The Islamic University – Gaza
Deanery of Higher Education
Faculty of Science
Master of Biological Science
Medical Technology

الجامعة الإسلامية- غزة
عمادة الدراسات العليا
كلية العلوم
ماجستير العلوم الحياتية
تحاليل طبية

Endothelial Nitric Oxide Synthase "eNOS" Gene Polymorphisms, Nitric Oxide and Progesterone levels in Idiopathic Recurrent Pregnancy Loss

Prepared by

Emad M. El-Gharably

Supervisor

Prof. Dr. Fadel A. Sharif

Submitted in Partial Fulfillment for the Requirement for the Master Degree of biological Sciences - Medical Technology

Department of Medical Technology

Faculty of Science

1433هـ - 2012م

بسم الله الرحمن الرحيم

الجامعة الإسلامية – غزة
The Islamic University - Gaza

عمادة الدراسات العليا
هاتف داخلي: 1150

الرقم Ref ج س غ/35/
التاريخ Date 2012/03/18

نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة عمادة الدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحث/ **عماد محمد سعيد الغرابلي** لنيل درجة الماجستير في **كلية *العلوم*/ قسم العلوم الحياتية– تحاليل طبية**، وموضوعها:

Endothelial Nitric Oxide Synthase "eNOS" Gene Polymorphisms, Nitric Oxide and Progesterone Levels in Idiopathic Recurrent Pregnancy loss

وبعد المناقشة التي تمت اليوم الأحد 24 ربيع آخر 1433هـ، الموافق 2012/03/18م الساعة الحادية عشرة صباحًا، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

أ.د. فضل أكرم الشريف	مشرفاً ورئيساً	.....
د. صلاح الدين يوسف جاد الله	مناقشاً داخلياً	.....
د. محمد أغا الريس	مناقشاً خارجيًا	.....

وبعد المداولة أوصت اللجنة بمنح الباحث درجة الماجستير في **كلية *العلوم*/قسم العلوم الحياتية–تحاليل طبية**.

واللجنة إذ تمنحه هذه الدرجة فإنها توصيه بتقوى الله ولزوم طاعته وأن يسخر علمه في خدمة دينه ووطنه.

والله ولي التوفيق،،،

عميد الدراسات العليا

أ.د. فؤاد علي العاجز

DECLARATION

I hereby declare that this submission is my own original work and that, to the best of my knowledge and belief, it contains no materials previously published or written by another person nor materials which to a substantial extent has been accepted for award of any other degree of the university or other institute, except where due acknowledgement has been made in the text.

Signature	Name	Date
Emad El-Gharably	Emad Mohammed El-Gharably	Feb.2012

Dedication

This work is dedicated to:

my father and my mother who taught me how to give

my wife who supported me wholeheartedly

my sons Ismail and abed-Allah

my brothers and sisters

all my teachers who supported me

all my friends who spare no effort to help

This work is also dedicated to:

the Palestinian people who have suffered and will be struggling with the persistence to have a free Palestine.

Acknowledgements

The practical part of this work was carried out at the Genetics Laboratory- Islamic University - Gaza.

Many individuals have contributed to the completion of this work, including the formation of this dissertation. I wish to thank all who made this work possible with their support, advice, and effort.

I would like to express my gratitude to my supervisor *Prof. Fadel A. Sharif* for his support, patience, special insights and encouragement throughout my research. The technical and editorial advice of *Prof. Sharif were* essential to the completion of this work and have taught me innumerable lessons and insights in the academic research in general.

I am very much thankful to the staff of the Palestinian Medical Relief society for their help in sample collection especially, *Mr. Marwan Darddona.*

I am also very much thankful to the staff of the Genetics Laboratory at the Islamic University of Gaza for their help in sample collection and PCR technical support. Especially, *Mr. Mohammed Ashour*, where his sincere contributions made this research attempt fruitful and possible.

I would like also to express my thanks to the central blood bank society represented by all the staff, where they permitted me to use their centrifuges to separate blood samples, refrigerators and deep freezers for serum storage.

I want to thank my colleague *Wael Abu-Ghal*i, thank you so much for allowing me to perform the Nitric Oxide Determination in your own laboratory. My deepest thanks are also extended to AL-Aqsa Medical Laboratory staff, *Mr. Adel khadir* and *Mr. Iyad Mortaja* for all their efforts to help me.

I want also to thank all the staff at the department of the biological sciences at the Islamic University- Gaza for their support and help, especially Mr. *Mohammed Awda*

Last but not least, I want to thank my beloved father and mother, thank you for always believing in me and loving me unconditionally, without your support and love I would not be where I am today. My heartfelt thanks go to my loving wife; your tender care and patience have not ceased even in my worst moments.

To all of these individuals I owe many thanks for their insights and unlimited support

Endothelial Nitric Oxide Synthase *"eNOS"* Gene Polymorphisms, Nitric Oxide and Progesterone levels in Idiopathic Recurrent Pregnancy Loss

Abstract (English)

Pregnancy is a hypercoagulable state with increased tendency for thrombus formation, a condition that is increased when combined with acquired or inherited risk factors that lead to thrombophilia. Recurrent pregnancy loss (RPL) is an important clinical and stressful problem that has been studied tremendously but the causes and treatment have not been fully resolved. RPL affects about 1-5% of women who conceiveand accounts for about 20% of clinically recognized pregnancy losses. Despite extensive researches to explain the causative effects of RPL, about 50%-60% of RPLs are still idiopathic. The association between endothelial nitric oxide synthase *(eNOS)* polymorphism, their haplotypes, serum nitric oxide (NO) levels and RPL, were studied in different ethnic populations. The results, however, were contradictory.

Objective: This study was conducted in order to determine the association between *promoter -786 T>C, exon 7 Glu298Asp (894 G>T) and intron 4 (4a4b) VNTR* polymorphismsof *eNOS* gene, serum NO and progesterone (P_4) levels, and idiopathic RPL in Palestinian women residing in Gaza strip.

Method: This study is an association study with a case-control design. The study population consisted of 45 (30 non-pregnant and 15 pregnant) women who suffered from unexplained RPL, and 45 (30 non-pregnant and 15 pregnant) healthy women matched for age and without previous history of RPL. Blood samples collection were carried out during the period from June2011 to September 2011. Two blood samples werecollected from each subject after fasting for 10-12 hours, one was whole blood and the other was serum.DNA was extracted from whole blood samples. The PCR products of *intron 4 (4a4b) VNTR* polymorphismwere analyzed by allele-specific PCR, where it separated electrophoretically using ethidium bromide-stained 2% agarose gel. However, the PCR products of *exon 7 Glu298Asp (894 G>T) and promoter -786T>C* polymorphismsby PCR-RFLP, where they digested using specific restriction enzymes and then separated electrophoretically using 2% agarose gel. Serum NO levels were measured spectrophotometrically, and P_4 levels were measured using Immulite 1000 Analyzer.

Results: The *C allele* carrierwhich represented by *(CC + CT)* genotypes and the *C* allele of the promoter *-786T>C* polymorphism are significantly associated with increased risk of RPL, where they presented with a higher frequency in RPL women and were associated with decreased serum NO levels in this group (all P-values <0.001). Neither *exon 7 Glu298Asp(894G>T)* nor *intron 4 (4a4b) VNTR* polymorphism was significantly associated with RPL risk in the study population. The serum NO levels were lower in RPL patients as compared to their respective controls (P-value =0.004). The study pointed to the presence of a positive proportional correlation between serum NO and P_4 levels in the study population (P-value= 0.002, Correlation coefficient= 0.319) that might be attributed to the presence of a putative progesterone receptor binding site in the upstream promoter region of *eNOS*. The study also showed that the *promoter -786T>C* polymorphism was not associated with P_4 level in the study population.

