Sudan University of Science and Technology
College of Graduate Studies

Assessment of protein C and protein S levels in Umbilical Cord Blood of Gestational Diabetic Mothers

قياس معدل بروتين سي و إس في دم الحبل السري في الامهات المصابات بسكر الحمل

A dissertation submitted in partial fulfillment of the requirement for the degree of M.Sc. In hematology and Immunohematology

Submitted by

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بسم الله الرحمن الرحيم

قال تعالى : (وَمَا خَلَقْتُ الْجِنَّ وَالإِنسَ إِلاَّ لِيَعْبُدُونِ)

صدق الله العظيم

سوره الذاريات الآية (٥٦)

Dedication

To my lovely and honorable parents whom pillar for all my dreams, specially my dad his words of encouragement and push for tenacity ring in my ears.

Special feeling of gratitude to my darling husband for his kindliness, supporting and god speed.

To my special gift in my womb who patience with me and carrying odds in this long march.

To my amazing supervisor: Dr. Hiba Badreldin Khalil for her longanimity to my tribulation and supporting me to master the leader dots.

To my special friends and colleagues who have supported me throughout the process, I always appreciate all they have done.

Thanks for all..

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Thanks to everyone who helped me to do this work.

My greatest thanks for all pregnant women whom the blood sample has been collected from in a difficult and hard moments.

GREAT THANKS…

Abstract

Introduction: Gestational diabetes mellitus (GDM) is one of dangerous and common disease in recent time and is increasing enormously worldwide in the recent decades especially in developing countries. The prevalence of Gestational Diabetes mellitus (GDM) differs depending on the regions and the country. Out of 25 pregnancies 1 develop GDM which is associated with complications in the period immediately before and after birth. It is one of the causes of maternal and fetal mortality and morbidity.

Objectives: To evaluate protein C and protein S levels in newborn Cord blood of GDM mothers and to compare with protein C and protein S levels of newborn cord blood of healthy pregnancy. Moreover, to correlate these levels according to the weight of newborns age and BMI of GDM mothers.

Materials and Methods: This is analytical case control study carried out in Alsaaha Specialized Hospital, Saad Aboulela University Hospital and Al_Qma Specialized Hospital, in the period from March to July 2018. 30 pregnant women diagnosed with GDM and thirty matched healthy pregnant women as control group were recruited in this study. Citrated new born Cord blood sample was collected from each patient and control for protein C and protein S level by immunoassay technique. Data was analyzed by SPSS version 20, and expressed as means, tables and figures.

Results: The mean of protein C and protein S in GDM cord blood were 56.9±8.6% and 61.5±8.7% respectively and in the healthy control were 104.4±13.4% and 113.4±12.3% , and there were statisically significant decreased of protein C and protein S levels in cord blood of newborns with GDM mothers compared with control group (P. value = 0.05). And there

was no correlation between protein C and protein S levels and FBG , age of mothers , BMI and weight of newborns from GDM mothers.

Conclusion: There were a significant decrease in protein C and protein S levels .And there was no association between protein C and protein S levels and FBG, age of GDM mothers , BMI and weight of newborns from GDM mothers. This study approved that the decreasing in protein C and protein S levels were used as alarm for thrombotic risk in newborn of mother with GDM mothers.

ملخص البحث

المقدمة: سكر الحمل من اخطر الأمراض واكثرها انتشارا في الوقت الحالي, وهو يزداد بشكل هائل عالميا في العقود الأخيرة خصوصا في الدول النامية. انتشار سكر الحمل يختلف اعتمادا على المناطق والدول . من اصل 25 حالة حمل لأول مرة توجد حالة واحدة تصاب بسكر الحمل, و تتعرض لمضاعفات قبل وبعد الولادة, وهو احد اسباب الوفيات والأمراض الجنينية والأمومية.

الأهداف: تحديد مستوى بروتين (سي) و(إس) في دم الحبل السري لمرضى سكر الحمل ومقارنتها مع مستوى بروتين (سي) و (إس) للحمل الطبيعي في مستشفى سعد ابو العلا الجامعي ومستشفى الساحة التخصصي ومستشفى القمة التخصصي ,و توضيح علاقة هذه المستويات مع وزن الطفل , عمر ومساحة كتلة جسم الامهات المصابات.

طرق البحث:هذه الدراسة لحالات وشواهد في مستشفى سعد أبو العلا الجامعي , مستشفى الساحة التخصصي و مستشفى القمة التخصصي في الفترة من مارس إلى يوليو2018. تهدف الدراسة لقياس قيمة بروتين سي و إس في الدم لدى النساء المرضى بسكر الحمل, تم اختيار ثلاثين امرأه تعانين من سكر الحمل كحالة دراسة و ثلاثين امرأه من الأصحاء كمجموعه ضبط. تم أخذ دم الحبل السري من كل مريضه و حالة ضابطة لقياس البروتين سي و إس بواسطه استخدام التقنية المناعية وتم تحليل النتائج بواسطة برنامج الحزم الاحصائية للعلوم الاجتماعية اصداره 20وتم توضيح النتائج في شكل متوسطات و جداول و رسومات توضيحيه.

النتائج :كان هناك فرق ذو دلالة إحصائية ف مستوى البروتين سي و إس بين الحالات المصابة وغير المصابة $p>0.05$,وأظهرت النتائج عدم وجود علاقة ذات دلالة إحصائية بين مستوى بروتين سي و إس وعمر الحالات تحت الدراسة , مساحة كتلة جسم الحالة و وزن الاطفال من الأمهات المصابات.

الخاتمة المستنتجة : أثبتت الدراسة ان هناك نقصا في معدل بروتين سي و إس في دم الحبل السري للحوامل المصابات بسكر الحمل مقارنة مع غير المصابات, و أنه لا توجد علاقة بين هذه المعدلات وسكر الدم عند الصيام , مؤشر كتلة جسم وعمر الامهات المصابات و وزن الاطفال من الامهات المصابات . النقصان في معدل بروتين سي و إس يمكن ان تستعمل كمؤشر لامكانية حدوث جلطة في الاطفال حديثي الولادة من امهات مصابات بسكرالحمل .

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Term	Abbreviation	Term	Abbreviation
GDM	Gestational Diabetes Mellitus	**DM**	Diabetes Mellitus
MDG	Millennium Development Goal	**OGTT**	Oral Glucose Tolerance Test
NGT	Normal Glucose Tolerance	**WHO**	World Health Organization
IFG	Impaired Fasting Glucose	**CRP**	C-Reactive Protein
PLt	Platelet	**PAI**	Plasminogen Activator Inhibitor
t-PA	Tissue Plasminogen Activator	**ATIII**	Anti-Thrombin III
APC	Activated Protein C	**KDa**	Kilo Dalton
TAFI	Thrombin Activatable Fibrinolysis Inhibitor	**AT**	Anti-Thrombin
EGF	epidermal growth factor	**Glu**	Glutamic Acid

PS	Protein S	**IDM**	Insulin Dependent diabetes Mellitus
PC	Protein C	**TMB**	Thermo Scientific Pierce
FBG	Fasting Blood Glucose	**HT**	Hyper Tension
SPSS	Statistical Package for Social Science	**TM**	Thrombomodulin
IADPSG	International Association of Diabetes and Pregnancy Study Group	**VWF**	Von Will brand Factor
IVH	Intra Vascular Hemorrhage	**WBC**	white Blood Cell
IGT	Impaired Glucose Tolerance	**IGF**	Intra Uterine Growth Factor
T1DM	Type 1 Diabetes Mellitus	**EPCR**	Endothelia Protein C Receptor.
T2DM	Type 2 Diabetes Mellitus	**BMI**	Body Mass Index
DIC	Disseminated Intra vascular Coagulopathy	**TFBI**	Tissue Factor Pathway Inhibitor

Chapter One

1.Introduction

1.1Gestational Diabetes Mellitus (GDM)

Gestational diabetes mellitus (GDM) is presence of diabetes in pregnant women which disappear after delivery. That means there is an elevated level of serum glucose (impaired glucose tolerance) in a pregnant woman who has no any evidence of diabetes before pregnancy.GDM has potential risk for mother as well as a fetus(Qazi *et al*,2016).

Out of the 8 goals stated by the United Nation Millennium Development Goal (MDG), 4th goal targets the reduction in child mortality and 5th goal states the upgrading the maternal health.GDM predict risk of for over diabetes in women, three to ten of all pregnancies are complicated by diabetes.The prevalence of diabetes is increasing worldwide, in every year 21 million people are reported. Decreasing level of physical activity, increasing obesity and changes in dietary patterns leading to more and increase the rate of GDM in developing countries including(Pakistan and India).GDM can lead to maternal and fetal complication also lead to fetus morbidity and mortality. There are two types of gestational diabetes mellitus either insufficient insulin production (Type 1) or sensitivity(Type2)(Raj,2012).The women with gestational diabetes have 35 to 60 % chance to developing diabetes in next ten to twenty years and infants with hyperglycemic conditions which may lead to metabolic disorder in later life(Qazi *et al.,2016*).

