



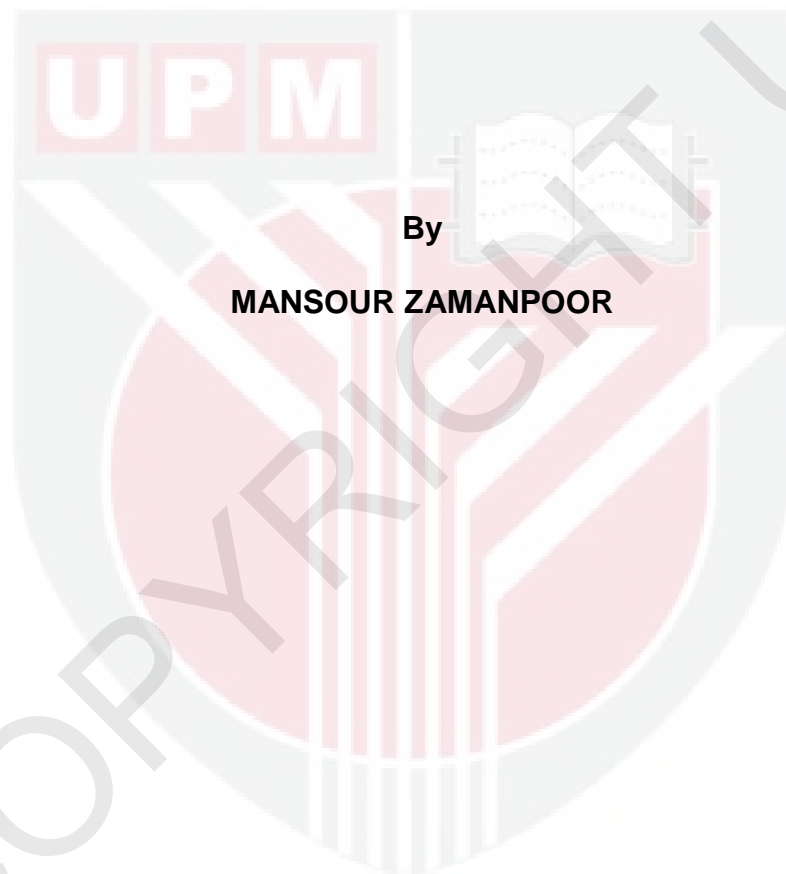
UNIVERSITI PUTRA MALAYSIA

***QUANTITATIVE ANALYSIS OF MALE FOETAL DNA IN MATERNAL
CIRCULATION IN GESTATIONAL DIABETES MELLITUS, IRON
DEFICIENCY ANAEMIA AND HYPERTENSIVE PREGNANCIES***

MANSOUR ZAMANPOOR

FPSK(m) 2009 18

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By

MANSOUR ZAMANPOOR

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of
Master of Science**

November 2009

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

QUANTITATIVE ANALYSIS OF MALE FOETAL DNA IN MATERNAL CIRCULATION IN GESTATIONAL DIABETES MELLITUS, IRON DEFICIENCY ANAEMIA AND HYPERTENSIVE PREGNANCIES

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November 2009

Chair: Thilakavathy A/P Karuppiah, PhD

Faculty: Medicine and Health Sciences

Advances in molecular genetics have allowed the investigation of the foetal genome through analysis of circulating foetal DNA in maternal plasma. Cell free foetal DNA (fDNA) in maternal plasma or serum is widely investigated as a source of foetal genetic materials, both in studies of pregnancy related disorders and in planning strategies for non-invasive prenatal diagnosis. Increased amount of circulating fDNA in maternal plasma has been found in adverse pregnancies such as preeclampsia, foetal chromosomal aneuploidies, placental abnormalities, preterm labour and hyperemesis gravidarum. It was suggested that elevation of fDNA in maternal plasma could be used for early identification of adverse pregnancies. To date, no study has been done to investigate the fDNA in gestational diabetes mellitus (GDM) and anaemia, considered as the more common pregnancy related complications. The aim of this study was to quantify circulating fDNA levels in normal healthy pregnant individuals and pregnant women with the following