Conclusion: The *(CC + CT)* genotypes *(C allele* carrier*)*and the *C* allele of the promoter *-786T>C* polymorphismare possible risk factors for RPL. The study showed that the *(C allele* carrier*)* which represented by *(CC + CT)* genotypesis associated with a decreased serum NO level that, in turn, is associated with RPL. Moreover, a positive proportional correlation between serum NO and P_4 levels was evident.Therefore, balancing P_4 and NO levels may be of benefit for maintaining pregnancy in those cases.

Keywords: *eNOS*, Polymorphism, PCR, RPL, Nitric oxide, Progesterone, Gaza Strip, Palestine.

العلاقة بين الأنماط المتعددة للجين المصنع لأكسيد النيتريك في البطانة، و مستويات أكسيد النيتريك و هرمون البروجستيرون و الإجهاض المتكرر الغير معروف السبب

ملخص الدراسة(Abstract (Arabic

الحمل هو حالة زيادة التجلط مع ميل متزايد لتشكيل خثرة، وتزداد هذه الحالة مع وجود عوامل الخطر المكتسبة أو الموروثة التي تؤدي إلى زيادة التخثر . يعتبر الإجهاض المتكرر مشكلة سريريه هامة ومجهدة وبالرغم من دراستها بشكل كبير إلا أن الأسباب والعلاج لم يتم حله ما بشكل كامل. فقدان الحمل المتكرريصيب حوالي 1-5٪ من النساء الراغبات في الإنجاب ويمثل حوالي 20٪ من حالات فقدان الحمل الم ثبتة سريريا. على الرغم من الأبحاث واسعة النطاق لشرح الآثار المسببة لفقدان الحمل المتكرر إلا أن حوالي 50٪ -60٪ من حالات الإجهاض المتكررلا تزال مجهولة السبب. تمت دراسة العلاقة بين الأنماط المتعددة للجين المصنع لأكسيد النيتريك في البطانة *(eNOS)*، و مستويات أكسيد النيتريك في مصل الدم، في المجتمعات العرقية المختلفة ومع ذلك كانت.النتائج متناقضة.

الهدف: دراسة العلاقة بين الأنماط المختلفة للجين المصنع لأكسيد النيتريك في البطانة*(eNOS)* وهي *promoter -786 T>C* ، *exon 7 Glu298Asp (894 G>T)* و *intron 4 (4a4b) VNTR*، مستويات أكسيد النيتريك وهرمون البروجستيرون في المصل وحالات فقدان الحمل المتكرر عند المرضى الفلسطينيين المقيمين بقطاع غزة.

الطريقة: تألف مجتمع الدراسة من 45 عينة (30غير حوامل و 15حوامل) يعانين من فقدان الحمل المتكرر، و 45 عينة أخرى (30غير حوامل و 15حوامل) لنساء أصحاء ولا يعانين من الإجهاض المتكرر كعينة ضابطة ومطابقة بالعمر لعينة المرضى . تم جمع عينات الدم خلال الفترة الزمنية (يونيو 2011 حتى سبتمبر 2011)، حيث تم سحب عينتين من كل شخص بعد صيام لمدة 10-12ساعة، احدهما كانت دم كامل، والأخرى كانت مصل. تم فصل المادة الوراثية (الـ DNA) من عينات الدم الكامل، تم تحديد الأنماط المختلفة لـ *intron 4 (4a4b) VNTR* باستخدام تقنية allele-specific PCR وهي (الإكثار من الجزء من الحمض النووي المحتوي على الطفرة و من ثم فصلة على جل اجاروز 2%)، ولكن تم تحديد الأنماط المختلفة لكل من *exon 7 Glu298Asp (894 G>T)* و *promoter -786 T>C* باستخدام تقنية PCR-RFLP وهي (الإكثار من الجزء من الحمض النووي المحتوي على الطفرة و من ثم قطعة بواسطة إنزيمات قاطعة متخصصة و من ثم فصلها على جل اجاروز 2%)، مستويات أكسيد النيتريك في المصل تم تحديدها باستخدام المطياف الضوئي Spectrophotometer، وتم قياس مستويات هرمون البروجسترون في المصل باستخدام جهاز Immulite Analyzer1000.

النتائج: أظهرت نتائج هذه الدراسة وجود ارتباط بين فقدان الحمل المتكرر و النمطين الجينيين *(CC + CT)* و الأليل *C* لـ *promoter -786T>C* polymorphism حيث ظهروا بترددات عالية عند النساء اللواتي يعانين

من فقدان الحمل المتكرر و كانت جميع قيم اختبار الدلالة الإحصائية (P-value <0.001)، حيث ترافقوا مع وجود مستويات منخفضة من أكسيد النيتريك في هذه المجموعة، كذلك أظهرت الدراسة بأنه لا يوجد ارتباط بين فقدان الحمل المتكرر والأنماط الجينية لكل من *exon 7 Glu298Asp (894G>T)* و *intron 4 (4a4b)VNTR*. كانت مستويات أكسيد النيرتيك بمصل المرضى الذين يعانون من فقدان الحمل المتكرراقل بالمقارنة مع العينات الضابطة التي تمثلهم (قيمة اختبار الدلالة = 0.004)، أشارت الدراسة لوجود علاقة طردية بين مستويات أكسيد النيتريك و البروجستيرون في المصل عند مجتمع الدراسة (قيمة اختبار الدلالة= 0.002، قيمة معامل الارتباط= 0.319). و اظهرت الدراسة عدم وجود ارتباط بين *promoter -786T>C* polymorphism ومستويات البروجستيرون في مصل الدم عند مجتمع الدراسة.

الخلاصة: الانماط الجينية *(CT + CC)* و الأليل *C* لـ *promoter -786T>C* polymorphism هي عوامل خطر محتملة لفقدان الحمل المتكرر. النمط الجيني *(CT + CC)* ترافق مع وجود مستويات منخفضة من أكسيد النيتريك في المصل والذي بدوره يرتبط بفقدان الحمل المتكرر كما انه توجد علاقة طردية بين مستويات أكسيد النيتريك و البروجستيرون في المصل. لذا تعديل مستوى البروجستيرون و أكسيد النيتريك قد يساعد في الحفاظ على اكتمال الحمل في مثل هذه الحالات.

الكلمات المفتاحية: الجين المصنع لأكسيد النيتويك في البطانة *eNOS*، نمط Polymorphism، تفاعل تسلسل البلمرة PCR، فقدان الحمل المتكرر RPL، أكسيد النيتريك، بروجستيرون، قطاع غزة، فلسطين.

Table of Contents

Chapter (4): Results

List of Figures

List of Tables

Abbreviations

ACE (I⁄D): Angiotensin Converting Enzyme (I= insertion, D= deletion).

Agt: Angiotensinogen.

AMP: Adenosine monophosphate.

AP-1: Activator protein 1.

bp: Base Pair.

BP: Blood Pressure.

Ca^{+2} ion: Calcium Ion.

Ca-dependent: Calcium dependent.

CaM: Calmodulin

cGMP: Cyclic Guanosine Monophosphate.

Chromosome 7q: The long arm of Chromosome 7.

cNOS: Constitutive Nitric Oxide Synthase.

