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# Variation in expression of fetal nucleic acids in maternal blood in pregnancies affected by congenital anomalies

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# Declaration of Author

I, Luisa F. Olaya A., declare that the contents of this thesis consist of original work carried out by the author unless otherwise stated and duly acknowledged. To the best of my knowledge no part of this thesis has been submitted in whole or in part for any other degree.

Luisa F. Olaya A.

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# Abstract

**Background:** The discovery of cell free fetal DNA has opened a new door for non-invasive prenatal screening and diagnosis. Other cell free nucleic acids, like microRNAs, may also be of value in detecting fetal anomalies. miRNAs (small non-coding RNAs) control developmental processes and may therefore be useful as biomarkers for fetal abnormalities and placental function. We aimed to develop the laboratory skills for reliable analysis of cell free miRNA and to collect some pilot data looking at variation in cell free fetal miRNA expression with fetal abnormality.

**Methods:** Optimisation studies were conducting collecting blood from normal pregnancies into three types of collection tubes (EDTA, STRECK and CITRATE) and keeping samples at $4^{0}$C or room temperature for time intervals ranging from 4 to 96 hours. Six miRNAs, previously defined as being placental specific, were measured using qRT-PCR. Samples were spiked with cell-miR-39 to allow normalisation. Further comparative studies were made between pregnancies affected by fetal abnormality and controls.

**Results:** Samples from 14 normal pregnancies (mean 21.3 weeks' gestation) were used for the optimisation experiments. There was very significant (up to 100 fold) variation in expression of all miRNAs between control specimens. Although there were no statistical significant differences in relative expression of miRNA between blood tubes, temperature or time of storage, there were trends suggesting that either STRECK tubes held at room temperature or EDTA tubes stored at $4^{0}$C showed less miRNA variation. Two miRNAs (miR-517 and miR-526) previously described as being placental specific could not be detected in any samples. Three miRNAs (miR-518, miR-520 and miR-525) that were previously described as being placental specific were also detected in plasma of three non-pregnant women; miR-525 appeared to be significantly up-regulated in pregnancy. Only one miRNA (miR-516) appeared to be both present and specific to pregnancy. miRNA expression from 16 normal (mean GA 22.8 weeks), 15 chromosomally abnormal (mean GA 13.6 weeks), 6 cardiac anomalies (mean GA 20.3 weeks) and 4 neural tube defects (mean GA 17.3 weeks) were compared. No significant differences in the relative expression of these six miRNAs were seen between control and anomalous pregnancies with the exception of significant up-regulation of miR-520 in two fetuses with hypoplastic left heart syndrome.

**Conclusion:** Optimisation studies suggest that cell free miRNAs are best extracted from either STECK (room temperature) or EDTA ($4^{0}$C) tubes and that plasma should be extracted within 72 hours. There appears to be significant variation in miRNA expression; some 'placental specific' miRNAs could not be detected at all and others are expressed in non-pregnant women. This variation may make quantitative comparison between normal and abnormal conditions difficult. Further work (using array or sequencing based technologies) is needed to determine whether specific miRNA signatures can be ascribed to individual congenital anomalies.

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# Dedication

I would like to dedicate this thesis to my family. Even though they were not present, they have supported me all along this path, especially to my little sister that has always helped and advised me and most importantly to my mother without whom nothing would be possible. I know that even from far away she was always with me.

Finally I would also like to dedicate this to my partner “cara de na”, your support during this whole process was priceless.

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# List of Abbreviations

| | |
|---|---|
| 1D | 1 day |
| 2D | 2 days |
| 3`UTR | 3` untranslated region |
| aCGH | Array comparative genomic hybridization |
| ACOG | American College of Obstetricians and Gynecologists |
| AFP | Alphafetoprotein |
| AGO | Argonaute |
| AGO2 | Argonaute 2 |
| B hCG | Beta Human chorionic gonadotropin |
| cffDNA | Cell free fetal DNA |
| cffmiRNA | Cell free fetal miRNA |
| CHD | Congenital heart defects |
| Cp | Crossing point |
| CRL | Crown Rump Length |
| Ct | Cycle threshold |
| CVS | Chorionic villus sampling |
| DS | Down syndrome |
| FGR | Fetal growth restriction |
| FISH | Fluorescent in situ hybridisation |
| FLT1 | fms-related tyrosine kinase 1 |
| GC | Guanine and cytosine |
| HT-qPCR | High-Throughput Quantitative PCR |
| ISH | In-situ hybridization |
| IUGR | Intrauterine Growth Restriction |
| miRNA | microRNAs |

| | |
|---|---|
| MPS | Multi-parallel sequencing |
| mRNA | messenger RNA |
| NGS | Next generation sequencing |
| NIPT | Non-invasive prenatal testing |
| NT | Nuchal translucency |
| NTD | Neural tube defects |
| PAPP-A | Pregnancy-associated plasma protein A |
| piRNA | PIWI-interacting RNA |
| PlGF | Placental growth factor |
| PP13 | Placental protein 13 |
| Pre-amp | Pre-amplification |
| pri-miRNA | primary miRNA |
| QF-PCR | Quantitative fluorescent PCR |
| qRT-PCR | Quantitative real time PCR |
| RISC | RNA-induced silencing complex |
| RPAH | Royal Prince Alfred Hospital |
| RT | Reverse-transcription |
| Rt | Room temperature |
| RT-PCR | Real time PCR |
| siRNA | Small interfering RNA |
| STR | Short tandem repeats, |
| T 21 | Trisomy 21 |
| T18 | Trisomy 18 |
| TGFβ1 | Transforming growth factor–β1 |
| TRBP | Tar RNA binding protein |
| Ts16Dn | Segmental trisomy 16 |
| uE3 | Unconjugated estriol |

# 1. Introduction

The process of prenatal diagnosis is most developed for chromosomal abnormality. It has traditionally involved population based screening to define a high-risk group. These women are offered invasive diagnostic testing to confirm the presence of an abnormality. Most screening processes are non-invasive, that is they do not place the pregnancy at risk. These screening tests commonly involve ultrasound and / or analysis of maternal serum proteins. Screening tests do not identify all affected cases and define some normal pregnancies as being high risk. In contrast, invasive tests, such as amniocentesis and chorionic villus sampling (CVS) are diagnostic, but the process of sampling amniotic fluid or placental tissue introduces a risk of fetal loss. Another limitation of invasive testing is the time taken to confirm the diagnosis – which may be a few weeks. During the last few years other non-invasive tools have been developed and introduced into clinical practice. These tests focus on direct identification of genomic abnormalities rather than on identification of surrogate ultrasound or surrogate markers. These tests are becoming more accessible to women and are being more widely used in clinical practice.

In 1997, Lo et al. demonstrated the presence of cell free fetal DNA (cffDNA) in maternal serum and plasma (Lo et al. 1997). This discovery provided the means for analysis of fetal genomic markers purely by collecting a maternal blood sample – effectively a non-invasive test that did not place the pregnancy at risk of miscarriage (Devaney et al. 2011; Lo et al. 1997; Legler et al. 2009). Further studies also demonstrated the presence

of fetal mRNA in the circulating plasma of pregnant women and this has also been shown to be potentially useful for the detection of fetal aneuploidies from maternal plasma (Ng et al. 2003; Ng et al. 2004). At present the commonest clinical application of this technology is screening for chromosomal abnormalities. There is, however, huge potential to evaluate other structural anomalies and placental function through investigation of cell free fetal nucleic acids circulating in maternal plasma.

Most recently, studies have shown that fetal microRNAs (miRNA) can also be detected in maternal plasma and can be used as molecular markers. An increase in some placental miRNAs in maternal plasma has been described in pregnancies affected by fetal growth restriction (FGR) (Mouillet et al. 2010) and there are differences in the expression of cell free fetal miRNA (cffmiRNA) in pregnant women with fetuses with neural tube defects (NTD) (Gu et al. 2012). The detection and quantification of these nucleic acids could play an important role as biomarkers for fetal abnormalities and placental function.

The main motivation for this project is to develop the first steps in the identification of cffmiRNA in maternal circulation that can provide us with more information about the state of the fetus. In the future, this knowledge would help us to make more informed decisions about the course of a pregnancy. We propose to identify differences in the expression of cffmiRNA in maternal plasma between normal pregnancies and a range of fetal abnormalities. We will study six known miRNAs that are associated with the placenta and will compare their expression in fetuses affected by trisomy 21, congenital heart defects (CHD) and NTD. We have also compared three different types of collection tubes to identify the best way of collecting and transporting samples for extraction of

small non-coding RNAs. This methodological optimization will help us in the identification of specific miRNAs to detect different abnormalities.

## 1.1. Prenatal testing

Most pregnant women undergo a number of different tests in early pregnancy which allow medical practitioners to define risks of common fetal abnormalities. These tests fall into two groups - screening and diagnostic tests, which help in the identification of congenital abnormalities and birth defects (aneuploidies, structural abnormalities and single gene disorders) (Latendresse & Deneris 2015; Jordan et al. 2013). These screening tests are typically offered to all pregnant women to identify a group of high-risk pregnancies. Diagnostic tests are then offered to the high risk group – limiting the risk of miscarriage from an invasive test to those that are considered to have a higher probability of being affected by the condition (Latendresse & Deneris 2015). Depending on these results women can consider their options and make informed decisions.

The time line for screening for chromosomal abnormalities is shown in Table 1-1. The Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) have published guidelines describing appropriate pathways for screening and diagnostic testing (RANZCOG 2015). Most women in Australia are offered combined first trimester screening – a test that uses both ultrasound and biochemical markers – at $11\text{-}13^{+6}$ weeks gestation (Snijders et al. 1998). Women who attend at a later stage are offered second trimester biochemical screening (between 14 and 20 weeks gestation) (Wald et al. 1988; Wald et al. 1999). Some women, deemed to be at high risk on the basis of family or obstetric history, may be offered a diagnostic test without initial screening.

Most screening tools define women as either being at high or at low risk – and the high risk cohort are subsequently offered invasive testing ( Table 1-1).

**Table 1-1.** Options for prenatal screening and diagnosis.

| | Test | Gestation age (weeks) | Measurements | Detection |
|---|---|---|---|---|
| **First trimester** | | | | |
| | **First trimester screening** | | | |
| **Combined first trimester screening (CFTS)** | Maternal age is take into account a priori to calculate the risk | | | |
| | Ultrasound | 11+0-13+6 | Crown Rump Length (CRL) | Defines viability, number of fetuses present and dates pregnancy. Aneuploidy and congenital abnormalities |
| | | | Nuchal translucency (NT) | |
| | Maternal serum | | Pregnancy-associated plasma protein A (PAPP-A) | Aneuploidy. Serum markers may also be deranged in pregnancies that develop pre-eclampsia and IUGR |
| | | | free B Human chorionic gonadotropin (free B hCG) | |
| | cfDNA screening | From 10 | cfDNA screening using maternal plasma | Aneuploidy |
| | **First trimester diagnostics** | | | |
| | CVS | 11-13 | | Aneuploidy and genetic mutations |
| | Amniocentesis | >15 | | Aneuploidies, NTD and genetic mutations |
| **Second trimester** | | | | |
| | **Second trimester screening** | | | |
| Quadruple test | Maternal age is take into acount a priori to calculate the risk | | | |
| | Maternal serum | 15-20 | alphafetoprotein (AFP)<br>BhCG<br>unconjugated estriol (uE3)<br>dimeric inhibin-A | Aneuploidy and congenital abnormalities |
| | Second trimester scan | 18-22 | Each chromosomal pattern has different syndromes that can be detected | Structural abnormalities<br>Markers of aneuploidy |

(Wald et al. 1988; Wald et al. 1999; Snijders et al. 1998; RANZCOG 2015)

Combined first trimester screening involves measurement of the ultrasound marker nuchal translucency thickness and the biochemical markers free BhCG and PaPP-A. Nuchal translucency and free BhCG are both typically increased in pregnancies affected

by Down syndrome. PaPP-A is typically decreased(Scott et al. 2009; Jaques et al. 2006). The risk for aneuploidy is computed by determining the background risk (based on maternal age, the gestational age of the pregnancy and a previous history of trisomic pregnancy) and then multiplying this by likelihood ratios derived through comparison of the absolute value of the measurement of these markers to known normal ranges (Snijders et al. 1998; Snijders & Nicolaides 1994; Royston & Wright 1998; Kagan et al. 2008). Some groups, such as the Fetal Medicine Foundation, have introduced other ultrasound markers, such as recognition of a hypoplastic (absent) nasal bone and / or reversed flow in the ductus venosus. These markers have been shown to increase the sensitivity and specificity of screening (Cicero et al. 2006; Maiz et al. 2012) These ultrasound and biochemical markers are also altered in trisomies 18 and 13 and in Turner syndrome – and can therefore be used to define risks for these chromosomal abnormalities in addition to trisomy 21.

Second trimester maternal serum screening involves measurement of AFP, hCG and oestriol (Wald – as above). This test is known as the triple test. Screening efficacy can be further improved by adding a fourth marker, Inhibin A (Wald et al. 1999). A triple or quadruple test can be offered from 14–20 weeks gestation and these tests have sensitivities of 65% and 75% for a fixed 5% false positive rate. The triple test typically gives risk results for trisomies 21 and 18 but not 13. The AFP is reduced in trisomic pregnancies, but will be raised in pregnancies that have a neural tube defect (Cuckle et al. 1987; Wald et al. 1988; Wald et al. 1977). Some algorithms also give risks for genetic conditions such as Smith Lemli Opitz or steroid sulfatase deficiency on the basis of a low oestriol (Schoen et al. 2003).

Both first and second trimester screening programs calculate risks for aneuploidy by taking the background (a priori) risk and adjusting this according to the markers that have been measured. If the risk is above a certain level (1 in 300 for combined first trimester screening) then the pregnancy is defined as being at high-risk for aneuploidy and an invasive diagnostic test is offered.

### 1.1.1. Invasive Prenatal diagnosis

Diagnostic molecular and cytogenetic tests require a sample of fetal or placental material. This can only be obtained by introducing a needle into the uterine cavity to 'capture' the sample. One of these tests, often considered to be the gold-standard, is amniocentesis. This test involves insertion of a very thin needle into the maternal abdomen and uterus under ultrasound control. Amniotic fluid that surrounds the fetus is collected and sent to the laboratory for analysis. The test is usually performed from 15 weeks' gestation (Shulman & Elias 1993). Fetal cells can either be isolated directly from the amniotic fluid, or the fluid can be cultured prior to analysis. (Shulman & Elias 1993; Norwitz & Levy 2013). The cellular content includes epithelial tissues that are in contact with the amniotic fluid (skin, bladder, lung, gastrointestinal tract). Cytogenetic analysis of this material therefore provides a sample that is considered to be very representative of the fetus. The sample can be contaminated with maternal cells – and the laboratory takes steps to identify this risk before issuing a result. The amniotic fluid sample can be analysed directly by, karyotyping, fluorescent in situ hybridisation (FISH), chromosomal microarray analysis or qfPCR to give rapid results (RANZCOG 2015). This result is normally restricted to chromosomal groups that commonly develop aneuploidy (21, 18, 13, X and Y). The remaining part of the sample is cultured and the karyotype is traditionally reported by staining and cytogenetic analysis but more recently this may

involve a molecular (comparative genomic hybridisation) technique. There is a 0.5-1% chance of finding mosaicism that may require further testing. A recent meta-analysis suggests that amniocentesis has a ≈1:900 risk of miscarriage (if is performed by a experience operator), although earlier RCTs suggested this was closer to 0.5%-1.0%, which is why amniocentesis is only recommended for high-risk pregnancies (Akolekar et al. 2015; Alfirevic et al. 2003; RANZCOG 2015)

Another invasive method of testing is chorionic villus sampling (CVS). This test is typically performed at earlier gestations - from 11 weeks gestation (Norwitz & Levy 2013; RANZCOG 2015) and is consequently the test of choice after early, combined first trimester screening. The sample for this test is taken from the chronic villus (the developing placenta). This sample of tissue can be aspirated with a needle or a catheter and is collected through either an abdominal or vaginal approach respectively. In the cytogenetic lab the sample is first dissected and cleaned (to avoid maternal cell contamination) to isolate the chorionic villi (Shulman & Elias 1993; Alfirevic et al. 2003). A 'direct prep' can be used for karyotyping, fluorescent in situ hybridisation (FISH), chromosomal microarray analysis or qfPCR and this is then followed by a culture used to confirm the initial findings and to examine all chromosomes rather than just 21, 18, 13, X and Y. Like amniocentesis, CVS can cause miscarriage – and there is approximately the same level of risk of fetal loss (Towner et al. 2007; Carter 1976). CVS has the advantage that it is performed earlier – potentially providing an earlier test result for decision making about termination of pregnancy or earlier reassurance in the event of a normal result. The prevalence of placental mosaicism is higher in CVS samples (1-2% rather than 0.5-1.0% for amniocentesis) – this can lead to more dilemmas about ongoing

management and the need for further testing (Akolekar et al. 2015; Schrijver et al. 2007).

A third method of sampling the feto-placental unit is cordocentesis (fetal blood sampling). This sample is taken by aspiration from the umbilical cord using a thin needle under ultrasound guidance. This can only be performed once the umbilical vein is of sufficient diameter to allow access – and is therefore typically reserved for karyotyping at later stages of pregnancy - after 20 weeks gestation (Tongsong et al. 2000). In this test there is no need for culture so results are available rapidly. This was a major advantage before the development of FISH and qfPCR as an amniocentesis performed at 20 weeks gestation would take 2-3 weeks to culture before a result was available. Because the test is also evaluating a different cell line (fetal lymphocytes) it may also, be useful if an amniocentesis has demonstrated a risk of mosaicism. On the other hand, the risk of fetal loss is slightly higher than for the other diagnostic tests (2%) (Tongsong et al. 2001).

Many laboratories have recently moved away from cytogenetic testing to use a molecular based approach. Similar molecular tools are used for all sample types, and include quantitative fluorescent PCR (QF-PCR), that is the quantification of specific DNA sequence (short tandem repeats, STR) with fluorescent primers (Atef et al. 2011; Badenas et al. 2010) and array comparative genomic hybridization (aCGH) which can detect unbalanced structural and numerical chromosomal abnormalities (Van den Veyver et al. 2009; Filges et al. 2012). The resolution of an array is approximately a hundred times higher than that of traditional cytogenetic analysis. The use of arrays has therefore led to identification of a number of pathological deletions / duplications that could not previously be seen through prenatal testing. The technology has also identified

a number of genomic variants that have not previously been recognized and are of unknown significance – that can make counseling difficult. The additional information available through aCGH typing may present an ethical dilemma as to whether this extra information should be reported or not and as to whether this information is relevant to the fetus.

All invasive tests introduce a risk of fetus loss, and most of them take more than a week to provide comprehensive results. With the isolation of fetal cells from maternal blood (Ross et al. 2000) and with the discovery of cell-free fetal DNA (Lo et al. 1997) in maternal circulation there have been advances in non-invasive prenatal screening and diagnosis that potentially present no risk to the fetus or the mother.

### 1.1.2. Non-invasive prenatal diagnostic (NIPT)

In 1997, Lo et al. demonstrated the presence of cell free fetal DNA (cffDNA) in maternal serum (Lo et al. 1997). During pregnancy, in the intervillous space of the placenta, maternal blood is in continuous contact with the trophoblast. This allows the exchange of oxygen and nutrients between the mother and the fetus. The placenta remodels continuously through apoptosis and cellular debris is released into the maternal circulation. This is recognized as cffDNA (Tjoa et al. 2006). The half life of cffDNA appears to be very short – so that no fetal load is detectable within two hours of the mother's placenta being delivered (Lo et al. 1999; Rijnders et al. 2004; Smid et al. 2003). Maternal plasma contains fragments of both maternal and fetal DNA. Approximately 10% of the cell free DNA load is fetal rather than maternal in origin – a significantly higher load than is seen when looking for fetal cells in the maternal circulation (Chiu et al. 2011; Simpson & Elias 1993). The relative concentration of cffDNA increases with

gestational age and is also affected by maternal weight, fetal (and placental) number, placental volume, and aneuploidies (Rava et al. 2014; Everett & Chitty 2015; Ashoor et al. 2012; Lo et al. 1998; Wang et al. 2013; del Mar Gil et al. 2014).

Circulating cell-free fetal DNA is considered to provide a valuable means of identifying genomic anomalies in the fetus. Tests have been developed that identify the presence of fetal genes not present in the mother (Rhesus D), the presence of genetic mutations that are paternally inherited (both X linked and autosomal) (Amicucci et al. 2000; Meaney & Norbury 2009; Saito et al. 2000) and cell free DNA testing is essentially diagnostic in these circumstances. Cell free DNA can also be examined to identify fetal aneuploidy – but should be considered as a highly sensitive and specific screening tool rather than a diagnostic tool. Other studies have suggested that there is an increase in the amount of cffDNA in the maternal circulation in pregnancies impacted by placental pathologies – including complications like pre-eclampsia (Leung et al. 2001; Lo et al. 1996) and placenta praevia (Sekizawa et al. 2002). An association between increased cffDNA in the maternal plasma and parturition (Phimister & Phillippe 2014) has also being hypothesized: When a pregnancy gets to term and the placenta is mature there is a greater release of cffDNA that activate leukocytes and macrophages that lead to parturition.

Identifying and quantifying very small amounts of cffDNA is a challenging process. There are now a number of technologies available. One example, next generation sequencing (NGS) counts millions of fragments of DNA and relatively small differences in the amounts of cffDNA can be identified in cases where there is fetal trisomy or monosomy. With this approach the whole genome found in the maternal plasma is sequenced (i.e.

both maternal and fetal cell free components), then the sequences are aligned with the known genome and those that correspond to the chromosome being studied are counted and compared to reference chromosomes. Techniques like multi-parallel sequencing (MPS), has shown sensitivity and specificities close to 100% in almost all studies that have examined cases affected by trisomy 21, (Webb et al. 2012; Fan et al. 2008; Palomaki et al. 2012; Dan et al. 2012) but the sensitivity and specificity is reported to be lower for trisomy's 18 and 13 (Table 1-2) (Bianchi et al. 2012; Liang et al. 2013; Chiu et al. 2011; Gil et al. 2015). These chromosomes have lower mean levels of guanine and cytosine (GC) content compared to chromosome 21 (Chen et al. 2011), which makes the recognition of the fragments of these chromosomes less accurate. Using a GC adjustment improves the detection of these two chromosomes (Palomaki et al. 2012; Jiang et al. 2012). Also, fetal fraction in the maternal circulation tends to be lower in trisomy 18 compared to trisomy 21 and euploid cases – so it is harder to differentiate between normal and trisomic cases (Ashoor et al. 2012). Finally, trisomy 13 and 18 pregnancies frequently have mosaic placentas – with a normal cell line included in placentation that potentially masks the presence of trisomy (Schuring-Blom et al. 2002). Targeted MPS uses similar principles - but is focused on sequencing specific chromosomal fragments instead of the whole genome. Similar levels of sensitivity and specificity seem to be reported using this technology – which has the potential advantage that it requires less sequencing time and so more samples can be run consecutively, reducing overall costs.

Next generation sequencing can also be used for the detection of sex chromosome aneuploidies. A 91.5% detection rate for monosomy X (45X) was reported in one series of 45 cases (Nicolaides et al. 2014). The specificity of detection of sex chromosome

aneuploidy is however poorer. This may in part be due to the presence of low level mosaicism in some phenotypically normal women but also because confined placental mosaicism involving sex chromosomes is relatively common (Bianchi et al. 2015).

**Table 1-2.** Studies of NIPT of trisomy's 21, 18 and 13 for MPS and Target MPS.

| Authors | Technique | Aneuploidie | Sensitivity(%) | Specificity (%) |
|---|---|---|---|---|
| Bianchi et al. | MPS | T21: 89 | 100 | 100 |
| | | T18: 36 | 97.2 | 100 |
| | | T13: 14 | 78.6 | 100 |
| Liang et al. | MPS | T21: 40 | 100 | 100 |
| | | T18: 14 | 100 | 100 |
| | | T13: 4 | 100 | 99.75 |
| Nicolaides et al. | Target MPS | T21: 25 | 100 | 100 |
| | | T18: 3 | 100 | 100 |
| | | T13: 1 | 100 | 100 |
| Ashoor et al. | Target MPS | T21: 50 | 100 | 100 |
| | | T18: 50 | 98 | 100 |

Measurement of cffDNA in maternal serum can be used as an alternative to the invasive tests for high-risk women (following traditional forms of aneuploidy screening) (RANZCOG 2015) – but application in this way fails to take advantage of the high sensitivity of NIPT test and also restricts the range of aneuploidy that will be detected to common trisomies and sex-linked disorders – the test is essentially similar to the rapid qfPCR component of a CVS rather than the full karyotype.

A variety of monogenic diseases that show autosomal dominant, recessive or X-linked inheritance have also been diagnosed by examination of cffDNA. Examples include testing for Huntington´s disease, myotonic dystrophy, early onset primary dystonia, haemophilia, Duchene muscular dystrophy and achondroplasia. The investigational tools used to detect these conditions are often less compliex than next generation sequencing and include conventional PCR (Amicucci et al. 2000; Meaney & Norbury

2009; Saito et al. 2000), quantitative florescent PCR (QF-PCR) (M. C. Gonzalez-Gonzalez et al. 2003) or RT-PCR (M.C. Gonzalez-Gonzalez et al. 2003). Detecting paternally inherited loci is more straight forward as these will not be present in maternal plasma unless the fetus has this variant. Lo and collaborators (1997) used the presence of the SRY gene to demonstrate the presence of cffDNA in their original work. Several groups have now shown that this loci can be detected with accuracy as early as the 5$^{th}$ week of gestation (Devaney et al. 2011; Lo et al. 1997).

Monogenic testing is also used to define fetal RHD genotype – and this has the potential to reduce the need for antenatal immuno-prophylaxis in RhD negative women and to alter the surveillance of women potentially at risk of alloimmune disease (Legler et al. 2009). A number of techniques for RHD genotyping have been reported including mass spectrophotometry, gel or capillary electrophoresis, but the most common technique is real-time PCR - which appears to be the most sensitive and specific of these (Dovč-Drnovšek et al. 2013; Grill et al. 2009; Al-Yatama et al. 2007; Kimura et al. 2008).