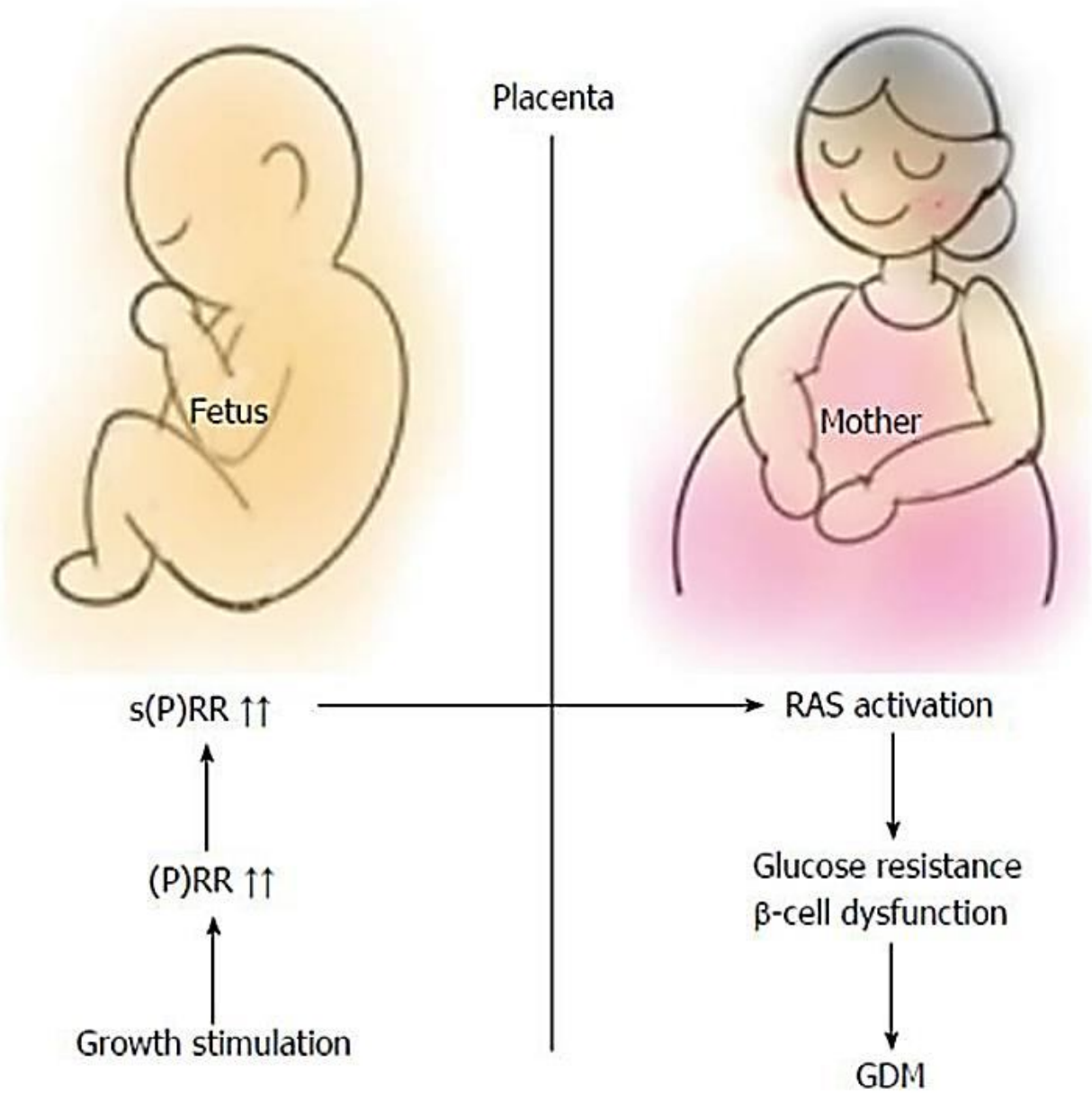

Figure 1.1: Mechanism of GDM, adopted by Siri L. Kjos and Ute M. Schaefer-Graf.2007

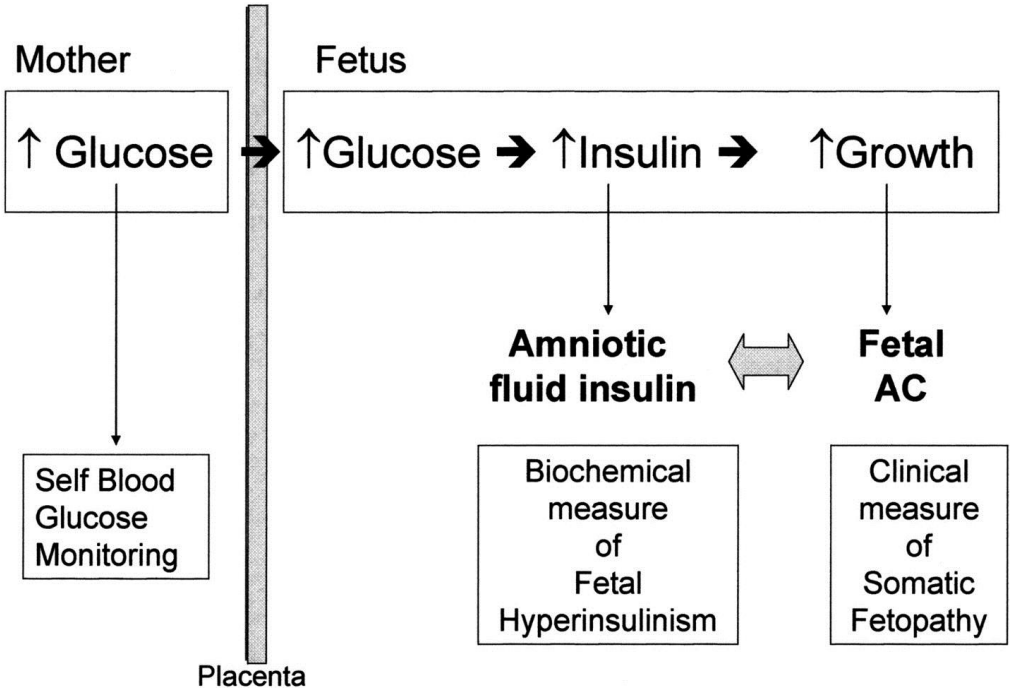

Figure 1.2 Mechanism of GDM in maternal and fetus, adopted by Siri L. Kjos and Ute M.Schaefer-Graf.2007

1.1.1 Physiology of GDM

The physiology of gestational diabetes mellitus is not completely clear until now (Daher,2013) .But generally, worldwide GDM is increasing due to old age pregnant women and due to obesity that resulting from life style change .Women with diabetes in pregnancy can be divided into those who were already have diabetes before pregnancy (overt diabetes), and those who developed it during pregnancy(gestational diabetes). Pregnancy is a normal phenomenon, there are many hormones act during pregnancy, Insulin resistance begins in mid of second trimester and continues to third trimester as well, and it is due to placental hormones and it increased in GDM pregnancy than normal pregnancy. Fetus of women with GDM has high insulin and blood cells level , and have high weight with insulin resistant in childhood which lead to diabetes in adulthood (Mohammed *et al.*,2014). In addition to insulin resistance , pancreatic beta-cell dysfunction might also play a role in

pathophysiology of GDM (Abdel Hameed and john, 2017). Finally decrease exercise and increased intake of caloric lead to relative glucose intolerance (Eman,2015).

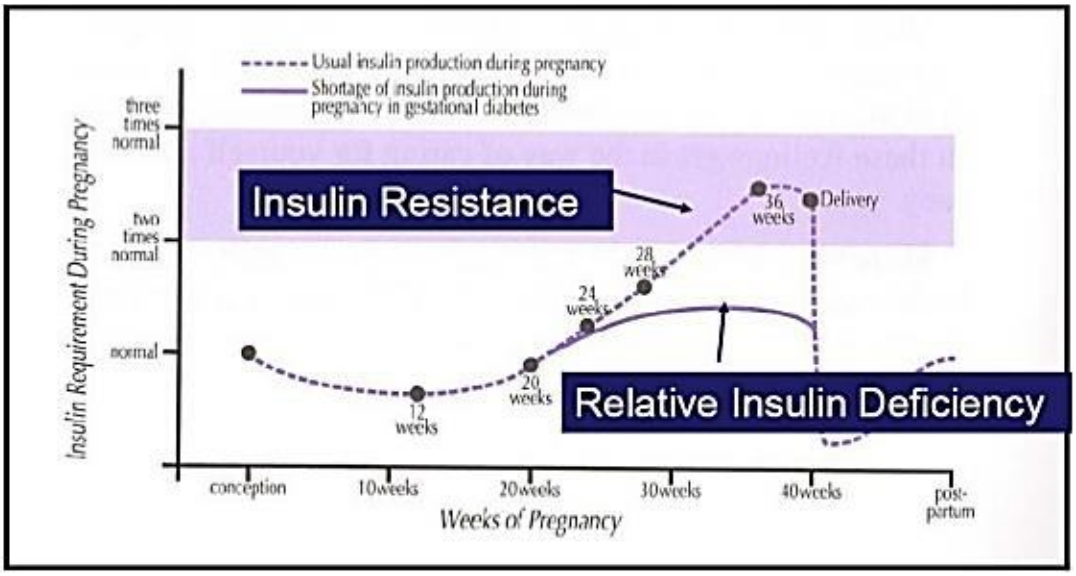

Figure 1.3 Physiology of GDM and Insulin requirement during pregnancy, adopted by Abdel Hameed and john, 2017

1.1.2 Clinical importance of GDM

Clinically GDM is a high risk factor for mother and child. If left undetected or untreated it may lead to serious complication to both of mother and the child. The immediate complication to the mother is preeclampsia, obstructed labor, caesarean delivery and in delayed complication due to rise in blood sugar lead to infection and delayed wound healing. The child may be hypoglycemic, macrosomic, shoulder dystocia or may lead to intrauterine death. Hence it becomes compulsory for all the pregnant women to undergo random blood glucose at the first antenatal visit to detect diabetes in pregnancy (Guanderson *et al.*,2009).

1.1.3 Effects of GDM on Maternal and Child Health

The Millennium Development Goal has clearly stated that 4 and 5 reduce child mortality and improve maternal health respectively. GDM is a

warning to both the mother and child. Once detected the treatment has to be started in order to be on a safer side to prevent any serious complication to occur during pregnancy and child birth. The delivery should be safe for the mother and the child as well because GDM causes problem to both (Esakoff *et al.*,2009).

The Maternal Consequences are:

Preeclampsia ,infection , polyhydramnios, postpartum bleeding, caesarean section, delayed wound healing or wound dehiscence if GDM is not controlled and long term effect is type 2 DM (England *et al.*, 2009 and O'Sullivan.1982).

The Fetal Consequences are:

• Congenital anomaly, macrosomia: It is defined as a birth weight greater than or equal to 4000 g. Incidence is 17-29% of pregnancies with GDM as compared with 10% in the non-diabetic population (Esakoff *et al.*,2009), hypoglycemia, hypocalcaemia, hyaline membrane disease, apnea , bradycardia ,Traumatic delivery, stillbirth and In long term complications of newborn of mother with GDM include hypertension, obesity and dyslipidemia (Mitanchez,2014).