C-terminal reductase domain: Carboxyle Group Terminal Reductase Domain.

DNAse: Deoxyribonuclease

EDTA: Ethylene Diamine Tetra Acetic Acid.

ELISA: Enzyme-Linked Immunosorbent Assay.

eNOS Gene: : Endothelial Nitric Oxide Syntahse Gene.

eNOS: Endothelial Nitric Oxide Synthase.

E_2: Estradiol 17-β.

FAD: Flavin Adenine Dinucleotide.

Fe^{2+}: Ferrous.

FMN: Flavin Mononucleotide.

GMP: Guanosine Monophosphate.

GnRH: Gonadotrophin-Releasing Hormone.

GTP: Guanosine Triphosphate.

H_2O: Water.

H_2O_2: Hydrogen Peroxide.

H_4Bip: Tetrahydrobiopterin.

HCG: Human Chorionic Gonadotrophin.

HES: Human Endometrial Surface Epithelial Cell Line.

HLA: Human Leukocyte Antigen.

HP: Healthy Pregnant.

HPLC: High-Performance Liquid Chromatography.

HWE: Hardy-Weinberg Equilibrium.

ICI 182,780: Estrogen Receptor Antagonist.

IFN-γ: Interferon-Gamma.

IL-1β: Interleukin-1Beta.

iNOS: Inducible Nitric Oxide Synthase.

IP_3: Inositol Triphosphate.

IRPL: Idiopathic Recurrent Ppregnancy Loss.

IUFD: Intrauterine Fetal Death.

IUGR: Intrauterine Growth Restriction.

Kb: Kilo base.

L-NAME: N^G-Nitro-L-arginine methyl ester.

L-NMMA: N^G-monomethyl-L-arginine.

LPS: Lipopolysaccharide.

LSD: Least Significant Difference.

M: Molar.

MAPK: Mitogen-Activated Protein Kinase.

MDA: Malondialdehyde.

mg: milligram.

miRNA: micro-RNA.

mRNA: Messenger Ribonucleic Acid.

ml: milliliter.

MTHFR: Methylenetetrahydrofolate Reductase.

NADPH: Nicotinamide Adenine Dinucleotide Phosphate.

NF-κB: Nuclear factor Kb

ng: Nanogram.

NOHA: N-hydroxy-L-arginine

nNOS: Neuronal Nitric Oxide Syntahse.

NO: Nitric Oxide.

NO_2^-: Nitrite.

NO_3^-: Nitrate.

NOS3 Gene: Nitric Oxide Synthase 3 Gene.

NOx: A stable (Inactive) end Products of Nitric Oxide Metabolism.

N-terminal oxygenase domain: Amino Group Terminal Oxygenase Domain.

O_2: Oxygen.

O_2^- : Superoxide Anion.

$ONOO^-$: Peroxynitrite.

OR: Odds Ratio.

(PAI-1) 4G⁄5G:(Plasminogen Activator Inhibitor-1 gene) 4Guanin/5Guanine.

PCR: Polymerase Chain Reaction.

PCR-RFLP: Polymerase Chain Reaction- Restriction Fragment Length Polymorphism.

PE: Preeclampsia.

PeNOS: Phosphorylation of eNOS.

P_4: Progesterone.

PH: Hydrogennumber (measure of acidity).

PI 3-kinase/Akt: Phosphoinositide 3 kinase/ Protein kinase B.

PR: Progesterone Receptor.

RBCs: Red Blood Cells.

RNAse: Ribonuclease.

RPA1: Replication protein A1.

RPL: Recurrent Pregnancy Loss.

rpm: Round per minute.

RT: Room Temperature.

sGC: Soluble Guanylate Cyclase.

SNPs: Single Nucleotide Polymorphisms.

TAE Buffer: Tris Acetate EDTA Buffer.

TNF-α: Tumor Necrosis Factor-Alpha.

tHcy: Homocysteine.

TSS: Translation Start Site.

VNTR: Variable-Number Tandem Repeat.

WBCs: White Blood Cells.

ZnS_4: Zink Sulfate.

β: Beta.

4a/4b: Intron 4 (a = deletion 393 base pair, b = insertion 420 base pair).

Chapter（1）
Introduction

Chapter (1) Introduction

1.1. Overview

The success of pregnancy depends, to a great extent, on events occurring during the early stages of gestation, such as the implantation of the blastocyst, trophoblast differentiation, invasion of the endometrium by the trophoblasts vis-a-vis establishment of feto-maternal vascular circuitry, enhanced blood supply through the maternal arteries to the placenta, immune protection of the fetus etc. Nitric oxide (NO) is a paracrine signaling molecule involved in the regulation of all these events either alone or in association with other neuroendocrine regulators[1].

Clinically recognized pregnancies end in miscarriage in 15–20% of cases. One to 5% of pregnant women experience recurrent pregnancy loss (RPL), of which 40 –55% are induced by unknown causes[2].

The causes of recurrent pregnancy loss can be divided into 4 categories: genetic, anatomic, endocrine (related to hormone abnormalities), and prothrombotic. Current medical literature suggests that causes are identified in only 50% of patients[3].

Successful implantation depends on the receptivity of maternal endometrium which is influenced by the synergistic actions of progesterone (P_4) and NO[1].

Early placental development occurs in a relatively hypoxic environment, and this low oxygen tension is necessary for the expression of several developmentally important genes by the embryo. The onset and the amount of maternal blood flow to the placenta are influenced by the vasodilatory effects of NO[1].

The formation of soluble NOis catalyzed by nitric oxise synthase (NOS) enzyme via a reaction including the conversion of L-arginine to L-citrulline, and involves the transfer of five electrons provided by nicotinamide adenine dinucleotide phosphate (NADPH)[4, 5]. NO is a short-lived free-radical gas synthesized by a family of NOS enzymes[2], with an extremely short half life of approximately 4 seconds[6]. The level of NO has been shown to be influenced by various polymorphisms in the eNOS gene[7, 8].

NOS enzymes are expressed in three isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). All of these isoforms are present in trophoblast cells of the first trimester and in various cells of the uterine cervix. The expression of NOS isoforms and the release of NO in the cervix have been shown to increase with advancing gestational age and during cervical ripening[2].

Endothelium-derived NO has a number of roles, including maintaining basal cerebral blood flow, cerebral vasodilation, and autoregulation, maintaining vascular integrity, and inhibiting smooth muscle proliferation[9].

The initial demonstration of the role of NO in penile erection led to investigation of its role in various reproductive processes. NO has been identified throughout the reproductive tract and is involved in ovarian folliculogenesis, ovulation, gonadotropin releasing hormone secretion, sperm motility, fertilization and embryo development. The identification of NO in the uterus and cyclic change in the endometrial levels of NOS suggest a role for this molecule in the events of implantation[10].

Nitric oxide (NO) contributes to maternal systemic vasodilation during pregnancy, regulates uterine and fetoplacental blood flow, and is involved in uterine quiescence before parturition[11].

Nitric Oxide (NO) is well-known to mediate vascular smooth muscle relaxation and lack of endothelial-derived NO is associated with vasospasm, and vascular infarction[12], Lack of NO has also been associated with the development of endothelial damage, hypertension, coronary spasm, myocardial infarction, coronary artery disease and ischemic stroke[2].

Exogenous NO promotes uterine relaxation and has prompted interest in the use of NO donors as tocolytic agents. Thus, endogenous production of NO may be involved in the regulation of myometrial tone in pregnancy, and a decline in NO production at term could play an important role in the initiation of, or preparation for, parturition[13].