clinical conditions: GDM, anaemia and hypertension (HTN). In this study, pregnant women carrying male singleton foetuses were recruited from the Maternity Hospital Kuala Lumpur as study subjects. A total of a hundred and sixteen samples consisting of GDM (n=40), anaemia (n=19), HTN (n=19), and normal pregnant women (n=38) carrying singleton male foetuses, were collected. The fDNA was extracted from maternal plasma. The fDNA concentrations were measured by quantitative real-time PCR amplification using TaqMan dual labelled probe system. The SRY gene which is located on Y chromosome was used as a unique foetal marker. The mean fDNA concentration for normal pregnancy samples was 41.14 GE/ml while the mean fDNA concentration for GDM pregnancy samples was 35.16 GE/ml, 30.96 GE/ml for anaemic pregnancy samples and 197.04 GE/ml for HTN pregnancy samples. No significant differences were observed in the mean fDNA concentration between normal and GDM pregnancy samples ($P=0.627$) and also between normal and anaemic pregnancy samples ($P=0.535$), but significant differences were observed between normal and HTN pregnancy samples ($P=0.001$). On the other hand, GDM and anaemia does not affect levels of fDNA in maternal plasma while HTN significantly elevate the levels of fDNA in maternal plasma. Hence, there is a potential of using fDNA measurements as a predictive marker for the development of HTN, but if fDNA is used as an additional marker in prenatal screening test in the future, the findings of this study suggests that fDNA quantity will not be as informative for GDM and anaemic pregnancies as it is for HTN. In conclusion, measuring the overall amount of circulating fDNA may be used as a general screening tool for pregnancy-associated disorders specifically

hypertensive disorders, and it is hoped that further developments over the next few years will enable us to move even closer to use non-invasive nucleic acid-based prenatal diagnosis.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**ANALISA KUANTITATIF DNA JANIN LELAKI DI DALAM DARAH IBU
MENGANDUNG YANG MENGALAMI KENCING MANIS, ANAEMIA
KEKURANGAN ZAT BESI DAN TEKANAN DARAH TINGGI**

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Perkembangan dalam genetik molekular membenarkan penyelidikan genom janin melalui analisa peredaran DNA janin di dalam plasma ibu. Sel bebas DNA janin di dalam plasma atau serum ibu dikaji secara meluas sebagai sumber bahan genetik janin, dalam kajian kehamilan yang berkaitan dengan penyakit dan pelan strategi untuk diagnosis prenatal yang tidak berbahaya. Peningkatan jumlah DNA janin yang beredar (fDNA) di dalam plasma ibu telah ditemui dalam kehamilan yang berbahaya seperti kekejangan, tekanan darah tinggi ketika kehamilan, aneuploidi kromosom janin, keluarbiasaan placenta, persalinan sebelum tempoh dan "hyperemesis gravidarum".

Adalah dicadangkan bahawa peningkatan fDNA dalam plasma ibu boleh digunakan dalam pengenalpastian awal dalam kehamilan berbahaya. Sehingga kini, tiada kajian yang telah dijalankan dalam penyelidikan fDNA dalam kencing manis semasa penghamilan dan anaemia di mana kebiasaannya berkaitan dengan komplikasi. Tujuan kajian ini dijalankan

