available:

……

Index Test

Describe the interventions for which this form is being used

……

Description of test: **a)complete** **b) incomplete c) can't tell**

Gestational age at test **i) average** **(…….)wks**

ii) range **(…….)wks**

Timing of measurement ……

Method of measurement ……

No of operators/experience ……

Reference standard/ outcome

Measured blind from diagnostic test yes/ no/ can't tell

Reference standard used ……

Threshold ……

Dataset used to establish threshold ……

Timing of measurement

Results

Population:	**Reference Test:** **Threshold:**			
Index test, Measurement:		Positive	Negative	**Total**
Threshold:	Positive	**TP**	**FP**	
	Negative	**FN**	**TN**	
	Total			

Other Information (ie. other statistics, measures of uncertainty etc)

Population:	**Reference Test:** **Threshold:**			
Index test, Measurement:		Positive	Negative	**Total**

Threshold:	Positive	**TP**	**FP**	
	Negative	**FN**	**TN**	
	Total			

Other Information (ie. other statistics, measures of uncertainty etc)

Population:	**Reference Test:** **Threshold:**			
Index test, Measurement:		Positive	Negative	**Total**
Threshold:	Positive	**TP**	**FP**	
	Negative	**FN**	**TN**	
	Total			

Other Information (ie. other statistics, measures of uncertainty etc)

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			

Absent			
Total			

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			
Absent			
Total			

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			
Absent			
Total			

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			
Absent			
Total			

Appendix 3

Study selection and data extraction form for diagnostic accuracy of ultrasound in the prediction of outcome of TTTS after diagnosis.

Reviewer ID …… **Date** …… **Paper no** ……

Year of publication ……... **Language** ……. **Region** ……...

Selection or rejection (must have all Y)

a) Population – (pregnant with MC twins and features of TTTS) Y/N
b) Index test : Y/N
c) Reference test/ outcome measure: Y/N
d) 2x2 table possible: Y/N

Select this study? Y/N If this is Y – complete this form

If N must describe why…….……

Data extraction

<u>Population</u>

Total number of patients/fetuses recruited (n)/(n) (……)/(…….)

Confirmation of chorionicity Y/N

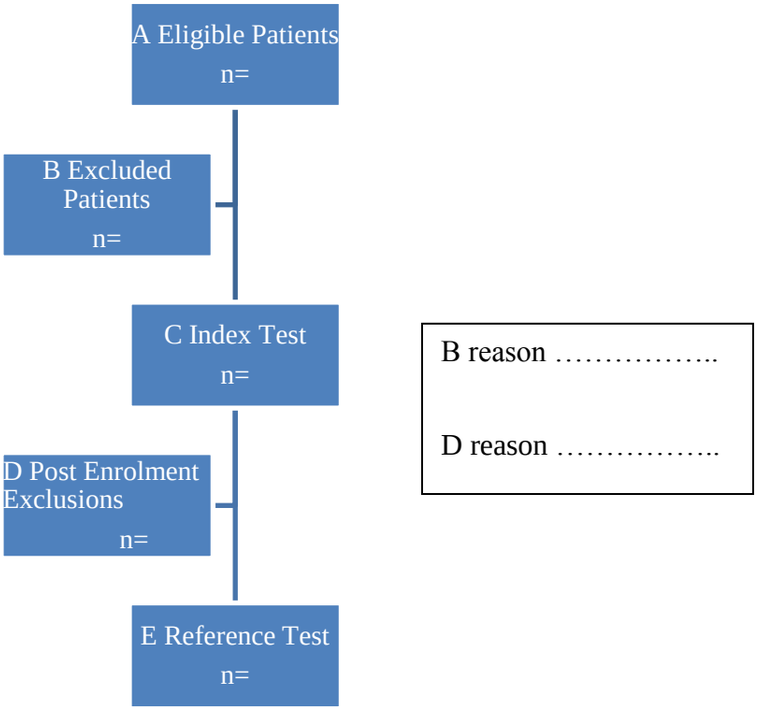
Method described Y/ N …….

<u>Study Design</u>

RCT/ Controlled observational study/ Uncontrolled study

Data collection retrospective/prospective/unreported/other

Patient enrolment consecutive/arbitrary/unreported/other



Outcomes

Complete follow up: yes/ no/ can't tell

Percentage of follow up **(>90%; 81-91%; <81%)** n (…….)/ % …….