Endothelial nitric oxide synthase (eNOS) is expressed in terminal villous vessels and in the syncytiotrophoblast of pregnant women. In mice, lipopolysaccharide (LPS)-induced abortion is mediated by placental NO production, and pharmacological inhibition of NO release by aminoguanidine successfully rescued LPS-induced abortion[2, 14, 15].

Alteration in NO metabolism may be a contributing factor in the pathogenesis of hypertension. Thus, abnormalities in the activity of the eNOS enzyme that synthesizes NO in endothelial cells may lead to NO deficiency with severe consequences[16].

Progesterone is a sex steroid essential for pregnancy and lactation. It is produced almost entirely by the ovarian corpus luteum (CL) and the placenta, it is essential for endometrial receptivity and successful establishment of pregnancy. Either an insufficient P_4 concentration or an insufficient response to P_4, therefore can lead to infertility and pregnancy loss[17].

In all species, including human, treatment with antiprogesterone initiates preterm labor, indicating the importance of P_4 in maintaining pregnancy[18]. Previous studies indicated that P_4 may regulate uterine relaxation responsiveness to the nitric oxide-cGMP system[18,19]. Therefore, in the presence of a full complement of P_4 action, inhibitors of NO may not be effective to produce preterm labor[18].

The most intensively studied eNOS Gene polymorphisms are -786T>C in the promoter region of the gene[2], the Glu298Asp missense mutation in exon 7 of the gene[8], and the 27 base pair variable number of tandem repeats (VNTR) in intron 4 (4a4b)[2, 21].

Investigations on the role of eNOS gene polymorphism and haplotypes as genetic determinants in idiopathic RPL in different populations have shown different results. The findings of those studies can be summarized as follows:

- Significant association between certain eNOS gene haplotypes and RPL[2, 14].

- Significant association between some but not all the investigated eNOS gene polymorphisms and RPL[1, 2, 21].
- Lack of association between eNOS gene polymorphisms and RPL[20, 22].

The etiology of RPL is often multi-factorial[11], regulated by multiple genetic pathways[15, 20], and different genes encoding for proteins involved in various biological pathways have been reported to be associated with RPL[15, 20, 23]. *Tempfer et al (2001*), in his discussion added further evidence to the concept of a polygenetic etiological background of women with RPL, when his study indicated that the heterozygous carrier of the eNOS gene polymorphism have a 1.6 fold increase the risk of RPL compared to the control population[14].

The frequency of eNOS polymorphisms has been shown to vary markedly among different ethnic groups[24]. Therefore ethnic variation need to be considered in an evaluation of the genetic background of RPL[14].

1.2. Problem

Recurrent pregnancy loss (RPL) is an important clinical and stressful problem that has been studied tremendously but the causes and treatment have not been fully resolved. No unequivocal cause is currently available for more than half of the cases suffering from RPL[12].

Since the cause of RPL can be identified in only 50% of cases, there are still many unresolved questions about the causes and treatment of RPL. Fortunately, the number of publications on this topic have substantially increased over the past 10 years, reflecting a growing interest among clinicians and scientists[25].

The effect of eNOS polymorphisms on the risk of RPL in the Palestinian population has not been explored yet. *Al-Sallout and Sharif (2010)* have shown that intron 4 (4a4b) VNTR polymorphism may be important in RPL[22]. However, the contribution of specific eNOS polymorphisms or haplotypes as an established risk factor for recurrent pregnancy loss has not been worked out.

1.3. Overall objective

The main objective of this study was to determine the association between promoter -786 T>C, exon 7 Glu298Asp (894 G>T) and intron 4 (4a4b) VNTR polymorphisms of eNOS gene, serum NO and P_4 levels, and idiopathic RPL in Palestinian patients residing in Gaza strip.

1.4. Specific objectives

1) To determine which of these eNOS gene polymorphisms contributes as a risk factor for recurrent pregnancy loss in Gaza strip-Palestine.
2) To determine the frequencies of the three [promoter -786 T>C, exon 7 Glu298Asp (894 G>T) and intron 4 (4a4b) VNTR] polymorphisms of eNOS gene in Palestinian women suffering from RPL.
3) To investigate the association between the three eNOS gene polymorphisms on the serum NO level in Palestinian women suffering from RPL.
4) To determine the correlation between the level of serum NO and serum P_4 levels in Palestinian women suffering from RPL.

1.5. Significance

This investigation may help elucidate one of the causes of unexplained RPL and open the way for new diagnostic and treatment strategies for such cases.

1.6. Limitations of the study

- Difficulties in specimens collection, since some women denied participation.
- Difficulties in obtaining material and kits for both NO determination and PCR, in addition to their high cost, made it impossible to increase the sample size.

Chapter (2)
Literature Review

2.1. Recurrent pregnancy loss

Recurrent pregnancy loss (RPL) is defined as three or more consecutive pregnancy losses before the 20th week of gestation[11, 14].

Clinical studies indicate that the risk of another miscarriage after 3 consecutive pregnancy losses is 30-45%. Furthermore, without any workup or treatment, the chance of a successful live birth in a couple with a history of RPL and no previous live birth is 55-60%. If the couple has a history of RPL and has had at least one previous normal pregnancy, the chance of a subsequent live birth is 70%. These percentages are based on studies of younger women, and it is important to keep in mind that the miscarriage rate increases with age[3].

Known etiologic factors of RPL include parental chromosome abnormalities, uterine abnormalities, hereditary thrombophilias, endocrinologic disorders, immunologic factors, infections, and nutritional and environmental factors[2, 26].

Whether RPL represents the common endpoint of independent etiologic factors or a distinct pathophysiological entity, is unknown. A wide variety of associated factors have been identified, among them uterine anomalies, luteal phase defect, hyperprolactinemia, hyperandrogenemia, hyperhomocysteinemia, genital infections, and maternal/paternal balanced translocations. Autoimmune dysfunctions, e.g. antiphospholipid syndrome, thyroid autoantibodies, and anti-single strand DNA autoantibodies, are found in 5–10% of affected women[14].

2.2. Hereditary thrombophilia

Thrombophilia or a predisposition for thrombosis may be inherited or acquired. While the most common thrombophilia is acquired and is manifested by elevated circulating antiphospholipid antibodies, about; 40% of cases presenting with thrombosis are inherited. Inherited thrombophilia has been shown to be a risk factor for cardiovascular disease such as deep venous thrombosis (DVT) as well as reproductive disorders including RPL[2, 27].

The most widely reported inherited thrombophilias associated with RPL include factor V Leiden (G506A), factor II prothrombin G20210A, methylenetetrahydrofolate reductase "MTHFR" (C677T), plasminogen activator inhibitor-1 (PAI-1) 4G⁄5G, factor-XIII V34L and angiotensin converting enzyme (ACE) I ⁄ D mutations[28]. DNA analyses for these six genes polymorphisms are currently requested by obstetricians for RPL cases of unknown etiology. The Department of Medical Laboratory Sciences at the Islamic University of Gaza is one of the few labs providing this service in Gaza Strip.

Administration of appropriate doses of anticoagulants such as "heparin, low molecular weight heparin or aspirin" during pregnancy has shown some success in maintaining pregnancy[29, 30, 31, 32, 33]. Statistical data about the anticoagulant success rate in maintaining pregnancy in Gaza strip are not available. However, RPL cases with normal six genes polymorphisms and those who are not responsive to anticoagulant administration need further investigation and at least some of them may be attributed to particular *eNOS* gene polymorphisms.

