Development and Characterisation of Circulating RNA Markers

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ABSTRACT

DEVELOPMENT AND CHARACTERISATION OF CIRCULATING RNA MARKERS

Circulating RNA was previously demonstrated through the identification of tumour-derived transcripts in the plasma and serum of cancer patients. This finding inspired the detection of cell-free fetal mRNA in maternal plasma which in turn facilitated the development of promising non-invasive prenatal assessment strategies applicable to pregnancies regardless of fetal sex.

Recently, in addition to maternal plasma, there have been studies by other research groups reporting the presence of placental/fetal mRNA in maternal whole blood. In the first part of this thesis, I studied a list of previously identified placental mRNA transcripts, including *chorionic somatomammotropin hormone 1 (CSH1)*, *KiSS-1 metastasis-suppressor (KISS1)*, *placenta-specific 4 (PLAC4)* and *placenta-specific 1 (PLAC1)* in maternal whole blood and determined if this whole blood-based approach offered advantages over maternal plasma analysis. The presence of *KISS1*, *PLAC4* and *PLAC1* in non-pregnant and post-partum blood samples as well as the

confirmed maternal contribution of *PLAC4* mRNA in maternal blood proved that most of the detected 'placental' mRNA molecules in maternal whole blood were of maternal origin. To explore if more pregnancy-associated circulating mRNA markers could be developed for maternal whole blood analysis, candidates were mined after performing gene expression microarray comparison of whole blood samples from pregnant and non-pregnant individuals. The pregnancy-specificity of the identified gene candidates was further investigated. However, their presence in non-pregnant whole blood and lack of clearance after pregnancy indicated that they were not "pregnancy-specific" markers. These data suggested that pregnancy specific transcripts could be more readily identified from maternal plasma than whole blood.

In section IV of this thesis, I reviewed and modified the blood sample processing and plasma RNA extraction protocols that were previously practised, in an attempt to enrich circulating fetal RNA in maternal plasma. Besides mRNA, extraction protocols for microRNAs (miRNAs), a new class of circulating nucleic acid markers, were also evaluated. The modifications in the protocols that I introduced led to significant improvements in RNA yield and enhanced the accuracy and reliability of circulating RNA detection in plasma, especially for those marginally detectable transcripts.

Finally, in the last study, I implemented what I have learnt from the analysis of circulating fetal RNA into the development of brain-derived RNA transcripts for detection in the plasma of patients who had sustained brain injuries. A systematic approach based on gene expression microarray analysis was adopted to search for circulating brain-specific mRNA markers. Transcripts showing high expression levels in brain tissues but low expression levels in peripheral blood were identified. However, the detectability of these brain-derived mRNA markers in both peripheral and jugular plasma was found to be low. Instead, concentrations of these mRNA markers were found to be higher in cerebral spinal fluid (CSF) from brain-injured than non-brain-injured patients.

The results presented in this thesis have not only provided a foundation facilitating the precise and accurate detection of fetal-specific RNA markers but have also improved the current understanding of the biology of circulating RNA.

摘要

游離核糖核酸 (RNA)，可以由癌細胞釋放到癌症病人的血漿及血清內。此項重大發現日後啓發了科學家在孕婦血漿中成功找到由胎兒產生的游離 RNA，繼而發展出各種可適用於所有胎兒的非損傷性產前測試。

近日，部份研究團隊提出在孕婦的全血中亦可找到胎兒或胎盤的傳信 RNA (mRNA)，是以本論文之第一部份是對孕婦全血中的胎盤 mRNA 作研究，當中包括絨膜生長催乳素激素 1 (*CSH1*)、*KiSS-1* 腫瘤轉移抑制基因 (*KISS1*)、 胎盤抗原基因 4 (*PLAC4*) 及胎盤抗原基因 1 (*PLAC1*)，從而決定孕婦全血是否比血漿更適合用作非損傷性產前測試。由於在非懷孕人士及產婦分娩後之全血樣本仍能檢測到 *KISS1*、*PLAC4* 及 *PLAC1*，加上證據顯示孕婦全血中的 *PLAC4* mRNA 中，有部份由母體所產生的，從此証明大部份孕婦全血中的所謂胎盤 mRNA 份子乃源自母體。本研究亦利用基因微陣列技術比較孕婦與非孕婦之全血樣本，務求徹底找出孕婦全血中跟懷孕有關及有機會發展成爲全血產前測試的游離 RNA。但是，進一步之懷孕專一性研究顯示這些候選基因並未能隨著分娩後從母親的血液中消失，亦同時存在於非懷孕人士之全血樣本內，因此並非懷孕獨有的標誌物。這些數據均証明利用孕婦血漿比全血更容易及更有效檢測到懷孕獨有的轉錄產物。

本論文之第二部份就血漿 mRNA 之自然特性重新檢閱血液樣本的處理過程

與血漿 RNA 的提取方法，以增加孕婦血漿中胎兒 RNA 的濃度；同時亦對近年新發現的游離糖核酸—微型核糖核酸 (miRNA) 之提取方法作評價。優化了提取方法能更精準地從血漿中檢驗游離 RNA。

最後，本人套用了研究游離胎兒 RNA 的經驗，利用基因微陣列技術有系統地找出由腦部衍生之 mRNA 標誌物，雖然此等標誌物在腦部受傷病人血漿之檢測率偏低，但與非腦部受傷病人比較，它們在腦部受傷病人的腦脊髓液中的濃度卻非常高。

綜上所述，本論文的研究不僅爲促進血漿中游離胎兒 RNA 檢驗之精準度建立了良好的基礎，亦使學界對游離 RNA 的生物學本質有更透徹的了解。

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LIST OF ABBREVIATIONS

AFP	α-fetoprotein
ANOVA	analysis of variance
BC	buffy coat
BI	brain-injured / brain injury
CEACAM8	*carcinoembryonic antigen-related cell adhesion molecule 8*
CGB	chorionic gonadotropin, beta polypeptide
CNS	central nervous system
CSF	cerebrospinal fluid
CRH	Corticotrophin releasing hormone
CSH1	*chorionic somatomammotropin hormone 1 (human placental lactogen)*
CSHL1	*chorionic somatomammotropin hormone-like 1*
CT	computed tomography
Ct	threshold cycle
CVS	chorionic villus sampling
DEFA4	*defensin 4*
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
FAM	6-carboxyfluorescein
FLCN	*folliculin*
GAPDH	*glyceraldehyde-3-phosphate dehydrogenase*
GFAP	*glial fibrillary acidic protein*
GPM6B	*glycoprotein M6B*
GSC	Glasgow coma scale
hME	homogenous MassEXTEND
HPLC	high performance liquid chromatography
ICP	intracranical pressure
IVT	*in-vitro* transcription
KISS1	*KiSS-1 metastasis-suppressor*
MALDI-TOF MS	matrix-assisted laser-desorption and ionisation time-of-flight mass spectrometry
MBP	*myelin basic protein*
MGB	minor-groove binding
$MgCl_2$	magnesium chloride

miRNA	microRNA
MMP8	*matrix metallopeptidase 8 (neutrophil collagenase)*
$Mn(OAc)_2$	manganese acetate
MPO	*myeloperoxidase*
MRI	magnetic resonance imaging
mRNA	messenger RNA
NPC	nasopharyngeal carcinoma
NSE	neurone-specific enolase
OLFM4	*olfactomedin 4*
ORM1	*orosomucoid 1*
PCR	polymerase chain reaction
PLAC1	*placenta-specific 1*
PLAC4	*placenta-specific 4*
PLP1	*proteolipid 1*
QPCR	real-time quantitative PCR
QRT-PCR	real-time quantitative reverse-transcriptase PCR
RT-PCR	reverse transcriptase-polymerase chain reaction
r*Tth*	recombinant *Thermus thermophilus*
SAP	shrimp alkaline phosphatase
SNP	single nucleotide polymorphism
SPARCL1	*SPARC-like 1 (hevin)*
STR	short tandem repeat
TAMRA	6-carboxytetramethylrhodamine
Taq	*Thermus aquaticus*
TBI	traumatic brain injury
UNG	uracil-N-glycosylase
WHO	World Health Organisation

SECTION I: Background

This section begins with a chapter which provides an overview of the history and developments of circulating nucleic acid markers, including their application in the field of prenatal diagnosis and cancer diagnostics. In the second chapter, the need of developing mRNA markers in the field of brain injury is presented. Both chapters serve as an introduction to the research questions addressed in this thesis.

CHAPTER 1: DEVELOPMENT OF CIRCULATING NUCLEIC ACIDS AS BIOMARKERS

1.1 Early studies on the presence of cell-free nucleic acids

The existence of cell-free circulating nucleic acids was first documented by Mandel and Metais (Mandel and Metais, 1948). Since late 1960s, clinical value of cell-free DNA in plasma/serum gradually emerged as its presence was demonstrated in different diseases including systemic lupus erythematosus (Tan *et al.*, 1966), rheumatoid arthritis (Koffler *et al.*, 1973), pulmonary embolism (Barada *et al.*, 1980) and haemodialysis (Steinman and Ackad, 1977). The clinical potential of cell-free circulating nucleic acids for cancer detection was first demonstrated in the late 1970s. The serum DNA concentration was higher in cancer patients than those with non-malignant diseases and the levels of serum DNA decreased in patients after successful anti-cancer therapy (Leon *et al.*, 1977). As the placenta features some characteristics of a malignant tumour and has been described as "pseudomalignant" (Strickland and Richards, 1992), the discovery of tumour-derived nucleic acids in the plasma/serum of cancer patients has prompted researchers to investigate the release of fetal nucleic acids into the plasma/serum of pregnant women. In 1997, Lo *et al.* discovered the presence of fetal DNA in maternal circulation by detecting Y-chromosome-specific sequences using plasma

samples taken from women conceived with male fetuses (Lo *et al.*, 1997).

Besides cell-free DNA, Kopreski *et al.* and Lo *et al.* simultaneously reported the presence of tumour-derived mRNA in the serum and plasma of cancer patients, respectively in 1999 (Kopreski *et al.*, 1999, Lo *et al.*, 1999a). Subsequently, Poon *et al.* employed the rationale for fetal DNA detection in maternal plasma (Lo *et al.*, 1997) and were the first to demonstrate the presence of fetal-specific mRNA in the plasma of pregnant women carrying male fetuses (Poon *et al.*, 2000). Recently, a new class of circulating cell-free nucleic acids, microRNA (miRNA), was discovered in plasma and serum by Chim *et al.* and Lawrie *et al.*, respectively (Chim *et al.*, 2008, Lawrie *et al.*, 2008). It was demonstrated that placental miRNA was detectable in maternal plasma and showed reduced detection rates in postpartum samples (Chim *et al.*, 2008).

In addition to plasma and serum, cell-free DNA was also reported to be present in other human bodily fluids such as urine (Botezatu *et al.*, 2000), amniotic fluid (Bianchi *et al.*, 2001), cerebrospinal fluid (Angert *et al.*, 2004), and peritoneal fluid (Cioni *et al.*, 2003). These encouraging findings revealed the potential of detecting cell-free nucleic acids for molecular diagnostics.

1.2 Circulating cell-free nucleic acids in plasma and serum

Whole blood is a convenient source of genetic materials for non-invasive diagnosis. However, the majority of nucleic acids obtained from whole blood are derived from blood cells. On the other hand, plasma and serum are prepared with removal of the blood cells, with their constituent intracellular nucleic acids, thus providing an environment with a low interference from blood-derived nucleic acids for the detection of target sequences emanated from other tissue sources.

In the field of cancer diagnosis and prenatal diagnosis, one of the major advantages of plasma nucleic acid-based diagnostics is that it does not require further cell enrichment techniques, such as for circulating tumour or fetal cells, which are relatively labour-intensive and time-consuming. Thus, the discovery of plasma nucleic acids triggered a growing tide of interest in studying their potential as a tool for molecular diagnostics.

1.2.1 Cancer detection

1.2.1.1 Circulating tumour-derived DNA

The levels of cell-free DNA was reported to be increased in plasma/serum of patients with different malignancies in early studies (Leon *et al.*, 1977, Shapiro *et*

al., 1983, Stroun *et al.*, 1987). Moreover, the levels of cell-free DNA in the serum of patients with metastases were higher than those without metastases and higher plasma/serum levels of cell-free DNA after treatment were associated with a poor prognosis (Leon *et al.*, 1977). These early findings suggested that circulating cell-free DNA in plasma/serum could play a role in cancer detection and monitoring.

The application of cell-free DNA in plasma/serum for cancer detection was accelerated by the development of sequence-specific DNA detection techniques, e.g. the polymerase chain reaction (PCR). Different types of tumour-derived mutated DNA sequences were identified in the plasma/serum of cancer patients. (i) Oncogene: Ras mutations were found in the plasma/serum of patients with pancreatic cancer (Mulcahy *et al.*, 1998, Yamada *et al.*, 1998, Castells *et al.*, 1999, Theodor *et al.*, 1999), colorectal cancer (Anker *et al.*, 1997, de Kok *et al.*, 1997, Kopreski *et al.*, 1997, Hibi *et al.*, 1998), myelodysplastic syndrome and acute myelogenous leukaemia (Vasioukhin *et al.*, 1994). (ii) Mutated sequences of tumour suppressor genes: Mutated p53 sequences were detected in patients with hepatocellular carcinoma (Kirk *et al.*, 2000) and colorectal cancer (Mayall *et al.*, 1998). (iii) DNA sequences with microsatellite alterations: These alterations were

reported in patients with primary head and neck squamous cell carcinoma (Nawroz *et al.*, 1996) and small cell lung cancer (Chen *et al.*, 1996). (iv) Hypermethylated promoter DNA sequences: These hypermethylated sequences were identified in patients with breast cancer (Hoque *et al.*, 2006), colorectal cancer (Bazan *et al.*, 2006), liver cancer (Wong *et al.*, 1999), lymphoma (Deligezer *et al.*, 2003), cervical cancer (Yang *et al.*, 2004), and melanoma (Marini *et al.*, 2006). (v) DNA sequences of different tumour-associated viruses: Tumour-associated viral DNA sequences were shown to be present in the circulation of patients suffering from nasopharyngeal carcinoma (NPC) (Mutirangura *et al.*, 1998), Hodgkin's lymphoma (Gallagher *et al.*, 1999), head and neck squamous cell carcinoma (Capone *et al.*, 2000), and cervical carcinoma (Pornthanakasem *et al.*, 2001). The successful detection of the various tumour-derived DNA sequences in the plasma/serum of cancer patients has laid the foundation for the non-invasive detection of cancers.

Furthermore, plasma DNA markers are potentially applicable for early cancer diagnosis and screening (Eisenberger *et al.*, 2006). Subsequent studies have also revealed their potential in prognostication (Castells *et al.*, 1999, Kawakami *et al.*, 2000, Lo *et al.*, 2000a, Taback *et al.*, 2001, Gautschi *et al.*, 2004), disease

recurrence (Lo *et al.*, 1999c, Gonzalgo *et al.*, 2002, Ryan *et al.*, 2003), and treatment outcome (Lo *et al.*, 2000b, Kimura *et al.*, 2004, Taback *et al.*, 2004). In brief, circulating tumour-derived DNA has demonstrated the feasibility of developing into a new class of non-invasive markers with unprecedented advantages over the more traditional cancer detection methods.

1.2.1.2 Circulating tumour-derived RNA

Besides tumour-derived DNA, scientists also studied circulating tumour-derived mRNA for cancer detection. Circulating tumour-derived mRNA markers may have various advantages when compared with circulating DNA detection. Firstly, each cell may express multiple copies of mRNA species while there are only two genome equivalents of DNA in a normal diploid cell. In addition, expression of mRNA can be tumour-, tissue- or even cell-specific. Therefore, the detection of carefully selected mRNA species in plasma not only opens up the possibility for highly sensitive non-invasive cancer detection, but also enables the development of tailor-made approaches for the management of certain cancer.

In 1999, Kopreski *et al.* and Lo *et al.* simultaneously reported the presence of tumour-derived mRNA in the serum and plasma of cancer patients, respectively

(Kopreski *et al.*, 1999, Lo *et al.*, 1999a). In particular, Kopreski *et al.* demonstrated that the tumour-derived tyrosinase mRNA detected in serum was free from contaminating tumour cells (Kopreski *et al.*, 1999). This was a surprising finding given the lability of RNA and the elevated level of serum RNases in cancer patients (Reddi and Holland, 1976). Subsequent studies also showed, in plasma/serum of cancer patients, the detection of different tumour-associated mRNA transcripts including different telomerase components (Chen *et al.*, 2001b, Dasi *et al.*, 2001, Miura *et al.*, 2003, Novakovic *et al.*, 2004), epithelial-expressed genes (Gal *et al.*, 2001, Silva *et al.*, 2001, Silva *et al.*, 2002, Kijima *et al.*, 2005), and beta-catenin (Wong *et al.*, 2004b). In particular, Miura *et al.* (2003) reported that the detection of serum telomerase reverse transcriptase protein (*TERT*) mRNA was more sensitive than either α-fetoprotein (*AFP*) or *AFP* mRNA alone in the diagnosis of hepatocellular carcinoma. These results demonstrated some of the advantages of circulating tumour-derived mRNA measurements over conventional diagnostic tumour markers.

In recent years, the use of real-time quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) has extended the scope and enabled the use of quantitative aberrations in circulating mRNA for cancer assessment. Dasi *et al.*

were the first to report that the plasma *TERT* mRNA levels were 7-fold and 10-fold higher in patients with colorectal cancer and follicular lymphoma, respectively, than in healthy controls (Dasi *et al.*, 2001). Subsequent studies also demonstrated the diagnostic use of the quantification of circulating tumour-derived mRNA in lung (Pelosi *et al.*, 2006, Sueoka *et al.*, 2005) and liver cancers (Miura *et al.*, 2003).

1.2.1.3 Circulating tumour-derived microRNA

MicroRNAs are a family of small (18-25 nucleotides), endogenous non-coding RNAs that target mRNAs largely for translational repression (Ambros, 2003, Bartel, 2004, Lai, 2003). miRNAs are involved in important homeostatic processes such as cellular proliferation and cell death (Cheng *et al.*, 2005), the dysregulation of which is an important trait in cancer progression (Evan and Vousden, 2001). The link between miRNAs and cancer was first highlighted by Calin *et al.* (Calin *et al.*, 2002). It was discovered that two miRNA genes, miR-16 and miR-15, were located in a region on chromosome 13 and were deleted in over 65% of chronic lymphocytic leukaemia patients. Several studies have shown that unique miRNA expression profiles are present in a number of cancers such as breast, lung, oesophageal, prostate and pancreatic cancer. Taken together, the

results reported in these studies are consistent with the hypothesis that miRNAs play a substantial role in the pathogenesis of human cancer and thus offer diagnostic and prognostic values for cancer assessment.

Indeed, scientists have begun to identify miRNAs as potential cancer biomarkers. The first serum miRNA biomarker for cancer was miR-21. Lawrie *et al.* determined that patients with diffuse large B cell lymphoma with increased relapse-free survival had high serum levels of miR-21 (Lawrie *et al.*, 2008). Moreover, Chen *et al.* looked at serum miRNAs from lung cancer, colorectal cancer, and diabetes patients and determined that each of these diseases had specific serum miRNA profiles (Chen *et al.*, 2008). In the sera of lung cancer patients, there were 63 miRNAs that were not found in normal sera. A serum-miRNA fingerprint was determined for colorectal cancer as well. While some of these miRNAs were identical to those found in lung cancer sera, there were 10 miRNAs specific to just lung or colorectal cancers. More specifically, the authors determined that miR-25 and miR-223, miRNAs previously shown to be involved in tumour formation, were highly expressed in lung cancer sera, as compared to normal sera, by both Solexa sequencing and QRT-PCR (Chen *et al.*, 2008). Furthermore, Resnick *et al.* showed that miRNAs-21, 92, 93, 126 and 29a

were significantly over-expressed in the sera from ovarian cancer patients compared to controls (Resnick *et al.*, 2009). These reports clearly showed that cancers affect miRNA levels in the bloodstream, although it is still unclear how tumour miRNAs make their way into the bloodstream. Tumour miRNAs may possibly be present as a result of tumour cells dying or tumour cells may actively release miRNAs into the surrounding environment.

1.2.2 Prenatal diagnosis

Conventional definitive prenatal diagnosis and assessment of fetal genetic diseases frequently require invasive procedures such as chorionic villus sampling and amniocentesis which may impose a definite risk of fetal miscarriage. In addition, non-invasive prenatal assessment techniques, such as biochemical screening and ultrasound scanning, are associated with a significant false positive rate. Thus, there is a long-standing need for a platform technology for the definitive and reliable non-invasive prenatal diagnosis.

Inspired by the "pseudomalignant" nature of the human placenta (Strickland and Richards, 1992), the existence of circulating cell-free fetal DNA and mRNA was demonstrated in pregnant women akin to the release of circulating tumour-derived

nucleic acids in cancer patients (Lo *et al.*, 1997, Poon *et al.*, 2000). In addition, the recent discovery of miRNA in placental tissues (Barad *et al.*, 2004, Bentwich *et al.*, 2005) and the successful detection of pregnancy-associated miRNA in maternal plasma (Chim *et al.*, 2008) offered yet another novel class of fetal/placental markers to the field. These discoveries have thus opened up new avenues for non-invasive prenatal diagnosis.

1.2.2.1 Circulating fetal DNA

By detecting Y-chromosomal sequences, the existence of fetal DNA in maternal plasma/serum was first demonstrated in women conceived with male fetuses (Lo *et al.*, 1997). A subsequent study demonstrated that fetal DNA constituted a mean percentage of 3.4% and 6.2% in maternal plasma in early and late pregnancies, respectively (Lo *et al.*, 1998b). Comparing with concentrations of fetal cells in maternal blood which was 0.0035% and 0.008% in the second and third trimesters of pregnancy, respectively (Hamada *et al.*, 1993), there was significant enrichment of extracellular fetal DNA in maternal plasma. Recently, with microfluidics digital PCR, fetal DNA concentrations amounting to 10-20% of all DNA in maternal plasma were reported (Lun *et al.*, 2008a). Moreover, the rapid clearance of circulating fetal DNA after delivery (Lo *et al.*, 1999e) and the

reliable detection of fetal DNA in maternal plasma/serum from as early as the fifth week of gestation (Samura *et al.*, 2003, Rijnders *et al.*, 2001) illustrated the feasibility of using circulating fetal DNA for non-invasive prenatal diagnosis in the first trimester of pregnancy.

Fetal gender determination

The discovery of circulating Y-chromosome-specific sequences in maternal plasma enabled the use of the technique for fetal sex determination (Lo *et al.*, 1997). Other studies further confirmed the high sensitivity and specificity of such an assessment of fetal sex by plasma DNA analysis (Costa *et al.*, 2001, Sekizawa *et al.*, 2001a, Wei *et al.*, 2001, Zhong *et al.*, 2001b). This highly accurate method was effective for stratifying families at risk of sex-linked disorders in terms of the appropriate clinical management options (Costa *et al.*, 2002) and has also been proven beneficial in the management of pregnancies at risk of congenital adrenal hyperplasia (Rijnders *et al.*, 2001).

Prenatal diagnosis of fetal genetic diseases

In maternal plasma, cell-free DNA are contributed by both the mother and the fetus. The majority of the DNA is identical between the mother and fetus.

However, paternally-inherited fetal DNA is distinguishable from maternal DNA at certain sites, such as polymorphisms and mutations. By detecting the paternally inherited mutations, it was feasible to offer non-invasive prenatal diagnosis for certain autosomal dominant diseases in maternal plasma. For example, detection of the expanded CTG trinucleotide repeats in the *dystrophia myotonica protein kinase* gene in maternal plasma was found to be useful for the prenatal diagnosis of myotonic dystrophy (Amicucci *et al.*, 2000). Moreover, circulating fetal DNA was also applied in the prenatal exclusion of autosomal recessive diseases, such as beta-thalassaemia major (Chiu *et al.*, 2002b), Huntington disease (Gonzalez-Gonzalez *et al.*, 2003), cystic fibrosis (Gonzalez-Gonzalez *et al.*, 2002), and other haemoglobinopathies (Fucharoen *et al.*, 2003). However, the application in these cases in the setting of autosomal recessive diseases is more complicated because the mere presence of a mutant allele cannot confirm or exclude the inheritance of the diseases. In light of this, Chiu *et al.* have demonstrated the feasibility of prenatal exclusion of congenital adrenal hyperplasia by detecting polymorphic markers associated with the paternally-inherited non-mutant fetal allele in maternal plasma (Chiu *et al.*, 2002a).

Prenatal assessment of fetal chromosomal aneploidy

In addition to mutations, several research groups also investigated the feasibility of using circulating fetal DNA as a tool for fetal chromosomal aneuploidies detection. Chen *et al.* demonstrated that STR sequences in circulating fetal DNA can be used for the diagnosis of paternally-inherited fetal chromosomal aneuploidies (Chen *et al.*, 2000, Chen *et al.*, 2001a). Also, quantitative aberrations of circulating fetal DNA can also aid the screening of fetal aneuploidies. Lo *et al.* and Lee *et al.* reported that in pregnant women conceived with fetuses with Down syndrome (trisomy 21), the plasma and serum level of fetal DNA has a 2-fold and a 1.7-fold increase, respectively, compared with fetal sex and gestational age matched healthy pregnant women (Lo *et al.*, 1999b, Lee *et al.*, 2002). Moreover, when the analysis was performed in conjunction with the quadruple test using maternal serum biochemical screening, fetal DNA can improve the detection rate from 81% to 86% at a 5% false-positive rate (Farina *et al.*, 2003). Recently, with the advancement of massively parallel genomic sequencing, Chiu *et al.* and Fan *et al.* introduced a new approach to quantify maternal plasma DNA sequences for non-invasive prenatal detection of fetal trisomy 21 (Chiu *et al.*, 2008, Fan *et al.*, 2008). Instead of targeting specific gene loci, the use of a locus-independent method has greatly increased the number of target molecules measurable from the

aneuploid chromosome. This, in turn, resulted in a single test that could identify trisomy 21 pregnancies at 100% accuracy.

Besides trisomy 21, an increased level of fetal DNA in maternal plasma was also demonstrated in pregnancies affected by trisomy 13 (Wataganara *et al.*, 2003). Also, using epigenetic allelic ratio (EAR) analysis of the *maspin* gene, Tong *et al.* successfully demonstrated the non-invasive prenatal analysis of of trisomy 18 (Tong *et al.*, 2006).

Indication of fetomaternal well-being

Rhesus D-negative pregnant women with rhesus D-positive fetuses carry an inherent risk of haemolytic disease of the newborn as a result of alloimmunisation. Faas *et al.* and Lo *et al.* were the first to demonstrate the use of circulating fetal DNA for the prenatal analysis of fetal rhesus D status (Faas *et al.*, 1998, Lo *et al.*, 1998a). As the haemolytic disease of newborn is more severe in male fetuses (Ulm *et al.*, 1999, Ulm *et al.*, 1998), the establishment of multiplex real-time PCR assay for fetal sex and rhesus D status determination further facilitated such a clinical application (Zhong *et al.*, 2001c). Since non-invasive fetal *RHD* genotyping is accurate and precise with lower associated costs, it has been

adopted as a routine test in the United Kingdom, France, and the Netherlands (Finning *et al.*, 2002, Bianchi *et al.*, 2005).

Aberrations of circulating fetal DNA concentration were observed in the plasma/serum of women with pathological pregnancies, e.g. preterm delivery (Leung *et al.*, 1998), polydramnios (Zhong *et al.*, 2000), fetomaternal haemorrhage (Lau *et al.*, 2000, Samura *et al.*, 2003), hyperemesis gravidarum (Sekizawa *et al.*, 2001b) and invasive placentation (Sekizawa *et al.*, 2002). Moreover, in the case of preeclampsia, the levels of circulating fetal DNA in maternal plasma were elevated compared with those in normal pregnancies (Lo *et al.*, 1999d, Smid *et al.*, 2001, Shimada *et al.*, 2004) and the levels correlated with disease severity (Zhong *et al.*, 2001d) and clinical onset (Leung *et al.*, 2001).

1.2.2.2 Circulating fetal messenger RNA

Subsequent to the discovery of circulating tumour-derived mRNA in cancer patients (Kopreski *et al.*, 1999), Poon *et al.* employed the rationale for fetal DNA detection in maternal plasma (Lo *et al.*, 1997) and were the first to demonstrate the presence of fetal-specific mRNA in the plasma of pregnant women (Poon *et al.*, 2000). The potential use of circulating fetal mRNA for prenatal diagnosis has

several advantages. Besides the high sensitivity and specificity of detection offered by the much higher copy number of mRNA than genomic DNA per cell, detection of circulating fetal mRNA in maternal plasma potentially bypasses the requirement of using sex or genetic polymorphism differences between the mother and the fetus. Moreover, circulating fetal RNA is detectable from as early as the fourth week of gestation (Chiu *et al.*, 2006). All these observations point to the potential of circulating fetal mRNA analysis for early prenatal assessment.

Speculations have been placed on the placental origin of circulating fetal mRNA. After demonstrating the presence of fetal-specific Y-linked zinc finger protein gene mRNA in maternal plasma (Poon *et al.*, 2000), the same group further developed QRT-PCR assays for the detection of two mRNA transcripts originated from the placenta, namely, *chorionic somatomammotropin hormone 1* (*CSH1*, also named as human placental lactogen) mRNA and the beta subunit mRNA of *human chorionic gonadotropin* (*CGB*) in maternal plasma (Ng *et al.*, 2003b). Moreover, Go *et al.* (2004) screened a panel of potential mRNA transcripts expressed in extraembryonic tissues and demonstrated the detection of placental transcription factor mRNA in maternal circulation (Go *et al.*, 2004). Furthermore, applying a systematic and high throughput oligonucleotide microarray approach,

Tsui *et al.* identified a number of circulating fetal-derived placenta-expressed mRNA transcripts that were detectable in maternal plasma. They further showed rapid clearance of these transcripts from the maternal circulation postpartum. These findings highlighted the clinical potential of applying circulating placental mRNA as fetal-specific markers in maternal plasma (Tsui *et al.*, 2004).

Other studies were conducted to demonstrate the association of quantitative aberrations of these circulating fetal mRNAs with complicated pregnancies. *Corticotropin-releasing hormone* (*CRH*) mRNA was shown to be 10-fold elevated in plasma collected from preeclamptic pregnancies when compared with control cases (Ng *et al.*, 2003a). Masuzaki *et al.* suggested the use of plasma mRNA analysis by QRT-PCR as a non-invasive diagnostic, prognostic, and follow-up test for gestational trophoblastic disease (Masuzaki *et al.*, 2005). In women carrying trisomy 18 fetuses, the serum concentration of *CGB* mRNA was 9.4-fold lower than that in pregnant women carrying euploid fetuses (Ng *et al.*, 2004). The presence of chromosome 21-encoded mRNA of placental origin in the plasma of women between the 9th and 13th weeks of pregnancy suggested the potential for early prenatal screening of Down syndrome (Oudejans *et al.*, 2003). Using mass spectrometry, Lo *et al.* demonstrated the use of plasma placental RNA allelic ratio

for the prenatal diagnosis of fetal trisomy 21 even in first trimester pregnancies (Lo *et al.*, 2007b). These studies demonstrated the feasibilities of adopting circulating fetal mRNA analysis for non-invasive prenatal diagnosis.

1.2.2.3 Circulating fetal microRNA

In 2000s, Barad *et al.* and Bentwich *et al.* identified a panel of miRNAs in human placental tissues (Barad *et al.*, 2004, Bentwich *et al.*, 2005). As discussed before, nucleic acids of placental origin were shown to be released into and detectable in maternal plasma (Ng *et al.*, 2003b, Chim *et al.*, 2005). Based on a similar hypothesis that miRNAs produced by the placenta would also be released into the maternal circulation, Chim *et al* demonstrated the presence of placental miRNA in maternal plasma (Chim *et al.*, 2008). Subsequently, Gilad *et al* reported the detection of a panel of pregnancy-associated miRNA in maternal serum (Gilad *et al.*, 2008). Also, since miRNAs are associated with various regulatory functions in humans (Bartel, 2004) and they exist at much higher copy numbers in cells than mRNAs (Lim *et al.*, 2003), this novel class of markers has the potential to be developed for non-invasive prenatal diagnostic purposes.

However, considerable challenges have hampered such potential use of miRNA.

First, 866 human mature miRNA species have been identified (Griffiths-Jones, 2004) but only a minor proportion of them has been studied in detail. Insights into the roles and hence their potential use as diagnostic miRNAs were lacking. Second, the rich protein content of plasma has posed difficulties for efficient RNA extraction. Third, most column-based protocols for RNA extraction result in great loss of short RNA molecules, which were previously considered as "contaminants" to the longer mRNA molecules. Fourth, special PCR techniques are required to specifically and sensitively detect these short RNA molecules. Since miRNAs offer new possibilities in the field of prenatal diagnosis, it would be one of the objectives of this thesis to investigate the possible ways to overcome the technical hurdles for miRNA analysis.

1.3 Circulating cell-free nucleic acids in other bodily fluids

Cell-free DNA and RNA were also detectable in bodily fluids other than plasma and serum, including urine, amniotic fluid, cerebrospinal fluid (CSF), and peritoneal fluid. This section gives a brief summary of the recent progress in these areas.

1.3.1 Urine

Cell-free DNA in urine is an attractive source of genetic material for clinical

analysis because it could be obtained in a potentially completely non-invasive manner. In recent years, interest has been focused on the study of transrenal DNA, which is cell-free DNA in urine derived from circulating cell-free DNA in plasma as it passes through the kidney barrier (Botezatu *et al.*, 2000). The postulated plasma sources of transrenal DNA suggested its origin from throughout the body, which offers a new diagnostic tool with interesting potentials (Umansky and Tomei, 2006).

Botezatu *et al.* were the first to demonstrate the existence of transrenal DNA in humans. They showed that male-specific sequences could be detected in the urine of women pregnant with male fetuses and the length of the transrenal DNA was between 150 and 250 bp (Botezatu *et al.*, 2000). Also, it was reported that the detection of fetal DNA in urine can be achieved from as early as the seventh week of gestation (Al-Yatama *et al.*, 2001). Moreover, K-ras mutations associated with colorectal carcinoma were detectable in the urine of the cancer patients (Su *et al.*, 2004b, Su *et al.*, 2008). These findings suggested that transrenal DNA could be an ideal tool for non-invasive diagnosis.

Nevertheless, not all workers in the field are convinced with the reliability of

detecting fetal-derived transrenal DNA. Zhong *et al.* reported their inability to detect male-specific DNA in urine samples obtained from women pregnant with male fetuses (Zhong *et al.*, 2001a). The same group was again unable to detect such fetal-derived transrenal DNA in pre-eclamptic pregnant women (Li *et al.*, 2003). These subjects were known to have elevated levels of circulating fetal DNA in their plasma (Zhong *et al.*, 2001d) and had increased renal permeability as evidenced by proteinuria (Hayashi *et al.*, 2002). Recently, Hung *et al.* demonstrated with a sex-mismatched haematopoietic stem cell transplant model that a significant proportion of the cell-free DNA detectable in urine originated from donor-derived cells, rather than from the transrenal passage of cell-free plasma DNA (Hung *et al.*, 2008). Therefore, further investigations are still needed to explore the possibility of using urine as a biological source for circulating nucleic acid analysis.

1.3.2 Amniotic fluid

The presence of cell-free fetal DNA in amniotic fluid was first documented by Bianchi *et al.* (Bianchi *et al.*, 2001). The reported concentration of fetal DNA in amniotic fluid was approximately 100- to 200-fold more than that in maternal plasma. However, unlike cell-free DNA in maternal plasma which is likely to be

originated from the placenta, Lun *et al.* demonstrated the non-placental origin of cell-free fetal DNA in amniotic fluid using an epigenetic approach (Lun *et al.*, 2007).

The large quantity of amniotic cell-free fetal DNA has prompted researchers to explore its diagnostic potentials. Larrabee *et al.* showed the use of amniotic cell-free fetal DNA in the diagnosis of fetal sex and aneuploidy (Down and Turner syndrome) using comparative genomic hybridisation analysis (Larrabee *et al.*, 2004). In addition, Lapaire *et al.* reported that the unique fragmentation signatures of amniotic cell-free fetal DNA were potentially indicative of different fetal aneuploidies (Lapaire *et al.*, 2007).

Larrabee *et al.* were the first to report the presence of cell-free fetal mRNA in amniotic fluid (Larrabee *et al.*, 2005). The level of cell-free fetal mRNA in amniotic fluid appeared to be much lower than that of cell-free fetal DNA (Larrabee *et al.*, 2005). Also, these mRNAs are not expressed in the placenta (Larrabee *et al.*, 2005) and thus may not be useful for diagnosing conditions involving placental pathology, e.g. pre-eclampsia and intra-uterine growth restriction. Although such cell-free nucleic acids may have value for prenatal

diagnosis, the collection of amniotic fluid involves invasive procedures and imposes a definite risk of miscarriage.

1.3.3 Cerebrospinal fluid

In pregnant women receiving spinal anesthesia for cervical cerclage, caesarean delivery or postpartum tubal ligation, Angert *et al.* demonstrated the presence of cell-free fetal DNA in CSF (Angert *et al.*, 2004). It was shown that the fetal DNA detected postpartum in CSF demonstrated a different clearance profile from that in the plasma/serum (Lo *et al.*, 1999e). Although the detection showed limited sensitivity where only four out of 26 cases had detectable fetal DNA, this study illustrated the passage of circulating cell-free fetal DNA across the blood brain barrier.

1.3.4 Peritoneal fluid

In a case study, Cioni *et al.* reported the presence of male cell-free fetal DNA in the peritoneal fluid of a pregnant woman with a male fetus (Cioni *et al.*, 2003). Further studies are required to confirm these findings and to precisely define the origin of these sequences.

1.4 Origin and biological characteristics of cell-free circulating nucleic acids

1.4.1 Extracellular fetal-derived nucleic acids

Apoptosis has been found to be associated with the presence of cell-free fetal nucleic acids in maternal plasma. As the trafficking of fetal cells such as fetal leukocytes and erythrocytes into the maternal circulation is a known biological phenomenon (Bianchi, 1999), it is hypothesise that death of these cells may contribute to the pool of cell-free fetal nucleic acids in the circulation. In fact, several studies have confirmed the existence of apoptotic fetal cells in maternal peripheral blood (Kolialexi *et al.*, 2001, Sekizawa *et al.*, 2000, van Wijk *et al.*, 2000). However, the rarity of circulating fetal cells is unlikely to be able to fully account for the high abundance of cell-free fetal DNA present in maternal plasma (Lo *et al.*, 1998b). Moreover, there is no demonstration of correlation between the two species in normal and diseased pregnancies (Bischoff *et al.*, 2002, Zhong *et al.*, 2002). It has thus been suggested that apoptosis of circulating fetal cells is unlikely to be the predominant source of cell-free fetal nucleic acids in maternal plasma.

There is growing evidence that cell-free fetal nucleic acids in maternal plasma are

derived directly from the placenta. In this regard, it has been suggested that a large amount of fetal genetic material is deported into the maternal circulation by the shedding of syncytial knots, which are apoptotic fragments of the syncytiotrophoblast released from placental villi, as part of the normal turnover process (Hahn *et al.*, 2005). Increased apoptosis has been observed in placentas complicated by either preeclampsia or intrauterine growth restriction (Ishihara *et al.*, 2002), while fetal DNA concentrations are increased in maternal plasma for such pregnancy-related diseases (Castells *et al.*, 1999, Lo *et al.*, 1999d). Moreover, the hypomethylated placental gene, *maspin*, which was hypermethylated in maternal blood cells, was detected in maternal plasma (Chim *et al.*, 2005). Furthermore, mRNA of placental origin has been detected in maternal plasma with high fetal-specificity (Ng *et al.*, 2003b, Tsui *et al.*, 2004). Taken together, these findings suggest that the origin of cell-free fetal nucleic acids in maternal plasma may involve direct placental release.

1.4.2 Molecular characterisation of circulating nucleic acids

1.4.2.1 Plasma DNA

Plasma Epstrin-Barr virus (EBV) DNA was shown to be a useful non-invasive marker for NPC detection (Lo *et al.*, 1999a). In 2003, Chan *et al.* explored in

detail the molecular characteristics of such circulating viral DNA species and found that 87% of the circulating EBV DNA molecules were shorter than 180 bp (Chan *et al.*, 2003). This observation supports the hypothesis that these viral molecules are released into the circulation by apoptosis of tumour cells, as DNA in apoptotic cells is cleaved by caspase-activated DNases into fragment lengths in multiples of nucleosomal DNA (Nagata, 2000).

Other studies have revealed the molecular characteristics of the different DNA species present in maternal plasma. Chiu *et al.* demonstrated that fetal DNA in maternal plasma was particle-free that they could pass through a 0.2 μm filter (Chiu *et al.*, 2001). By amplification of different sized-amplicons targeting the *leptin* gene and *sex determining region Y* (*SRY*) gene, Chan *et al.* have showed that DNA fragments in the plasma of pregnant women were significantly longer than those in the plasma of non-pregnant women and maternal-derived DNA molecules were longer than the fetal-derived ones (Chan *et al.*, 2004). They also showed that more than 99% of the fetal-derived DNA molecules were shorter than 313 bp in length. Furthermore, Koide *et al.* have demonstrated that the size distribution of fetal DNA in maternal plasma is relatively stable despite prolonged storage of plasma samples (Koide *et al.*, 2005).

1.4.2.2 Plasma mRNA

Considering the well-known instability of RNA in nature, it was surprising that cell-free RNA could be detected in plasma. Tsui *et al.* demonstrated the relative stability of endogenous plasma RNA and the rapid degradation of exogenous RNA added in plasma (Tsui *et al.*, 2002). Such observation has pointed in favour to the hypothesis that endogenous circulating RNA is protected by an unknown mechanism in plasma. In 2000, Halicka *et al.* showed that cells undergoing apoptosis would package DNA and RNA into separate apoptotic bodies and thus protected from nuclease activities in the circulation (Halicka *et al.*, 2000). Furthermore, Ng *et al.* demonstrated the association of circulating mRNAs in plasma with subcellular particles (Ng *et al.*, 2002). Also, it was speculated that placental mRNAs were possibly associated with syncytiotrophoblast microparticles (Gupta *et al.*, 2004). Concerning the integrity of the circulating mRNAs, it was shown that placental mRNAs in the maternal plasma appeared to have a preponderance in the 5' end of the fragments (Wong *et al.*, 2005). This finding facilitated the development of new assays targeting fetal RNA markers for non-invasive prenatal diagnosis and monitoring.

1.4.2.3 Plasma miRNA

MicroRNAs are a family of small (18-25 nucleotides), endogenous non-coding RNAs that target mRNAs largely for translational repression (Ambros, 2003, Bartel, 2004, Lai, 2003). To investigate the potential of plasma miRNA as a clinical diagnostic tool, Chim *et al.* studied the physical nature of a placental miRNA, miR-141 (Chim *et al.*, 2008). Unlike plasma mRNA, plasma miR-141 could not be filtered out by a 0.22 μm filter indicating that it was unlikely to be associated with subcellular particles. Despite of this, miR-141 was demonstrated to be more stable in human plasma compared with placental mRNA. Further investigation would be needed to elucidate the underlying mechanisms for their higher stability.

1.5 Conclusion

Circulating nucleic acids is a convenient source of genetic material for non-invasive diagnosis of diseases. It is envisioned that additional research on the physical characteristics of the different circulating molecular species would be forthcoming and would thus improve the current understanding of the biology of plasma nucleic acids.

CHAPTER 2: ASSESSMENT OF BRAIN INJURY

2.1 Brain injury

In this thesis, brain injury is defined as haemorrhage in the brain requiring open craniotomy for blood clot evacuation. The haemorrhage could be due to traumatic brain injury or spontaneous intra-cerebral haemorrhage.

2.1.1 Traumatic brain injury (TBI)

The World Health Organisation (WHO) defines TBI as any injury to the brain resulting from the application of external forces to the skull (WHO, 2006). Traumatic brain injuries which are commonly caused by vehicle crashes, falls, sports injuries, violence and gunshots, can lead to a spectrum of problems including concussion, contusion, or diffuse injuries that cause more severe neurological damage (WHO, 2004). TBI could be classified into 2 categories, open and closed, based on the nature of the injury (WHO, 2004).

2.1.1.1 Open head brain injury

Open head injuries occur when an object penetrates the skull or when the skull is broken. In addition to physically damaging the brain, open head injuries invite

infection in the open wound, which can complicate the condition. There are two main types of open head injuries: skull fractures and penetrating injuries.

Fractures can occur at any area of the skull. The most common type is a linear fracture, which includes cracks or breaks in the skull. Some linear fractures are minor, but if a piece of the bone pushes into the brain, called a depressed skull fracture, it can cause serious damage. Fractures can also occur at the suture lines, the areas between the cranial bore plates that are fused together during childhood. The most serious skull fracture, however, is one that occurs at the base of the skull.