adalah untuk mengukur tahap peredaran DNA bebas secara kuantitatif dalam individu hamil yang sihat dan wanita hamil yang mengalami keadaan klinikal termasuk kencing manis anaemia dan tekanan darah tinggi ketika hamil. Dalam kajian ini, wanita hamil yang mengandung janin lelaki dipilih dari klinik persalinan Hospital Kuala Lumpur sebagai subjek penyelidikan. Sebanyak seratus enam belas sampel adalah wanita mengandung yang mengalami kencing manis (n=40), anaemia (n=19), tekanan darah tinggi (n=19), dan wanita hamil yang sihat (n=38) telah dikumpulkan. Dalam kajian ini, fDNA telah diekstrak melalui plasma ibu. Kepekatan fDNA telah diukur dengan menggunakan kuantitatif amplifikasi gen SRY dengan menggunakan PCR dalam masa sebenar yang merupakan penanda unik janin yang terdapat di kromosom Y dengan menggunakan sistem alat dwi label TaqMan. Purata kepekatan fDNA untuk sampel biasa ialah 41.14 genom seimbang/ml sementara purata untuk kepekatan fDNA untuk sampel GDM ialah 35.16 genom seimbang/ml. Sementara itu, purata untuk kepekatan fDNA sampel anaemia ialah 30.96 genom seimbang/ml dan purata untuk kepekatan fDNA bagi sampel tekanan darah tinggi ialah 197.04 genom/ml. Tiada perbezaan yang ketara dalam purata kepekatan fDNA di antara sampel biasa kencing manis ketika mengandung ($P=0.627$) dan juga di antara sampel biasa dan anaemia ($P=0.535$). Akan tetapi terdapat perbezaan yang ketara antara purata kepekatan fDNA bagi sampel biasa dan tekanan darah tinggi ($P=0.001$). Sementara itu, penyakit kencing manis ketika mengandung dan anaemia tidak mempunyai kesan terhadap tahap fDNA di dalam plasma ibu manakala tekanan darah tinggi secara jelas meningkatkan tahap fDNA di dalam plasma ibu. Oleh hal yang demikian adalah menarik untuk

menggunakan pengukuran fDNA sebagai penanda ramalan untuk wanita hamil yang mengalami tekanan darah tinggi. Akan tetapi, pengukuran fDNA tidak diperlukan sebagai penanda tambahan dalam prosedur diagnosis prenatal pada masa hadapan memandangkan data kami mencadangkan bahawa kuantiti fDNA tidak memberi perbezaan ketara antara wanita hamil yang sihat dan wanita hamil yang mengalami GDM dan anaemia. Kesimpulannya, pengukuran keseluruhan jumlah fDNA boleh digunakan sebagai alat umum untuk prosedur diagnosis yang berkaitan dengan penyakit dan diharapkan bahawa perembangan dalam beberapa tahun akan datang dalam penggunaan prosedur diagnosis yang selamat adalah berasaskan nukleik asid.

ACKNOWLEDGEMENTS

My greatest and ultimate debt and gratitude is due to Allah, the Most Beneficent and the Most Merciful. May He pardon and forgive my weaknesses and endow me with knowledge and help.

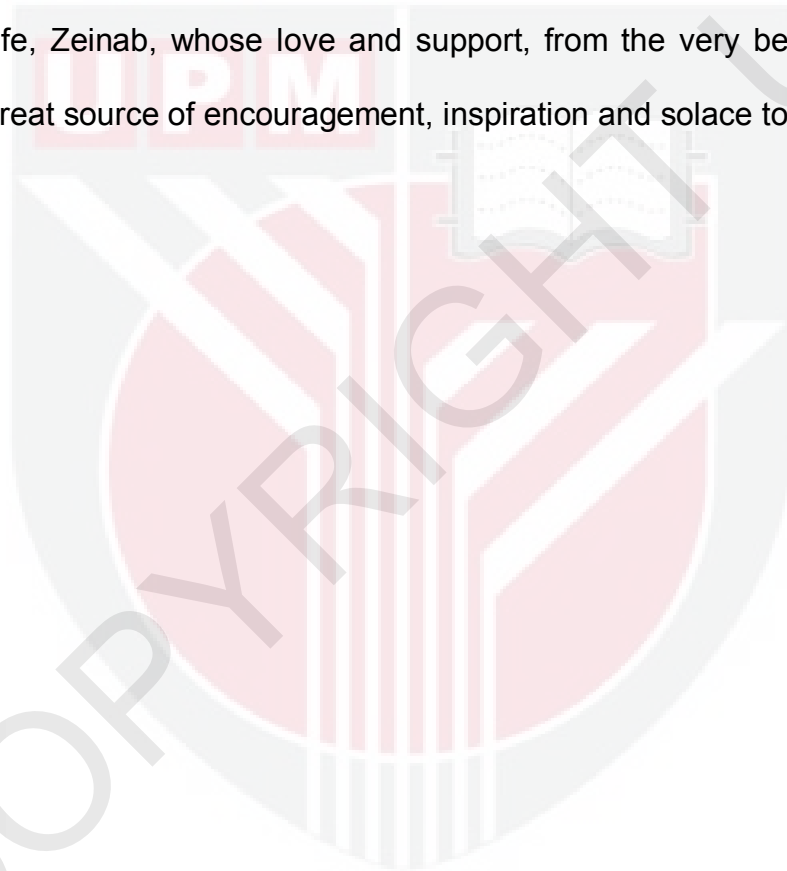
My gratitude is to Dr. Thilakavathy Karupiah for her guidance, suggestions, patience and encouragement throughout the project. I am also grateful to my co-supervisors Associate Professor Dr. Rozita Rosli, Associate Professor Dr. Mohd Nazri Yazid and Associate Professor Dr. Zaheed Husain, for their help, support and valuable discussions.