Other possible abnormalities leading to hypercoagulable states that may be associated with RPL include the antithrombin III deficiency, protein C deficiency, protein S deficiency, and elevated factor VIII[34](Table 2.1).

Table 2.1. **Common thrombophilia-factors associated RPL.**

Thrombophilias	Inheritance	Prevalence	Risk of DVT
Factor V Leiden G1691A mutation (activated protein C Resistance)	Autosomal dominant	2%-15%	3-8x
Factor II prothrombin G20210A (Prothrombin mutation)	Autosomal dominant	2%-3%	3x
MTHFR C677T mutation (Hyperhomocysteinemia)	Autosomal recessive	11%	2.5-4x
Antithrombin deficiency	Autosomal dominant	0.02%	25-50x
Protein C deficiency	Autosomal dominant	0.2%-0.3%	10-15x
Protein S deficiency	Autosomal dominant	0.1%-0.2%	2x
Elevated factor VIII	X- Linked	5%-15%	5x

DVT, deep venous thrombosis.
*Prevalence is in the general population however; significant ethnic differences are known. Risk of DVT in the non pregnant individual with listed throbophilia compared with a nonpregnant individual without thromobophilia.

Adapted from Reference (34)

2.3. Genetic association studies

Association studies are valuable for understanding the contribution of specific genetic factors to the development of RPL. A role for the HLA system, the pathway of folic acid metabolism, and the blood clotting cascade all have been elucidated through various association studies. Moreover, targeted mutations in experimental animals have also been used to define the contribution of specific genes to the pathophysiology of RPL[14, 28].

Factor V Leiden G506A and the G20210A prothrombin gene polymorphisms are both among the leading genetic risk factors to enhanced blood coagulation, and both were significantly associated with RPL by several investigators. Additionally, polymorphisms in the *MTHFR* gene, which cause elevation in the level of homocysteine, have been identified as risk factor for thrombosis and RPL. An association with RPL has also been reported for the *V34L* polymorphism *of the FXIII* gene, *4G⁄5G* polymorphism *in the plasminogen activator inhibitor-1 (PAI-1)* gene and in *the I⁄D* polymorphism *in the angiotensin converting enzyme (ACE)* gene[28].

Several genetic studies have revealed associations between recurrent miscarriage and genetic polymorphisms related to thrombophilia, anticoagulation factors, human lymphocyte antigen, metabolic enzymes, cytokines, hormones, and vasodilators[28] (Table 2.2).

Table 2.2. **Genes Involved in recurrent miscarriage.**

Gene	Polymorphism or mutation	Criteria of recurrent miscarriage	Burden	Reference
Thrombophilia				
Factor V	Leiden mutation	≥ 2	Early RM: OR, 2.0; Late RM: OR, 7.8	Rey et al, 2003
Prothrombin	G20210A mutation	≥ 2	Early RM: OR, 2.4	Rey et al, 2003
Anticoagulation				
PAI-1 and Factor XIII	PAI-1 4G/5G and FXIII Val34Leu	≥ 2, unexplained	Early RM: OR, 2.4	Dossenbach-Glaninger et al, 2003
HLA				
HLA-G	*01013/*0105N *0104/*0105N	≥ 3, no uterine abnormality, no translocation, APL ≥ 3, unexplained	— Subsequent miscarriage: OR, 3.6	Pfeiffer et al, 2001 Aldrich et al, 2001
Detoxification enzyme				
GSTM1	null	≥ 2, no uterine abnormality, no translocation	≥ 2: OR, 2.2 ≥ 3: OR, 2.9	Sata et al, 2003
Cytokine				
IL-1b	IL 1-511C, IL 1B-31T	≥ 3, unexplained	—	Wang et al, 2002
IL-1RN	IL-1RN*3	≥ 3, unexplained	OR, 5.6	Karhukorpi et al, 2002
IL-6	IL-1RN*2	≥ 3, unexplained	OR, 7.4	Unfried et al, 2001
	-634G	≥ 2, no uterine abnormality, no translocation	≥ 2: OR, 0.46	Saijo et al, 2004
Hormone				
CYP17	A2 allele	≥ 2, no uterine abnormality, no translocation	OR, 1.7 (heterozygosity) OR, 2.3 (homozygosity)	Sata et al, 2003
Vasodilator				
NOS3	Allele A/B heterozygous	≥ 3, unexplained	OR, 1.6	Tempfer et al, 2001

Adapted from reference number (28)

2.4. History of Nitric Oxide

In the early 1980s it was established that NO was produced in the human body. Shortly afterward it became clear that NO had important functions in the regulation of vascular tone, and it was demonstrated that NO was identical to endothelium-derived relaxing factor, a factor derived from endothelial cells that induced relaxation of smooth muscle cells. NO was selected as the 1992 "Molecule of the Year" by the *Science Journal*, and the 1998 Nobel Prize for Medicine or Physiology was awarded to Louis

Ignarro, Ferid Murad, and Robert Furchgott, the founders of NO research. Since its discovery, the gaseous radical NO has elicited much attention from the scientific community, and NO has been implicated in many diverse processes ranging from the regulation of vascular tone and male erectile function to neurotransmission and microbiocidal activity[35].

2.5. Endothelial nitric oxide synthase *(eNOS)* gene structure and isoforms

Three quite *NOS* distinct isoforms have been identified, products of different genes, with different localization, regulation, catalytic properties and inhibitor sensitivity, and with 51-57% homology between the human isoforms[5].

These isoforms referred to by the most common nomenclature: *nNOS* (also known as *Type I, NOS-I or NOS-1*) being the isoform first found (and predominating) in neuronal tissue, *iNOS* (also known as *Type II, NOS-II or NOS-2)* being the isoform which is inducible in a wide range of cells and tissues and *eNOS* (also known as *Type III, NOS-III or NOS-3)* being the isoform first found in vascular endothelial cells. These isoforms have in the past been also differentiated on the basis of their constitutive *(eNOS and nNOS)* versus inducible *(iNOS)* expression[5](Figure 2.1).

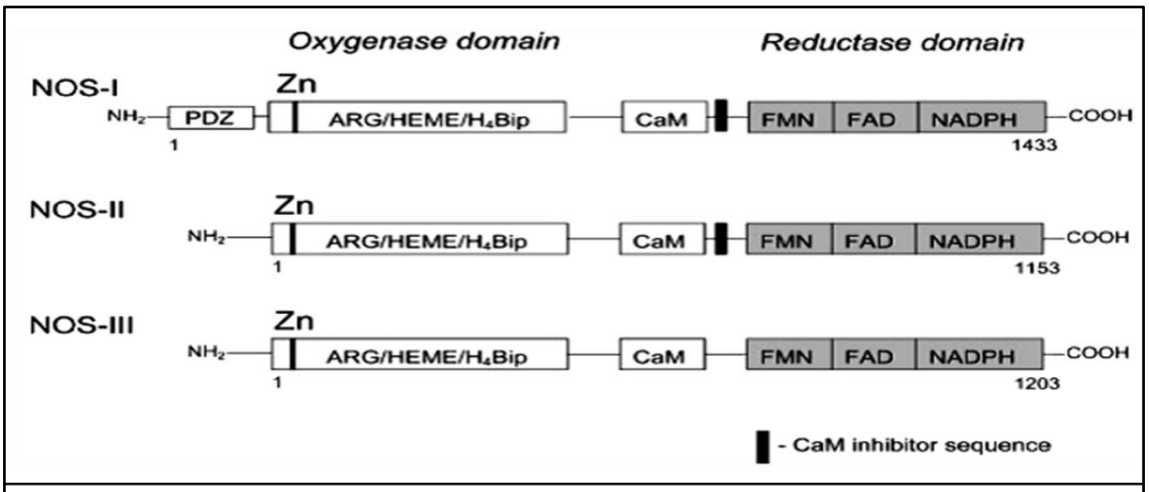


Figure Key: ARG: Argentine, CaM: Calmodulin, COOH: Carboxyl group terminal domain, FAD: Flavin adenine dinucleotide, FMN: Flavin mononucleotids, H_4Bip: Tetrahydrobiopterin, NADPH: Nicotinamide adenine dinucleotide phosphate, NH2: Amino group terminal domain, NOS: Nitric oxide synthase, Zn: Zink.