Penetrating injuries occur when an object enters the skull and damages the brain, which is common with gunshot injuries and in car accidents when a person's head breaks the windshield.

2.1.1.2 Closed head brain injury

Closed head injuries occur when the brain hits the inside of the skull. These types of injuries may result from whiplash or when the head strikes the windshield of a car. Although there may be no physical signs of injury, the brain can swell inside the

skull, which can put pressure on delicate tissues and nerves, causing permanent damage.

2.1.2 Spontaneous intra-cerebral haemorrhage

Spontaneous intracerebral haemorrhage accounts for 10–20% of all stroke-related neurological deficits of sudden onset (Broderick *et al.*, 1999). The most important cause of it seems to be a pathophysiological alteration in the vessel wall leading to a rupture of the small penetrating arteries and arterioles of 50–200 μm in diameter, that originate from the major cerebral arteries (Anderson *et al.*, 1994, Challa *et al.*, 1992, Fisher, 1971, Takebayashi *et al.*, 1984). Degenerative changes induced by chronic hypertension or cerebral amyloid angiopathy also increase the likelihood of rupture of these vessels (Sessa, 2008, Qureshi *et al.*, 2001)

2.2 Clinical assessment of brain injury

The clinical assessment of brain injury could be based on clinical features, imaging findings, physiological and biochemical features.

2.2.1 Clinical features

Level of consciousness and brainstem reflexes have strong influence on survival and the potential for recovery. The Glasgow Coma Scale (GCS), which is used to

objectively assess the degree of impaired consciousness, is a powerful predictor of prognosis and a low initial score is associated with a poor outcome (Teasdale and Jennett, 1974, Choi *et al.*, 1988). This score is composed of observing eye opening, motor function and verbal performance in response to external stimuli. However, controversy exists as to the timing of GCS score determination. Earlier scoring can reduce the predictive value of the GCS (Marion and Carlier, 1994). Also, in some patients the eye and verbal scores cannot be assessed due to swelling of the eyes and endotracheal intubation (Marion and Carlier, 1994, Gale *et al.*, 1983).

Since abnormalities of brainstem reflexes including papillary function, extraocular movements and motor response patterns like extensor or flexor posturing predict a poor outcome following severe brain injury (Davis and Cunningham, 1984), the Glasgow-Liege scale, which combines the GCS score with an assessment of brainstem function was developed (Born *et al.*, 1985).

2.2.2 Imaging findings

The common imaging techniques used for brain injury diagnosis and assessment are computed tomography (CT) and magnetic resonance imaging (MRI).

2.2.2.1 Computed tomography

The findings of initial CT of the brain are important when assessing prognosis following brain injury. A completely normal CT scan at the time of admission after severe head injury is associated with a lower mortality and a better functional recovery than for patients whose scans are abnormal, even among patients with an initial GCS score of 3 or 4 (Lobato *et al.*, 1986). However, CT is insensitive to lesions within the brainstem due to the small size of the structures involved and their proximity to the surrounding bone and such lesions are often associated with a poor outcome (Mittl *et al.*, 1994).

2.2.2.2 Magnetic resonance imaging

MRI can demonstrate lesions of the white matter and brainstem that often are missed by CT scans (Mittl *et al.*, 1994, Orrison *et al.*, 1994). However, it could not be applied to patients with pacemakers and certain ferromagnetic appliances.

2.2.3 Physiological features

2.2.3.1 Intracranial pressure (ICP) monitoring

ICP is the pressure in the cranium and thus in the brain tissue and cerebrospinal fluid. ICP is normally 7–15 mm Hg for a supine adult. For head-injured adults, the ICP

threshold is between 20 and 25 mm Hg, above which outcome will be affected negatively (Steiner and Andrews, 2006). Patients with persistently elevated, escaped, or uncontrollable ICP tended to have a much poorer outcome than those in whom ICP was easily controlled using standard treatment measures (Conroy and Kraus, 1988, Kelly *et al.*, 1997, Rosner *et al.*, 1995).

2.2.3.2 Cerebral blood flow and metabolism

Abnormalities in cerebral blood flow measurements (Kelly *et al.*, 1997, Robertson *et al.*, 1992), oxygen metabolism and arteriovenous oxygen difference (Le Roux *et al.*, 1997, Robertson *et al.*, 1992) that indicate diminished blood flow, deranged oxygen metabolism and persistent or repeated episodes of relative cerebral ischaemia predict a poor outcome.

2.2.4 Biochemical features

The biomarkers that have been researched for the clinical assessment of BI are mainly protein markers that reflect damage of and release from the major cell types or structures in the brain parenchyma including astrocytes, neurons and axons.

2.2.4.1 Neurone-specific enolase (NSE)

NSE is located in the cytoplasm of neurones and is probably involved in increasing neuronal chloride levels during the onset of neural activity (Ingebrigtsen and Romner, 2002). It is the only marker which specifically indicates traumatic damage to neurones. The serum levels of NSE are increased in patients with severe brain injury but the correlation with CT findings and GCS scores is weak (Skogseid *et al.*, 1992).

2.2.4.2 S100B protein

The S100 proteins are involved in the Ca^{2+}-dependent regulation of a variety of intracellular processes. Several studies reported the correlation between serum S100B measurements and GCS scores, CT findings, ICP as well as long-term outcome (Herrmann *et al.*, 1999, Raabe *et al.*, 1999, Woertgen *et al.*, 1999). Patients with intracranial pathologies revealed by CT scan showed increased serum S100B levels. A cut-off value to distinguish between patients with good and bad clinical outcomes was about 2 μg/L with specificity of well over 90% (Raabe *et al.*, 1999, Woertgen *et al.*, 1999).

Recently, with the development of molecular diagnostics, Chan *et al.* attempted to detect *S100B* mRNA in the plasma of stroke patients and healthy individuals (Chan

et al., 2007). However, the plasma *S100B* mRNA concentrations showed no statistically significant difference in stroke patients compared with controls and no statistically significant correlation was observed between plasma *S100B* mRNA concentrations and GCS scores. Furthermore, by employing the bone marrow transplantation model system for ascertaining the tissue origin of plasma RNA species, they confirmed that plasma *S100B* mRNA was predominantly of haematopoietic origin and could not be used as a brain-specific plasma marker.

2.2.4.3 Myelin basic protein (MBP)

MBP is found in growing oligodendroglial cells and is specific to myelin. It was shown that in patients with a poor outcome, the mean serum MBP levels between two and six days after injury were significantly higher than in those with a good outcome (Thomas *et al.*, 1978, Yamazaki *et al.*, 1995).

2.2.4.4 Glial fibrillary acidic protein (GFAP)

GFAP constitutes the major part of the cytoskeleton of astrocytes and is found only in astroglial cells of the central nervous system. It was demonstrated that the serum GFAP levels increased in 12 of 25 head-injured patients and declined rapidly after 6

hours indicating that it may be a very specific marker for primary brain injury (Missler *et al.*, 1999).

2.3 Conclusion

The assessment of brain injury largely relies on clinical features, imaging findings and physiological fetures of the patient. Appropriate biomarkers should be able to provide additional objective information to aid clinical decision making. Researches have been focused on protein markers that reflect damage of and release from the major cell types or structures in the brain parenchyma. However, mature markers have not yet been established. There is still a need to develop biomarkers for clinical assessment of brain injury.

2.4 Aims of this thesis

This thesis focuses on the development of approaches and protocols for the detection of circulating RNA markers so as to facilitate their application in molecular diagnostics and enhance the current understanding of the biological nature of circulating RNA.

As discussed, the detection of placenta-derived mRNA in maternal plasma has been successfully applied for non-invasive prenatal diagnosis. However, recently, there have been studies by other research groups reporting the presence of placental/fetal mRNA in maternal whole blood. Chapter 4 describes a study which aims to investigate the detection of fetal mRNA in maternal whole blood and determine if it offered advantages over maternal plasma analysis.

Optimal protocols are essential for the precise and accurate detection of RNA markers. Chapter 5 reviews the blood sample processing and plasma RNA extraction protocols in respect to the known physical characteristics of plasma mRNA, in an attempt to enrich circulating fetal RNA in maternal plasma. In Chapter 6, extraction protocols for microRNAs, a new class of circulating nucleic acid markers, are also evaluated. Modifications of the protocols have been introduced to significantly improve the RNA yield in plasma.

The earlier chapters focus on the detection of fetal RNA in maternal circulation. In Chapter 7, I describe the implementation of what I have learnt in the analysis of circulating fetal RNA into the development of brain-derived RNA transcripts for detection in plasma of patients who sustained traumatic brain injuries. A systematic

approach based on gene expression microarray analysis was adopted to search for circulating brain-specific mRNA markers.

Concluding remarks and a speculation on the future perspectives of circulating RNA markers are presented in Chapter 8.

SECTION II: Materials and Methodology

This section provides methodology details of the experiments involved in this thesis.

CHAPTER 3: QUANTITATIVE AND QUALITATIVE ANALYSIS OF CIRCULATING NUCLEIC ACIDS

3.1 Preparation of samples

3.1.1 Preparation of whole blood

For quantitative analysis, peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing blood tubes. 0.3 mL blood samples were transferred into plain polypropylene tubes and 0.9 mL Trizol LS reagent (Invitrogen, CA, USA) was added. The samples were stored frozen at -80°C until RNA extraction. Details of whole blood sample collection for microarray analysis are described in Chapter 3.2.2.

3.1.2 Preparation of plasma

Peripheral blood was collected into ethylenediaminetetraacetic acid (EDTA)-containing blood tubes. Plasma was prepared by 3 different protocols according to the need of the experiment.

Protocol I

The blood samples were centrifuged at 1,600 *g* for 10 min at 4°C (Centrifuge 5810R, Eppendorf, Hamburg, Germany). Plasma was then transferred into plain

polypropylene tubes with care not to disturb the underlying cell layer. The plasma was further centrifuged at 16,000 *g* for 10 min at 4°C (Centrifuge 5415R, Eppendorf) and the supernatant was collected into fresh polypropylene tubes with care not to disturb the underlying cell pellet. 2 mL of Trizol LS reagent (Invitrogen) was added into 1.6 mL plasma. The storage of plasma with Trizol LS (Invitrogen) is critical for preservation of RNA integrity. Wong *et al.* have demonstrated the superior integrity of RNA in plasma that had been stored with Trizol (Invitrogen) for three years, comparing with the same plasma samples which had been stored alone without the Trizol LS (Invitrogen) (Wong *et al.*, 2004a). The samples were stored frozen at -80°C until RNA extraction.

Protocol II

The blood samples were centrifuged at 100 *g* for 10 min at 4°C (Centrifuge 5810R, Eppendorf) without brake. Plasma was then transferred into plain polypropylene tubes and passed through a 5-μm filter (Millipore, MA, USA) to remove contaminating cells. The filtrate was collected into fresh polypropylene tubes. 2 mL of Trizol LS reagent (Invitrogen) was added into 1.6 mL filtrate. The samples were stored frozen at -80°C until RNA extraction.

Protocol III

The blood samples were collected in EDTA-containing tubes and centrifuged at 1,600 *g* for 10 min at 4°C (Centrifuge 5810R, Eppendorf). Plasma was then transferred into plain polypropylene tubes with care not to disturb the underlying cell layer and passed through a 5 μm-filter (Millipore). Filtrate of 2.8 mL was collected into polyallomer centrifuge tubes and ultracentrifuged at 99,000 *g* for 30 min at 4°C (Ultracentrifuge XL-70, Beckman, CA, USA). The ultracentrifuged plasma, separated into 3 different layers, was collected in order: 1.6 mL uppermost supernatant, 0.9 mL middle layer and 0.3 mL lowest layer. Each layer was collected carefully without disturbing the other layers and transferred to a fresh polypropylene tube. Trizol LS reagent (Invitrogen) of 2 mL, 1 mL and 0.9 mL was added into the uppermost, middle and lowest layers, respectively. The samples were stored frozen at -80°C until RNA extraction.

3.1.3 Preparation of blood cells

Peripheral blood collected in EDTA-containing tubes was subjected to centrifugation at 1,600 *g* for 10 min at 4°C (Centrifuge 5810R; Eppendorf). Buffy coat is the fraction of a centrifuged blood sample that contains most of the white blood cells and platelets, settling between plasma (top layer) and red blood cells

(bottom layer). After centrifugation, the buffy coat was carefully transferred into fresh polypropylene tubes and re-centrifuged again at 2,300 *g* for 5 min at room temperature (Centrifuge 5415R, Eppendorf). The upper residual plasma was removed to obtain the lower blood cell layer.

For DNA studies, the blood cell fraction was stored at -20℃ until DNA extraction. For RNA studies, 900 μL of Trizol LS reagent (Invitrogen) was mixed thoroughly with 300 μL of the blood cell fraction before storage at -80℃.

3.1.4 Preparation of cerebrospinal fluid

Cerebrospinal fluid (CSF) was collected in plain tubes. When less than 2 mL of CSF was collected, 4.8 mL of Trizol LS reagent (Invitrogen) was added into 1.6 mL CSF in polypropylene tubes. When more than 2 mL of CSF was collected, the CSF sample was passed through a 5 μm-filter (Millipore) before addition of the Trizol LS reagent (Invitrogen). The samples were stored frozen at -80°C until RNA extraction.

3.1.5 Preparation of placental tissue

Placental tissues were obtained from pregnant women immediately after elective

caesarean delivery. For DNA studies, they were stored in plain polypropylene tubes. For RNA studies, they were stored in RNAlater™ solution (Ambion®, Austin, TX, USA) according to manufacturer's instructions immediately upon collection. The samples were stored at –80°C until nucleic acid extraction.

3.1.6 Preparation of brain tissue

Brain tissues were obtained from patients admitted to the Prince of Wales Hospital for neurosurgical operation. A small part of their brain tissues discarded due to the operation was collected and stored in RNAlater™ solution (Ambion®), according to manufacturer's instructions immediately upon collection. The samples were stored at –80°C until nucleic acid extraction.

3.2 Nucleic acid extraction

3.2.1 Extraction of total RNA for quantitative analysis

Plasma, whole blood, buffy coat and CSF RNA was isolated by a modified procedure combining the Trizol LS reagent (Invitrogen) and RNeasy Mini Kit (Qiagen, Hilden, Germany) (Ng *et al.*, 2002). Of note, extraction based on either the Trizol LS reagent (Invitrogen) or RNeasy Mini Kit (Qiagen) alone has proven to yield insufficient amount of RNA for detection (Wong *et al.*, 2004a). The Trizol

LS reagent (Invitrogen) is a solution of phenol and guanidine isothiocyanate, which is used to denature proteins including ribonucleases while maintaining the integrity of the ribonucleic acids. This technique is an improvement to the single-step RNA extraction method developed previously (Chomczynski and Sacchi, 1987). The RNeasy Mini Kit (Qiagen) is a column-based RNA isolation technology, combining the binding properties of a silica-gel-based membrane and the ease of a microspin protocol. This approach does not involve the use of caesium chloride gradient ultracentrifugation or alcohol precipitation, which is relatively time-consuming and labour-intensive, therefore providing a fast and simple alternative for the preparation of total RNA from blood samples.

For isolation of total RNA from plasma, whole blood, buffy coat and CSF, chloroform was added into the samples stored in Trizol LS reagent (Invitrogen) in a chloroform-to- Trizol LS reagent ratio of 1:3. The mixture was separated into different phases by centrifugation at 12,000 *g* for 15 min at 4°C (Centrifuge 5415R; Eppendorf): RNA in the aqueous phase, DNA in the organic phase, and proteins at the interphase. The aqueous layer was carefully removed and transferred into fresh polypropylene tubes.

For isolation of total RNA from placental and brain tissues, 50-100 mg of tissue was

homogenised in 1.5 mL Trizol reagent (Invitrogen), and 0.3 mL chloroform was added. Cell-debris was removed by centrifugation at 12,000 *g* for 15 min at 4°C (Centrifuge 5415R; Eppendorf). To 1.5 mL of homogenate, 0.4 mL of chloroform was added and mixed. The mixtures were separated into different phases by centrifugation at 12,000 *g* for 15 min at 4°C (Centrifuge 5415R; Eppendorf): RNA in the aqueous phase, DNA in the interphase, and proteins at the organic phase. The aqueous layer was carefully removed and transferred into fresh polypropylene tubes.

Total RNA in the aqueous layer was then extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. First, for adjusting the column binding conditions, one volume of 70% ethanol was added to one volume of the aqueous layer. The mixture was applied to an RNeasy spin column (Qiagen) followed by centrifugation at 8,000 *g* for 15 sec (Centrifuge 5415D; Eppendorf), during which total RNA was adsorbed to the membrane. After all mixture was passed through the column, 700 μL of Buffer RW1 (proprietary to the manufacturer) was loaded onto the column to wash away contaminants by further spin using the same conditions. Afterwards, the column was subjected to two additional washes each using 500 μL of Buffer RPE (proprietary to the manufacturer). High-speed centrifugation at 8,000 *g* for 2 min (Centrifuge 5415D;

Eppendorf) was then performed for the complete removal of residual buffer from the membrane. Lastly, to elute RNA from the column, 50 µL of RNase-free water was added directly onto the membrane and incubated at room temperature for 5 min, followed by centrifugation at 8,000 *g* for 1 min (Centrifuge 5415D; Eppendorf). This elution step was repeated by reloading the eluate onto the column in order to obtain a higher total RNA concentration.

At this stage, to ensure isolation of pure RNA from the samples, DNase I digestion was performed using the Amplification Grade DNase I (Invitrogen). Each whole blood, buffy coat and tissue RNA sample was quantified using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For digestion, the following four components were added to fresh microcentrifuge tubes on ice: 1 µg of RNA sample, 1 µL of DNase I, 1 µL of 10X Reaction Buffer and RNase-free water to 10 µL. This reaction mixture was incubated at room temperature for 15 min, followed by the addition of 1 µL of 25 mM EDTA solution and incubation at 65°C for 10 min to inactivate DNase I. The treated RNA samples were then stored at -80°C until use.

3.2.2 Extraction of whole blood RNA for microarray analysis

For microarray analysis, whole blood RNA was isolated by two methods, PAXgene™ (PreAnalytiX) or RiboPure™-Blood Kit (Ambion) according to the individual project need.

3.2.2.1 Whole blood RNA extraction with PAXgene™

For whole blood sample to be extracted with PAXgene ™ (PreAnalytiX), 5 mL of peripheral blood was collected directly into PAXgene Blood RNA Tubes (PreAnalytiX) and incubated at room temperature for overnight followed by centrifugation at 4,000 *g* for 10 min. The supernatant was then removed and 4 mL of RNase-free water was added to re-suspend the pellet. After centrifuging at 4,000 *g* for 10 min, the supernatant was discarded and 350 µL Buffer BR1 was added to dissolve the pellet. The sample was transferred to 1.5-mL microcentrifuge tube followed by the addition of 300 µL Buffer BR2 and 40 µL proteinase K and incubation for 10 min at 55°C using a shaker–incubator at 1,400 rpm. The lysate was transferred into a PAXgene Shredder spin column and centrifuged at maximum speed for 3 min. The entire supernatant of the flow-through fraction was transferred to a fresh 1.5 mL microcentrifuge tube and 350 µL of absolute ethanol was added. The mixture was applied to the PAXgene RNA spin column and centrifuged at 8,000 *g* for 1 min. The column was washed with 350 µL of Buffer

BR3 and centrifuged at 8,000 *g* for 1 min. DNase I incubation mix was prepared with 10 µL of DNase I stock solution and 70 µL of Buffer RDD, added onto the column and incubated at room temperature for 15 min. After DNase I digestion, the column was washed once with 350 µL of Buffer BR3 and twice with 500 µL of Buffer BR4 followed by centrifuged at 8,000 *g* for 1 min. after each wash. The membrane was then dried by spinning the empty column at maximum speed for 1 min. The RNA was eluted with 40 µL of Buffer BR5 and centrifuged at 16,000 *g* for 1 min. This elution step was repeated by reloading the eluate onto the column in order to obtain a higher total RNA concentration. The eluate was incubated for 5 minutes at 65°C and stored at -80°C.

3.2.2.2 Whole blood RNA extraction with RiboPure™-Blood Kit

For whole blood RNA to be extracted with RiboPure™-Blood Kit (Ambion, Austin, TX), 2 mL peripheal blood collected EDTA tube was transferred to 5.2 mL RNA*later* solution. The sample was stored at -80°C until RNA extraction. When isolating the RNA, the sample was first centrifuged at 16,000 *g* for 1 min. The pellet was then re-suspended with 3.2 mL of Lysis Solution and 200 µL sodium acetate solution and 2 mL of acid-phenol:chloroform was added, vortexed and incubated at room temperature for 5 min followed by centrifugation at 8,000 *g* for

1 min. The aqueous phase was transferred into a new tube with the addition of 2.4 mL absolute ethanol. The mixture was applied to a filter cartridge assembly, and centrifuged for 10 sec to pass the liquid through the filter. The filter cartridge assembly was washed once with 700 μL of Wash Solution 1 and twice with 700 μL of Wash Solution 2/3 followed by centrifugation for 10 sec after each wash. The empty filter cartridge assembly was dried by a one-minute centrifugation at maximum speed followed by the elution with 40 μL pre-heated Elution Solution. This elution step was repeated by reloading the eluate onto the column in order to obtain a higher total RNA concentration.

3.2.3 Extraction of small RNA-containing total RNA

Plasma and blood cells RNA and small RNA was isolated by a method modified from the procedure used for plasma and buffy coat RNA isolation (Ng *et al.*, 2003a, Ng *et al.*, 2002). It was a combination of the use of the Trizol LS reagent (Invitrogen) and the mircroRNA isolation kit: *mir*Vana™ miRNA isolation kit (Ambion®) (Chim *et al.*, 2008) or Qiagen miRNeasy mini kit (Qiagen).

3.2.3.1 Extraction of small RNA-containing total RNA with *mir*Vana™ miRNA isolation kit

The *mir*Vana™ miRNA Isolation Kit (Ambion) is a column-based RNA isolation

technology. Unlike other RNA extraction columns which were unable to retain very small RNA sized below 200 nucleotides, the filter cartridge (Ambion) was specially designed for purification of small RNAs, inculding miRNA, which are about 21-25 nucleotides long. It involves an organic extraction followed by immobilisation of RNA on glass-fiber filters to purify small RNA-containing total RNA using a microspin protocol. Placental tissue RNA was isolated by a method similar to that in plasma and blood cells. However, Trizol reagent (Invitrogen) was used instead of Trizol LS reagent (Invitrogen) for solid tissue RNA extraction.

Details of the total RNA-containing aqueous layer preparation for buffy coat and plasma with Trizol LS reagent (Invitrogen) and that for placental tissue with Trizol reagent (Invitrogen) were described in Chpater 3.2.1.

Total RNA in the aqueous layer was then extracted using the *mir*Vana™ miRNA Isolation Kit (Ambion) according to the manufacturer's protocols. First, for adjusting the filter cartridge binding conditions, 1.25 volumes of absolute ethanol were added to one volume of the aqueous layer. The mixture was applied to *mir*Vana™ filter cartridges (Ambion) followed by centrifugation at 10,000 *g* for 10 sec (Centrifuge 5415D; Eppendorf), during which total RNA was adsorbed to the

membrane. After all mixture was passed through the filter cartridge, 700 µL of miRNA Wash Solution 1 (proprietary to the manufacturer) was loaded onto the filter cartridge to wash away contaminants by further spins under the same conditions. Afterwards, the filter cartridge was subjected to another 500 µL of miRNA Wash solution 2/3 (proprietary to the manufacturer). High-speed centrifugation at 10,000 *g* for 1 min (Centrifuge 5415D; Eppendorf) was then performed for the complete removal of residual buffer from the membrane. Lastly, to elute RNA from the filter cartridge, 100 µL of pre-heated RNase-free water (at 95°C) was added directly onto the membrane and incubated at room temperature for 1 min, followed by centrifugation at 10,000 *g* for 1 min (Centrifuge 5415D; Eppendorf).

To ensure isolation of pure RNA from the samples, DNase I digestion was performed using the Amplification Grade DNase I (Invitrogen) as described in Chapter 3.2.1.

3.2.3.2 Extraction of small RNA-containing total RNA with Qiagen miRNeasy mini kit

This Qiagen miRNeasy mini kit was used to isolate small RNA-containing total

RNA in plasma only aiming to compare its performance with the previously published protocol as described in Chapter 6. Details of the total RNA-containing aqueous layer preparation for plasma with Trizol LS reagent (Invitrogen) were described in Chpater 3.2.1.

Total RNA in the aqueous layer was then extracted using the Qiagen miRNease mini kit (Qiagen) according to the manufacturer's instruction. In particular, in order to adjust for the column binding conditions, 1.5 volume of absolute ethanol was added into the aqueous layer. The mixture was applied to a miRNeasy spin column (Qiagen) followed by centrifugation at 8,000 *g* for 15 sec (Centrifuge 5415D; Eppendorf), during which total RNA was adsorbed to the membrane. The mixture was applied to an RNeasy spin column (Qiagen) followed by centrifugation at 8,000 *g* for 15 sec (Centrifuge 5415D; Eppendorf), during which total RNA was adsorbed to the membrane.

After all mixture was passed through the column, for cases subjecting to on-column DNase treatment, 350 μL of Buffer RWT (proprietary to the manufacturer) was loaded onto the column to wash away contaminants by further spin using the same conditions. 10 μL of DNase I stock solution (Qiagen) was

mixed with 70 μL of Buffer RDD (proprietary to the manufacturer). The mixture was applied to the membrane of the column and incubated on the benchtop at room temperature for 15 min followed by the addition of 350 μL of Buffer RWT (proprietary to the manufacturer).

As for other cases subjecting to off-column DNase treatment, 700 μL of Buffer RWT (proprietary to the manufacturer) was loaded onto the column to wash away contaminants by further spin using the same conditions.

Afterwards, the column regardless of the DNase treatment was subjected to two additional washes each using 500 μL of Buffer RPE (proprietary to the manufacturer). High-speed centrifugation at 8,000 *g* for 2 min (Centrifuge 5415D; Eppendorf) was then performed for the complete removal of residual buffer from the membrane. Lastly, to elute RNA from the column, 30 μL of RNase-free water was added directly onto the membrane and incubated at room temperature for 5 min, followed by centrifugation at 8,000 *g* for 1 min (Centrifuge 5415D; Eppendorf). This elution step was repeated by reloading the eluate onto the column in order to obtain a higher total RNA concentration.

For off-column DNase I treatment, the digestion was performed using the Amplification Grade DNase I (Invitrogen) as described in Chapter 3.2.1.

3.2.4 Extraction of DNA

DNA was extracted from plasma, blood cells and urine cell pellet using the QIAamp DNA Mini Kit (Qiagen) according to the 'blood and bodily fluid spin protocol' (Lo *et al.*, 1998b). For plasma DNA extraction, 800 μL of plasma was used. For blood cell DNA extraction, 300 μL of buffy coat was used. For urine cell pellet DNA extraction, urine cell pellet was first resuspended in 200 μL of 1X phosphate buffered saline (Invitrogen) before DNA extraction. For each 400 μL of fluid sample, 40 μL of QIAGEN Protease and 400 μL of AL buffer were added and the mixture was incubated at 56℃ for 10 min. After that, 400 μL of cold absolute ethanol was added. The mixture was transferred onto a QIAamp Spin Column and centrifuged at 16,000 *g* for 1 min. The extraction column was washed twice with AW1 and AW2 buffers, at 16,000 *g* for 1 min and 3 min, respectively. DNA was finally eluted in 50 μL of deionised water, by centrifugation at 16,000 *g* for 1 min. The extracted DNA was stored at -20℃ until analysis.

3.3 Quantitative measurements of nucleic acids

3.3.1 Principle of real-time quantitative PCR

Real-time quantitative PCR systems allow robust and accurate detection of nucleic acids in plasma and other human bodily fluids. In this thesis, the real-time quantitative PCR (QPCR) (Heid *et al.*, 1996) was employed for quantitative measurement of DNA. QRT-PCR (Gibson *et al.*, 1996), real-time quantitative reverse transcriptase-polymerase chain reaction, was employed for the quantitative measurement of mRNA and microRNA. A typical TaqMan® based real-time quantitative PCR relies on the detection of the fluorescence generated by the TaqMan® probe which is a single-stranded synthetic oligonucleotide with a dual-labeled fluorescent reporter dye at the 5' end and a quencher dye at the 3' end. In a reaction mixture of QPCR or QRT-PCR, if the probe is not annealed to the target sequence, it would be intact. The proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence because of energy transfer. If the target sequence is present, the probe specifically hybridises to the target sequence. The DNA polymerase, which possesses the 5' to 3' nuclease activity, cleaves the probe. The reporter and the quencher become separated, resulting in an increased fluorescence emission by the reporter. Accumulation of PCR products was detected directly by monitoring the increase in fluorescence of the reporter. The use of this

type of fluorogenic probe allows great specificity as only the amplification of the intended sequence is measured, while primer-dimers and non-specific PCR products are not detected.

3.3.2 One-step QRT-PCR assays for mRNA markers

3.3.2.1 Principle

Messenger RNA was measured by one-step QRT-PCR using components provided by the TaqMan® EZ RT-PCR Kit (Applied Biosystems, Foster City, CA, USA). This is a single-tube, single enzyme quantitative system enabled with the use of the recombinant *Thermus thermophilus* (r*Tth*) DNA polymerase, which functions both as a reverse transcriptase and a DNA polymerase (Myers and Gelfand, 1991). RNA is first reverse transcribed to cDNA by sequence-specific primer. Then, in the PCR amplification, the 5' to 3' nucleolytic activity of the r*Tth* DNA polymerase cleaves hybridised TaqMan® probe separating the reporter dye and quencher dye on the same probe (Holland *et al.*, 1991). This results in an increase in fluorescence signal which is subsequently detected and analysed by the sequence detector.

3.3.2.2 Quantification of mRNA markers

RT-PCR was set up in a reaction volume of 25 uL with components provided in the TaqMan EZ RT-PCR Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequences of primers, probes and calibrators are listed in Table 3.1. Each reaction was run with 20 ng of buffy coat RNA or 1 ng of placental tissue RNA or 3 or 5 μL of extracted plasma RNA.

Table 3.2 illustrates the preparation of the RT-PCR mixture and the RT-PCR thermal profile using *CSH1* system as an example. The reaction was initiated at 50 °C for 2 min for uracil N-glycosylase (UNG) to act, followed by reverse transcription at 60°C for 30 min. After a 5-min denaturation at 95°C, 45 cycles of PCR were carried out with denaturation at 94°C for 20 s and 1 min of annealing/extension at 56°C. The RT-PCR mix preparation and thermal cycling procedures were the same for the other genes, with the exception that the primer and probe concentrations as well as the annealing/extension temperature were adjusted through optimisation to give maximum fluorescence signal in each individual system (Table 3.1).

For absolute quantification of mRNA in terms of copy numbers, a calibration

curve specifying the studied amplicon was run alongside with the tested samples. The calibration curves were prepared by serial dilutions of high performance liquid chromatography (HPLC)-purified single-stranded synthetic DNA oligonucleotides (Proligo, Helios) with concentrations ranging from 2.5 x 10^6 copies to 2.5 copies according to the manufacturer's reported concentration. Such single-stranded oligonucleotides have been demonstrated to reliably mimic the products of the reverse transcription step and produce calibration curves that are identical to those obtained using T7-transcribed RNA (Bustin, 2000). Each sample was analysed in duplicate with multiple negative water blanks in every analysis. The assays were carried out on an ABI Prism 7900 Sequence Detector.

The absolute mRNA concentration in each sample was calculated using one of the following formulas:

$$C_{plasma} = Q \times (V_{RNA} / V_{ext})$$

$$C_{buffy\ coat/placental\ tissue} = Q / C_{input\ RNA}$$

where C_{plasma} is the mRNA concentration in plasma (copies/mL of plasma); Q is the mRNA quantity determined by sequence detector (copies/µL of RNA sample); V_{RNA} is the total volume of RNA solution after the RNA extraction and DNase I treatment (typically 39 µL); V_{ext} is the volume of plasma used for extraction (typically 1.6 mL); $C_{buffy\ coat/placental\ tissue}$ is the mRNA concentration in buffy coat

or placental tissue (copies/ng of total RNA); and $C_{\text{input RNA}}$ is the concentration of the input total RNA (ng/μL).

Table 3.1: Primer, probe and calibrator sequences and reaction conditions for quantitative real-time RT-PCR assays

Transcripts	Sequence		Annealing Temp (°C)	Final concentration for RT-PCR (nM)
CSH1	F primer	5'- CATGACTCCCAGACCTCCTTC -3'	56	300
	R primer	5'- TGCGGAGCAGCTCTAGATTG -3'		300
	Probe	5'-(FAM) TTCTGTTGCGTTTCCTCCATGTTGG (TAMRA)-3'		100
	Calibrator	5'- TGCGGAGCAGCTCTAGATTGGATTTCTGTTGCGTTTCCTCCATGTTGGAG-GGTGTCGGAATAGAGTCTGAGAAGCAGAAGGAGGTCTGGGAGTCATGC -3'		
KISS1	F primer	5'- GCCCAGGCCAGGACTGA -3'	57	300
	R primer	5'- GCCAAGAAACCAGTGAGTTCATC -3'		300
	Probe	5'-(FAM) CCTCAAGGCACTTCTAGGACCTGGCTCTTC (TAMRA)-3'		100
	Calibrator	5'- CTGCCCAGGCCAGGACTGAGGCAAGCCTCAAGGCACTTCTAGGACCTGG-CTCTTCTCACCAAGATGAACTCACTGGTTTCTTGGCAG -3'		
PLAC4	F primer	5'- CCTTTCCCCCTTATCCAACT -3'	60	400
	R primer	5'- GTACTGGTTGGGCTCATTTTCT -3'		400
	Probe	5'-(FAM) CCCTAGCCTATACCC (MGBNFQ)-3'		100
	Calibrator	5'- ACCTTTCCCCCTTATCCAACTAGCCCTAGCCTATACCCTCTGCTGCCCAAG-AAAATGAGCCCAACCAGTACAC -3'		

Table 3.1: Primer, probe and calibrator sequences and reaction condition for quantitative real-time RT-PCR assays (continued)

Transcripts	Sequence		Annealing Temp (°C)	Final concentration for RT-PCR (nM)
PLAC1	F primer	5'- ATTATCCCCAGCTGCCAGAA -3'	56	400
	R primer	5'- GCAGCCAATCAGATAATGAACCA -3'		400
	Probe	5'-(FAM) AAGAAATCCTCACTGGACGGCTTCCTG (TAMRA)-3'		200
	Calibrator	5'- CAAATTATCCCCAGCTGCCAGAAGAAGAAATCCTCACTGGACGGCTTCCT-GTTTCCTGTGGTTCATTATCTGATTGGCTGCAGG -3'		
DEFA4	F primer	5'- TCTATTTCCTTTGCATGGGATAAAA -3'	56	300
	R primer	5'- GCAGACCATGCCCCTTG -3'		300
	Probe	5'-(FAM) CTTCAGGTTTCAGGCTCA (MGBNFQ)-3'		100
	Calibrator	5'- TATCTATTTCCTTTGCATGGGATAAAAGCTCTGCTCTTCAGGTTTCAGGCT-CAACAAGGGGCATGGTCTGCTC -3'		
CEACAM8	F primer	5'- ATCTTATGAGTGAAGAAGTAACTGGC -3'	58	400
	R primer	5'- TTGTTGCTGGAGATGGAGG -3'		400
	Probe	5'-(FAM) CGTACATCCGGAGACT (MGBNFQ)-3'		100
	Calibrator	5'- TAAATCTTATGAGTGAAGAAGTAACTGGCCAGTTCAGCGTACATCCGGAG-ACTCCCAAGCCCTCCATCTCCAGCAACAACTC -3'		

Table 3.1: Primer, probe and calibrator sequences and reaction condition for quantitative real-time RT-PCR assays (continued)

Transcripts	Sequence		Annealing Temp (°C)	Final concentration for RT-PCR (nM)
OLFM4	F primer	5'- GCTCCAGCCGCAGCTTAG -3'	60	300
	R primer	5'- GGAGCCGGTGAAATTGGAA -3'		300
	Probe	5'-(FAM) AGGTTCTGTGTCCCAGTTG (MGBNFQ)-3'		100
	Calibrator	5'- CCAGCTCCAGCCGCAGCTTAGGCAGCGGAGGTTCTGTGTCCCAGTTGTTT-TCCAATTTCACCGGCTCCGTG -3'		
FLCN	F primer	5'- TGCAGCTAAGCAGCCAACTG -3'	60	200
	R primer	5'- CATTCATGGTGCCTTGGAGACT -3'		200
	Probe	5'-(FAM) AACGTCAGGCCTGTTG (MGBNFQ)-3'		100
	Calibrator	5'- CGTGCAGCTAAGCAGCCAACTGCAGAAACGTCAGGCCTGTTGCAGTCTCC-AAGGCACCATGAATGCC -3'		
ORM1	F primer	5'- TTGCGCATTCCCAAGTCA -3'	60	300
	R primer	5'- CAGTGGCTCACACTTATCCTTTTTC -3'		300
	Probe	5'-(FAM) TGTCGTGTACACCGATT (MGBNFQ)-3'		100
	Calibrator	5'- GCTTGCGCATTCCCAAGTCAGATGTCGTGTACACCGATTGGAAAAAGGAT-AAGTGTGAGCCACTGGAG -3'		

Table 3.1: Primer, probe and calibrator sequences and reaction condition for quantitative real-time RT-PCR assays (continued)

Transcripts	Sequence		Annealing Temp (°C)	Final concentration for RT-PCR (nM)
MMP8	F primer	5'- AGGCAGATATCAACATTGCTTTTTAC -3'	60	600
	R primer	5'- GATTCCATTGGGTCCATCAAA -3'		600
	Probe	5'-(FAM) AAAGAGATCACGGTGACAAT (MGBNFQ)-3'		150
	Calibrator	5'- GAGAGGCAGATATCAACATTGCTTTTTACCAAAGAGATCACGGTGACAATT-CTCCATTTGATGGACCCAATGGAATCCTT -3'		
MPO	F primer	5'- CCACCAAAACCGATCACCAT -3'	56	300
	R primer	5'- CACTCCTCGCCTGCATCAT -3'		300
	Probe	5'-(FAM) CTTCCTGAACTGGGTACC (MGBNFQ)-3'		100
	Calibrator	5'- TCCCACCAAAACCGATCACCATCCCGGAGCTTCCTGAACTGGGTACCGAT-GATGCAGGCGAGGAGTGGG -3'		
MBP	F primer	5'- TTTTTAAGCTGGGAGGAAGAGATAGT -3'	60	300
	R primer	5'- CGGAACCAGGTGGGTTTTC -3'		300
	Probe	5'-(FAM) TCTGGATCACCCATGGC (MGBNFQ)-3'		100
	Calibrator	5'- AATTTTTAAGCTGGGAGGAAGAGATAGTCGCTCTGGATCACCCATGGCTA-GACGCTGAAAACCCACCTGGTTCCGGA -3'		

Table 3.1: Primer, probe and calibrator sequences and reaction condition for quantitative real-time RT-PCR assays (continued)

Transcripts	Sequence		Annealing Temp (°C)	Final concentration for RT-PCR (nM)
GPM6B	F primer	5'- CCAGTGACCATGCCTTGCT -3'	60	300
	R primer	5'- GGACGCAATTCCATAGATGACA -3'		300
	Probe	5'-(FAM) AGCGAGGTGATACAACT (MGBNFQ)-3'		100
	Calibrator	5'- CGCCAGTGACCATGCCTTGCTGAGCGAGGTGATACAACTGATGCAGTATG-TCATCTATGGAATTGCGTCCTT -3'		
GFAP	F primer	5'- CTGGAGGTTGAGAGGGACAAT -3'	60	300
	R primer	5'- CTTCCAGCCTCAGGTTGGTT -3'		300
	Probe	5'-(FAM) TGAGGCAGAAGCTCC (MGBNFQ)-3'		100
	Calibrator	5'- GGCTGGAGGTTGAGAGGGACAATCTGGCACAGGACCTGGCCACTGTGAG-GCAGAAGCTCCAGGATGAAACCAACCTGAGGCTGGAAGCC -3'		
PLP1	F primer	5'- CCTTCTGTCCATCTGCAAAACA -3'	60	300
	R primer	5'- GCAGCCCCCACAAATGC-3'		300
	Probe	5'-(FAM) TGAGTTCCAAATGACCTTC (MGBNFQ)-3'		100
	Calibrator	5'- AACCTTCTGTCCATCTGCAAAACAGCTGAGTTCCAAATGACCTTCCACCTG-TTTATTGCTGCATTTGTGGGGGCTGCAG -3'		

FAM, 6-carboxyfluorescein

MGNNFQ, minor groove binding non-fluorescent quencher

TAMRA, 6-carboxytetramethylrhodamine

Table 3.2 One-step QRT-PCR assay for quantification of *CSH1* mRNA.

(A) One-step QRT-PCR reaction mixture (EZ r*Tth* RNA PCR Reagent Kit; Applied Biosystems)

Components	**Volume per reaction (μL)**	**Final concentration**
5X TaqMan EZ Buffer	5.00	1X
$Mn(OAc)_2$ (25 mM)	3.00	3 mM
dATP (10 mM)	0.75	300 μM
dCTP (10 mM)	0.75	300 μM
dGTP (10 mM)	0.75	300 μM
dUTP (20 mM)	0.75	600 μM
Forward Primer (10 μM)	0.75	300 nM
Reverse Primer (10 μM)	0.75	300 nM
TaqMan Probe (5 μM)[a]	0.50	100 nM
AmpErase UNG (1 U/μL)[b]	0.25	0.01 U/μL
r*Tth* DNA Polymerase (2.5 U/μL)	1.00	0.1 U/μL
RNase-free water	7.75	-
RNA sample	3.00	-
Total	25.00	-

[a] Dual-labeled fluorescent probe containing a reporter at the 5′ end (FAM, 6-carboxyfluorescein) and a quencher at the 3′ end (TAMRA, 6-carboxytetramethylrhodamine).
[b] UNG, uracil-N-glycosylase

(B) Thermal profile of one-step QRT-PCR reaction

Step		**Temperature**	**Time**
UNG treatment		50 ℃	2 min
Reverse Transcription		60 ℃	30 min
UNG deactivation		95 ℃	5 min
45 Cycles	Denaturation	94 ℃	20 sec
	Annealing / Extension	56 ℃	1 min

3.3.3 Two-step QRT-PCR assays for microRNA quantification

3.3.3.1 Principles

MicroRNA in RNA samples was measured by a two-step QRT-PCR assay using components provided by the TaqMan® MicroRNA Reverse Transcription Kit and the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA, USA). This two-step QRT-PCR system involves two separate procedures (Figure 3.1).

Reverse Transcription (RT)

In this step, a stem-loop primer hyrbridises to the 3' end of the mature miRNA. It can distinguish between other highly similar mature miRNA sequences (Figure 3.1). The primer can also distinguish between mature miRNAs with single base difference (Applied Biosystems; Product bulletin on TaqMan MicroRNA Assays: Quantitate microRNAs with the specificity and sensitivity of TaqMan assay chemistry; accessed 3 October 2006). Upon RT, cDNA of the microRNA is generated as an extension from the 3' end of the stem-loop primer, resulting in a cDNA-DNA hybrid for subsequent PCR amplification.

The Multiscribe™ reverse transcriptase used in the RT step is capable of generating cDNA from both microRNA and single-strand DNA. Hence, standard curve for absolute quantification was constructed by serial dilutions of single-stranded synthetic DNA oligonucleotides specifying the studied miRNAs .

PCR Amplification

The quantity of cDNA-DNA hybrid generated is directly proportional to the initial quantity of miRNA present in the RNA sample. Hence, the assay quantifies the

miRNA using primers and probe amplifying the cDNA-DNA hybrid from the RT step (Figure 3.1). The forward primer hybridises specifically to the cDNA portion of the microRNA in the hybrid; the reverse primer hybridises specifically to the unfolded stem-loop sequence of the stem-loop primer in the hybrid; the probe hybridises at the junction within the hybrid. Such design further enhances the specificity of the assay to miRNA detection. It is also capable of generating a dynamic range of detection of up to seven logs.

3.3.3.2 Advantages

The two-step QRT-PCR system for microRNA quantification involves the use of a stem-loop primer and a set of specific primers and probes which has several important features for the detection of microRNA.

Detection of the very short microRNA

QRT-PCR or QPCR requires a template of at least 60 nucleotides for primers and probe hybridisation and efficient PCR amplification. However, mature miRNAs are very short, of about 21-25 bases long. They, alone, cannot provide enough target sequence length for primers and probe hybridisation. The use of a hairpin primer for the generation of a cDNA-DNA hybrid provides a target template long enough for the subsequent PCR amplification, which is otherwise impossible.