1.1.4 Diagnosis of GDM

Gestational diabetes mellitus is one of common metabolic problem during pregnancy that increases perevelance of Type 2 diabetes and pregnancies complications (Mohammed *et al.*, 2014). Biochemical laboratory tests are an essential part of human disease diagnosis and monitoring by using various biological fluids or tissues, however, blood is still the most commonly used diagnostic material. Unfortunately, blood collection is an invasive procedure that may involve some risk to the health of medical staff and patient. The ideal period to screen for GDM is around 16 weeks of gestation and even earlier in high-risk groups with a history of fetal wastage. It is safe to screen for GDM during early weeks of pregnancy as

by early detection of glucose intolerance during pregnancy and adequate care to the antenatal women, a good fetal outcome can be achieved similar to that of NGT pregnant women (Seshiah *et al.*,2008).If a woman is found to have normal glucose tolerance test in the first trimester, she should be tested for GDM around 24th -28th weeks and around 32nd-34th weeks and also in later weeks if necessary, particularly when rapid weight gain occurs or fetal macrosomia is suspected (Franks *et al.*,2016).

Increased in plasma glucose level is first alarm to be care about healthy of pregnant women when one of the following is diagnosis:

- Fasting plasma glucose=5.1_6.9 mmol/l(92_125 mg/dl).
- 1h post 75 gm oral glucose load >=10 mmol/l(180 mg/dl).
- 2-h post 75 gm oral glucose load= 11 mmol/l (153_199 mg/dl) (Franks *et al.*,2016).

The recommended glucose cut-off value for GDM corresponds to those proposed by IADPSG and is lower than those recommended by earlier guidelines. Unlike earlier guidelines, they are based on the association of plasma glucose and adverse maternal and neonatal outcomes during pregnancy, at birth and immediately following it. The difference from IADPSG guidelines is that these new WHO guidelines set a range of plasma glucose levels to distinguish diabetes in pregnancy and GDM (WHO,2013).

Although the ADA adopted this new scheme, the American College of Obstetricians and Gynecologists (2013) declined to endorse the single 75-gram oral glucose tolerance test. Instead, the College continues to recommend a two-step approach to screen and diagnose gestational diabetes (Franks *et al*,.2015).

Many international and regional guidelines have lagged behind the current research.

Table 1.1 Classification of glucose intolerance by 75gm 2 hour oral glucose tolerance test(OGTT).Adopted by Franks *et al.*,2016.

Diagnosis	Fasting plasma glucose (mg/dl)	2-hour plasma glucose (mg/dl)
Normal glucose tolerance (NGT)	<100	<140
Impaired fasting glucose (IFG)	100-125	
Diabetes mellitus (DM)	≥126	≥200

Leptin is one of measurable marker in umbilical cord of pregnant women with gestational diabetes that affected fetal growth, It has been studied(Mohammed *etal.*, 2014).Insulin-like growth factor-1 (IGF-1), which has effects similar to insulin, reduces blood glucose level, improves insulin sensitivity and may play an important role in the pathogenesis of gestational diabetes (GDM). Insulin_ like growth factor_1, glucose, insulin, CRP, fibrinogen and lipids concentrations were significantly higher in women with GDM(Matusezk *et al.*, 2011).

1.1.5 Management of GDM

Efficacy medical treatment is one of important way to manage the risk of gestational diabetes mellitus in pregnant lady, but the first and basic step is diet modification which use by pregnant lady to avoid the increasing in plasma glucose above 95 mg/dL, although there is no control experiment to identify ideal glucose level that to be risk or harmful for fetal. In some countries glyburide has been used for managing gestational diabetes mellitus, It's inexpensive and safety (Eman,2015).

Aim of management is to:

Maintain euglycemia ,Prevent obstetrical complications ,Fix optimal time and appropriate mode of delivery.

Management includes:

1. Counseling of the patient: to care about his exercise and diet control, self- monitoring of blood glucose and identification and treatment of hypoglycemia.
2. Treatment of blood glucose control (Luis,2013).

1.2Haemostasis

Greek word, haem meaning blood and stasis mean to stop. This balance process maintained by complication of coagulation system, fibrinolytic system, Platelets and vessel wall. Normally, coagulation process is controlled by several inhibitors to limit the clot formation and avoid the thrombus (Previtali *et al.*, 2011).

1.2.1Primary Hemostasis

Primary hemostasis is basic and series step result in initial platelet plug that make by interaction of platelets, vessel walls and adhesion proteins.

1.2.2 Secondary Hemostasis

Secondary hemostasis consists of the cascade of coagulation serine proteases (Table: 1.2) that culminates in cleavage of soluble fibrinogen by thrombin. Thrombin cleavage generates insoluble fibrin that forms a cross linked fibrin mesh at the site of an injury. It has been traditionally classified into intrinsic and extrinsic pathways, both of which converge on factor X activation (Owens and Mackman,2010) (Figure: 1.4).

Table 1.2: Nomenclature of the coagulation proteins/clotting factors (Palta S *et al.* 2010)

Clotting factor number	Clotting factor name	Function	Plasma half-life (h)	Plasma concentration (mg/L)
I	Fibrinogen	Clot formation	90	3000
II	Prothrombin	Activation of I, V, VII, VIII, XI, XIII, protein C, platelets	65	100
III	TF	Co factor of VIIa	-	-
IV	Calcium	Facilitates coagulation factor binding to phospholipids	-	-
V	Proacclerin, labile factor	Co-factor of X-prothrombinase complex	15	10
VI	Unassigned			
VII	Stable factor, proconvertin	Activates factors IX, X	5	0.5
VIII	Antihaemophilic factor A	Co-factor of IX-tenase complex	10	0.1
IX	Antihaemophilic factor B or Christmas factor	Activates X: Forms tenase complex with factor VIII	25	5
X	Stuart-Prower factor	Prothrombinase complex with factor V: Activates factor II	40	10
XI	Plasma thromboplastin antecedent	Activates factor IX	45	5
XII	Hageman factor	Activates factor XI, VII and prekallikrein		-
XIII	Fibrin-stabilising factor	Crosslinks fibrin	200	30
XIV	Prekallikerin (F Fletcher)	Serine protease zymogen	35	
XV	HMWK- (F Fitzgerald)	Co factor	150	
XVI	vWf	Binds to VIII, mediates platelet adhesion	12	10 µg/mL
XVII	Antithrombin III	Inhibits IIa, Xa, and other proteases	72	0.15-0.2 mg/mL
XVIII	Heparin cofactor II	Inhibits IIa	60	-
XIX	Protein C	Inactivates Va and VIIIa	0.4	-
XX	Protein S	Cofactor for activated protein C		-

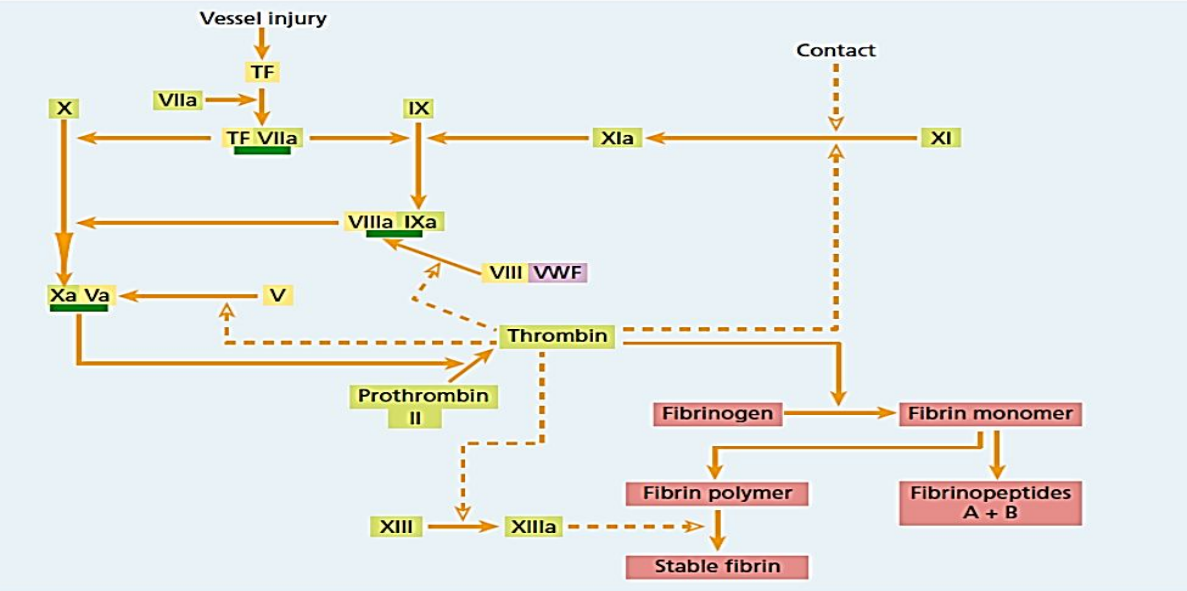

Figure 1.4: The pathway of blood coagulation cascade, adopted by A.V. Hoff brand and P.A.H. Moss, 2011

1.2.3Extrinsic Pathway

Its primary step of plasma hemostasis begins by tissue factor in sub endothelial tissue which bind with FVIIa and calcium to activate FX(Owens and Mackman , 2010).

1.2.4Intrinsic Pathway

It begins with factor XII, HMW kininogen, prekallekerin and factor XI (contact family) to activate factor XI which activate factor IX, which then acts with factor VIII to form tenase complex on a phospholipid surface to activate factor X (Hall, 2010).

1.2.5Common Pathway

Activated factor X together with FV, tissue phospholipid, calcium and plt phospholipid form prothrombinase complex to convert prothrombin to thrombin. Thrombin convert soluble fibrin to an insoluble fibrin gel and activated FXIII which crosslinks fibrin polymers and form a fibrin mesh that traps circulating plt, red cells and leucocytes (Kumar *et al.*,2010).