Figure 2.1. **The domain structure of nitric oxide synthase *(NOS)* isoforms. (adapted from reference number 37).**

The constitutive *(nNOS and eNOS)* are Ca^{2+}/Calmodulin- dependent enzymes, while the inducible *(iNOS)* is a Ca^{2+} -independent enzyme that is transcriptionally regulated by several cytokines[36].

The human *NOS* genes are located on chromosome 12 for *NOS-I (nNOS or neuronal isoform*: 29 exons, 28 introns; 150 kbp), 17 for *NOS-II (iNOS or inducible isoform:* 26 exons, 25 introns; 37 kbp), and 7 for *NOS-III (endothelial isoform or eNOS:* 26 exons, 25 introns, 21–22 kbp)[37].

The endothelial nitric oxide synthase *(eNOS*) gene was cloned in 1993 and was localized to chromosome 7q35-36[16, 38]. the gene comprises 26 exons spanning approximately 21 kb of genomic DNA[1, 16, 39]. and encodes an mRNA of 4052 nucleotides[39], that encode a 135-kD protein containing 1,203 amino acids. Approximately, 1,500 base pairs of upstream promoter sequence have also been characterized and contain transcription factor-binding sites that mediate regulation by shear stress and estrogens, among others[4].

Each *NOS* isoform has the same layout of catalytic domains: a C-terminal reductase with one binding site each for *Flavin* adenine dinucleotide (FAD), Flavin mononucleotide (FMN) and *Nicotinamide adenine dinucleotide phosphate* (NADPH), and an N-terminal oxygenase section. The oxygenase domain contains bound heme and the binding site for the cofactor tetrahydrobiopterin (H_4Bip). H_4Bip is essential for the coupling of NADPH-dependent O_2 activation to NO synthesis. Each isoenzyme has a different N-Terminal extension, which is not essential for catalysis and probably functions in the intracellular localization of the enzyme[40].

The constitutive *NOS* and *iNOS* exist as dimeric and tetrameric complexes, respectively. Each unit contains two identical subunits. Each unit has a reductase and oxidase domain linked by a calmodulin (CaM) binding site[41].

The binding of calmodulin promotes electron transfer from the reductase domain to the oxygenase domain. Importantly, only the homodimeric form of *NOS* is able to

metabolize L-arginine and a single inter-subunit ZnS_4 cluster might be important for dimer and H_4Bip binding site stabilization[37].

The eNOS is localized mainly in vascular endothelial cells. It regulates blood pressure homeostasis by inhibiting platelet aggregation and relaxing the underlying vasculature. Interestingly, it was found to be colocalized with *nNOS* in neuronal areas of rat brain[37].

Neural nitric oxide synthase *(nNOS*) is found in neurons, skeletal muscle, epithelial cells and modulates neurotransmission, gastrointestinal motility, and penile erection. The so-called *"inducible" NOSisoform (iNOS)* is expressed in numerous tissues in response to endotoxin/cytokines and is involved in immune response. In contrast to *eNOS,* its activity is not dependent on changes in free intracellular Ca^{2+} concentrations. Thus, it is a ‘‘high-output’’ NO-generating system that might be essential for eliminating pathogens[37].

2.6. Biosynthesis of nitric oxide

Nitric oxide is not stored but rather released upon its synthesis. Thus, NO generation is regulated through alterations in the expression or activity of the *eNOS* enzyme itself or through changes in the availability of activating cofactors or endogenous inhibitor molecules[4].NO formation catalyzed by means of *NOS* which implicates the formation of N-hydroxy-L-arginine (NOHA) by means of reduced nicotinamide adenine dinucleotide phosphate (NADPH)- and tetrahydrobiopterine (H_4Bip)-dependent monooxygenation, this occurs in two steps*;* the first one consists of an N-oxygenation of the guanidino terminal group of L-arginine to N-hydroxy-L-arginine (NOHA), and the second one which consists of an oxidative cleavage of the C = N bond of NOHA leading to citrulline and NO formation[36]. The only known intermediate is N-hydroxy-L-arginine, a product of the initial step, which involves the initial N-hydroxylation of the guanidinium nitrogen atom[37].

Endothelial nitric oxide synthase enzyme is competitively inhibited by N^G-monomethyl-L arginine (L-NMMA) and other L-arginine analogues[36, 42]. *NOS* is also inhibited by flavoprotein binders, and calmodulin binders[42].

2.7. Mechanism of *eNOS* action

The quest to identify the so-called endothelium-derived relaxing factor led to the discovery in the vasculature of an enzyme. *NOS,* that generates NO from the amino acid L-arginine. This enzyme is constitutive, is calcium- and calmodulin-dependent, and releases picomoles of NO in response to receptor stimulation. The identification of a competitive inhibitor of this enzyme, the methylated L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA), provided an important tool to investigate the relevance of NO in biologic processes[43].

The discovery of this vasodilator tone indicated the existence of an endogenous nitrovasodilator system, the actions of which are imitated by compounds such as nitroglycerin and sodium nitroprusside. These compounds, which have long been recognized as clinically efficacious, act after their conversion into NO. The reaction of NO with the ferrous iron in the heme prosthetic group of the soluble guanylate cyclase in vascular smooth-muscle cells increases the concentration of cyclic GMP, leading to vascular relaxation. Hemoglobin, a potent inactivator of NO, binds to it by a similar mechanism[43].

Nitric oxide also inhibits platelet aggregation by a mechanism dependent on cyclic GMP and synergizes with prostacyclin, which inhibits the aggregation of platelets by increasing their concentrations of cyclic AMP. Unlike prostacyclin, NO also inhibits platelet adhesion. Furthermore, platelets themselves generate NO, which acts as a negative-feedback mechanism to inhibit platelet activation. Thus, platelet aggregation *in-vivo* may be regulated by platelet-derived NO as well as by NO and prostacyclin released from the vascular endothelium. Nitrovasodilators, in combination with prostacyclin or its analogues, may therefore provide a useful antithrombotic therapy[43].

Nitric oxide synthase is activated by increases in intracellular Ca^{2+} concentration, Intracellular Ca^{2+} binds to calmodulin to form a complex that is crucial for enzyme activity. In vascular endothelial cells, stimulation with vasoactive agonists (e.g. acetylcholine, bradykinin, thrombin) activates membrane phospholipases through G protein-linked receptors which, in turn, generate inositol triphosphate (IP_3) as a second messenger. The increased IP_3 concentration elicits Ca^{2+} release from intracellular storesby binding to IP_3 receptors on the endoplasmic reticulum. Further increases in intracellular concentrations of Ca^{2+} involve the influx of extracellular Ca^{2+}. Whereas many oxidative enzymes employ a single electron donor, the oxidative enzyme *NOS* uses multiple oxidative cofactors with associated binding sites[41].