Increased specificity

The use of hairpin primer for the QRT-PCR has increased the specificity of the assay in several aspects. First, the hairpin primer recognises and hybridises to RNA with the exact 3' end sequence. Even a single base extension at the 3' end

will result in a delayed threshold cycle in the detection. This allows the primer to distinguish the mature miRNA target from its miRNA precursors or any genomic DNA contaminants in the RNA samples. Second, the primers and probes used in the PCR amplifications are very specific to the cDNA-DNA hybrid. This has greatly enhanced the specificity of the assay to the target microRNA and allows the assay to differentiate between highly similar microRNA sequences with even a single nucleotide difference.

3.3.3.3 TaqMan® MicroRNA Assay

TaqMan® MicroRNA Assays (Applied Biosystems) were used for quantification of mature miR-16 sequences. The assays were designed by and purchased from Applied Biosystems for different microRNA targets. For the TaqMan® probes of the assays, the dual-labeled fluorescent probe contained 6-carboxyfluorescein (FAM) as the reporter dye at the 5' end and minor-groove binding (MGB) non-fluorogenic quencher (NFQ) at the 3' end.

The first step, reverse transcription (RT), was set in a reaction volume of 15 µL using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Table 3.3 A and C summarises the preparation of the RT reaction mixture and the thermal profile, respectively. To each 15 µL of the reaction mixture, 5 µL of extracted plasma RNA was added as template. In each analysis, a calibration curve was included for absolute quantification of the target RNA. The standard curve was constructed using serial dilution of synthetic DNA oligonucleotides (Proligo) on the target microRNA with concentrations ranging between 2.5 x 10^6 and 2.5 copies per RT reaction.

The second step, PCR amplification, was set up in a reaction volume of 20 µL using TaqMan 2X Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA). Table 3.3 B and D summarise the preparation of the PCR amplification reaction mixture and the thermal profile, respectively. To each 20 µL of reaction mixture, 3 µL of RT product from all the reactions was added as template. At the same time, the analysis was run with another four no template control reactions to monitor the level of contamination in the reagents used in the PCR reaction.

The absolute concentration of the target sequences in each sample was calculated using one of the following formulas:

$$C_{plasma} = Q \times (V_{RNA} / V_{ext})$$

$$C_{buffy\ coat/placental\ tissue} = Q / C_{input\ RNA}$$

where C_{plasma} is the target sequence concentration in plasma (copies/mL of plasma); Q is target sequence quantity determined by sequence detector (copies/µL of RNA sample); V_{RNA} is the total volume of RNA solution after the RNA extraction and DNase I treatment; V_{ext} is the volume of plasma used for extraction; $C_{buffy\ coat/placental\ tissue}$ is the target sequence concentration in buffy coat or placental tissue (copies/ng of total RNA); $C_{input\ RNA}$ is the concentration of the input total RNA determined by the sequence detector (copies/ng of RNA sample).

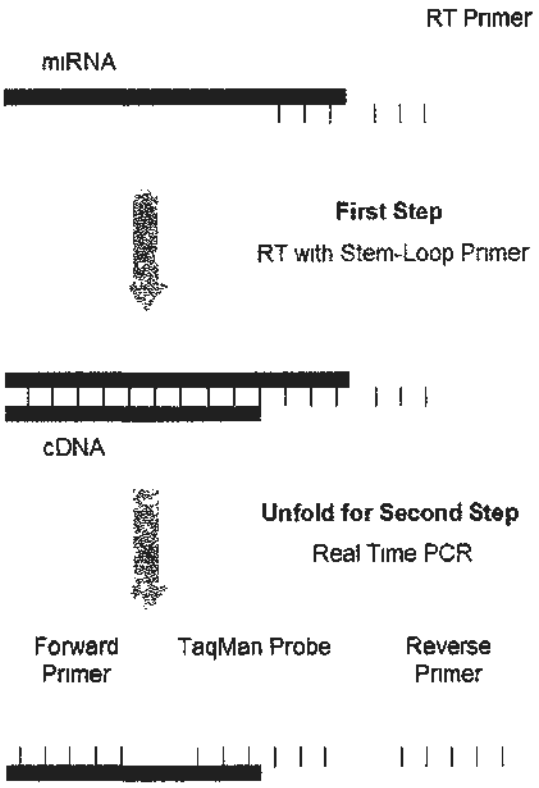


Figure 3.1 Two-step QRT-PCR assays for miRNA quantification. In the RT step, a stem-loop primer hyrbridises specifically to the 3' end of the mature miRNA Upon reverse transcription, cDNA of the miRNA is generated as an extension from the 3' end of the stem-loop primer, resulting in a cDNA-DNA hybrid for subsequent PCR amplification The forward primer hybridises specifically to the cDNA of the miRNA in the hybrid, the reverse primer hybridises specifically to the unfolded stem-loop sequence of the stem-loop primer in the hybrid, the probe hybridizes at the junction within the hybrid QPCR was then performed for real-time detection (Modified from TaqMan® MicroRNA Assays instruction manual, Applied Biosystems, 2005)

Table 3.3 Two-step QRT-PCR assay for microRNA quantification.

(A) Composition of reverse transcription (RT) reaction mixture

Component	Volume for one reaction (μL)	Final concentration
10X RT Buffer[a]	1.5	1X
dNTPs (with dTTP; 100 mM)[a]	0.15	13.33 mM
5X Stem-loop RT Primer[b]	3	1X
RNase Inhibitor (20 U/μL)[a]	0.19	2.67 U/μL
MultiScribe™ Reverse Transcriptase (50 U/μL)[a]	1.0	6.67 U/μL
Nuclease-free water	4.16	-
RNA sample	5	-
Total	7.5	-

[a] Components provided by TaqMan® MicroRNA Reverse Transcription Kit, Applied Biosystems
[b] Components provided by TaqMan® MicroRNA Assay, Applied Biosystems

(B) Composition of PCR reaction mixture

Component	Volume for one reaction (μL)	Final concentration
TaqMan 2X Universal PCR Master Mix (No AmpErase UNG)[b,c]	10	1X
TaqMan MicroRNA Assay Mix (10X)[d]	1	1X
Nuclease-free water	6	-
RT Product	3	-
Total	20	-

[a] Dual-labeled fluorescent probe containing a reporter at the 5′ end (FAM, 6-carboxyfluorescein) and a quencher at the 3′ end (MGB NFQ, minor-groove binding non-fluorogenic quencher).
[b] UNG = uracil-N-glycosylase
[c] Applied Biosystems
[d] Components provided by TaqMan® MicroRNA Assay, Applied Biosystems

(C) Thermal profile of reverse transcription (RT)

Step	Temperature	Time
Initial incubation	4 ℃	5 min
Annealing of stem-loop RT primers	16 ℃	30 min
Reverse transcription	42 ℃	30 min
Deactivation of MultiScribe™ Reverse Transcriptase	85 ℃	5 min
Final incubation	4 ℃	∞

(D) Thermal profile of PCR amplification

Step		Temperature	Time
Activation of AmpliTaq Gold®		95 ℃	10 min
40 Cycles	Denaturation	95 ℃	15 secg
	Annealing / Extension	60 ℃	1 min

3.3.4 One-step QPCR assays for *leptin* DNA quantification

3.3.4.1 Principle

DNA in samples was measured by QPCR using components provided by the TaqMan® PCR Core Reagent Kit (Applied Biosystems, Foster City, CA). The principle behind the quantification is similar to that of QRT-PCR, except, in QPCR, the AmpliTaq Gold® DNA polymerase is used and there is no reverse transcription step is involved. Moreover, standard curve for absolute quantification was constructed by serial dilutions of human male genomic DNA extracted from blood cells.

3.3.4.2 Quantification of the *leptin* gene

QPCR assays were used for the quantification of the *leptin (LEP)* gene. The sequence information for the primers (Integrated DNA Technology) and probes (Applied Biosystems) is given in Table 3.4. For the TaqMan® probes, the dual-labeled fluorescent probe contained 6-carboxyfluorescein (FAM) as the reporter dye at the 5' end and the minor-groove binding (MGB) non-fluorogenic quencher (NFQ) at the 3' end.

QPCR was set up in a reaction volume of 50 µL using the TaqMan® PCR Core Reagent Kit (Applied Biosystems). Table 3.4 summarises the preparation of the QPCR reaction mix and the thermal profile of the amplification. To each 50 µL of reaction mixture, 5 µL of extracted urine/plasma DNA was added as a template. In each analysis, a calibration curve was included for absolute quantification of the target DNA. The standard curve was constructed using serial dilutions of male blood cells genomic DNA with concentrations ranging between 0.78 and 10,000

genome equivalents per five microlitres. At the same time, the analysis was run with at least four no template control reactions to monitor the level of contamination. The reaction was initiated with 2 min incubation at 50°C for contamination control with UNG. After deactivation of UNG at 95°C for 10 min, the reaction was cycled for 50 times with denaturation at 95°C for 30 sec and 1 min of annealing/extension at 60°C.

The absolute concentration of the DNA in each sample was calculated using the following formula:

$$C_{plasma/urine} = Q \times (V_{DNA} / V_{ext})$$

where $C_{plasma/urine}$ is the DNA concentration in plasma/urine (copies/mL of plasma/urine); Q is DNA quantity determined by the sequence detector (copies/µL of DNA sample); V_{DNA} is the total volume of DNA solution after the DNA extraction; V_{ext} is the volume of plasma used for extraction.

Table 3.4 QPCR assay for DNA quantification of *LEP* and *SRY* DNA.

(A) Composition of QPCR reaction mixture (TaqMan® PCR Core Reagent Kit, Applied Biosystems)

Component	Volume for one reaction (µL)	Final concentration
10X TaqMan Buffer A	5.0	1X
$MgCl_2$ (25 mM)	8.0	4 mM
dATP (10 mM)	1.0	200 µM
dCTP (10 mM)	1.0	200 µM
dGTP (10 mM)	1.0	200 µM
dUTP (20 mM)	1.0	400 µM
Forward Primer (10 µM)	5.0	1 µM
Forward Primer (10 µM)	5.0	1 µM
Probe (10 µM)[a]	2.5	500 nM
AmpliTaq Gold® (5 U/µL)	2.0	0.025 U/ µL
AmpErase UNG (1 U/µL)[b]	0.5	0.01 U/ µL
Dimethyl sulfoxide (DMSO)	2.5[c]	50 nL/ µL
Deionised water	10.5	-
DNA sample	5.0	-
Total	50.0	-

[a] Dual-labeled fluorescent probe containing a reporter at the 5′ end (FAM, 6-carboxyfluorescein) and a quencher at the 3′ end (MGB NFQ, minor-groove binding non-fluorogenic quencher).
[b] UNG = uracil-N-glycosylase
[c] DMSO is added in all *LEP* assays; DMSO is replaced with deionised water in all *SRY* assays

(B) Thermal profile

Step		Temperature	Time
UNG treatment		50 ℃	2 min
Deactivation of UNG and Activation of AmpliTaq Gold®		95 ℃	10 min
50 Cycles	Denaturation	95 ℃	30 sec
	Annealing / Extension	58 ℃	1 min
Final incubation		72 ℃	1 min

3.4 Qualitative analysis of nucleic acids

3.4.1 Principle of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

The MassARRAY® system (Sequenom, San Diego, CA, USA) is an accurate and high-throughput technology for DNA/RNA genotyping. It is capable of resolving single nucleotide polymorphisms (SNPs) of DNA/RNA samples quantitatively, by utilising the MALDI-TOF MS to differentiate mass difference of products generated from the homogeneous MassEXTEND™ (hME) assay (Sequenom, San Diego, CA, USA).

The hME assay consists of an initial PCR and a primer extension reaction. In the initial PCR, a single pair of primers flanking the region with the SNPs is used. Upon amplification, the PCR products of different genotypes of the SNP are produced in proportion to the genotypic ratio in the original nucleic acid sample. In the primer extension reaction, an oligonucleotide primer (extension primer) anneals to a position right beside the SNP of interest. Thermal cycling was then performed using a reaction mixture with a DNA polymerase along with a mixture of terminator nucleotides, a combination of different deoxyribonucleotides (dNTP) and dideoxynucleotides (ddNTP), which allows extension of the hME primer through the polymorphic site and generates allele-specific extension products with

different unique molecular masses. MALDI-TOF MS is then used to analyse the resultant masses of the extension products and to report genotype of the nucleic acid samples (Tang *et al.*, 1995).

3.4.2 DNA genotyping analysis for informative cases screening

DNA was extracted from placental tissues and maternal buffy coat for determination of the fetal and maternal genomic genotypes of a single nucleotide polymorphism (SNP) on placental genes *CSHL1*, *PLAC4* and *KISS1*. Cases where the fetal and maternal genotypes differed were considered as informative.

Determination of the genomic genotype involved steps of PCR amplification, base extension and analysis of the extension products by a mass spectrometer. For each 25 μL PCR reaction, 25 ng of DNA from maternal buffy coat or placental tissue was amplified. Each reaction contained 1x HotStar *Taq* PCR buffer with 1.5 M $MgCl_2$ (Qiagen), an additional 1 mM MgCl2 (Qiagen), 50 M each of dATP, dGTP, and dCTP, 100 M dUTP (Applied Biosystems), 200 nM each of the forward and reverse primers (Integrated DNA Technologies; Table 3.5), and 0.1 U of HotStar *Taq* Polymerase (Qiagen). The PCR reaction was initiated at 95°C for 15 min, followed by 94°C for 20 s, 56°C for 30 s, and 72°C for 1 min for 45 cycles, and a final

incubation at 72°C for 3 min.

Homogenous MassEXTEND (hME) assay (Sequenom) was used to determine the genotype of the SNP. The hME assay was based on the annealing of an extension primer adjacent to the polymorphic site. The primer was then extended through the polymorphic site for 1 or 2 bases, depending on the SNP allele. The molecular masses of the allele-specific extension products were then measured by the MassARRAY Compact (Sequenom).

To perform the hME analysis, PCR products were subjected to shrimp alkaline phosphatase (SAP) treatment to dephosphorylate any remaining dNTPs and prevent their incorporation in the subsequent primer extension assay. 0.34 μL of MassARRAY hME buffer (Sequenom) and 3.06 μL of water were added into the PCR product. The mixture was incubated at 37°C for 40 min followed by 85°C for 5 min. SNP genotyping were performed with hME assays (Sequenom). Briefly, 4 μL of base extension reaction cocktail containing 771 nM extension primer (Integrated DNA Technologies; Table 3.6), 1.15 U of Thermosequenase (Sequenom), and 64 μM of dideoxynucleotide/deoxynucleotide mix (Sequenom) were added to 10 μL of the PCR products. The dideoxynucleotide/deoxynucleotide mix for *PLAC4* and

KISS1 assays was ddATP, ddCTP, ddTTP and dGTP while that for *CSHL1* assay was ddATP, ddCTP, ddGTP and dTTP. The reaction conditions were 94 °C for 2 min, followed by 94 °C for 5 sec, 52 °C for 5 sec, and 72 °C for 5 sec for 75 cycles. The primer extension assays were designed such that the allele-specific extension products for each SNP demonstrated distinct masses that were readily resolvable by MALDI-TOF MS analysis (Table 3.6). The final base extension product was cleaned up by addition of 12 mg of the Clean Resin (Sequenom) and 24 μL of water. The mixtures were mixed in a rotator for 20 min. After centrifugation at 361 *g* for 5 min, 10 nL of reaction solution was dispensed onto a 384-format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by a MassARRAY Nanodispenser S (Sequenom). A MassARRAY Analyzer Compact Mass Spectrometer (Sequenom) was used for data acquisition from the SpectroCHIP. Mass spectrometric data were automatically imported into a MassARRAY Typer (Sequenom) database for analysis (Tsui *et al.*, 2005).

Table 3.5 Primer sequences for reverse transcription and PCR amplification of the *PLAC4*, *CSHL1* and *KISS1* SNP

Gene	Primer	Sequence
PLAC4	Gene-specific primer for reverse transcription	5' -GTATATAGAACCATGTTTAGGCCAG-3'
(rs8130833)	Forward PCR primer	5' -**ACGTTGGATG**GTATTGCAACACCATTTGGG-3'
	Reverse PCR primer	5' -**ACGTTGGATG**TAGAACCATGTTTAGGCCAG-3'
CSHL1	Gene-specific primer for reverse transcription	5' -GCACTGGGGAGGGGTCA-3'
(rs2246207)	Forward PCR primer	5' -**ACGTT**AGAAATCCAACTTAGAGCTGCTCCA-3'
	Reverse PCR primer	5' -**ACGTTGGATG**GAGGGTCTGCCCAGTCAGGT-3'
KISS11	Gene-specific primer for reverse transcription	5'-GCCTACCTTGCCTCAGTCCTGGCCT-3'
(rs3924587)	Forward PCR primer	5'-**ACGTTGGATG**ACCCTCTGGACATTCACCCA-3'
	Reverse PCR primer	5'-**ACGTTGGATGT**GTGCCTACCTTGCCTCAGT-3'

Bold fonts indicate the tag. The tags on the 5' ends of each PCR primers increase the masses of unused PCR primers so it would fall outside the analytical mass range in the MALDI-TOF analysis.

Table 3.6 Sequences and molecular masses of the extension primer and the expected extension products for each of the alleles of the *PLAC4*, *CSHL1* and *KISS1* SNPs

Gene	Expected peak	Sequence (5'-3')	Molecular weight (Da)
PLAC4	Unextended primer	AGGCCAGATATATTCGTC	5498.6
(rs8130833)	Extension product for allele A	AGGCCAGATATATTCGTC**A**	5795.8
	Extension product for allele G	AGGCCAGATATATTCGTC**GT**	6116.0
CSHL1	Unextended primer	TATGACACCTCGGACAG	5179.4
(rs2246207)	Extension product for allele C	TATGACACCTCGGACAG**C**	5452.6
	Extension product for allele T	TATGACACCTCGGACAG**TG**	5796.8
KISS1	Unextended primer	5'-CCCAGCCAGGTGGTCTC-3'	5147.3
(rs3924587)	Extension product for allele A	5'-CCCAGCCAGGTGGTCTC**A**-3'	5444.6
	Extension product for allele G	5'-CCCAGCCAGGTGGTCTC**GT**-3'	5764.7

Bold fonts indicate the extended dNTPs and ddNTPs.

3.4.3 RNA-SNP genotyping analysis

RNA extracted from placental tissue, maternal whole blood and maternal plasma was subjected to RNA-SNP genotyping analysis to investigate the origin of *CSHL1*, *PLAC4* and *KISS1* mRNA transcripts. Determination of the RNA-SNP involved the steps of reverse transcription of RNA, PCR amplification, base extension and analysis of the extension products by a mass spectrometer. Sequences of the oligonucleotides used in these reactions are shown in Table 3.5 and 3.6.

For *PLAC4* and *KISS1* SNP assay, placental RNA (625 ng), or 48 μL plasma RNA was reversed transcribed in a reaction volume of 20 μL or 100 μL, respectively, using the ThermoScript reverse transcriptase (Invitrogen), according to the manufacturer's recommendation. Each reaction contained 50 nM gene-specific primer (Integrated DNA Technologies), 1 mM each of dATP, dTTP, dCTP and dGTP mix (Promega), 1x cDNA synthesis buffer, 5 mM DTT, 0.1 U/μL RNaseOUT and 0.0375 U/μL Thermoscript reverse transcriptase. After preincubating the mixture of RNA, primer and dNTP at 65°C for 10 min, the reaction was carried out at 55°C for 60 min followed by 85°C at 5 min. The resultant cDNA samples were then PCR amplified.

All placental cDNA or plasma cDNA was added to a total PCR volume of 40 μL or 200 μL, respectively. Each reaction contained 0.6X HotStar *Taq* PCR buffer with 0.9 mM $MgCl_2$ included (Qiagen), 25 μM each of dATP, dGTP and dCTP, 50 μM of dUTP (Applied Biosystems), 200 nM each of forward and reverse primers (Integrated DNA Technologies) and 0.02 U/μL of HotStar *Taq* Polymerase (Qiagen). The PCR was initiated at 95°C for 7 min, followed by denaturation at 95°C for 40 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min for 55 cycles and a final incubation at 72°C for 3 min.

Procedures for *CSHL1* SNP assay were the same as *PLAC4* except that 454 ng placental RNA, 2.5 ng whole blood RNA and 48 μL plasma RNA was reverse transcribed at 60°C for 60 min followed by 85°C for 5 min. Placental cDNA of 2.5 μL, plasma or whole blood cDNA of 100 μL was added to a total PCR volume of 25 μL for placenta or 100 μL for plasma or whole blood. The PCR reaction mix was the same as that for *PLAC4* except 1X HotStar *Taq* PCR buffer was used for placental cDNA. The PCR was initiated at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 65°C for 30 sed and extension at 72°C for 1 min with final incubation at 72°C for 3 min. 75 cycles were performed in the primer extension reaction for *CSHL1*.

No product was observed when placental RNA samples were subjected to PCR without reverse transcription. To dephosphorylate any remaining dNTPs and prevent their incorporation in the subsequent primer extension assay, PCR products were subjected to shrimp alkaline phosphatase (SAP) treatment as described in Chapter 3.4.2.

For primer extension, I added 5 μL treated PCR product to 9 μL base extension reaction cocktail containing 342.6 nM extension primer (Integrated DNA Technologies), 0.51 U Thermosequenase (Sequenom) and 28.4 μM each of ddATP, ddCTP, ddTTP and dGTP (Sequenom). The thermal profile for the primer extension reaction was 94°C for 2 min, followed by 100 cycles of 94°C for 5 sec, 52°C for 5 sec and 72°C for 5 sec. The extension product was cleaned up and dispensed onto SpectroCHIP (Sequenom) as described in Chapter 4.3.2. A MassARRAY Analyzer Compact Mass Spectrometer (Bruker) was used for data acquisition from the SpectroCHIP. Mass spectrometric data were automatically imported into a MassARRAY Typer (Sequenom) database for analysis (Lo *et al.*, 2007b).

3.5 Analysis of gene expression profiles

3.5.1 Principles of Affymetrix microarray

The Affymetrix GeneChip® (Affymetrix, Santa Clare, CA) system uses millions of oligonucleotide probes, each designed to hybridise to a particular part of a transcript. They are synthesised *in situ* on the array using photolithography, a printing process that results in every microarray having up to 1.3 million individual probe-cells, each 18 μm across. The Affymetrix GeneChip® Human Genome U133 Set (HG-U133A and HGU133B) is made up of two microarrays comprised of over 1,000,000 unique oligonucleotide features covering over 39,000 transcript variants which in turn represent 33,000 human genes. Sequences used in the design of the array were selected from GenBank®, dbEST, and RefSeq. Sequence clusters were created from Build 133 of UniGene (April 20, 2001).

The chips are designed so that every transcript is represented by 11 probes that match different parts of the 3' end of the mRNA sequence. Every probe consists of a pair of 25-residue oligonucleotides, one a perfect match to the transcript, the other a mismatch in which the middle residue has been changed. This probe-pairing strategy helps minimise the effects of non-specific hybridisation and background signal.

3.5.2 RNA sample and array processing

The total RNA is firstly converted to cDNA, labelled, fragmented and presented to the microarray. Labelled RNA hybridises to its complementary probe sequence on the chip. Since there are millions of oligo nucleotides for each probe-sequence, the amount of labelled RNA that binds corresponds to the amount in solution. After hybridisation, each array was washed and stained in a GeneChipH Fluidics Station 400 (Affymetrix). The chips were scanned with the GeneArray Scanner (Affymetrix) and analysed using the GeneChipH Microarray Suite 5.0 (Affymetrix).

3.5.2.1 RNA preparation and labeling

Globin reduction was performed to reduce the α- and β-globin in whole blood RNA samples from pregnant and non-pregnant females before reverse transcription. The 10X Oligo Hyb Buffer was prepared with 100 mM of Tris-HCl, pH 7.6 and 200 mM KCl. 10X RNase H buffer was mixed 100 mM of Tris-HCl, pH 7.6 with 10 mM DTT and 20 mM $MgCl_2$. 10X Globin Reduction Mix consisted of 7.5 μM of α1 oligo, 7.5 μM of α2 oligo and 20 μM of β oligo. Sequences of the globin reduction oligo nucleotides are summarised in Table 3.7.

Firstly, 7 μg of whole blood total RNA was mixed with 2 μL of 10X Globin Reduction Mix, 1 μL of 10X Oligo Hyb Buffer to a final volume of 10 μL and incubated in a thermal cycler at 70°C for 5 min. and then cooled to 4°C. 10 μL of RNase H Reaction Mix which was prepared with 2 μL of 10X RNase H Buffer (Ambion), 1 μL of SUPERase-In (Ambion), 2 μL of RNase H (1U/μL, Ambion) and 5 μL of Nuclease-free water was added into the RNA-Globin Reduction Oligo hybridisation sample and incubated at 37 °C for 10 min. and then cooled to 4°C followed by the addition of 1 μL of 0.5 M EDTA.

The RNase H-treated RNA samples were purified with the IVT cRNA Cleanup Spin Column from GeneChip Sample Cleanup Module. 80 μL of RNase-free water was added to the RNase H-treated RNA sample and and mixed by vortexing for 3 seconds followed by the addition of 350 μL IVT cRNA Binding Buffer (proprietary to the manufacturer) to the sample and vortexing for 3 sec. 250 μL of absolute ethanol was added to the lysate and mixed by pipetting. The sample was applied to the IVT cRNA Cleanup Spin Column and centrifuged for 15 sec at 8,000 *g* (Centrifuge 5415D; Eppendorf). 500 μL of IVT cRNA Wash Buffer (proprietary to the manufacturer) was transferred onto the spin column and centrifuged for 15 sec at 8,000 *g* to wash. 500 μL of 80% (v/v) ethanol was added

onto the spin column and centrifuged for 15 sec at 8,000 *g* followed by centrifugation of the open-cap spin column 5 min at maximum speed. 14 µL of RNase-free water was then added directly onto the spin column membrane and centrifuged 1 min at maximum speed to elute.

When performing the One-Cycle Eukaryotic Target Labeling Assay, total RNA was first reverse transcribed using a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA was purified and serves as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets were then cleaned up, fragmented, and hybridised to GeneChip expression arrays.

Total RNA, 2 µL of 50 µM T7-Oligo(dT) primer (Affymetrix) and RNase-free water were added into a PCR tube to a final volume of 12 µL and incubated at 4°C for at least 2 min. For first strand synthesis, 4 µL of 5X 1st Strand Reaction Mix (Invitrogen), 2 µL of 0.1 M DTT (Invitrogen) and 1 µL of 10 mM dNTP was

added into each RNA/T7-Oligo(dT) Primer mix and immediately placed at 42°C for 2 min. 1 μL of SuperScript II (Invitrogen) was added to each RNA samples for a final volume of 20μL. The mixture was incubated at 42°C for 1 hour and then cooled at 4°C for at least 2 min.

Second-strand master mix was prepared by mixing 30 μL of 5X 2nd strand reaction mix (Invitrogen), 3 μL of 10 mM dNTP (Invitrogen), 1 μL of 10 U/μL E. coli DNA ligase (Invitrogen), 4 μL of 10 U/μL E. coli DNA Polymerase I (Invitrogen), 1 μL of 2 U/μL RNase H (Invitrogen) and 91 μL of RNase-free water. 130 μL of second-strand master mix was added to each first-strand synthesis sample and incubated at 16°C for 2 hours. 2 μL of 5 U/μL T4 DNA Polymerase (Invitrogen) was added to each sample and incubated for 5 min at 16°C. After incubation, 10 μL of 0.5 M EDTA was then added to the sample followed by the cleanup of double-stranded cDNA with Sample Cleanup Module (Affymetrix) according to the manufacturer's instruction.

600 μL of cDNA Binding Buffer (proprietary to the manufacturer) was added to the double-stranded cDNA synthesis preparation, mixed by vortexing for 3 seconds. The yellow mixture formed was applied to the cDNA Cleanup Spin

Column and centrifuged for 1 min at 8,000 *g* (Centrifuge 5415D; Eppendorf). 750 µL of the cDNA Wash Buffer (proprietary to the manufacturer) was added onto the spin column and centrifuged for 1 min at 8,000 *g*. The flow-through was discarded. The cap of the spin column was opened to dry the membrane completely. The column was centrifuged for 5 min at maximum speed. The spin column was transferred into a 1.5 mL collection tube. 14 µL of cDNA Elution Buffer (proprietary to the manufacturer) was applied directly onto the spin column membrane and incubated for 1 min at room temperature followed by centrifuging the column for 1 min at maximum speed (≤ 25,000 *g*) to elute.

Biotin-labelled cRNA was synthesised with GeneChip In-vitro Transcription (IVT) Labeling Kit (Affymetrix). 4 µL of 10X IVT Labeling Buffer (proprietary to the manufacturer), 12 µL of IVT Labeling NTP Mix, 4 µL of IVT Labeling Enzyme Mix (proprietary to the manufacturer) and 8 µL of RNase-free water was added into 12 µL of resultant cDNA. The mixture was incubated at 37°C for 16 hours.

The biotin-labeled cRNA was cleaned up with Sample Cleanup Module (Affymetrix). 60 µL of RNase-free water was added into the IVT reaction, mixed by vortexing for 3 s following the procedures described in section 3.5.2.1 for

purification of RNase-H treated RNA samples. Finally, 11 μL of RNase-free water was then added directly onto the spin column membrane and centrifuged 1 min at maximum speed to elute. The elution step was repeated with the addition of another 10 μL of RNase-free water.

The cRNA sample was quantified using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The cRNA yield was calculated as the formula below:

$$\text{adjusted cRNA yield} = \text{RNAm} - (\text{total RNAi})\ (y)$$

where RNAm is the amount of cRNA measured after IVT (μg); total RNAi is the starting amount of total RNA (μg); y is the fraction of cDNA reaction used in IVT.

The cRNA was fragmented before hybridisation onto GeneChip probe arrays (Affymetrix). The Affymetrix Fragmentation Buffer has been optimised to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis. 12 μL of 5X Fragmentation Buffer (proprietary to the manufacturer) and RNase-free water was added to 30 μg of cRNA to a final volume of 60 μL. The mixture was incubated at 94°C for 35 min and then put on ice. Fragmented sample cRNA was stored frozen at -80°C until hybridisation.

Table 3.7 Sequences of globin reduction oligonucleotides

Globin Reduction Oligos	Sequences
α1	5'- TGCAGGAAGGGGAGGAGGGGCTG-3'
α2	5'- TGCAAGGAGGGGAGGAGGGCCCG-3'
β	5'- CCCCAGTTTAGTAGTTGGACTTAGGG-3'

3.5.2.2 Target hybridisation

Prior to hybridisation, the 12X MES stock buffer was prepared by mixing 64.61 g of MES hydrate (Sigma) and 193.3 g of MES Sodium Salt (Sigma) in molecular biology grade water to a final volume of 1 L. The buffer was adjusted to pH 6.5 – 6.7 and filtered through a 0.2 μm filter. The 2X hybridisation buffer was prepared by mixing 83 mL of 12X MES Stock Buffer, 177 mL of 5 M NaCl, 40 mL of 0.5 M EDTA, 1 mL of 10% Tween-20 and 199 mL of molecular biology grade water (Invitrogen). The buffer was stored at 4°C and shielded from light.

The GeneChip probe array (Affymetrix) was equilibrated to room temperature immediately before use, filled with 1X hybridisation buffer and incubated at 45°C for 10 minutes with rotation. The hybridisation cocktail was prepared by mixing 5 μL of 3 nM control oligonucleotide B2 (Affymetrix), 15 μL of 20X eukaryotic hybridisation control (Afftmetrix), 3 μL of 10 mg/mL herring sperm DNA (Invitrogen), 3 μL of 50 mg/mL acetylated BSA (Invitrogen), 150 μL of 2X

hybridisation buffer and 94 µL of molecular biology grade water (Invitrogen). The cocktail was heated to 99°C for 5 min and then 45°C for 5 min followed by spinning at maximum speed in a microcentrifuge (Centrifuge 5415R, Eppendorf, Germany) for 5 min to remove any insoluble material from the hybridisation mixture. The buffer solution was removed from the probe array cartridge which was then filled with 250 µL of the clarified hybridisation cocktail and incubated in the hybridisation oven at 45°C for 16 hours with rotation of 60 rpm.

3.5.2.3 Array washing, staining and scanning

After hybridisation, the probe array was washed and stained according to the Affymetrix fluidics protocol EukGE-WE2v4 in the GeneChipH Fluidics Station 400 (Affymetrix). Preparation of buffers and staining reagent is summarised in Table 3.8. The post-hybridisation wash involved 10 cycles of wash buffer A at 25°C and then 4 cycles of wash buffer B at 50°C. The probe array was then stained for 10 min in SAPE solution at 25°C followed by 10 cycles of post-stain wash with wash buffer A at 25°C. The array was further stained for 10 min in antibody solution and then 10 min in SAPE solution at 25°C. After staining, the array was washed with 15 cycles of wash buffer A at 30°C

The chips were scanned with the GeneArray Scanner (Affymetrix) and analysed using the GeneChipH Microarray Suite 5.0 (Affymetrix). The data were further analysed with GeneSpring v 7.2 (Agilent Technologies, Palo Alto, CA).

Table 3.8 Preparation of staining and antibody solution

(A) Preparation of the SAPE solution mix

Components	Volume (μL)	Final Concentration
2X Stain Buffer	600	1X
50 mg/mL BSA	48	2 mg/mL
1 mg/mL Streptavidin Phycoerythrin (SAPE)	12	10 μg/mL
DI H_2O	540	
Total volume	1200	

(B) Preparation of the antibody solution mix

Components	Volume (μL)	Final Concentration
2X Stain Buffer	300.0	1X
50 mg/mL BSA	24.0	2 mg/mL
10 mg/mL Goat IgG Stock	6.0	0.1 mg/mL
0.5 mg/mL biotinylated antibody	3.6	3 μg/mL
DI H_2O	266.4	
Total volume	600.0	

3.6 Statistical analysis

Statistical analyses were performed with SigmaStat 3.0 software (Systat).

SECTION III: FETAL mRNA IN MATERNAL CIRCULATION

The study presents in this section compared the detection of placenta-derived mRNA in maternal plasma and whole blood to elucidate the appropriate medium for fetal RNA detection in non-invasive prenatal diagnosis.

CHAPTER 4: DETECTION OF PLACENTA-DERIVED mRNA IN MATERNAL PLASMA AND WHOLE BLOOD

4.1 Introduction

The analysis of fetal nucleic acids in maternal plasma holds much promise for non-invasive prenatal diagnosis (Wright and Burton, 2009, Chiu *et al.*, 2008, Lun *et al.*, 2008b). Many investigations conducted to date were based on the detection of Y chromosomal sequences in maternal plasma (Lo *et al.*, 1997), thus, limiting the utility of such applications to pregnancies involving male fetuses only. Circulating fetal mRNA analysis, on the other hand, offers a means for non-invasive prenatal assessment that is applicable to pregnancies regardless of the fetal gender (Farina *et al.*, 2004, Ng *et al.*, 2003b, Tsui *et al.*, 2004).

Through the detection of the mRNA of placental expressed hormones, namely *CSH1* and *CGB*, the placenta was shown to be a source for fetal mRNA release into maternal plasma (Ng *et al.*, 2003b). This observation facilitated the development of a microarray-based strategy to systematically identify placental expressed mRNA markers that were detectable in maternal plasma (Tsui *et al.*, 2004). Furthermore, aberrant concentrations of placental expressed mRNA species have been shown to be associated with pregnancy complications, such as the

elevation of *CRH* mRNA in maternal plasma of preeclamptic pregnancies (Farina *et al.*, 2004, Ng *et al.*, 2003a). In 2007, Lo *et al.* demonstrated that fetal chromosomal aneuploidy, such as trisomy 21, can be detected non-invasively from maternal plasma analysis by RNA-single nucleotide polymorphism (RNA-SNP) allelic ratio determination (Lo *et al.*, 2007b). These studies suggest that circulating placental mRNA detection offers much opportunity for the development of non-invasive prenatal diagnostic or assessment applications.

Besides maternal plasma, maternal whole blood has been reported to be another medium for fetal mRNA detection (Concu *et al.*, 2005, Farina *et al.*, 2005, Maron *et al.*, 2007, Okazaki *et al.*, 2006). *CSH1*, *CGB* and *PLAC4* mRNA concentrations were reported to be much higher in maternal whole blood than plasma (Okazaki *et al.*, 2006). Indeed, the total RNA content of whole blood is higher than that of plasma. Hence, this study was to investigate the detection of fetal mRNA in maternal whole blood and determine if it offered advantages over maternal plasma analysis.

4.2 Materials and Methods

4.2.1 Study design

Using QRT-PCR, the concentrations of four previously studied placental expressed transcripts, *CSH1*, *KISS1*, *PLAC1* (Ng *et al.*, 2003b, Tsui *et al.*, 2004) and *PLAC4* (Lo *et al.*, 2007a) were compared in maternal plasma and whole blood. Our previous microarray study (Tsui *et al.*, 2004) showed that these transcripts had much higher expression in placental tissues than blood cells. The relative placental-specificity of these transcripts are also supported by data in the public database, SymAtlas v1.2.4, Genomics Institute of the Novartis Research Foundation (Figures 4.1 – 4.3) (Su *et al.*, 2004a). Figures 4.1 – 4.3 show that those transcripts are predominantly expressed in placental tissues as compared with the many other human tissues assessed by gene expression microarrays. The pregnancy-specificity of *CSH1*, *KISS1*, *PLAC1* and *PLAC4* was then determined by assessing their disappearance in post-delivery blood samples and detectability in blood samples of non-pregnant females and males. The fetal specificity of *PLAC4* and *CSHL1* mRNA was further confirmed by studying their genotypes in maternal plasma and whole blood using the RNA-SNP approach by matrix-assisted laser-desorption and ionisation time-of-flight mass spectrometry. *CSHL1* is one of the potentially pregnancy-specific placental expressed transcripts identified in our previous microarray study (Tsui *et al.*, 2004). Lastly, to explore if

more pregnancy-related circulating mRNA markers could be developed for maternal whole blood analysis, candidates were mined after performing gene expression microarray comparison of whole blood samples from pregnant and non-pregnant individuals.

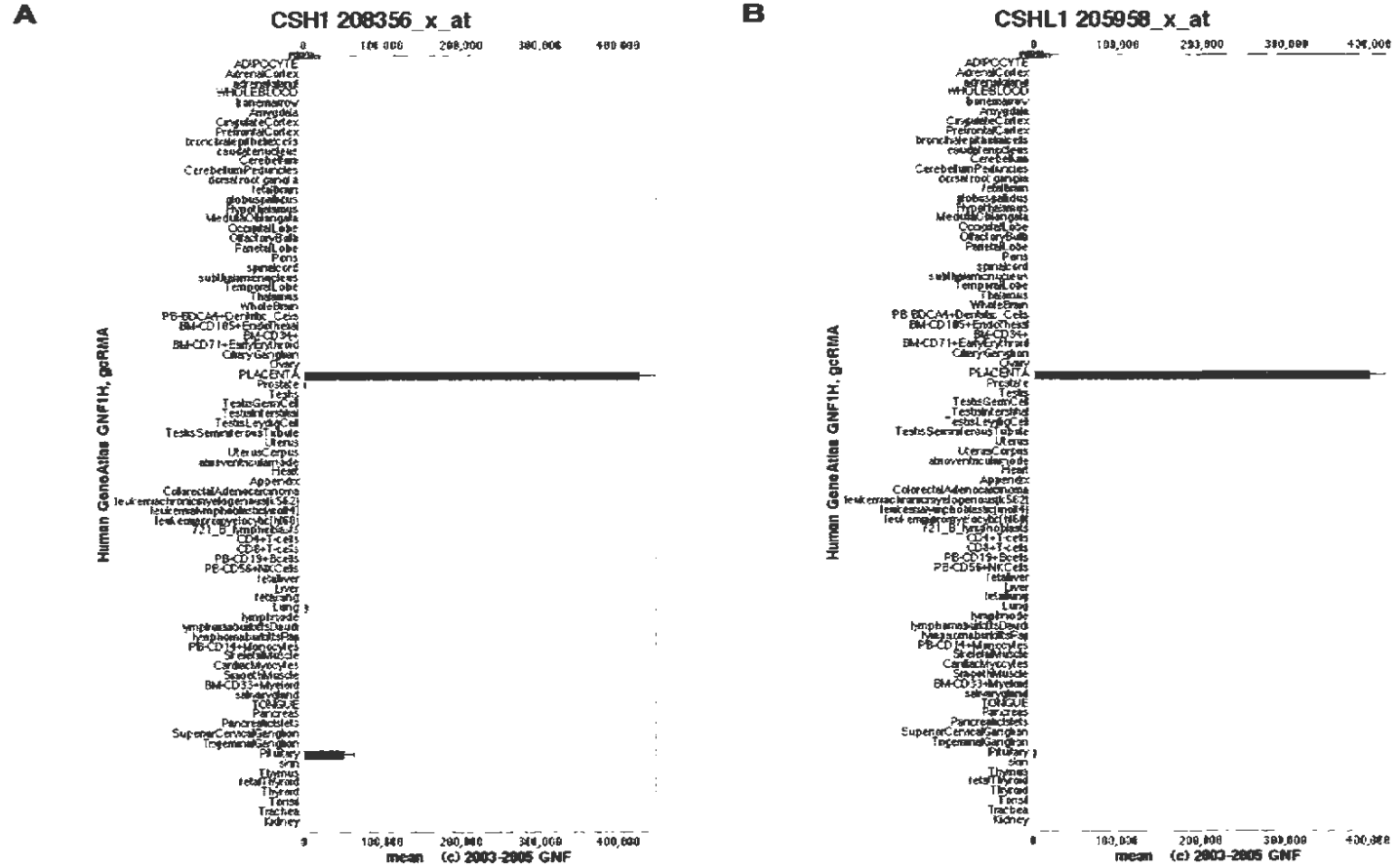


Figure 4.1 Bar-charts adopted from Human GeneAtlas GNF1H showing expression of (A) *CSH1* (208356_x_at) and (B) *CSHL1* (205958_x_at) in different human tissues (http://symatlas.gnf.org/SymAtlas).