Comments on data available:

……

Index Test

Describe the interventions for which this form is being used

……

Description of test: **a)complete** **b) incomplete c) can't tell**

Gestational age at test **i) average** **(…….)wks**

ii) range **(……)wks**

Timing of measurement ……

Method of measurement ……

No of operators/experience ……

Reference standard/ outcome

Measured blind from diagnostic test yes/ no/ can't tell

Reference standard used ……

Threshold ……

Dataset used to establish threshold ……

Timing of measurement

Results

Population:	**Reference Test:** **Threshold:**			
Index test, Measurement:		Positive	Negative	**Total**
Threshold:	Positive	**TP**	**FP**	
	Negative	**FN**	**TN**	
	Total			

Other Information (ie. other statistics, measures of uncertainty etc)

Population:	**Reference Test:** **Threshold:**			
Index test, Measurement: **Threshold:**		Positive	Negative	**Total**
	Positive	**TP**	**FP**	
	Negative	**FN**	**TN**	
	Total			
Other Information (ie. other statistics, measures of uncertainty etc)				

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			
Absent			
Total			

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			

Absent			
Total			

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			
Absent			
Total			

Specify outcome ……..

	Test 1 ……	Test 2 ……..	Total
Present			
Absent			
Total			

Specify outcome ……

	Test 1 ……	Test 2 ……..	Total
Present			
Absent			
Total			

Appendix 4

Study selection and data extraction form for the review of effectiveness of FLA and serial amnioreduction in the treatment of TTTS.

Reviewer ID …… **Date** …… **Paper no** ……

Year of publication ……... **Language** ……. **Region** ……...

Selection or rejection (must have a) Y and b) two Y)

a) Population – (pregnant with MC twins and features of TTTS) Y/N
b) Interventions
   i. Amnioreduction Y/N
   ii. Laser Y/N

Select this study? Y/N If this is Y – complete this form

If N must describe why rejected

……

Data extraction

Population

Total number of patients/fetuses recruited (n)/(n) (…….)/(…….)

Data collection retrospective/prospective/unreported/other

Patient enrolment consecutive/arbitrary/unreported/other

Confirmation of chorionicity Y/N

Method described Y/ N …….

Interventions

Describe the interventions for which this form is being used

……

Description of interventions: **a)complete** **b) incomplete** **c) can't tell**

Gestational age at intervention **i) average** **(…….)wks**

ii) range **(…….)wks**

Outcomes

Complete follow up: yes/ no/ can't tell

Percentage of follow up (>90%; 81-91%; <81%) n (…….)/ % …….

Blinding of intervention from outcome yes/ no/ unreported

Comments on data available:

……

Did anyone not get the intended treatment or who got more than one treatment and why

……..

Study Design

RCT/ Controlled observational study/ Uncontrolled study

Specify outcome … SURVIVAL- overall

	Intervention 1 ……	Intervention 2 ……..	Total
Present			
Absent			
Total			

Specify outcome …SURVIVAL- of at least one

	Intervention 1 ……	Intervention 2 ……..	Total
Present			
Absent			
Total			

Specify outcome …SURVIVAL- donor

	Intervention 1	Intervention 2	Total

	……	……..	
Present			
Absent			
Total			

Specify outcome …SURVIVAL- recipient

	Intervention 1 ……	Intervention 2 ……..	Total
Present			
Absent			
Total			

Specify outcome ……

	Intervention 1 ……	Intervention 2 ……..	Total
Present			
Absent			
Total			

Appendix 5

Studies excluded from the review diagnostic accuracy of ultrasound in the first trimester for the prediction of which MC twin pregnancies will be affected by TTTS.

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Appendix 6

Studies excluded from the review of the diagnostic accuracy of ultrasound after diagnosis of TTTS for the prediction of outcome.

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Appendix 7

Studies excluded from the review of the effectiveness of treatments for TTTS.