Nitric oxide can freely diffuse across cellular membranes into adjacent cells and serve as a signaling agent[10], it exerts its effects by binding to heme group of guanylate cyclase enzyme resulting in profound (50-200 times) increase in rate of conversion of guanosine 5' triphosphate (GTP) to cyclic guanosine monophosphate (cGMP)[44](Figure 2.2).

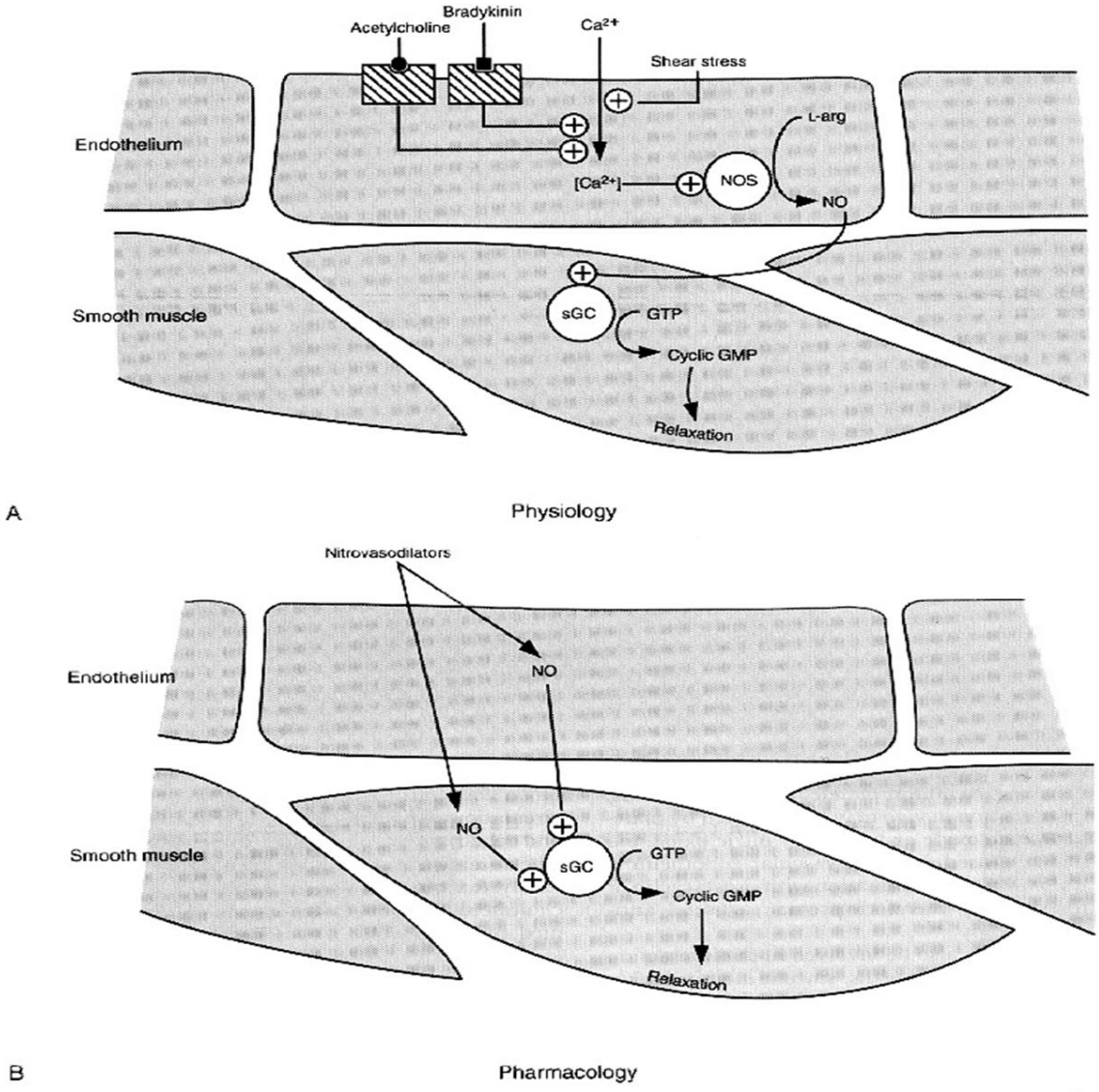
Cyclic guanosine monophosphate (cGMP) activates protein kinases and leads in turn to the phosphorylation changes and consequence to smooth muscle relaxation[42, 44, 45].

There is increasing evidence that NO can directly regulate gene expression by modulating the activity of transcription factors such as nuclear factor κB (NF-κB) and the activator protein 1 (AP-1). Since NF-κB inhibits progesterone receptor (PR) action via protein-protein interaction, NO may, therefore, modulate P_4 responses in the reproductive tract[46].

At high concentrations NO plays a role in apoptotic cell death. An increased apoptosis following exogenous application of NO donors or *iNOS* induction has been described in different cell types, such as macrophages and mesangial cells. NO-induced apoptosis was accompanied by the accumulation of the tumour suppressor protein p53 and activation of caspases[46].

Nitric oxide may also be involved in the interaction of leukocytes with vessel walls, since it inhibits leukocyte activation. Furthermore, it inhibits the proliferation of smooth-muscle cells. It participates in the general homeostatic control of the vasculature[43].

Nitric oxide is an important mediator of physiological processes, but it also has a cytotoxic role when the superoxide anion (O_2^-) is present. In fact, NO is able to combine with superoxide to generate peroxynitrite ($ONOO^-$), a reactive oxidant that is known to produce relevant peroxidative damage. Normally, O_2^- is metabolized by the superoxide dismutase to hydrogen peroxide (H_2O_2), which is then metabolized by catalase to H_2O and O_2. As the amount of O_2^- increases, $ONOO^-$ also increases, while the availability of NO decreases. In this regard, placental oxidant-antioxidant imbalance and the consequent peroxynitrite production could play an important role in this gestational pathology[11].



In Panel A, shear stress or receptor activation of vascular endothelium by bradkyinin or acetylcholine results in a influx of calcium. The consequent increase in intracellular calcium stimulates the constitutive nitric oxide synthase (NOS). The nitric oxide (NO) formed from L-arginine (L-arg) by this enzyme diffuses to nearby smooth-muscle cells, in which it stimulates the soluble guanylate cyclase (sGC), resulting in enhanced synthesis of cyclic GMP, from guanosine triphosphate (GTP). This increase in cyclic GMP in the smooth muscle cells lead to their relaxation. **In Panel B,** nitrovasodialators such as sodium nitroprusside and nitroglycerine release nitric oxide, spontaneously or through an enzymatic reaction. The liberated nitric oxide stimulates the soluble guanylate cyclase (sGC) in the smooth muscle cell, resulting in relaxation

Figure 2.2. **Nitric oxide- mediated vascular relaxation. (adapted from reference 43).**

2.8. Metabolism of nitric oxide

Nitric oxide is labile species with a half life of only few seconds in biologic systems, in cell culture systemic NO degrades rapidly to nitrite, but in the presence of Fe^{2+} heme, or certain other transition metals, nitrite is converted to the more stable product nitrate. Thus *in-vivo*, nitrite is unstable and has a short half life and the major ion is nitrate[47].