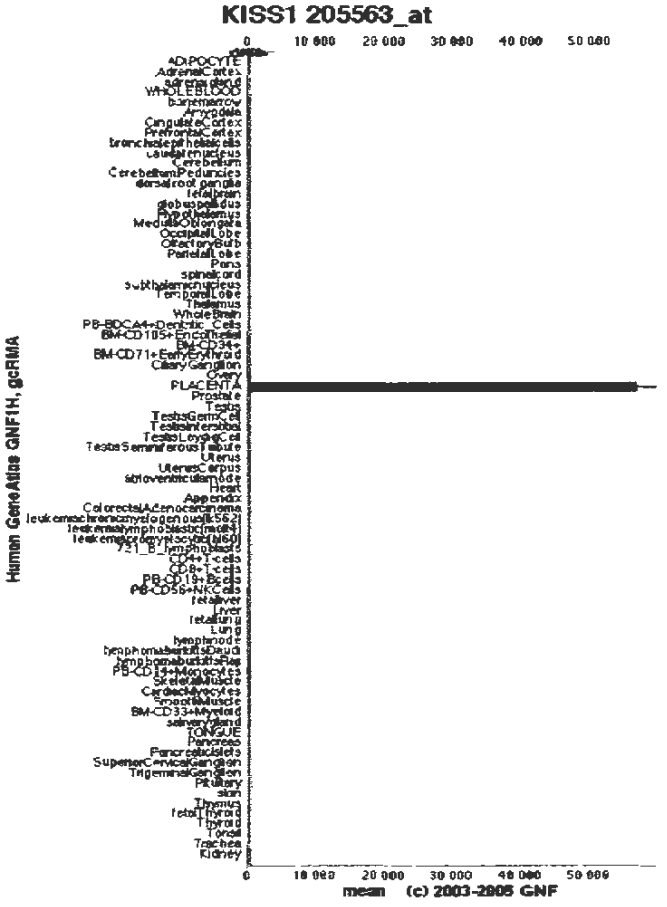


Figure 4 2 Bar-charts adopted from Human GeneAtlas GNF1H showing expression of *KISS1* (205563_at) in different human tissues (http //symatlas gnf org SymAtlas)

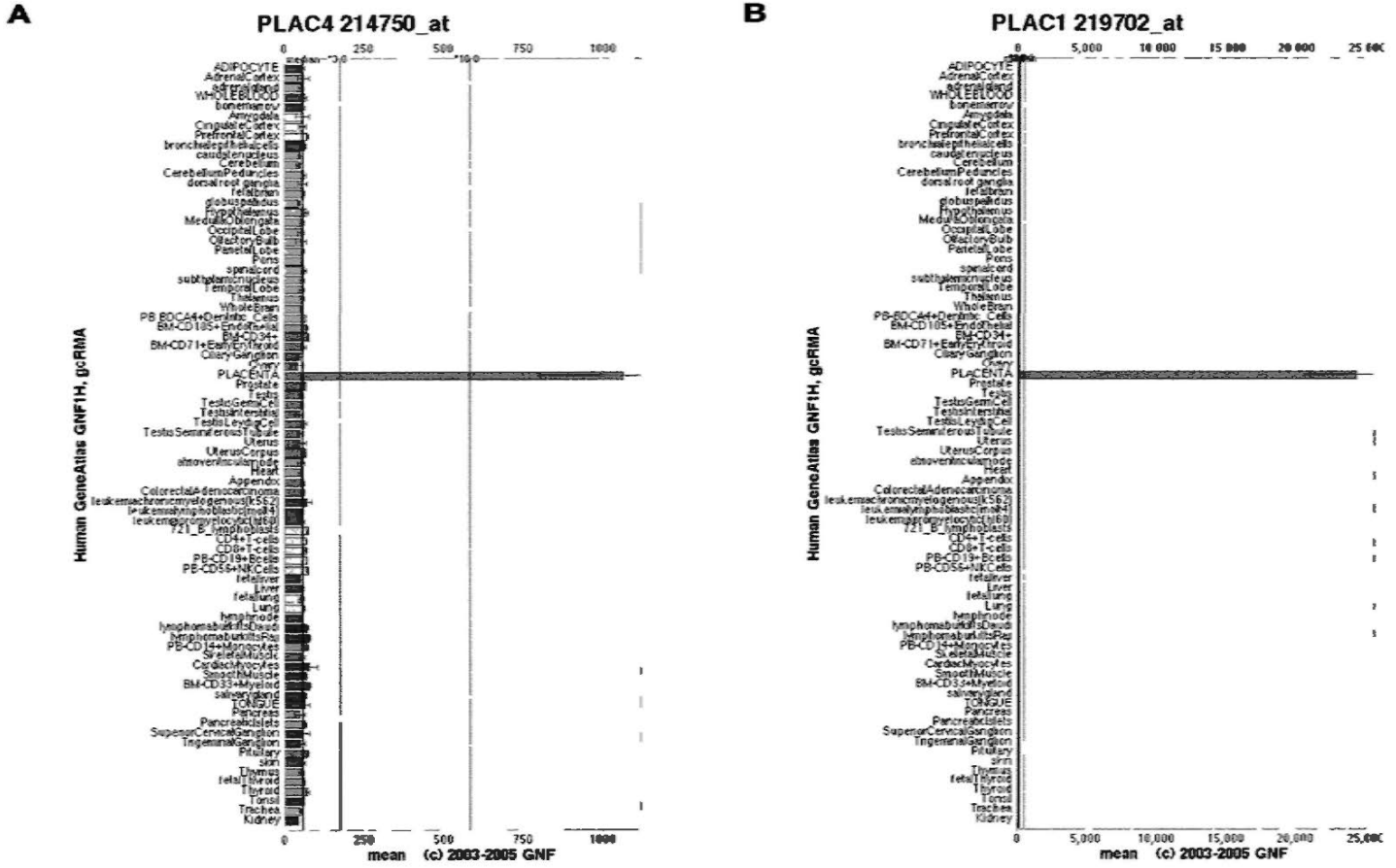


Figure 4.3 Bar-charts adopted from Human GeneAtlas GNF1H showing expression of (A) *PLAC4* (214750_at) and (B) *PLAC1* (219702_at) in different human tissues (http //symatlas gnf org/SymAtlas).

4.2.2 Subjects and sample collection

Ethics approval from the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee was obtained. Third trimester healthy women with singleton uncomplicated pregnancies (gestational age range, 38 – 39 weeks) were recruited with written informed consent from the Prince of Wales Hospital, Hong Kong. 12 mL of peripheral blood was collected into EDTA tubes before and at 24 hours after delivery. Placental tissues were collected immediately after delivery. Age-matched healthy non-pregnant individuals were recruited from the community.

4.2.3 Sample processing

Blood samples were processed within 6 hours according to blood processing protocol I described in Chapter 3.1.2. Whole blood, plasma and buffy coat samples were stored as details mentioned in Chapter 3.1. Placental tissue samples were handled as illustrated in Chapter 3.1.5.

4.2.4 RNA and DNA extraction

RNA and DNA of the samples were isolated according to the protocols described in Chapter 3.2.

4.2.5 QRT-PCR

All mRNA transcripts were quantified using one-step QRT-PCR. Calibration curves were prepared by serial dilutions of HPLC-purified single-stranded synthetic DNA oligonucleotides (Proligos, Singapore) with the amplicon specified. The sequence information of the primers, probes and calibrators as well as the reaction conditions are summarised in Table 3.1. QRT-PCR was set up as previously described in Chapter 3.3.2.2 (Tsui *et al.*, 2004, Lo *et al.*, 2007a).

4.2.6 RNA-SNP genotyping

Genotyping of *PLAC4* SNP, rs8130833, and *CSHL1* SNP, rs2246207, was performed involving steps of reverse transcription of RNA, PCR amplification, base extension and mass spectrometric analysis of the extension products (Lo *et al.*, 2007a). The experiment was set up as described in Chapter 3.4. DNA was extracted from placental tissues and maternal buffy coat for determination of the fetal and maternal genomic genotypes. The circulating *PLAC4* and *CSHL1* mRNA genotypes were then determined in maternal plasma and whole blood samples and further compared with the fetal and maternal genomic genotypes to confirm if they indeed originated from the fetus.

4.2.7 Gene expression microarray analysis

From each of five third trimester pregnant women and five non-pregnant healthy females, 5 mL of peripheral blood was collected into PAXgene^{TM} blood RNA tubes (PreAnalytiX) with further processing and RNA extraction according to manufacturer's instructions described in Chapter 3.2.2.1. DNase treatment was performed with RNase-Free DNase Set (Qiagen). 7 μg of extracted RNA from each sample was treated following the globin-reduction protocol recommended by Affymetrix (Affymetrix, 2003) described in Chapter 3.5.2.1.

The globin-reduced RNA was labelled and hybridised to the GeneChipH Human Genome U133A and U133B Arrays (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. After hybridisation, each array chip was washed, stained and scanned as reported (Tsui *et al.*, 2004). The details of the procedures were included in Chapter 3.5. The data were analysed with GeneSpring v 7.2 (Agilent Technologies, Palo Alto, CA).

The microarray data in the .CEL format was imported and normalised using the following steps in sequence: (1) raw data processing by Robust Multi-chip Average, with GC-content background correction (GC-RMA); (2) data transformation

whereby microarray data with values below 0.001 were set to 0.001; and (3) the signal intensity for each gene was divided by the median of its measurements in all samples. After filtering the gene-list with 70% confidence interval and non-parametric t-test analysis (P = 0.05), 2,246 gene transcripts demonstrated differential expression between pregnant and non-pregnant blood samples. The gene list was further filtered by selecting transcripts with 1.5-fold higher expression in the pregnant compared with non-pregnant blood samples, and resulted in 247 gene transcripts. These genes were sorted in descending order of the fold difference.

4.2.8 Statistics

Statistical analyses were performed using Sigma Stat (Systat).

4.3 Results

4.3.1 Placental mRNA concentrations in third trimester maternal plasma and whole blood

Whole blood and plasma samples from ten third trimester pregnancies were analysed. The median (range) concentrations for *CSH1, KISS1, PLAC4* and *PLAC1* mRNA in whole blood were 90,673 (9,324 – 387,010), 14,712 (1,000 – 71,544), 344,330 (88,940 – 552,686) and 12,498 (3,250 – 54,328) copies/mL,

respectively. The median (range) concentrations for *CSH1, KISS1, PLAC4* and *PLAC1* mRNA in plasma were 11,338 (1,771 – 27,754), 535 (undetectable – 2,283), 4,317 (2,779 – 10,737) and 498 (undetectable – 1,276) copies/mL, respectively. The median concentrations of *CSH1, KISS1, PLAC4* and *PLAC1* mRNA were 8-fold ($P = 0.001$, Mann-Whitney test), 27-fold ($P = 0.017$, Mann-Whitney test), 80-fold ($P < 0.001$, Mann-Whitney test) and 25-fold ($P < 0.001$, Mann-Whitney test) higher, respectively, in whole blood than in plasma. Using the Spearman correlation test, no statistically significant correlation was observed between the whole blood and plasma mRNA signals for *CSH1* ($R^2 = 0.045$, $P = 0.583$), *KISS1* ($R^2 = 0.003$, $P = 0.365$), *PLAC4* ($R^2 = 0.013$, $P = 0.631$), and *PLAC1* ($R^2 = 0.041$, $P = 0.631$).

4.3.2 Post-delivery clearance of placenta-derived mRNA

Whole blood and plasma samples were also collected 24 hours after delivery from the ten cases discussed above. The four transcripts were no longer detectable in all postpartum plasma samples. The plasma concentrations detected before and after delivery were statistically significant for all transcripts ($P < 0.001$, Wilcoxon test). For the post-delivery whole blood samples, *CSH1* and *KISS1* mRNA became undetectable in 8 and 9 cases, respectively. However, *PLAC4* mRNA remained

detectable in all cases at a median concentration of 162,203 copies/mL. *PLAC1* mRNA was detected in 7 post-delivery whole blood samples at a median of 10,081 copies/mL (Figure 4.4). Using the Wilcoxon test, the whole blood concentrations detected before and after delivery were shown to be significantly different for *CSH1* and *KISS1* ($P < 0.001$) but not for *PLAC4* ($P = 0.160$) and *PLAC1* ($P = 0.105$).

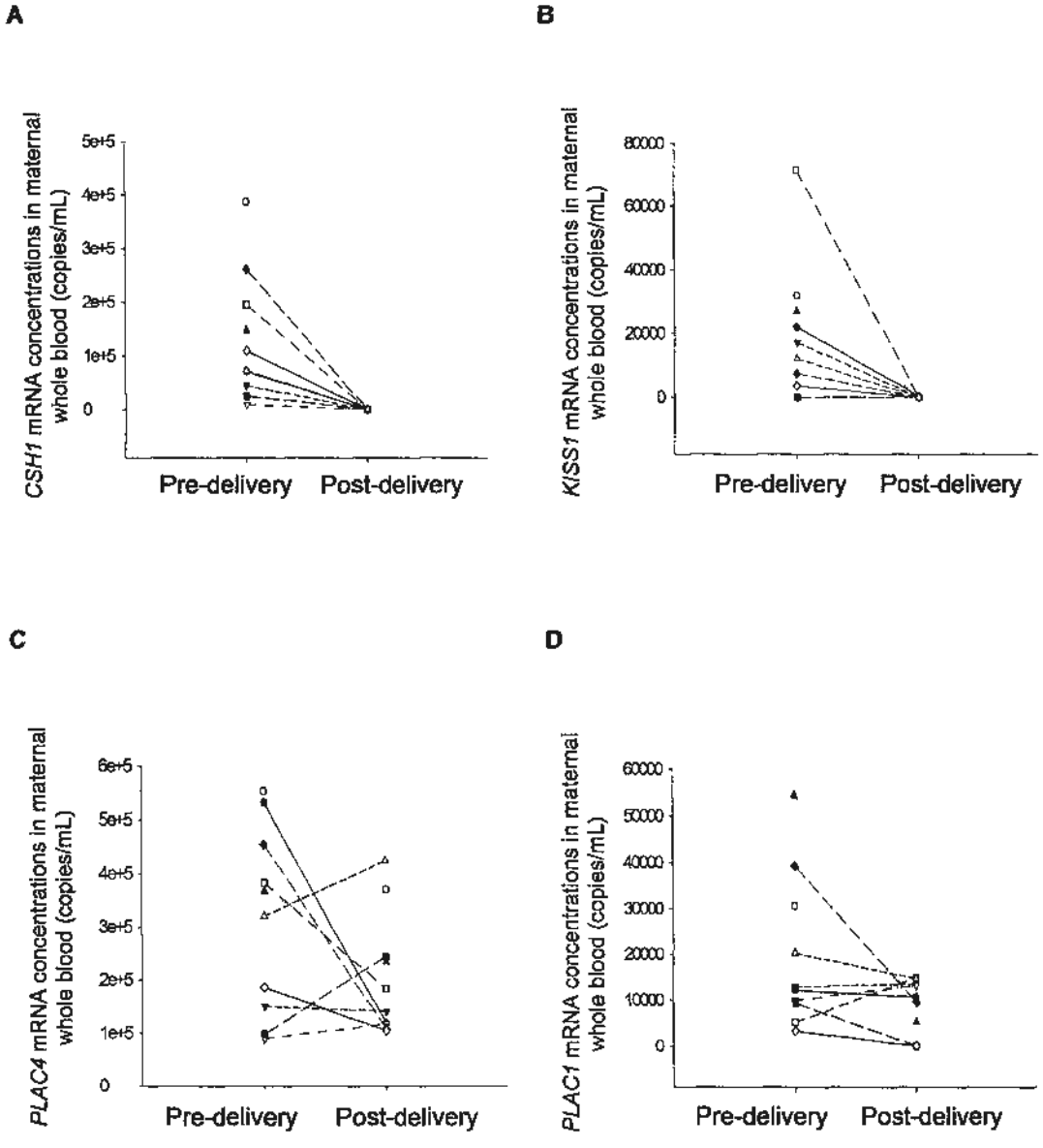


Figure 4.4 **Concentrations of placenta-derived mRNA in pre- and 24-hour-post-delivery maternal whole blood.** Scatter plots of (A) *CSH1* (B) *KISS1* (C) *PLAC4* (D) *PLAC1* mRNA. Corresponding samples from each individual are connected by a line.

4.3.3 Detection of placenta-derived mRNA in non-pregnant individuals

CSH1, *KISS1*, *PLAC1* and *PLAC4* mRNA were not detectable in the plasma of 10 non-pregnant females and 10 males. Whole blood samples from these 20 individuals showed absence of *CSH1* mRNA. *KISS1* mRNA was detected in whole blood of 2 each of the non-pregnant females and males at 96 and 1,315 copies/mL in the former group and 264 and 300 copies/mL in the males. *PLAC4* mRNA was detected in all 20 whole blood samples with median concentrations of 244,498 copies/mL and 592,372 copies/mL, respectively, in non-pregnant females and males. *PLAC1* mRNA could be detected in whole blood of 9 non-pregnant females and 8 males at median concentrations of 12,024 copies/mL and 7,959 copies/mL, respectively. These concentrations were not significantly different from that of the third trimester whole blood samples for *PLAC4* ($P = 0.065$, Kruskal-Wallis one way analysis of variance (Kruskal-Wallis ANOVA) test) and *PLAC1* ($P = 0.368$, Kruskal-Wallis ANOVA test) (Figure 4.5).

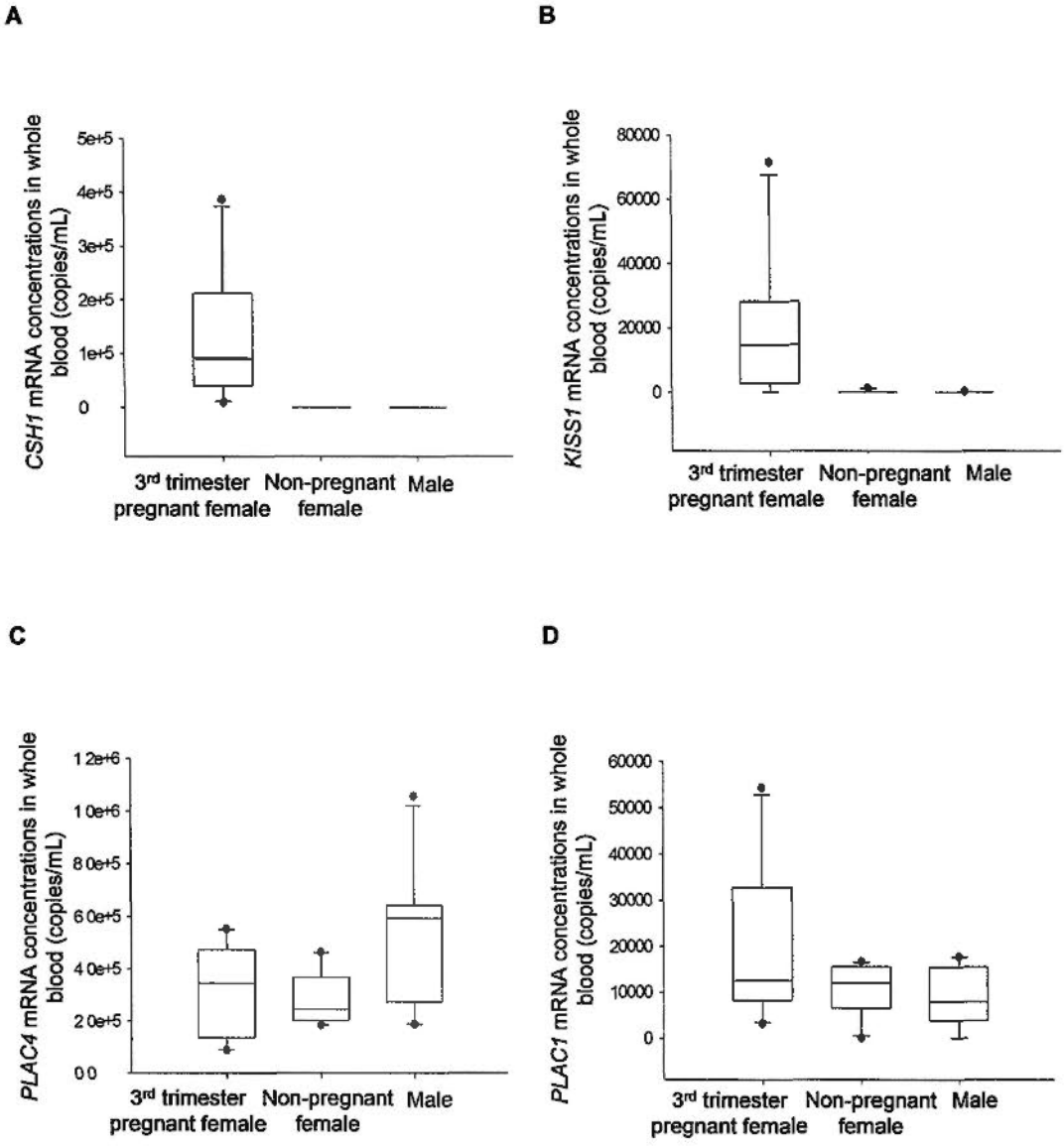


Figure 4.5 **Concentrations of placenta-derived mRNA in third trimester pregnant and non-pregnant female and male whole blood.** Box plots of (A) *CSH1* (B) *KISS1* (C) *PLAC4* (D) *PLAC1* mRNA. The line inside each box denotes the median. The lower and upper limits denote the 25th and 75th percentiles, respectively. The lower and upper whiskers denote the 10th and 90th percentiles, respectively. Filled circles denote the outliers.

4.3.4 Genotyping of *PLAC4* and *CSHL1*

Third trimester placental tissues, maternal whole blood, buffy coat and plasma samples were collected. The genotypes of the placental mRNA transcripts, *PLAC4* and *CSHL1*, in maternal plasma and whole blood were compared with the genomic genotypes of the fetal-maternal pairs to confirm if the circulating transcripts were truly of fetal origin. *PLAC4* has a SNP, rs8130833, within the coding region (Lo *et al.*, 2007b). *CSHL1* was targeted because it demonstrated high placental tissue expression at similar levels like *CSH1* which was highly homologous with other genes of the growth hormone cluster and that a target-specific RNA-SNP assay could not be developed (Tsui *et al.*, 2004).

The fetal and maternal genotypes for each pregnancy were first determined using placental tissue and maternal buffy coat DNA. Though nucleated fetal cells may be present at a concentration of 1 to 6 cells per milliliter of maternal blood in the samples (Bianchi, 1999, Krabchi *et al.*, 2001), such a level of "contaminant" would unlikely affect the predominant maternal genotype detected in maternal blood cells. Cases where the fetal and maternal genotypes differed were considered as informative and included 7 out of 28 cases for *PLAC4* and 11 out of 50 cases for *CSHL1*. The *PLAC4* and *CSHL1* mRNA genotypes were then

determined in maternal plasma and whole blood RNA samples of the informative pregnancies. Representative mass spectra for *PLAC4* and *CSHL1* RNA-SNP genotyping are shown in Figures 4.6 and 4.7. The genotyping data of all informative cases are summarised in Table 4.1. For *PLAC4* mRNA, the genotype in maternal plasma corresponded to that of the fetus while the genotype in whole blood was always heterozygous indicating both fetal and maternal contributions. For *CSHL1* mRNA, the genotypes in both the plasma and whole blood samples were identical to the fetus.

Table 4.1 *PLAC4* and *CSHL1* RNA-SNP genotyping in third trimester maternal plasma and whole blood.

		DNA Genotype		RNA-SNP Genotype	
	Sample No.	**Fetus**	**Mother**	**Plasma**	**Whole Blood**
PLAC4	668	AG	AA	AG	AG
	696	AG	AA	AG	AG
	770	AG	AA	AG	AG
	774	AG	GG	AG	AG
	917	AG	AA	AG	AG
	1160	AA	AG	AA	AG
	1359	AA	AG	AA	AG
CSHL1	1172	CC	CT	CC	CC
	2432	CC	CT	CC	CC
	137	CT	CC	CT	CT
	140	CT	CC	CT	CT
	846	CT	CC	CT	CT
	847	CT	TT	CT	CT
	929	CT	CC	CT	CT
	2437	CT	TT	CT	CT
	2451	CT	TT	CT	CT
	2468	CT	TT	CT	CT
	2417	TT	CT	TT	TT

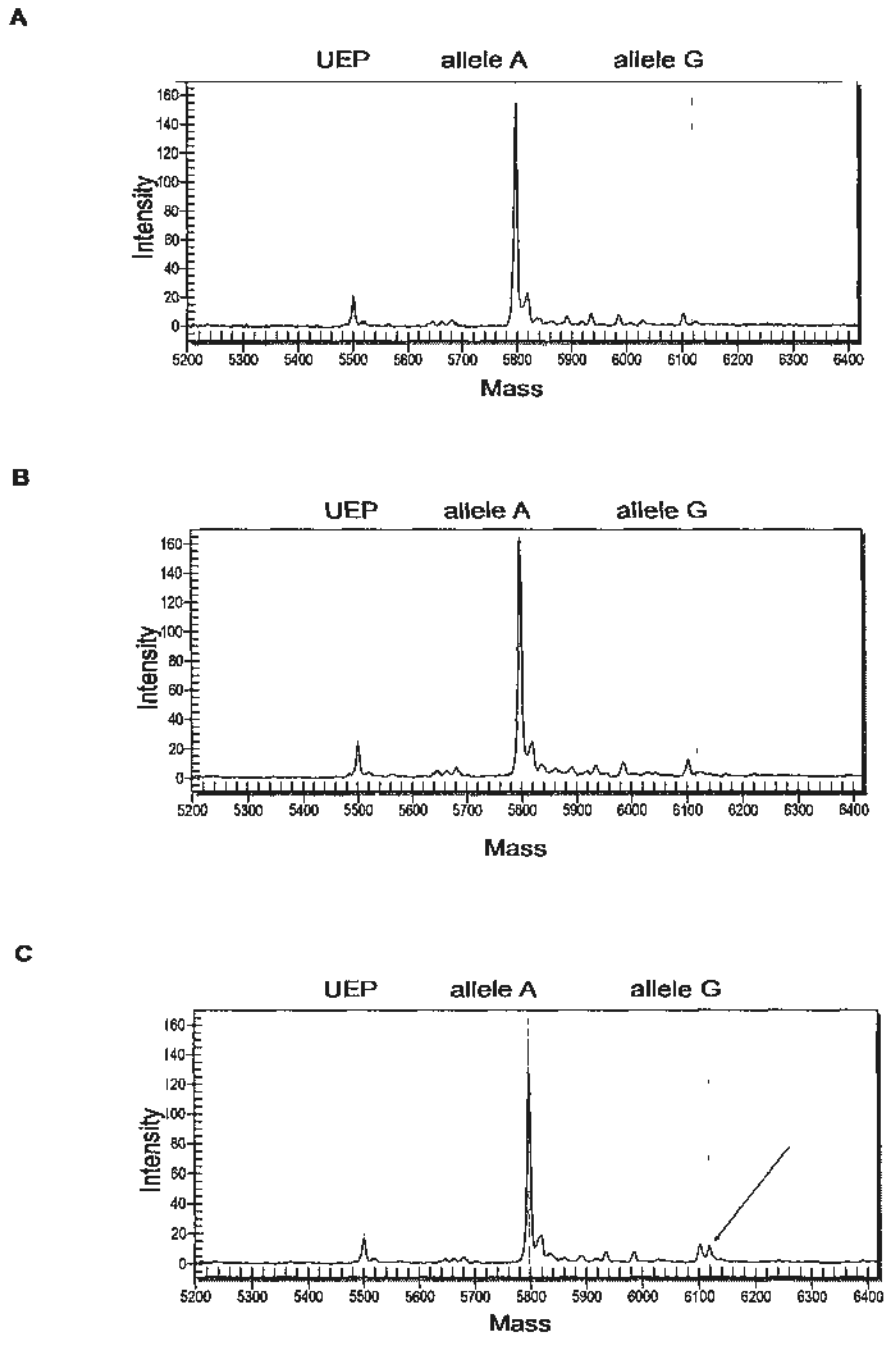


Figure 4.6 **Mass spectra showing *PLAC4* RNA-SNP genotypes of placenta, third trimester maternal plasma and whole blood samples.** (A) a homozygous placental RNA sample showing a single peak A with unextended primers (UEP). (B) third trimester maternal plasma RNA showing a single peak A as the placental RNA sample. (C) third trimester maternal whole blood RNA showing a peak A and a minor peak G as indicated by the arrow.

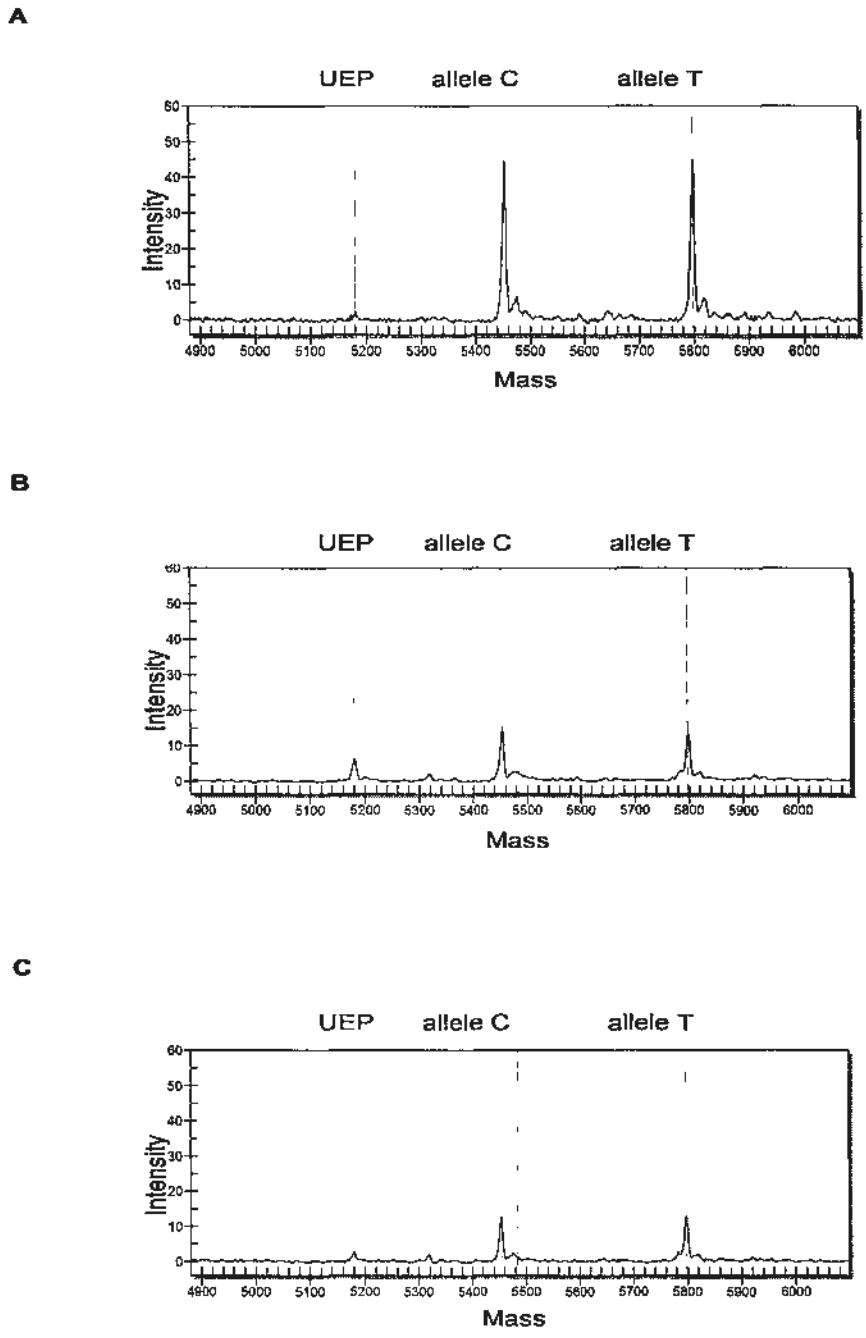


Figure 4.7 **Mass spectra showing *CSHL1* RNA-SNP genotypes of placenta, third trimester maternal plasma and whole blood samples.** (A) a heterozygous placental RNA sample showing 2 peaks, allele C and allele T with unextended primers (UEP). (B) third trimester maternal plasma RNA also showing 2 peaks as the placental RNA sample. (C) third trimester maternal whole blood RNA showing both peaks of allele C and allele T.

4.3.5 Gene expression profile comparison between pregnant and non-pregnant female blood samples

The gene expression profiles of five third trimester and five non-pregnant female whole blood samples were compared using GeneSpring® v 7.2, (Agilent Technologies) software. The final gene list was generated by selecting transcripts with 1.5-fold higher expression in the pregnant than non-pregnant blood samples and resulted in 247 gene transcripts. These genes were sorted in descending order of the fold difference (Table 4.2). Twenty of the top 25 gene transcripts were found to be related to blood cell functions and more specifically the functions of neutrophils (13/25) (Table 4.2). However, placental expressed transcripts previously identified, namely *CSH1*, *CSHL1*, *KISS1*, *PLAC4* and *PLAC1* were not present on the list. *DEFA4, CEACAM8, OLFM4, FLCN, ORM1, MMP8* and *MPO* which represented transcripts with large, medium and small differences in expression between pregnant and non-pregnant blood were further analysed (Table 4.2).

Table 4.2 List of genes more highly expressed in whole blood of third trimester pregnant females than non-pregnant females

[a]Transcripts	[a]Gene Name	Probe Set ID	Genbank Accession No.	[b]Fold Change	Expression in Pregnant Blood	Expression in Non-pregnant Blood
Defensin, alpha 4, corticostatin	***DEFA4***	***207269_at***	***NM_001925***	***26.5***	***212.1***	***8.0***
Carcinoembryonic antigen-related cell adhesion molecule 8	***CEACAM8***	***206676_at***	***M33326***	***22.9***	***373.9***	***16.3***
Transcribed sequences		231688_at	AW337833	21.4	206.2	9.6
Lactotransferrin	LTF	202018_s_at	NM_002343	17.2	541.1	31.5
Lipocalin 2	LCN2	212531_at	NM_005564	17.1	344.1	20.1
Olfactomedin 4	***OLFM4***	***212768_s_at***	***AL390736***	***15.6***	***141.3***	***9.1***
Defensin, alpha 1, myeloid-related sequence	DEFA1	205033_s_at	NM_004084	14.0	12060.0	864.5
Cathelicidin antimicrobial peptide	CAMP	210244_at	U19970	13.7	531.9	38.8
Cysteine-rich secretory protein 3	CRISP3	207802_at	NM_006061	12.2	80.7	6.6
Folliculin	***FLCN***	***235250_at***	***AA992036***	***6.2***	***43.9***	***7.0***
Chitinase 3-like 1	CHI3L1	209396_s_at	M80927	5.5	126.1	23.1
Orosomucoid 1	***ORM1***	***205041_s_at***	***NM_000607***	***4.6***	***16.1***	***3.5***
Polycythemia rubra vera 1	PRV1	219669_at	NM_020406	4.5	115.8	25.7
Annexin A3	ANXA3	209369_at	M63310	3.8	275.8	73.0
POU domain, class 6, transcription factor 1	POU6F1	216332_at	L14482	3.7	39.2	10.5
Transcobalamin I	TCN1	205513_at	NM_001062	3.7	87.9	23.8

(to be continued)

(continued)

[a]Transcripts	[a]Gene Name	Probe Set ID	Genbank Accession No.	[b]Fold Change	Expression in Pregnant Blood	Expression in Non-pregnant Blood
Carcinoembryonic antigen-related cell adhesion molecule 6	CEACAM6	211657_at	M18728	3 6	112 3	31 2
Orosomucoid 2	ORM2	205040_at	NM_000607	3 6	67 9	19 1
Thyroglobulin	TG	214977_at	AK023852	3 3	18 8	5 7
T-cell receptor interacting molecule	TRIM	217147_s_at	AJ240085	3 1	183 1	58 5
Matrix metalloproteinase 8	***MMP8***	***207329_at***	***NM_002424***	***3.1***	***23.9***	***7.7***
S100 calcium binding protein A12	S100A12	205863_at	NM_005621	3 0	2963 0	982 4
Hemogen	HEMGN	223669_at	AF130060	3 0	593 0	196 9
S100 calcium binding protein P	S100P	204351_at	NM_005980	3 0	1576 0	530 5
Matrix metalloproteinase 9	MMP9	203936_s_at	NM_004994	2 8	428 2	155 7
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
Neural proliferation, differentiation and control, 1	NPDC1	218086_at	NM_015392	1 5	29 9	19 6
Motile sperm domain containing 2	MOSPD2	221895_at	AW469184	1 5	118 2	77 4
Myeloperoxidase	***MPO***	***203949_at***	***NM_000250***	***1.5***	***8.5***	***5.6***
LOC401505	LOC401505	225036_at	BF969806	1 5	89 7	58 8

[a] Bold and italic fonts indicate the whole blood markers selected for QRT-PCR analysis [b] Fold change is the fold difference in expression level between the pregnant and non-pregnant blood samples according to the microarray data

4.3.5.1 Quantification of the whole blood mRNA markers in third trimester whole blood and placental tissues

To assess if the pregnancy-related whole blood mRNA transcripts were likely to have originated from the placenta, placental tissue and whole blood samples were collected from ten pregnancies. The median mRNA concentrations for *OLFM4, FLCN, MMP8* and *MPO* were 3, 60, 3 and 2 copies per ng placental RNA, respectively while *DEFA4, CEACAM8* and *ORM1* was not detectable in placental RNA. The median mRNA concentrations for *DEFA4, CEACAM8, OLFM4, FLCN, ORM1, MMP8* and *MPO* were 125, 1181, 781, 207, 338, 386 and 200 copies per ng whole blood RNA, respectively, in third trimester cases.

4.3.5.2 Concentrations of whole blood mRNA markers in pre- and post-delivery maternal whole blood

After normalizing with *GAPDH* to adjust for the leukocyte concentration in whole blood, a statistically significant decreasing trend was observed in the post-delivery maternal blood samples for transcripts *DEFA4, CEACAM8, OLFM4* and *MMP8* comparing with the corresponding pre-delivery blood samples according to the Wilcoxon test ($P < 0.05$). No significant difference was observed for *FLCN* ($P = 1.00$), *ORM1* ($P = 0.63$) and *MPO* ($P = 0.06$) (Figure 4.8).

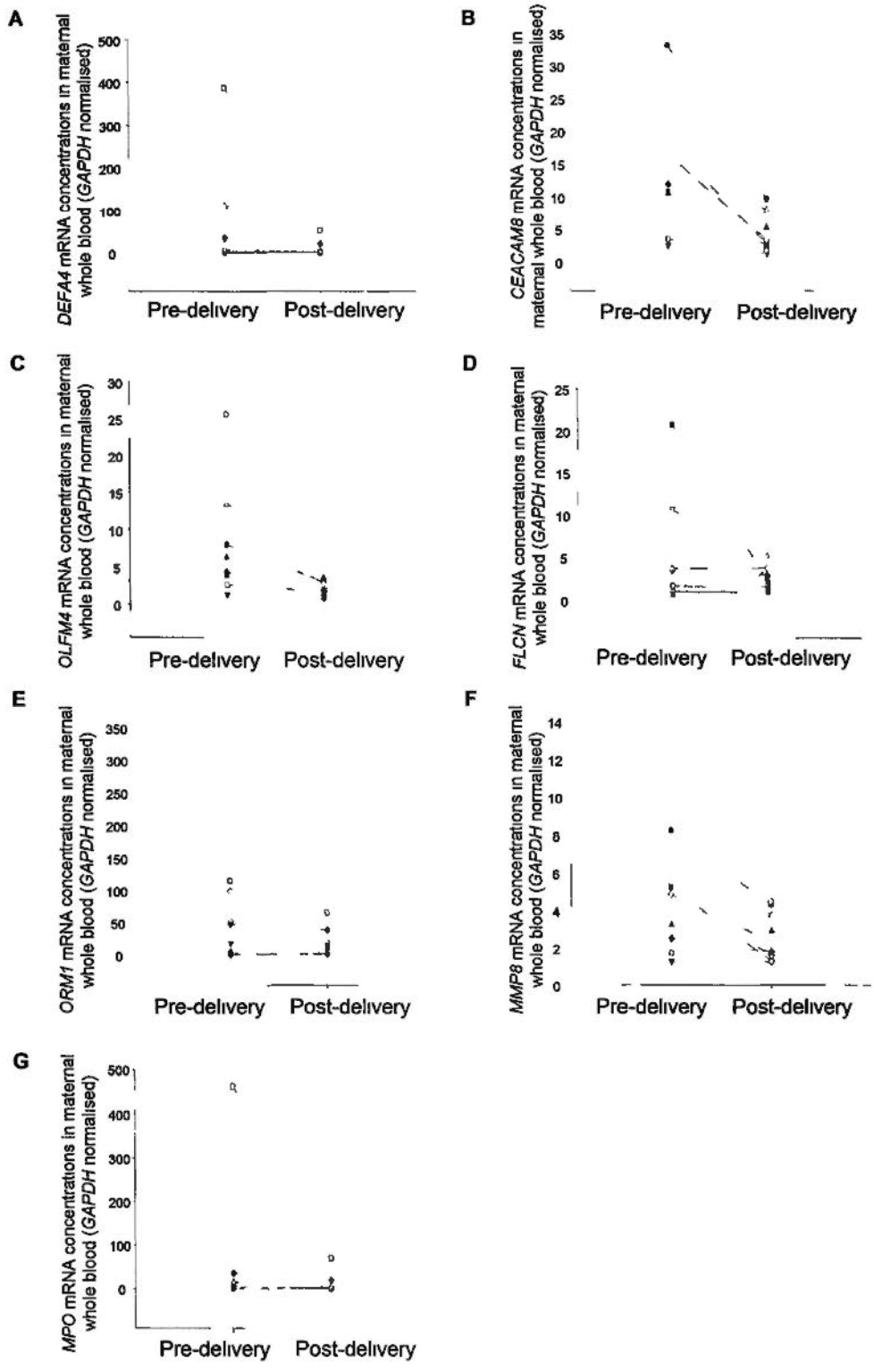


Figure 4.8 **Quantitative analysis of identified whole blood mRNA markers in pre- and 24-hour-post-delivery maternal whole blood.** (A) *DEFA4*. (B) *CEACAM8*. (C) *OLFM4*. (D) *FLCN*. (E) *ORM1*. (F) *MMP8*. (G) *MPO* mRNA. Corresponding samples from each individual are connected by a line.

4.3.5.3 Concentrations of whole blood mRNA markers in third trimester and non-pregnant whole blood

When comparing the absolute concentrations of *DEFA4, CEACAM8, OLFM4, FLCN, ORM1, MMP8* and *MPO* mRNA in whole blood samples from third trimester pregnant, non-pregnant female and male, statistical significant differences were observed in all markers ($P < 0.001$, Kruskal-Wallis ANOVA test) except *FLCN* ($P = 0.231$, Kruskal-Wallis ANOVA test) (Figure 4.9).

However, after normalisation with *GAPDH* to adjust for the leukocyte concentration in whole blood (data not shown), significant differences among third trimester pregnant, non-pregnant female and male whole blood were only found for transcripts *OLFM4* and *FLCN* ($P < 0.05$, Kruskal-Wallis ANOVA test).

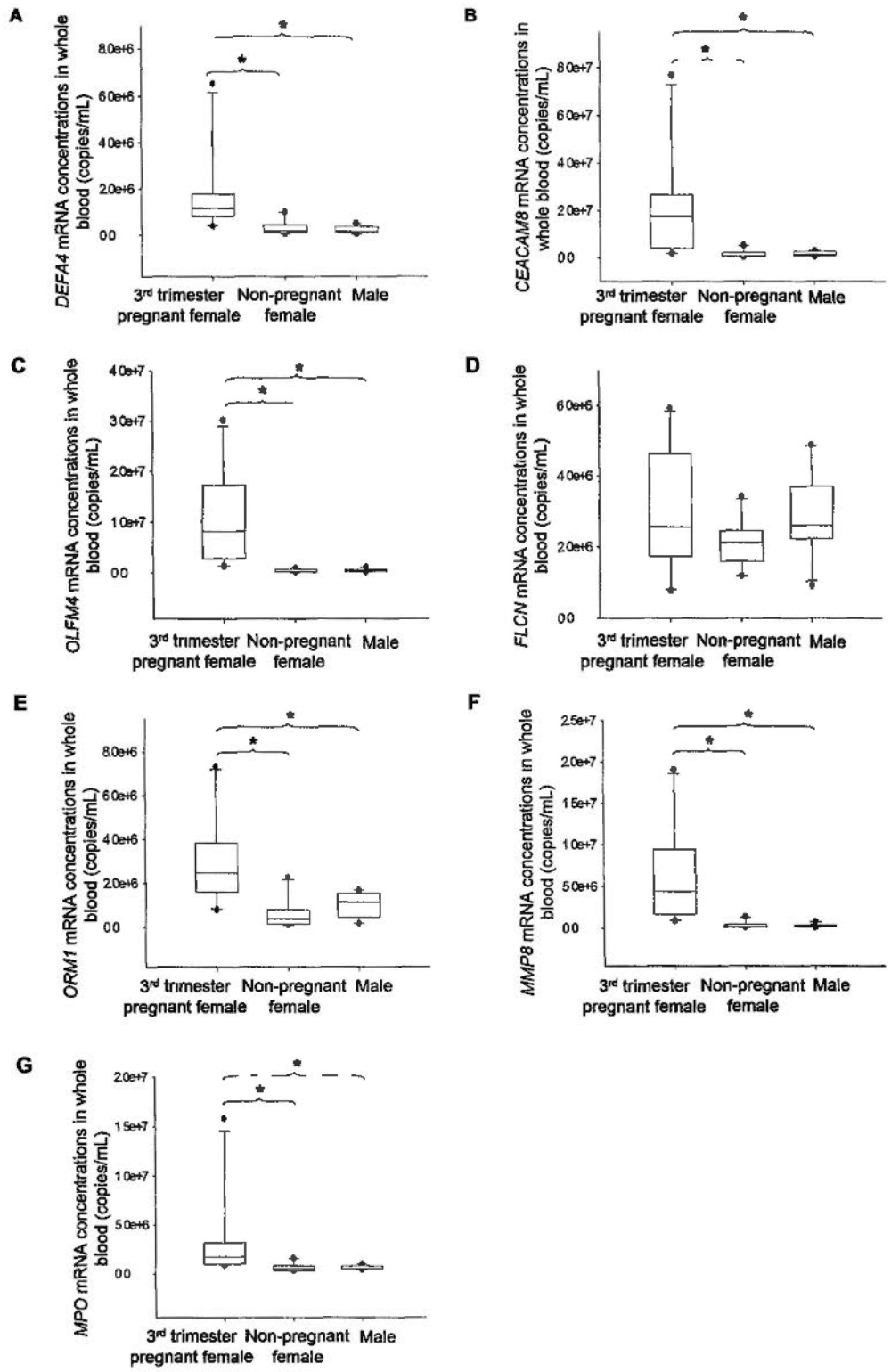


Figure 4.9 **Concentrations of identified whole blood mRNA in third trimester pregnant and non-pregnant whole blood.** Box plots of (A) *DEFA4* (B) *CEACAM8* (C) *OLFM4* (D) *FLCN* (E) *ORM1* (F) *MMP8* (G) *MPO* mRNA. The line inside each box denotes the median. The lower and upper limits denote

the 25th and 75th percentiles, respectively. The lower and upper whiskers denote the 10th and 90th percentiles, respectively. Filled circles denote the outliers. Asterisks indicate the groups with significant differences in mRNA concentrations of the marker ($P < 0.05$, Kruskal-Wallis test followed by pairwise comparison using the Student-Neuman-Keuls test).