### 1.1.3. NIPT and Models of care

In addition to using NIPT as an investigational tool following recognition of a high risk of aneuploidy, two other models for clinical application of NIPT for aneuploidy screening have been suggested. The first would be to offer all women NIPT as a primary screening test for Down syndrome. As NIPT has been shown to be the most sensitive and specific means of screening for common trisomies (Norton et al. 2015; Bianchi et al. 2014) this would appear to be a very sensible long term goal. The issue with this in the short term is that the test is expensive and if this is included in the health care budget by exclusion of another test, say the 12 week scan, then other anomalies excepting the common

trisomies may be missed. An alternative strategy that has received much interest is one that is described as contingent screening. In this model of care, women continue to have combined first trimester screening as the standard of care in screening for Down syndrome and other common forms of aneuploidy. If they are very high risk (> 1 in 50) then invasive testing (CVS) is recommended – allowing other atypical chromosomal abnormalities that would be missed with NIPT to be detected. If the risk is very low (<1 in 1000) then these women would be reassured that no further testing is necessary. The intermediate risk group (1 in 50 to 1 in 1000) would be offered NIPT as a second level of screening – providing further reassurance in most cases but identifying some aneuploidy cases that would have been missed using a 1 in 50 cut-off for invasive testing (Hui & Hyett 2013). Contingent screening takes advantage of the higher level of sensitivity that NIPT offers, but restricts its application to 10-20% of women that have an intermediate level of risk – making it more cost effective. The model also restricts invasive testing to a far smaller number of women – so is an effective means of reducing the false positive rate – and the risk of procedural related miscarriage.

#### 1.1.3.1. Other cell free fetal nucleic acid products

Other nucleic acids that are derived from the fetal / placental compartment can also be isolated from maternal plasma. Placental specific fragments of messenger RNA (mRNA) were first identified by Ng and collaborators (2003). mRNA is known to be rapidly degraded and the researchers' were surprised to find the product appeared to be relatively stable within maternal plasma. It has been suggested that this is because the degraded parts of trophoblast are protected by particles (Ng et al. 2002; Gupta et al. 2004) known as microvesicles (Tsui et al. 2014) or apoptotic bodies (Ng et al. 2003). Studies have shown that mRNA is rapidly cleared after delivery – in the same way as

cffDNA – this means that investigators can be confident that any product identified during a pregnancy is specifically related to that event (Ng et al. 2003). mRNA could potentially be used in the investigation and surveillance of a number of non-genetic obstetric complications such as for the detection of preeclampsia and fetal growth restriction (Farina et al. 2010; Mouillet et al. 2010; Takenaka et al. 2014). Researchers who are interested in predicting / defining pre-eclampsia have examined the role of cffmRNAs such as fms-related tyrosine kinase 1 (FLT1), endoglin, placental growth factor (PlGF), transforming growth factor–β1 (TGFβ1), and placental protein 13 (PP13) (Farina et al. 2010).

Lo et. al also demonstrated the potential value of quantifying cffmRNA for the prediction of fetal aneuploidy – although cffDNA is being used as the clinical tool for these conditions (Lo et al. 2007). In this work, that focused on cffmRNA they use SNPs directed to *PLAC4*, a gene located in chromosome 21 to identify and quantify expression of this mRNA and then showed the presence of trisomy by calculating chromosome dosage using the allelic ratio (Lo et al. 2007). Using this cffmRNA biomarker the author's demonstrated 90% sensitivity and 96.5% specificity for trisomy 21. The RNA-SNP approach relies on being able to recognize polymorphisms and detection is only possible in heterozygous fetuses; in this study it could only be applies in 40% of pregnancies (Lo et al. 2007) meaning a broader range of markers (with more SNPs) would be needed to make this practical for clinical use. Other potential advantages of using cffmRNA in prenatal diagnosis include the fact that these biomarkers are gender and polymorphism independent, and that as they are transcribed from genes that are active in the placenta there are multiple copies – and therefore potentially higher concentrations of product. A big disadvantage of this technique is that it is limited by the variation in the

heterozygosity rate, and due to the high number of homozygotes it wouldn´t be useful for the whole population.

The demonstration of the presence of fetal nucleic acids in maternal plasma 20 years ago has allowed the development of a range of screening and diagnostic tests which are now being applied clinically (RANZCOG 2015). It is likely that there will further major technical developments and that the application of molecular genomic techniques in reproductive medicine will continue to expand. One novel area of research involves identification and quantification of cell free miRNAs (Keck-Wherley et al. 2011). Fetal miRNAs have been recognized in the maternal circulation and have the potential to provide more information about the aetiology of some anomalies.

### 1.2. miRNA

One of the most studied RNAs in the past few decades is miRNA, an endogenous nucleic acid that is a small non-coding RNA molecule, which is involved in the regulation of gene expression and plays an important role in the cleavage or translational repression of mRNA.

There are multiple types of small RNAs that are divided into groups by their length and by the interaction that they have with the different Argonaute (AGO) family proteins. They are classified into three classes in animals: miRNA, small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA) (Ha & Kim 2014). These small RNA have similar functions but different mechanisms of action, and of these non-coding RNA, miRNA is the most highly expressed in somatic tissues (Ha & Kim 2014).

These RNA molecules are between 18 and 22 nucleotides in length, and they have been reported to have an important roll in developmental timing, differentiation, proliferation, cell death and metabolism (Gangaraju & Lin 2009). They were first described in 1993 by Lee *et.al* and Wightman *et.al* who detected lin-4 (a gene known to regulate the timing of larva development in *C. elegans)* (Lee et al. 1993; Wightman et al. 1993). Lee *et.al* also found that the action of this non-coding RNA was to bind to the 3` untranslated region (3`UTR) of the mRNA of lin-14, and as a consequence of this they observed a decrease in the amount of LIN-14 protein but not a change in the amount of mRNA of lin-14 (Wightman et al. 1993).

Afer this discovery, research focused on undestanding the function of these molecules. One of these studies showed that the target recognition of miRNA is the "miRNA seed" (at the 5` of the miRNA which extends from the second nucleotide to the seventh nucleotide). The 3`UTR region appears to be highly conserved and 60% of human protein-coding genes have a conserved region for miRNA binding (Friedman et al. 2009). From the moment that a miRNA is transcribed until it regulates mRNA the molecule undergoes a series of regulated steps. This biogenesis is facilitated by the cell involving a number of different proteins and factors (Ha & Kim 2014).

miRNA play a very important role in different biological pathways. They are in charge of regulating the expression of proteins and it are consequently involved in the regulation of different diseases and anomalies. They also play a role in determination the fate of cells including processes of cell proliferation, cell differentiation and cell death. For example, some miRNA can accelerate proliferation and prevent apoptosis by regulating the pro-apoptotic gene *hid* (Kloosterman & Plasterk 2006). miRNAs have also been

shown to play an important role in myogenesis and cardiogenesis, they are needed for the maintenance and growth of tissue (Sokol & Ambros 2005) and are important for brain function (for example miR-134 can control the expression of a protein that controls the development of dendritic spines (Schratt et al. 2006)). These non-coding molecules have significant potential as diagnostic and therapeutic tools, which is why understanding and detecting these molecules is so important.

### 1.2.1. miRNA Biogenesis

miRNA are transcribed by RNA polymerase II or RNA polymerase III (Borchert et al. 2006), the transcription can go though a canonical pathway first producing primary miRNA (pri-miRNA) transcripts. pri-miRNA has a hairpin structure in which the miRNA is inserted with a passenger strand that is then removed during the process of maturation, it can also encode one or more stem-loops. The maturation of pri-miRNA takes place inside the nucleus; it is initiated by the RNase III Drosha and DiGorge critical region 8 proteins, these complexes cleave the 5` and 3` arms of the pri-miRNA hairpin (Gregory et al. 2004) (Figure 1-1). It cleaves approximately 11 base pairs away from the basal junction between the single-stranded RNA and the double stranded RNA, converting it into pre-miRNA of 70 to 100 nucleotides in length. In some cases the action of factors like p68 and p72 are required by some miRNA for more efficient cleavage (Winter et al. 2009).

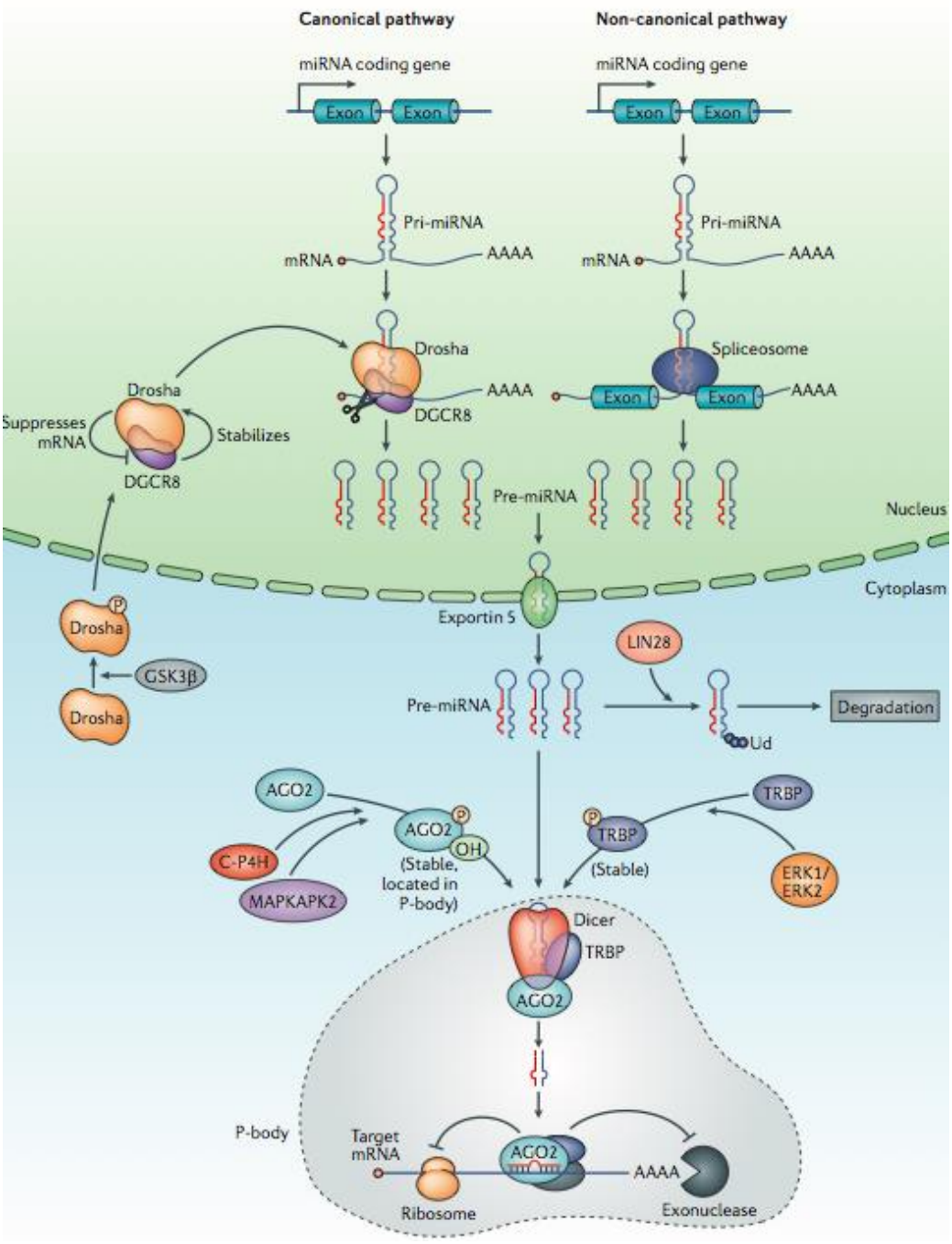



**Figure 1-1.** Biogenesis of miRNA. Canonical and non-canonical pathways.
(Figure from Li & Rana 2014)

After the cleavage the pre-miRNA is exported by Exportin-5 with Ran-GTP to the cytoplasm where it can continue the maturation process, these proteins are also in charge of protecting the pre-miRNA in the nucleus (Figure 1-1), and for a successful

binding of the Exportin-5 the pre-miRNA has to have a defined length of the double-stranded stem (Winter et al. 2009) that is independent of the sequence.

In the cytoplasm, an enzyme called Dicer cleaves pre-miRNA close to the terminal loop and creates a 22-nucleotide miRNA duplex (this endonuclease is regulated by its product let-7). The RNA duplex is loaded into an AGO protein to form the RNA-induced silencing complex (RISC) (Figure 1-1) that is composed of the RNase Dicer, the Tar RNA binding protein (TRBP), the protein activator of PKR and Argonaute 2 (AGO2) which also helps to mediate the RISC effects on the mRNA targets (Winter et al. 2009). The TRBP and the PACT are not vital in the cleavage performed by Dicer but they make cleavage easier. The passenger strand is removed from the pre-miRNA by the AGO protein, and the complex becomes a mature RISC. When the RISC is mature the interaction between the miRNA and its mRNA target is possible.

Once the mature miRNA is in the RISC complex and binds to the target mRNA the complex is activated. This complex can act through different mechanisms including translational repression, inhibition of translation initiation, inhibition of translation post-initiation and induction of mRNA destabilization decay (Ha & Kim 2014) (Figure 1-2). For the inhibition of translation initiation, the complex (miRISC) acts on the eukaryotic translation initiation factor 4F(elF4F), and represses the 40S small ribosomal subunit, 60S subunit incorporation and the formation of the 80S ribosomal complex. For the inhibition of translation post-initiation, the complex miRISC inhibits the elongation of the ribosome so the ribosome drops the mRNA where it is degraded. Finally for the induction of mRNA destabilization decay, the binding of the complex miRISC recruits enzymes that causes the mRNA to destabilize (Li & Rana 2014).

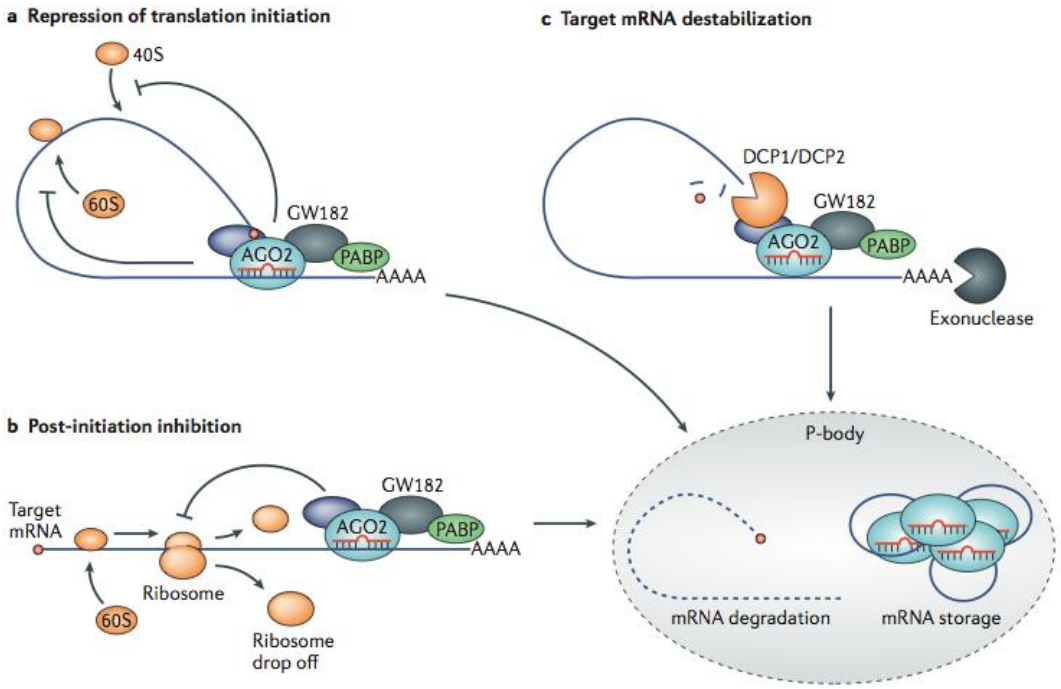



**Figure 1-2.** Potential mechanisms of miRNAs to repress mRNA.

a) miRNA can inhibit the initiation of translation by affecting initiation factors. b) They can inhibit the elongation of ribosomes making them to drop off the mRNA. c) They can bind to the target mRNA and recruit enzymes that destabilize it. All of these mechanisms could end up in mRNA degradation. (Figure from Li & Rana 2014).

An alternative non-canonical pathway involves transcribed genes that are located in the introns (Mirtrons), which make pre-miRNAs directly through splicing and debranching. These are then they transported by Exportin-5. Some of this type of miRNA present 5`or 3` single-stranded RNA tails that need to be trimmed before they are processed by Dicer or these processes would be suboptimal. 7-methylguanosine ($m^7G$)-capped, pre-miRNA can also be directly produced by transcription without the need of Drosha and transported to the cytoplasm by Exportin-1 (Ha & Kim 2014). These two non-canonical pathways finish by loading pre-miRNA in the AGO2 where they act as mature miRNAs.

Although this process occurs inside the cell there are studies that show that these molecules can be found in the circulation as well. This makes the detection and identification of miRNA much simpler – and potentially suitable for use as a clinical tool.

#### 1.2.1.1. Cell free miRNA or circulating miRNA

In tumour biology, several cancers have been found to have variable expression of cell free tumor miRNA and research continues into developing the value of these markers in detection and marking progression of these diseases (Pritchard et al. 2012). Intracellular miRNA has been found in various extracellular fluids, but the function and the origin of cell free miRNA is not clear. What is known is that these molecules are transported in microvesicles or exosomes (Cheng et al. 2014), which protect them from being degraded and allow them to remain relatively stable. Some studies looking into the communication between cells suggest that the transportation of miRNA results in expression in the cell that will receive it rather than in the cell that it originated from (Mostert et al. 2011). Cell free miRNAs are found in fluids and are being transported from one cell to another, meaning that by simulating cell free miRNA transport therapeutic uses could be developed. These findings also make miRNA good candidates as diagnostic markers.

#### 1.2.1.2. Detection and measurement of miRNA

The expression of the initial transcript does not always match the expression of the mature miRNAs (van Rooij 2011). For this reason is better to study and determine the quantity of mature miRNA, i.e. the mature molecules are the ones that inform that a specific miRNA is present, with potential to act on a target mRNA. Some studies detect

pre-miRNA, intracellular mature miRNA and circulating miRNA. Most of these molecules are quantified using the same techniques, but require different methods for extraction. For NIPT measurement of circulating miRNA is required because of the low numbers of fetal cells for pre-miRNA or intracellular mature miRNA.

The method used for detection and measurement of miRNA depends on the type of investigation and the interest of the investigator. For example if a study focuses on the expression profile of miRNAs, this can be achieved by microarray or sequencing. Whilst for studies to determine the quantity of specific miRNAs the technics of choice are Northern blott, quantitative real time PCR (qRT-PCR) and in-situ hybridization (ISH). Northen Blot has the disadvantage of longer time to produce results and lower throughput. More recently qRT-PCR has become the method of choice (Zhao & Fernald 2005). Results determined by microarray or sequencing have to be verified with other protocols for specific targets.

- Microarrays

This method is used for the detection of several targets and to detect the presence and regulation of those miRNAs. The first step of this method is to extract the miRNA or the total RNA from cells, tissue or fluids. It is better to use a kit with reagents that allow the enrichment of small RNAs because small RNAs are only 0.01% of total RNAs (Shingara et al. 2005). After the isolation the mature miRNA is labeled using T4 RNA ligase to attach the fluorophore-labeled nucleotides which are then directly hybridized to the arrays which contain the specific probes for those miRNAs and after some washes the miRNAs

are detected (van Rooij 2011; Li & Ruan 2009). In the analysis the findings have to be normalized and verified by qRT-PCR or Northern Blot.

- Deep Sequencing

Similar to arrays, this technique is used to detect all the miRNA that is present in a sample. It uses Next Generation Sequencing, in which all the small RNA molecules in a sample are sequenced. In this technique it is possible to measure the absolute abundance and with the help of RNA-folding prediction software, the identification of miRNA is possible (Winter et al. 2009). Next Generation Sequencing has a big advantage over microarrays because the information obtained from this technique is much more complete, but the analysis of the result that MPS creates is much more difficult to interpret.

- Real time PCR

RT-PCR is used for the detection and quantification of specific miRNA. It is the most commonly used method to verify results or detect specific targets. In this method the miRNA is extracted and will be converted into cDNA by a reverse transcription reaction. This can be done using two different primers, one containing a stem-loop and the second with a universal primer (Winter et al. 2009). The specificity and sensitivity of this method depends on the specificity of the primers.

- Northern Blot

This technique requires a lot of time and large amount of RNA, it allows the visualization of pre-miRNA and the expression of miRNA. In this method the extracted miRNA is separated by size in an electrophoresis gel and then is transferred to a membrane in which it is fixed by UV or heat. The membrane is hybridized with labeled probes and the probes that where hybridized to the target miRNA are detected with streptavidin (biotin) or anti-digoxigenin, which will be detected by a chemiluminescent reaction (Winter et al. 2009).

- In-situ hybridization

This method allows the detection of longer precursors of miRNA but not the mature miRNA because of the small size of the later. It can show miRNA that are expressed in a specific tissue or a specific cell during early embryonic development until a certain stages of pregnancies (Winter et al. 2009). This technique can shed light on the particular roles of miRNA in different processes (Nielsen 2012).

These techniques have been implemented in various clinical contexts in the last few years. They can be used by a variety of medical specialties to provide a wide range of information on different biological processes.

### 1.2.2. Clinical application of miRNA

Due to the capacity of these molecules to regulate different pathways they have been being investigated for application in different specialties in Medicine. Because these

molecules regulate two-thirds of the human genome they provide a large amount of data. Because of the role of miRNA in the regulation of gene expression these molecules have the capacity to regulate many pathways and a number of different types of diseases. For this reason much effort is being expended to identify miRNAs and their role in regulation of disease. The particular focus is where there may be a option for therapeutic intervention(Nana-Sinkam & Croce 2013).

miRNA have been widely studied in the regulation of a wide range of cancers. One such study by Liu et al. has shown that specific miRNA could redirect basic functions and pathways to tumor development and progression (Liu et al. 2008). Another use for measurements of miRNA is the identification of biomarkers which could help in the early detection of metastases, allowing an earlier therapeutic response (Nana-Sinkam & Croce 2013). For breast cancer there are many studies that have found different miRNAs that can be used as biomarkers for the detection of various forms of this particular cancer, but the results are not always consistent. Case-control studies have found that miR-21 is up regulated in the blood (Wu et al. 2011) and tissue (Wang et al. 2010) of women with breast cancer (these results were obtain by RT-PCR and by sequencing) (Zhao et al. 2010). Another similar study based on sequencing didn´t find this change (Leidner et al. 2013). Furthermore, miR-155 is an other example with conflicting results. It was found to be up regulated in tissue (Wang et al. 2010) collected from breast cancer patients and also in blood (Leidner et al. 2013) and breast cancer cell lines (Roth et al. 2010) by qRT-PCR. But this was not proven by another group using a sequencing approach (Wu et al. 2011). Why these differences occur is uncertain, but may be explained by variation in ethnicity, sampling etc. (Zhao et al. 2010; Yao et al. 2013).

miRNAs have also been used in the studies of lung cancer(Hennessey et al. 2012), as markers for leukemia (Tanaka et al. 2009; Schotte et al. 2012) and for recognition of head and neck carcinomas (Chang et al. 2008). Other studies have focused on cardiovascular disease. As with the research done in cancer, the studies in this field are trying to find biomarkers that can help in the detection of disease. Additionally, they are searching for direct miRNA-based therapeutics. In this regard of myocardial infarction there have been two miRNAs consistently reported that can be used as biomarkers; miR-499 (Corsten et al. 2010; Adachi et al. 2010) and miR-133a (Corsten et al. 2010; Kuwabara et al. 2011). As in the cancer research, these studies found additional miRNA that are down or up regulated but without consistency across research groups; examples include miR-1 and miR-208b. In cardio vascular diseases one miRNA has been used as a therapeutic miRNA (miR-21); this miRNA prevents the further progression of fibrosis and heart failure, and has been shown to lead to an improvement in heart function in cardiac disease models (Srinivasan et al. 2013). There are more studies in cardiac disease including recognition of the important roll of miR-210 and the miR-146a family in atherosclerosis (Raitoharju et al. 2011) and the down regulation of miR-29 in myocardial fibrosis (van Rooij et al. 2008).

Non-coding RNA molecules have been studied in a lot of other fields of medicine such as neurodegenerative disorders, which are focusing the research on the possibility of using miRNA as a therapeutic tool. In Parkinson's diseases the analysis of brain tissue post mortem of patients with the disease, compared to those without, reveal differential expression of miRNA. miR-133b is normally abundant in the brain but in patients with Parkinson's is less abundant, and this miRNA is found to be involved in dopaminergic neuron differentiation (Maciotta et al. 2013). Also, they are being studied in metabolic

diseases, and their involvement in the regulation of lipid and glucose metabolism (Fernández-Hernando et al. 2013). The therapeutic capacity of these molecules is as yet unclear. In some fields there are concerns about using miRNA as therapeutic drugs, because one miRNA can regulate more that one target mRNA. Therefore alteration of one miRNA can affect many pathways (Gaynullina et al. 2015). For this reason it is better to have a full understanding of the different paths that one miRNA regulates so we can identify all the possible outcomes. It seems a lot more information is required before these molecules can be safely used in a therapeutic setting.

As discussed, miRNAs can affect many mRNAs and has a big impact in the field of obstetrics. In particular much research has focused on the involvement of these molecules in developmental biology. This research is discussed in the following section.

### 1.3.miRNA in Obstetrics

Detection of cell free fetal DNA in maternal circulation has become a well-established method for screening for Down syndrome, other common aneuploidies, fetal rhesus-D status and to establish fetal gender (Hyett et al. 2005; Hyland et al. 2009). Research has also identified changes in miRNA expression in the serum / plasma of pregnant women carrying fetuses affected by different abnormalities as well as in pregnancy with adverse outcomes (Sankaran et al. 2011; Tang et al. 2013). Several groups are also attempting to describe how certain miRNA supress genes and to identify which gene(s) are being supressed. These molecules could potentially be used as an indirect means of detecting these congenital abnormalities or pregnancies with adverse outcomes. Studies related to miRNA in pregnancy have focused on prenatal diagnosis of aneuploidies, cardiac

abnormalities, adverse pregnancy outcomes (pre-eclampsia / IUGR / preterm delivery / stillbirth), neural tube defects (NTD) and other abnormalities.