My special gratitude is to Associate Professor Dr. Rozita Rosli for her guidance, suggestions, patience and encouragement throughout the thesis writing. I am also grateful to Prof Dato Dr. Lye Munn Sann for his guidance and valuable discussions in the biostatistics part of this study.

I thank my friends in the Molecular Genetics Laboratory; Dr. Reza, Wendy, Nadine, Radha, Pushpa, Behnam, Chan, Chin, Razieh, Narges, Dr. Lama, Zahrah, Eunice, Herson, Fatim, low, Farhana, and late Dhurai Raj, for all moral support and friendship. I also thank Dr Syahrilnizam Abdullah, Dr Abhimanyu Veerakumarasivam and Dr Norshariza Nordin for all the discussions. I would like to thank Puan Salimah Mohd Sain, Puan Hazlen Salleh and Puan Pushpaleela for all the kindness and assistance they have rendered me. I thank Dr. Niraj and Dr. Haw for their kind collaboration in

sampling from the Maternity Hospital Kuala Lumpur. I also appreciate the staff in the Deputy Dean's office for post graduate studies.

To my parents, who have taught me to trust myself and to love all things both great and small, I am grateful for their constant love, support, encouragement and for raising me the way I am today. To my brothers and sisters, thank you in believing in me. Last but not least, my grateful thanks go to my wife, Zeinab, whose love and support, from the very beginning, has been a great source of encouragement, inspiration and solace to me.



I certify that an Examination Committee has met on **11 November 2009** to conduct the final examination of **Mansour Zamanpoor** on his **Master of Science** thesis entitled “**Quantitative Analysis of Male Foetal DNA in Maternal Circulation in Gestational Diabetes Mellitus, Iron Deficiency Anaemia and Hypertensive Pregnancies**” in accordance with Universiti Pertanian Malaysia (Higher Degree) act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the Master of Science.

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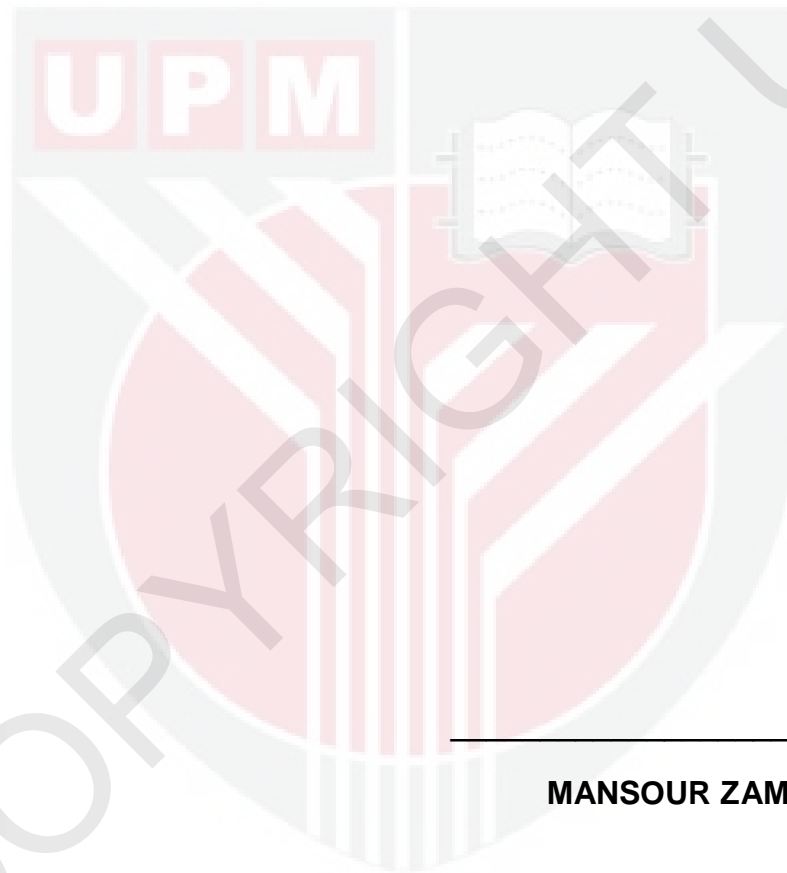
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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institutions.