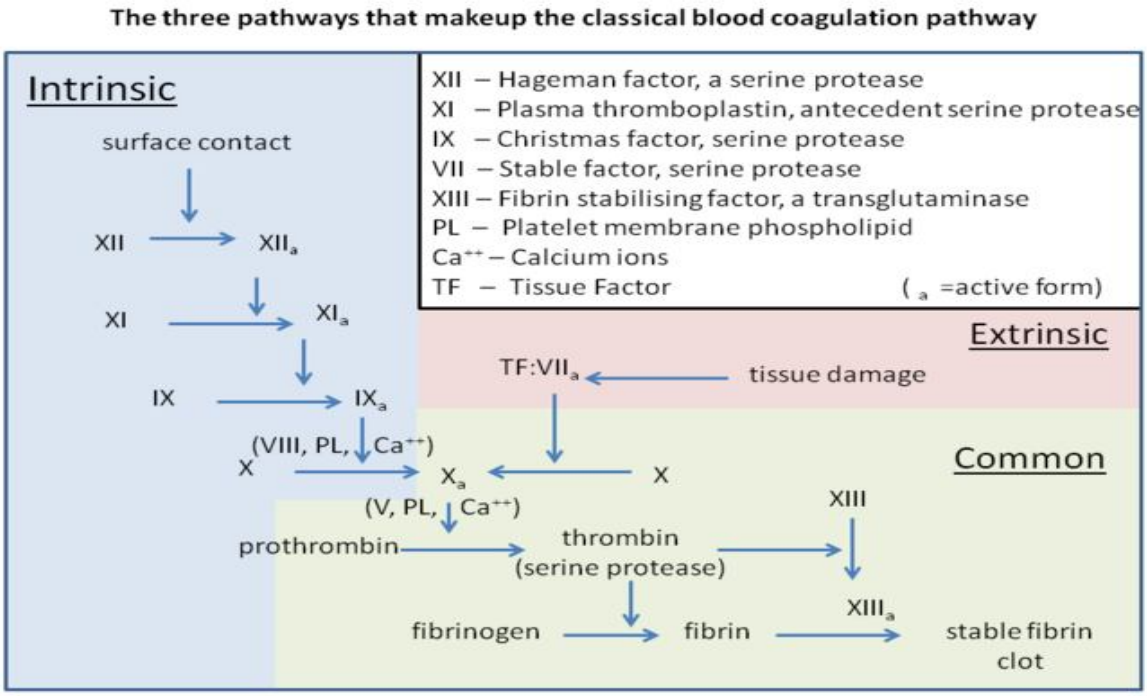

Figure 1.5 Classical pathway, adopted by A.V. Hoff brand and P.A.H. Moss. 2011.

1.2.6Fibrinolytic System

Fibrinolysis is aprocess that remove clots to prevent uncontrolled thrombosis and embolism.

Primary Fibrinolysis

Normally occurs after clot retraction .The main enzyme in primary fibrinolysis is plasmin that degrades fibrin mesh.

Plasmin is produced in an inactive form (plasminogen) in the liver, and cannot cleave fibrin, and circulates in the bloodstream. Instead, it is incorporated into the clot when it is formed and then activated into plasmin later. Plasminogen is activated to plasmin by tissue plasminogen activator (t-PA) and urokinase, an enzyme found in the urine. T-PA is released into the blood very slowly by the damaged endothelium of the blood vessels. T-PA and urokinase are themselves inhibited by plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2 (PAI-1 and PAI-2). In contrast, plasmin further stimulates plasmin generation by producing more active forms of both tissue plasminogen activator (t-PA) and urokinase. Following fibrin degradation by plasmin, old activated platelets from the platelet plug are phagocytized and destroyed by macrophages (Hoff brand and Moss, 2011).

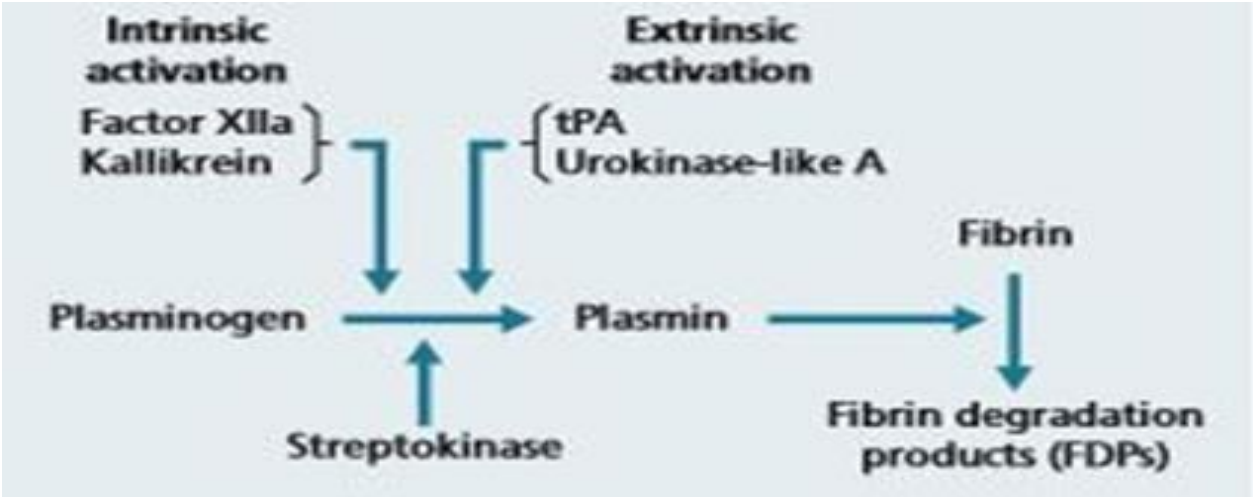

Figure 1.6:The fibrinolytic system , adopted by A.V. Hoff brand and P.A.H. Moss. 2011

Secondary Fibrinolysis

Secondary fibrinolysis generally refers to treatment of pathological thromboembolism. If blood clots embolize to different parts of the body, they can cause tissue death by blocking off blood flow to those tissues. This is a common cause of heart attacks, pulmonary embolism, and strokes. Several medications exist to help treat and prevent these conditions (Hoff Brand,2011).

1.2 Cord Blood

Its blood from umbilical cord and placenta after baby born and the cord is cut. Cord blood contain stem cells , so some parents decided to store it and using as treatment or alternative source of stem cells in patient with no working bone marrow and patient with cancer. Placenta also contains whole human blood as it connects mother and fetus and there is selective feto-maternal circulation at utero-placental interface throughout pregnancy. APC has important role in maintaining of uretro-placental circulation , and also is a major anticoagulant in placenta . Placenta make cord blood pure and free from bacterial and protozoal contamination so Its transfusion is associated with a lower risk of infection transmission in comparison with bone marrow transplantation and adult blood transfusion.The cord blood has a high concentration of cytokines and growth factors in its plasma, which eventually helps in gene switching mechanism after birth of baby. Stem cells in placental blood are 0.01% of the cellular content of the umbilical cord whole blood (Baer *etal.*, 2013).In the case of a healthy neonate, umbilical cord blood does not contain any acquired antibodies (Nidhi,2016).

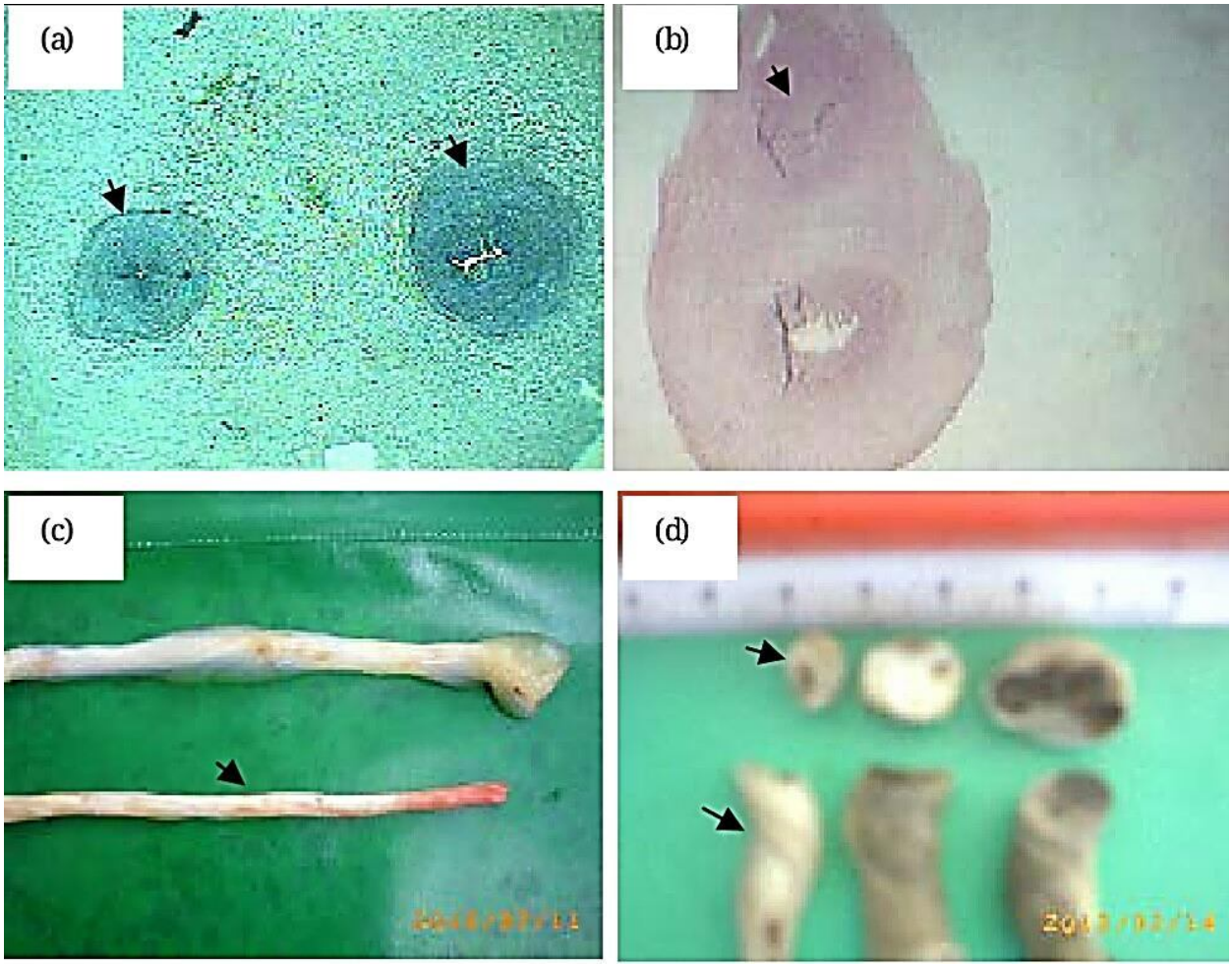

Figure1.7 Comparison of lean umbilical cord of GDM group with normal cord (a) Cross section of normal umbilical cord show two umbilical arteries, (b) SUA with one vein (full section), (c) Lower cord with SUA of GDM group shows lean umbilical cord in comparison with upper normal cord and (d) Left cord with SUA shows the cross section area of lean cord less than 1 cm in comparison with cords without SUA , (Lateef R., H., 2015)

1.3.1 Maternal and Cord Blood Hemostasis

Coagulation system of neonate differ from that of the adult in many ways. There is a reduction of factors II to XIII (except VIII), fibrinogen, ATIII, protein C and heparin cofactor II .