These non-coding molecules have not just been studied in the maternal blood, but also within the tissue of fetal or placental origin and also in mouse models (Keck-Wherley et al. 2011). As invasive testing is associated with a risk of miscarriage, recent research has focused on the identification of cell free nucleic acids, which can be investigated without putting the mother or fetus at risk.

A number of placenta specific miRNA have been identified in normal pregnancies (Liang et al. 2007; Kotlabova et al. 2011), however no research has been done in relation to expression of these placenta specific miRNA in pregnancies where the fetus is affected by a congenital anomaly. Further work is needed to determine whether these placental specific markers are of value in detecting pregnancies that are affected by fetal abnormality.

We have previously discussed how placental trophoblast breaks down, releasing cffDNA into the maternal circulation. Placental miRNA is shown schematically in Figure 1-3 believed to behave in a similar manner. The miRNA is released by the trophoblast and transported out of the cell via a number of different pathways including as: - cell fragments / microparticles, associated with lipoproteins or RNA-binding proteins (AGO2), - apoptotic bodies or microvesicles that are released when the cell is going though apoptosis, - nanovesicles (exosomes)(Ouyang et al. 2014) that are derived from endosomes and are released from the cell or - from multivesicular bodies, being released when they are combined with the cell membrane(Kamhieh-Milz et al. 2014)(Figure 1-3).

This provides enormous potential for being able to generate information about gene regulation in both the fetus and the placenta. Furthermore, if we can find informative markers (like with cffDNA) that identify fetuses affected by structural anomalies this would facilitate earlier diagnosis providing pregnant women with an opportunity to make informed decisions about their pregnancy.

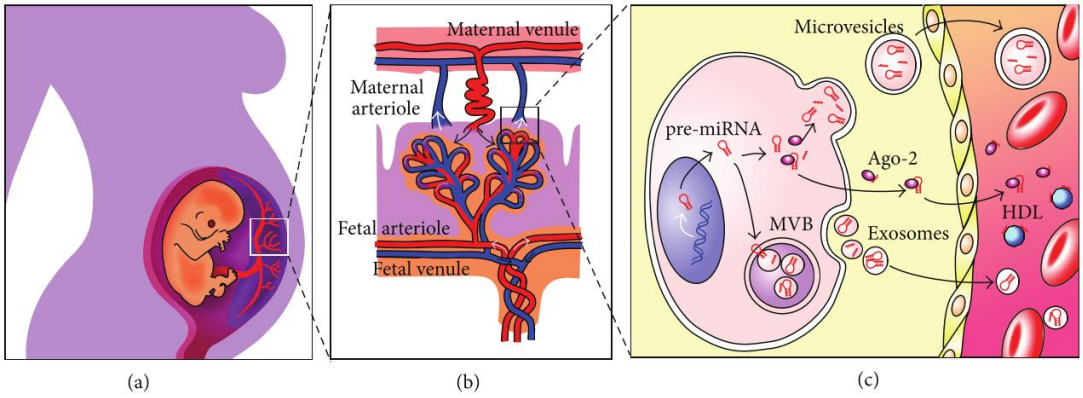



**Figure 1-3**. How miRNA is released in to the maternal circulation.
a) Shows the placenta (the connection between the mother and the fetus). b) Is showing the chorionic villi, which allow the transport of nutrients and oxygen form the maternal blood. c) Is showing how the miRNA are transported from fetal cells to the circulation of the mother. (Figure from Kamhieh-Milz et al. 2014)

### 1.3.1. miRNA expression in Aneuploidy

The efficacy of cffDNA based non-invasive prenatal testing for common chromosomal abnormalities such as trisomy 21 is so high that there is no real clinical need to develop a new cell free miRNA based approach. Changes in copy number do, however, alter miRNA expression.

One study, based on the segmental trisomy 16 (Ts16Dn) mouse model, a well characterized animal model of Down Syndrome identified overexpression of miR-155 and miR-802 in the hippocampus, and in whole blood, compared to controls(Keck-Wherley et al. 2011). This study defined differential miRNA expression by miRNA array then confirmed these findings with RT-PCR Western Blots. The results contrast with

those of Kotlabova et al.(Kotlabova et al. 2013) who found overexpression of miR-155 in amniotic fluid derived from DS samples (using RT-PCR). A third study, based on High-Throughput Quantitative PCR (HT-qPCR), a technique that allows detection of differential expression in 1043 human miRNA (Kamhieh-Milz et al. 2014), found four miRNA that were under- or over-expressed (miR-27b, miR-483.5p, miR-486.50 and miR-888.3p) in trisomy 21 compared to euploid fetuses. The three studies looked at different sample types, from different models, using different techniques – so it is not surprising that such variable results were described. These studies do, however, confirm that there is differential miRNA expression between trisomic samples and controls; suggesting that there is potential for these products to be used as potential markers for DS – once the biological variability is better defined.

Another interesting example of differential expression of miRNA by microarray involves neonates affected by trisomy 13. Individuals affected by trisomy 13 continue to produce fetal hemoglobin (HbF) and do not switch to production of adult hemoglobin. This study examined miRNAs associated with regulation of HbF (Sankaran et al. 2011). They discovered that miR-15a and miR-16-1 play an important role in silencing the fetal and embryonic hemoglobin genes. In this study they cultured cells from newborns with trisomy13 and found that this miRNA targeted the MYB transcription factor, which plays an important role in hematopoiesis. Normal expression of these miRNAs moderates the expression of this factor. This is not the case for trisomy 13 where the levels of these miRNAs are elevated, which leads to greater down regulation of the MYB transcription factor, resulting in the switch of fetal to adult haemoglobin being delayed. One consequence of this work is that the research team has developed a better understanding of the miRNAs that regulate hematopoiesis. This may in turn help with

the development of therapeutic interventions that can be applied in other clinical scenarios.

### 1.3.2. miRNA expression in adverse pregnancy outcomes

The main causes of perinatal death are congenital abnormality, preterm delivery – 8.3 % incidence in NSW (3.867 of 25.432 births), placental insufficiency (intrauterine growth restriction) - 4.5 % incidence in NSW (1.151 of 25.432 births) and pre-eclampsia - 9.3% incidence in NSW (2.374 of 25.432 births) (Li et al. 2010). Stillbirth also continues to be a significant problem, affecting 0.9 % of pregnancies (NSW, 222 of 25.432 births). Placental pathologies are often implicated in all four of these complications – so differential expression of miRNAs involved in placental function may provide a means of identifying pregnancies at risk of these adverse outcomes.

Preterm delivery is defined by birth at <37 weeks of pregnancy. Preterm births can be sub-divided by gestation and some studies focus on extreme (<28 weeks) or early (28-34 weeks) cohorts of births. Two studies focusing on understanding and finding markers for preterm labor found that miR-388 is expressed in chorioamniotic membranes of patients with preterm labor compared to patients at term both without labor and in labor, they discovered that this miRNA inhibits the expression of PLA2G4B (a phospholipase linked to parturition)(Montenegro et al. 2009). Another study found that miR-483-5p has a lower expression in placentas of preterm delivery compared to normal term delivery pregnancies (Mayor-Lynn et al. 2011). Both of these studies used arrays to find the most significant markers, Montenegro and collaborators use computation analysis to find the targets of the miRNAs detected, while Mayor-Lynn and collaborators coupled arrays with bioinformatics analysis to identify mRNA and then

predict the target genes. There are no data that have been published that demonstrate that these miRNA changes can also be determined non-invasively as a cell free fetal fraction in maternal plasma.

Pre-eclampsia is a hypertensive disorder, where hypertension is develop after 20 weeks gestation in addition to one or more of these condition: proteinuria, maternal organ dysfunction and uteroplacental dysfunction (Tranquilli et al. 2014). There are four studies reporting differential expression of miRNAs in women with pre-eclampsia. Zhu et al recruited 15 women who had severe pre-eclampsia and 8 women with mild pre-eclampsia (using the diagnostic criteria of the guidelines of the International Society for the Study of Hypertension in Pregnancy). They took samples from the placenta at the time of delivery and performed a microarray and RT-PCR, which showed 34 miRNA that are down-regulated in pre-eclemptic women. They concluded that miRNAs like miR-210 could be a potential marker, because increases in this miRNA can indicate high degrees of hypoxia (poor placentation in pre-eclamtic pregnancies can cause hypoxia). Similarly Pineles et al. found an increase miR-210 in 9 women who had pre-eclampsia compared to controls. Zhang et al. tested serum samples from 30 women and found up-regulation of miR-210 in those –who subsequently developed mild or sever pre-eclampsia. In contrast to the study of Zhang et al., Gunet et al. found that miR-152 was down-regulated in pre-eclamptic pregnancies but also confirmed that miR-210 is up-regulated. The consistent finding that this miRNA was an up-regulated marker in both placental tissues and in blood makes it a very good candidate for improving predictive methods for this particular disease.

Intrauterine Growth Restriction (IUGR) is traditionally defined as a fetus that has an abdominal circumference or estimated fetal weight <10$^{th}$ centile on ultrasound and that shows evidence of haemodynamic compromise (for example has absent flow in the umbilical artery Doppler) (Lausman et al. 2013). In a study of 45 pregnancies affected by IUGR leading to delivery at a mean of 37.8 weeks of infants weighing mean 2749.3g, placental tissue was investigated by next-generation sequencing. The researchers demonstrated changes in the expression of seven miRNA, but when they tried to demonstrate the same findings in maternal serum they were unable to do so (Higashijima et al. 2013). In a second study by Tang et al. miR-141 was found to be up-regulated in the placenta of IUGR pregnancies. This miRNA is known to suppress PLAG1 genes, which are implicated in IUGR by regulating the expression of insulin-like growth factor 2, which is critical in the early development of the placenta and in fetal growth (Tang et al. 2013).

Stillbirth is defined as fetal death in utero at >20 weeks gestation (PSANZ 2009). One potential cause is related to poor placentation with defective trophoblast growth or a reduce penetration of the trophoblast in the decidua (Hustin et al.). Ventura and collaborators studied miR-17 and miR-19b in placentas from women that had early pregnancy loss, they showed that these miRNAs were down regulated compared to the controls, this study suggest that these miRNAs are involved in the placental invasion (Ventura et al. 2013) by the repression of PTEN (a gene involved in the trophoblast invasion).

### 1.3.3. Congenital heart defects (CHD)

In Australia the prevalence of congenital heart defects is 7.8 in 1,000 births in the general population and is the cause of approximately 20% of prenatal deaths (Leggat 2011). There are a large range of malformations, most of which are very difficult to detect by traditional methods. (the fetuses don't show visible signs of the CHDs) (Hunter & Simpson 2014). Ultrasound is the most reliable method used to detect CHDs, but this technique relies heavily on the experience of the medical practitioner which can lead to malformations being missed in the screening process and also cannot identify all types of congenital heart defect (He et al. 2013). Some of the more common CHDs in Australia are: transposition of great vessels (103 new cases in 2003), Tetralogy of Fallot (82 new cases in 2003) and hypoplastic left heart syndrome (37 new cases in 2003)(Leggat 2011).

Transposition of great vessels (or arteries) is when the aorta leaves from the right ventricle and the pulmonary artery leaves from the left ventricle. (the aorta carries low-oxygen blood to the body and the pulmonary artery take oxygen-rich blood back to the lungs). This CHD can be fixed by a surgery to switch the major arteries (Anon 2013b; Anon n.d.). Tetralogy of Fallot causes cyanosis and is characterized by a ventricular septal defect (a hole in the part of the septum that separates the ventricles), a pulmonary stenosis (thickened and narrowed pulmonary valve), a right ventricular hypertrophy (increase in the size of the right ventricle) and an overriding aorta (the aorta lies directly over the ventricular septal defect) (Hunter & Simpson 2014). Hypoplastic left heart syndrome (HLHS) is when the left side of the heart didn´t develop properly, so the body doesn´t receive enough oxygenated blood, it can be fixed by a series of surgeries (Hunter & Simpson 2014).

Some miRNA are involved in the development of the heart, such as miR-1 and miR-133, both of which are involved in the mesoderm differentiation of stem cells and suppress endodermal and ectodermal cell fates (Lo et al. 1999). Later in development these miRNa also promote and repress cardiomyocyte differentiation (Twiss et al. 2014; Small & Olson 2011; Dr. Estefania Lozano-Velasco 2011; Liu & Olson 2010; Thum et al. 2008). Other miRNAs are involved in heart disease like miR-29 and miR-21 that can lead to fibrosis by repressing extracellular components and stimulating mitogen-activated protein kinase that promotes fibrosis (Badenas et al. 2010; Anon 2013b)One recent study was done in the tissue of the right ventricle of hearts donated by 15 HLHS patients of less than or equal to 13 years of age. Performing an array analysis (confirmed by rt-PCR) they found 9 down-regulated and 4 up-regulated miRNA compared to the control(Sucharov et al. 2015). These miRNA could be useful as marker, but as of yet no studies of them have been done in circulation.

Studies made on circulating miRNA for the identification of some heart diseases have been performed in adults and they have found some potential markers that can help with the identification of the diseases (Voellenkle et al. 2010; Fichtlscherer et al. 2010). Fichtisherer and collaborators investigated miRNAs involved in coronary heart diseases form eight patients and found four miRNAs that were reduced compared to the controls. In comparison Vollenkle and collaborators found three different miRNAs relating to chronic heart failure. miRNAs found in adult patients with specific cardiac diseases should be investigated in terms of fetuses with cardiac malformations as it could be the case that miRNAs involved in these diseases could also be involved in the development of congenital heart defects.

One study made by Shasha and collaborators found four miRNA in the serum of pregnant women that can be potential markers. They used sequencing of three pooled samples of women with fetuses affected by CHD and three controls and the results where verified with qRT-PCR. With the validation they found that miR-19b and miR-29c are up-regulated in fetuses with ventricular septal defects. miR-375 was also up-regulated for fetuses presenting arterial septal defects. These three miRNAs, plus miR-22, were up-regulated in fetuses with Tetralogy of Fallot (Zhu et al. 2013). This study makes a very good first approach in the identification of markers for CHD using NIPT, however these markers need to be tested in more women carrying fetuses with different types of CHD to identify any differences in the expression of these miRNAs This will help us to understand if the same miRNA can be regulating different types of CHD or/ and if there are specific miRNAs for each type of CHD. In addition looking at miRNAs expressed in adults with heart disease may lead us to genes or pathways that can also be affected in the development of the heart.

### 1.3.4. Neural tube defects (NTD)

These congenital defects have a prevalence of 10.1 per 10,000 pregnancies in Australia (2005)(EA 2008). NTD are birth defects that involve the brain, spine and spinal cord, which are originated during the early embryonic development when the neural tube doesn´t close completely. In a normal pregnancy the neural tube is developed and closed 22-24 days after conception. The type of NTD depends on the site at which the neural tube failed to close. The most common NTDs are spina bifida (open or closed) and anencephaly. Iniencephaly, encephalocele and craniorachischisis are the other, less common, types of neural tube defects (Botto et al. 1999).

Spina bifida can be one of two main types, open or closed. The defects associated with spina bifida are: Meningocele (when the spinal cord extends out of the spine like a sac containing nerve fluids, typically this does not lead to nerve damage), Myelomeningocele (when the spinal cord extends out of the spine but is not contained in a sac, this can cause nerve damage and fluid in the brain), Spina bifida occulta (which has no visible signs), Anencephaly (the total or partial absence of the brain and malformed cranial vault), Craniorachischisis (a congenital fissure of the skull and spine), Iniencephaly (the partial or total absence of cervico-thoracic vertebrae) and Encephalocele (when the brain extends out of the skull)(Botto et al. 1999).

During embryonic development there are a large number of miRNAs that play important roles in neural tube development. This process is very complex, highly coordinated and very regulated. Over the course of neurulation a range of different miRNA that are involved can be suppressed or overexpressed at different stages of the development (Mukhopadhyay et al. 2011). A mouse model has been used for the detection of the miRNAs that are involved in the development of neural pathways. Studies using this model have shown that miR-9 is involved in the development of the brain and is specifically expressed in the nervous system(Krichevsky 2003). miR-124 and miR-9 play an important role in the differentiation of the neural stem cells by regulating the REST complex (which silences neuronal genes in non-neuronal cells) (Krichevsky 2003; Shenoy & Blelloch 2014). There are a lot of miRNA that help in the development of the neural tube, like the let-7 family that helps regulates the cell cycle and differentiation (Mukhopadhyay et al. 2011; Maller Schulman et al. 2008). None of these miRNA have been identified in maternal circulation.

A study made by Gu and collaborators found 6 miRNA in the maternal circulation that have a different expression between pregnancies with fetuses with spina bifida compared to normal pregnancies (Gu et al. 2012). They performed a miRNA microarray in 3 pregnancies with NTDs to detect the differential expression, and then they confirmed the finding with qRT-PCR. One of these miRNAs that was up-regulated is miR-144, which is reported to target PAX3 (a gene required in the closure of the neural tube)(Phelan et al. 1997). This study is the first step in the identification of cffmiRNA in pregnancies with NTDs, however work still need to be done with a larger cohort of patients, so there is better detection of new miRNA, which can allow us to detect this abnormality faster and easier.

Current diagnosis of fetuses with congenital abnormalities relies on the ability to define the anomaly on ultrasound – which may not occur before the 20 week scan. A non-invasive prenatal test would potentially allow earlier detection of anomalies by defining a group who were high risk and needed a focused scan. Given the fact that potential markers (i.e. miRNAs) have been identified for a range of structural anomalies and diseases we hypothesize that it will be possible to detect some of these in maternal serum.

# 2. Hypothesis and aims of this study

## 2.1.Hypothesis

The developmental processes of fetuses affected by chromosomal abnormality (trisomy 21) or structural anomalies (cardiac abnormalities and neural tube defects) differ from those seen in normal pregnancies. This includes differential expression of miRNAs that control developmental genes. We hypothesise that changes that occur in miRNA expression are detectable in maternal serum, as cell free miRNA. Further, we propose that these changes can be used to distinguish between normal fetuses and those that have chromosomal or structural defects.

## 2.2.Aims of this study

To demonstrate that cell free fetal (or placental) miRNAs can be successfully extracted from maternal serum and quantified.

To determine which blood tubes are most suitable for collection of maternal blood, maximising our ability to detect and quantify fetal (or placental) specific cell free miRNA with a collection system that is robust in clinical practice. This will test effects of storage time and temperature.

To determine whether a series of six placental specific miRNAs are differentially expressed in maternal plasma collected from pregnancies affected by chromosomal abnormality (trisomy 21) or structural anomalies (cardiac and neural tube defects) in comparison to chromosomal and structurally normal pregnancies.

# 3. Methodology

## 3.1.Sample collection and processing

Blood samples were collected from 55 pregnant women attending the RPA Women and Babies clinic of Royal Prince Alfred Hospital (RPAH) between April 2014 and June 2015 Each women signed a consent form approved by the Human Ethics Committee of the Royal Prince Alfred Hospital (RPAH) (protocol No X13-0350 & HREC/13/RPAH/484).

Blood samples were collected in three different tubes (EDTA, STRECK or CITRATE). Approximately 10ml of whole blood was collected into EDTA and STRECK tubes while 5ml of whole blood was collected in CITRATE tubes. To enable establishment and validation of the methodology the samples were collected from two different groups of women. The first group acted as the control group, and blood from these women was collected between 12 and 29 weeks' of gestation. The second group was a small group of women whose fetuses had been diagnosed with anomalies. These anomalies included Neural Tube Defects (NTD), Congenital Heart Defects (CHD) and Trisomy 21 (T 21). Each of these diagnoses was made using non-invasive and invasive tests such as ultrasound and serum markers. For the second group the samples were obtained between the 11th and 28th week of gestation. Details of the anomalies and of the clinical features that were recognised at the time of presentation were obtained from the Fetal Medicine Unit medical record. Women were recruited on an opportunistic basis dependent on the timing of their presentation to the fetal medicine unit at Royal Prince Alfred Hospital.

## 3.2.Experimental design analysis

To analyse miRNA stability, samples were collected from women with normal pregnancies between the 18th and 20th week of gestation. To examine the effect of collection tube on miRNA stability the samples were collected into three different types of blood tube (i.e. EDTA, STRECK and CITRATE). The experimental plan for the studies is shown schematically in Figure 3-1 and Figure 3-2.

For the comparison of conditions (shown schematically in Figure 3-3) we analysed blood from 15 pregnancies carrying fetuses with congenital abnormalities, 4 with Neural tube defects and 6 affected by different types of CHD.

Each series of studies was performed on samples which were collected and stored at 4°C or at room temperature as described in detail below.

### 3.2.1. Samples at 4°C

Prior to aliquoting seven of the control samples were stored for 3 to 6 hours at room temperature. After this time the whole blood was aliquoted into tubes of 2 ml for all the samples, irrespective of tube type (i.e. approximately 5 aliquots for EDTA and STRECK tubes and 2 aliquots for the CITRATE tubes). Two tubes of each tube type were then centrifuged at 1950rpm for 20 min and 650µl of plasma was removed and stored at -80°C. The remaining blood was then placed at 4°C. After 24 and 48 hours the aliquots for each tube type were centrifuged and the plasma removed and stored as described above. As the number of aliquots was smaller for the CITRATE tubes the effect of 4°C

storage was only examined at 3-6 hours as baseline and at 48 hours. This information is shown schematically in Figure 3-1.

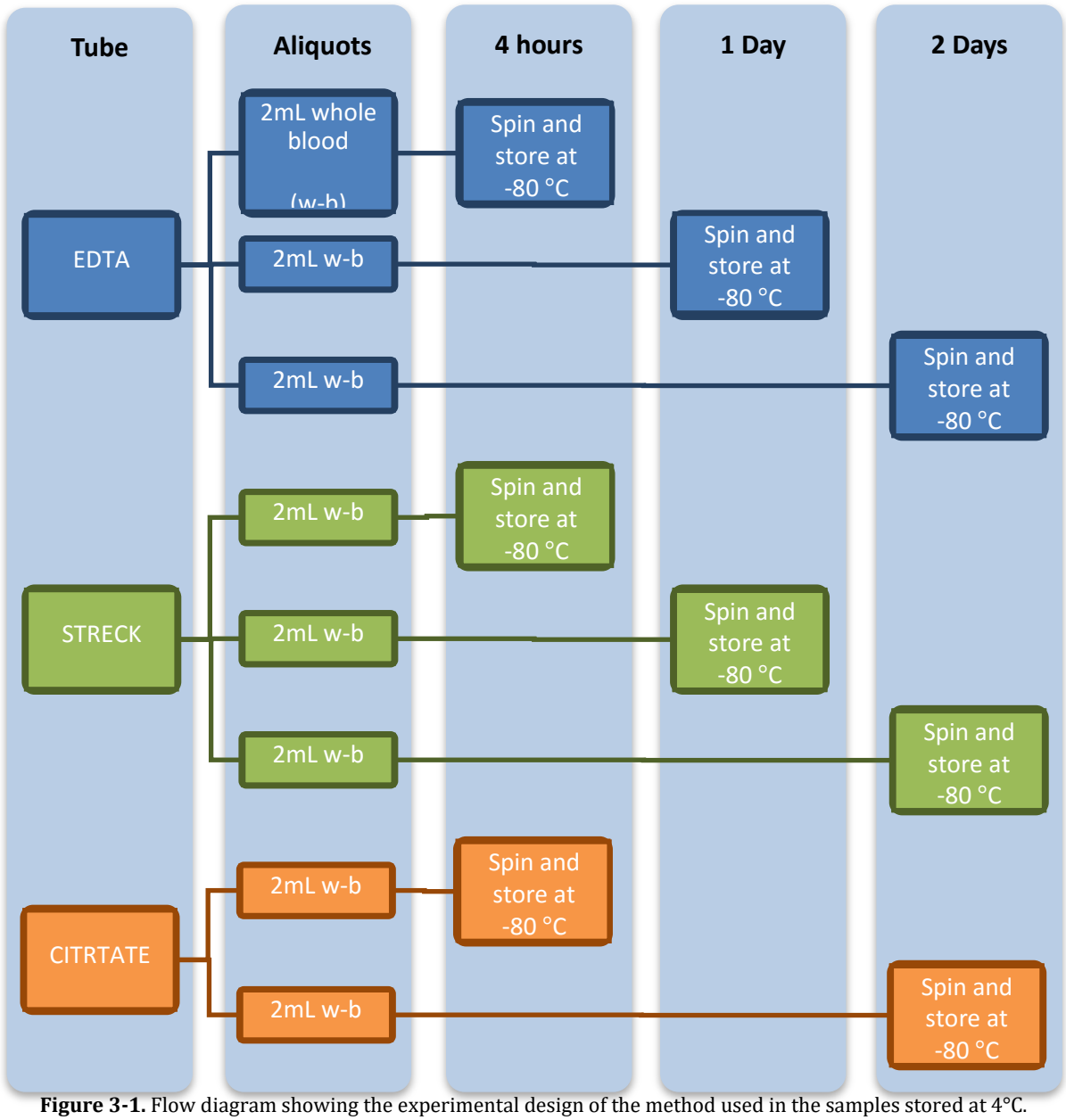



**Figure 3-1.** Flow diagram showing the experimental design of the method used in the samples stored at 4°C.

### 3.2.2. Samples at room temperature (Rt)

To investigate the viability of collecting and shipping samples from Vietnam (where the fetal medicine group see many more fetal anomalies) the effect of a worst-case scenario (i.e. no cold storage and long delivery time) on the measurement of fetal miRNA was investigated.