MANSOUR ZAMANPOOR

Date:

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LIST OF ABBREVIATIONS

μl	Microliter
μM	Micromolar
ADA	American Diabetes Association
AFP	Alpha-fetoprotein
BMI	Body mass index
bp	Base pair
CA	Cancer antigen
CD71	Transferrin receptor antigen
CT	Cycle threshold
CV	Coefficient of variations
CVS	Chorionic villous sampling
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dl	Decilitre
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediaminetetraacetic acid
FAM™ dye	6-carboxyfluorescein
fDNA	Foetal DNA
FISH	Fluorescent <i>in situ</i> hybridisation
G	Gauge
<i>g</i>	G-force
g/dl	Gram per decilitre
GDM	Gestational diabetes mellitus

GE	Genomic equivalence
Hb	Haemoglobin
hCG	Human chorionic gonadotropin
Hct	Haematocrit
Hg	Mercury
HLA-A	Human leukocyte antigen A serotype group
HTN	Hypertension
IHV	Intrahepatic vein
LBW	Low birth weight
LED	Light emitting diode
MgCl ₂	Magnesium chloride
mM	Millimolar
NB	Nasal Bone
ng	Nanogram
nM	Nanomolar
NRBC	Nucleated erythrocyte
NT	Nuchal translucency
NTC	Non Template Control
OGTT	Oral Glucose Tolerance Test
PAPP-A	Pregnancy-associated plasma protein A
PCR	Polymerase chain reaction
PE	Pre-eclampsia
pg	Picogram
PGD	Pre-gestational diabetes
PIH	Pregnancy-induced hypertension
PUBS	Percutaneous Umbilical Blood Sampling
QF-PCR	Quantitative fluorescence polymerase chain reaction

qPCR	Quantitative real time PCR
Rh	Rhesus antigen
RN	Fluorescence intensity
SRY	Sex Determining Region Y
TAMRA™ dye	6-carboxy-tetramethyl-rhodamine
U	Unit
uE3	Unconjugated oestriol
UNG	Uracil N-glycosylase



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CHAPTER 1

INTRODUCTION

The need for prenatal diagnosis and monitoring is expanding and has become an established modern obstetric practice in many countries. There are multiple advantages of understanding the prenatal status of the foetus. The first advantage of prenatal diagnosis of foetal malformations is that genetic counselling can be provided. In addition, the parents, obstetrician, geneticist, and other specialists can discuss options ranging from abortion to intrauterine medical and surgical treatments. The optimal time, mode, and place of delivery can be determined, and a postnatal treatment plan can be formulated.

Secondly, the well-being of the foetus can be revealed before it is born. Prenatal diagnosis aims to detect or exclude a selected group of morphological, structural, functional, chromosomal and molecular defects in the unborn foetus (Papp and Papp, 2003). Because of delay in child-bearing age and smaller family size of modern societies, the request for safe, accurate and timely prenatal diagnostic services is increasing.