4.4 Discussion

For decades, to develop non-invasive prenatal diagnosis, investigators have focused on the search for intact fetal cells in maternal blood (Brown, 1963, Mavrou *et al.*, 2007). However, the rarity of such cells has hindered their robust identification in maternal blood (Bianchi *et al.*, 2002). The discoveries of cell-free fetal DNA (Lo *et al.*, 1997) and later placental mRNA (Ng *et al.*, 2003b) in maternal plasma have offered new opportunities for the development of non-invasive prenatal diagnosis (Lo *et al.*, 2007b). More recently, placental expressed and pregnancy-associated transcripts have been reported to be detectable in maternal whole blood (Concu *et al.*, 2005, Okazaki *et al.*, 2006, Maron *et al.*, 2007, Farina *et al.*, 2006). As the RNA yield in whole blood far exceeds that of plasma, this study addresses whether the maternal whole blood might represent a better specimen type for the detection of fetal specific placenta-derived mRNA.

This study confirmed that placental expressed mRNA concentrations in third trimester whole blood were indeed higher than that in plasma for *CSH1, KISS1, PLAC4* and *PLAC1*. The observation was consistent with previous reports (Okazaki *et al.*, 2006, Banzola *et al.*, 2008). While the four transcripts were not detectable in all post-delivery maternal plasma samples, there were no significant reductions in *PLAC4* and *PLAC1* mRNA concentrations in maternal whole blood samples collected 24-hour after delivery. In non-pregnant females and males, all four placental transcripts were absent in plasma but *PLAC4* and *PLAC1* could be detected in the whole blood of both non-pregnant females and males. These data suggested that the *PLAC4* and *PLAC1* mRNA in whole blood could be derived from tissues or organs other than the placenta. This could also explain the lack of clearance of *PLAC4* and *PLAC1* mRNA from maternal whole blood after delivery. This is further supported by the lack of statistical significant correlations between the whole blood and plasma signals for *CSH1, KISS1, PLAC4* and *PLAC1* suggesting that the mRNA species detected in the two specimen types possibly have originated from different tissue sources.

It is believed that fetal or placental RNA is released into the maternal circulation due to apoptosis of the trophoblast cells (Halicka *et al.*, 2000, Ng *et al.*, 2003b). If

our targeted RNA transcripts were indeed placenta-derived, they should carry the same genotype as the fetus. The concordance between the placental tissue and plasma RNA-SNP genotyping results for *PLAC4* and *CSHL1* confirmed the fetal origin of such mRNA molecules in maternal plasma. As for maternal whole blood mRNA, the *CSHL1* genotype was concordant with that of the fetus while the *PLAC4* genotype was always heterozygous even when the fetus was homozygous (cases 1160 and 1359 in Table 4.1). This latter observation suggests that there is maternal contribution to the detectable *PLAC4* mRNA in maternal whole blood. This finding confirms those reported by Go *et al* (Go *et al.*, 2007a). As can be appreciated from Table 4.1, pregnancies involving a homozygous fetus but a heterozygous mother (cases 1160 and 1359) are more useful for confirming the fetal specificity (i.e. absence of maternal transcript contamination) of the targeted transcript. The presence of a fetal-specific allele in maternal plasma (e.g. G-allele in case 668) merely suggests the existence of fetal contribution to the mRNA pool but does not indicate that there is no maternal transcript contamination. Yet, it is the absence of the maternal specific allele (e.g. G-allele in case 1160) that confirms the fetal-specificity of the transcript.

Concluding from our data, *CSH1*, *CSHL1* and *KISS1* appear to be

pregnancy-specific in both plasma and whole blood. On the contrary, *PLAC4* and *PLAC1* are pregnancy-specific only in plasma but not whole blood which has both fetal and maternal contributions. Thus, it is not surprising that Banzola *et al* reported a lack of quantitative difference in whole blood *PLAC4* (located on chromosome 21) mRNA between euploid and trisomy 21 pregnancies (Banzola *et al.*, 2008). Our data showed that there was no statistical significant difference in whole blood *PLAC4* mRNA concentrations even between pregnant and non-pregnant individuals. One possible explanation of the maternal contribution to these apparently "placenta-derived" transcripts in whole blood may be related to the phenomenon of illegitimate expression that lymphocytes had been shown to express transcripts irrelevant to their functions (Gala *et al.*, 1998, Kimoto, 1998, Ko *et al.*, 2000). Microarray analyses do not reveal these expression profiles because microarray expression data are often presented in a relative scale where the extremely low abundance signals will be dwarfed by the high abundance signals. These low abundance signals in blood cells may become a significant contaminant in terms of absolute quantities when whole blood is the biological sample analysed.

Nonetheless, *CSH1, CSHL1* and *KISS1* appeared to be pregnancy-specific in

whole blood. Thus, the possibility of whether other pregnancy-specific whole blood mRNA markers could be developed based on microarray comparison of pregnant and non-pregnant whole blood samples was investigated systematically. 247 genes which showed at least 1.5-fold elevation in the former than the latter group were found. Thirteen of the top 25 transcripts on the list were related to blood cell functions, especially that of neutrophils. This is not surprising as pregnancy has been reported to induce inflammatory change in peripheral blood and neutrophils are major contributors to inflammation (Germain *et al.*, 2007, Sacks *et al.*, 1998). Unexpectedly, none of the previously studied placenta-derived transcripts, *CSH1, KISS1, PLAC4* and *PLAC1*, appeared on the gene list. This was also the case as reported by Maron *et al.* (Maron *et al.*, 2007). According to our QRT-PCR data on the pregnancy-related whole blood markers such as *DEFA4*, the median expression levels of these genes were much higher than that of the placenta-derived transcripts in whole blood (Figures 4.5 and 4.9). Therefore, it is possible that *CSH1, CSHL1* and *KISS1* were ranked much lower than 247. Furthermore, as *PLAC4* and *PLAC1* were readily detectable in both pregnant and non-pregnant whole blood and possibly without the 1.5-fold threshold difference in expression, they would not have appeared on the sorted gene list.

The pregnancy-specificity of 7 transcripts with differences in expression was validated between pregnant and non-pregnant whole blood as determined by the microarray analysis. *DEFA4* is involved in host defense and found in neutrophils (Schneider *et al.*, 2005). *CEACAM8* is expressed only in neutrophils and eosinophils in humans with undetermined function (Zhao *et al.*, 2004b, Zhao *et al.*, 2004a). *OLFM4* is previously recognised as human granulocyte colony stimulating factor stimulated clone-1 with high expression in cancerous tissues (Kobayashi *et al.*, 2007, Koshida *et al.*, 2007). *FLCN* is speculated to be a tumour suppressor gene with unclear function (Adley *et al.*, 2006). *ORM1* encodes a key acute phase plasma protein (Fournier *et al.*, 2000, Hochepied *et al.*, 2003). During pregnancy, *ORM1* is suggested to be involved in the maintenance of homeostasis between maternal and fetal systems within the chorioallantoic placenta (Thomas, 1993). *MMP8* is expressed in diverse cell types including neutrophils, macrophages, T cells, epithelial cells and endothelial cells in inflammatory conditions (Hanemaaijer *et al.*, 1997, Prikk *et al.*, 2001). *MMP8* was also detected in the chorion during labor and was proposed to be a predictive marker for preterm delivery (Arechavaleta-Velasco *et al.*, 2004, Nien *et al.*, 2006). *MPO* is a heme protein primarily hosted in human polymorphonuclear neutrophils (Klebanoff, 1999). During pregnancy, MPO was observed on the surface of neutrophils

(Kindzelskii *et al.*, 2006).

Our QRT-PCR validation of the whole blood markers showed a statistically significant difference between the expression levels among third trimester pregnant, non-pregnant female and male individuals for *OLFM4* and *FLCN* only, after normalisation with the corresponding *GAPDH* data. All markers, except *FLCN*, were related to the haematopoietic system, especially the neutrophils. It has also been reported that the total leukocyte count would rise during pregnancy with neutrophils accounting for most of the increased leukocyte count (Kuhnert *et al.*, 1998, Pitkin and Witte, 1979). Hence, the increased expression level observed from the microarray data was probably due to the augmented leukocyte number instead of the transcript expression level in the cells. This may also explain why significant difference in concentrations of those transcripts was found only when directly comparing the pregnant and non-pregnant whole blood samples but not after *GAPDH* normalisation.

The whole blood microarray study identified transcripts whose functions were related to the physiological changes of pregnancy. However, their presence in non-pregnant whole blood and lack of clearance after pregnancy suggested that

they were not "pregnancy-specific" markers, albeit being "pregnancy-related". As they were also detectable in blood of non-pregnant individuals, they were therefore not fetal-specific either. Concluding from all the data in this study, it appears that placenta-derived fetal-specific transcripts can be more readily identified from maternal plasma than whole blood. While some transcripts are fetal-specific in maternal whole blood, e.g. *CSHL1*, due care is needed to validate each new potential whole blood transcript that is meant to be used as a circulating fetal RNA marker. Illegitimate expression by maternal blood cells would need to be excluded before adopting such a marker.

SECTION IV: IMPROVEMENTS ON DETECTION OF CIRCULATING RNA IN PLASMA

Two chapters are included in this section. Each chapter presents different protocol improvements to enrich and increase the yield of circulating mRNA and microRNA in plasma.

CHAPTER 5: DEVELOPMENT OF EXTRACTION PROTOCOLS TO IMPROVE THE YIELD FOR FETAL mRNA IN MATERNAL PLASMA

5.1 Introduction

Circulating fetal mRNA analysis offers a means for non-invasive prenatal assessment that is applicable to pregnancies regardless of the fetal gender (Farina *et al.*, 2004, Ng *et al.*, 2003b, Tsui *et al.*, 2004). Quantitative aberrations in circulating placental mRNA have been reported for pregnancy-associated complications (Farina *et al.*, 2004). Non-invasive prenatal detection of fetal trisomy 21 has been achieved by single nucleotide polymorphism (SNP) allelic ratio determination in circulating placental mRNA (Lo *et al.*, 2007b). These studies affirmed the potential of circulating placental mRNA analysis for non-invasive prenatal assessment.

Since low recovery of placental mRNA from maternal plasma may lead to false negative results, such as for trisomy 21 detection (Lo *et al.*, 2007b), there is a need to develop protocols that would improve circulating mRNA yield and ideally be practical to perform by most laboratories. Our current blood processing protocol, which involves high speed centrifugation, was previously evaluated by Chiu *et al.*

for maternal plasma fetal DNA analysis for the purpose of minimising the chance of cellular DNA contamination (Chiu *et al.*, 2001). However, circulating DNA and RNA demonstrate different physical characteristics. The majority of circulating fetal DNA in maternal plasma is cell-free while fetal RNA is particle-associated (Chiu *et al.*, 2001, El-Hefnawy *et al.*, 2004, Halicka *et al.*, 2000, Ng *et al.*, 2002). Thus, the same blood processing protocol may not be equally ideal for the capture of circulating fetal DNA and RNA. In addition, our current plasma RNA extraction protocol was adopted from Wong *et al.* (Ng *et al.*, 2003b, Wong *et al.*, 2004a) and there may be room for improvement. Consequently, the objective of this part of the thesis is to review and modify the blood processing and RNA extraction protocols with consideration to the known physical characteristics of plasma RNA.

It is hypothesised that particle-associated RNA may be pelleted and discarded if plasma supernatant was harvested after harsh centrifugation speeds as currently practiced (Ng *et al.*, 2003b). Thus, recovery of such plasma RNA molecules may be improved if plasma supernatant is harvested after gentle centrifugation or alternatively be concentrated into the infranatant when subjected to extreme speeds of centrifugation, such as ultracentrifugation. The study also confirms the fetal-specificity of the enriched particles.

5.2 Materials and Methods

The study comprised of two modules. The first module (module 1) investigated the effects of centrifugation on the concentration of fetal RNA and the fetal specificity of the enriched RNA particles. The second module (module 2) focused on the modification of the plasma RNA extraction protocol. Trizol LS reagent (Invitrogen) was demonstrated to be effective in preserving plasma RNA (Wong *et al.*, 2004a). The effect of modifying the Trizol LS reagent (Invitrogen) to plasma ratio on fetal mRNA yield was investigated.

Module 1:

Blood samples were processed to harvest plasma from healthy pregnant women attending the Prince of Wales Hospital in Hong Kong before and after delivery with different centrifugation protocols: (I) 1,600 *g* then 16,000 *g* centrifugation as previously reported (Chiu *et al.*, 2001, Ng *et al.*, 2003b); (II) 100 *g* centrifugation with 5 μm filtration; and (III) 1,600 *g* centrifugation, 5 μm filtration then ultracentrifugation at 99,990 g. Details of the protocols were described in Chapter 3.1.2.

The quantitative difference in *CSH1, PLAC4* and *KISS1* mRNA concentration

were compared in maternal plasma processed with our standard blood processing protocol (Protocol I) and a protocol with gentle spinning of 100 *g* (Protocol II) by QRT-PCR. The same comparison was also performed in samples processed with protocol I and a protocol involved ultracentrifugation of 99,000 *g* (protocol III). RNA/DNA extraction procedures and QRT-PCR conditions were reported in Chapter 3.2 and 3.3 respectively.

The pregnancy-specificity of the enriched mRNA particles was assessed by observing the clearance of placenta-expressed transcripts in plasma collected 24 hours after delivery.

In addition, fetal specificity of the enriched *PLAC4* and *KISS1* mRNA was further confirmed by studying their genotypes in maternal plasma using the RNA-SNP approach by MALDI-TOF MS (Lo *et al.*, 2007b). Coding SNPs of rs8130833 in *PLAC4* (Lo *et al.*, 2007b) and rs3924587 in *KISS1* were investigated. *CSH1* was highly homologous with other genes of the growth hormone cluster that a target-specific RNA-SNP assay could not be developed. DNA was extracted from placental tissues and maternal buffy coat to determine the fetal and maternal genotypes. Cases where the fetal and maternal genotypes differed were considered

as informative. The *PLAC4* and *KISS1* mRNA genotypes in maternal plasma of informative cases were determined to confirm the origin of the enriched RNA molecules. Protocols for *PLAC4* and *KISS1* SNP genotyping assay were the same as previously described in Chapter 3.4 (Lo *et al.*, 2007b). Primer sequences are shown in Table 3.5 and 3.6.

Module 2:

Blood samples were processed according to protocol I (Chapter 3.1.2) to harvest plasma from ten healthy second trimester pregnant women attending the Prince of Wales Hospital in Hong Kong (gestational age range, 15-19 weeks). Trizol LS reagent (Invitrogen) was added into 1.6 mL plasma aliquots with 1.25 parts of Trizol LS (protocol E1) as well as with 3 parts Trizol LS (protocol E2) for every part of plasma volume as per originally described (Ng *et al.*, 2003b). These plasma-Trizol LS mixtures were stored frozen at -80°C until RNA extraction. In addition, a third plasma aliquot from each case were collected initially as per protocol E1 but topped up the Trizol LS volumes to 3 times the plasma volumes immediately before RNA extraction (protocol E3).

The effect of modifying the Trizol LS reagent (Invitrogen) to plasma ratio on fetal

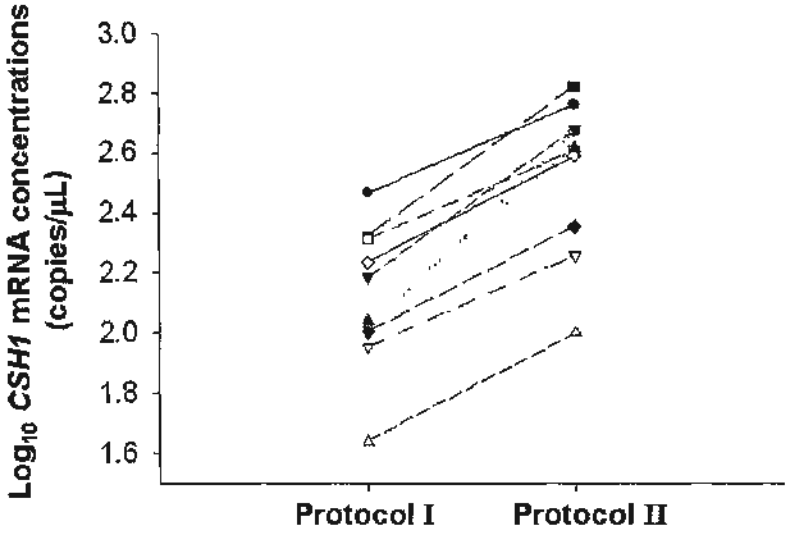
mRNA yield of *CSH1*, *PLAC4* and *KISS1* was examined quantitatively by QRT-PCR.

5.3 Results

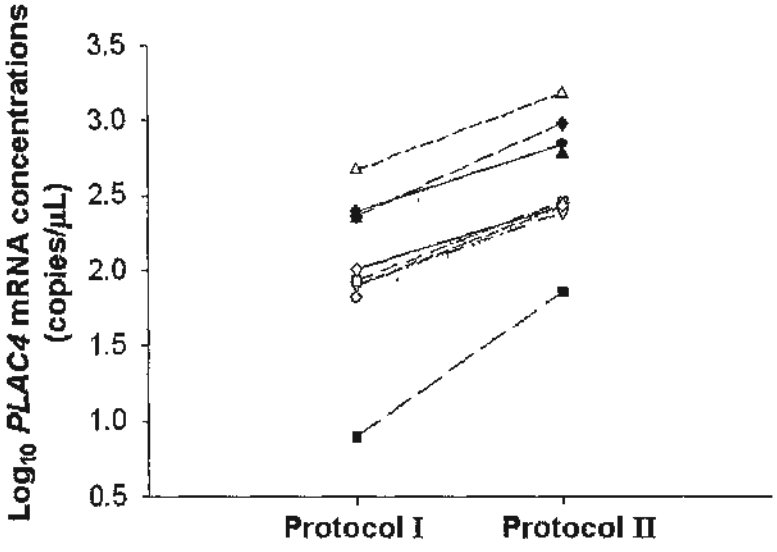
Module 1:

The quantitative mRNA concentration differences in *CSH1*, *PLAC4* and *KISS1* were compared between ten maternal blood samples (all at gestational age 38 weeks) processed with protocols I and II. Median (interquartile range (IQR)) concentrations of *CSH1*, *PLAC4* and *KISS1* mRNA were, respectively, 132 (102 – 197), 94 (79 – 228) and 1 (undetectable – 4) copies/μL in RNA extracted from plasma processed with protocol I while they were 409 (267 – 473), 280 (267 – 673) and 11 (3 – 19) copies/μL, respectively, in plasma RNA from protocol II. The differences were statistically significant ($P < 0.05$, Wilcoxon test) (Figure 5.1).

(A)


Log10 CSH1 mRNA concentrations (copies/µL)
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
Protocol I
Protocol II

(B)


Log10 PLAC4 mRNA concentrations (copies/µL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Protocol I
Protocol II

(C)

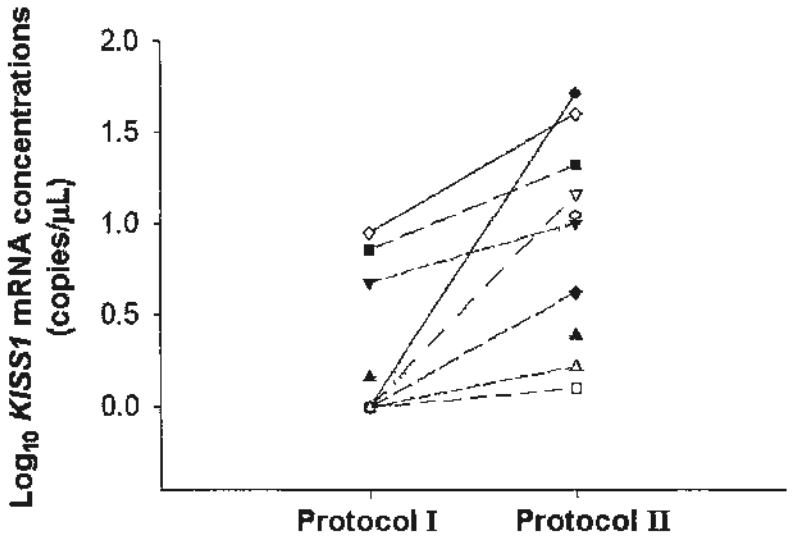


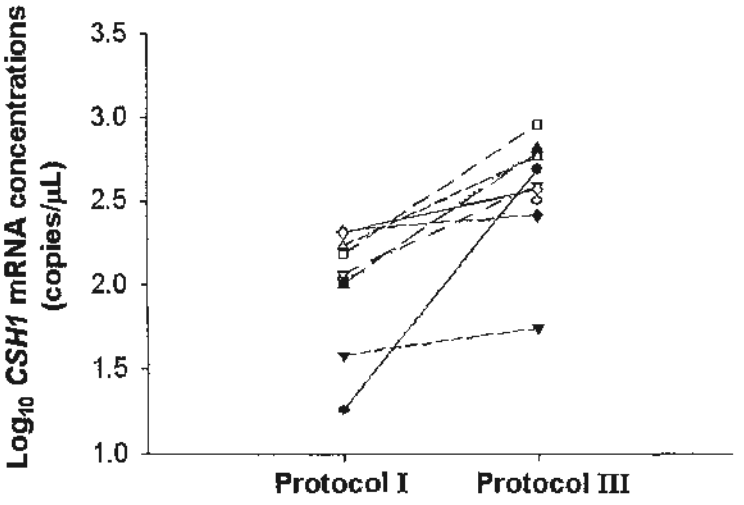
Figure 5.1 **Concentrations of placenta-derived mRNA in maternal plasma processed with different protocols.** Line and scatter plots of (A) *CSH1*. (B) *PLAC4*. (C) *KISS1* mRNA concentrations in third-trimester maternal plasma processed with protocols I and II.

When comparing the *CSH1* and *KISS1* mRNA concentrations in ten third-trimester maternal blood samples (gestational age range, 38-39 weeks) handled with protocols I and III, the median (IQR) mRNA concentrations were 113 (102 – 167) and 4 (undetectable – 7) copies/μL, respectively, in plasma RNA extracts prepared with protocol I. After ultracentrifugation in protocol III, a gradient was observed and the bottom-most 300 μL were collected for RNA extraction. Almost 90% of

the mRNA transcripts detected in a corresponding aliquot without ultracentrifugation were recovered in this plasma compartment. Median (IQR) *CSH1* and *KISS1* mRNA concentrations were, respectively, 440 (332 – 603) and 30 (12 – 39) copies/μL in RNA extracts derived from this plasma compartment from protocol III which were significantly higher than the RNA concentrations obtained from protocol I ($P < 0.05$, Wilcoxon test) (Figure 5.2). As a larger input volume of plasma was required for protocol III, there was insufficient volume for the testing of *PLAC4*.

As a major concern for prenatal diagnosis, the pregnancy-specificity of the enriched mRNA particles was assessed by observing the clearance of placenta-expressed transcripts in plasma collected 24h after delivery. Among ten postpartum plasma samples processed with protocol II, *CSH1* and *PLAC4* mRNA were detected in one case with concentrations lower than 1 copy/μL RNA and no *KISS1* transcript was detected. For postpartum plasma samples processed with protocol III, *CSH1* was undetectable while *KISS1* was detected in one case with less than 1 copy/μL RNA.

(A)



(B)

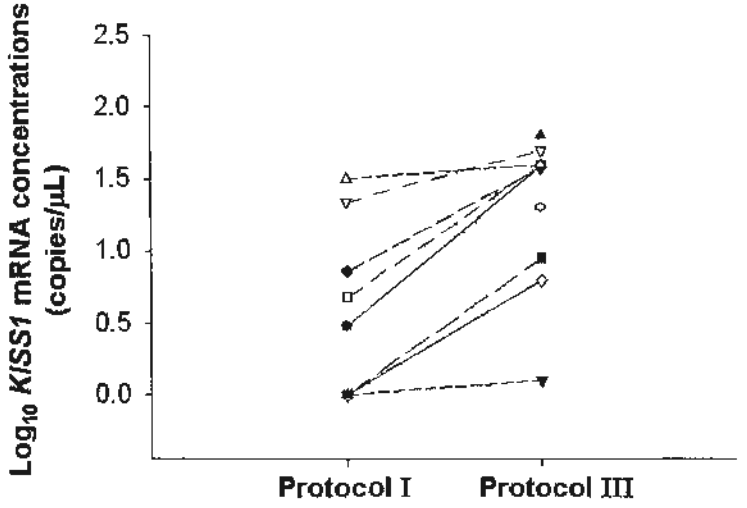


Figure 5.2 **Concentrations of placenta-derived mRNA in maternal plasma processed with different protocols.** Line and scatter plots of (A) *CSH1*. (B) *KISS1* mRNA concentrations in third-trimester maternal plasma processed with protocols I and III.

In addition, fetal specificity of the enriched *PLAC4* and *KISS1* mRNA was further confirmed by studying their genotypes in maternal plasma using the RNA-SNP approach by MALDI-TOF MS (Lo *et al.*, 2007b). Sixty pairs of maternal buffy coat and placental tissue DNA samples were screened for informative fetal-maternal pairs as explained in Chapter 3.4.2. Nine and four cases were found to be informative for *PLAC4* and *KISS1*, respectively. Maternal plasma from half of the informative cases were processed with protocol II while the other half was handled with protocol III and subjected to RNA-SNP genotyping as described in Chapter 3.4.3. Genotyping data of all informative cases are summarised in Table 5.1 and 5.2. The *PLAC4* and *KISS1* mRNA genotypes in maternal plasma samples were identical to placental tissue DNA, i.e. the fetus, for all informative cases for both protocols II and III, confirming the fetal origin of the enriched RNA molecules.

Table 5.1 *PLAC4* and *KISS1* RNA-SNP genotyping in third-trimester maternal plasma processed with Protocol II.

			DNA Genotype		RNA-SNP Genotype
Protocol	**Gene**	**Sample No.**	**Fetus**	**Mother**	**Maternal Plasma**
II	***PLAC4***	1888	AA	AG	AA
		1970	AA	AG	AA
		1954	AG	AA	AG
		1977	AG	AA	AG
	KISS1	1861	AG	GG	AG
		1979	GG	AG	GG

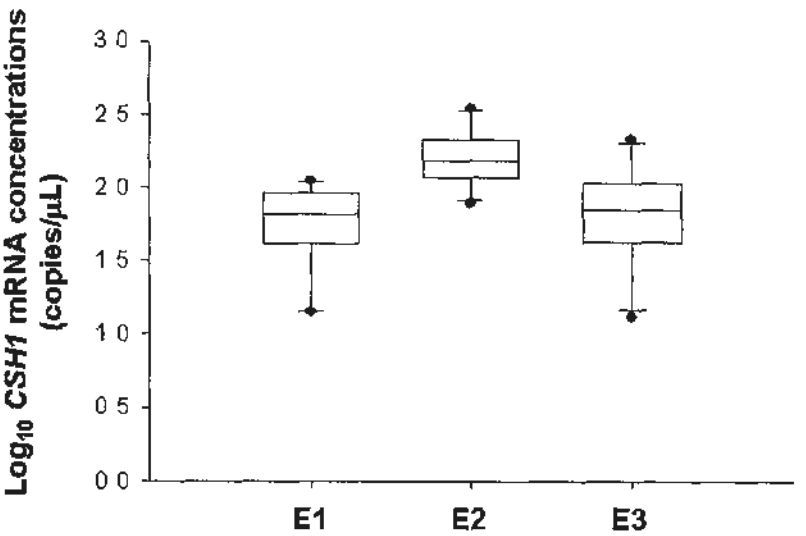
Table 5.2 *PLAC4* and *KISS1* RNA-SNP genotyping in third-trimester maternal plasma processed with Protocol III.

			DNA Genotype		RNA-SNP Genotype
Protocol	**Gene**	**Sample No.**	**Fetus**	**Mother**	**Maternal Plasma**
III	***PLAC4***	0153	AG	AA	AG
		1103	AG	AA	AG
		1126	AG	GG	AG
		1815	AG	AA	AG
		1156	GG	AG	GG
	KISS1	1144	GG	AG	GG
		1557	GG	AG	GG

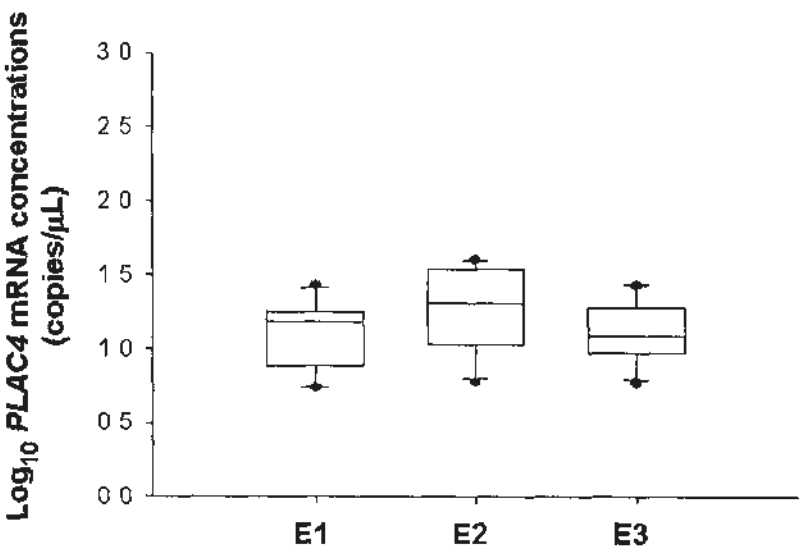
Module 2:

The quantitative differences among plasma RNA samples extracted with protocol E1 (1.25 parts Trizol LS), protocol E2 (3 parts Trizol LS) and protocol E3 (topped up to 3 parts Trizol LS immediately before RNA extraction) were examined. The median (IQR) concentrations of *CSH1*, *PLAC4* and *KISS1* mRNA were significantly increased from 66 (55 - 86) to 150 (122 - 199), from 15 (9 - 17) to 21 (11 - 34) and from 4 (3 - 6) to 18 (14 - 23) copies/μL, respectively, in plasma RNA samples extracted with protocol E2 when compared with protocol E1 ($P < 0.05$, Friedman Analysis of Variance (Friedman ANOVA) test). When extra Trizol LS amount was added into the plasma prior to RNA extraction (E3), there was no statistically significant difference when compared with E1 as shown in Figure 5.3 ($P > 0.05$, Friedman ANOVA test).

(A)


Log10 CSH1 mRNA concentrations (copies/µL)
30
25
20
15
10
05
00
E1
E2
E3

(B)


Log10 PLAC4 mRNA concentrations (copies/µL)
30
25
20
15
10
05
00
E1
E2
E3

(C)

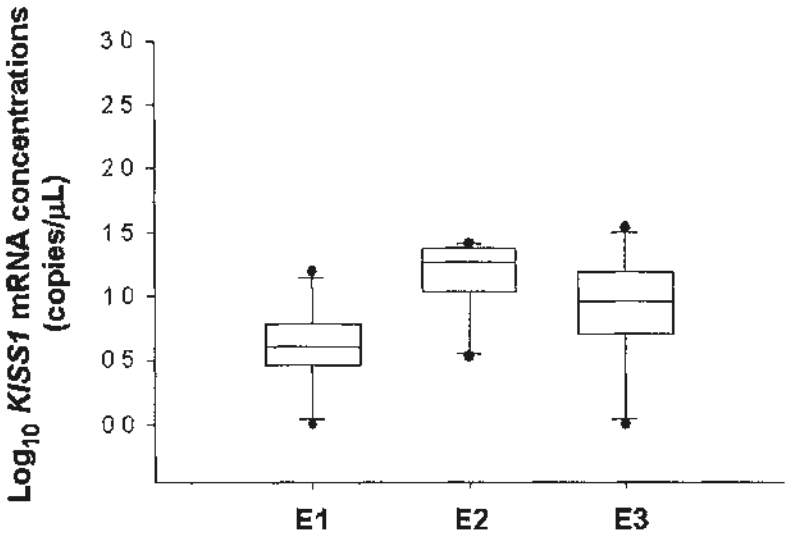


Figure 5.3 **Concentrations of placenta-derived mRNA in maternal plasma processed with different protocols.** Box plots of (A) *CSH1*. (B) *PLAC4*. (C) *KISS1* mRNA concentrations in second-trimester maternal plasma extracted with protocols E1, E2 and E3. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. The filled circles mark the data points outside the 10th and 90th percentiles.

5.4 Discussion

Plasma RNA concentration was first documented by Mandel and Metais (Mandel and Metais, 1948). It is further shown characteristics of particle-associated with surprising high stability more than 50 years after the first discovery (Ng *et al.*, 2002, Tsui *et al.*, 2002). Currently, in the field of prenatal diagnosis, there is no

consensus in the blood processing protocol for preparation of plasma for RNA analysis, nor a systematic evaluation on the issue. The most widely used protocol is the one that has been thoroughly evaluated by Chiu *et al.* confirming its advantages in comparing maternal plasma for DNA analysis (Go *et al.*, 2007b, Lo *et al.*, 2007b, Ng *et al.*, 2002, Ge *et al.*, 2005, Chiu *et al.*, 2001). However, circulating DNA and RNA demonstrate different physical characteristics. The majority of circulating fetal DNA in maternal plasma is cell-free while fetal RNA is particle-associated (Chiu *et al.*, 2001, El-Hefnawy *et al.*, 2004, Halicka *et al.*, 2000, Ng *et al.*, 2002). Thus, the same blood processing protocol may not be equally ideal for the capture of circulating fetal DNA and RNA.

Analysis of the data from module 1 revealed that the particle-associated fetal RNA molecules could be effectively enriched in plasma processed with gentle spinning. As shown in Figure 5.1, the concentration of the placental mRNA was increased in every single case in plasma prepared from protocol II when comparing that from protocol I. This observation is also consistent with Wong *et al.* that increasing the centrifugation force from 800 *g* to 4,500 *g* resulted in the decrease in the detection of tumour markers including *β-catenin*, *P-selectin* and *CK20* mRNA in plasma of cancer patients (Wong *et al.*, 2007). The gentle centrifugation force enabled the

particle-associated fetal RNA molecules remaining in the plasma phase without being spun down together with the blood cells. However, this gentle force may also increase the chance of cell contamination to the plasma. As demonstrated in the previous studies that plasma RNA molecules could pass through 5 μm filter but not 0.22 μm filter (Ng *et al.*, 2002) and the majority of the blood cell diameter ranges from 6 – 20 μm (Martini and Timmons, 1997). The 5 μm filter became necessary for preparing gently-spun plasma with minimal adulterating blood cells.

These fetal RNA molecules not only could be enriched by gentle spinning, they could also be further concentrated into a small volume as demonstrated in Figure 5.2. The increase in the *CSH1* and *KISS1* mRNA concentration was significantly higher in plasma processed with protocol III than protocol I. This pointed to the possibility and potential of developing a protocol to concentrate large volume of plasma into a small volume for detection of rare transcripts.

More importantly, the enriched placenta-derived RNA molecules in the maternal plasma became undetectable in almost all cases after the fetus and the placenta were delivered. This manifested the pregnancy-specificity of the enriched RNA particles. In spite of this, this is still an indirect proof. The fetal-specificity of these

RNA molecules was further investigated with the RNA-SNP approach (Lo *et al.*, 2007b). The complete concordance between the plasma RNA genotypes and the fetal genomic genotypes in all cases provided concrete evidence to the origin of the enriched RNA particles.

Besides blood processing protocols, the RNA extraction protocols were also evaluated in module 2 of this study. As illustrated in Figure 5.3, increasing the Trizol LS reagent to plasma ratio increased the yield of plasma mRNA extraction. Trizol LS reagent (Invitrogen) is a monophasic solution of phenol and guanidine isothiocyanate, which is capable of denaturing proteins, including ribonucleases, thus preserving the integrity of ribonucleic acids. Because plasma is a protein-rich bodily fluid, increasing the amount of Trizol LS reagent (Invitrogen) might enhance the protein denaturation so that more RNA could possibly be released from its binding cellular proteins.

In this study, we have demonstrated that particle-associated fetal RNA molecules could be enriched in plasma processed with gentle spinning or be concentrated into a small volume. These observations further supported that circulating fetal RNA is particle-associated in nature. With effective modification of plasma RNA

extraction protocols, these improvements may enhance the accuracy and reliability of detecting circulating fetal mRNA in maternal plasma, especially for those marginally detectable transcripts. However, as ultracentrifuge is not a common instrument in many diagnostic laboratories, protocol III may not be practical for all users. Protocol II requires low speed centrifugation without braking which lengthens the resident time of a sample in a centrifuge to 30 minutes and reduces the throughput. Hence, modification in extraction protocols was explored and confirmed the use of extra Trizol LS addition as a convenient protocol to improve circulating placental mRNA yield. In summary, three options that may be used to improve circulating mRNA yield have been presented and laboratories may have differences in preference depending on the local setting and needs.

CHAPTER 6: IMPROVEMENTS ON THE DETECTION OF PLASMA MICRORNA

6.1 Introduction

MicroRNAs are a family of small (18-25 nucleotides), endogenous non-coding RNAs that target mRNAs for regulation of gene expression largely through translational repression (Ambros, 2003, Bartel, 2004, Lai, 2003). Barad *et al.* and Bentwich *et al.* have identified miRNAs in human placental tissues (Barad *et al.*, 2004, Bentwich *et al.*, 2005). Recently, Chim *et al.* were the first to demonstrate the presence of placental miRNA in maternal plasma (Chim *et al.*, 2008) and Gilad *et al.* subsequently reported the detection of a panel of pregnancy-associated miRNAs in maternal serum (Gilad *et al.*, 2008). Also, since miRNAs are associated with various regulatory functions in humans (Bartel, 2004) and exist at much higher copy numbers in cells than mRNAs (Lim *et al.*, 2003), this novel class of markers has the potential to be developed for non-invasive prenatal diagnostic purposes.

Chapter 5 of this thesis has highlighted the importance of optimal protocols on the yield of nucleic acid extraction. It has been demonstrated that increasing the Trizol LS-to-plasma ratio boosted the mRNA yield in maternal plasma. Since plasma

mRNAs and miRNAs possess different physical characteristics whereby mRNAs are particle-associated (Ng *et al.*, 2002) while miRNAs could pass through 0.22 μm filter (Chim *et al.*, 2008), increasing the Trizol LS-to-plasma ratio may or may not have the same beneficial effect on the miRNA yield. In this study, the effect of Trizol LS-to-plasma ratio on miRNA yield was investigated. Also, the performance of the two commonly used miRNA commercial extraction protocols practised in the field, namely *mir*Vana™ miRNA isolation kit (Ambion®, Austin, TX) and Qiagen miRNeasy (Qiagen, Hilden, Germany) kits, were evaluated in combination with different DNase I treatment options with both technical and biological replicates.

6.2 Materials and Methods

6.2.1 Study design

This study comprised of 3 modules. In the first module, the effect of Trizol LS-to-plasma ratio was evaluated. In chapter 5 of this thesis, it was demonstrated that increasing the Trizol LS-to-plasma ratio boosted the mRNA yield in maternal plasma. The effect of increasing such a ratio from 1.25 to 3 on miRNA yield was investigated by comparing the quantitative difference of miR-16 in plasma samples stored in different ratios. For example, a ratio of 1.25 is defined as the

mixing of Trizol LS reagent 1.25 times the given volume of plasma.

In module 2, the performance of the two commonly used miRNA commercial extraction protocols from mirVana™ miRNA isolation kit (Ambion®, Austin, TX) (V) and Qiagen miRNeasy (Q) kit were evaluated in combination with different DNase I treatment options, namely no DNase treatment (D0), Invitrogen off-column DNase I treatment (D1) and Qiagen on-column DNase treatment (D2), with 5 technical replicates per condition. Plasma samples were pooled and re-divided into aliquots of 1.6 mL each. Five aliquots were each subject to extraction by the following methods: mirVana column without DNase treatment (VD0), mirVana column with Invitrogen off-column DNase I treatment (VD1), Qiagen miRNeasy column without DNase treatment (QD0), Qiagen miRNeasy column with Invitrogen off-column DNase I treatment (QD1) and Qiagen miRNeasy column with Qiagen on-column DNase treatment (QD2). miR-16 concentration was measured by QRT-PCR in these samples. *Leptin* DNA concentrations were measured to test for DNA contamination. Moreover, the extracted RNA samples were digested with RNase A to test the substrate specificity of the miR-16 TaqMan microRNA assay.

The experimental design for the third module was similar to module 2 except that 10 biological replicates were tested and due to the DNA contamination observed in the non-DNase-treated RNA samples in module 2, only DNase-treated groups, i.e. VD1, QD1 and QD2, were included in module 3.

6.2.2 Subjects and sample processing

Healthy individuals were recruited from the community and 12 mL peripheral blood was collected into EDTA tubes. The blood samples were processed with *protocol I* as described in Chapter 3.1.2.

Module 1

Ten healthy individuals were recruited. 1.25 parts of Trizol LS reagent was added into 1.6 mL plasma aliquots. Another 1.6 mL plasma aliquot from each individual was mixed with 3 parts Trizol LS. These plasma-Trizol LS mixtures were stored frozen at -80°C until miRNA extraction.

Module 2

Five healthy individuals were recruited. The plasma obtained were pooled together and divided into 15 aliquots of 1.6 mL plasma. 4.8 mL Trizol LS reagent was

added into each aliquot of plasma and stored frozen at -80°C until miRNA extraction.

Module 3.

Ten healthy individuals were recruited. Each 4.8 mL plasma sample was added to 14.4 mL Trizol LS reagent and stored frozen at -80°C until miRNA extraction.

6.2.3 miRNA extraction

For module 1, the miRNA was extracted with the *mir*Vana™ miRNA isolation kit (Ambion®, Austin, TX) according to the manufacturer's instructions with details in Chapter 3.2.3.1.

For module 2, five plasma aliquots were extracted with the *mir*Vana™ miRNA isolation kit (Ambion) and the RNA was eluted with 30 μL RNase-free water. Off-column DNase I treatment (Invitrogen) was performed on 15 μL of the resultant RNA (VD1) while there was no DNase treatment for the remaining 15 μL of extracted RNA (VD0). The other 10 plasma aliquots were extracted with the Qiagen miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions mentioned in Chapter 3.2.3.2. On-column DNase

treatment (Qiagen) was done in 5 aliquots (QD2). For the remaining 5 aliquots, after elution with 30 μL RNase-free water, off-column DNase I (Invitrogen) was treated on 15 μL of the resultant RNA (QD1) while there was no DNase treatment for the remainder (QD0).

For module 3, the 4.8 mL plasma from each of the 10 individuals was divided into 3 aliquots for VD1, QD1 and QD2, i.e. with DNase treatments. The samples for VD1 were extracted with the *mir*Vana™ (Ambion) miRNA isolation kit and those for QD1 and QD2 were extracted with Qiagen miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

6.2.4 Quantification of miRNA and *leptin* DNA

For modules 1 – 3, the two-step QRT-PCR assay for miRNA quantification was set up following the procedures described in Chapter 3.3.3. TaqMan® MicroRNA Assay of miR-16 was purchased from Applied Biosystems. Serial dilutions of synthetic oligonucleotides were used for the absolute quantifications of the miRNAs. For modules 2 and 3, the QPCR assay for *leptin* quantification was set up following the procedures described in Chapter 3.3.4. The standard curve was constructed using serial dilutions of male blood cell genomic DNA with

concentrations ranging between 0.78 and 10,000 genome equivalents per five microlitres.

6.3 Results

Module 1

The differences in miR-16 concentrations between the 10 plasma samples stored in 1.25 part Trizol LS reagent and those stored in 3 parts were compared. The median (range) concentrations of miR-16 were 1.28×10^6 (5.51×10^5 – 1.94×10^6) and 7.97×10^6 (4.16×10^6 – 1.94×10^7) copies/mL respectively. The median concentration increased 6.2 fold in the plasma samples stored in more Trizol LS reagent. The differences were statistically significant (P = 0.002, Wilcoxon test). As shown in Figure 6.1, the miR-16 concentration was elevated in every single case extracted with 3 parts of Trizol LS reagent.

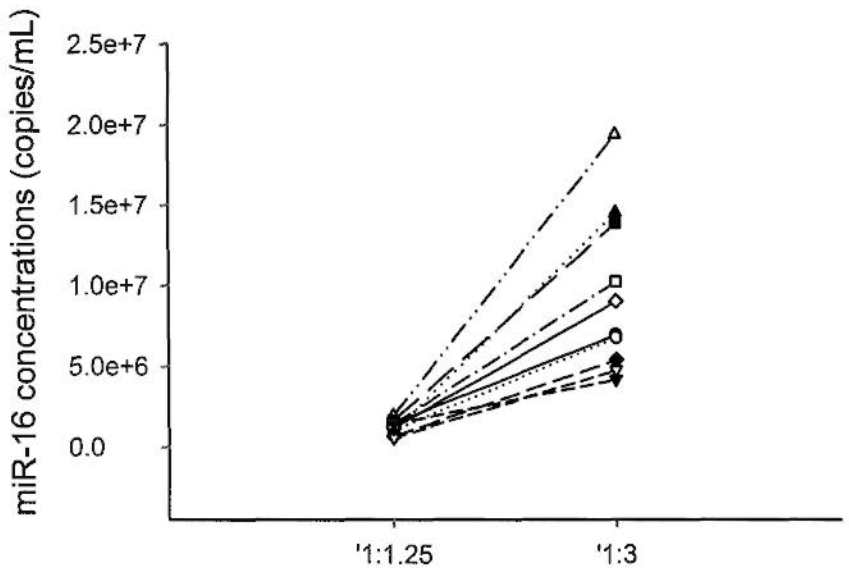


Figure 6.1 Concentrations of miR-16 in maternal plasma extracted from plasma stored in different plasma-to-Trizol LS ratios. Plasma aliquots from the same individual are shown by the same symbol inter-connected by a line.