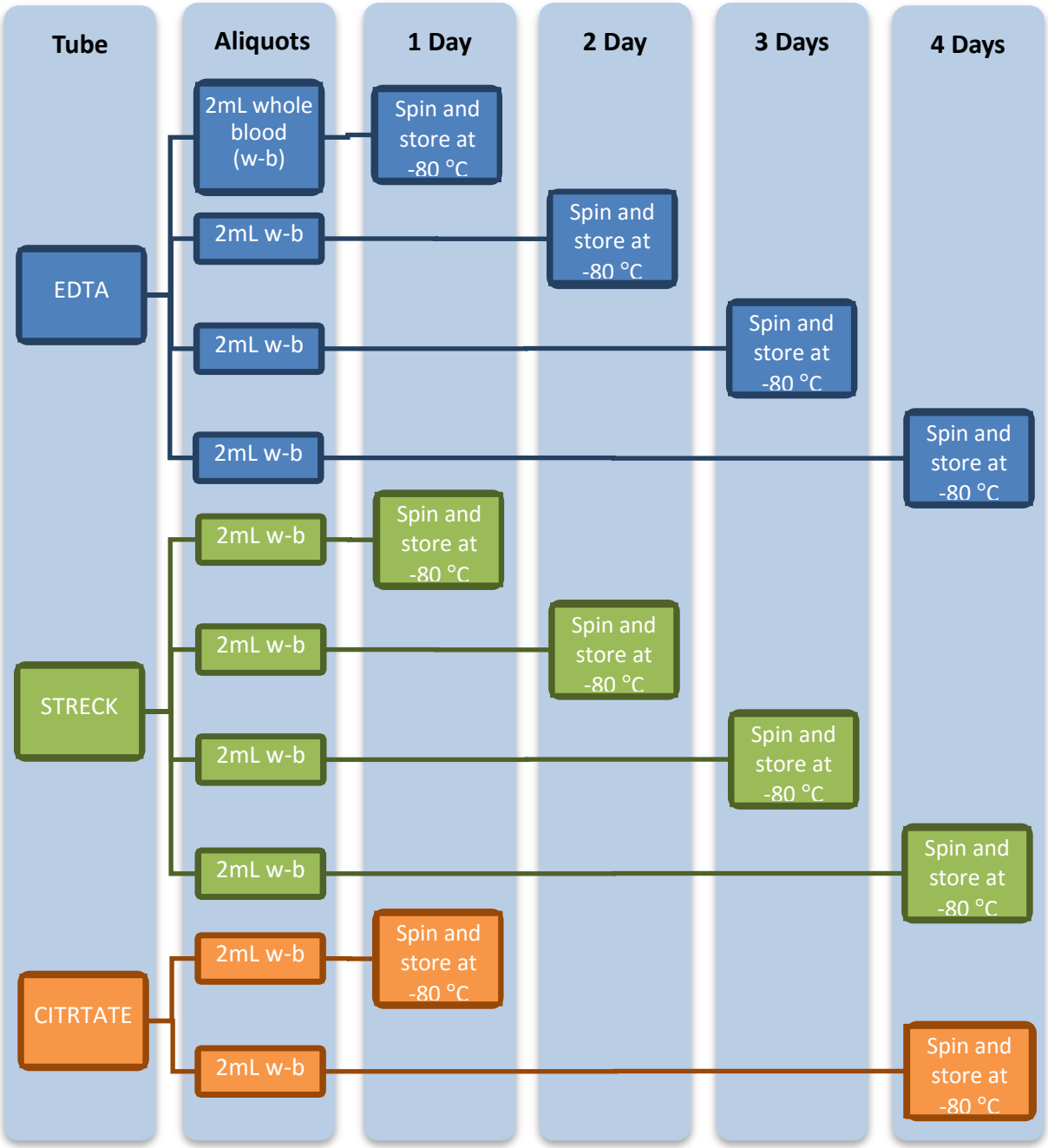



**Figure 3-2.** Flow diagram showing the experimental plan for examination of the effect of storage at Rt and collection tube in miRNA expression

In a design similar to that used for the cold storage experiments, control samples were collected into each tube type and then held for approximately 24 hours at room temperature. The whole blood was then aliquoted (approximately 2 ml/aliquot was then centrifuged at room temperature at 1950rpm for 20 min and the plasma (650µl) was removed and stored at -80°C. The remaining blood samples were kept Rt, and after

approximately 48 hours one of the EDTA and STERCK were processed as above and stored. This process was repeated for the final time point (i.e. 72 hours) for all three types of tube (Figure 3-2).

### 3.2.3. Samples for the comparison of the effect of fetal abnormality on miRNA expression

These samples were collected and stored at room temperature until processing (4-24 hours). In total samples were collected from twenty-five pregnancies with diagnosed fetal abnormalities (anomalies) and fourteen normal pregnancies (controls). Shown schematically Figure 3-3, plasma for extraction of miRNA was obtained as described in the previous sections.

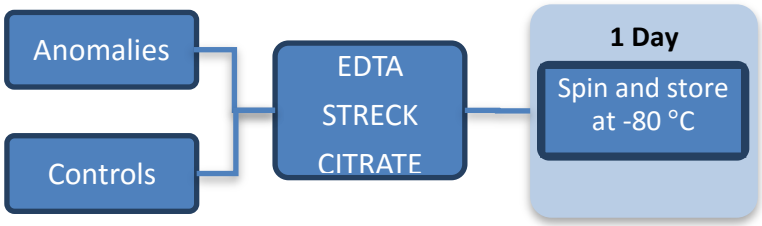



**Figure 3-3.** Flow diagram of the experimental plan for examination of the effect of fetal abnormality presence and collection tube type.

## 3.3.Description of the miRNAs studied

The miRNA selected for measurement in this work have been described by Kotlabova and collaborators (Kotlabova et al. 2011) as the six most placental specific miRNA. Some of these miRNAs were also found by Liang and collaborators to be related to the placenta (Liang et al. 2007). The miRNA used were hsa-miR-516-5p, hsa-miR-517*, hsa-miR-518b, hsa-miR-520a*, hsa-miR-525 and hsa-miR-526b (Table 3-1).

To analyze expression, TaqMan® MicroRNA Assays (Life Technologies, Carlsbad, California 92008), were used. For this analysis primers were purchased. Each kit is supplied with 5X RT primer, a 20X mix of forward and reverse primers, and the miRNA-specific probes (which were used in the pre-amplification and RT-PCR steps).

**Table 3-1.** miRNAs studied and their sequences

| Assay Name | Mature miRNA Sequence |
|---|---|
| hsa-miR-516-5p | CAUCUGGAGGUAAGAAGCACUUU |
| hsa-miR-517* | CCUCUAGAUGGAAGCACUGUCU |
| hsa-miR-518b | CAAAGCGCUCCCCUUUAGAGGU |
| hsa-miR-520a* | CUCCAGAGGGAAGUACUUUCU |
| hsa-miR-525 | CUCCAGAGGGAUGCACUUUCU |
| hsa-miR-526b | CUCUUGAGGGAAGCACUUUCUGUU |

### 3.4.

### 3.5.Controls used for validation

In each study a number of different controls were used. Firstly, to control for RT and PCR efficiency we used a spike in control. In this study it was the *C.elegans* miR-39 miRNA. This was used to assess the validity of the whole protocol and as it is added to the tube prior to the RT step. This spike in control has the additional advantage in that it has the potential for use as an internal control for the normalization of the data. This internal control is necessary as to date there are no well described miRNAs which can be used as a normalization control for placental miRNA. In this study the *C.elegans* miR-39 miRNA, at a concentration of $1.6x10^8$ copies/µl (2.2µl, Qiagen) was added to all samples studied just prior to the reverse-transcription step. As for the other miRNAs, its expression in the samples was determined using Taqman probes (TaqMan® MicroRNA Assay, cel- miR-39).

In addition to the cel-miR-39 (as spike in control), for the analysis of the effect of the different fetal anomalies we also included a reference sample in each run. This reference sample was made from a pool of miRNA isolated from blood samples obtained from women with pregnancies with normal fetuses as well as pregnancies with abnormal fetuses. This reference sample was included to enable us to determine the variability between runs, for the subset of the miRNAs being investigated.

## 3.6.Optimization for the processing of the samples

The processing and analysis of the samples followed a series of steps (shown schematically in Figure 3-4) and described in detail in the following sections. The first of these steps was to extract the miRNA from the blood of the pregnant women. Once extracted the miRNA concentration was determined using spectroscopic methods. A reverse transcription step was then applied to convert the miRNA to cDNA and the samples were then pre-amplified, and the miRNAs were quantified using RT-PCR.

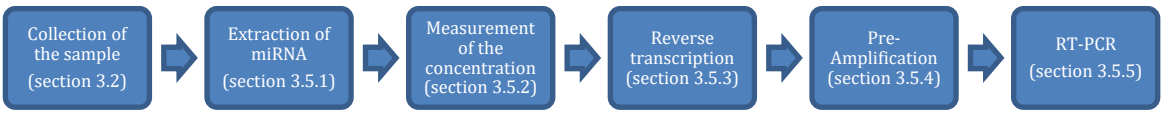



**Figure 3-4.** Flow diagram of the basic processing methodology used in this study

### 3.6.1. miRNA extraction

The extraction of the miRNA from plasma was performed using the Life Technology Kit (mirVana™ Paris™ Kit. Life Technologies, Carlsbad, California 92008) according to the manufacturers instructions. This kit uses a series of washes to collect total RNAs. The total RNAs are then added to a column and the small RNAs are isolated using an ethanol

gradient. The extracted small RNAs are then purified further using a second column and eluted with nuclease-free water.

In the studies described in this thesis we are examining the expression of fetal miRNA, which likely makes up a very small percentage of the total miRNA in the maternal circulation. For this reason optimization of the extraction procedure was required. Using the methodology described in the Life Technology Extraction kit there are two possible places to optimise, the first being the volume of plasma used for the extraction and the second being the final step where the volume used to eluted the small RNAs from the column could be manipulated. The standard protocol allows extraction of up to 625µl of plasma. The first optimization was done using 250µl and 500µl of plasma of two of the control samples and measuring the concentration. The second optimization, investigated reducing the amount of nuclease-free water from the volume that the protocol recommends to elute the small RNA (100µl) to 50 µl.

Based on the results of these optimization steps (described in detail in the results section 4.2.1.) all further extractions used 620µl of plasma and 35µl of nuclease free water to elute miRNA from the column.

### 3.6.2. miRNA concentration assessment

After isolation the RNA concentration in each sample was analysed using the NanoDrop (ND1000 spectrophotometer) and the Qubit (MyQubit miRNA Assay, Qubit® 2.0 Fluorometer.) assays. The Nanodrop was used to enable measurement of total RNA and the Qubit enabled measurement of the quantity of the small RNAs present in the sample.

The operation of the NanoDrop is based on two fiber optic cables that have a gap in which a minimum of 1µl of sample is loaded. Light emitted by one cable passes through the sample and is then 'detected' by the other cable. The Purity of the samples is measured by calculating the ratio of absorbance at different wave lengths (280, 260 and 230 nm). This technique measures the concentration of total RNA is by loading 1µl of each sample into the sampling arm.

The measurements in the Qubit were done using a kit for miRNA (the Qubit® microRNA Assay Kit) this assay is highly selective for quantity but not quality of small RNA, based on the quantification of fluorescence in comparison with a reference standard. For this procedure the working solution was prepared by mixing the Qubit® microRNA reagent (1:200) with Qubit® microRNA buffer Two standards (supplied by the manufacturer) were included in each run. For the analysis of samples, the volume required is between 1 and 20µl and was adjusted to 200µl with the working solution prior to analysis using the Qubit® 2.0 Fluorometer

As the Qubit measurement is a more accurate determinate of small RNA concentration it was routinely used for standardisation of the small RNA concentration in the samples to be analysed. Each sample was analysed at two volumes (2.5µl and 5µl).

### 3.6.3. Reverse-transcription (RT)

Following the measurement of miRNA a reverse-transcription (RT) step was performed. The RT step converts the miRNA in to cDNA. This step was performed in two ways, depending on whether a pre-amplification step was included in the sample preparation. The experimental design for this is shown schematically in Figure 3-5.

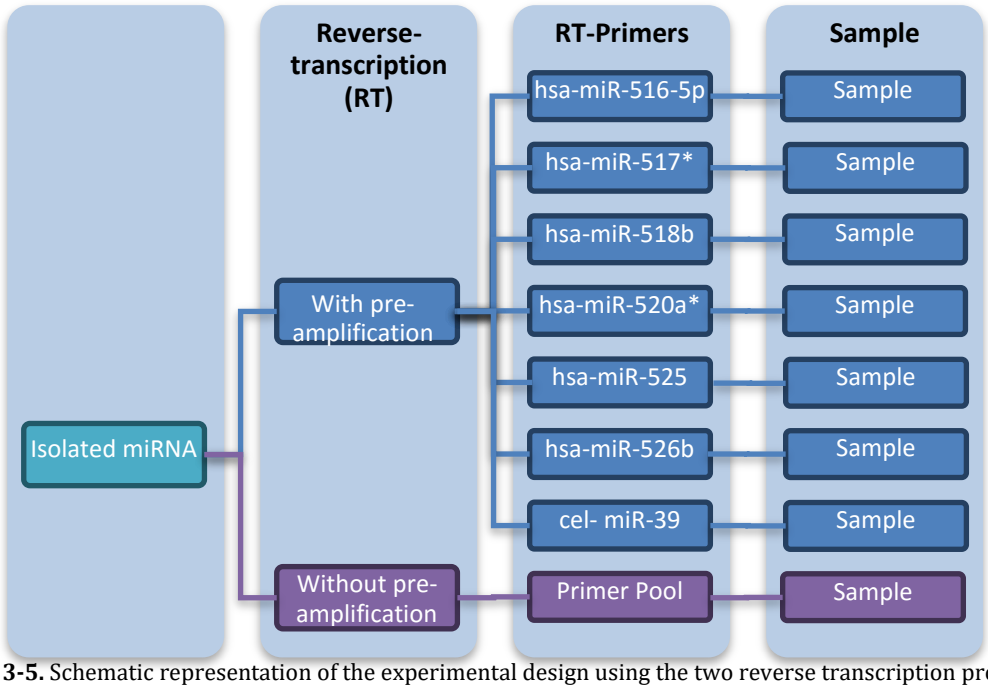



**Figure 3-5.** Schematic representation of the experimental design using the two reverse transcription protocols.

To measure the sample miRNA levels in the absence of pre-amplification the RT process proceeded as follows: a master mix was prepared (Table 3-2. a), and divided based on the number of miRNAs being investigated (Figure 3-5). To each portion 5µl of the isolated miRNA was added, followed by 3µl of primer specific for the miRNA being investigated. The samples were then run in the thermo cycler using the cycling parameters described in Table 3-2. b.

**Table 3-2.** Reverse-transcription without pre-amplification.
Shown are **a.** the contents of the master mix; **b.** the thermal cycler amplification program

**a.**

| | **Per sample(µl)** |
|---|---|
| 100mM dNTPs (with dTTP) | 0.15 |
| MultiScribeTM Reverse Transcriptase, 50 U/µL | 1 |
| 10×Reverse Transcription Buffer | 1.5 |
| RNase Inhibitor, 20 U/µL | 0.19 |
| Nuclease-free water | 4.16 |
| Total volume | 7 |

**b.**

| **Step** | **Time (min)** | **Temp ºC** |
|---|---|---|
| Hold | 30 | 16 |
| Hold | 30 | 42 |
| Hold | 5 | 85 |
| Hold | ∞ | 4 |

In some cases a pre-amplification was performed as follows: the master mix (Table 3-3. b) consisted of a primer pool (Table 3-3. a.) (Figure 3-5) which consisted of 5.83µL of each 5X miRNA primer being investigated. The isolated miRNA (2ul) was added to 13µl of the master-mix/primer-pool and the samples were amplified using the conditions described in Table 3-2. b.

**Table 3-3.** Reverse-transcription step. When performing the pre-amplification.
**a.** Reverse-transcription primers pool; **b.** Content of the master mix

**a.**

| **5X miRNA Primers(**µl) | **# Of assay** | **Total pooled volume(**µl) | **Volume of 1X TE(**µl) | **Total of RT(**µl) |
|---|---|---|---|---|
| 5.83 | 7 | 40.81 | 542.19 | 583.00 |

**b.**

| | **Per Sample(**µl) |
|---|---|
| RT Primer Pool | 6.75 |
| 100mM dNTPs (with dTTP) | 0.34 |
| MultiScribeTM Reverse Transcriptase, 50 U/µL | 3.38 |
| 10✕Reverse Transcription Buffer | 1.69 |
| RNase Inhibitor, 20 U/µL | 0.21 |
| Nuclease-free water | 1.14 |
| Total volume | 13.51 |

### 3.6.4. Pre-amplification (pre-amp) testing

Pre-amplification is an optional step between the RT and the real time-PCR. This step amplifies the miRNA of interest and is useful in cases where miRNA concentrations are low. This step is only performed when following the "with pre-amplification" branch of Figure 3-5. The final step of the standard protocol (TaqMan®Pre-amplification, Life Technologies, Carlsbad, California 92008) was optimised.

5.83 µL of each 20X miRNA (mix of forward, reverse primers, and the miRNA-specific probes) were combined to make the pre-amp pool (Table 3-4. a.). This pool was added a

master mix (Table 3-4. b.). 2.5µl of the cDNA was added to 22.5µl of the master mix. Subsequently the samples were run in the thermo cycler following the parameters in Table 3-4c.

**Table 3-4.** Pre-amplification step.
**a.** Pre-amp primers pool **b.** Content of the master mix **c.** Parameters values to program the thermal cycler

**a.**

| **20X miRNA(**µl) | **# Of assay** | **Total pooled volume(**µl) | **Volume of 1X TE(**µl) | **Total of Pre-amp(**µl) |
|---|---|---|---|---|
| 5.83 | 7 | 40.81 | 542.19 | 583.00 |

**b.**

| | **Per Sample(**µl) |
|---|---|
| TaqMan Pre-amp Master Mix (2X) | 14.06 |
| Pre-amp Primer Pool | 4.22 |
| Nuclease-free water | 7.03 |
| Total volume | 25.31 |

**c.**

| **Step Type** | **Time (min)** | **Temp ºC** |
|---|---|---|
| Hold | 10 | 95 |
| Hold | 2 | 55 |
| Hold | 2 | 72 |
| Cycle (14 Cycles) | 15 s | 95 |
| | 4 | 60 |
| Hold | 10 | 99.9 |
| Hold | ∞ | 4 |

The final step of the standard protocol is to add 175µl of 0.1X TE buffer (pH 8.0) to each sample to give a final volume of 200µl. For the optimization of this step, instead of the addition of a 175µl of 0.1X TE buffer, 4 serial dilutions of the pre-amp product were prepared (Figure 3-6, 1:8 being the standard dilution).

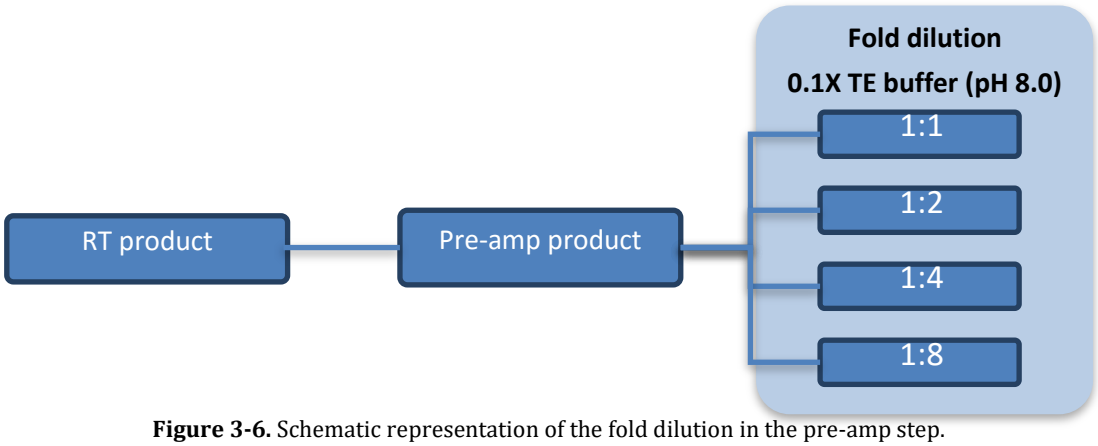



**Figure 3-6.** Schematic representation of the fold dilution in the pre-amp step.

### 3.6.5. Real time Polymerase Chain Reaction (RT-PRC)

After the RT and pre-amp steps the RT-PCR was performed following the TaqMan® Fast Advanced Master Mix protocol (Life Technologies, Carlsbad, California 92008). A master mix was prepared as described (Table 3-5) and diluted pre-amp product or RT product (3ul) were added to the plate with 7µl of master mix. Subsequently the samples were run in LightCycler®480 following the parameters in the Table 3-5. b. The LightCycler®480 calculated the threshold cycle value (or crossing point, Ct or Cp).

**Table 3-5.** Details of RT-PCR
**a.** Content of the master mix; **b.** thermocycling parameters used to amplify miRNAs of interest on the Roche LightCycler®480.

**a.**

| | **384- well plates (µl)** |
|---|---|
| TaqMan® Fast Advanced Master Mix (2✕) | 5 |
| TaqMan® Gene Expression Assay (20✕) | 0.5 |
| Nuclease-free water | 1.5 |
| Total volume | 7 |

**b.**

| **Step Type** | **Time (min)** | **Temp ºC** | **Cycle** |
|---|---|---|---|
| Hold | 2 | 50 | |
| Hold | 0.2 | 95 | |
| Denature | 0.03 | 95 | 40 |
| Anneal/extend | 0.3 | 60 | |

## 3.7.Data analysis

The data was analysed by calculating the ΔΔCt values from the Ct values generated by the LightCycler®480. This calculation takes into account the Ct-values of each sample, the Ct values of the spike in control and the Ct-values of reference sample (Equation 3-1). In the case of the time and temperature experiments the data obtained from the baseline sample (i.e. the earliest time point at 4°C) was used as the reference sample. The studies investigating the expression of miRNAs in the fetal anomaly cohort used the pooled sample included in each run as the reference sample.

**Equation 3-1.** Equation of the ΔΔCt.
CT.GOI: Ct value of the miRNA of the sample of interest; GM.HK: Ct value of the spike-in of the sample of interest; CT.GOI.QC: Ct value of the sample of reference; GM.HK.QC: Ct value of the spike-in of the sample of reference.

$$\Delta\,\Delta Ct = 2^{-((CT.GOI-GM.HK)-(CT.GOI.QC-GM.HK.QC))}$$

.

The data was analysed using SPSS software version 22.0 (SPSS, Ing, Chicago, USA) and the graphics where generated in GraphPad Software (GraphPad Prism version 6.00, La Jolla California USA, www.graphpad.com). A t-test was performed for the detection of differences in the optimization. A Mann-Whitney U test was used to compare the relative expression level of the miRNA in the time and temperature experiment, and for the analysis of the effect of fetal anomalies on miRNA expression.

# 4. Results

## 4.1.Description of the samples

A total of 42 blood samples collected from pregnant women were analysed in this study. The range of abnormalities and the way they are grouped for this study is described in Table 4-1. The characteristics of the sample population, in particular the screening criteria for fetal abnormalities, are reported in Table 4-2. The measurements obtained at first trimester screening such as crown-rump length (CRL: mm), nuchal translucency (NT: mm), free beta human chorionic gonadotrophin (free BhCG: multiple of the median (MoM)) and pregnancy associated placental protein A (PAPP-A: MoM) are included. Some women did not have first trimester screening – so this information is only available for a subgroup of cases.

The different variables for the groups with genetic abnormalities were compared to the pregnancies with normal fetuses (controls) (Table 4-2). When the group with congenital abnormalities as a whole were compared with those with normal pregnancies there was no difference in age or gestational age. There is a significant difference between the controls and the neural tube defect group for the CRL and the NT ($p<0.01$). Furthermore, there is a significant difference between the controls and congenital heart defect group for the NT ($p<0.01$).

**Table 4-1.** Congenital abnormalities studied.

| Condition | | N | Specific Abnormalities |
|---|---|---|---|
| **Congenital abnormalities (C-A)** | **T21** | 14 | Trisomy 21 |
| | **T18** | 1 | Trisomy 18 |
| **Neural Tube Defects (NTD)** | **S-B** | 3 | Sacral Spina Bifida |
| | **Anen** | 1 | Anencephaly |
| **Congenital Heart Defects (CHD)** | **HLHS** | 2 | Hypoplastic left heart syndrome |
| | **Other** | 1 | Corrected Transposition of great aeries |
| | | 1 | Tetratology of Fallot with absent pulmonary syndrome |
| | | 1 | Ventricular septal defect, overriding aorta |
| | | 1 | Arch Hypoplasia |

**Table 4-2.** Characteristics of the various patient populations studied.

| | Controls | Chromosomal abnormalities (C-A) | | | Neural Tube Defects (NTD) | | | Congenital Heart Defects (CHD) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | All C-A | T21 | T18 | All NTD | Spina Bifida | Anen | All CHD | HLHS | Other |
| **N** | 16 | 15 | 14 | 1 | 4 | 3 | 1 | 6 | 2 | 4 |
| **Age** | 31.4 ± 3.5 | 37.2 ± 3.8 | 37.1 ± 4 | 39 | 29.5 ± 3.5 | 28.3 ± 3.2 | 33 | 31.2 ± 1.9 | 31 ± 2.8 | 31.3 ± 1.9 |
| **Gestational age (Week)** | 22.8 ± 7.4 | 13.6 ± 1.9 | 13.7 ± 1.9 | 12 | 17.3 ± 2.9 | 18.7 ± 0.6 | 13 | 20.3 ± 3.9 | 19.5 ± 0.7 | 20. 8 ± 4.9 |
| **$N^+$** | 11 | 12 | 11 | 1 | 2 | 1 | 1 | 4 | 1 | 3 |
| **Crown-rump length (CRL) (mm)** | 64 ± 6.3 | 64.9 ± 9.9 | 66.2 ± 9.1 | 50 | 66.3 ± 2.3* | 67.9 | 64.6 | 68.0 ± 8.3 | 77.0 | 65 ± 7 |
| **Nuchal translucency (NT) (mm)** | 1.9 ± 0.5 | 3.2±1.5* | 3.3 ± 1.5 | 2.2 | 1.4 ± 0.3* | 1.6 | 1.15 | 2.8 ± 1.8* | 2 | 3.1 ± 2.1 |
| **Free beta hCG. (MoM)** | 1.6 ± 1 | 2.7 ± 2.3* | 2.9 ±2.4 | 0.2 | 1.4 ± 0.1 | 1.49 | 1.31 | 0.9 ± 0.2 | 0.82 | 0.9 ± 0.2 |
| **PAPP-A. (MoM)** | 1.6 ± 1 | 0.55 ± 0.4* | 0.5 ± 0.4 | 0.32 | 0.6 ± 0.4 | 0.8 | 0.3 | 1.4 ± 0.7 | 1.8 | 1.4 ± 0.7 |

Rt: Experiment at room temperature, 4°C: Experiment at 4°C; $N^+$: total number of samples used for the analysis of the first trimester screening; Anen: Anencephaly; T21: Trisomy 21; T18: Trisomy 18; HLHS: Hypoplastic Lefts Heart Syndrome. (Values are shown as mean ± SD).