Also there is lower level of vWF and PAI with higher D-dimer levels of newborn in compared with their mother, all this factors lead to increase tendency for bleeding in premature infants. The newborn is at risk for vitamin k deficiency with bleeding due to poor transport of vitamin k

across the placenta and low levels of coagulation factors (Gruel,2010). After all that the real pathologic mechanism of GDM on hemostasis balance still unclear (Lemkes *et al.*,2010).

1.4 Protein C

Known as autoprothrombin IIA, produce in liver. It is a vitamin K_dependent proenzyme of a serine protease composed of multiple domains of 62 KDa, that work in concert as a natural anticoagulant system.Human protein C is structurally similar to other vitamin K-dependent proteins affecting blood clotting, such as prothrombin, Factor VII, Factor IX and Factor X. Mature protein C has light and heavy chain linked by disulfide bond. The light chain consist of Gla domain and two epidermal growth factor like domain (EGF).Thrombin activate protein C from inactive form (zymogen) to active form (serine protease), but it has poorly affinity to activate it without binding with endothelial cell thrombomodulin(TM and endothelial protein C receptor(EPCRs).Because of EPCR's role, activated protein C is found primarily near endothelial cells.Mature human protein C contains 419 amino acids, and the human gene (PROC) on chromosome 2 (2p13-14) contains 9 exons (Orstrom and Scolar, 2011).

1.4.1 Protein C Functions

Activated protein C (with phospholipid, calcium and its cofactor, protein S, and factor V)degrades thrombin activated factor (Va and VIIIa)responses. APC degrades clotting factors Va and VIIIa in tenase and prothrombinase complexes, in this way attenuating thrombin generation, and in addition it transforms pro-TAFI into TAFI (Thrombin Activatable Fibrinolysis Inhibitor) (Cabello-Gutierrez *et al* .,2009), (*Isermann et al.*,2003) (Figure 1.4).

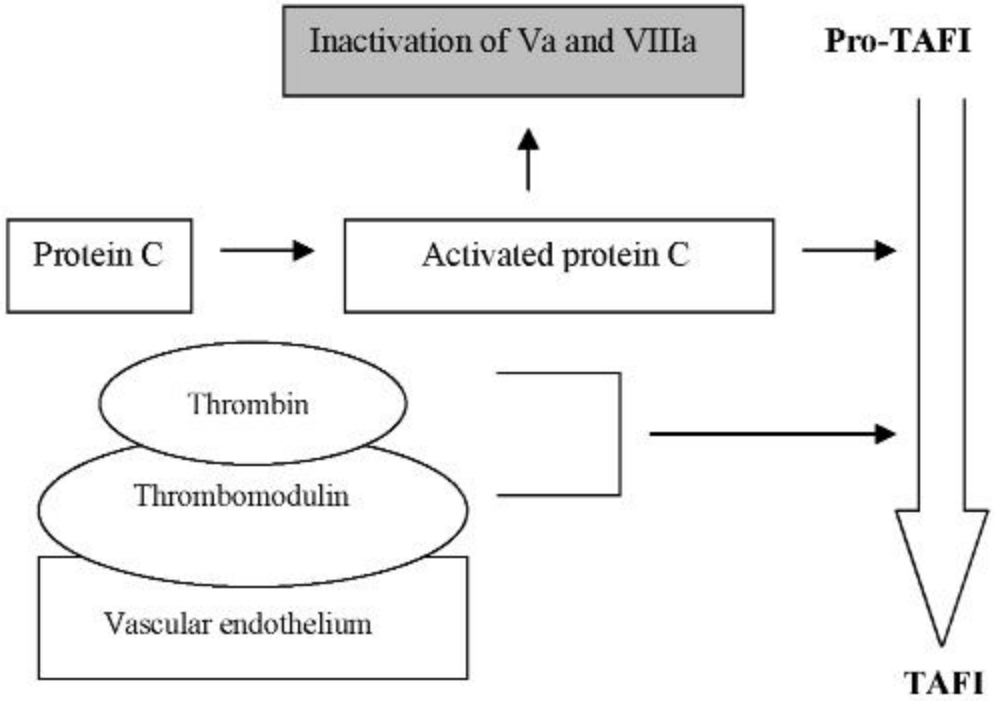

Fig. 1.8 Protein C pathway generation of activated protein C(APC) under the effect of the thrombin/thrombomodulin complex activity of APC: (a) inactivation of Va and VIII a factors, (b)generation of TAFI (thrombin activatable fibrinolysis inhibitor), adopted by U Mieczyslaw et al .,2010

Activated protein C (APC) in pregnant women play a role in maintenance of the uteroplacental circulation and development of the fetus as well as in pathogenesis of preeclampsia. APC is the most important vaso-protective protein due to its anti-thrombotic and anti -inflammatory as well as anti-apoptotic properties. Due to high TM level in the placenta and myometrium as well as to the placental concentration of EPCR receptors, APC is probably the major anticoagulant of the uteroplacental circulation potentiating the anti thrombogenic effect of APC .Also in general circulation, APC plays a role of an important anticoagulant – the second after anti thrombin (AT) or even the first one, making up the antithrombogenic activity of the vascular wall. In a pregnant woman, a decrease in vascular anti thrombogenic activity (a drop in AT and APC) may lead to preeclampsia (assumption of a preeclampsia hypothesis.

According to diabetologists, the pathogenesis of diabetic nephropathy indicates a pathogenetic relationship with reduced APC activity in the kidney) a direct effect of TM deficiency in glomerular endothelium (Gilbert *etal.*,2008).

1.5 Protein S (PS)

Protein S is a vitamin K–dependent anticoagulant protein that was first discovered in Seattle, Washington in 1979 and arbitrarily named after the city of its discover (TenKate and VanderMeer, 2008).

1.5.1Genetics, structure and biochemistry

Human PS is a single-chain (75-kDa) glycoprotein, encoded by the gene PROSI on chromosome 3. The mature form of the protein has 635 amino acids and is organized into eight domains: An NH2-terminal Gla domain (residues 1 to 45), an aromatic stack, a 29-residue thrombin-sensitive domain (residues 46 to 75), four EGF domains (residues 76 to 242), and a COOH-terminal domain homologous to the sex hormone-binding globulin and androgen-binding protein. Like other vitamin K-dependent proteins, PS requires carboxylation of its glutamic acid (Glu) residues to become biologically active. This process involves a γ-glutamyl carboxylase and a reduced form of vitamin-K resulting in the addition of CO2molecules to the γ-carbon of glutamic acids forming γ-carboxyglutamic acid (Gla) residues on the N-terminal Gla domain. Plasma PS is synthesized mainly in the liver and endothelial cell, but is also stored in the endothelial cell and the alpha granules of platelets. Approximately 40% of protein S circulates in the free form, and the remaining 60% circulatesas a 1:1 complex with C4b-binding protein (C4bBP), a regulatory protein of the complement system(Suleiman *et al.,* 2013).

1.5.2 Functions of protein S

Protein S serves its function as anticoagulant by tow mechanisms, one dependent on activating protein C and other independent on activating protein C (Malar and Gausman, 2011).

Protein S enhances APC inactivation of factors Va and VIIIa. The interaction between protein S and APC alters the structure of APC and moves the APC active site closer to the membrane surface.PS has other functions not associated with APC. PS can directly inhibit both factor X and prothrombin activation, serve as a cofactor for tissue factor pathway inhibitor (TFPI) function, enhance fibrinolysis by decreasing thrombin-activatable-fibrinolytic-inhibitor (TAFI)Protein S has been reported to bind to factor Xa, factor VIII, and factor Va to compete for pro - thrombinase-binding sites on the membrane surface**696,** and to stimulate the inhibition of factor Xa by TFPI. These interactions serve to inhibit prothrombin activation in vitr. The C4bBP-protein S complex may inhibit factor X activation as well.Protein S also has additional potential roles outside of anticoagulation. Protein S interaction with T cells promotes T cell aggregation and proliferation and may serve to regulate inflammatory processes (Suleiman *et al.,* 2013).

1.5.3 Regulation of protein S

α-Thrombin cleavage of protein S at Arg^{49}, Arg^{60}, or Arg^{70} in the thrombin-sensitive domain inhibits the ability of protein S to act as a cofactor for APC. Protein S activity is also regulated by interaction with C4bBP. The 1:1 complex between protein S and C4bBP neutralizes the anticoagulant capacity of protein S (Greer *et al.*, 2014).

1.5.4 Clinical aspects of protein S

Hereditary and acquired deficiencies of PS predispose individuals to increased risk of thrombosis. Acquired decreased levels of plasma PS are caused by several mechanisms: PS consumption (thrombosis, surgery and

DIC), decreased synthesis of PS (liver disease, vitamin K deficiency, warfarin therapy, newborn), and redistribution of complexed PS(chronic inflammation, acute phase reaction, oral contraceptives, estrogen replacement therapy and pregnancy) (Malar and Gausman, 2011).

1.6 Previous Studies

Neary *et al.*,2015 in the Rotunda Hospital, Dublin, Ireland, showed and approved that protein C was significantly lower in cord plasma than in maternal and control plasma (Neary *et al.*,2015).