Module 2

To evaluate the performance of the two commonly adopted commercial miRNA extraction protocols, the miR-16 and *leptin* DNA concentrations were compared among 5 groups extracted with 2 different columns (V and Q) and digested with different DNase I options or with no DNase I using 5 technical replicates per extraction condition. The descriptive statistics of the 5 groups are summarised in Table 6.1. Briefly, the median (range) concentrations of miR-16 for VD0, VD1, QD0, QD1 and QD2 were 1.90×10^7 ($1.12 \times 10^7 - 2.44 \times 10^7$), 1.02×10^7 ($9.50 \times 10^6 - 1.90 \times 10^7$), 6.39×10^7 ($5.04 \times 10^7 - 8.49 \times 10^7$), 1.70×10^6 ($7.51 \times 10^5 - 5.00 \times 10^6$) and 4.34×10^6 ($3.63 \times 10^6 - 4.69 \times 10^6$) copies/mL, respectively (Figure

6.2). After RNase A treatment, the readings became 14 (undetectable – 136) for VD0 as well as undetectable in all cases for VD1 and QD1. For QD0 and QD2, miR-16 could be detected in, respectively, 1 case with concentration of 35 copies/mL and 2 cases with concentrations of 15 as well as 117 copies/mL. As for *leptin* DNA concentration, the median (range) concentrations were 135 (85 – 185) and 336 (318 – 530) genome equivalent/mL, repectively, for VD0 and QD0. *Leptin* DNA was not detectable in other groups. Since DNA contamination existed in groups V0 and QD0, they were excluded from further statistical comparison. When comparing the miR-16 concentrations among the groups VD1, QD1 and QD2, significant differences were observed (Kruskal-Wallis ANOVA test, $P = 0.005$) and further statistical analysis indicated that the differences were found between VD1 and QD1, as well as VD1 and QD2 (Student-Newman-Keuls method, $P < 0.05$).

Module 3

When the experiments were performed for VD1, QD1 and QD2 with 10 biological replicates, the median (range) concentrations of miR-16 were 6.38×10^6 ($2.44 \times 10^6 - 1.44 \times 10^7$), 3.73×10^5 ($1.97 \times 10^3 - 3.90 \times 10^6$) and 3.11×10^6 ($8.46 \times 10^5 - 4.98 \times 10^6$) copies/mL, respectively (Figure 6.3). Table 6.1 summarises the

descriptive statistics. The differences were statistically significant (Kruskal-Wallis ANOVA test, $P < 0.001$) and further analysis demonstrated that such significant differences could be observed in every pairwise comparison among the three groups (Student-Newman-Keuls method, $P < 0.05$). After RNase A treatment, the miR-16 could only be detected in 3, 2 and 2 cases, respectively, for VD1, QD1 and QD2 with concentrations of less than 100 copies/mL. As for *leptin* DNA, all samples in VD1 and QD1 demonstrated undetectable level of *leptin* while in QD2, 2 cases showed the presence of *leptin* at a concentration of 1 genome equivalent/mL.

Table 6.1 Descriptive statistics of miR-16 concentrations in different extraction groups.

Module 2 (technical replicates)

Extraction group	Median	Mean	Minimum	Maximum	Standard Error
VD0	1.90×10^7	1.78×10^7	1.12×10^7	2.44×10^7	2.32×10^6
VD1	1.02×10^7	1.20×10^7	9.50×10^6	1.90×10^7	1.80×10^6
QD0	6.39×10^7	6.44×10^7	5.04×10^7	8.49×10^7	5.90×10^6
QD1	1.70×10^6	2.08×10^6	7.51×10^5	5.00×10^6	7.52×10^5
QD2	4.34×10^6	4.22×10^6	3.63×10^6	4.69×10^6	2.06×10^5

Module 3 (biological replicates)

Extraction group	Median	Mean	Minimum	Maximum	Standard Error
VD1	6.38×10^6	6.75×10^6	2.44×10^6	1.44×10^7	1.10×10^6
QD1	3.73×10^5	8.26×10^5	1.97×10^3	3.90×10^6	3.83×10^5
QD2	3.11×10^6	3.08×10^6	8.46×10^5	4.98×10^6	4.17×10^5

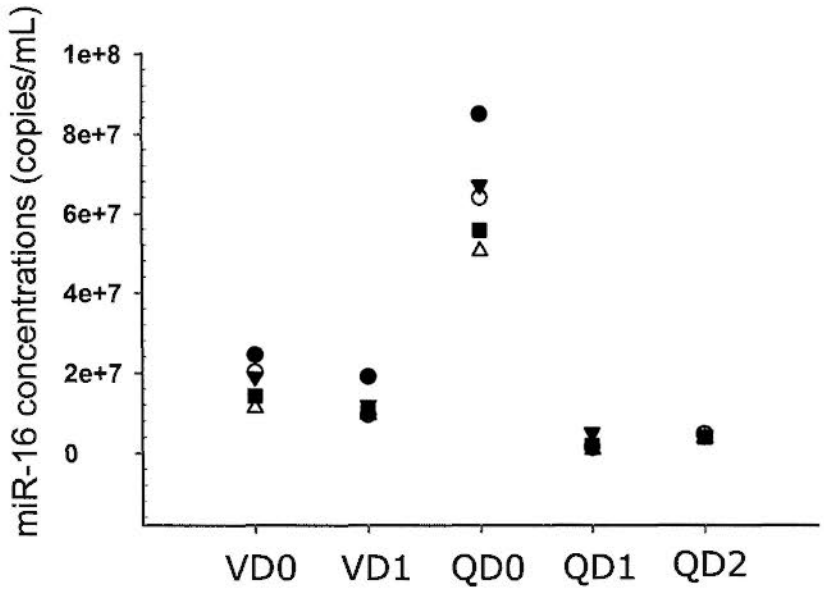


Figure 6.2 Concentrations of plasma miR-16 among technical replicates in different extraction groups (module 2).

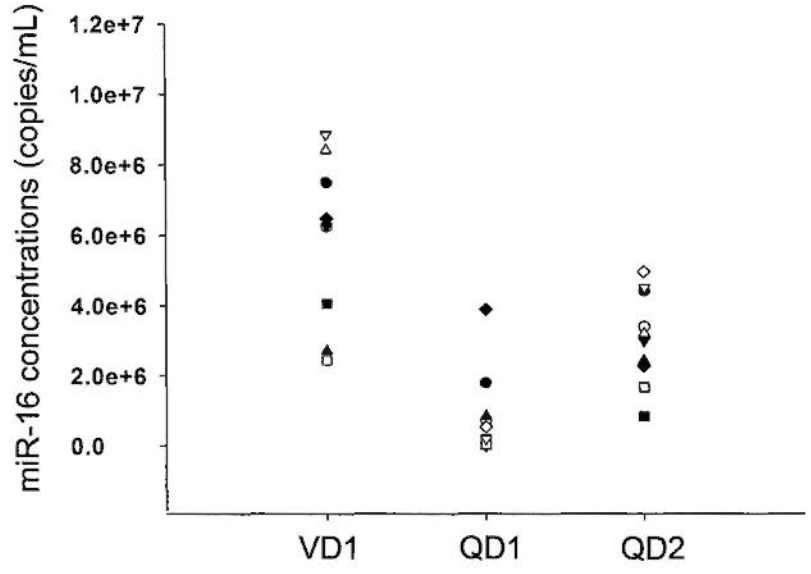


Figure 6.3 Concentrations of plasma miR-16 among biological replicates in different extraction groups (module 3).

6.4 Discussion

The successful detection of placental miRNA in maternal circulation has opened up new possibilities for non-invasive prenatal diagnosis (Chim *et al.*, 2008, Gilad *et al.*, 2008). Past experiences revealed that pre-analytical issues were critical for the detection of biomarkers in plasma which in turn may influence the accuracy and reliabilities of the assays (Chiu *et al.*, 2001, Heung *et al.*, 2009). Since plasma mRNAs and miRNAs possess different physical characteristics (Ng *et al.*, 2002, Chim *et al.*, 2008), effective modifications in plasma mRNA extraction protocols may or may not be equally beneficial for plasma miRNA isolation.

Analysis of the data from module 1 revealed that miRNA yield could also be effectively improved in plasma stored in 3 parts of Trizol LS reagent. Trizol LS reagent is a monophasic solution of phenol and guanidine isothiocyanate, which is capable of denaturing proteins, including ribonucleases, thus preserving the integrity of ribonucleic acids. Because plasma is a protein-rich bodily fluid, increasing the amount of Trizol LS reagent (Invitrogen) might enhance the protein denaturation so that more RNA could possibly be released from its binding cellular proteins. Therefore, despite the fact that the physical nature of plasma mRNAs and miRNAs are different, for example plasma mRNAs were demonstrated to be

associated with particles (Ng *et al.*, 2002) while miRNAs could pass through 0.22 μm filters (Chim *et al.*, 2008), increasing the Trizol LS reagent-to-plasma ratio to 3 was beneficial for enhancing the miRNA concentrations in plasma as for mRNA (reported in Chatper 5).

Besides the preservation of RNA in plasma, the efficiency of the extraction columns used would also be a critical factor for determining the miRNA yield. In module 2 of the study, the miR-16 yield was compared in RNA preparations using 2 different common extraction columns with different DNase treatment options. Among the 5 groups, VD0, VD1, QD0, QD1 and QD2, the groups without DNase treatment (VD0 and QD0) resulted in the highest miR-16 yield. However, the QPCR data of *leptin* demonstrated that the DNA contamination existed in these 2 groups. Thus, these two groups were excluded from further analysis. Among the remaining groups, as shown in Figure 6.2, the scatter points for group QD2 were the tightest with the lowest standard error of 2.06×10^5 which was 4.88% of its mean. However, the miR-16 concentration in group VD1 was significantly higher than the other groups with standard error of 1.80×10^6 which was 15.02% of its mean. The substantial decrease in the miR-16 concentration after the RNase A treatment indicated that the TaqMan miR-16 assay was specific to RNA. These

data suggested that the group VD1 resulted in the highest miR-16 yield in plasma samples with a reasonable standard error.

When the experiments were performed in 10 biological replicates for VD1, QD1 and QD2 in module 3, the conclusion was similar to that in module 2 whereby VD1 showed statistically significantly higher miR-16 yield. In module 2, the QD2 group showed lower standard error. However, in the current experiment, although the standard error of QD2 remained the lowest among the 3 groups, it became 13.53% of its mean whereas that of VD1 was 16.34% of its mean which was similar to that found in module 2. The substantial decrease in the miR-16 concentration after the RNase A treatment in both module 2 and module 3 indicated that the TaqMan miR-16 assay was specific to RNA detection.

Concluding from these data, VD1, i.e. the *mir*Vana miRNA isolation columns with off-column DNase I treatment, showed statistically significantly higher miR-16 yield compared with the other protocols. Furthermore, the RNA preparations from VD1 were confirmed to be free from DNA contamination. There are mounting evidence that miRNA plays an important role in regulating gene expression. With the development of the miRNA microarray technology, more disease-associated

miRNA species may possibly be identified in near future. It is hopeful that this evaluation work may facilitate the detection of circulating miRNA candidate markers in plasma for non-invasive diagnosis of various diseases.

SECTION V: SYSTEMATIC IDENTIFICATION OF CIRCULATING BRAIN-DERIVED mRNA

The study presented in this section integrates and adopts the technologies and protocols described in the previous chapters to identify brain-derived mRNA markers in a systematic manner.

CHAPTER 7: SYSTEMATIC IDENTIFICATION OF CIRCULATING BRAIN-DERIVED mRNA TRANSCRIPTS AS BIOMARKERS OF BRAIN INJURY

7.1 Introduction

In developed counties, injuries are the major cause of death under the age of 45 years and brain injury causes up to half of all trauma deaths (Shackford *et al.*, 1993, Jennett, 1996, Acosta *et al.*, 1998, Sundstrom *et al.*, 2007). Current assessment of brain injury largely relies on clinical features, imaging findings and physiological features of the patient. The Glasgow Coma Scale (GCS), which is commonly used to objectively assess the degree of impaired consciousness, is a powerful predictor of prognosis and a low initial score is associated with a poor outcome (Teasdale and Jennett, 1974, Choi *et al.*, 1988). However, controversy exists as to the timing of GCS score determination (Marion and Carlier, 1994). Computed tomography (CT) is insensitive to lesions within the brainstem due to the small size of the structures involved and their proximity to the surrounding bone (Mittl *et al.*, 1994). Magnetic resonance imaging (MRI) can demonstrate lesions of the white matter and brainstem that often are missed by CT scans (Mittl *et al.*, 1994, Orrison *et al.*, 1994). However, it could not be applied to patients with pacemakers and certain ferromagnetic appliances.

Appropriate biomarkers should be able to provide additional objective information to aid clinical decision making. Some researchers believe that insults such as traumatic and ischaemic brain injury are routinely accompanied by either regional or global energy failure, and associated with disturbed protein synthesis, so their focus has mainly been placed on protein markers (Kochanek *et al.*, 2008). At the moment, S100B protein has been the mo

st well studied marker and its elevation in serum of brain-injured patient was reported to be associated with poor outcome (Raabe *et al.*, 1999, Woertgen *et al.*, 1999, Townend and Ingebrigtsen, 2006). However, the sensitivity of the test has not been satisfactory. S100B protein concentration below the threshold value did not necessarily indicate that the patient had a better outcome (Savola and Hillbom, 2003). Besides protein biomarkers, circulating plasma DNA concentration was demonstrated to be elevated in traumatic brain injury patients (Campello Yurgel *et al.* 2007) and was shown to be correlated with stroke severity (Rainer *et al.* 2003). Yet, the optimal plasma DNA cutoff values for diagnosis or prognosis remained uncertain. Further investigations would be needed to validate and refine the cutoff values for clinical applications.

Studies have indicated that brain injury could activate both necrotic and apoptotic cell death (Raghupathi *et al.*, 2000, Tashlykov *et al.*, 2007). Actually, apoptosis in placenta has been speculated to be the source of fetal mRNAs in maternal circulation (Hahn *et al.*, 2005, Smith *et al.*, 1997). Based on the successful experience of applying placental mRNA detection for non-invasive prenatal diagnosis, our group hypothesised that brain-derived mRNAs could be released into the circulation during brain injury. Since serum S100B protein was previously reported to be elevated in brain-injured patients, Chan *et al.*, attempted to detect its mRNA in the plasma of stroke patients and healthy individuals (Chan *et al.*, 2007). Surprisingly, the *S100B* mRNA was detectable in both groups. The authors further proved with a bone marrow transplantation model that the detectable plasma *S100B* mRNA molecules were predominantly of haematopoietic origin.

In light of these previous findings, in this part of thesis, I aimed to identify brain-specific mRNA transcripts systematically and to investigate their detectability in plasma. Brain injury in this study was defined as haemorrhage in the brain including traumatic brain injury and spontaneous hypertensive intra-cerebral haemorrhage that required open craniotomy for clot evacuation.

7.2 Materials and Methods

7.2.1 Study design

Using microarray analysis, the gene expression profiles of brain tissue and peripheral blood from brain-injured patients were compared. Transcripts with high expression level in brain tissue but low expression level in peripheral blood were identified and further validated by QRT-PCR. Their detectability in peripheral and jugular plasma of brain-injured patients as well as in peripheral plasma of non-brain-injured patients was studied. As CSF is an important part of the nervous system, these markers were also tested in CSF.

7.2.2 Subjects and sample processing

Fourteen brain-injured patients admitted to the Prince of Wales Hospital for neurosurgical operation were recruited. A small part of the brain tissue that would otherwise be discarded due to the procedural need of the operation was collected and processed following procedures described in Chapter 3.1.6. Peripheral and jugular blood was collected into EDTA tubes and processed as per protocol I described in Chapter 3.1.2 and 4.8 mL of Trizol LS reagent was added into 1.6 mL plasma samples. The first four among the 14 cases were included for microarray analysis. For these cases, 2 mL of peripheral blood collected in EDTA tubes was

processed as mentioned in Chapter 3.2.2.2. For cases with CSF available, the samples were processed as detailed in Chapter 3.1.4.

Fourteen patients without brain injury but subjected to spinal anaethesia were also recruited as non-brain-injured controls in the Prince of Wales Hospital. Peripheral blood was collected into EDTA tubes and processed with protocol I as described in Chapter 3.1.2. CSF was collected into plain tubes and handled following the procedures described in Chapter 3.1.4. Two filtered CSF samples and two unfiltered CSF samples were collected.

7.2.3 RNA extraction

Plasma, whole blood, buffy coat and CSF RNA were isolated as described in Chapter 3.2.2.

7.2.4 Microarray analysis

From each of the four brain-injured patients, 2 mL of peripheral blood, 1 piece of grey matter brain tissue and 1 piece of white matter brain tissue were collected for microarray analysis. 5 μg of extracted RNA from each sample was reverse transcribed, labeled and hybridised to the GeneChipH Human Genome U133A and U133B Arrays (Affymetrix, Santa Clara, CA) according to manufacturer's

instructions. After hybridisation, each array chip was washed, stained and scanned as reported *(Tsui et al.*, 2004). The experimental details were described in Chapter 3.5. The data were analysed with GeneSpring v 7.2 (Agilent Technologies, Palo Alto, CA).

The microarray data in the .CEL format were imported and normalised using the following steps in sequence: (1) raw data processing by Robust Multi-chip Average, with GC-content background correction (GC-RMA); (2) data transformation whereby microarray data with values below 0.001 were set to 0.001; and (3) the signal intensity for each gene was divided by the median of its measurements in all samples. After filtering the gene-list with 95% confidence interval and non-parametric t-test analysis (P = 0.05), 9,197 gene transcripts demonstrated differential expression between grey matter brain tissue and peripheral blood samples. 5,105 gene transcripts demonstrated differential expression between white matter brain tissue and peripheral blood samples.

To mine for brain-specific transcripts, the gene lists were further filtered by selecting transcripts with 10-fold higher expression in the brain tissues compared with peripheral blood samples. The genes were sorted in descending order of

fold-difference.

7.2.5 Quantitative real-time RT-PCR

Myelin basic protein (MBP), glycoprotein M6B (GPM6B), glial fibrillary acidic protein (GFAP) and *proteolipid 1 (PLP1)* mRNA transcripts were quantified using one-step QRT-PCR. Calibration curves were prepared by serial dilutions of HPLC-purified single-stranded synthetic DNA oligonucleotides (Proligos, Singapore) with the amplicon specified and as described in Chapter 3.3.2. The sequence information of the primers, probes and calibrators as well as the reaction conditions are summarised in Table 3.1. QRT-PCR was set up as described earlier in Chapter 3.3.2.2.

7.2.6 Statistics

Statistical analyses were performed using Sigma Stat (v. 3.0, Systat).

7.3 Results

7.3.1 Gene expression profile analysis

The gene expression profiles of four sets of grey or white matter brain tissues and four peripheral blood samples were compared using GeneSpring® v 7.2, (Agilent

Technologies) software. For brain-specific marker mining, the final gene lists were generated by selecting transcripts with 10-fold higher expression in the brain tissue than peripheral blood samples and resulted in 918 and 897 gene transcripts in the gene list for grey matter and white matter respectively. These genes were sorted in descending order of fold-difference. Among the top 15 genes in the two gene lists, 12 of them were found in both lists with similar ranking (Tables 7.1 and 7.2). Therefore the top five gene transcripts including *MBP, SPARCL1, GPM6B, GFAP* and *PLP1* were selected for further analysis. Besides, the microarray data for these five transcripts from SymAtlas v1.2.4, which is a public database comparing the expression of Affymatrix U133A probesets in different tissues, were also taken as reference (Figures 7.1 – 7.3) (Su *et al.*, 2004a). Since *SPARCL1* was shown to be expressed in a variety of tissue types, it was excluded from downstream analysis.

Table 7 1 List of genes more highly expressed in white matter than whole blood of brain-injured patients

Probe Set ID	Fold Change	Abs. Expression	Gene Name	Description	RefSeq
209072_at	1597 0	8,516	MBP	Myelin basic protein	NM_001025081
200795_at	1571 0	11,258	SPARCL1	Islet cell autoantigen 1, 69kDa	NM_004684
209170_s_at	922 1	7,752	GPM6B	Glycoprotein M6B	NM_001001994
203540_at	653 7	4,664	GFAP	Glial fibrillary acidic protein	NM_002055
209167_at	640 6	6,611	GPM6B	Glycoprotein M6B	NM_001001994
210198_s_at	537 4	6,036	PLP1	Proteolipid protein 1	NM_000533
225491_at	477 4	8,183	SLC1A2	Solute carrier family 1, member 2	NM_004171
226228_at	460 6	6,797	AQP4	Aquaporin 4	NM_001650
201893_x_at	453 6	2,384	DCN	Decorin	NM_001920
201667_at	443 7	4,404	GJA1	Gap junction protein, alpha 1	NM_000165
218309_at	404 0	1,723	CaMKIINalpha	G protein-coupled receptor 30	NM_018584
201242_s_at	402 9	4,707	ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	NM_001001787
201117_s_at	386 2	3,181	CPE	Carboxypeptidase E	NM_001873
201387_s_at	346 3	1,879	UCHL1	Ubiquitin carboxyl-terminal esterase L1	NM_004181
202508_s_at	313 4	3,511	SNAP25	Synaptosomal-associated protein	NM_003081

Table 7.2 List of genes more highly expressed in grey matter than whole blood of brain-injured patients.

Probe Set ID	Fold Change	Abs. Expression	Gene Name	Description	RefSeq
209072_at	2624.0	13,989	MBP	Myelin basic protein	NM_001025081
200795_at	1390.0	9,959	SPARCL1	Islet cell autoantigen 1, 69kDa	NM_004684
209170_s_at	1267.0	10,651	GPM6B	Glycoprotein M6B	NM_001001994
210198_s_at	869.6	9,769	PLP1	Proteolipid protein 1	NM_000533
209167_at	843.1	8,700	GPM6B	Glycoprotein M6B	NM_001001994
203540_at	672.1	4,795	GFAP	Glial fibrillary acidic protein	NM_002055
225275_at	503.6	4,042	EDIL3	EGF-like repeats and discoidin I-like domains 3	NM_005711
225491_at	446.1	7,646	SLC1A2	Solute carrier family 1, member 2	NM_004171
203400_s_at	438.9	2,970	TF	Transferrin	NM_001063
226228_at	432.4	6,380	AQP4	Aquaporin 4	NM_001650
218309_at	392.5	1,674	CaMKIINalpha	G protein-coupled receptor 30	NM_018584
201893_x_at	388.4	2,041	DCN	Decorin	NM_001920
201667_at	382.9	3,801	GJA1	Gap junction protein, alpha 1	NM_000165
223699_at	368.7	1,488	CNDP1	Carnosine dipeptidase 1	NM_032649
201242_s_at	366.3	4,280	ATP1B1	ATPase, Na+/K+ transporting, beta 1	NM_001001787

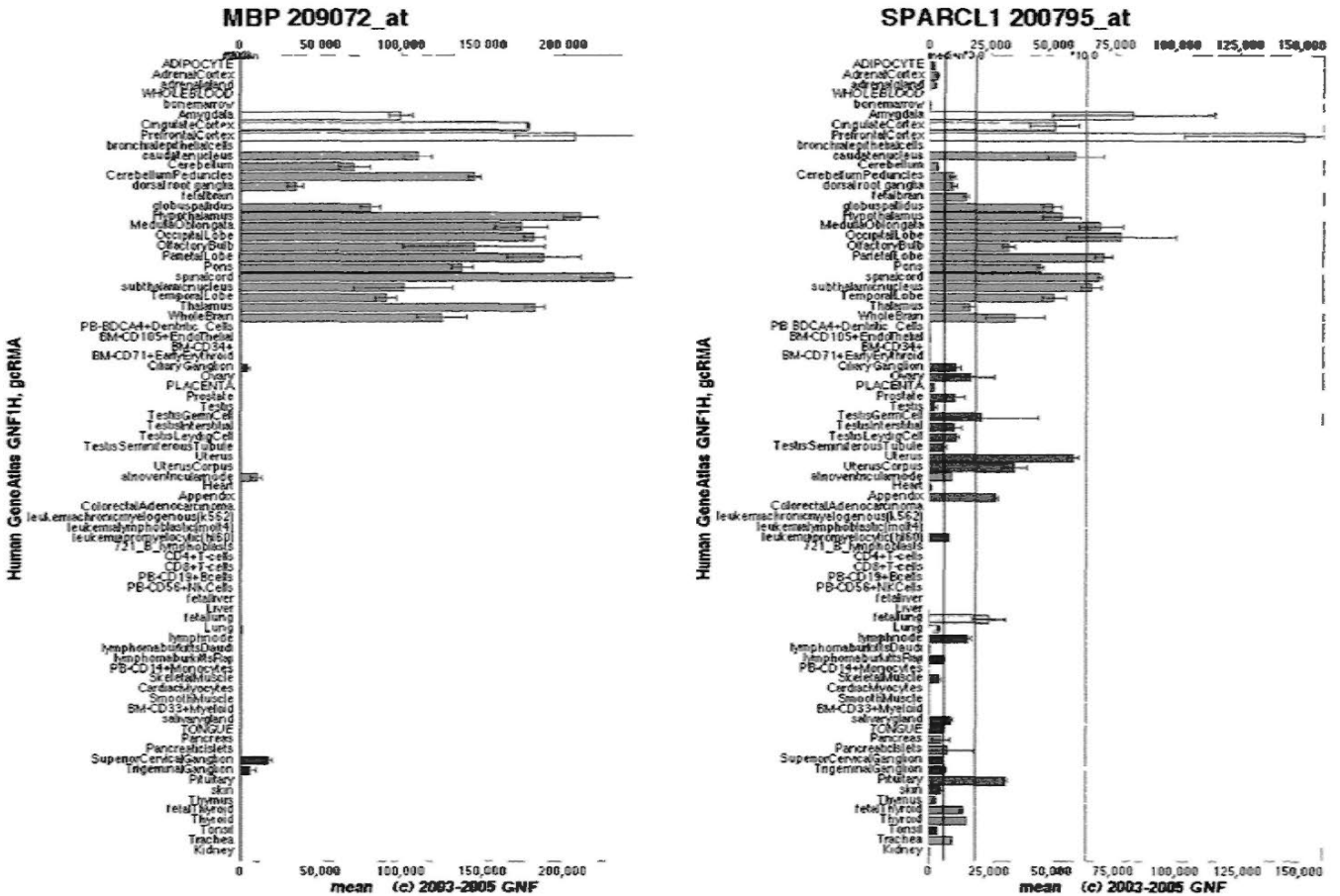


Figure 7.1 Bar-charts adopted from Human GeneAtlas GNF1H showing expression of *MBP* (209072_at) and *SPARCL1* (200795_at) in different human tissues (http //symatlas gnf org/SymAtlas).

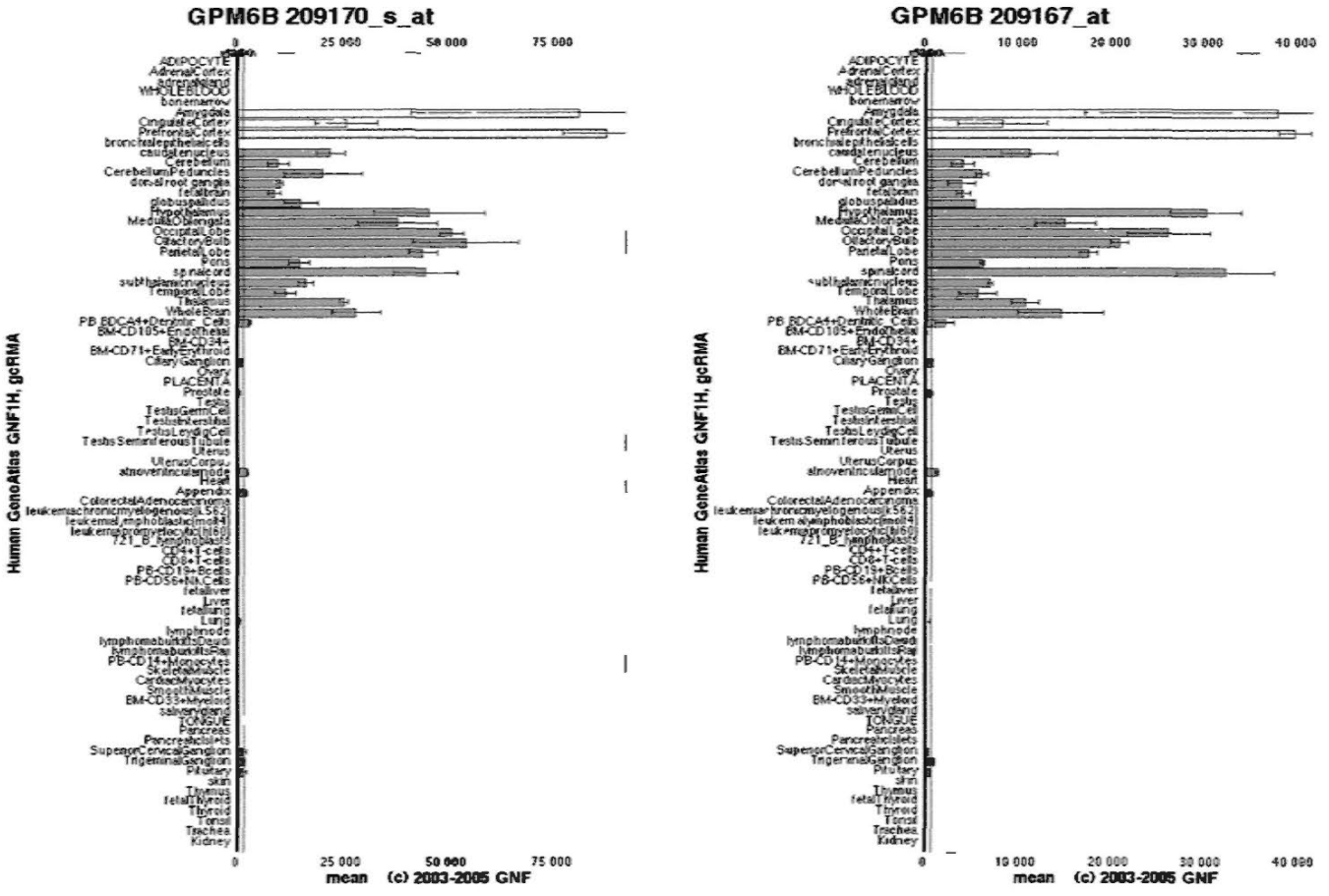


Figure 7 2 Bar-charts adopted from Human GeneAtlas GNF1H showing expression of *GPM6B* (209170_s_at) and (209167_at) in different human tissues (http://symatlas.gnf.org/SymAtlas)

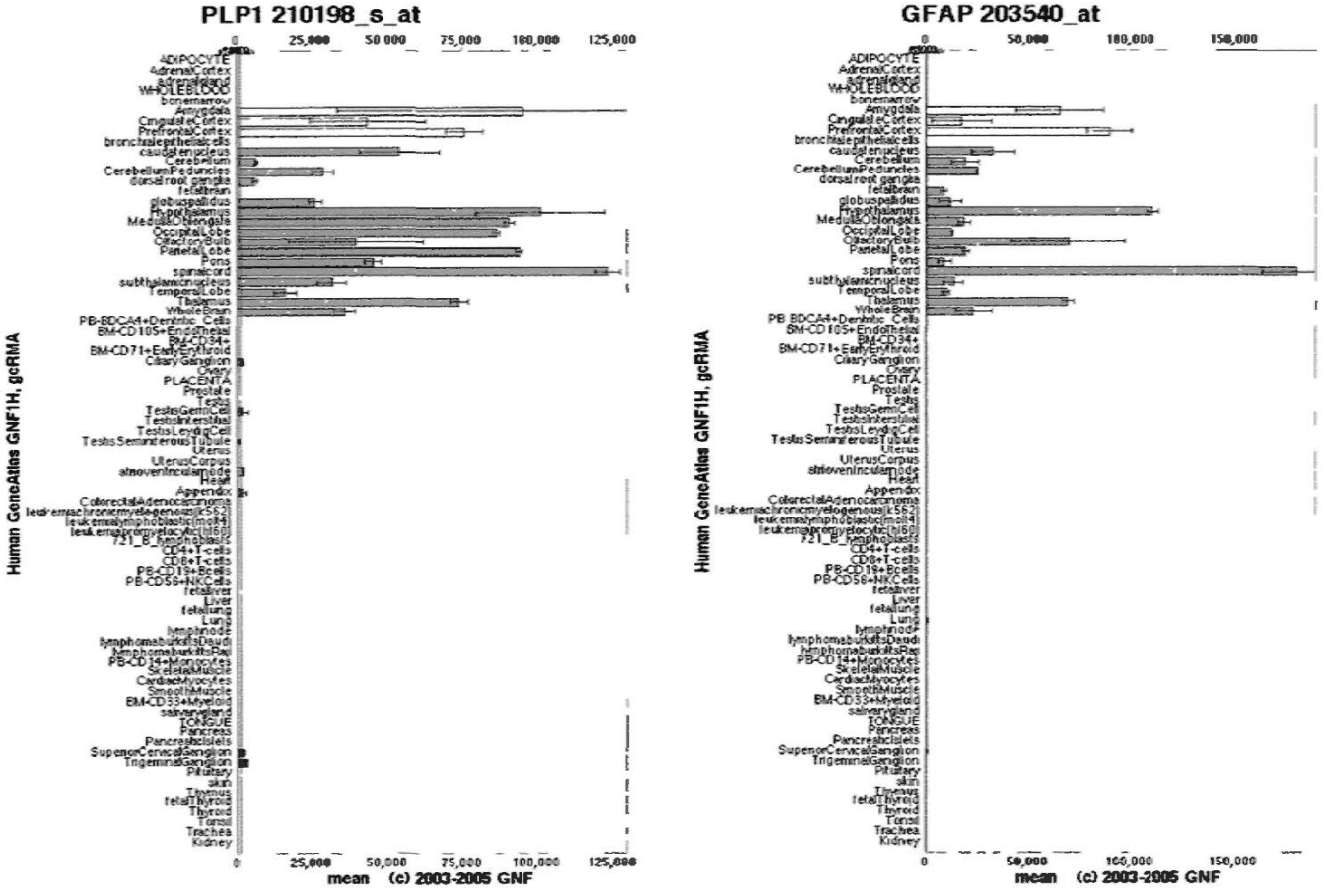
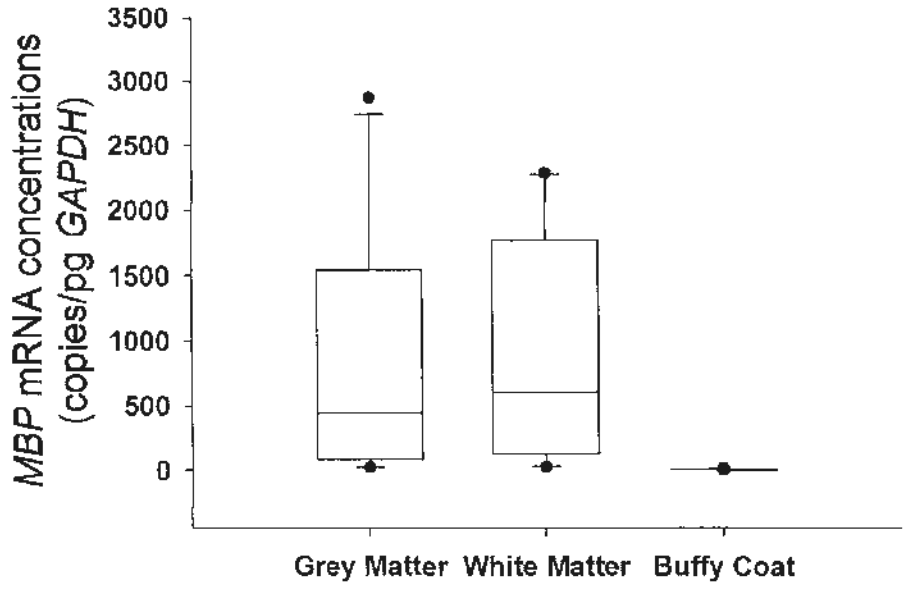


Figure 7.3 Bar-charts adopted from Human GeneAtlas GNF1H showing expression of *PLP1* (210198_s_at) and *GFAP* (203540_at) in different human tissues (http //symatlas gnf org/SymAtlas).

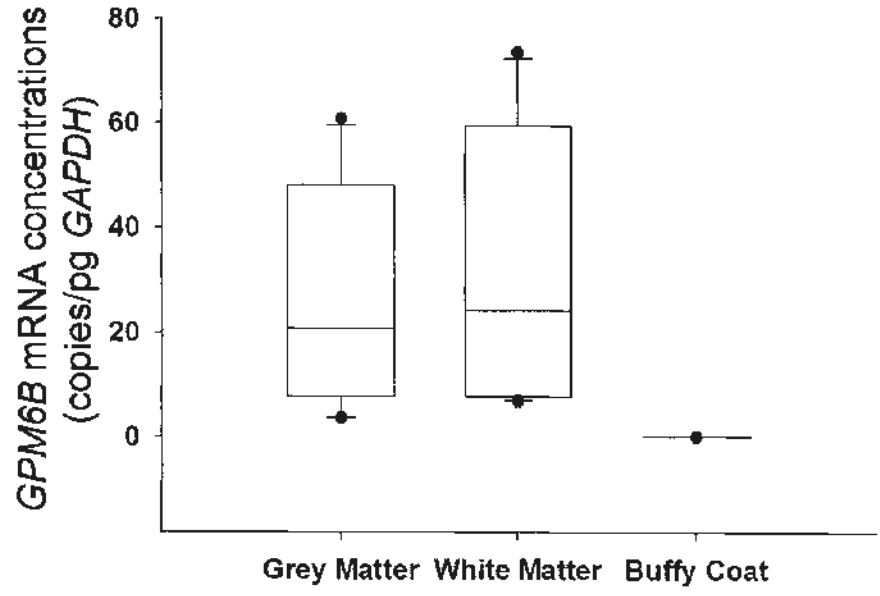
7.3.2 Expression of the brain-derived mRNA in brain tissues and blood cells

Brain tissues of grey matter and white matter as well as blood cells from ten brain-injured patients were analysed. The median (range) concentrations for *MBP*, *GPM6B*, *GFAP* and *PLP1* mRNA in grey matter were 443 (20 – 2,869), 21 (4 – 61), 142 (13 - 629) and 253 (3 – 1,235) copies/pg *GAPDH*, respectively. The median (range) concentrations for *MBP*, *GPM6B*, *GFAP* and *PLP1* mRNA in white matter were 600 (24 – 2,287), 24 (7 – 73), 152 (26 – 768) and 428 (10 – 1,608) copies/pg *GAPDH*, respectively. In blood cells, *GPM6B*, *GFAP* and *PLP1* mRNA was undetectable at all cases while *MBP* was detectable in five cases but with a median (range) concentration of undetectable (undetectable – 7) copies/ pg *GAPDH* (Figure 7.4).

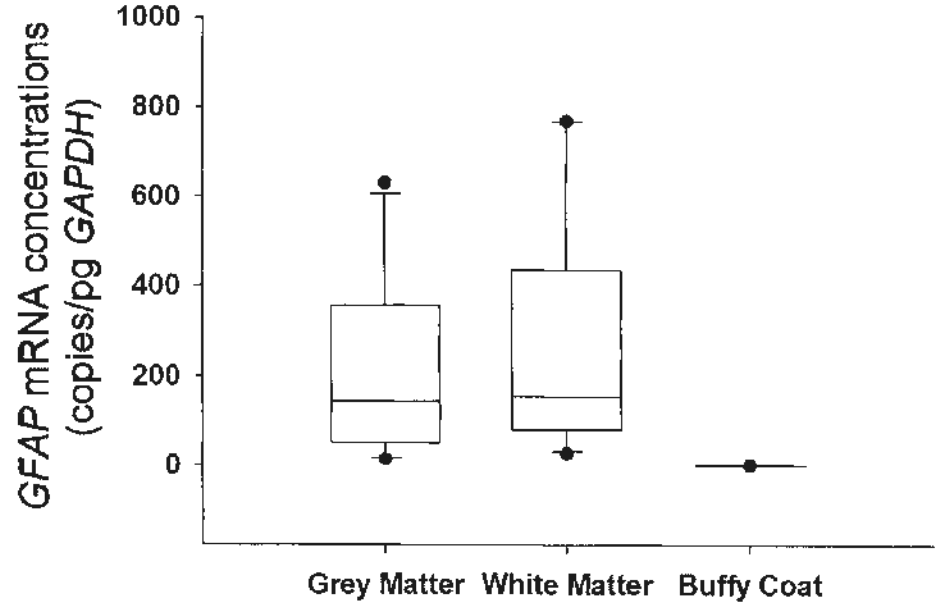
(A)



(B)


GPM6B mRNA concentrations (copies/pg GAPDH)
80
60
40
20
0
Grey Matter
White Matter
Buffy Coat

(C)


GFAP mRNA concentrations (copies/pg GAPDH)
1000
800
600
400
200
0
Grey Matter
White Matter
Buffy Coat

(D)

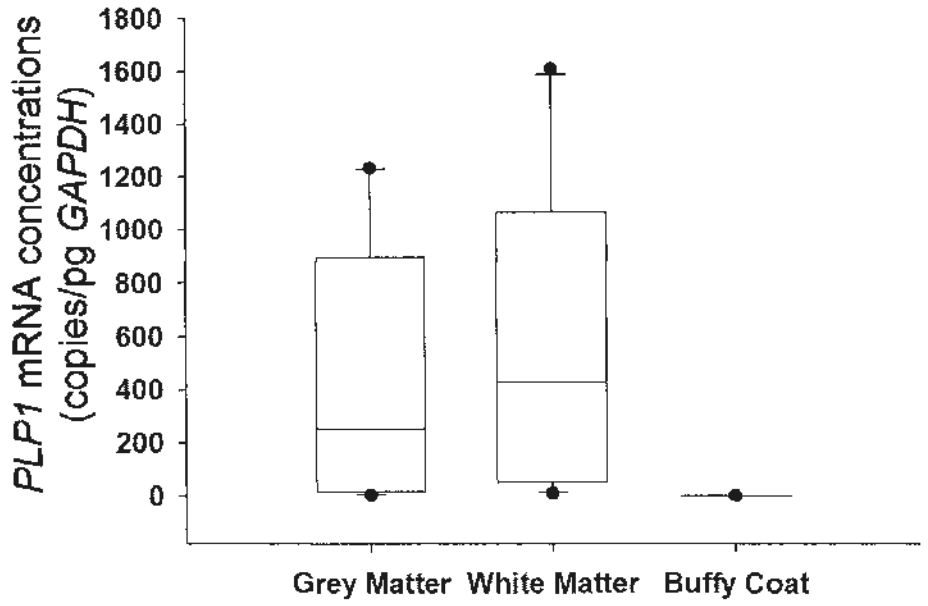


Figure 7.4 **Concentrations of brain-derived mRNA in grey matter, white matter brain tissue and buffy coat of brain-injured patients.** Box plots of (A) *MBP* (B) *GPM6B* (C) *GFAP* (D) *PLP1* mRNA. The line inside each box denotes the median. The lower and upper limits denote the 25th and 75th percentiles, respectively. The lower and upper whiskers denote the 10th and 90th percentiles, respectively. Filled circles denote the outliers.