* Significantly different from control ($p$<0.05)

## 4.2.Optimization of sample processing

The characteristics of patient samples used for these optimisation studies are shown in Table 4-3. All samples were obtained from women with normal pregnancies. Additionally no differences in fetal characteristics at the first trimester screening were observed for the samples analysed at after incubation at room temperature or 4 °C.

**Table 4-3.** Characteristics of the controls studied.

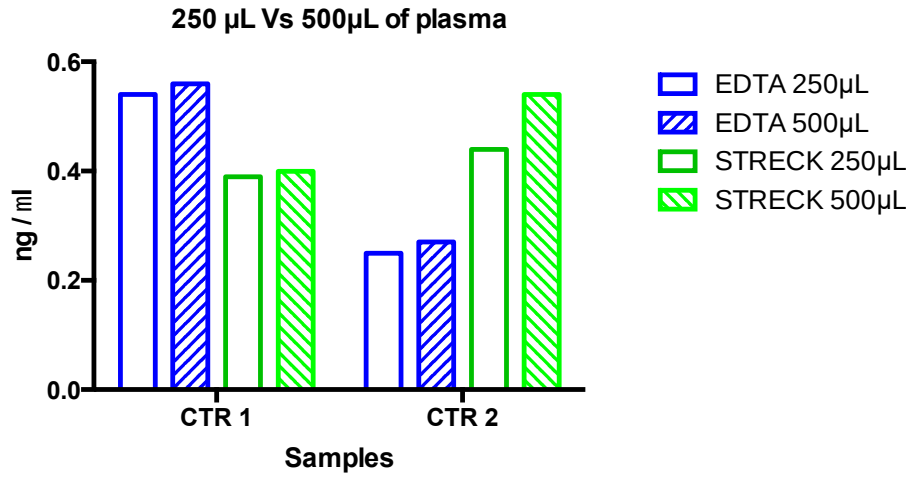
| | Controls | | |
|---|---|---|---|
| | **All controls** | **Rt** | **4°C** |
| **N** | 29 | 7 | 7 |
| **Age** | 31.5 ± 3.6 | 32 ± 3.8 | 31.1 ± 4 |
| **Gestational age (Week)** | 21.3 ± 5.6 | 19.6 ± 1.0 | 19.3 ± 0.5 |
| **$N^+$** | 23 | 6 | 6 |
| **Crown-rump length (CRL) (mm)** | 64.6 ± 7.2 | 60.8 ± 7.7 | 65.5 ± 8.8 |
| **Nuchal translucency (NT) (mm)** | 1.8 ± 0.4 | 1.8 ± 0.3 | 1.6 ± 0.4 |
| **Free beta hCG. (MoM)** | 1.4 ± 0.8 | 1.3 ± 0.5 | 1.4 ± 0.7 |
| **PAPP-A. (MoM)** | 1.5 ± 1 | 1.5 ± 1.3 | 1.3 ± 0.7 |

Rt: Experiment at room temperature, 4°C: Experiment at 4°C; $N^+$: total number of samples used for the analysis of the first trimester screening

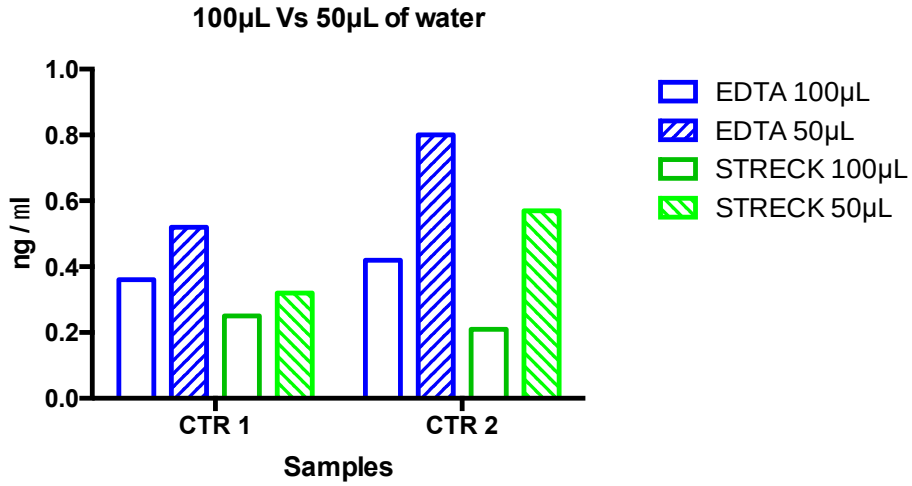
### 4.2.1. miRNA extraction

For the optimization of the volume of blood to be used for nucleic acid extraction we compared 250µl and 500µl of starting material (plasma). These volumes were eluted with 100µl of nuclease-free water and the miRNA concentration in the eluent was measured using Qubit. We observed (Figure 4-1. a.) that there was little difference in the concentration of the eluted miRNA, but there was a difference in miRNA concentration between samples.

**a.**




**b.**




c.

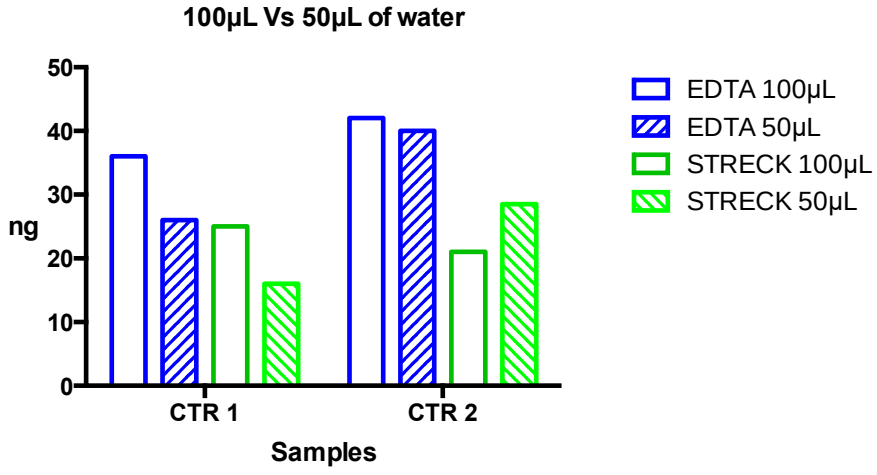



**Figure 4-1.** Optimization of the extraction step
**a.** Difference between the initial amount of plasma added in the extraction; **b.** Difference in concentration between the volume of eluent added (nuclease-free water) at the end of the extraction to elute the miRNA c. difference in amount between the volume of eluent added (nuclease-free water) at the end of the extraction to elute the miRNA.

For the second optimization test we used a starting volume of 620µl of plasma and two different volumes of nuclease-free water to elute the miRNA. As before, the miRNA concentration was measured using the Qubit. The results are expressed as ng/ul and total miRNA extracted (Figure 4-1. b. and c. respectively). Again the results were variable between the samples with, as expected, a lower concentration of miRNA with the addition of more nuclease-free water. There was no difference between tube types and in particular for CTR 2 the amount of miRNA eluted was the same irrespective of elution volume. (For these two optimization steps no statistical analysis was performed as there was only one datum for each variable).

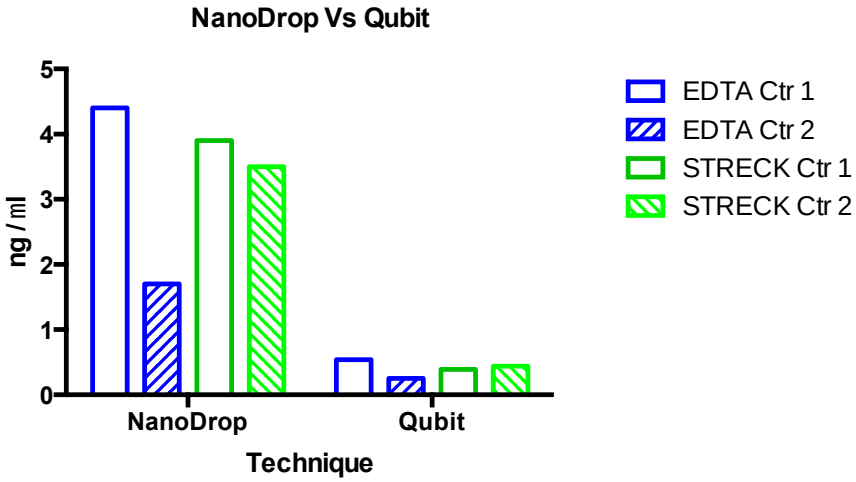
### 4.2.2. miRNA concentration assessment

To investigate the optimal method to determine the miRNA concentration two different techniques were used. The parameters for these tests were: 620µl of starting material (plasma), 100µl of eluent (nuclease-free water). Each sample was measured in the NanoDrop (1µl of sample) and the Qubit (2.5µl of sample). Irrespective of tube type, and not unexpectedly the concentrations of RNA measured by the NanoDrop were much higher than the concentration of small or miRNAs determined using the Qubit (Figure 4-2. a.).

The effect of volume loaded (i.e. 2.5µl and 5µl) was also examined in the Qubit assay system. The parameters for these readings were: 620µl of starting material (plasma), samples were eluted with 50µl of nuclease-free water and read using the Qubit. Increasing the volume loaded did not increase miRNA concentration and was not

affected by tube type (Figure 4-2. b.). (As before no statistical analysis was performed, as just one datum was collected for each variable).

**a.**




**b.**

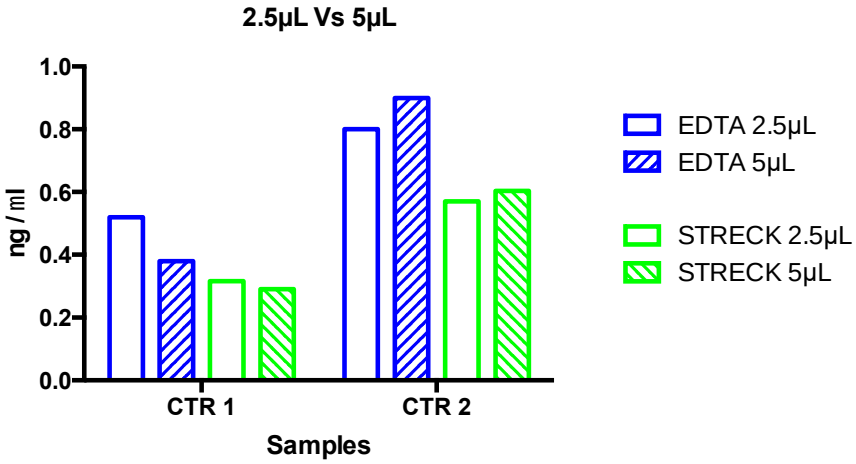



**Figure 4-2.** Optimization of the measurement step.
**a.** Difference between NanoDrop and Qubit for the measurements of the concentration; **b.** Difference between the miRNA concentrations with different initial amounts of extracted miRNA (2.5µl and 5µl) measured in the Qubit

### 4.2.3. miRNA primers and probes

Initially the cycle threshold (Ct) of cel-miR-39 was used to ensure reverse transcription and amplification, dilution curves are shown in Figure 4-3. For cel-miR-39 the Ct-values increased linearly, representing a decrease in concentration, with increasing dilution (Figure 4-3. a). Dilution curves for the other miRNAs examined were performed to

establish the linear range (Figure 4-3. b). Unfortunately, due to low concentrations, miR-526 and miR-517 failed to amplify before Ct 35 and for this reason were not included in any further analysis. The dilution curves for the remaining miRNAs are shown in Figure 4-3. b.

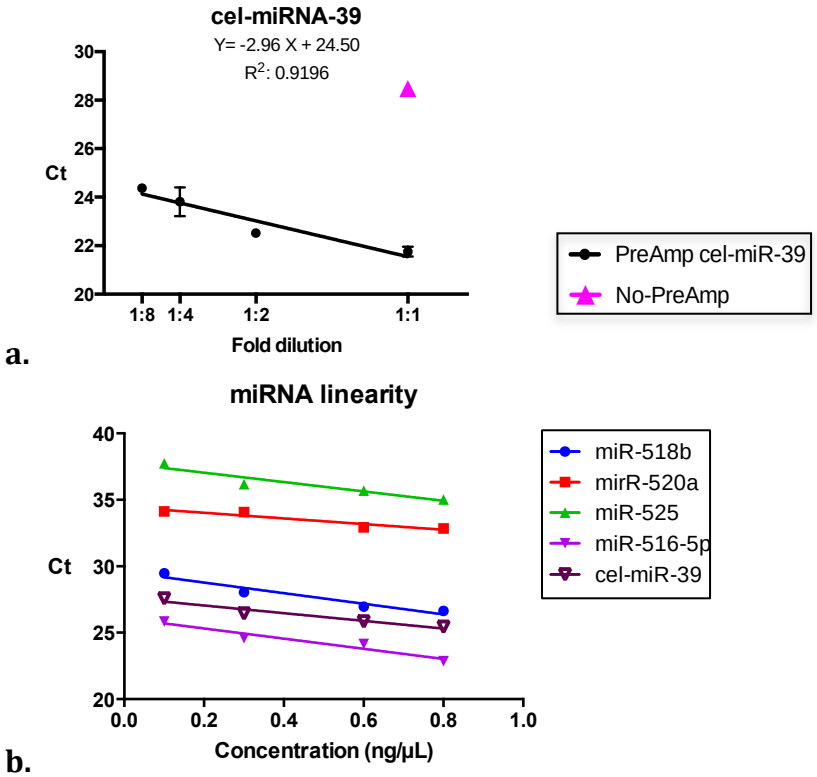



**Figure 4-3.** Efficiency of miRNA primers and probes.
**a.** Efficiency of primers and probes with out Pre-amp; **b.** Efficiency and optimization cel-miR-39, with Pre-amp and without.

### 4.2.4. Pre-amplification testing

To ensure that the pre-amp was working correctly and not interfering with the RT-PCR process, and to find the optimum dilution of the pre-amp product, a second set of serial dilutions were preformed and the results were compared to those obtained from samples which had not undergone the pre-amp step. The linear trend observed in the previous section was also observed for the pre-amplified products for all the miRNA in the study (Figure 4-4. a-d).

The results also appear to show a difference in the Ct-values between the pre-amp samples and the non-pre-amp ($p<0.05$). In all cases the pre-amp samples amplified at an earlier Ct reflecting an increase in miRNA. Additionally for all the miRNAs studied, the non-pre-amp has an even later Ct-value (i.e. lower concentration) than the most dilute sample. Furthermore as shown the Ct-values for all the miRNAs studied decreased in a linear fashion with dilution (Figure 4-4. a-d).

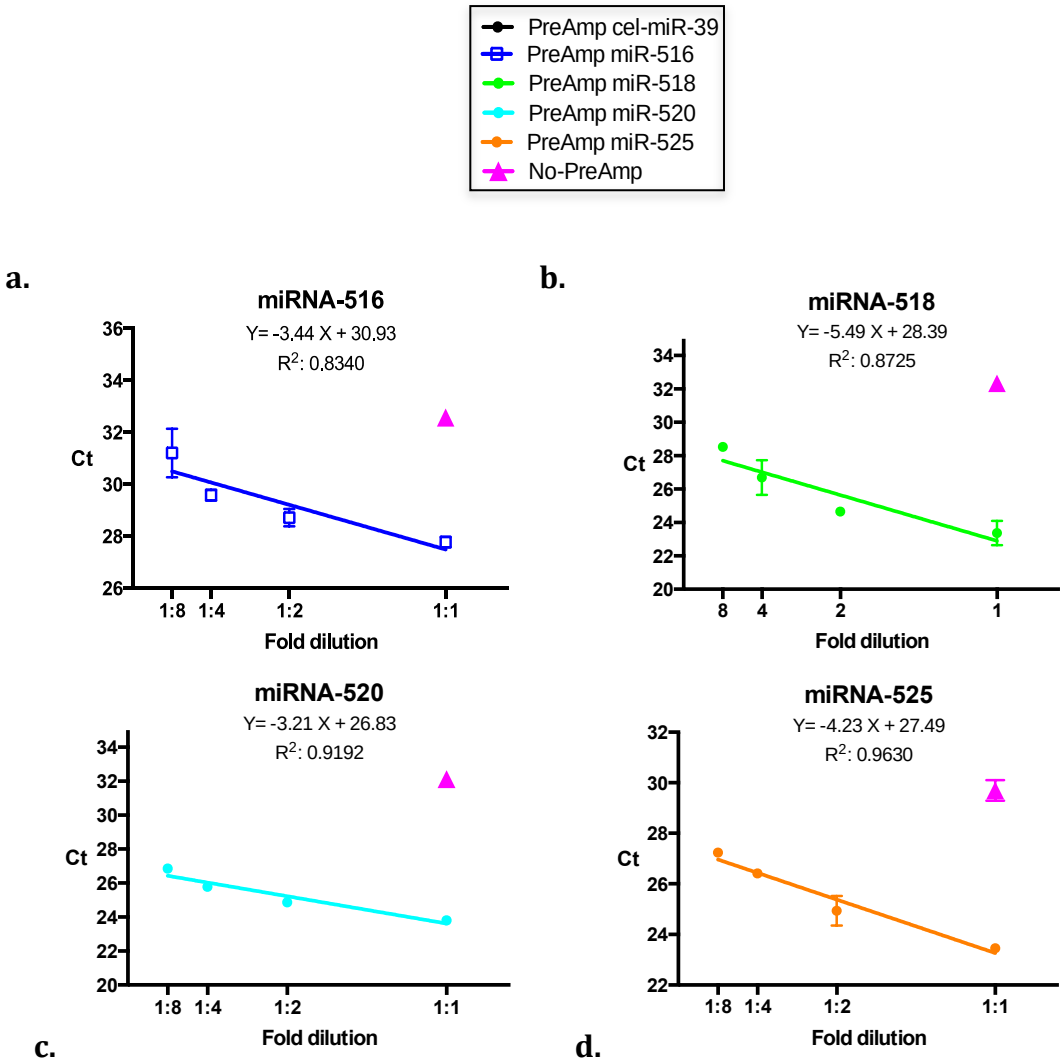



**Figure 4-4.** Optimization of Pre-amp and efficiency of miRNA primers and probes.
**a.** miR-516 with Pre-amp and with-out; **b.** miR-518 with Pre-amp and with-out**; c.** miR-520 with Pre-amp and with-out; **d.** miR-525 with Pre-amp and with-out.

## 4.3. Results of the experiments to test the stability miRNA in each tube in different conditions.

### 4.3.1. Control samples at 4°C

The results of the miRNA analysis for the seven control samples kept for 4 hours, 1 day (1D) and 2 days (2D) at 4°C are shown in Figure 4-5. Data was only analysed if there were more than two time points measured. As shown the Ct values were very variable between the samples analysed and across the time points studied. In general due to the high variability there was no observable differences between the tubes for each time point. However for the STECK tube, the miR-520 at 2 days appeared different to the other time points analysed. (Figure 4-5. a). Additionally for the EDTA and STRECK tubes there tended to be less variability in Ct at the 4 hours point when compared with the 1D or 2D analysis. This pattern was not seen for the CITRATE tubes.

The results were then expressed as the level of expression relative to the internal control (Cel-miR-39) and the results are shown in Figure 4-6. As shown the plasma concentration of miRNAs were affected differently depending on the duration between collection and extraction and the tube type. Interestingly the citrate tube showed the least variability between time points and the STRECK tube the greatest. Additionally miRNA-520 and miRNA 525 appeared to be more stable than miRNA-516. In these experiments the miRNA-518 did not amplify using the parameters described in the methods section and was not used for these analyses.

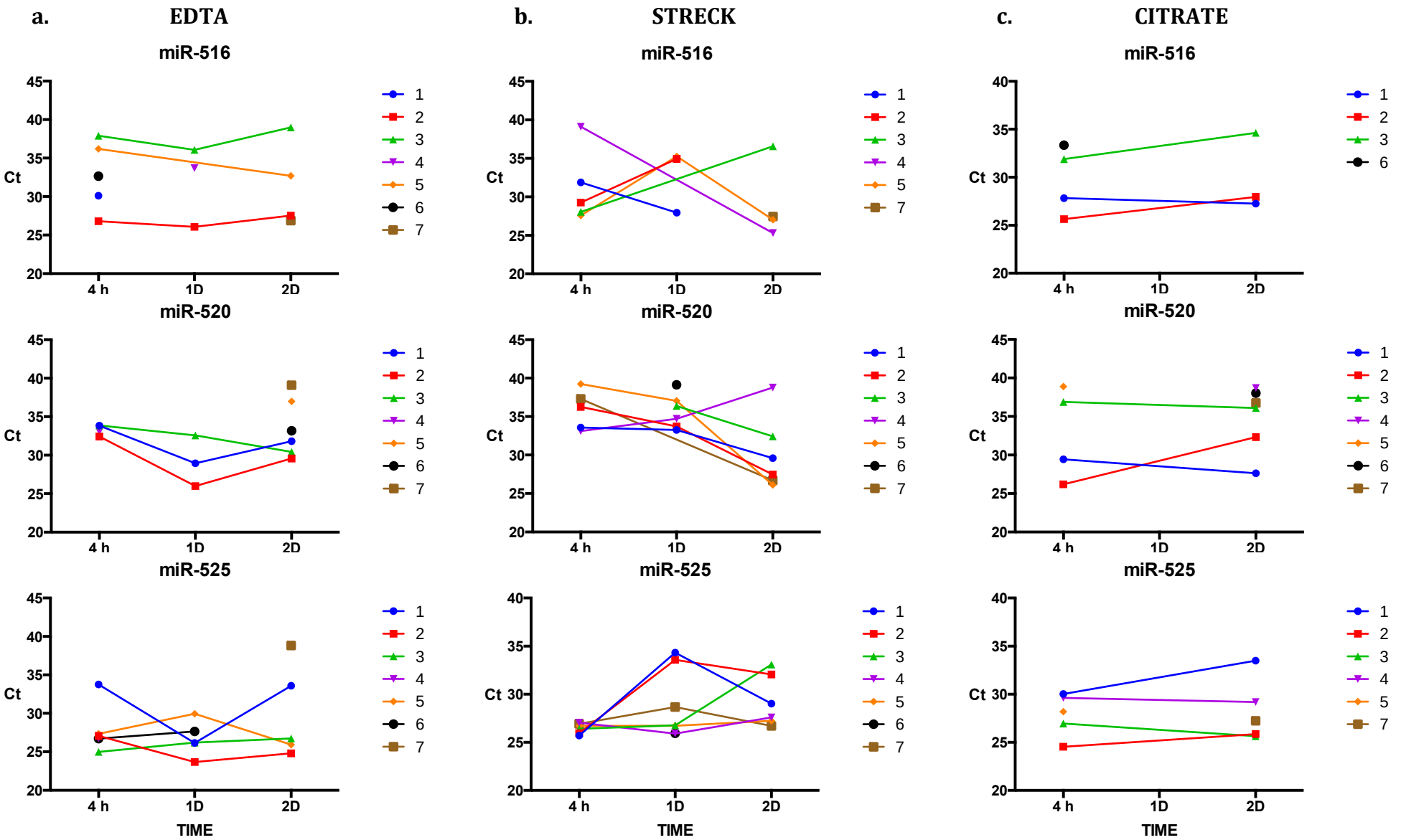



**Figure 4-5.** Raw Ct-value. Variability between the 7 control samples, showing the effect of collection tube over three time points at 4°C.
a. EDTA; b. STECK; c. CITRATE.

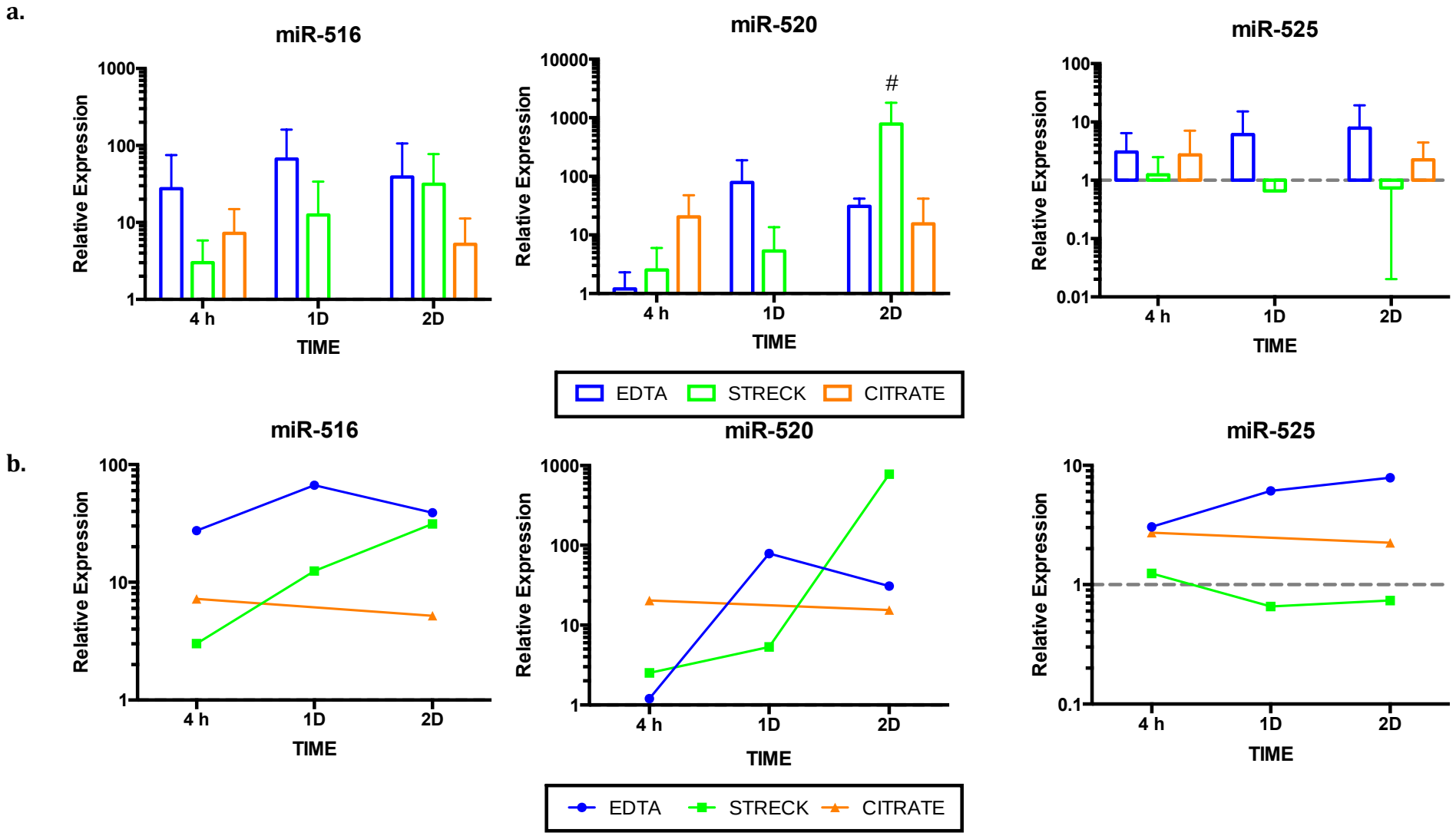



**Figure 4-6.** Relative expression of all the miRNA over time for each tube.
**a.** Effect of time; **b.** Trend of each tube.
# $p<0.05$ Significant difference for the tube compared to the first and second time points.