In Poland, **Mieczyslaw *et al.*, 2010** have done a study determined that, the concentration of protein C antigen in mother's plasma was 135.11±1.05%, whereas in cord plasma was 57.60±10.32%, thus it was lower by over a half(42.67%) in the fetus as compared to the mother,and also they approved that the level of total protein S in mother's plasma was 92.49±13.24%, whereas in cord plasma 33.19±4.96%,thus being almost three fold lower (35.88%) in the fetus than in the mother (Mieczyslawm *et al.*,2010).

Jordi *et al.*, in 1998 did their study in normal pregnant women *(n* = 60) and pregnant women with gestational diabetes (n = 15) formed the study population. Coagulation and fibrinolysis parameters were estimated using commercial tests. Protein C and protein S activities in normal and gestational diabetes pregnancies were determined .And their result conducted that in the third trimester and during labor, lower functional PC levels were observed in the GD group, and also during the active phase of labor and after delivery, total and free PS levels were lower in the GDM group (jordi *et al.*,1998).

In addition **Hassan *et al.*,1989** in KSA, cord Blood samples were collected randomly from 300 Saudi mothers and their babies at the time of delivery. And their result showed that protein C was significantly

lower in cord plasma than in maternal and control plasma (Hassan *et al.*,1989).

1.7 Rationale

GDM is most developing disease that affect wide range of pregnant women whose delivered an infant with abnormal complication such as hypoglycemia, hypertrophy, inflammation and may lead to increase infant mortality and morbidity. Some pregnant women may at risk for hypercoaguble status during pregnancy, and presence of GDM will progress the pregnancy and infant for poor quality of life. Some evidence, shows that thrombosis has a tendency to progress in neonate of diabetic mothers (Edstrom and Christensen,2000). Cord blood sample will provide a source of material analysis for newborn instead of venous blood at the time of delivery. Analysis and investigation of diseases from cord blood samples is a non-invasive protocol rather than consider that cord blood is a waste blood . Protein C and protein S estimation has a predictor role for thrombotic event and this study may help to follow the newborns health for future and risk of thrombosis .

1.8Objectives

1.8.1General objective

- To determine of protein C and protein S in newborn cord blood samples among mother with GDM.

1.8.2Specific Objective

- To estimate the protein C and protein S levels in newborn cord blood of healthy pregnant and newborn cord blood of GDM mothers.

- To compare the levels of protein C and protein S between healthy and GDM pregnant women.
- To correlate the cord blood protein C and protein S levels with the FBG levels among mothers.
- To associate the protein C and protein S levels of newborns cord blood GDM mothers to their gestational weeks ,age, BMI and weight of newborns.

Chapter Two

2. Materials and Methods

2.1 Materials

Analytical case control study conducted and approved by Sudan University of Science and Technology, College of Medical Laboratory Science and the samples were collected from Alsaaha Specialized Hospital, Saad Aboulela University Hospital and Al_Qma Specialized Hospital from March to May 2018.The samples were collected from 30 healthy pregnant women as control and another 30 samples from pregnant women with GDM after an informed consent.

2.1.1 Inclusion criteria

- Volunteer healthy pregnant women.
- Volunteer GDM pregnant women.

2.1.2 Exclusion criteria

- New born delivered with renal or hepatic dysfunction, heart and inflammatory disease.
- Premature newborn.
- Women with coagulation problems.

2.1.3 Ethical consideration

Ethical committee of research in the College of Medical Laboratory Science was approved the study. The purpose and objectives of the study was explained to each one of participants, the participant has right to voluntary informed consent, has right to with draw at any time without any deprivation, assured them that the data collected will remain confidential and it's not allowed for any person to identify it. The questionnaire was filled, and participant has right to benefit from the researcher knowledge and skills. Samples were coded and confidentiality

of patient data was maintained throughout the study by locking hard copies and password protecting electronic files.

2.1.4 Study variables

2.1.4.1 Dependent variable

- Protein C
- Protein S

2.1.4.2 Independent variables

- Gestational diabetes mellitus (GDM) (Infants of Diabetic Mothers).
- Gestational age.
- Age.
- FBG.
- BMI.

2.2 Methods

2.2.1 Sampling

Cord blood newborns samples from volunteering pregnant women were collected immediately after delivery and before delivered of placenta by sterile plastic siring (5 ml) and added to citrated anticoagulant tube and gently mixed, then used for measurement of protein C and protein S by using BIOBASE-EL-10 Microplate Reader.

2.2.2 Protein C and protein S by BIOBASE-EL-10 Microplate Reader

2.2.2.1 Principle

The plasma\serum is incubated in a well, and each well contains a different plasma\serum. A positive control plasma\ serum and a negative control plasma\ serum would be included among the 96 samples being tested. Antibodies or antigens present in plasma\ serum are captured by corresponding antigen or antibody coated on to the solid surface. After some time, the plate is washed to remove plasma and unbound antibodies

or antigens with a series of wash buffer. To detect the bound antibodies or antigens, secondary antibodies that are attached to an enzyme such as peroxidase or alkaline phosphatase are added to each well. After an incubation period, the unbound secondary antibodies are washed off. When a suitable substrate is added, the enzyme reacts with it to produce a color. This color produced is measurable as a function or quantity of antigens or antibodies present in the given sample. The intensity of color/ optical density is measured at 450nm. The intensity of the color gives an indication of the amount of antigen or antibody.

2.2.2.2 Reagent and component

Sample buffer , Reference plasma, washing buffer, Conjugate, TMB substrate and Stop solution**.**

2.2.2.3 Procedure, samples and controls

Firstly we should wash the samples and put 100 micro liters from samples into micro wells, then add 100 micro liters of reference plasma and diluted controls into wells and incubate for 30 minutes in room temperature. Wash 3X with 300 micro liter washing buffer and add 100 micro liter conjugates into each wells and incubate for 30 minutes in room temperature and wash 3X, add 100 micro liter of TMB into each wells and incubate for 30 minutes with protected from lights, finally put 100 micro liter of stop solution and incubate 5 minutes, agitate plate for 5 seconds and read absorbance at 450 nm.

Interpretation of the results

BIOBASE-EL-10 Microplate Reader calculates the test result automatically and displays protein C and protein S concentration of the test sample as %.

Reference Values

protein C: 70-160 %.

Protein S: 60-150%.

2.2.3 Data analysis

The collected data proceed for analysis using SPSS version 20 computerized program by independent T test and correlation, and the data presented in form of tables and figures.

Chapter Three

3. Results

3.1 Descriptive Analysis

3.1.1The age groups

The GDM patients were divided into 2 age groups; 8 patients (27%) their age more than 35 years and 22 patients (73%) less than 35 years (Figure3.1).

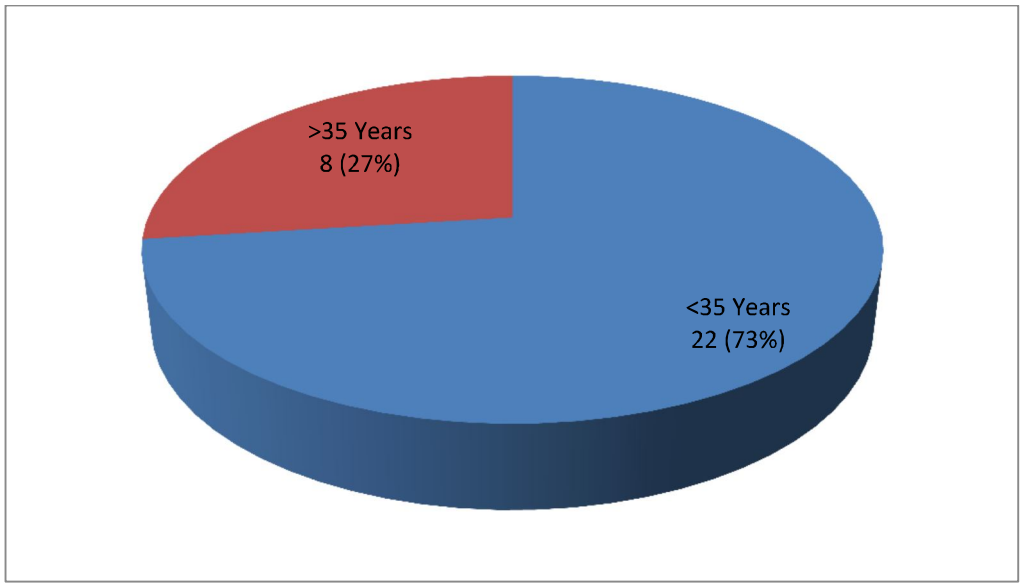

Figure 3.1 Distributions of patients according to group

3.1.2 The History of GDM

There were 16(53%) patients had a history of GDM whereas 14(47%) were not(Figure 3.2).

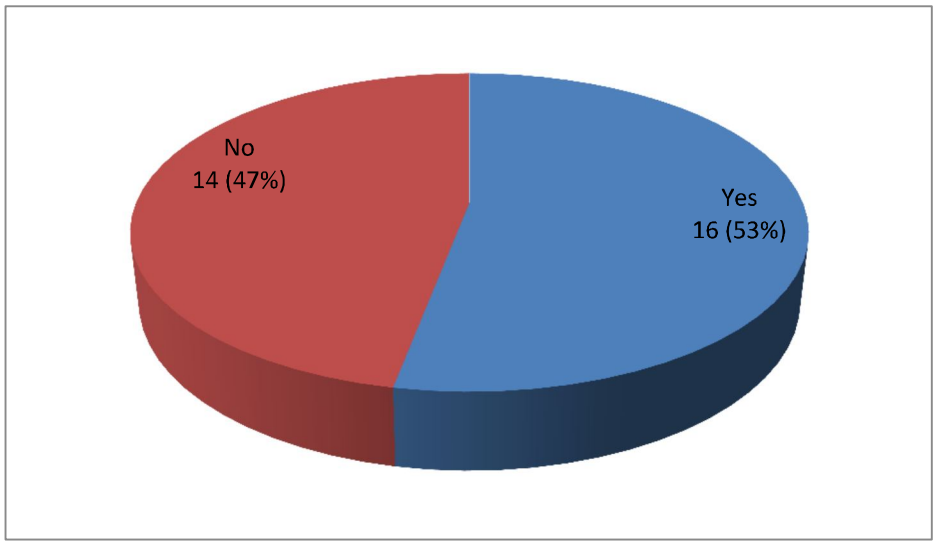

Figure 3.2Distribution of patients according to history of GDM

3.1.3 The Other disease

GDM patient divided into 4 groups according to their medical history into: 12 patients (40%) had no any other disease with GDM, 8 patients (27%) with DM, 7 patients (23%) with hypertension and 3 patients (10%) with hypertension and DM (Figure 3.3).