7.3.3 Plasma detectability of the brain mRNA markers

The plasma detectability of *MBP*, *GPM6B*, *GFAP* and *PLP1* mRNA was determined by analysing the presence of the brain mRNA in both the jugular and peripheral plasma from 14 brain-injured patients. *MBP* mRNA was detectable in

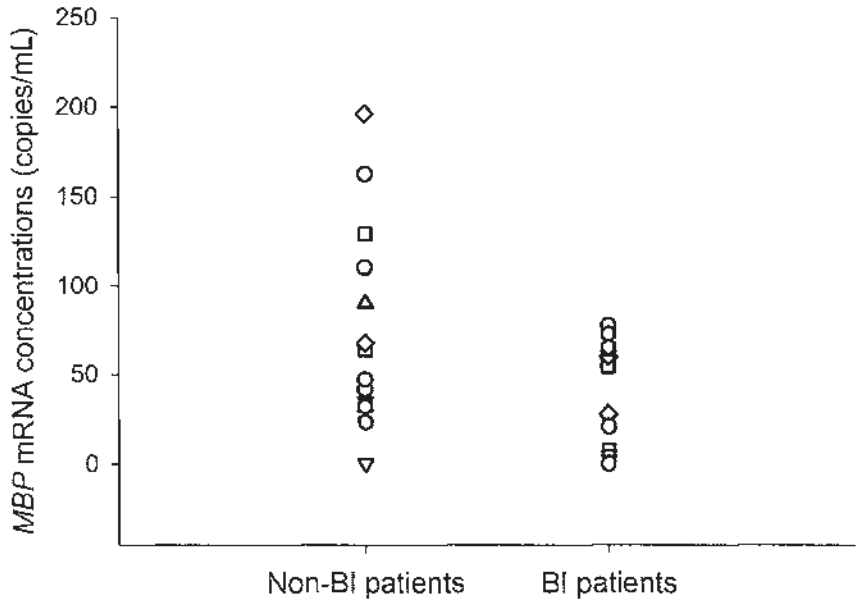
13 cases in both peripheral and jugular plasma with median (range) concentrations of 54 (undetectable – 77) and 28 (undetectable– 605) copies/mL, respectively. Only six peripheral plasma samples and 2 jugular plasma samples showed the presence of *GPM6B* mRNA with median (range) concentrations of 13 (4 – 87) copies/mL for peripheral plasma as well as 3 and 147 copies/mL for jugular plasma. For *GFAP* mRNA, it could be detected in 3 peripheral and 4 jugular plasma samples in a median (range) concentrations of 73 (29 – 225) and 111 (20 – 169) copies/mL, respectively. *PLP1* mRNA was detected in 2 and 4 cases of peripheral and jugular plasma respectively. The concentrations were 23 and 25 copies/mL in peripheral plasma while the median (range) concentrations were 44 (19 – 583) copies/mL in jugular plasma. *GAPDH* mRNA was detectable in all the samples with median (range) concentrations of 305 (2 – 1134) and 225 (13 – 2892) pg *GAPDH*/mL in peripheral and jugular plasma, respectively.

7.3.4 Comparison in the brain-derived mRNA expression between peripheral plasma from brain-injured and non-brain-injured patients

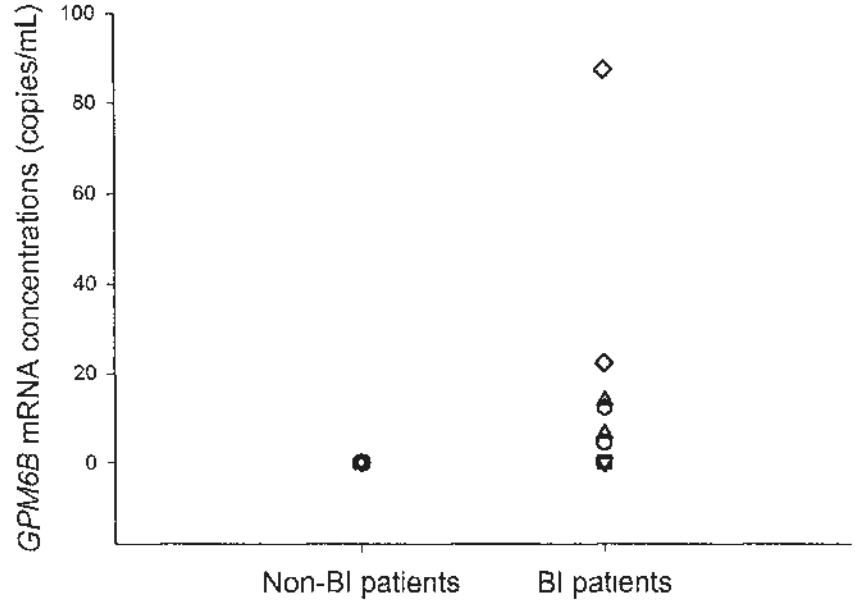
The concentrations of *MBP*, *GPM6B*, *GFAP* and *PLP1* mRNA were compared in peripheral plasma samples from 14 brain-injured and 14 non-brain-injured patients. *MBP* mRNA was detectable in 13 cases in plasma from each patient groups with

median (range) concentrations of 54 (undetectable – 77) copies/mL in brain-injured patients and 55 (undetectable – 196) copies/mL in non-brain-injured patients. The difference between the two groups was not statistically significant (P = 0.183, Mann-Whitney test). *GPM6B* and *GFAP* mRNA was not detectable at all in non-brain-injured plasma while in brain-injured patients, they were detectable in 6 cases with a median (range) concentration of undetectable (undetectable – 87) copy/mL for *GPM6B* and in 3 cases with a median (range) concentration of undetectable (undetectable –73) copy/mL for *GFAP*. Two brain-injured and five non-brain-injured peripheral plasma samples showed the presence of *PLP1* mRNA with median (range) concentrations of undetectable (undetectable – 25) and undetectable (undetectable – 363) copy/mL, respectively (Figure 7.5). The plasma concentration differences between the brain-injured and non-brain-injured patients were statistical significant for *GPM6B* mRNA (P = 0.003, Mann-Whitney test) but not for both *GFAP* mRNA (P = 0.079, Mann-Whitney test) and *PLP1* mRNA (P = 0.156, Mann-Whitney test).

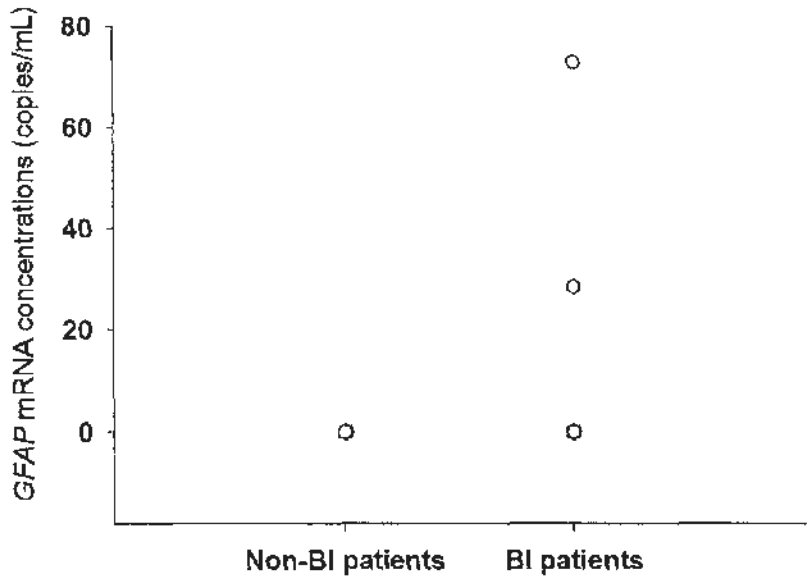
(A)


250
200
150
100
50
0
MBP mRNA concentrations (copies/mL)
Non-BI patients
BI patients

(B)


100
80
60
40
20
0
GPM6B mRNA concentrations (copies/mL)
Non-BI patients
BI patients

(C)



(D)

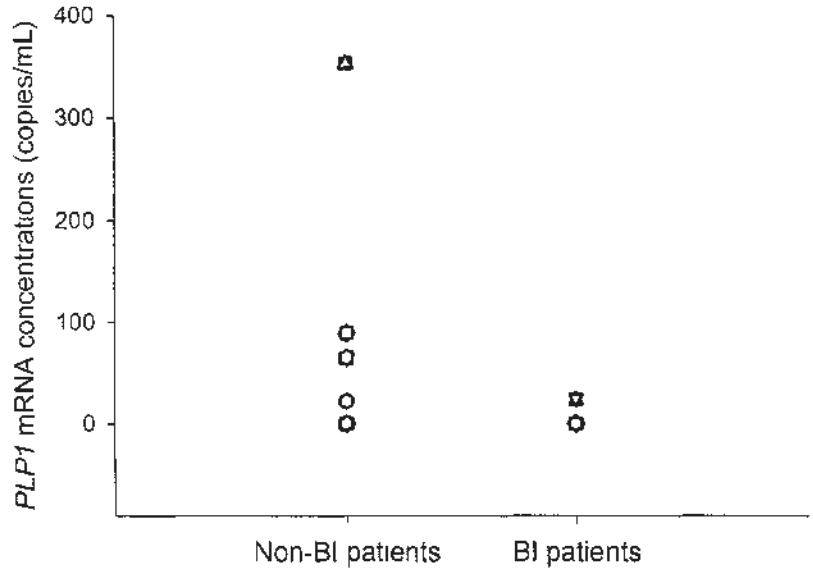


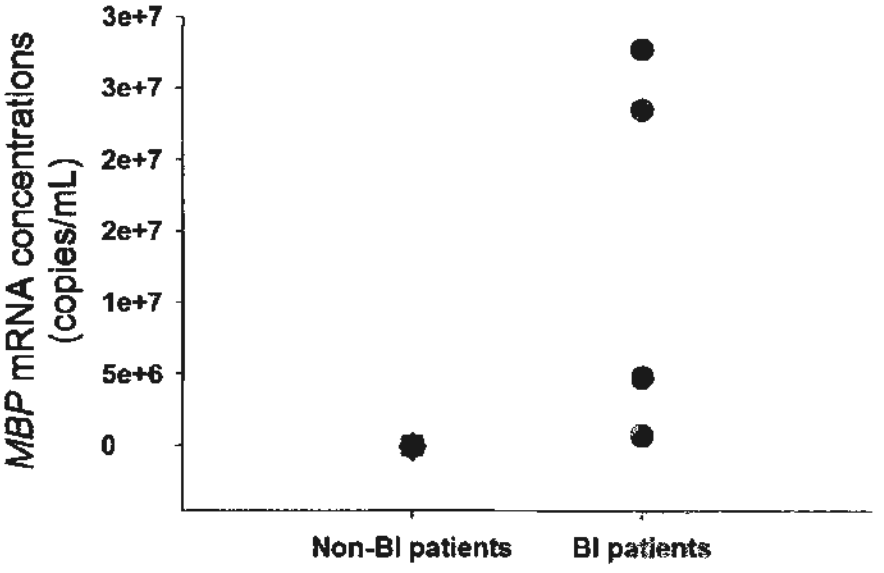
Figure 7.5 Concentrations of brain-derived mRNA in peripheral plasma of brain-injured (BI) and non-brain-injured (Non-BI) patients. Scatter plots of (A) *MBP* (B) *GPM6B* (C) *GFAP* (D) *PLP1* mRNA.

7.3.5 Detection of the brain-derived mRNA transcripts in CSF

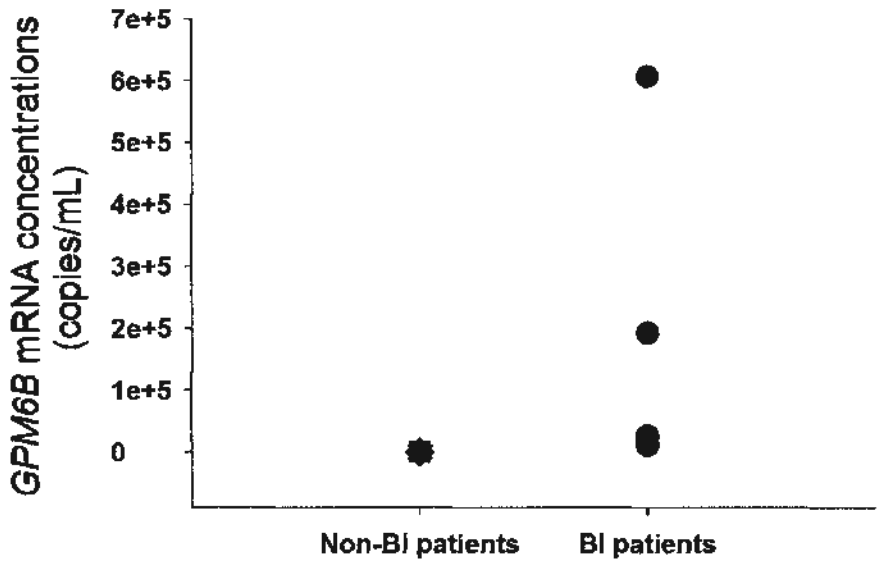
The concentrations of *MBP*, *GPM6B*, *GFAP* and *PLP1* mRNA were assessed in 10 filtered CSF samples from non-brain-injured patients as well as 2 filtered CSF and 2 unfiltered CSF samples from brain-injured patients. *MBP* and *GFAP* mRNA could be detected in all CSF samples. The median (range) *MBP* mRNA concentration was 252 (135 – 1,291) copies/mL in CSF from non-brain-injured patients. In CSF from brain-injured patients, the *MBP* mRNA concentrations were 23,494,894 and 27,723,870 copies/mL in the unfiltered samples as well as 759,787 and 4,832,704 copies/mL in the filtered samples which were 84,420 and 536,967 times higher than the median concentration of non-brain-injured CSF respectively. As for *GFAP* mRNA, the median (range) concentration was 137 (14 – 314) copies/mL in CSF from non-brain-injured patients. The *GFAP* mRNA concentrations were 6,880,050 and 7,717,148 copies/mL in unfiltered CSF from brain-injured patients while they were 1,120,551 and 312,694 copies/mL in the filtered brain-injured samples which were 8,179 and 2,282 times higher than the median concentration of non-brain-injured CSF respectively. *GPM6B* mRNA was undetectable in non-brain-injured CSF with 606,233 and 191,803 copies/mL in unfiltered brain-injured CSF as well as 25,219 and 11,762 copies/mL in the filtered portion. In the case of *PLP1* mRNA, it could be detected in 6 non-brain

injured CSF samples with a median (range) concentration of 20 (undetectable – 128) copies/mL while it was 8,311,590 and 1,298,710 copies/mL in unfiltered brain-injured CSF as well as 252,930 and 181,335 copies/mL in filtered brain-injured CSF which were 12,646 and 9,066 times higher than the median of concentration of non-brain-injured CSF respectively (Figure 7.6). Table 7.3 showed that more than 500 µL of blood cells were needed to contribute to the amount of *MBP* mRNA detected in brain-injured CSF.

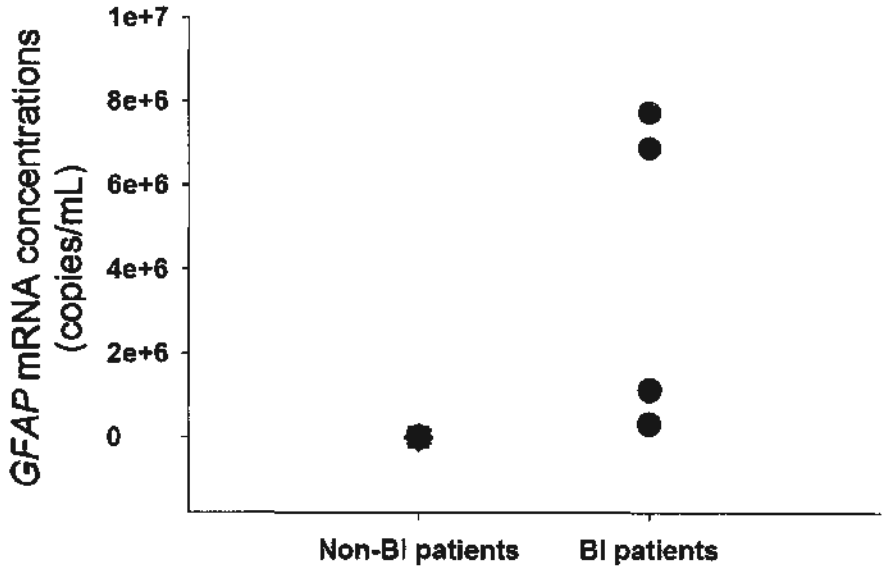
(A)



(B)


GPM6B mRNA concentrations (copies/mL)
7e+5
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
Non-BI patients
BI patients

(C)


GFAP mRNA concentrations (copies/mL)
1e+7
8e+6
6e+6
4e+6
2e+6
0
Non-BI patients
BI patients

(D)

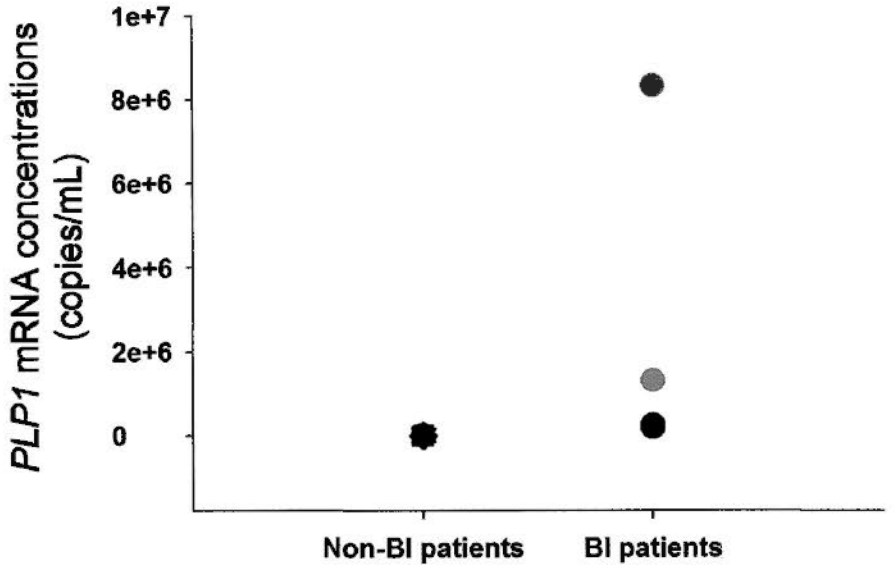


Figure 7.6 **Concentrations of brain-derived mRNA in CSF of brain-injured (BI) and non-brain-injured (Non-BI) patients.** Black dots and blue dots indicate filtered and unfiltered CSF, respectively. Scatter plots of (A) *MBP* (B) *GPM6B* (C) *GFAP* (D) *PLP1* mRNA.

Table 7.3 Estimation of buffy coat RNA equivalent to *MBP* mRNA in CSF

	Sample No.	*MBP* (copies/mL)	Equivalent to BC RNA (ng)	Equivalent to BC vol. (μL)
Unfiltered CSF	1	27723870	449514	20124
	2	23494894	380945	70545
Filtered CSF	3	759787	12319	551
	4	4832704	78357	9515

* BC denotes for buffy coat.

7.4 Discussion

Based on the experience in prenatal diagnosis, circulating tissue-specific mRNA markers may possible be an alternative to protein markers in the clinical assessment of brain injury. As a biomarker for brain injury, the mRNA transcript needs to be originated from brain and be detectable in plasma. Therefore, the gene profiles of brain tissue and peripheral blood were first compared systematically attempting to find out transcripts that were highly expressed in the brain but with low expression in blood cells. 918 and 897 gene transcripts that demonstrated a 10-fold higher expression in the brain tissue than peripheral blood samples were in the gene list generated from grey matter and white matter respectively. Among the top 15 genes in the two gene lists, 12 of them were found in both lists with similar rankings (Tables 7.1 and 7.2). The five genes demonstrated the largest fold difference in intensity between the two tissue types were selected including *MBP, SPARCL1, GPM6B, GFAP* and *PLP1*. Since *SPARCL1* was also shown to be expressed in a variety of tissues for example uterus, appendix, pituitary and thyroid according to data in the public database, SymAtlas v1.2.4, Genomics Institute of the Novartis Research Foundation (Figure 7.1), it was excluded for further analysis. *MBP* is a major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the nervous system (Barbarese *et al.*, 1999,

Simons and Trotter, 2007). *GPM6B* is expressed in oligodendrocytes and neurons (Olinsky *et al.*, 1996, Werner *et al.*, 2001, Yan *et al.*, 1993). *GFAP* encodes one of the major intermediate filament proteins of mature astrocytes (Laping *et al.*, 1994) and its protein concentration in CSF was increased in cerebrospinal ischaemia (Anderson *et al.*, 2003) while *PLP1* is present in the central nervous system and functions in myelination (Nave *et al.*, 1987).

The brain predominance of the transcripts *GFAP, GPM6B* and *PLP1* was further confirmed by the QRT-PCR data that the expression level in both grey and white matter was high and was absent in blood cells. Though *MBP* could be detected in some blood cells, it demonstrated a very low level of expression.

Although these transcripts were proven to be highly expressed in brain tissues, their detectability in plasma was sub-optimal. The detectability of *GFAP, GPM6B* and *PLP1* mRNA in peripheral plasma from brain-injured patients was less than 36% but they were absent in peripheral plasma from non-brain-injured patients. Since jugular vein carries the venous return from the brain and is the first of such veins accessible external to the cranium, the concentrations of the brain-derived transcripts may possibly be higher. Nevertheless, our data showed that the jugular

plasma detectabilities of these transcripts were less than 30%. The detectability was not higher in jugular than peripheral plasma.

For *MBP*, the plasma detectabilities for both peripheral and jugular plasma samples from brain-injured patients were 93%. However, it was also present in the peripheral plasma from non-brain-injured patients at equally high detectability with no significant difference in concentration. This suggested that the detected *MBP* transcripts may not be originated from the brain. This could be due to the non-specificity of the assay. Grima *et al* reported a *MBP*-related transcript, namely *Golli-MBP,* which is expressed in the bone marrow and the immune system. *Golli-MBP* contains 3 additional exons located upstream of the exons of classic *MBP* which is expressed in the nervous system (Grima *et al.*, 1992, Zelenika *et al.*, 1993, Grima *et al.*, 1994, Campagnoni *et al.*, 1993). Hence the assay designed for *MBP* in this study could also amplify transcripts of *Golli-MBP.*

Since CSF completely surrounds and bathes the exposed surfaces of the central nervous system (CNS) and free exchange occurs between the interstitial fluid and CSF, changes in CNS may produce changes in the composition of the CSF. The *MBP*, *GFAP, GPM6B* and *PLP1* mRNA were also tested in CSF. All of the

transcripts were detectable in all the available samples. Though *MBP, GFAP* and *PLP1* mRNA was also present in non-brain-injured CSF, the median concentration was less than 0.02% of its concentration in filtered CSF from brain-injured patients. The high concentration of *MBP*, *GFAP, GPM6B* and *PLP1* in brain-injured CSF suggested that CSF could be an appropriate medium for studying the pathology of brain injury. They may also possibly be applied to differentiate CSF rhinorrhoea from simple rhinorrhoea but further investigation is needed.

Using systematic microarrary analysis, I identified *MBP*, *GFAP, GPM6B* and *PLP1* mRNA to be potentially brain-specific transcripts. The successful mining was further confirmed by QRT-PCR data. Their higher concentrations in CSF from BI than non-BI patients suggested that they should probably be derived from brain tissue and leaked into CSF during injury. Unfortunately, their detectability in plasma was sub-optimal. This is possibly because of the small size of the brain as an organ and the once-off release of mRNA into the circulation which promptly gets diluted.

SECTION VI: CONCLUDING REMARKS

This section summarises the implications of the studies in this thesis. The prospects for future work are also discussed.

CHAPTER 8: CONCLUSIONS AND FUTURE PERSPECTIVES

8.1 Placenta-derived fetal specific mRNA is more readily detectable in maternal plasma than in whole blood

Circulating fetal mRNA analysis offers a means for non-invasive prenatal assessment that is applicable to pregnancies regardless of the fetal sex (Farina *et al.*, 2004, Ng *et al.*, 2003b, Tsui *et al.*, 2004). Quantitative aberrations in circulating placental mRNA have been reported for pregnancy-associated complications (Farina *et al.*, 2004). Non-invasive prenatal detection of fetal trisomy 21 has been achieved by single nucleotide polymorphism (SNP) allelic ratio determination in circulating placental mRNA (Lo *et al.*, 2007b). These studies affirmed the potential of circulating placental mRNA analysis for non-invasive prenatal assessment.

Besides maternal plasma, maternal whole blood has been reported to be another medium for fetal mRNA detection (Concu *et al.*, 2005, Farina *et al.*, 2005, Maron *et al.*, 2007, Okazaki *et al.*, 2006). *CSH1* and *CGB* concentrations were reported to be much higher in maternal whole blood than plasma (Okazaki *et al.*, 2006). The study in Chapter 4 of this thesis demonstrated that placental expressed mRNA

concentrations in third trimester whole blood were indeed higher than that in plasma for *CSH1, KISS1, PLAC4* and *PLAC1*. The observation was consistent with that of Okazaki *et al* (Okazaki *et al.*, 2006). *CSH1, CSHL1* and *KISS1* appear to be pregnancy-specific in both plasma and whole blood. On the contrary, *PLAC4* and *PLAC1* are pregnancy-specific only in plasma but not whole blood which has both fetal and maternal contributions. One possible explanation of the maternal contribution to these apparently "placenta-derived" transcripts in whole blood may be related to the phenomenon of illegitimate expression that lymphocytes had been shown to express transcripts irrelevant to their functions (Gala *et al.*, 1998, Kimoto, 1998, Ko *et al.*, 2000).

Besides the comparison in detection of the placenta-derived transcripts in maternal plasma and whole blood, Chapter 4 also demonstrated another approach in searching for possible pregnancy-specific mRNA markers in maternal blood. The new approach was to compare the blood expression profiles between pregnant and non-pregnant females. However, QRT-PCR validation of the 7 identified transcripts showed that significant differences in the concentrations of those transcripts was found only when directly comparing the pregnant and non-pregnant whole blood samples but not after *GAPDH* normalisation. In fact, all

markers, except *FLCN*, were related to the haematopoietic system, especially the neutrophils. It has been reported that the total leukocyte count would rise during pregnancy with neutrophils accounting for most of the increased leukocyte count (Kuhnert *et al.*, 1998, Pitkin and Witte, 1979). Hence, the increased expression level observed from the microarray data was probably due to the augmented leukocyte number instead of a genuine increase in transcript expression level in the cells.

The whole blood microarray study identified transcripts whose functions were related to the physiological changes of pregnancy. However, their presence in non-pregnant whole blood and lack of clearance after pregnancy suggested that they were not "pregnancy-specific" markers, albeit being "pregnancy-related". As they were also detectable in blood of non-pregnant individuals, they were therefore not fetal-specific either. Concluding from all the data in this study, it appears that placenta-derived fetal-specific transcripts can be more readily identified from maternal plasma than whole blood. While some transcripts are fetal-specific in maternal whole blood, e.g. *CSHL1*, due care is needed to validate each new potential whole blood transcript that is meant to be used as a circulating fetal RNA marker. Illegitimate expression by maternal blood cells would need to

be excluded before adopting such markers.

8.2 Plasma RNA yield could be improved by modification of extraction protocols

As discussed, non-invasive prenatal diagnosis by circulating fetal RNA analysis is feasible. However, low recovery of placental mRNA from maternal plasma may lead to false negative results, such as for trisomy 21 detection (Lo *et al.*, 2007b). There is a need to develop protocols that would improve circulating mRNA yield and ideally be practical to perform by most laboratories. Chapter 5 of this thesis reviewed and modified the blood processing and RNA extraction protocols with consideration to the known physical characteristics of plasma RNA.

The work in this thesis has demonstrated that particle-associated fetal mRNA molecules could be enriched in plasma processed with gentle spinning or be concentrated into a small volume. These observations further supported that circulating fetal RNA is particle-associated in nature. With effective modification of the plasma RNA extraction protocol by adding extra Trizol LS reagent, these improvements may enhance the accuracy and reliability of detecting circulating fetal mRNA in maternal plasma, especially for those marginally detectable

transcripts

These findings highlighted the importance of using optimal protocols to improve the yield of nucleic acids extraction Since another novel class of markers, miRNA, has been shown to have potential to be developed for non-invasive prenatal diagnosis and possess different physical characteristics as placental mRNA, Chapter 6 evaluated different extraction protocols for miRNA isolation Despite of the different physical natures of plasma mRNAs and miRNAs, increasing the Trizol LS reagent-to-plasma ratio to 3 was demonstrated to be beneficial for preserving both mRNA and miRNA in plasma The comparison of the miR-16 concentration among different extraction systems and DNase I treatment revealed that the use of *mir*Vana miRNA isolation columns with off-column DNase I treatment, showed statistically significant higher miR-16 yield than others while being free from DNA contamination

There is mounting evidence that miRNA plays an important role in regulating gene expression With the development of the miRNA microarray technology, more disease-associated miRNA may possibly be identified in near future It is hoped that this evaluation work would facilitate the detection of both the circulating

mRNA and miRNA candidate markers in plasma for non-invasive prenatal diagnosis as well as other plasma-based RNA diagnostics.

8.3 Brain-derived mRNA transcripts for the assessment of brain injury.

Current assessment of brain injury largely relies on clinical features, imaging findings and the physiologic state of the patient. Researchers in the field have focused on investigating value of the potential protein markers such as S100B protein (Kochanek *et al.*, 2008). However, there is still no sensitive and brain-injury-specific biomarker identified so far that could provide additional objective information to aid clinical decisions. Adopting the experience in prenatal diagnosis, circulating tissue-specific mRNA markers may possibly be an alternative to the use of protein markers in the assessment of brain injury. Chapter 7 of this thesis aimed to identify brain-derived mRNA transcripts systematically and to investigate its feasibility to be developed as biomarkers for brain-injury.

This study has successfully identified the brain-derived mRNA markers, *MBP*, *GFAP*, *GPM6B* and *PLP1*. Though *GFAP* and *GPM6B* were undetectable in plasma from non-brain-injured patients indicating they are possibly specific for

brain injury, their low detectability (36%) in plasma from brain-injured patients hindered their development as blood markers for brain injury. On the other hand, *GPM6B* mRNA was detectable in brain-injured but not control CSF suggested its possible role to serve as a CSF mRNA marker for brain injury assessment.

8.4 Future perspectives

The detection of circulating RNA in plasma has much potential in transforming non-invasive diagnostics. Over the past years, encouraging findings have been reported on the detection and possible clinical applications of circulating RNA. Further development in this field will be facilitated by a better understanding of the physiological and pathological characteristics of these circulating mRNA and miRNA species.

In the first part of this thesis, I confirmed that placenta-derived fetal-specific mRNA species were more readily deteactable in maternal plasma than in whole blood. The availability of placental mRNA in maternal plasma offered the possibility to study the transcriptome of the placenta from the maternal circulation. With the development of massively parallel sequencing technologies, such as Solexa sequencing, which has been demonstrated to be applicable to the analysis

of fetal DNA both qualitatively and quantitatively (Chiu *et al.*, 2008), investigation of the fetal RNA trancriptome in maternal plasma would be possible. The comparison of such expression profiles between plasma from normal pregnancies and those with placental disorders such as pre-eclampsia, may identify disease-associated placental mRNA markers.

Moreover, it is demonstrated that some well known placenta-derived transcripts such as *CSH1, CSHL1* and *KISS1* appear to be pregnancy-specific in both plasma and whole blood whereas some others such as *PLAC4* and *PLAC1*, are pregnancy-specific only in plasma but not whole blood. It is intriguing that these placenta-derived fetal-specific *CSH1, CSHL1* and *KISS1* transcripts could be detected in maternal whole blood at high quantities. In future studies, the biological characteristics and the release mechanism of placenta-derived transcripts in maternal whole blood can be further investigated.

In the second part of this thesis, I have optimised the miRNA isolation protocols in plasma. Placental miRNAs are associated with various regulatory functions in humans (Bartel, 2004) and exist at much higher concentrations in cells than mRNAs (Lim *et al.*, 2003). They therefore have the potential to be developed for

non-invasive prenatal diagnostic purposes. It would be exciting to investigate if the aberrant plasma levels of these placental-derived miRNAs can be observed in pathological pregnancies. For example, a recent study performed by Pineles *et al.* demonstrated that a distinct subset of miRNAs is differentially expressed in the placentas of pregnancies with pre-eclampsia and small-for-gestational age fetuses (Pineles *et al.*, 2007). It would be useful to investigate if the aberration of these miRNAs expressed in the pathological placentas would also be reflected in maternal plasma. Further experimentation might be performed to look for plasma miRNAs whose levels are altered in these pathological conditions. This would represent another step forward to developing circulating miRNAs as markers for non-invasive prenatal diagnosis. On the other hand, with more advanced molecular techniques and high throughput technologies, such as miRNA microarray, the number of miRNAs discovered in humans would continue to increase. It is of interest to apply this approach, which requires no prior knowledge of the function of miRNAs to identify more circulating placental miRNAs. Finally, because miRNAs are important regulators of gene expression, further investigations might be conducted to address the question of whether circulating miRNA possesses any functional implication in terms of feto-maternal communication.

In the last part of this thesis, the brain-derived transcripts were identified by systematic microarray analysis. The mining strategy was devised to discover transcripts with high expression level in brain tissue and low expression level in blood cells. This approach has been demonstrated to be successful in Chapter 7 and in discovering placenta-specific markers. In future studies, the same rationale could also be applied to screening for other tissue-specific mRNA transcripts, such as liver-specific mRNA markers for early diagnosis of liver cirrhosis. Moreover, the detectability of brain-derived *GFAP, GPM6B* and *PLP1* mRNA in peripheral plasma from brain-injured patients was less than 36%. In terms of future study, it is of interest to boost the detectability of these markers by using a more sensitive and advanced technology such as microfluidics digital PCR. In addition, the high concentration of these brain-specific mRNA markers in CSF suggested the possibility of further investigation on the pathology of brain injury and other neurological disorder such as stroke. Moreover, the biological characteristics and physical nature of the CSF mRNA remain unknown. It would be another interesting area requiring further investigation and the knowledge on this would enrich our understanding in the biology of circulating nucleic acids.

REFERENCES

Acosta JA, Yang JC, Winchell RJ, Simons RK, Fortlage DA, Hollingsworth-Fridlund P, Hoyt DB 1998. Lethal injuries and time to death in a level I trauma center. *J Am Coll Surg,* **186**: 528-533.

Adley BP, Smith ND, Nayar R, Yang XJ 2006. Birt-Hogg-Dube syndrome: clinicopathologic findings and genetic alterations. *Arch Pathol Lab Med,* **130**: 1865-1870.

Affymetrix 2003. Globin reduction protocol: a method for processing whole blood RNA samples for improved array results. *Affymetrix Technical Note*: 1-9.

Al-Yatama MK, Mustafa AS, Ali S, Abraham S, Khan Z, Khaja N 2001. Detection of Y chromosome-specific DNA in the plasma and urine of pregnant women using nested polymerase chain reaction. *Prenat Diagn,* **21**: 399-402.

Ambros V 2003. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell,* **113**: 673-676.

Amicucci P, Gennarelli M, Novelli G, Dallapiccola B 2000. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clin Chem,* **46**: 301-302.

Anderson CS, Chakera TM, Stewart-Wynne EG, Jamrozik KD 1994. Spectrum of primary intracerebral haemorrhage in Perth, Western Australia, 1989-90: incidence and outcome. *J Neurol Neurosurg Psychiatry,* **57**: 936-940.

Anderson RE, Winnerkvist A, Hansson LO, Nilsson O, Rosengren L, Settergren G, Vaage J 2003. Biochemical markers of cerebrospinal ischemia after repair of aneurysms of the descending and thoracoabdominal aorta. *J Cardiothorac Vasc Anesth,* **17**: 598-603.

Angert RM, Leshane ES, Yarnell RW, Johnson KL, Bianchi DW 2004. Cell-free fetal DNA in the cerebrospinal fluid of women during the peripartum period. *Am J Obstet Gynecol,* **190**: 1087-1090.

Anker P, Lefort F, Vasioukhin V, Lyautey J, Lederrey C, Chen XQ, Stroun M, Mulcahy HE, Farthing MJ 1997. K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. *Gastroenterology,* **112**: 1114-1120.

Arechavaleta-Velasco F, Marciano D, Diaz-Cueto L, Parry S 2004. Matrix metalloproteinase-8 is expressed in human chorion during labor. *Am J Obstet Gynecol,* **190**: 843-850.

Banzola I, Rusterholz C, Zannoni L, Rizzo N, Zhong XY, Caramelli E, Holzgreve W, Farina A, Hahn S 2008. PLAC4 and beta-HCG mRNA levels are not altered in the maternal circulation of pregnancies with trisomy 21. *Prenat Diagn,* **28**: 1262-1267.

Barad O, Meiri E, Avniel A, Aharonov R, Barzilai A, Bentwich I, Einav U, Gilad S, Hurban P, Karov Y, Lobenhofer EK, Sharon E, Shiboleth YM, Shtutman M, Bentwich Z, Einat P 2004. MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. *Genome Res,* **14**: 2486-2494.

Barada FA, Jr., Suratt PM, Davis JSt, Sipes JN, Castle CA, Taylor RP, Godfrey SM 1980. Free plasma DNA in patients with pulmonary embolism. *South Med J,* **73**: 345-346, 350.

Barbarese E, Brumwell C, Kwon S, Cui H, Carson JH 1999. RNA on the road to myelin. *J Neurocytol,* **28**: 263-270.

Bartel DP 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell,* **116**: 281-297.

Bazan V, Bruno L, Augello C, Agnese V, Calo V, Corsale S, Gargano G, Terrasi M, Schiro V, Di Fede G, Adamo V, Intrivici C, Crosta A, Rinaldi G, Latteri F, Dardanoni G, Grassi N, Valerio MR, Colucci G, Macaluso M, Russo A 2006. Molecular detection of TP53, Ki-Ras and p16INK4A promoter methylation in plasma of patients with colorectal cancer and its association with prognosis. Results of a 3-year GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study. *Ann Oncol,* **17 Suppl 7**: vii84-90.

Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P,

Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z 2005. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet,* **37**: 766-770.

Bianchi DW 1999. Fetal cells in the maternal circulation: feasibility for prenatal diagnosis. *Br J Haematol,* **105**: 574-583.

Bianchi DW, LeShane ES, Cowan JM 2001. Large amounts of cell-free fetal DNA are present in amniotic fluid. *Clin Chem,* **47**: 1867-1869.

Bianchi DW, Simpson JL, Jackson LG, Elias S, Holzgreve W, Evans MI, Dukes KA, Sullivan LM, Klinger KW, Bischoff FZ, Hahn S, Johnson KL, Lewis D, Wapner RJ, de la Cruz F 2002. Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. *Prenat Diagn,* **22**: 609-615.

Bianchi DW, Avent ND, Costa JM, van der Schoot CE 2005. Noninvasive prenatal diagnosis of fetal Rhesus D: ready for Prime(r) Time. *Obstet Gynecol,* **106**: 841-844.

Bischoff FZ, Sinacori MK, Dang DD, Marquez-Do D, Horne C, Lewis DE, Simpson JL 2002. Cell-free fetal DNA and intact fetal cells in maternal blood circulation: implications for first and second trimester non-invasive prenatal diagnosis. *Hum Reprod Update,* **8**: 493-500.

Botezatu I, Serdyuk O, Potapova G, Shelepov V, Alechina R, Molyaka Y, Ananev V, Bazin I, Garin A, Narimanov M, Knysh V, Melkonyan H, Umansky S, Lichtenstein A 2000. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin Chem,* **46**: 1078-1084.

Broderick JP, Adams HP, Jr., Barsan W, Feinberg W, Feldmann E, Grotta J, Kase C, Krieger D, Mayberg M, Tilley B, Zabramski JM, Zuccarello M 1999. Guidelines for the management of spontaneous intracerebral hemorrhage: A statement for healthcare professionals from a special writing group of the Stroke Council, American Heart Association. *Stroke,* **30**: 905-915.

Brown ES 1963. Foetal erythrocytes in the maternal circulation. *Br Med J,* **1**: 1000-1001.

Bustin SA 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol,* **25**: 169-193.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A,* **99**: 15524-15529.

Campagnoni AT, Pribyl TM, Campagnoni CW, Kampf K, Amur-Umarjee S, Landry CF, Handley VW, Newman SL, Garbay B, Kitamura K 1993. Structure and developmental regulation of Golli-mbp, a 105-kilobase gene that encompasses the myelin basic protein gene and is expressed in cells in the oligodendrocyte lineage in the brain. *J Biol Chem,* **268**: 4930-4938.

Campello Yurgel V, Ikuta N, Brondani da Rocha A, Lunge VR, Fett Schneider R, Kazantzi Fonseca AS, Grivicich I, Zanoni C, Regner A 2007. Role of plasma DNA as a predictive marker of fatal outcome following severe head injury in males. *J Neurotrauma*, **24**: 1172-1181.

Capone RB, Pai SI, Koch WM, Gillison ML, Danish HN, Westra WH, Daniel R, Shah KV, Sidransky D 2000. Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. *Clin Cancer Res,* **6**: 4171-4175.

Castells A, Puig P, Mora J, Boadas J, Boix L, Urgell E, Sole M, Capella G, Lluis F, Fernandez-Cruz L, Navarro S, Farre A 1999. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. *J Clin Oncol,* **17**: 578-584.

Challa VR, Moody DM, Bell MA 1992. The Charcot-Bouchard aneurysm controversy: impact of a new histologic technique. *J Neuropathol Exp Neurol,* **51**: 264-271.

Chan KCA, Zhang J, Chan AT, Lei KI, Leung SF, Chan LYS, Chow KCK, Lo YMD 2003. Molecular characterization of circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. *Cancer Res,* **63**: 2028-2032.

Chan KCA, Zhang J, Hui AB, Wong N, Lau TK, Leung TN, Lo KW, Huang DW, Lo YMD 2004. Size distributions of maternal and fetal DNA in maternal plasma. *Clin Chem,* **50**: 88-92.

Chan RWY, Graham CA, Rainer TH, Lam NY, Chiu RWK, Chik KW, Lee V, Lo YMD 2007. Use of a bone marrow transplantation model system to demonstrate the hematopoietic origin of plasma S100B mRNA. *Clin Chem,* **53**: 1874-1876.

Chen CP, Chern SR, Wang W 2000. Fetal DNA in maternal plasma: the prenatal detection of a paternally inherited fetal aneuploidy. *Prenat Diagn,* **20**: 355-357.

Chen CP, Chern SR, Wang W 2001a. Fetal DNA analyzed in plasma from a mother's three consecutive pregnancies to detect paternally inherited aneuploidy. *Clin Chem,* **47**: 937-939.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M 2001b. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell,* **107**: 451-464.

Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J, Zhang CY 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res,* **18**: 997-1006.

Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, Lederrey C, Anker P 1996. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med,* **2**: 1033-1035.

Cheng AM, Byrom MW, Shelton J, Ford LP 2005. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res,* **33**: 1290-1297.

Chim SS, Tong YK, Chiu RW, Lau TK, Leung TN, Chan LY, Oudejans CB, Ding C, Lo YM 2005. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc Natl Acad Sci U S A,* **102**: 14753-14758.

Chim SSC, Shing TKF, Hung ECW, Leung TY, Lau TK, Chiu RWK, Lo YMD 2008. Detection and characterization of placental microRNAs in maternal plasma.

Clin Chem, **54**: 482-490.

Chiu RWK, Poon LLM, Lau TK, Leung TN, Wong EM, Lo YMD 2001. Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. *Clin Chem,* **47**: 1607-1613.

Chiu RWK, Lau TK, Cheung PT, Gong ZQ, Leung TN, Lo YMD 2002a. Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. *Clin Chem,* **48**: 778-780.

Chiu RWK, Lau TK, Leung TN, Chow KCK, Chui DH, Lo YMD 2002b. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. *Lancet,* **360**: 998-1000.

Chiu RWK, Lui WB, Cheung MC, Kumta N, Farina A, Banzola I, Grotti S, Rizzo N, Haines CJ, Lo YMD 2006. Time profile of appearance and disappearance of circulating placenta-derived mRNA in maternal plasma. *Clin Chem,* **52**: 313-316.

Chiu RWK, Chan KCA, Gao Y, Lau VYM, Zheng W, Leung TY, Foo CH, Xie B, Tsui NBY, Lun FMF, Zee BC, Lau TK, Cantor CR, Lo YMD 2008. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc Natl Acad Sci U S A,* **105**: 20458-20463.

Choi SC, Narayan RK, Anderson RL, Ward JD 1988. Enhanced specificity of prognosis in severe head injury. *J Neurosurg,* **69**: 381-385.

Chomczynski P, Sacchi N 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem,* **162**: 156-159.

Cioni R, Bussani C, Scarselli B, Mello G, Mecacci F, Scarselli G 2003. Detection of fetal DNA in the peritoneal cavity during pregnancy. *Eur J Obstet Gynecol Reprod Biol,* **107**: 210-211.

Concu M, Banzola I, Farina A, Sekizawa A, Rizzo N, Marini M, Caramelli E, Carinci P 2005. Rapid clearance of mRNA for PLAC1 gene in maternal blood after delivery. *Fetal Diagn Ther,* **20**: 27-30.

Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P, Dumez Y 2001. First-trimester fetal sex determination in maternal serum using real-time PCR. *Prenat Diagn,* **21**: 1070-1074.

Costa JM, Benachi A, Gautier E 2002. New strategy for prenatal diagnosis of X-linked disorders. *N Engl J Med,* **346**: 1502.