### 4.3.2. Control samples at room temperature (Rt)

Analysis of the effect of maintaining samples at room temperature for four days is shown in Figure 4-7. As before no analysis was performed on samples where there were less than two time points.

As previously shown, there was variability in the Ct values obtained for each sample. Overall there was no significant difference in the CT value between the tube types for each time point. However for the EDTA tubes there was a significant decrease in the level of miR-525 between the third and fourth days ($p<0.05$)_ (Figure 4-8. a. miR-525). The data were then expressed relative to controls (Cel-miR-39 and first time point). As shown (Figure 4-9) there were different trends for each tube type over the 4 days of the study. The EDTA tubes appeared to have an increasing relative expression level of miR-516. The level of miR-520 was increased in the samples collected in EDTA tubes, whilst the pattern for miR-525 was different with decreased relative expression at day 4. This pattern was less obvious for the STRECK tubes and for the CITRATE tubes Figure 4-7. b.

It is best to use EDTA or CITRATE tubes if the samples can be stored at 4°C and is better to store the plasma at -80°C before the third day. On the other hand if the samples are going to be left at room temperature before the plasma is separated it is best to used STRECK tube and they shouldn't be left more than three days.

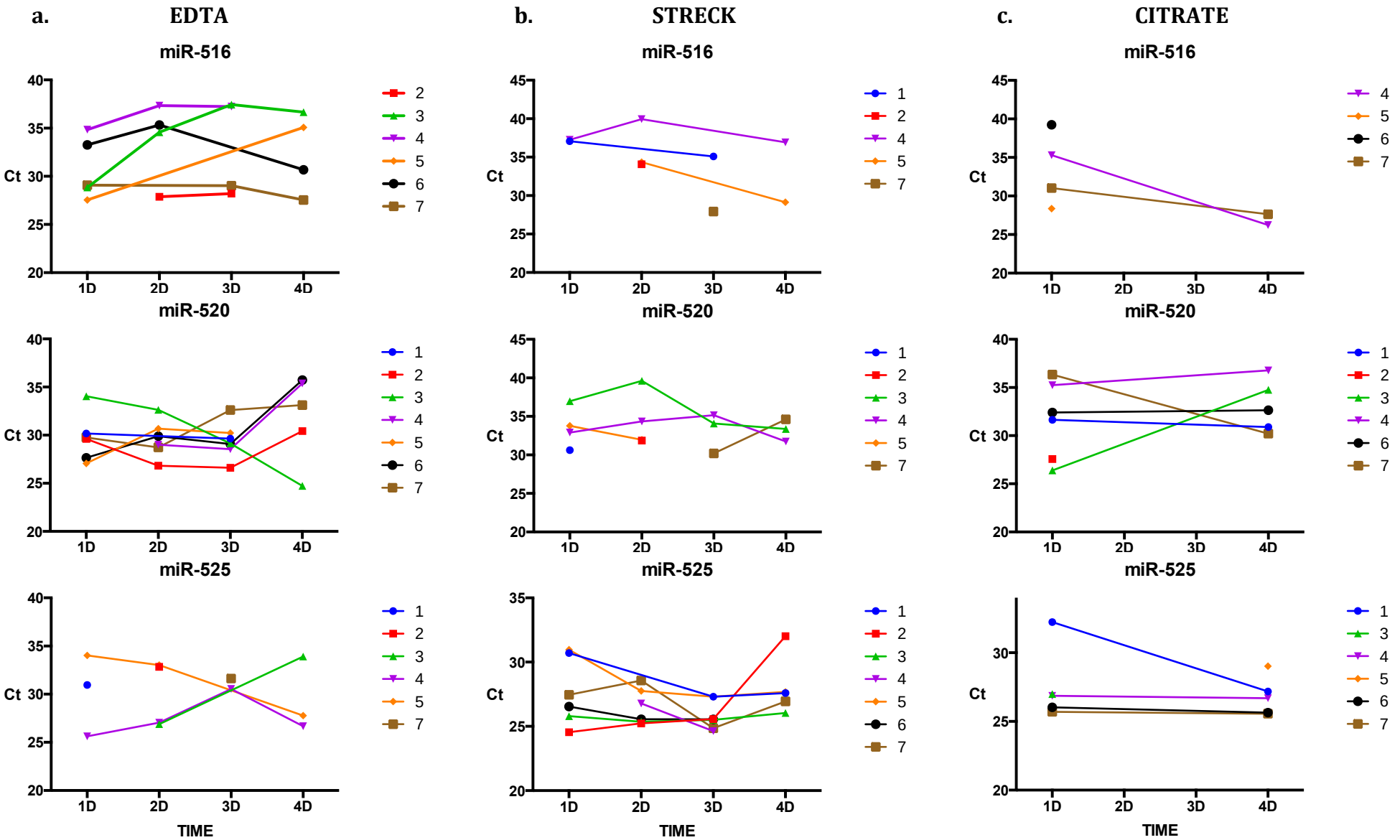
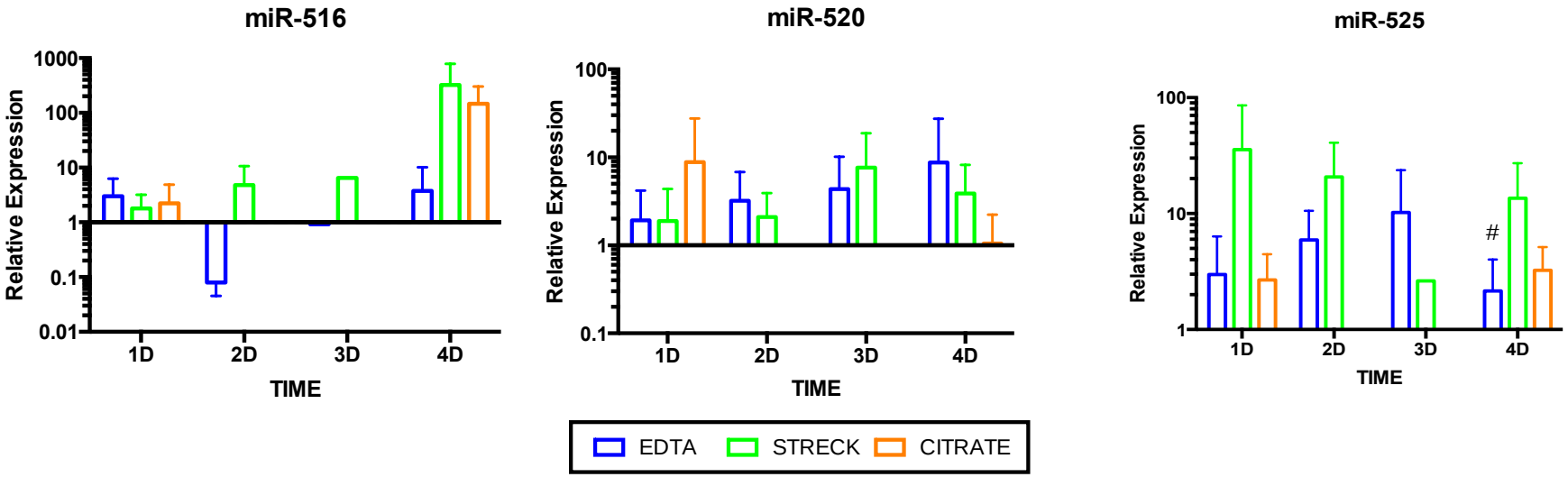



**Figure 4-7.** Raw Ct-values. Variability between the 7 control samples, showing the effect of collection tube and time though three time points at Rt.
**a.** EDTA; **b**. STECK; **c.** CITRATE.

a.




b.

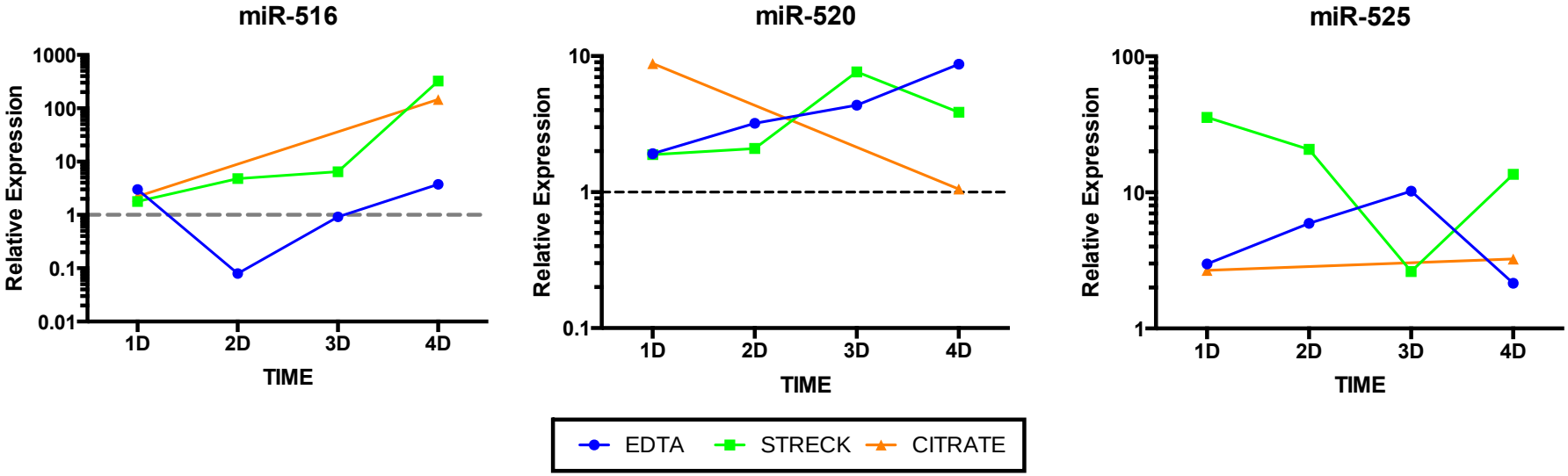


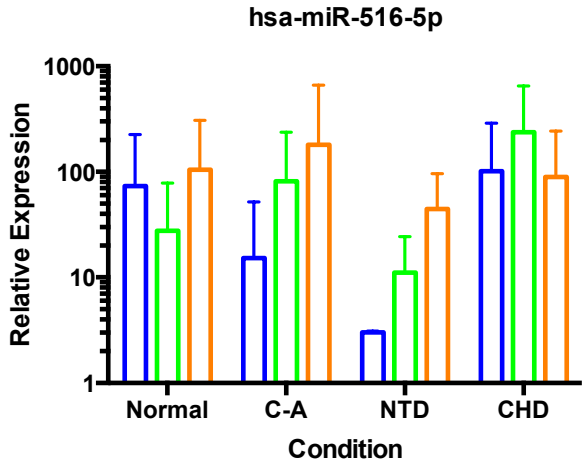

**Figure 4-8.** Relative expression of all the miRNA during time for each tube left at Rt.
**a.** Effect of time; **b.** Trend of each tube.
# $p<0.05$ Significant difference for the tube compared to the third time points.

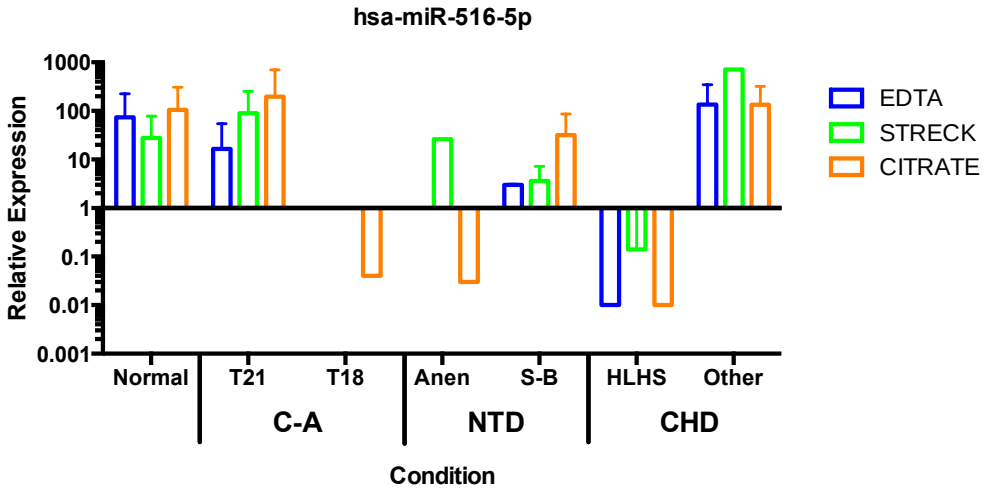
### 4.3.3. Samples for the comparison of different fetal anomalies

In the analysis of different conditions we first compared all the congenital anomalies against the controls. For these studies, samples were collected in the three different tubes, left at room temperature and the plasma was stored within 24 hours. On the whole the pattern of expression of each miRNA was similar irrespective of the collection tube used.

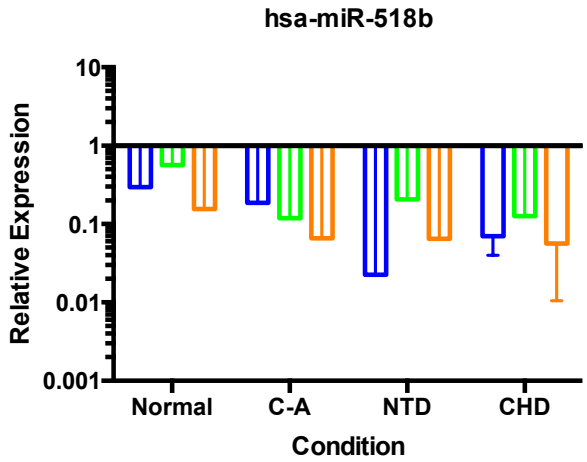
When the data obtained from the different groups of congenital abnormalities as a whole were compared with the controls no major differences in levels of any of the miRNAs was observed. There appeared to be a trend toward decreased levels of miRNA 525 in the NTD group and the CHD group but due to the small numbers this failed to reach significance. In contrast, when the specific conditions within each group were compared to controls, significant differences were observed for miR-520 that had a higher level of expression in the HLHS group compared to the normal pregnancies ($p<0.05$) (Figure 4-9, c).

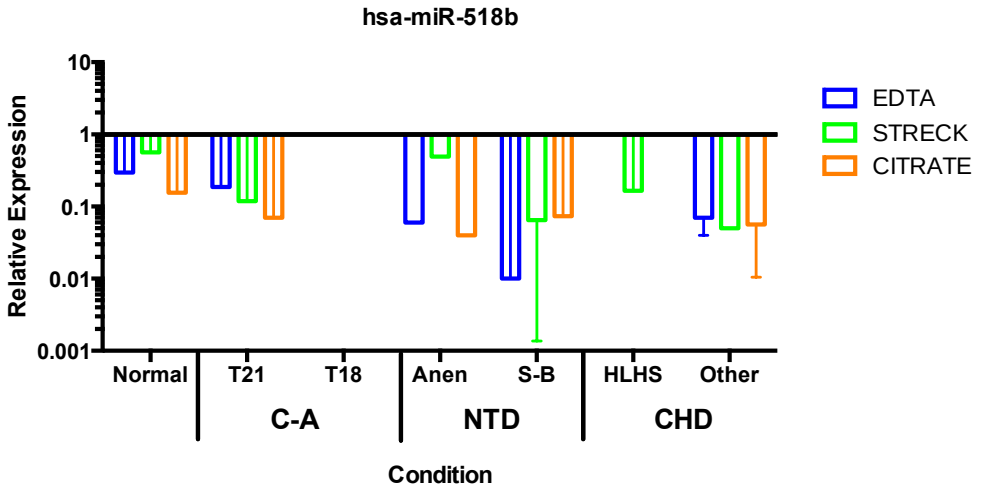
**a.**


hsa-miR-516-5p
Relative Expression
1000
100
10
1
Normal
C-A
NTD
CHD
Condition

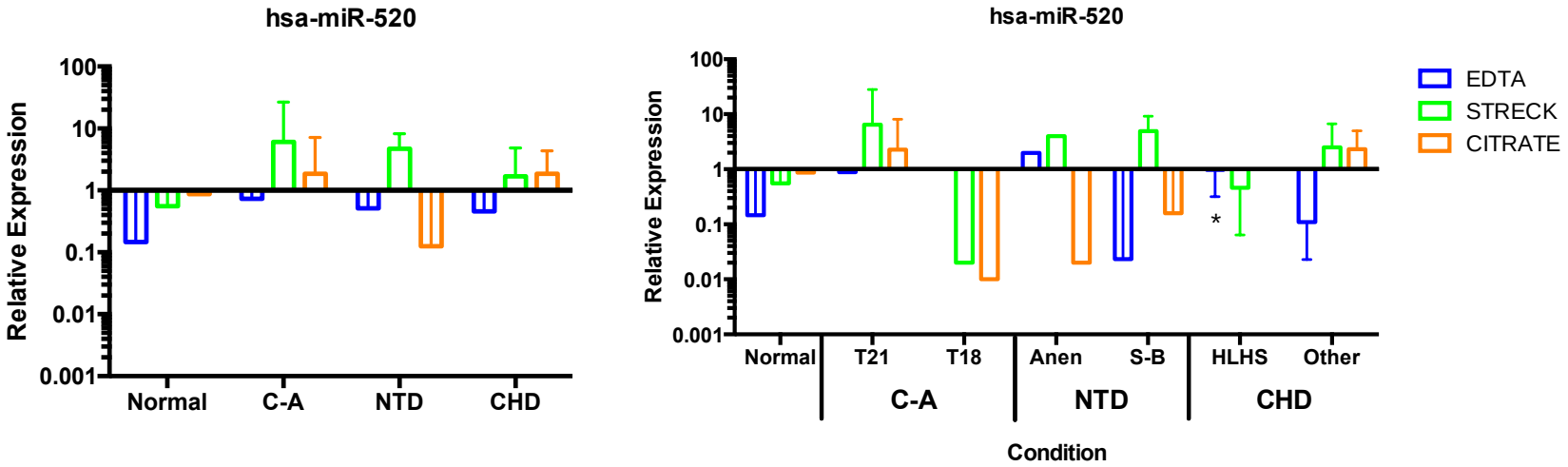

hsa-miR-516-5p
Relative Expression
1000
100
10
1
0.1
0.01
0.001
Normal
T21
T18
Anen
S-B
HLHS
Other
C-A
NTD
CHD
Condition
EDTA
STRECK
CITRATE

**b.**


hsa-miR-518b
Relative Expression
10
1
0.1
0.01
0.001
Normal
C-A
NTD
CHD
Condition


hsa-miR-518b
Relative Expression
10
1
0.1
0.01
0.001
Normal
T21
T18
Anen
S-B
HLHS
Other
C-A
NTD
CHD
Condition
EDTA
STRECK
CITRATE

**c.**




**d.**

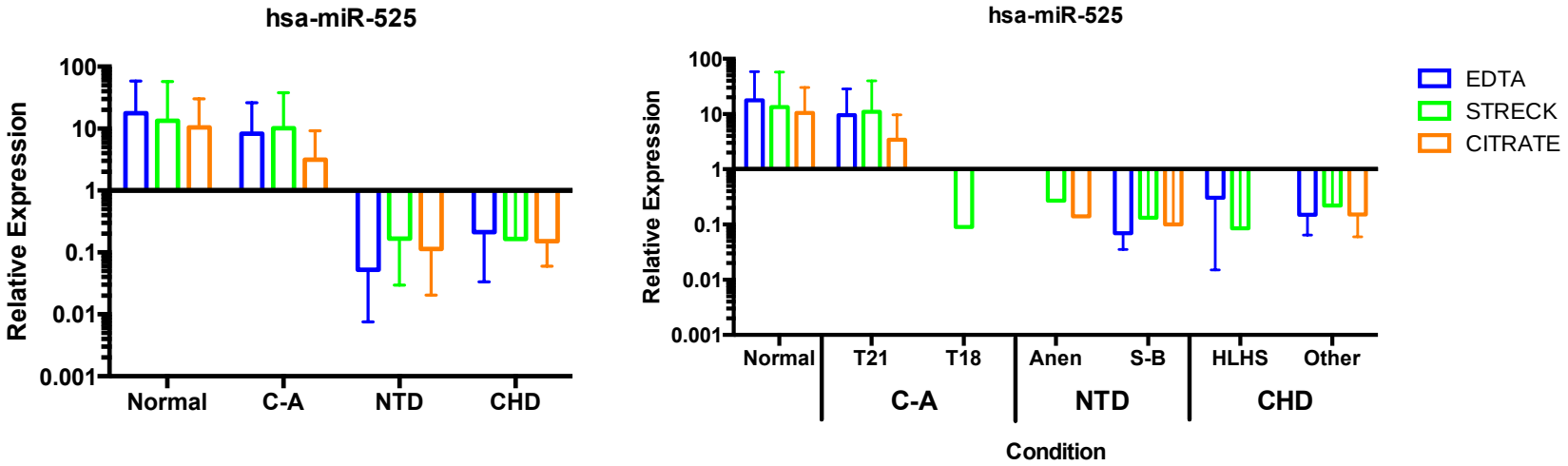



**Figure 4-9.** Relative expression of all the miRNA for the different conditions and for the different specific conditions.
**a.** Relative expression for miR-516; **b.** Relative expression for miR-518. **c.** Relative expression for miR-520; **d.** Relative expression for miR-525.
* $p<0.05$ Significant difference between the conditions and the non-pregnant women.

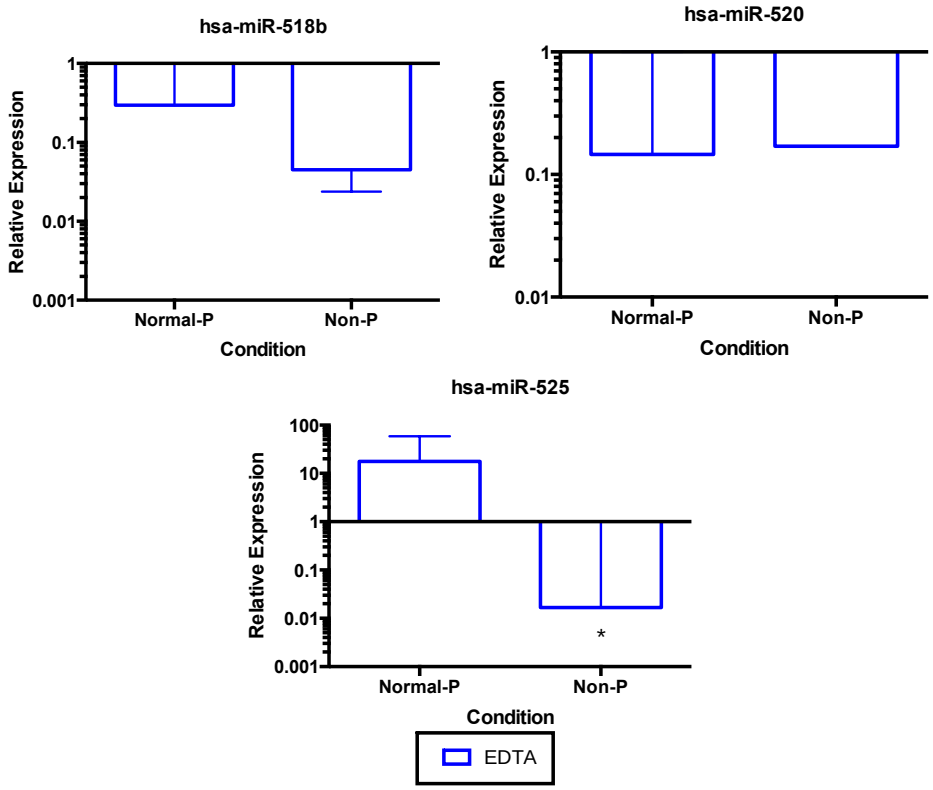



**Figure 4-10.** Comparison between normal pregnancies and non-pregnant women. Normal-P: normal pregnant women; Non-P: non-pregnant women. * $p<0.05$ Significant differences.

As some of the miRNA in the study didn´t amplify, we compared the normal pregnancies with three non-pregnant women, to see if they are expressed in non-pregnant women. For these studies blood was collected only in EDTA tubes. As with the pregnant women miR-517 and miR-526 failed to amplify. The circulating miRNA level of miR-525 was significantly lower ($p<0.05$) in non-pregnant women than pregnant women (Figure 4-10). There was, however, definite evidence of the presence of miR-525 in non-pregnant women – which contrasts to previous data suggesting this miRNA is placental specific. Similarly, miR-518 and moR-520 appear to be non-specific – and were in fact found in similar levels in pregnant and non-pregnant women. In the non-pregnant women miR-516, failed to amplify suggesting that this miRNA is indeed fetal in origin. miR-516 was therefore the only miRNA that we tested that proved to be both present and placental specific.