Prenatal diagnosis includes all aspects of embryonic and foetal diagnosis (Connor and Ferguson-Smith, 1997). Specifically, prenatal diagnosis is helpful for (1) managing the remaining weeks of the pregnancy, (2) determining the outcome of the pregnancy, (3) planning for possible

complications with the birth process, (4) planning for problems that may occur in the newborn infant, (5) deciding whether to continue the pregnancy, (6) finding conditions that may affect future pregnancies (Klatt, 1994).

Prenatal screening or diagnostic tests are offered to women who are at greater risk to develop an adverse pregnancy. The ability of being informed of the possible prospective problems of the foetus can let parents to prepare in advance financially, emotionally and practical challenges that they would be facing for the affected birth as well as making decision whether they wish to continue with the pregnancy or not (Lowry *et al.*, 1995).

The definitive prenatal diagnosis requires the analysis of foetal genetic material, which is obtained through invasive techniques such as amniocentesis and chorionic villus sampling. Because these techniques are associated with risk of foetal miscarriage, alternative non-invasive methods are being actively explored to sample for foetal genetic material. The ideal prenatal diagnostic test should be safely performable in early pregnancy, with the guarantee of an accurate and timely diagnosis to prevent undesired stress to the parents and incorrect medication to the foetus.

Due to advances in molecular genetics, detection and isolation of foetal DNA in the maternal circulation have been made possible which opens up a new approach in the non-invasive assessment of foetal-maternal health (Lo *et al.*, 1997). Therefore, foetal genome can be investigated through analysis of free foetal DNA (fDNA) in maternal plasma. High concentrations of foetal DNA in

maternal circulation have been reported in pregnancy related complications such as chromosomal aneuploidies, pre-eclampsia, hyperemesis gravidarum, preterm labour and invasive placentation (Farina *et al.*, 2003; Wataganara *et al.*, 2003; Lee *et al.*, 2002; Sekizawa *et al.*, 2002; Sekizawa *et al.*, 2001b; Zhong *et al.*, 2000a; Lo *et al.*, 1999c; Lo *et al.*, 1999b; Leung *et al.*, 1998). Taken together, these data suggest that it might be possible to use fDNA in maternal plasma or serum for predicting at-risk pregnancies.

Gestational diabetes mellitus (GDM) is the most common medical complication and metabolic disorder of pregnancy, occurring in 1-14% of patients depending on the population described and the criteria used for diagnosis. GDM is defined as carbohydrate intolerance that begins or is first recognised during pregnancy. Screening and diagnosing pregnancies complicated by GDM is important for preventing adverse prenatal outcomes (Carr and Gabbe, 1998). On the other hand, iron deficiency anaemia in childbearing women increases maternal mortality, prenatal infant loss, and prematurity (Schorr and Hediger, 1994). Forty percent of all maternal prenatal deaths are linked to anaemia. Besides that, hypertensive disorders of pregnancy include preeclampsia which complicate 10% of pregnancies are a leading cause of maternal and infant illness and death (World Health Organisation, 2002).

In this study, GDM, iron deficiency anaemia and hypertension (HTN) were studied as pregnancy related complications. Prior to this study, no study has addressed the quantitative aspects of circulating foetal DNA (fDNA) in GDM

and anaemia. The outcome of the project can help us better understand of GDM, anaemia and HTN, which will be useful in developing non-invasive prenatal screening or diagnosis of these complications for early detection by further studies in the future. Thus, the study outcome will be made available to expectant mothers as a screening method for complicated pregnancies once the technique is established. Early detection of these complications would enable not only the mother but also the family to mentally as well as financially prepare for the decision that has to be made with regards to the pregnancy.

Objectives

The objectives of this study are:

1. To optimise the quantitative real time PCR assay using self-designed primers to detect the SRY gene in maternal plasma of pregnant women bearing male foetus.
2. To investigate the concentrations of fDNA in maternal circulation in three groups of pregnancy-related complications including GDM, anaemia and HTN as well as healthy normal pregnant women.
3. To compare the concentrations of fDNA in maternal plasma of the complicated pregnancy groups with healthy normal pregnant women.

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