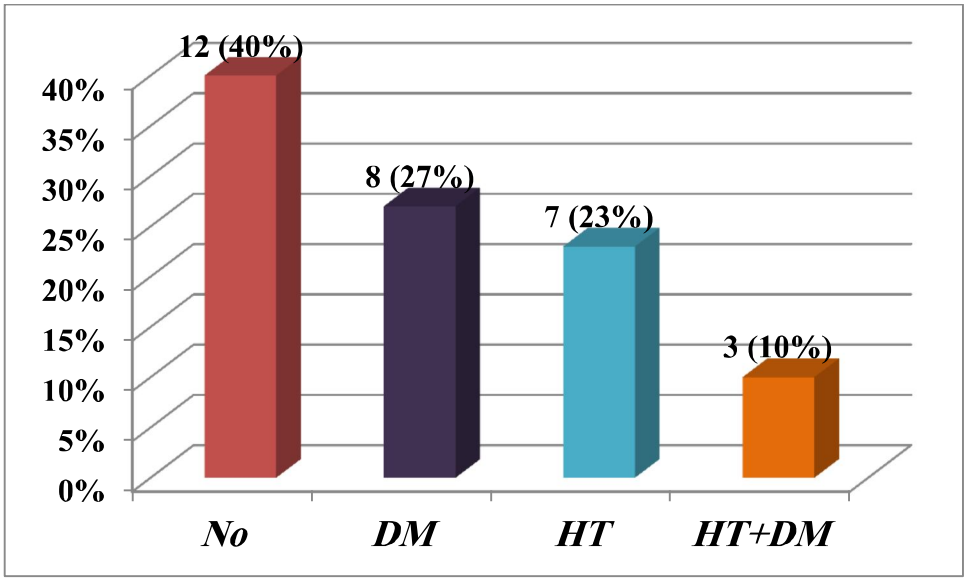

Figure 3.3 Distribution of patients according to clinical history.

3.1.4 Age, Weights, FBS, Gestational Weeks and BMI

The result showed significant increased in weight of baby (P.value=0.003), BMI (P.value=0.001) and FBG (P.value=0.002) of pregnant women with GDM compare to healthy pregnant women, while, insignificant increased in age of mothers among GDM mother and gestational weeks among control mother (Table 3.1).

Table (3.1) mean comparison of study variables in case versus the control group

Variables	Case (Mean±SD)	Control (Mean±SD)	*P-value*
Age	31.03±3.55	29.10±5.66	0.112
Weight of baby	3.47±0.47	3.07±0.51	0.003
Gestational weeks	37.70±0.65	37.77±0.68	0.699
BMI	28.22±3.67	24.67±4.05	0.001
FBG	110.53±19.15	85.57±8.98	0.002

3.2Protein C and Protein S level

The means of protein C and protein S levels in GDM cord blood were significant decreased among newborn from mother with GDM compared to control group(P.value<0.05) (Table 3.2).

Table (3.2) Mean of PC and PS level among cases(GDM) and control group

Parameters	Case (Mean±SD)	Control (Mean±SD)	*P-value*
PC (%)	56.9±8.6	104.4±13.4	0.000
PS (%)	61.5±8.7	113.4±12.3	0.000

3.3GDM- FBG and Protein C, protein S level

Protein C level in GDM pregnant with FBG <100 (mg/dl) was (58.3±5.5%) compared to pregnant women with FBG>100 (mg/dl) was (56.3±9.7%), and also in protein S level as (62.0±5.3%) for GDM

pregnant with FBG <100 (mg/dl) and 61.3±10.0% for GDM pregnant women with FBG>100 (mg/dl) (Table 3.3).

Table (3.3) Means Comparison of protein C and protein S according to FBG level among GDM

Parameters	<100 (mg/dl) (Mean±SD)	>100 (mg/dl) (Mean±SD)	*P-value*
PC (%)	58.3±5.5	56.3±9.7	0.483
PS (%)	62.0±5.3	61.3±10.0	0.843

3.4 Correlation between protein C, protein S and BMI in women with gestational diabetes

In women who had Gestational diabetes mellitus the protein C and protein S had insignificant correlation with BMI (P.value>0.05) (Figure 3.4 and Figure 3.5).

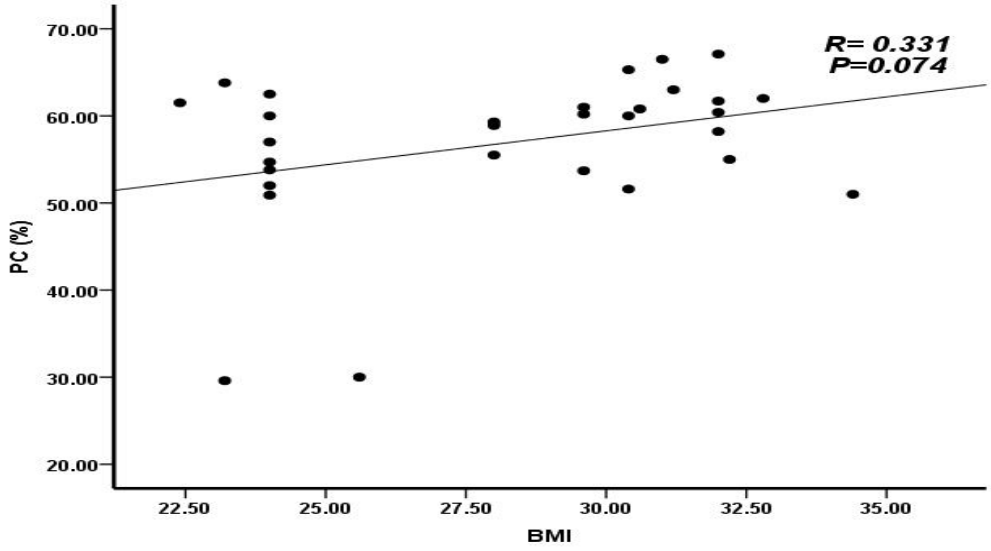

Figure 3.4 Correlation between protein C (PC) and body mass index (BMI)in women with GDM

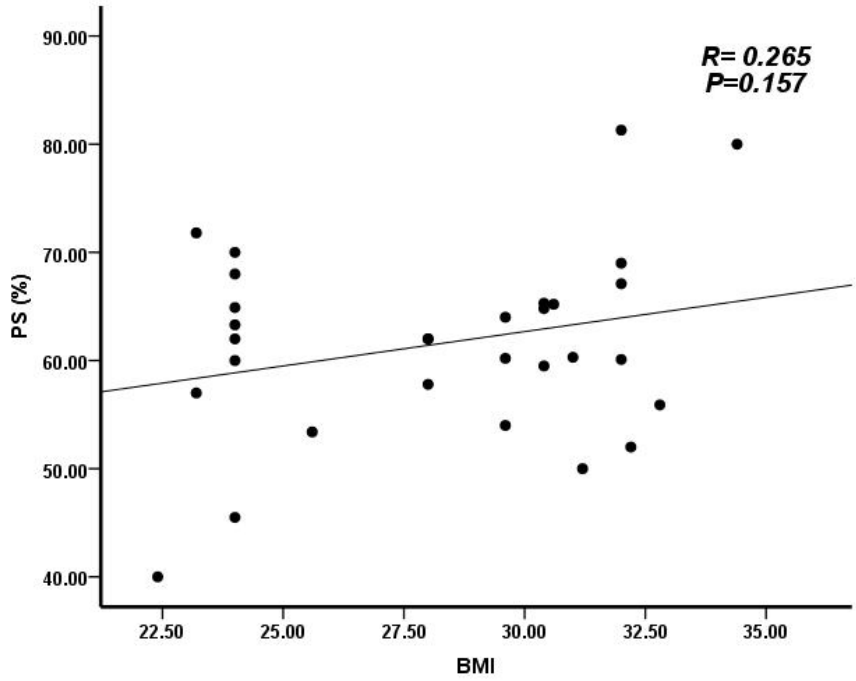

Figure3.5 Correlation between protein S (PS) and body mass index (BMI) in women with GD

3.5 Correlation between protein C, protein S and age of women with GDM

Protein C and protein S level were showed insignificant correlation with age of women with GDM (P>0.05) (Figure 3.6 and Figure 3.7).