# 5. Discussion

## 5.1.Optimization of sample processing

In addition to using cell free fetal DNA to screen for chromosomal abnormalities or genetic anomalies, it may be possible to use other cell free nucleic acids to screen for other functional anomalies of the fetus and placenta. Cell free fetal DNA is the most studied cell free nucleic acid to date. Several groups recognised that, in order to be able to use this test in clinical circumstances, it was first necessary to define how the laboratory process was affected by different sampling and storage conditions. In establishing research in cell free fetal miRNAs we therefore felt that it was first important to define criteria for sample collection so that this process was appropriately controlled in subsequent experiments.

There are two published studies that document the impact of transport conditions / sampling tubes on cell free fetal DNA (Barrett et al. 2011; Wong et al. 2013). These studies were performed using blood from pregnant women, collected at 10 to 16 weeks of gestation. The blood was collected into either EDTA or cffDNA STRECK tubes. Samples were then stored at a range of temperatures and for various time intervals before being processed, with nucleic acid extraction from the plasma. These studies showed that the concentration of cell free DNA in EDTA tubes increases over time; and consequently the relative proportion of fetal to maternal product decreases – thus making subsequent analysis more difficult. In comparison, cell free DNA levels were stable in the cell stabilizing, STRECK tube for a longer time period. As a consequence, the STRECK tube

has become the universal product used for cffDNA analysis performed clinically to screen for chromosomal abnormalities. Other groups have, however, described that fetal DNA is stable in EDTA tubes for up to nine days (Clausen et al. 2013). In this study, whilst the total DNA in the circulation increases over the first four days, it then remained stable for a further five days more. These three studies used different techniques to process their samples but all authors suggested that cell free fetal DNA concentrations would vary depending on the conditions in which the samples were stored and transported.

There are no studies describing sample stability of cell free fetal mRNA and / or miRNA. Moving away from cffDNA towards miRNA to identify new biomarkers would expand the range of abnormalities that can be detected. Placental specific miRNAs have been detected (Mouillet et al. 2010), but the change in the expression of these miRNAs with respect to different abnormalities has not been extensively studied. To draw any conclusions a large number of samples need to be collected and studied. To enable this and due to the low prevalence of many of these abnormalities it is likely that the samples will need to be collected from a number of clinical sites. For this reason, the samples will be coming from a range of locations often some distance from the molecular lab. Therefore, before such studies are undertaken, the methodology for sample collection, transport, storage and processing needs to be confirmed. To make this possible, the first aim of this study was to identify the effect of storage condition and collection tube on the expression of fetal miRNAs. In this study we have reported a number of experiments used to optimise sampling and lab preparation technique.

From a laboratory perspective, the first step in optimisation involved deciding on the optimal amount of plasma required to extract sufficient quantities of miRNA for analysis. This is particularly important when the miRNA of interest, in this case fetal miRNA, are likely to be in low abundance compared to other sources of miRNA (here maternal circulating miRNA). In this study a comparison between two different volumes of starting plasma showed no overall differences in the quantity of miRNA extracted. We used the maximum volume of plasma possible according to the extraction kit manufacturers' instructions; but note that this volume was lower than that reported by Gu and collaborators in the detection of cffmiRNA (Gu et al. 2012). It would be expected that when the amount of plasma used in an extraction is doubled the amount of small RNA collected would also be doubled. This was not observed during the optimization. A possible explanation for this result could be that the column became saturated with small miRNA and so any additional miRNA were not fully retained. The amount of miRNA did increase slightly (1.5 fold) when the starting volume of plasma was increased so the maximum possible volume was used.

Although it was not statistically significant, there did appear to be some variation in the Ct and relative expression of miRNAs dependent on the type of tube used for collection. We therefore feel that it will be appropriate to standardise tubes across collection centres for future work. Although the STRECK tubes are more expensive ($10 each!) they do appear to be more robust when samples are transported at room temperature. This is similar to some of the findings in relation to cffDNA (Barrett et al. 2011; Wong et al. 2013). We do note, however, that this trend was not consistent across miRNA – with higher within sample variability in miR-516 than the other miRNAs – and this was the only miRNA that proved to be placental specific. Once specific miRNA candidates for

prenatal diagnosis have been identified it will be necessary to repeat optimisation studies. When the final volume used to elute the small RNA is halved the final concentration approximately doubles in most of the cases. Meaning that the smaller the volume used to elute, the higher the concentration of small RNA that can be used in the rest of the process (the same total quantity of miRNA). The lowest practical amount that can be used for the downstream experiments was 35µl and for this reason it was established as the standard eluent volume and used for all later experiments.

To determine the concentration of miRNA the values measured by the Qubit were more informative than the NanoDrop. Not unexpectedly the NanoDrop yielded values an order of magnitude higher than the Qubit because it measures total RNA. To more accurately quantify miRNA the Qubit was used and volumes of 2.5µl- 5µl of the isolated miRNA yielded values above the lower detection limit of Qubit, and in a linear fashion. In this study to minimize volume of sample used the quantitation of miRNA concentration was typically performed using 2.6µl of undiluted sample.

Six previously described placental specific miRNAs were used for the optimisation experiments. These were chosen from the limited amount of literature available that describes placental specific miRNAs. Liang et. al examined 40 different human tissues with the objective of defining specific miRNAs for each (Liang et al. 2007). Within the tissue bank they used was one placental sample. They tested a range of miRNAs, using qRT-PCR – although it's not clear how these were selected. Fifty-six miRNA's appeared to be placental specific including miR-526b.

Kotlabova et al. (2011) examined plasma samples from 23 pregnant and 10 non-pregnant women with an aim of defining placental specific cell free miRNAs in maternal circulation. The plasma samples were collected at 12, 16 and 36 weeks gestation. Term placentas were used as positive controls. This group performed qRT-PCR for 22 miRNAs that had been described previously as either being 'pregnancy associated' or 'placental specific' (the later group taken from the results of Liang et al. (2007)). Kotlabova et al. (2011) confirmed that only six of these 50 identified miRNAs were indeed placental specific and could be identified in maternal plasma at all three time points. These were the six miRNAs that we used – and the same primers were used as those described in the Kotlabova paper.

Two of the miRNA selected for this study (miR-517* and miR-526b) did not amplify in any of our specimens. This was the case even when cycling was increased to 50 cycles. This may be because they were not present in high enough concentrations in the sample (unlikely) or because of inefficiencies in the amplification protocol. Another explanation could be that there are ethnic variations in miRNA expression – although as the previous publication was conducted in central European population and as our own patients are predominantly European – this seems unlikely. miR-526b did in fact show some variation within the Kotlabova paper and it may have been better to use the shorter primer miRNA-526a that was more consistent. It is also possible that these two miRNAs are placental tissue specific but are not always expressed in maternal plasma as had initially been thought.

cel-miR-39 was used as to spike samples to act as a control for the efficiency of the qRT-PCR and for normalization of the data. The strategy that should be followed to normalize

the data for biological variability is to use a miRNA that is not variable but that is endogenous to the sample. Currently such an endogenous miRNA hasn´t been identified (Tiberio et al. 2015). Studies done in cfmiRNA in cancer also use *C.elegans* as a spike control (Sita-Lumsden et al. 2013; Kroh et al. 2010). The lack of an internal control presents a problem in trying to define anomalies on the basis of variable miRNA expression.

In the same set of experiments the remaining miRNAs (miR-516-5p, miR-518b, miR-520a* and miR-525) including the spike in control (cel-miR-39) were detected at the expected cycle threshold. These miRNAs showed strong linear relationships between raw Ct-values and concentrations of small RNA determined by the Qubit, indicating that the primers and the probes worked as expected even without the use of the pre-amplification step. In a similar manner the miRNAs post pre-amplification showed a similar linear relationship when investigated using a serial dilution. In all cases the pre-amplified miRNA concentrations are significantly higher than the non-pre-amp samples even at the highest dilution. This demonstrates the utility of the pre-amplification step when investigating miRNA that is present in small quantities, such as cell-free-fetal miRNAs.

In both stability experiments (at 4°C and Rt) there is high level of variability between samples at each time point. It would not be expected for different women to have the same levels of expression of miRNA. These natural levels of variability can by caused by potential confounding variables. Such as maternal age, gestational age, ethnicity etc. which were not possible to study in this cohort. Cell contamination, the presence of protein and the presence of lipids can affect the efficiency of the isolation of miRNA

which can be the confounding variables(Sita-Lumsden et al. 2013). Also, individual factors can influence in the variability of the miRNA such as race, life-style, exercise, smoking habits, diet, gestational age and others (Tiberio et al. 2015). To account for this variability an average of the relative expression was used to compare the trends between tubes, miRNAs and times.

miRNAs are known to be transported by various structures (microparticules, proteins, apoptotic bodies and multivesicular bodies to name a few) and released by trophoblasts and the shedding of placental cells (Kamhieh-Milz et al. 2014). These transported miRNAs can be found in maternal blood. For both of the experiments performed in this study, whole blood was stored and the plasma was separated at the time point of interest. The whole blood includes not just cell-free fetal nucleic acids but also the microparticles, white cells and platelets that can contain these nucleic acids. Over the time frame of these experiments these structures can break down and released additional miRNA into the plasma. Some white cells like neutrophils have a half life of approximately seven hours in the circulation (Blumenreich 1990), other structures that can release miRNAs are platelets, which have a half life of approximately four days (Cesar & Vecino 2009). For the other structures that can transport miRNA there is not much information of their half life but there is proof that these structures can help to maintain the integrity of miRNA and extend it´s half life (Cheng et al. 2014). This study shows in general that the amount of miRNA in the whole blood samples left either at 4°C or Rt increases with time. In this study this release is expressed as an increase towards the last time point, not just in the concentration but also in the standard deviation. This agrees with the theory that structures break down over time and release additional miRNA into the whole blood. The increase of the concentration is also reflected in the

relative expression of each miRNA. Interestingly despite this trend, higher expression is not observed in all the tube types or all the miRNAs being studied.

In addition to the tubes we also showed that the relative expression of the miRNAs is changed with time and storage condition. For example miR-525 is fairly stable with time, while the relative expression of mir-516 and miR-520 increased over time, as does the variability of their measurement. A possible explanation for this could be the origin of the miRNAs. If mir-516 and miR-520 are present in different types of structures (microparticules, exososomes, cells and platelets), when the structures degraded the additional mir-516 and miR-520 is released into the whole blood. If miR-525 is only present as cell free miRNA or in some microparticles, there is little additional source for miR-525 to be released from.

This experiment used three different types of tubes to collect the whole blood. The first two types of tubes, CITRATE and EDTA, have different compounds that are used for the same role, as anticoagulants. As such they stabilize whole blood allowing further investigation to be delayed to a later time. According to the literature CITRATE tubes have lower miRNA counts than EDTA tubes (Anon 2013a). Both tubes are designed to be stored at 4°C. In our study, the EDTA tubes that were stored at 4°C showed no significant change in miRNA concentrations over the course of the experiment. In contrast, tubes stored at Rt showed significant changes in miRNA concentrations indicating that miRNA levels are not stable in this circumstance. Based on the real time-PCR data the CITRATE tubes appeared to be more stable, but the strength of this conclusion has to be tempered by the fact that, due to the smaller sample size, only two time points could be assessed. The STRECK tubes are commercially prepared and specifically formulated for cell free

RNA stability. These tubes contain EDTA and nuclease inhibitors, metabolic inhibitors and a cell preservative in a liquid medium (Streck 2014). This is a proprietory product and the exact formulation of these materials is not provided. These additional preservatives are designed to improve the stability of cell-free RNA at Rt. This study showed that the samples stored in this tube were more stable at Rt compared to the samples at 4°C, as in studies done in cffDNA these types of tubes are useful for long periods of storages (Barrett et al. 2011; Wong et al. 2013). At Rt it takes three days before the concentration varies, while at 4°C after one day there is an increase. These generalized trends are seen in the three miRNA studied in this stability experiment, but the magnitude of the variability changes over time.

The miRNA studied are according to the literature placental specific miRNA (Liang et al. 2007; Kotlabova et al. 2011). In this study we detected that some of these miRNAs can also be measured in the circulation of non-pregnant women. In particular miR-525 was expressed in non-pregnant women albeit at much lower levels than detected in the circulation of pregnant women. On the other hand, the relative expression of miR-518 and miR-520 was not different between the pregnant and non-pregnant women. The miRNAs which were not detected in the plasma of non-pregnant women are miR-516, miR-517* and miR-526b. Of these miRNAs only miR-516 was detected in pregnant women. Whether this miRNA is truly placental specific or merely only expressed in the circulation at very low concentrations is as yet not clear and requires further investigation.

## 5.2.Comparison of normal pregnancies to those with fetal abnormality

In the time available for this project, relatively small numbers of fetal abnormality were seen in the clinical centre (Royal Prince Alfred Hospital). This included cohorts with chromosomal abnormality (predominantly Down syndrome), cardiac anomalies (a variety of defects) and neural tube defects (spina bifida or anencaphaly). The expression of these six miRNAs was compared between pregnancies carrying normal and abnormal fetuses.

Most of these anomalous fetuses were detected through first trimester screening – and there is a significant difference in the gestational age of those that had trisomy 21 and the control samples – which could impact on miRNA expression. In addition, fetuses with Down syndrome typically have different nuchal translucency, free-BhCG and PaPP-A levels. These differences are indicative of differences in cardiovascular physiology and placental function and changes in miRNA expression may merely be related to these factors rather than been novel independent variables that would be of additional value from a screening perspective. Neither the gestational age nor the first trimester screening features were so varied in the structural anomalies (compared to controls) and miRNA changes here may be a true representation of differing developmental regulation.

Although there were no significant differences in miRNA expression between groups of abnormalities and controls, there was a difference in miR-520 expression in neural tube defects (NTD) – although this was only seen in CITRATE based samples. In these samples, miR-520 was down regulated compared to the control. It is not clear whether this is a 'real' result given the fact that only small numbers of samples affected by neural

tube defects were available and that there was a non-significant increase in miRNA-520 expression in the EDTA and STRECK samples. This is the most extreme case of differences between tubes within a condition, but there is also indication that this sort of variance is present in other results. This indicates that care needs to be taken when selecting the type of tubes. More over, for miR-525, whilst not reaching statistical significance, there does appear to be down regulation between the neural tube defects (NTD), congenital heart defects (CHD) and controls. The data may become significant if greater numbers of cases were examined.

As mentioned previously, when comparing the different types of condition (Chromosomal abnormalities, C-A, congenital heat defects, CHD and neural tube defects, NTD) with the control there are no significant change, however when the condition are divided into specific categories some variations start to emerge. The study made by Zhu et al. also shows that the expression of miRNA is different when comparing different types of CHD Vs controls, but not if the CHD are treated as a whole and are compared to the control (Zhu et al. 2013). We observed this in miR-525 in the C-A group, when looking at specific chromosomal-abnormalities, T21 is up regulated and T18 is down regulated. This is also observed in miR-520 in the CHD group in which when looking at specific defects, HLHS is significantly different from the control group but when is analysed as a whole there is no significant differences. It is not possible to draw conclutions from this small study. However, the observation in this work and that oF Zhu et al. 2013 suggest larger studies are warranted.

## 5.3.Limitations of this study

Whilst we have shown that we can isolate cell free fetal miRNA from maternal plasma, and have demonstrated that either STRECK tubes (at room temperature) or EDTA tubes (at 4°C) have minimal impact on stability of miRNA for a 48-72 hour period, there are some limitations in this study and in the potential applicability of this technology for prenatal diagnosis.

One of the main disadvantages of this study is the use of qRT-PCR as a discovery tool for markers to identify different congenital abnormalities. qRT-PCR is generally used as a method for validation, due to the limited amount of miRNAs that can be studied. Microarrays or next generation sequencing can help in the discovery of new markers for each of the different abnormalities studied (Git et al. 2010) by analysing a wide range of miRNA in a single experiment. More over, the miRNA analysed in this study can help more in the identification of problems with placental function than in the identification of congenital abnormalities, for example miR-518b that was elevated in pre-eclamptic placentas compared to normal placentas (Zhu et al. 2009).

Another limitation of this study is the use of cel-miR-39 as a control for normalization of the data instead of an endogenous control that could correct for the biological variability. All though, as mentioned before there is not an endogenous control for plasma/serum samples, so the use of cel-miR-39 is necessary to correct for the efficiency of the qRT-PCR (Tiberio et al. 2015). Also, the scope of the study was limited by the number of samples for each condition collected over the course of this work. The acquisition of more samples would have allowed the confirmation of these findings and will give more statistical power to the conclusion.

Furthermore, a second or third centrifugation of the plasma was not done; this could affect the isolation of miRNA due to the contamination of platelets and white cells that can cause the presence of proteins, that can inhibit the efficiently of the extraction protocol or the qRT-PCR (Tiberio et al. 2015). Additionally, the collection and processing of additional samples post delivery or termination of pregnancy would have allow us to have more information to confirm that the miRNAs studied were truly placental specific miRNAs - as some other studies in the literature have done (Gu et al. 2012).

Another factor that was not analysed in this study is the effect of the collection of the samples post CVS, which can increase the amount of miRNA found in circulation due to the invasive procedure done to the placenta. This has been analysed in cffDNA where there wasn´t a significant difference between the analysis of maternal plasma following CVS compared to maternal plasma prior to CVS (Di Tommaso et al. 2013).

## 5.4.Future studies

This study is the first approach in the identification placental specific miRNA that could one day, be used as a biomarker to assist in the detection of different congenital abnormalities. In addition they may add to a better understanding of the role that particular miRNAs play in congenital abnormalities. Thorough investigation should be undertaken so abnormalities can be detected earlier in pregnancy and more informed decisions about the course of a pregnancy could be taken.

First of all there is the need for a larger number of samples for each type of congenital abnormality. The collection of these samples will be probably taken in different sites,

which means that all samples will have to be processed at the same time points regardless of the distance that the samples have to travel, samples will have to be processed with in three days.

Samples of blood for each type of congenital abnormality should be collected after first trimester screening if the pregnant women is part of the high risk group and another blood sample should be taken after the confirmation of the abnormalities (after CVS or Amniocentesis). These pre and post blood samples will allow us to confirm if there is a influence in the amount and relative expression of miRNAs caused by the invasive diagnosis, in a similar manner to the work done in cffDNA (Di Tommaso et al. 2013) . More over, it would be beneficial to have a blood bank of samples that are collected before the first trimester screening to be analysed if the fetus is diagnosed with an abnormality in a later stage. These samples will help us to determine if the miRNA identified as biomarkers for a specific abnormality can also be detected early in pregnancy.

For all patients included in this future study, (pregnancies with normal and abnormal fetuses) samples of blood after delivery or termination should be taken. This step will help us to determine if the miRNA identified as biomarkers are truly specific to the fetus or not, this was performed by Gu et al. in the identification of miRNA in NTD and the miRNA selected as biomarkers were not present in maternal circulation after a couple of hours (Gu et al. 2012). Is also important to confirm if the miRNA being investigated are present or not in a large cohort of non-pregnant women, which will allow us to confirm the findings as abnormality specific miRNA.

In addition to blood samples, placental tissue would be collected after the delivery or termination. Further more the amniotic fluid and the chorionic villi would be collected after testing. These extra samples will test the robustness of these markers. This has been done in previous studies in which the detection in blood of some miRNA are not confirmed to be in the placenta or vice-versa (Liang et al. 2007; Kotlabova et al. 2011).

For the processing of the all the different types of samples collected, miRNA would be isolated. Some of the blood samples collected from different types of abnormalities after diagnosis and some controls will be sequenced to detect the differentially express miRNA between the samples. The findings will be validated by qRT-PCR of all the other types of samples collected (Git et al. 2010). After the identification of the miRNAs by the application of bioinformatics the pathways that the miRNA are involve in will be identified and analysed.

Finally, if possible it will be interesting to investigate the different paths of the miRNAs identified in mouse models (Keck-Wherley et al. 2011; Krichevsky 2003). The study of mouse models embryos with abnormalities will also help us in the understanding of the development of some abnormalities, and in the identification of the regulation pathways that some of the miRNA can be involved during development.

## 5.5.Conclusions

Optimization studies suggest that either STECK (room temperature) or EDTA (4OC) tubes should be used and plasma should be extracted within 72 hours. To allow for standardisation of results, in further studies the type of tube and time to processing should be constant.

There appears to be significant variation in miRNA expression. This variation may make quantitative comparison between normal and abnormal conditions difficult. Some 'placental specific' miRNAs could not be detected at all and others are expressed in non-pregnant women. The two exceptions were miR-525 (increased expression in pregnant women) and miR-516 (not expressed in non-pregnant women).

The largest cohort of the abnormal samples tested were the ones affected by trisomy 21. There was no obvious difference in miRNA expression in the six miRNAs used in this study between these chromosomally abnormal pregnancies and normal controls. There was evidence that miR-520 is upregulated in fetuses affected by hypoplastic left heart syndrome – but this is currently based on evidence in two affected cases. Examination of samples from larger cohorts with fetal abnormalities, paying attention to grouping by the specific nature of the anomaly, is needed. Further work (using array or sequencing based technologies) is needed to determine whether specific miRNA signatures can be ascribed to individual congenital anomalies.

# References


Adachi, T. et al., 2010. Plasma microRNA 499 as a biomarker of acute myocardial infarction. *Clinical chemistry*, 56(7), pp.1183–5.

Akolekar, R. et al., 2015. Procedure-related risk of miscarriage following amniocentesis and chorionic villus sampling: a systematic review and meta-analysis. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, 45(1), pp.16–26.

Al-Yatama, M.K. et al., 2007. Polymerase-chain-reaction-based detection of fetal rhesus D and Y-chromosome-specific DNA in the whole blood of pregnant women during different trimesters of pregnancy. *Medical principles and practice : international journal of the Kuwait University, Health Science Centre*, 16(5), pp.327–32.

Alfirevic, Z., Sundberg, K. & Brigham, S., 2003. Amniocentesis and chorionic villus sampling for prenatal diagnosis. *The Cochrane database of systematic reviews*, (3), p.CD003252.

Amicucci, P. et al., 2000. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clinical chemistry*, 46(2), pp.301–2.

Anon, 2013a. TECH NOTE: nCounter miRNA Analysis in Plasma and Serum Samples | NanoString Technologies. Available at: http://www.nanostring.com/forms/miRNA_plasma_serum/download [Accessed August 29, 2015].

Anon, 2013b. Types of CHD and their Descriptions. Available at: http://www.congenitalheartdefects.com/typesofCHD.html#TGA [Accessed May 7, 2015].

Anon, Types of Congenital Heart Defects - NHLBI, NIH. Available at: http://www.nhlbi.nih.gov/health/health-topics/topics/chd/types [Accessed May 7, 2015].

Ashoor, G. et al., 2012. Fetal fraction in maternal plasma cell-free DNA at 11-13 weeks' gestation: effect of maternal and fetal factors. *Fetal diagnosis and therapy*, 31(4), pp.237–43.

Atef, S.H. et al., 2011. Prenatal diagnosis of fetal aneuploidies using QF-PCR: the egyptian study. *Journal of prenatal medicine*, 5(4), pp.83–9.

Badenas, C. et al., 2010. Assessment of QF-PCR as the first approach in prenatal diagnosis. *The Journal of molecular diagnostics : JMD*, 12(6), pp.828–34.

Barrett, A.N. et al., 2011. Implementing prenatal diagnosis based on cell-free fetal DNA: accurate identification of factors affecting fetal DNA yield. *PloS one*, 6(10), p.e25202.

Bianchi, D.W. et al., 2014. DNA sequencing versus standard prenatal aneuploidy screening. *The New England journal of medicine*, 370(9), pp.799–808.

Bianchi, D.W. et al., 2015. Fetal sex chromosome testing by maternal plasma DNA sequencing: clinical laboratory experience and biology. *Obstetrics and gynecology*, 125(2), pp.375–82.

Bianchi, D.W. et al., 2012. Genome-wide fetal aneuploidy detection by maternal plasma DNA sequencing. *Obstetrics and gynecology*, 119(5), pp.890–901.

Blumenreich, M.S., 1990. The White Blood Cell and Differential Count.

Borchert, G.M., Lanier, W. & Davidson, B.L., 2006. RNA polymerase III transcribes human microRNAs. *Nature structural & molecular biology*, 13(12), pp.1097–101.

Botto, L.D. et al., 1999. Neural-Tube Defects. *New England Journal of Medicine*, 341(20), pp.1509–1519.

Carter, C.O., 1976. Risks of miscarriage after amniocentesis. *Journal of medical genetics*, 13(5), p.351.

Cesar, J.M. & Vecino, A.M., 2009. Survival and function of transfused platelets. Studies in two patients with congenital deficiencies of platelet membrane glycoproteins. *Platelets*, 20(3), pp.158–62.

Chang, S.S. et al., 2008. MicroRNA alterations in head and neck squamous cell carcinoma. *International journal of cancer. Journal international du cancer*, 123(12), pp.2791–7.

Chen, E.Z. et al., 2011. Noninvasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma DNA sequencing. *PloS one*, 6(7), p.e21791.

Cheng, L. et al., 2014. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. *Journal of Extracellular Vesicles*, 3.

Chiu, R.W.K. et al., 2011. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. *BMJ (Clinical research ed.)*, 342(jan11_1), p.c7401.

Cicero, S. et al., 2006. Nasal bone in first-trimester screening for trisomy 21. *American journal of obstetrics and gynecology*, 195(1), pp.109–14.

Clausen, F.B. et al., 2013. Pre-analytical conditions in non-invasive prenatal testing of cell-free fetal RHD. *PloS one*, 8(10), p.e76990.

Corsten, M.F. et al., 2010. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circulation. Cardiovascular genetics*, 3(6), pp.499–506.

Cuckle, H.S., Wald, N.J. & Thompson, S.G., 1987. Estimating a woman's risk of having a pregnancy associated with Down's syndrome using her age and serum alpha-fetoprotein level. *British journal of obstetrics and gynaecology*, 94(5), pp.387–402.