Dasi F, Lledo S, Garcia-Granero E, Ripoll R, Marugan M, Tormo M, Garcia-Conde J, Alino SF 2001. Real-time quantification in plasma of human telomerase reverse transcriptase (hTERT) mRNA: a simple blood test to monitor disease in cancer patients. *Lab Invest,* **81**: 767-769.

de Kok JB, van Solinge WW, Ruers TJ, Roelofs RW, van Muijen GN, Willems JL, Swinkels DW 1997. Detection of tumour DNA in serum of colorectal cancer patients. *Scand J Clin Lab Invest,* **57**: 601-604.

Deligezer U, Yaman F, Erten N, Dalay N 2003. Frequent copresence of methylated DNA and fragmented nucleosomal DNA in plasma of lymphoma patients. *Clin Chim Acta,* **335**: 89-94.

Eisenberger CF, Stoecklein NH, Jazra S, Hosch SB, Peiper M, Scheunemann P, Am Esch JS, Knoefel WT 2006. The detection of oesophageal adenocarcinoma by serum microsatellite analysis. *Eur J Surg Oncol,* **32**: 954-960.

El-Hefnawy T, Raja S, Kelly L, Bigbee WL, Kirkwood JM, Luketich JD, Godfrey TE 2004. Characterization of amplifiable, circulating RNA in plasma and its potential as a tool for cancer diagnostics. *Clin Chem,* **50**: 564-573.

Evan GI, Vousden KH 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature,* **411**: 342-348.

Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE 1998. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet,* **352**: 1196.

Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR 2008. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci U S A,* **105**: 16266-16271.

Farina A, LeShane ES, Lambert-Messerlian GM, Canick JA, Lee T, Neveux LM, Palomaki GE, Bianchi DW 2003. Evaluation of cell-free fetal DNA as a second-trimester maternal serum marker of Down syndrome pregnancy. *Clin Chem,* **49**: 239-242.

Farina A, Chan CW, Chiu RWK, Tsui NBY, Carinci P, Concu M, Banzola I, Rizzo N, Lo YMD 2004. Circulating corticotropin-releasing hormone mRNA in maternal plasma: relationship with gestational age and severity of preeclampsia. *Clin Chem,* **50**: 1851-1854.

Farina A, Rizzo N, Concu M, Banzola I, Sekizawa A, Grotti S, Carinci P 2005. Lower maternal PLAC1 mRNA in pregnancies complicated with vaginal bleeding (threatened abortion <20 weeks) and a surviving fetus. *Clin Chem,* **51**: 224-227.

Farina A, Sekizawa A, Purwosunu Y, Rizzo N, Banzola I, Concu M, Morano D, Giommi F, Bevini M, Mabrook M, Carinci P, Okai T 2006. Quantitative distribution of a panel of circulating mRNA in preeclampsia versus controls. *Prenat Diagn,* **26**: 1115-1120.

Finning KM, Martin PG, Soothill PW, Avent ND 2002. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion,* **42**: 1079-1085.

Fisher CM 1971. Pathological observations in hypertensive cerebral hemorrhage. *J Neuropathol Exp Neurol,* **30**: 536-550.

Fournier T, Medjoubi NN, Porquet D 2000. Alpha-1-acid glycoprotein. *Biochim Biophys Acta,* **1482**: 157-171.

Fucharoen G, Tungwiwat W, Ratanasiri T, Sanchaisuriya K, Fucharoen S 2003. Prenatal detection of fetal hemoglobin E gene from maternal plasma. *Prenat Diagn,* **23**: 393-396.

Gal S, Fidler C, Lo YMD, Chin K, Moore J, Harris AL, Wainscoat JS 2001. Detection of mammaglobin mRNA in the plasma of breast cancer patients. *Ann N Y Acad Sci,* **945**: 192-194.

Gala JL, Heusterspreute M, Loric S, Hanon F, Tombal B, Van Cangh P, De Nayer P, Philippe M 1998. Expression of prostate-specific antigen and prostate-specific

membrane antigen transcripts in blood cells: implications for the detection of hematogenous prostate cells and standardization. *Clin Chem,* **44**: 472-481.

Gallagher A, Armstrong AA, MacKenzie J, Shield L, Khan G, Lake A, Proctor S, Taylor P, Clements GB, Jarrett RF 1999. Detection of Epstein-Barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease. *Int J Cancer,* **84**: 442-448.

Gautschi O, Bigosch C, Huegli B, Jermann M, Marx A, Chasse E, Ratschiller D, Weder W, Joerger M, Betticher DC, Stahel RA, Ziegler A 2004. Circulating deoxyribonucleic Acid as prognostic marker in non-small-cell lung cancer patients undergoing chemotherapy. *J Clin Oncol,* **22**: 4157-4164.

Ge Q, Liu Q, Bai Y, Wen T, Lu Z 2005. A semi-quantitative microarray method to detect fetal RNAs in maternal plasma. *Prenat Diagn,* **25**: 912-918.

Germain SJ, Sacks GP, Soorana SR, Sargent IL, Redman CW 2007. Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *J Immunol,* **178**: 5949-5956.

Gibson UE, Heid CA, Williams PM 1996. A novel method for real time quantitative RT-PCR. *Genome Res,* **6**: 995-1001.

Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A 2008. Serum microRNAs are promising novel biomarkers. *PLoS ONE,* **3**: e3148.

Go AT, Visser A, Mulders MA, Blankenstein MA, Van Vugt JM, Oudejans CB 2004. Detection of placental transcription factor mRNA in maternal plasma. *Clin Chem,* **50**: 1413-1414.

Go AT, Visser A, Mulders MA, Blankenstein MA, van Vugt JM, Oudejans CB 2007a. 44 single-nucleotide polymorphisms expressed by placental RNA: assessment for use in noninvasive prenatal diagnosis of trisomy 21. *Clin Chem,* **53**: 2223-2224.

Go AT, Visser A, Mulders MA, Twisk JW, Blankenstein MA, van Vugt JM, Oudejans CB 2007b. C21ORF105, A chromosome 21-encoded mRNA, is not a discriminative marker gene for prediction of Down syndrome in maternal plasma.

Prenat Diagn, **27**: 146-149.

Gonzalez-Gonzalez MC, Garcia-Hoyos M, Trujillo MJ, Rodriguez de Alba M, Lorda-Sanchez I, Diaz-Recasens J, Gallardo E, Ayuso C, Ramos C 2002. Prenatal detection of a cystic fibrosis mutation in fetal DNA from maternal plasma. *Prenat Diagn,* **22**: 946-948.

Gonzalez-Gonzalez MC, Trujillo MJ, Rodriguez de Alba M, Garcia-Hoyos M, Lorda-Sanchez I, Diaz-Recasens J, Ayuso C, Ramos C 2003. Huntington disease-unaffected fetus diagnosed from maternal plasma using QF-PCR. *Prenat Diagn,* **23**: 232-234.

Gonzalgo ML, Eisenberger CF, Lee SM, Trock BJ, Marshall FF, Hortopan S, Sidransky D, Schoenberg MP 2002. Prognostic significance of preoperative molecular serum analysis in renal cancer. *Clin Cancer Res,* **8**: 1878-1881.

Griffiths-Jones S 2004. The microRNA Registry. *Nucleic Acids Res,* **32**: D109-111.
Grima B, Zelenika D, Pessac B 1992. A novel transcript overlapping the myelin basic protein gene. *J Neurochem,* **59**: 2318-2323.

Grima B, Zelenika D, Javoy-Agid F, Pessac B 1994. Identification of new human myelin basic protein transcripts in the immune and central nervous systems. *Neurobiol Dis,* **1**: 61-66.

Gupta AK, Holzgreve W, Huppertz B, Malek A, Schneider H, Hahn S 2004. Detection of fetal DNA and RNA in placenta-derived syncytiotrophoblast microparticles generated in vitro. *Clin Chem,* **50**: 2187-2190.

Hahn S, Huppertz B, Holzgreve W 2005. Fetal cells and cell free fetal nucleic acids in maternal blood: new tools to study abnormal placentation? *Placenta,* **26**: 515-526.

Halicka HD, Bedner E, Darzynkiewicz Z 2000. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. *Exp Cell Res,* **260**: 248-256.

Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H 1993. Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. *Hum Genet,* **91**: 427-432.

Hanemaaijer R, Sorsa T, Konttinen YT, Ding Y, Sutinen M, Visser H, van Hinsbergh VW, Helaakoski T, Kainulainen T, Ronka H, Tschesche H, Salo T 1997. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. *J Biol Chem,* **272**: 31504-31509.

Hayashi M, Ueda Y, Hoshimoto K, Ota Y, Fukasawa I, Sumori K, Kaneko I, Abe S, Uno M, Ohkura T, Inaba N 2002. Changes in urinary excretion of six biochemical parameters in normotensive pregnancy and preeclampsia. *Am J Kidney Dis,* **39**: 392-400.

Heid CA, Stevens J, Livak KJ, Williams PM 1996. Real time quantitative PCR. *Genome Res,* **6**: 986-994.

Herrmann M, Curio N, Jost S, Wunderlich MT, Synowitz H, Wallesch CW 1999. Protein S-100B and neuron specific enolase as early neurobiochemical markers of the severity of traumatic brain injury. *Restor Neurol Neurosci,* **14**: 109-114.

Heung MMS, Tsui NBY, Leung TY, Lau TK, Lo YMD, Chiu RWK 2009. Development of Extraction Protocols to Improve the Yield for Fetal RNA in Maternal Plasma. *Prenat Diagn*, **29**: 277-279

Hibi K, Robinson CR, Booker S, Wu L, Hamilton SR, Sidransky D, Jen J 1998. Molecular detection of genetic alterations in the serum of colorectal cancer patients. *Cancer Res,* **58**: 1405-1407.

Hochepied T, Berger FG, Baumann H, Libert C 2003. Alpha(1)-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties. *Cytokine Growth Factor Rev,* **14**: 25-34.

Holland PM, Abramson RD, Watson R, Gelfand DH 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A,* **88**: 7276-7280.

Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, Wood T, Jeronimo C, Rosenbaum E, Stern J, Yu M, Trink B, Kiviat NB, Sidransky D 2006. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. *J Clin Oncol,* **24**: 4262-4269.

Hung ECW, Shing TKF, Chim SSC, Yeung PC, Chan RWY, Chik KW, Lee V, Tsui NBY, Li CK, Wong CS, Chiu RWK, Lo YMD 2008. Presence of Donor-Derived DNA and Cells in the Urine of Sex-Mismatched Hematopoietic Stem Cell Transplant Recipients: Implication for the Transrenal Hypothesis. *Clin Chem*, **55**: 715-722.

Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez JB, Samoto T, Maruo T 2002. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. *Am J Obstet Gynecol*, **186**: 158-166.

Jennett B 1996. Epidemiology of head injury. *J Neurol Neurosurg Psychiatry*, **60**: 362-369.

Kawakami K, Brabender J, Lord RV, Groshen S, Greenwald BD, Krasna MJ, Yin J, Fleisher AS, Abraham JM, Beer DG, Sidransky D, Huss HT, Demeester TR, Eads C, Laird PW, Ilson DH, Kelsen DP, Harpole D, Moore MB, Danenberg KD, Danenberg PV, Meltzer SJ 2000. Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. *J Natl Cancer Inst*, **92**: 1805-1811.

Kijima M, Togo S, Ichikawa Y, Miura M, Yamagishi S, Matsuo K, Tanaka K, Masui H, Ishikawa T, Ike H, Shimada H 2005. Clinical significance of serum CEA protein and CEA mRNA after resection of colorectal liver metastases. *Anticancer Res*, **25**: 1327-1332.

Kimoto Y 1998. A single human cell expresses all messenger ribonucleic acids: the arrow of time in a cell. *Mol Gen Genet*, **258**: 233-239.

Kimura T, Holland WS, Kawaguchi T, Williamson SK, Chansky K, Crowley JJ, Doroshow JH, Lenz HJ, Gandara DR, Gumerlock PH 2004. Mutant DNA in plasma of lung cancer patients: potential for monitoring response to therapy. *Ann N Y Acad Sci*, **1022**: 55-60.

Kindzelskii AL, Clark AJ, Espinoza J, Maeda N, Aratani Y, Romero R, Petty HR 2006. Myeloperoxidase accumulates at the neutrophil surface and enhances cell metabolism and oxidant release during pregnancy. *Eur J Immunol*, **36**: 1619-1628.

Kirk GD, Camus-Randon AM, Mendy M, Goedert JJ, Merle P, Trepo C, Brechot C, Hainaut P, Montesano R 2000. Ser-249 p53 mutations in plasma DNA of patients with hepatocellular carcinoma from The Gambia. *J Natl Cancer Inst,* **92**: 148-153.

Klebanoff SJ 1999. Myeloperoxidase. *Proc Assoc Am Physicians,* **111**: 383-389.

Ko Y, Grunewald E, Totzke G, Klinz M, Fronhoffs S, Gouni-Berthold I, Sachinidis A, Vetter H 2000. High percentage of false-positive results of cytokeratin 19 RT-PCR in blood: a model for the analysis of illegitimate gene expression. *Oncology,* **59**: 81-88.

Kobayashi D, Koshida S, Moriai R, Tsuji N, Watanabe N 2007. Olfactomedin 4 promotes S-phase transition in proliferation of pancreatic cancer cells. *Cancer Sci,* **98**: 334-340.

Kochanek PM, Berger RP, Bayir H, Wagner AK, Jenkins LW, Clark RS 2008. Biomarkers of primary and evolving damage in traumatic and ischemic brain injury: diagnosis, prognosis, probing mechanisms, and therapeutic decision making. *Curr Opin Crit Care,* **14**: 135-141.

Koffler D, Agnello V, Winchester R, Kunkel HG 1973. The occurrence of single-stranded DNA in the serum of patients with systemic lupus erythematosus and other diseases. *J Clin Invest,* **52**: 198-204.

Koide K, Sekizawa A, Iwasaki M, Matsuoka R, Honma S, Farina A, Saito H, Okai T 2005. Fragmentation of cell-free fetal DNA in plasma and urine of pregnant women. *Prenat Diagn,* **25**: 604-607.

Kolialexi A, Tsangaris GT, Antsaklis A, Tzortzatou F, Amentas C, Koratzis A, Mavrou A 2001. Apoptosis in maternal peripheral blood during pregnancy. *Fetal Diagn Ther,* **16**: 32-37.

Kopreski MS, Benko FA, Kwee C, Leitzel KE, Eskander E, Lipton A, Gocke CD 1997. Detection of mutant K-ras DNA in plasma or serum of patients with colorectal cancer. *Br J Cancer,* **76**: 1293-1299.

Kopreski MS, Benko FA, Kwak LW, Gocke CD 1999. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. *Clin Cancer Res,* **5**: 1961-1965.

Koshida S, Kobayashi D, Moriai R, Tsuji N, Watanabe N 2007. Specific overexpression of OLFM4(GW112/HGC-1) mRNA in colon, breast and lung cancer tissues detected using quantitative analysis. *Cancer Sci,* **98**: 315-320.

Krabchi K, Gros-Louis F, Yan J, Bronsard M, Masse J, Forest JC, Drouin R 2001. Quantification of all fetal nucleated cells in maternal blood between the 18th and 22nd weeks of pregnancy using molecular cytogenetic techniques. *Clin Genet,* **60**: 145-150.

Kuhnert M, Strohmeier R, Stegmuller M, Halberstadt E 1998. Changes in lymphocyte subsets during normal pregnancy. *Eur J Obstet Gynecol Reprod Biol,* **76**: 147-151.

Lai EC 2003. microRNAs: runts of the genome assert themselves. *Curr Biol,* **13**: R925-936.

Lapaire O, Bianchi DW, Peter I, O'Brien B, Stroh H, Cowan JM, Tantravahi U, Johnson KL 2007. Cell-free fetal DNA in amniotic fluid: unique fragmentation signatures in euploid and aneuploid fetuses. *Clin Chem,* **53**: 405-411.

Laping NJ, Teter B, Nichols NR, Rozovsky I, Finch CE 1994. Glial fibrillary acidic protein: regulation by hormones, cytokines, and growth factors. *Brain Pathol,* **4**: 259-275.

Larrabee PB, Johnson KL, Pestova E, Lucas M, Wilber K, LeShane ES, Tantravahi U, Cowan JM, Bianchi DW 2004. Microarray analysis of cell-free fetal DNA in amniotic fluid: a prenatal molecular karyotype. *Am J Hum Genet,* **75**: 485-491.

Larrabee PB, Johnson KL, Lai C, Ordovas J, Cowan JM, Tantravahi U, Bianchi DW 2005. Global gene expression analysis of the living human fetus using cell-free messenger RNA in amniotic fluid. *JAMA,* **293**: 836-842.

Lau TK, Lo KW, Chan LYS, Leung TY, Lo YMD 2000. Cell-free fetal deoxyribonucleic acid in maternal circulation as a marker of fetal-maternal hemorrhage in patients undergoing external cephalic version near term. *Am J Obstet Gynecol,* **183**: 712-716.

Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH,

Pezzella F, Boultwood J, Wainscoat JS, Hatton CS, Harris AL 2008. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol,* **141**: 672-675.

Lee T, LeShane ES, Messerlian GM, Canick JA, Farina A, Heber WW, Bianchi DW 2002. Down syndrome and cell-free fetal DNA in archived maternal serum. *Am J Obstet Gynecol,* **187**: 1217-1221.

Leon SA, Shapiro B, Sklaroff DM, Yaros MJ 1977. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res,* **37**: 646-650.

Leung TN, Zhang J, Lau TK, Hjelm NM, Lo YM 1998. Maternal plasma fetal DNA as a marker for preterm labour. *Lancet,* **352**: 1904-1905.

Leung TN, Zhang J, Lau TK, Chan LYS, Lo YMD 2001. Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia. *Clin Chem,* **47**: 137-139.

Li Y, Zhong XY, Kang A, Troeger C, Holzgreve W, Hahn S 2003. Inability to detect cell free fetal DNA in the urine of normal pregnant women nor in those affected by preeclampsia associated HELLP syndrome. *J Soc Gynecol Investig,* **10**: 503-508.

Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP 2003. The microRNAs of Caenorhabditis elegans. *Genes Dev,* **17**: 991-1008.

Lo KW, Lo YMD, Leung SF, Tsang YS, Chan LYS, Johnson PJ, Hjelm NM, Lee JC, Huang DP 1999a. Analysis of cell-free Epstein-Barr virus associated RNA in the plasma of patients with nasopharyngeal carcinoma. *Clin Chem,* **45**: 1292-1294.

Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, Haines CJ, Redman CW 1999b. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem,* **45**: 184-188.

Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet,* **350**: 485-487.

Lo YMD, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, Poon PM, Redman CW, Wainscoat JS 1998a. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med,* **339**: 1734-1738.

Lo YMD, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, Wainscoat JS, Johnson PJ, Chang AMZ, Hjelm NM 1998b. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet,* **62**: 768-775.

Lo YMD, Chan LYS, Lo KW, Leung SF, Zhang J, Chan AT, Lee JC, Hjelm NM, Johnson PJ, Huang DP 1999c. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res,* **59**: 1188-1191.

Lo YMD, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, Haines CJ, Redman CW 1999d. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem,* **45**: 184-188.

Lo YMD, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM 1999e. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet,* **64**: 218-224.

Lo YMD, Chan AT, Chan LYS, Leung SF, Lam CW, Huang DP, Johnson PJ 2000a. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. *Cancer Res,* **60**: 6878-6881.

Lo YMD, Leung SF, Chan LYS, Chan AT, Lo KW, Johnson PJ, Huang DP 2000b. Kinetics of plasma Epstein-Barr virus DNA during radiation therapy for nasopharyngeal carcinoma. *Cancer Res,* **60**: 2351-2355.

Lo YMD, Tsui NBY, Chiu RWK, Lau TK, Leung TN, Heung MMS, Gerovassili A, Jin Y, Nicolaides KH, Cantor CR, Ding C 2007b. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nat Med,* **13**: 218-223.

Lun FMF, Chiu RWK, Leung TY, Leung TN, Lau TK, Lo YMD 2007. Epigenetic analysis of RASSF1A gene in cell-free DNA in amniotic fluid. *Clin Chem,* **53**: 796-798.

Lun FMF, Chiu RWK, Chan KCA, Leung TY, Lau TK, Lo YMD 2008a.

Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. *Clin Chem,* **54**: 1664-1672.

Lun FMF, Tsui NBY, Chan KCA, Leung TY, Lau TK, Charoenkwan P, Chow KCK, Lo WY, Wanapirak C, Sanguansermsri T, Cantor CR, Chiu RWK, Lo YMD 2008b. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. *Proc Natl Acad Sci U S A,* **105**: 19920-19925.

Mandel P, Metais P 1948. Les acides nucleiques du plasma sanguin chez l'homme. *C R Seances Soc Biol Fil,* **142**: 241-243.

Marini A, Mirmohammadsadegh A, Nambiar S, Gustrau A, Ruzicka T, Hengge UR 2006. Epigenetic inactivation of tumor suppressor genes in serum of patients with cutaneous melanoma. *J Invest Dermatol,* **126**: 422-431.

Marion DW, Carlier PM 1994. Problems with initial Glasgow Coma Scale assessment caused by prehospital treatment of patients with head injuries: results of a national survey. *J Trauma,* **36**: 89-95.

Maron JL, Johnson KL, Slonim D, Lai CQ, Ramoni M, Alterovitz G, Jarrah Z, Yang Z, Bianchi DW 2007. Gene expression analysis in pregnant women and their infants identifies unique fetal biomarkers that circulate in maternal blood. *J Clin Invest,* **117**: 3007-3019.

Martini FH, Timmons MJ 1997. The cardiovascular system: Blood. In *Human Anatomy,* BRAKE, D (ed.)^(eds.). 2nd Ed. ed. Prentice-Hall International, Inc.: Illinois.

Masuzaki H, Miura K, Yamasaki K, Miura S, Yoshiura K, Yoshimura S, Nakayama D, Mapendano CK, Niikawa N, Ishimaru T 2005. Clinical applications of plasma circulating mRNA analysis in cases of gestational trophoblastic disease. *Clin Chem,* **51**: 1261-1263.

Mavrou A, Kouvidi E, Antsaklis A, Souka A, Kitsiou Tzeli S, Kolialexi A 2007. Identification of nucleated red blood cells in maternal circulation: a second step in screening for fetal aneuploidies and pregnancy complications. *Prenat Diagn,* **27**: 150-153.

Mayall F, Jacobson G, Wilkins R, Chang B 1998. Mutations of p53 gene can be detected in the plasma of patients with large bowel carcinoma. *J Clin Pathol,* **51**: 611-613.

Missler U, Wiesmann M, Wittmann G, Magerkurth O, Hagenstrom H 1999. Measurement of glial fibrillary acidic protein in human blood: analytical method and preliminary clinical results. *Clin Chem,* **45**: 138-141.

Mittl RL, Grossman RI, Hiehle JF, Hurst RW, Kauder DR, Gennarelli TA, Alburger GW 1994. Prevalence of MR evidence of diffuse axonal injury in patients with mild head injury and normal head CT findings. *AJNR Am J Neuroradiol,* **15**: 1583-1589.

Miura N, Shiota G, Nakagawa T, Maeda Y, Sano A, Marumoto A, Kishimoto Y, Murawaki Y, Hasegawa J 2003. Sensitive detection of human telomerase reverse transcriptase mRNA in the serum of patients with hepatocellular carcinoma. *Oncology,* **64**: 430-434.

Mulcahy HE, Lyautey J, Lederrey C, qi Chen X, Anker P, Alstead EM, Ballinger A, Farthing MJ, Stroun M 1998. A prospective study of K-ras mutations in the plasma of pancreatic cancer patients. *Clin Cancer Res,* **4**: 271-275.

Mutirangura A, Pornthanakasem W, Theamboonlers A, Sriuranpong V, Lertsanguansinchi P, Yenrudi S, Voravud N, Supiyaphun P, Poovorawan Y 1998. Epstein-Barr viral DNA in serum of patients with nasopharyngeal carcinoma. *Clin Cancer Res,* **4**: 665-669.

Myers TW, Gelfand DH 1991. Reverse transcription and DNA amplification by a Thermus thermophilus DNA polymerase. *Biochemistry,* **30**: 7661-7666.

Nagata S 2000. Apoptotic DNA fragmentation. *Exp Cell Res,* **256**: 12-18.

Nave KA, Lai C, Bloom FE, Milner RJ 1987. Splice site selection in the proteolipid protein (PLP) gene transcript and primary structure of the DM-20 protein of central nervous system myelin. *Proc Natl Acad Sci U S A,* **84**: 5665-5669.

Nawroz H, Koch W, Anker P, Stroun M, Sidransky D 1996. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med,* **2**:

1035-1037.

Ng EKO, Tsui NBY, Lam NY, Chiu RWK, Yu SC, Wong SC, Lo ES, Rainer TH, Johnson PJ, Lo YMD 2002. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. *Clin Chem,* **48**: 1212-1217.

Ng EKO, Leung TN, Tsui NBY, Lau TK, Panesar NS, Chiu RWK, Lo YMD 2003a. The concentration of circulating corticotropin-releasing hormone mRNA in maternal plasma is increased in preeclampsia. *Clin Chem,* **49**: 727-731.

Ng EKO, Tsui NBY, Lau TK, Leung TN, Chiu RWK, Panesar NS, Lit LCW, Chan KW, Lo YMD 2003b. mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci U S A,* **100**: 4748-4753.

Ng EKO, El-Sheikhah A, Chiu RWK, Chan KCA, Hogg M, Bindra R, Leung TN, Lau TK, Nicolaides KH, Lo YMD 2004. Evaluation of human chorionic gonadotropin beta-subunit mRNA concentrations in maternal serum in aneuploid pregnancies: a feasibility study. *Clin Chem,* **50**: 1055-1057.

Nien JK, Yoon BH, Espinoza J, Kusanovic JP, Erez O, Soto E, Richani K, Gomez R, Hassan S, Mazor M, Edwin S, Bahado-Singh R, Romero R 2006. A rapid MMP-8 bedside test for the detection of intra-amniotic inflammation identifies patients at risk for imminent preterm delivery. *Am J Obstet Gynecol,* **195**: 1025-1030.

Novakovic S, Hocevar M, Zgajnar J, Besic N, Stegel V 2004. Detection of telomerase RNA in the plasma of patients with breast cancer, malignant melanoma or thyroid cancer. *Oncol Rep,* **11**: 245-252.

Okazaki S, Sekizawa A, Purwosunu Y, Iwasaki M, Farina A, Okai T 2006. Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood. *J Med Genet,* **43**: e47.

Olinsky S, Loop BT, DeKosky A, Ripepi B, Weng W, Cummins J, Wenger SL, Yan Y, Lagenaur C, Narayanan V 1996. Chromosomal mapping of the human M6 genes. *Genomics,* **33**: 532-536.

Orrison WW, Gentry LR, Stimac GK, Tarrel RM, Espinosa MC, Cobb LC 1994. Blinded comparison of cranial CT and MR in closed head injury evaluation. *AJNR*

Am J Neuroradiol, **15**: 351-356.

Oudejans CB, Go AT, Visser A, Mulders MA, Westerman BA, Blankenstein MA, van Vugt JM 2003. Detection of chromosome 21-encoded mRNA of placental origin in maternal plasma. *Clin Chem,* **49**: 1445-1449.

Pelosi G, Schianchi E, Dell'orto P, Veronesi G, Spaggiari L, Pasini F, Sozzi G, Brambilla E, Griso C, Viale G 2006. Detecting cell-free circulating hTERT mRNA in the plasma may identify a subset of nonsmall cell lung cancer patients. *Virchows Arch,* **448**: 7-15.

Pineles BL, Romero R, Montenegro D, Tarca AL, Han YM, Kim YM, Draghici S, Espinoza J, Kusanovic JP, Mittal P, Hassan SS, Kim CJ 2007. Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia. *Am J Obstet Gynecol,* **196**: 261 e261-266.

Pitkin RM, Witte DL 1979. Platelet and leukocyte counts in pregnancy. *JAMA,* **242**: 2696-2698.

Poon LLM, Leung TN, Lau TK, Lo YMD 2000. Presence of fetal RNA in maternal plasma. *Clin Chem,* **46**: 1832-1834.

Pornthanakasem W, Shotelersuk K, Termrungruanglert W, Voravud N, Niruthisard S, Mutirangura A 2001. Human papillomavirus DNA in plasma of patients with cervical cancer. *BMC Cancer,* **1**: 2.

Prikk K, Maisi P, Pirila E, Sepper R, Salo T, Wahlgren J, Sorsa T 2001. In vivo collagenase-2 (MMP-8) expression by human bronchial epithelial cells and monocytes/macrophages in bronchiectasis. *J Pathol,* **194**: 232-238.

Qureshi AI, Tuhrim S, Broderick JP, Batjer HH, Hondo H, Hanley DF 2001. Spontaneous intracerebral hemorrhage. *N Engl J Med,* **344**: 1450-1460.

Raabe A, Grolms C, Seifert V 1999. Serum markers of brain damage and outcome prediction in patients after severe head injury. *Br J Neurosurg,* **13**: 56-59.

Raghupathi R, Graham DI, McIntosh TK 2000. Apoptosis after traumatic brain injury. *J Neurotrauma,* **17**: 927-938.

Rainer TH, Wong LK, Lam W, Yuen E, Lam NY, Metreweli C, Lo YMD 2003. Prognostic use of circulating plasma nucleic acid concentrations in patients with acute stroke. *Clin Chem*, **49**: 562-569

Reddi KK, Holland JF 1976. Elevated serum ribonuclease in patients with pancreatic cancer. *Proc Natl Acad Sci U S A*, **73**: 2308-2310.

Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, Cohn DE 2009. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. *Gynecol Oncol*, **112**: 55-59.

Rijnders RJ, van der Schoot CE, Bossers B, de Vroede MA, Christiaens GC 2001. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. *Obstet Gynecol*, **98**: 374-378.

Ryan BM, Lefort F, McManus R, Daly J, Keeling PW, Weir DG, Kelleher D 2003. A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in postoperative follow up. *Gut*, **52**: 101-108.

Sacks GP, Studena K, Sargent K, Redman CW 1998. Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis. *Am J Obstet Gynecol*, **179**: 80-86.

Samura O, Miharu N, Hyodo M, Honda H, Ohashi Y, Honda N, Hara T, Ohama K 2003. Cell-free fetal DNA in maternal circulation after amniocentesis. *Clin Chem*, **49**: 1193-1195.

Savola O, Hillbom M 2003. Early predictors of post-concussion symptoms in patients with mild head injury. *Eur J Neurol*, **10**: 175-181.

Schneider JJ, Unholzer A, Schaller M, Schafer-Korting M, Korting HC 2005. Human defensins. *J Mol Med*, **83**: 587-595.

Sekizawa A, Samura O, Zhen DK, Falco V, Farina A, Bianchi DW 2000. Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. *Prenat Diagn*, **20**: 886-889.

Sekizawa A, Kondo T, Iwasaki M, Watanabe A, Jimbo M, Saito H, Okai T 2001a.

Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clin Chem,* **47**: 1856-1858.

Sekizawa A, Sugito Y, Iwasaki M, Watanabe A, Jimbo M, Hoshi S, Saito H, Okai T 2001b. Cell-free fetal DNA is increased in plasma of women with hyperemesis gravidarum. *Clin Chem,* **47**: 2164-2165.

Sekizawa A, Jimbo M, Saito H, Iwasaki M, Sugito Y, Yukimoto Y, Otsuka J, Okai T 2002. Increased cell-free fetal DNA in plasma of two women with invasive placenta. *Clin Chem,* **48**: 353-354.

Sessa M 2008. Intracerebral hemorrhage and hypertension. *Neurol Sci,* **29 Suppl 2**: S258-259.

Shackford SR, Mackersie RC, Holbrook TL, Davis JW, Hollingsworth-Fridlund P, Hoyt DB, Wolf PL 1993. The epidemiology of traumatic death. A population-based analysis. *Arch Surg,* **128**: 571-575.

Shapiro B, Chakrabarty M, Cohn EM, Leon SA 1983. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer,* **51**: 2116-2120.

Shimada K, Murakami K, Shozu M, Segawa T, Sumitani H, Inoue M 2004. Sex-determining region Y levels in maternal plasma: Evaluation in abnormal pregnancy. *J Obstet Gynaecol Res,* **30**: 148-154.

Silva JM, Dominguez G, Silva J, Garcia JM, Sanchez A, Rodriguez O, Provencio M, Espana P, Bonilla F 2001. Detection of epithelial messenger RNA in the plasma of breast cancer patients is associated with poor prognosis tumor characteristics. *Clin Cancer Res,* **7**: 2821-2825.

Silva JM, Rodriguez R, Garcia JM, Munoz C, Silva J, Dominguez G, Provencio M, Espana P, Bonilla F 2002. Detection of epithelial tumour RNA in the plasma of colon cancer patients is associated with advanced stages and circulating tumour cells. *Gut,* **50**: 530-534.

Simons M, Trotter J 2007. Wrapping it up: the cell biology of myelination. *Curr Opin Neurobiol,* **17**: 533-540.

Skogseid IM, Nordby HK, Urdal P, Paus E, Lilleaas F 1992. Increased serum creatine kinase BB and neuron specific enolase following head injury indicates brain damage. *Acta Neurochir (Wien)*, **115**: 106-111.

Smid M, Vassallo A, Lagona F, Valsecchi L, Maniscalco L, Danti L, Lojacono A, Ferrari A, Ferrari M, Cremonesi L 2001. Quantitative analysis of fetal DNA in maternal plasma in pathological conditions associated with placental abnormalities. *Ann N Y Acad Sci,* **945**: 132-137.

Smith SC, Baker PN, Symonds EM 1997. Placental apoptosis in normal human pregnancy. *Am J Obstet Gynecol,* **177**: 57-65.

Steinman CR, Ackad A 1977. Appearance of circulating DNA during hemodialysis. *Am J Med,* **62**: 693-697.

Strickland S, Richards WG 1992. Invasion of the trophoblasts. *Cell,* **71**: 355-357.

Stroun M, Anker P, Lyautey J, Lederrey C, Maurice PA 1987. Isolation and characterization of DNA from the plasma of cancer patients. *Eur J Cancer Clin Oncol,* **23**: 707-712.

Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB 2004a. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A,* **101**: 6062-6067.

Su YH, Wang M, Brenner DE, Ng A, Melkonyan H, Umansky S, Syngal S, Block TM 2004b. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. *J Mol Diagn,* **6**: 101-107.

Su YH, Wang M, Brenner DE, Norton PA, Block TM 2008. Detection of mutated K-ras DNA in urine, plasma, and serum of patients with colorectal carcinoma or adenomatous polyps. *Ann N Y Acad Sci,* **1137**: 197-206.

Sueoka E, Sueoka N, Iwanaga K, Sato A, Suga K, Hayashi S, Nagasawa K, Nakachi K 2005. Detection of plasma hnRNP B1 mRNA, a new cancer biomarker, in lung cancer patients by quantitative real-time polymerase chain reaction. *Lung Cancer,* **48**: 77-83.

Sundstrom T, Sollid S, Wentzel-Larsen T, Wester K 2007. Head injury mortality in the Nordic countries. *J Neurotrauma,* **24**: 147-153.

Taback B, Giuliano AE, Hansen NM, Hoon DS 2001. Microsatellite alterations detected in the serum of early stage breast cancer patients. *Ann N Y Acad Sci,* **945**: 22-30.

Taback B, O'Day SJ, Boasberg PD, Shu S, Fournier P, Elashoff R, Wang HJ, Hoon DS 2004. Circulating DNA microsatellites: molecular determinants of response to biochemotherapy in patients with metastatic melanoma. *J Natl Cancer Inst,* **96**: 152-156.

Takebayashi S, Matsuo K, Kaneko M 1984. Ultrastructural studies of cerebral arteries and collateral vessels in moyamoya disease. *Stroke,* **15**: 728-732.

Tan EM, Schur PH, Carr RI, Kunkel HG 1966. Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J Clin Invest,* **45**: 1732-1740.

Tang K, Fu D, Kotter S, Cotter RJ, Cantor CR, Koster H 1995. Matrix-assisted laser desorption/ionization mass spectrometry of immobilized duplex DNA probes. *Nucleic Acids Res,* **23**: 3126-3131.

Tashlykov V, Katz Y, Gazit V, Zohar O, Schreiber S, Pick CG 2007. Apoptotic changes in the cortex and hippocampus following minimal brain trauma in mice. *Brain Res,* **1130**: 197-205.

Teasdale G, Jennett B 1974. Assessment of coma and impaired consciousness. A practical scale. *Lancet,* **2**: 81-84.

Theodor L, Melzer E, Sologov M, Idelman G, Friedman E, Bar-Meir S 1999. Detection of pancreatic carcinoma: diagnostic value of K-ras mutations in circulating DNA from serum. *Dig Dis Sci,* **44**: 2014-2019.

Thomas DG, Palfreyman JW, Ratcliffe JG 1978. Serum-myelin-basic-protein assay in diagnosis and prognosis of patients with head injury. *Lancet,* **1**: 113-115.

Thomas T 1993. Distribution of alpha 2-macroglobulin and alpha 1-acid

glycoprotein mRNA shows regional specialization in rat decidua. *Placenta,* **14**: 417-428.

Tong YK, Ding C, Chiu RW, Gerovassili A, Chim SS, Leung TY, Leung TN, Lau TK, Nicolaides KH, Lo YM 2006. Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: Theoretical and empirical considerations. *Clin Chem,* **52**: 2194-2202.

Townend W, Ingebrigtsen T 2006. Head injury outcome prediction: a role for protein S-100B? *Injury,* **37**: 1098-1108.

Tsui NBY, Ng EKO, Lo YMD 2002. Stability of endogenous and added RNA in blood specimens, serum, and plasma. *Clin Chem,* **48**: 1647-1653.

Tsui NBY, Chim SSC, Chiu RWK, Lau TK, Ng EKO, Leung TN, Tong YK, Chan KCA, Lo YMD 2004. Systematic micro-array based identification of placental mRNA in maternal plasma: towards non-invasive prenatal gene expression profiling. *J Med Genet,* **41**: 461-467.

Tsui NBY, Chiu RWK, Ding C, El-Sheikhah A, Leung TN, Lau TK, Nicolaides KH, Lo YMD 2005. Detection of trisomy 21 by quantitative mass spectrometric analysis of single-nucleotide polymorphisms. *Clin Chem,* **51**: 2358-2362.

Ulm B, Ulm MR, Panzer S 1998. Fetal sex and hemolytic disease from maternal red-cell alloimmunization. *N Engl J Med,* **338**: 1699-1700.

Ulm B, Svolba G, Ulm MR, Bernaschek G, Panzer S 1999. Male fetuses are particularly affected by maternal alloimmunization to D antigen. *Transfusion,* **39**: 169-173.

Umansky SR, Tomei LD 2006. Transrenal DNA testing: progress and perspectives. *Expert Rev Mol Diagn,* **6**: 153-163.

van Wijk IJ, de Hoon AC, Jurhawan R, Tjoa ML, Griffioen S, Mulders MA, van Vugt JM, Oudejans CB 2000. Detection of apoptotic fetal cells in plasma of pregnant women. *Clin Chem,* **46**: 729-731.

Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M 1994. Point mutations of the N-ras gene in the blood plasma DNA of patients with

myelodysplastic syndrome or acute myelogenous leukaemia. *Br J Haematol,* **86**: 774-779.

Wataganara T, LeShane ES, Farina A, Messerlian GM, Lee T, Canick JA, Bianchi DW 2003. Maternal serum cell-free fetal DNA levels are increased in cases of trisomy 13 but not trisomy 18. *Hum Genet,* **112**: 204-208.

Wei C, Saller DN, Sutherland JW 2001. Detection and quantification by homogeneous PCR of cell-free fetal DNA in maternal plasma. *Clin Chem,* **47**: 336-338.

Werner H, Dimou L, Klugmann M, Pfeiffer S, Nave KA 2001. Multiple splice isoforms of proteolipid M6B in neurons and oligodendrocytes. *Mol Cell Neurosci,* **18**: 593-605.

WHO 2004. Rehabilitation for Persons with Traumatic Brain Injury. CENTRE. World Health Organization.

WHO 2006. Helmets: a road safety manual for decision-makers and practitioners. World Health Organization: Geneva, Switzerland.

Woertgen C, Rothoerl RD, Metz C, Brawanski A 1999. Comparison of clinical, radiologic, and serum marker as prognostic factors after severe head injury. *J Trauma,* **47**: 1126-1130.

Wong BCK, Chiu RWK, Tsui NBY, Chan KCA, Chan LW, Lau TK, Leung TN, Lo YMD 2005. Circulating placental RNA in maternal plasma is associated with a preponderance of 5' mRNA fragments: implications for noninvasive prenatal diagnosis and monitoring. *Clin Chem,* **51**: 1786-1795.

Wong IH, Lo YMD, Zhang J, Liew CT, Ng MH, Wong N, Lai PB, Lau WY, Hjelm NM, Johnson PJ 1999. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. *Cancer Res,* **59**: 71-73.

Wong SC, Lo ES, Cheung MT 2004a. An optimised protocol for the extraction of non-viral mRNA from human plasma frozen for three years. *J Clin Pathol,* **57**: 766-768.

Wong SC, Lo SF, Cheung MT, Ng KO, Tse CW, Lai BS, Lee KC, Lo YMD 2004b.

Quantification of plasma beta-catenin mRNA in colorectal cancer and adenoma patients. *Clin Cancer Res,* **10**: 1613-1617.

Wong SC, Ma BB, Lai PB, Ng SS, Lee JF, Hui EP, Lam MY, Chan CM, Chan AT 2007. The effect of centrifugation on circulating mRNA quantitation opens up a new scenario in expression profiling from patients with metastatic colorectal cancer. *Clin Biochem,* **40**: 1277-1284.

Wright CF, Burton H 2009. The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis. *Hum Reprod Update,* **15**: 139-151.

Yamada T, Nakamori S, Ohzato H, Oshima S, Aoki T, Higaki N, Sugimoto K, Akagi K, Fujiwara Y, Nishisho I, Sakon M, Gotoh M, Monden M 1998. Detection of K-ras gene mutations in plasma DNA of patients with pancreatic adenocarcinoma: correlation with clinicopathological features. *Clin Cancer Res,* **4**: 1527-1532.

Yamazaki Y, Yada K, Morii S, Kitahara T, Ohwada T 1995. Diagnostic significance of serum neuron-specific enolase and myelin basic protein assay in patients with acute head injury. *Surg Neurol,* **43**: 267-270; discussion 270-261.

Yan Y, Lagenaur C, Narayanan V 1993. Molecular cloning of M6: identification of a PLP/DM20 gene family. *Neuron,* **11**: 423-431.

Yang HJ, Liu VW, Wang Y, Chan KY, Tsang PC, Khoo US, Cheung AN, Ngan HY 2004. Detection of hypermethylated genes in tumor and plasma of cervical cancer patients. *Gynecol Oncol,* **93**: 435-440.

Zelenika D, Grima B, Pessac B 1993. A new family of transcripts of the myelin basic protein gene: expression in brain and in immune system. *J Neurochem,* **60**: 1574-1577.

Zhao L, Furebring M, Xu S, Venge P 2004a. Subcellular localization and mobilization of carcinoembryonic antigen-related cell adhesion molecule 8 in human neutrophils. *Br J Haematol,* **125**: 666-673.

Zhao L, Xu S, Fjaertoft G, Pauksen K, Hakansson L, Venge P 2004b. An enzyme-linked immunosorbent assay for human carcinoembryonic antigen-related cell adhesion molecule 8, a biological marker of granulocyte activities in vivo. *J*

Immunol Methods, **293**: 207-214.

Zhong XY, Holzgreve W, Li JC, Aydinli K, Hahn S 2000. High levels of fetal erythroblasts and fetal extracellular DNA in the peripheral blood of a pregnant woman with idiopathic polyhydramnios: case report. *Prenat Diagn,* **20**: 838-841.

Zhong XY, Hahn D, Troeger C, Klemm A, Stein G, Thomson P, Holzgreve W, Hahn S 2001a. Cell-free DNA in urine: a marker for kidney graft rejection, but not for prenatal diagnosis? *Ann N Y Acad Sci,* **945**: 250-257.

Zhong XY, Hahn S, Holzgreve W 2001b. Prenatal identification of fetal genetic traits. *Lancet,* **357**: 310-311.

Zhong XY, Holzgreve W, Hahn S 2001c. Risk free simultaneous prenatal identification of fetal Rhesus D status and sex by multiplex real-time PCR using cell free fetal DNA in maternal plasma. *Swiss Med Wkly,* **131**: 70-74.

Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W, Hahn S 2001d. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. *Am J Obstet Gynecol,* **184**: 414-419.

Zhong XY, Holzgreve W, Hahn S 2002. Cell-free fetal DNA in the maternal circulation does not stem from the transplacental passage of fetal erythroblasts. *Mol Hum Reprod,* **8**: 864-870.