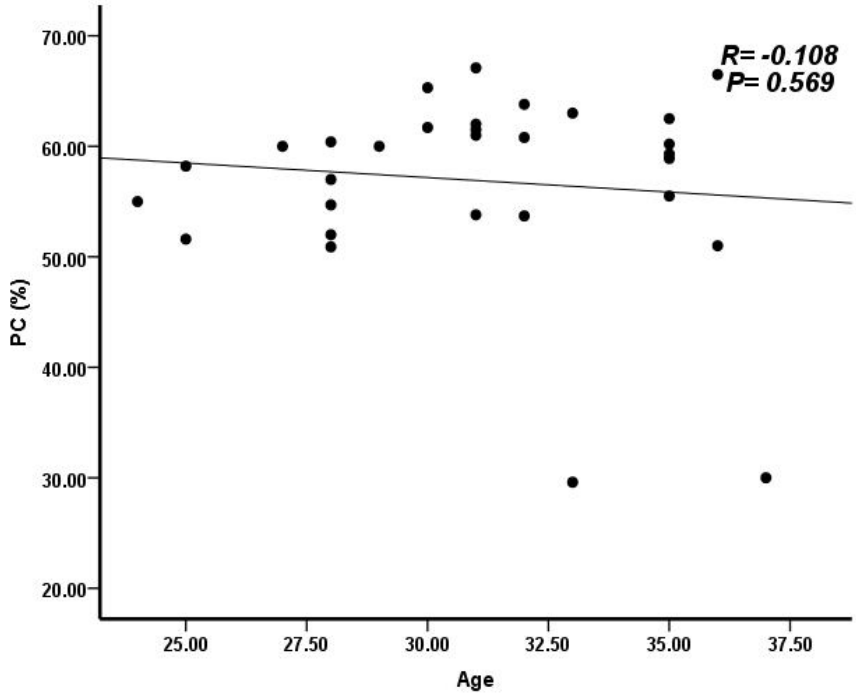

Figure 3.6 Correlation between protein C and age of women with GDM

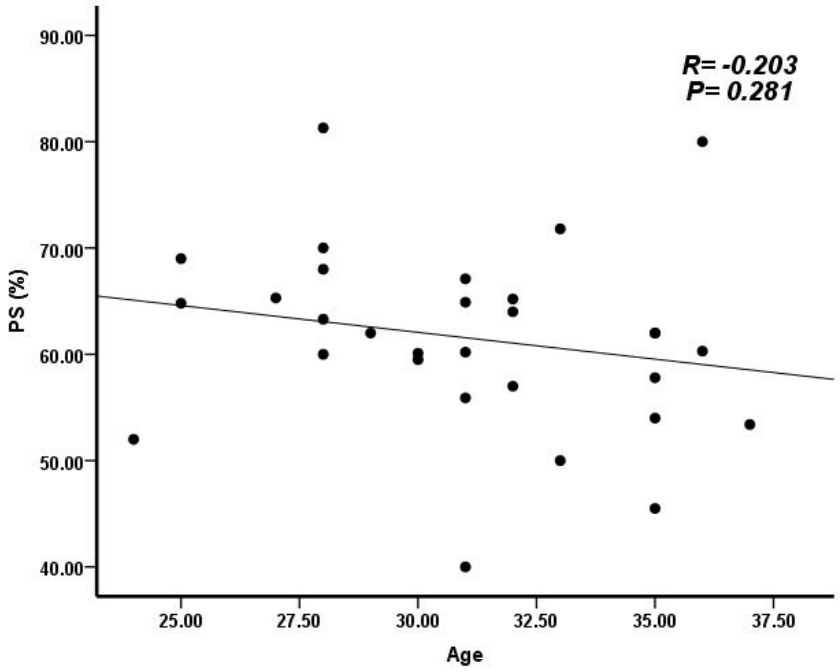

Figure 3.7 Correlation between protein S and age of women with GDM

3.6 Correlation between protein C, protein S and weight of baby

In women who had Gestational diabetes mellitus the protein C and protein S levels were showed insignificant correlation with weight of baby (P>0.05) (Figure 3.8 and Figure 3.9).

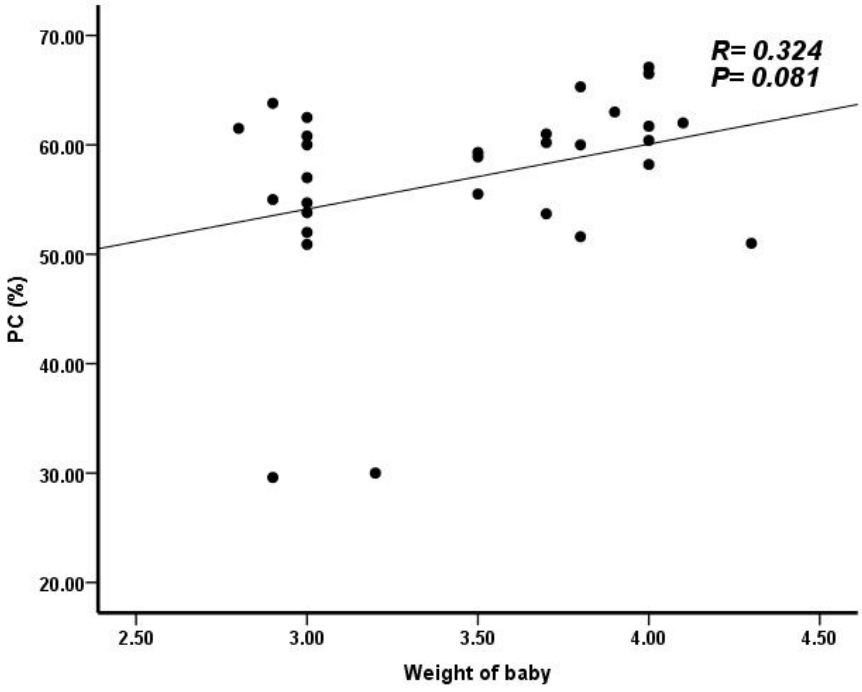

Figure 3.8 Correlation between protein C and weight of baby

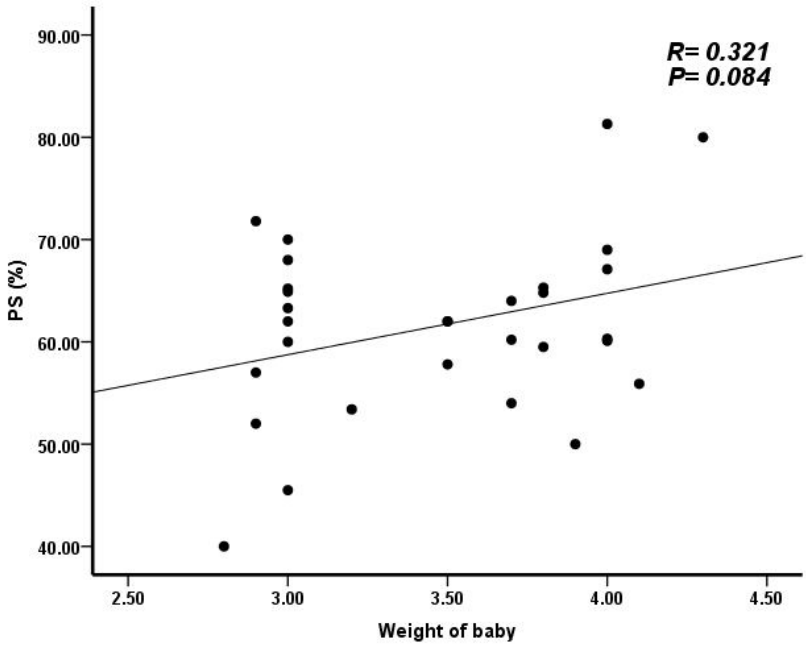

Figure 3.9 Correlation between protein S and weight of baby

Chapter Four

4. Discussion, Conclusions and Recommendations

4.1 Discussion

GDM is a systemic disease that affects both the mother and fetus. These mothers are more likely to develop Type 2 DM in the following 10 years increases by 20-30%; hence, they must be monitored closely (Mohammed *et al.*, 2014) . There are few studies in the available literature on protein C and protein S in mothers with GDM. GDM remains a significant cause of an increment in perinatal mortality. (Qazi *et al.*,2016).

The present study showed that the means of protein C and protein S levels in GDM patients were significantly decreased when compared with the control group, and there is no correlation between this levels and weight of baby , age and BMI of GDM mothers. And also the result showed significant increased in weight of baby ,BMI and FBG of pregnant women with GDM compare to healthy pregnant women, while, insignificant increased in age of mothers and gestational weeks among GDM mothers compare to control mother. All previous studies results indicated that protein C and protein S levels in GDM patients was going decreased and they were a potential risk for thrombosis especially if the disease getting severe. This observation is agreed with study of **NEARY *et al* .2015,** who concluded that all infants from mother with GDM had decreased protein C and S levels. Moreover, another research supported our result in control group **by Mieczyslaw *et al* in 2010** , who observed that protein C antigen in mother's plasma was 135.11±1.05%, where's in cord plasma was 57.60±10.32%,where in thus it was lower by over a half(42.67%) in the fetus when compared to the mother, also they approved that the level of total protein S in mother's plasma was

92.49±13.24%, where's in cord plasma was 33.19±4.96%, thus being almost three fold lower (35.88%) in the fetus than in the mother.

Moreover a study done in **1998 by Jordi Bellart *et al*** supported this result, and concluded that in the third trimester and during labor, lower functional PC levels were observed in the GD group .During delivery, APC levels were also lower in GDM than in normal pregnancies and after delivery, normal levels were recovered. And also during the active phase of labor and after delivery, total and free PS levels were lower in the GDM group.

Moreover, **Hassan and *et al* in 1989** admitted that protein C was significantly lower in cord plasma than in maternal and control plasma.

These findings of this study could play an important role in the health impact of newborn of diabetic mothers. Furthermore, this study could be used as a reference or a benchmark study for related studies.

4.2 Conclusions

- Significant decreased in Protein C and protein S levels in cord blood gestational diabetes mellitus was observed.
- There was insignificant association between FBG level, BMI, age and weight of newborns from GDM mothers and Protein C and S levels.
- Newborn of GDM mother are at risk for thrombotic disorders.

4.3 Recommendations

- Protein C and S levels are not easily or routinely order in follow up of GDM, this fact should be change.
- Protein C and Protein S level may be one of the valuable markers of hemostatic disorders in cord blood GDM, and estimation has a predictor role for thrombotic event.
- Estimation of protein C and S level may be considered as an easy, reliable, economic and rapid method.
- Beside the clinical and physiological application cord blood has many benefits.
- Sample size should be increase.
- More researches should be performed for monitoring the complete coagulation profile.

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Questionare

Assessment of protein C and protein S in Umbilical Cord Blood of Gestational Diabetic Mothers

Name: …… ID code ……

Age: ……

History of diabetes? .....

History of thrombosis? .....

Number of previous pregnancy? .....

Weight of the infant? .....

Glucose level ……..

Medical condition ……

Medication of mother……..

Protein c level ……..%

Protein s level……..%

Informed consent:

انا المذكوره أعلاه أوافق على استخدام دم الحبل السري لغرض البحث العلمي ولي الحق بالانسحاب متى ما شئت ولي الحق في الحصول على النتائج الخاصة بي. وألا يتم استخدام هذه العينات لأغراض التجارب الانسانية والحيوانية.