Dan, S. et al., 2012. Clinical application of massively parallel sequencing-based prenatal noninvasive fetal trisomy test for trisomies 21 and 18 in 11,105 pregnancies with mixed risk factors. *Prenatal diagnosis*, 32(13), pp.1225–32.

Devaney, S.A. et al., 2011. Noninvasive fetal sex determination using cell-free fetal DNA: a systematic review and meta-analysis. *JAMA*, 306(6), pp.627–36.

Dovč-Drnovšek, T. et al., 2013. Reliable Determination of Fetal RhD Status by RHD Genotyping from Maternal Plasma. *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fuŕ Transfusionsmedizin und Immunhaḿatologie*, 40(1), pp.37–43.

Dr. Estefania Lozano-Velasco, M.D.F.D.A.A., 2011. microRNAs in myogenesis shared roles in cardiac and skeletal muscle. *European society of cardiology*. Available at: http://www.escardio.org/The-ESC/Communities/Working-Groups/Working-Group-on-Development,-Anatomy-&-Pathology/Publications/microRNAs-in-myogenesis-shared-roles-in-cardiac-and-skeletal-muscle.

EA, A.S.& S., 2008. Neural tube defects in Australia (AIHW). *An epidemiological report. Cat. no. PER 45. Sydney: AIHW National Perinatal Statistics Unit.* Available at: http://www.aihw.gov.au/publication-detail/?id=6442468181 [Accessed May 7, 2015].

Everett, T.R. & Chitty, L.S., 2015. Cell-free fetal DNA: the new tool in fetal medicine. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, 45(5), pp.499–507.

Fan, H.C. et al., 2008. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proceedings of the National Academy of Sciences of the United States of America*, 105(42), pp.16266–71.

Farina, A. et al., 2010. Performance of messenger RNAs circulating in maternal blood in the prediction of preeclampsia at 10-14 weeks. *American journal of obstetrics and gynecology*, 203(6), pp.575.e1–7.

Fernández-Hernando, C. et al., 2013. MicroRNAs in metabolic disease. *Arteriosclerosis, thrombosis, and vascular biology*, 33(2), pp.178–85.

Fichtlscherer, S. et al., 2010. Circulating microRNAs in patients with coronary artery disease. *Circulation research*, 107(5), pp.677–84.

Filges, I. et al., 2012. Array comparative genomic hybridization in prenatal diagnosis of first trimester pregnancies at high risk for chromosomal anomalies. *Molecular cytogenetics*, 5(1), p.38.

Friedman, R.C. et al., 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome research*, 19(1), pp.92–105.

Gangaraju, V.K. & Lin, H., 2009. MicroRNAs: key regulators of stem cells. *Nature reviews. Molecular cell biology*, 10(2), pp.116–25.

Gaynullina, D. et al., 2015. Alteration of mRNA and microRNA expression profiles in rat muscular type vasculature in early postnatal development. *Scientific reports*, 5, p.11106.

Gil, M.M. et al., 2015. Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: updated meta-analysis. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, 45(3), pp.249–66.

Git, A. et al., 2010. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *RNA (New York, N.Y.)*, 16(5), pp.991–1006.

Gonzalez-Gonzalez, M.C. et al., 2003. Early Huntington disease prenatal diagnosis by maternal semiquantitative fluorescent-PCR. *Neurology*, 60(7), pp.1214–1215.

Gonzalez-Gonzalez, M.C. et al., 2003. Huntington disease-unaffected fetus diagnosed from maternal plasma using QF-PCR. *Prenatal Diagnosis*, 23(3), pp.232–234.

Gregory, R.I. et al., 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature*, 432(7014), pp.235–40.

Grill, S. et al., 2009. High throughput non-invasive determination of foetal Rhesus D status using automated extraction of cell-free foetal DNA in maternal plasma and mass spectrometry. *Archives of gynecology and obstetrics*, 279(4), pp.533–7.

Gu, H. et al., 2012. Diagnostic role of microRNA expression profile in the serum of pregnant women with fetuses with neural tube defects. *J Neurochem*, 122(3), pp.641–649.

Gupta, A.K. et al., 2004. Detection of fetal DNA and RNA in placenta-derived syncytiotrophoblast microparticles generated in vitro. *Clinical chemistry*, 50(11), pp.2187–90.

Ha, M. & Kim, V.N., 2014. Regulation of microRNA biogenesis. *Nature reviews. Molecular cell biology*, 15(8), pp.509–524.

He, Y. et al., 2013. Application of spatio-temporal image correlation technology in the diagnosis of fetal cardiac abnormalities. *Experimental and therapeutic medicine*, 5(6), pp.1637–1642.

Hennessey, P.T. et al., 2012. Serum microRNA biomarkers for detection of non-small cell lung cancer. *PloS one*, 7(2), p.e32307.

Higashijima, A. et al., 2013. Characterization of placenta-specific microRNAs in fetal growth restriction pregnancy. *Prenatal diagnosis*, 33(3), pp.214–22.

Hui, L. & Hyett, J., 2013. Noninvasive prenatal testing for trisomy 21: challenges for implementation in Australia. *The Australian & New Zealand journal of obstetrics & gynaecology*, 53(5), pp.416–24.

Hunter, L.E. & Simpson, J.M., 2014. Prenatal screening for structural congenital heart disease. *Nature reviews. Cardiology*, 11(6), pp.323–34.

Hustin, J., Jauniaux, E. & Schaaps, J.P., Histological study of the materno-embryonic interface in spontaneous abortion. *Placenta*, 11(6), pp.477–86.

Hyett, J.A. et al., 2005. Reduction in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early pregnancy. *Prenatal Diagnosis*, 25(12), pp.1111–1116.

Hyland, C.A. et al., 2009. Evaluation of non-invasive prenatal RHD genotyping of the fetus. *The Medical journal of Australia*, 191(1), pp.21–5.

Jaques, A.M. et al., 2006. Using record linkage and manual follow-up to evaluate the Victorian maternal serum screening quadruple test for Down's syndrome, trisomy 18 and neural tube defects. *Journal of medical screening*, 13(1), pp.8–13.

Jiang, F. et al., 2012. Noninvasive Fetal Trisomy (NIFTY) test: an advanced noninvasive prenatal diagnosis methodology for fetal autosomal and sex chromosomal aneuploidies. *BMC medical genomics*, 5, p.57.

Jordan, R.G. et al., 2013. Prenatal and Postnatal Care.

Kagan, K.O. et al., 2008. Screening for trisomy 21 by maternal age, fetal nuchal translucency thickness, free beta-human chorionic gonadotropin and pregnancy-associated plasma protein-A. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, 31(6), pp.618–24.

Kamhieh-Milz, J. et al., 2014. Differentially expressed microRNAs in maternal plasma for the noninvasive prenatal diagnosis of Down syndrome (trisomy 21). *BioMed research international*, 2014, p.402475.

Keck-Wherley, J. et al., 2011. Abnormal microRNA expression in Ts65Dn hippocampus and whole blood: contributions to Down syndrome phenotypes. *Developmental neuroscience*, 33(5), pp.451–67.

Kimura, M. et al., 2008. Noninvasive fetal RHD genotyping by maternal plasma with capillary electrophoresis. *Transfusion*, 48(6), pp.1156–63.

Kloosterman, W.P. & Plasterk, R.H.A., 2006. The diverse functions of microRNAs in animal development and disease. *Developmental cell*, 11(4), pp.441–50.

Kotlabova, K. et al., 2013. Extracellular chromosome 21-derived microRNAs in euploid & aneuploid pregnancies. *The Indian journal of medical research*, 138(6), pp.935–43.

Kotlabova, K., Doucha, J. & Hromadnikova, I., 2011. Placental-specific microRNA in maternal circulation--identification of appropriate pregnancy-associated microRNAs with diagnostic potential. *Journal of reproductive immunology*, 89(2), pp.185–91.

Krichevsky, A.M., 2003. A microRNA array reveals extensive regulation of microRNAs during brain development. *RNA*, 9(10), pp.1274–1281.

Kroh, E.M. et al., 2010. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods (San Diego, Calif.)*, 50(4), pp.298–301.

Kuwabara, Y. et al., 2011. Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circulation. Cardiovascular genetics*, 4(4), pp.446–54.

Latendresse, G. & Deneris, A., 2015. An Update on Current Prenatal Testing Options: First Trimester and Noninvasive Prenatal Testing. *Journal of Midwifery & Women's Health*, 60(1), pp.24–36.

Lausman, A. et al., 2013. Intrauterine growth restriction: screening, diagnosis, and management. *Journal of obstetrics and gynaecology Canada : JOGC = Journal d'obstétrique et gynécologie du Canada : JOGC*, 35(8), pp.741–57.

Lee, R.C., Feinbaum, R.L. & Ambros, V., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75(5), pp.843–854.

Leggat, S., 2011. Childhood Heart Disease in Australia - Current Practices and Future Needs - February. *HeartKids Australia*. Available at: http://heartkids.org.au/wp-content/uploads/2012/01/White_Paper_A4_March_2011_web_doc.pdf [Accessed May 6, 2015].

Legler, T.J. et al., 2009. Prenatal RhD Testing: A Review of Studies Published from 2006 to 2008. *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie*, 36(3), pp.189–198.

Leidner, R.S., Li, L. & Thompson, C.L., 2013. Dampening enthusiasm for circulating microRNA in breast cancer. *PloS one*, 8(3), p.e57841.

Leung, T.N. et al., 2001. Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia. *Clinical chemistry*, 47(1), pp.137–9.

Li, W. & Ruan, K., 2009. MicroRNA detection by microarray. *Analytical and bioanalytical chemistry*, 394(4), pp.1117–24.

Li, Z. et al., 2010. *Australia's mothers and babies*, Canberra: AIHW National Perinatal Epidemiology and Statistics Unit.

Li, Z. & Rana, T.M., 2014. Therapeutic targeting of microRNAs: current status and future challenges. *Nature Reviews Drug Discovery*, 13(8), pp.622–638.

Liang, D. et al., 2013. Non-invasive prenatal testing of fetal whole chromosome aneuploidy by massively parallel sequencing. *Prenatal diagnosis*, 33(5), pp.409–15.

Liang, Y. et al., 2007. Characterization of microRNA expression profiles in normal human tissues. *BMC genomics*, 8(1), p.166.

Liu, C.G. et al., 2008. MicroRNA expression profiling using microarrays. *Nat Protoc*, 3(4), pp.563–578.

Liu, N. & Olson, E.N., 2010. MicroRNA regulatory networks in cardiovascular development. *Developmental cell*, 18(4), pp.510–25.

Lo, Y.M. et al., 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet*, 350(9076), pp.485–487.

Lo, Y.M. et al., 1998. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *American journal of human genetics*, 62(4), pp.768–75.

Lo, Y.M. et al., 1996. Two-way cell traffic between mother and fetus: biologic and clinical implications. *Blood*, 88(11), pp.4390–5.

Lo, Y.M.D. et al., 2007. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nature medicine*, 13(2), pp.218–23.

Lo, Y.M.D. et al., 1999. Rapid Clearance of Fetal DNA from Maternal Plasma. *The American Journal of Human Genetics*, 64(1), pp.218–224.

Maciotta, S., Meregalli, M. & Torrente, Y., 2013. The involvement of microRNAs in neurodegenerative diseases. *Frontiers in cellular neuroscience*, 7, p.265.

Maiz, N. et al., 2012. A mixture model of ductus venosus pulsatility index in screening for aneuploidies at 11-13 weeks' gestation. *Fetal diagnosis and therapy*, 31(4), pp.221–9.

Maller Schulman, B.R. et al., 2008. The let-7 microRNA target gene, Mlin41/Trim71 is required for mouse embryonic survival and neural tube closure. *Cell Cycle*, 7(24), pp.3935–3942.

Del Mar Gil, M. et al., 2014. Cell-free DNA analysis for trisomy risk assessment in first-trimester twin pregnancies. *Fetal diagnosis and therapy*, 35(3), pp.204–11.

Mayor-Lynn, K. et al., 2011. Expression profile of microRNAs and mRNAs in human placentas from pregnancies complicated by preeclampsia and preterm labor. *Reproductive sciences (Thousand Oaks, Calif.)*, 18(1), pp.46–56.

Meaney, C. & Norbury, G., 2009. Noninvasive prenatal diagnosis of early onset primary dystonia I in maternal plasma. *Prenatal diagnosis*, 29(13), pp.1218–21.

Montenegro, D. et al., 2009. Expression patterns of microRNAs in the chorioamniotic membranes: a role for microRNAs in human pregnancy and parturition. *The Journal of pathology*, 217(1), pp.113–21.

Mostert, B. et al., 2011. Diagnostic applications of cell-free and circulating tumor cell-associated miRNAs in cancer patients. *Expert review of molecular diagnostics*, 11(3), pp.259–75.

Mouillet, J.F. et al., 2010. The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction. *Placenta*, 31(9), pp.781–784.

Mukhopadhyay, P. et al., 2011. MicroRNA gene expression signatures in the developing neural tube. *Birth Defects Res A Clin Mol Teratol*, 91(8), pp.744–762.

Nana-Sinkam, S.P. & Croce, C.M., 2013. Clinical applications for microRNAs in cancer. *Clinical pharmacology and therapeutics*, 93(1), pp.98–104.

Ng, E.K. et al., 2004. Evaluation of human chorionic gonadotropin beta-subunit mRNA concentrations in maternal serum in aneuploid pregnancies: a feasibility study. *Clin Chem*, 50(6), pp.1055–1057.

Ng, E.K.O. et al., 2003. mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci U S A*, 100(8), pp.4748–4753.

Ng, E.K.O. et al., 2002. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. *Clinical chemistry*, 48(8), pp.1212–7.

Nicolaides, K.H. et al., 2014. Assessment of fetal sex chromosome aneuploidy using directed cell-free DNA analysis. *Fetal diagnosis and therapy*, 35(1), pp.1–6.

Nielsen, B.S., 2012. MicroRNA in situ hybridization. *Methods in molecular biology (Clifton, N.J.)*, 822, pp.67–84.

Norton, M.E. et al., 2015. Cell-free DNA analysis for noninvasive examination of trisomy. *The New England journal of medicine*, 372(17), pp.1589–97.

Norwitz, E.R. & Levy, B., 2013. Noninvasive prenatal testing: the future is now. *Reviews in obstetrics & gynecology*, 6(2), pp.48–62.

Ouyang, Y. et al., 2014. MiRNA nano-packages for maternal-placental-fetal communication. *Placenta*, 35(9), p.A5.

Palomaki, G.E. et al., 2012. DNA sequencing of maternal plasma reliably identifies trisomy 18 and trisomy 13 as well as Down syndrome: an international collaborative study. *Genetics in medicine : official journal of the American College of Medical Genetics*, 14(3), pp.296–305.

Phelan, S.A., Ito, M. & Loeken, M.R., 1997. Neural tube defects in embryos of diabetic mice: role of the Pax-3 gene and apoptosis. *Diabetes*, 46(7), pp.1189–97.

Phimister, E.G. & Phillippe, M., 2014. Cell-free Fetal DNA — A Trigger for Parturition. *New England Journal of Medicine*, 370(26), pp.2534–2536.

Pritchard, C.C. et al., 2012. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer prevention research (Philadelphia, Pa.)*, 5(3), pp.492–7.

PSANZ, 2009. Clinical Practice Guideline for Perinatal Mortality. Available at: http://www.stillbirthalliance.org.au/doc/Section_1_Version_2.2_April_2009.pdf [Accessed February 18, 2016].

Raitoharju, E. et al., 2011. miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study. *Atherosclerosis*, 219(1), pp.211–7.

RANZCOG, 2015. Prenatal screening and diagnosis of chromosomal and genetic abnormalities in the fetus in pregnancy. Available at: http://www.ranzcog.edu.au/component/search/?searchword=Prenatal screening and diagnosis of chromosomal a&searchphrase=all&Itemid=1 [Accessed February 11, 2016].

Rava, R.P. et al., 2014. Circulating fetal cell-free DNA fractions differ in autosomal aneuploidies and monosomy X. *Clinical chemistry*, 60(1), pp.243–50.

Rijnders, R.J.P. et al., 2004. Cell-free fetal DNA is not present in plasma of nonpregnant mothers. *Clinical chemistry*, 50(3), pp.679–81; author reply 681.

Van Rooij, E. et al., 2008. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proceedings of the National Academy of Sciences of the United States of America*, 105(35), pp.13027–32.

Van Rooij, E., 2011. The art of microRNA research. *Circ Res*, 108(2), pp.219–234.

Ross, R.K. et al., 2000. Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. *J Natl Cancer Inst*, 92(4), pp.328–332.

Roth, C. et al., 2010. Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. *Breast Cancer Research*, 12(6), p.R90.

Royston, P. & Wright, E.M., 1998. How to construct "normal ranges" for fetal variables. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, 11(1), pp.30–8.

Saito, H. et al., 2000. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. *Lancet*, 356(9236), p.1170.

Sankaran, V.G. et al., 2011. MicroRNA-15a and -16-1 act via MYB to elevate fetal hemoglobin expression in human trisomy 13. *Proceedings of the National Academy of Sciences of the United States of America*, 108(4), pp.1519–24.

Schoen, E. et al., 2003. Maternal serum unconjugated estriol as a predictor for Smith-Lemli-Opitz syndrome and other fetal conditions. *Obstetrics and gynecology*, 102(1), pp.167–72.

Schotte, D., Pieters, R. & Den Boer, M.L., 2012. MicroRNAs in acute leukemia: from biological players to clinical contributors. *Leukemia*, 26(1), pp.1–12.

Schratt, G.M. et al., 2006. A brain-specific microRNA regulates dendritic spine development. *Nature*, 439(7074), pp.283–9.

Schrijver, I., Cherny, S.C. & Zehnder, J.L., 2007. Testing for maternal cell contamination in prenatal samples: a comprehensive survey of current diagnostic practices in 35 molecular diagnostic laboratories. *The Journal of molecular diagnostics : JMD*, 9(3), pp.394–400.

Schuring-Blom, G.H. et al., 2002. Trisomy 13 or 18 (mosaicism) in first trimester cytotrophoblast cells: false-positive results in 11 out of 51 cases. *European journal of obstetrics, gynecology, and reproductive biology*, 101(2), pp.161–8.

Scott, F., Coates, A. & McLennan, A., 2009. Pregnancy outcome in the setting of extremely low first trimester PAPP-A levels. *The Australian & New Zealand journal of obstetrics & gynaecology*, 49(3), pp.258–62.

Sekizawa, A. et al., 2002. Increased cell-free fetal DNA in plasma of two women with invasive placenta. *Clinical chemistry*, 48(2), pp.353–4.

Shenoy, A. & Blelloch, R.H., 2014. Regulation of microRNA function in somatic stem cell proliferation and differentiation. *Nature reviews. Molecular cell biology*, 15(9), pp.565–76.

Shingara, J. et al., 2005. An optimized isolation and labeling platform for accurate microRNA expression profiling. *RNA (New York, N.Y.)*, 11(9), pp.1461–70.

Shulman, L.P. & Elias, S., 1993. Amniocentesis and chorionic villus sampling. *The Western journal of medicine*, 159(3), pp.260–8.

Simpson, J.L. & Elias, S., 1993. Isolating fetal cells from maternal blood. Advances in prenatal diagnosis through molecular technology. *JAMA*, 270(19), pp.2357–61.

Sita-Lumsden, A. et al., 2013. Circulating microRNAs as potential new biomarkers for prostate cancer. *British journal of cancer*, 108(10), pp.1925–30.

Small, E.M. & Olson, E.N., 2011. Pervasive roles of microRNAs in cardiovascular biology. *Nature*, 469(7330), pp.336–42.

Smid, M. et al., 2003. No evidence of fetal DNA persistence in maternal plasma after pregnancy. *Human genetics*, 112(5-6), pp.617–8.

Snijders, R. et al., 1998. UK multicentre project on assessment of risk of trisomy 21 by maternal age and fetal nuchal-translucency thickness at 10–14 weeks of gestation. *The Lancet*, 352(9125), pp.343–346.

Snijders, R.J. & Nicolaides, K.H., 1994. Fetal biometry at 14-40 weeks' gestation. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology*, 4(1), pp.34–48.

Sokol, N.S. & Ambros, V., 2005. Mesodermally expressed Drosophila microRNA-1 is regulated by Twist and is required in muscles during larval growth. *Genes & development*, 19(19), pp.2343–54.

Srinivasan, S. et al., 2013. MicroRNAs -the next generation therapeutic targets in human diseases. *Theranostics*, 3(12), pp.930–42.

Streck, 2014. Cell-Free RNA BCT. Available at: http://www.streck.com/resources%5CCell_Stabilization%5CCell-Free_RNA_BCT%5C01_Instructions_(IFU)%5C01_IFU_Cell-Free_RNA_BCT_IFU.pdf [Accessed August 24, 2015].

Sucharov, C.C. et al., 2015. Micro-RNA expression in hypoplastic left heart syndrome. *Journal of cardiac failure*, 21(1), pp.83–8.

Takenaka, S. et al., 2014. Prediction of Fetal Growth Restriction by Analyzing the Messenger RNAs of Angiogenic Factor in the Plasma of Pregnant Women. *Reproductive sciences (Thousand Oaks, Calif.)*, p.1933719114557895–.

Tanaka, M. et al., 2009. Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. *PloS one*, 4(5), p.e5532.

Tang, Q. et al., 2013. miR-141 contributes to fetal growth restriction by regulating PLAG1 expression. *PloS one*, 8(3), p.e58737.

Thum, T., Catalucci, D. & Bauersachs, J., 2008. MicroRNAs: novel regulators in cardiac development and disease. *Cardiovascular research*, 79(4), pp.562–70.

Tiberio, P. et al., 2015. Challenges in using circulating miRNAs as cancer biomarkers. *BioMed research international*, 2015, p.731479.

Tjoa, M.L. et al., 2006. Trophoblastic oxidative stress and the release of cell-free feto-placental DNA. *The American journal of pathology*, 169(2), pp.400–4.

Di Tommaso, M. et al., 2013. Cell-free fetal DNA in maternal circulation after chorionic villous sampling. *Prenatal diagnosis*, 33(7), pp.695–9.

Tongsong, T. et al., 2000. Cordocentesis at 16-24 weeks of gestation: experience of 1,320 cases. *Prenatal diagnosis*, 20(3), pp.224–8.

Tongsong, T. et al., 2001. Fetal loss rate associated with cordocentesis at midgestation. *American journal of obstetrics and gynecology*, 184(4), pp.719–23.

Towner, D. et al., 2007. Miscarriage risk from amniocentesis performed for abnormal maternal serum screening. *American journal of obstetrics and gynecology*, 196(6), pp.608.e1–5; discussion 608.e5.

Tranquilli, A.L. et al., 2014. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. *Pregnancy hypertension*, 4(2), pp.97–104.

Tsui, N.B.Y. et al., 2014. Investigation of biological factors influencing the placental mRNA profile in maternal plasma. *Prenatal diagnosis*, 34(3), pp.251–8.

Twiss, P. et al., 2014. Non-invasive prenatal testing for Down syndrome. *Seminars in fetal & neonatal medicine*, 19(1), pp.9–14.

Ventura, W. et al., 2013. Placental expression of microRNA-17 and -19b is down-regulated in early pregnancy loss. *European journal of obstetrics, gynecology, and reproductive biology*, 169(1), pp.28–32.

Van den Veyver, I.B. et al., 2009. Clinical use of array comparative genomic hybridization (aCGH) for prenatal diagnosis in 300 cases. *Prenatal diagnosis*, 29(1), pp.29–39.

Voellenkle, C. et al., 2010. MicroRNA signatures in peripheral blood mononuclear cells of chronic heart failure patients. *Physiological genomics*, 42(3), pp.420–6.

Wald, N.J. et al., 1988. Maternal serum screening for Down's syndrome in early pregnancy. *BMJ (Clinical research ed.)*, 297(6653), pp.883–7.

Wald, N.J. et al., 1977. Maternal serum-alpha-fetoprotein measurement in antenatal screening for anencephaly and spina bifida in early pregnancy. Report of U.K. collaborative study on alpha-fetoprotein in relation to neural-tube defects. *Lancet (London, England)*, 1(8026), pp.1323–32.

Wald, N.J., Watt, H.C. & Hackshaw, A.K., 1999. Integrated screening for Down's syndrome on the basis of tests performed during the first and second trimesters. *The New England journal of medicine*, 341(7), pp.461–7.

Wang, E. et al., 2013. Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. *Prenatal diagnosis*, 33(7), pp.662–6.

Wang, F. et al., 2010. Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor. *Gynecologic oncology*, 119(3), pp.586–93.

Webb, A. et al., 2012. Non invasive prenatal diagnosis of aneuploidy: Next generation sequencing or fetal DNA enrichment? *Balkan Journal of Medical Genetics*, 15(SUPPL.), pp.17–26.

Wightman, B., Ha, I. & Ruvkun, G., 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*, 75(5), pp.855–862.

Winter, J. et al., 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nature cell biology*, 11(3), pp.228–34.

Wong, D. et al., 2013. Optimizing blood collection, transport and storage conditions for cell free DNA increases access to prenatal testing. *Clinical biochemistry*, 46(12), pp.1099–104.

Wu, Q. et al., 2011. Next-generation sequencing of microRNAs for breast cancer detection. *Journal of biomedicine & biotechnology*, 2011, p.597145.

Yao, S. et al., 2013. Genetic variants in microRNAs and breast cancer risk in African American and European American women. *Breast cancer research and treatment*, 141(3), pp.447–59.

Zhao, H. et al., 2010. A pilot study of circulating miRNAs as potential biomarkers of early stage breast cancer. *PloS one*, 5(10), p.e13735.

Zhao, S. & Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *Journal of computational biology : a journal of computational molecular cell biology*, 12(8), pp.1047–64.

Zhu, S. et al., 2013. Identification of maternal serum microRNAs as novel non-invasive biomarkers for prenatal detection of fetal congenital heart defects. *Clinica chimica acta; international journal of clinical chemistry*, 424, pp.66–72.

Zhu, X. et al., 2009. Differential expression profile of microRNAs in human placentas from preeclamptic pregnancies vs normal pregnancies. *American journal of obstetrics and gynecology*, 200(6), pp.661.e1–7.