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Appendix 8

Morbidity, gestational age and birth weight data for review of effectiveness of FLA and serial amniodrainage for treatment of TTTS:

(where p values are not stated the differences are not statistically significant, p=0.05 taken to be significant)

RCT – inclusion after qualifying amnioreduction

Crombleholme et al

- Gestational age at delivery (wks) – Mean – laser 30.67; AR 30.29
- Delivery <32 wks – data not known
- Fetal morbidity – data not known
- Maternal morbidity –

    Mini-laparotomy in 7 cases in laser group to expose the surface of the uterus

    Uterine bleeding n=1 Laser (no maternal transfusion needed but procedure had to be stopped due to poor visualisation; n=0 AR

    Spinal headache n=1 (group not specified)

    Maternal hospitalisation (including for preterm labour, short cervix, preterm prelabour rupture of membranes (PPROM) or to monitor fetal growth) 9.5% each arm

    PPROM prior to 28/40 laser 4.8%, AR 0; requirement for additional tocolysis laser 0, AR 4.8%; delivery <28/40 laser 9.5% (n=2), AR 4.8% (n=1).

RCT – inclusion at diagnosis

Senat et al

- Gestational age at delivery (wks) – Median- laser 33.3; SA 29.0 p=0.004

- Delivery <32 wks – Laser 30/72 (41.7%); SA 48/70 (68.6%)

- Fetal morbidity

    Alive without major neurological morbidity at 6/12 overall- Laser 75/144 (52.1%); SA 44/140 (31.4%) p=0.003

    Alive without major neurological morbidity at 6/12 donor- Laser 36/72 (50%); SA 25/70 (357%) p=0.09

    Alive without major neurological morbidity at 6/12 recipient- Laser 39/72 (54.2%); SA 19/70 (27.1%) p=0.001

- Maternal morbidity

    Intra-abdominal fluid leak n=2 Laser; n=0 SA

    Abruptio placenta n=1 Laser; n=2 SA

Observational studies – inclusion at diagnosis

Middledorp et al (recruitment only after 26 weeks)

- Gestational age at delivery (wks) – Median - laser 31; SA 29

- Interval between intervention and delivery (days) – Median – laser 31, SA 9

- Birth weight (g) – Mean (SD) – laser 1615 (516); SA 1472 (634) p=0.43

- Fetal morbidity:

    Major neonatal morbidity (necrotising enterocolitis (NEC) grade III, chronic lung disease, terminal renal failure) – laser 0, SA 6 (27%) p=0.02

    Severe cerebral injury – laser 3 (15%), SA 5 (23%) p=0.7

    Adverse outcome (IUD, neonatal death, major neonatal morbidity or severe cerebral injury) – laser 3 (15%), SA 8 (36%) p=0.17

- Maternal morbidity:

Need for additional tocolysis – laser 2 (20%), SA 9 (82%) p=0.009

PPROM within 2 weeks of procedure – laser 0, SA 1 (9%)

Hecher et al

- Gestational age at delivery (wks) – Median/Range – laser 33.7 / 24.9-40.3; SA 30.7 / 27.7-37.3 p=0.018

- Interval between intervention and delivery (days) – Median (range) – laser 90 (2-134); SA 72 (27-131) p=0.022

- Birth weight (g)

Donor median (range) – laser 1750 (470-2960); SA 1145 (660-2660) p=0.034

Recipient median (range) – laser 2000 (460-3460); SA 1560 (870-2660) p=0.076

- Fetal morbidity:

Incidence of abnormal ultrasound findings in the brain in neonates Laser 5/89 (6%); SA 8/44 (18%) p=0.03

Quintero et al

- Gestational age at delivery (wks) – Median/range- Laser 32 / 16.7-40.3); SA 29 / 18.4-38 p=0.005

- Interval between intervention and delivery (wks) – Median/range- Laser 10.3 / 0-21.4; SA 6.9 / 0-19 p<0.001

- Mean birth weight (g)

Donor- laser 1781 $\pm$ 734; SA 1219 $\pm$ 644 p<0.001

Recipient- laser 1940 $\pm$ 773; SA 1612 $\pm$ 724 p=0.019

- Fetal morbidity:

Neurological morbidity (at least 1 fetus per pregnancy) Laser 4/95 (4.2%); SA 19/78 (24.4%) including 4 neurological damage both fetuses= total with neurological morbidity 23

Donor neurological morbidity Laser 3/65 (4.7%); SA 12/66 (18.2%) p=0.014

Recipient neurological morbidity Laser 1/71 (1.4%); SA 11/64 (17.2%) p=0.001

Intact neurological survival (≥1NN survivor per pregnancy, neither twin with neurological damage) Laser 75/95 (78.9%); SA 40/78 (51.3%) p<0.001

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