The metabolic fate of endogenous NO is surprisingly poorly understood. Putative intermediate metabolites include an array of low and high molecular weight thiol-nitrosoglutathione, nitrosoalbumin, and nitrosohemoglobin- some of which might be present in sufficient quantities to exert the biologic effects. Furthermore, NO reacts with another endogenous radical, superoxide anion (O_2^-), to form peroxynitrite ($ONOO^-$). Peroxynitrite may isomerize to yield nitrate or may lead to nitration of tyrosine residues on proteins. The extent to which this occurs *in-vivo*, whether it is a major route of metabolism for endogenous NO, and the routes of metabolism of nitrated proteins remain unknown[47].

Because NO_2^- plus NO_3^- (termed NO_x) are relatively stable in blood, the concentration of NOx in blood may be an indicator of the endogenous formation of NO. In fasting individuals, as much as 90% of the circulating NO_2^- is derived from the L-arginine- NO pathway, and NO_2^- is a valid indicator of NO production[48].

2.9. Physiologic role of nitric oxide in the body

Nitric oxide is a multifunctional signal and important modulator of cellular responses in a variety of tissues including those involved in human reproduction[49]. There is considerable evidence that local production of NO contributes to the maintenance of low vascular resistance in the fetoplacental circulation. Since umbilical cord and chorionic plate vessels are unlikely to contribute greatly to the regulation of fetoplacental blood flow because of their large calibre, stem villous arterioles of the placenta are thought to be the major site of resistance[49].

Nitric oxide is an endothelial vasodilator with additional antithrombotic and atheroprotective properties[50]. In the last decade, NO has assumed an important functional role in a variety of physiological systems and different pathways, therefore it is indisputable that such a polyvalent molecule should also play a decisive role in the reproductive system[51].

Endothelial nitric oxide synthase is the main enzyme required for vascular NO production[2].The *eNOS* gene is expressed in the endothelium of a variety of tissues, as well as in cardiac and myometrial myocytes, platelets, and in airway epithelium[39].

Trophoblast cells of first trimester express high amounts of *NOS* activity. Recent studies in humans indicated that more than 90% of the *NOS* activity in the trophoblast is Ca^{+2} dependant, and that the biochemical and immunological characteristics point out to the isoform e*NOS*. NO synthesized by placenta, trophoblast and to some extent the fetal membranes inhibit the uterine myometrial contractions either directly or through an interaction with cyclooxygenase, thus playing a role in maintenance of uterine quiescence early in gestation and preventing preterm labor[1].

In the vascular system, NO induces vasodilation, inhibits platelet aggregation, prevents neutrophil/platelet adhesion to endothelial cells, inhibits smooth muscle cells proliferation and migration, and maintains endothelial cell barrier function. In the neural system, NO acts as a neurotransmitter, whereas increased expression of *iNOS* plays a key role under several pathological conditions[51].

The endothelial *(eNOS)* and neural *(nNOS)* isoforms have so far been associated with blood pressure regulation and neurotransmission, whereas the inducible *(iNOS)* isoform is suggested to be involved in macrophage defense mechanisms against infections. Thus, induction of the NO pathway by cytokines such as interferon (IFN-γ), tumor necrosis factor (TNF-α), and interleukin (IL-1β) has been detected in a variety of cell types, including murine macrophages, endothelial cells, and β-pancreatic cells. Although there is evidence suggesting that NO induction is protective *in-vivo* against intracellular infections (e.g.; leishmaniasis, listeriosis, and blood-stage malaria)[52].

Since neurones, vasculature and cells of the immune system are an integral part of the reproductive organs, it is obvious that NO is an important regulator of the biology and physiology of the reproductive system[51].

2.10. Association between *NOS* and Pregnancy

Normal pregnancy is associated with an increase in blood volume and cardiac output and a fall of blood pressure (BP) in the first half of pregnancy caused by systemic arteriolar vasodilation. It has been proposed that the enhanced endothelial synthesis of the NO is responsible for this vasodilation, and several studies have shown that flow mediated vasodilation of the brachial artery (an NO-dependent response) is enhanced from early gestation[50].

The potent vasodilator properties of NO in the resistance arteries, coupled with the genetic basis of hypertension, suggests that mutations affecting the endothelial NO *(eNOS)* gene, and consequently impairing NO release, might contribute to increased vascular resistance and in turn an elevation in systemic blood pressure[53].

Nitric oxide was recently implicated as an important regulatory agent in various female reproductive processes, such as ovulation, implantation, pregnancy maintenance, labor and delivery. Animal studies clearly indicate that during pregnancy, NO is up-regulated in the myometrium and placenta. It contributes to uterine quiescence and controls utero-fetoplacental blood flow[46].

Nitric oxide is also involved in cervical ripening during labor. Moreover, these studies also indicate that the regulation of NO production in the female reproductive tract is mainly controlled by steroid hormones in a tissue-specific manner[46].

Prior to the onset of labor, the cervix undergoes physical changes, which are necessary for vaginal delivery. These changes, which occur during the last weeks of pregnancy, include softening, effacement and dilatation of the cervix and are given the term cervical ripening. The timing of these changes requires careful regulation. Premature cervical ripening may lead to preterm labor, a condition that is associated with considerable morbidity and mortality. Alternatively, failure of ripening leads to

delay in the onset of labor and an associated increase in the Caesarean section rate and birth asphyxia. In humans, cervical ripening is an inflammatory reaction involving leukocytic infiltration, changes in the water content of the extracellular matrix and rearrangement of collagen fibres. The control of this process remains poorly understoodalthough a number of mediators have been implicated, including P_4 withdrawal, prostaglandins, relaxin, and various inflammatory cytokines[25].

Recently, The inflammatory mediator, NO has been implicated in cervical ripening[25]. The importance of NO in the context of pregnancy is further substantiated by co-localization and inhibitor studies showing its effect on human chorionic gonadotrophin (HCG) release. Reports indicate that NO results in transient but prompt release of HCG by the placenta. Abnormal NO levels were reported in placenta from pre-eclamptic pregnancies as well as from term pregnancies with fetal growth retardation. NO exhibits its effect on release of GnRH, an important neuroendocrine regulator inside the placenta, from hypothalamic neurons. Thus, in human placenta NO, *eNOS* possibly helps to maintain pregnancy by controlling both endocrine function and vascular tone[1].

The endothelial nitric oxide synthase appears to be the most abundant isoform in early placenta, other isoforms such as *iNOS* are predominantly expressed throughout pregnancy in the uterus[54].

Bansal et al. (1997) reported that myometrial *iNOS* expression, assessed by immunohistochemistry and Western blotting, was greater in the early third trimester (26–34 weeks gestation) than either the late third trimester (37–41 weeks gestation) or in the non-pregnant state. These data suggest that an increase in myometrial *iNOS* expression might contribute to the maintenance of uterine quiescence during pregnancy. The role of the constitutive isoforms of *NOS(eNOS and nNOS)* has not been determined[55].

Estrogens upregulate *NOS* in animals[56, 57], and therefore, the huge rise in circulating estradiol concentration during early pregnancycould stimulate increased NO synthesis[56].

Experimental data in mice and previously published results in humans point to a crucial role of NO in the course of pregnancy with respect to induced abortion. Experimental data in mice have shown that lipopolysaccharide (LPS)- induced abortion is medicated by placental NO Production [2, 15, 52]. Pharmacological inhibition of NO release by aminoguanidine successfully rescues LPS-Induced abortion[2, 15].

The physiological and biological relevance of NO in pregnancy and labor can be deduced from the finding that inhibition of NO synthesis by administration of N^G-nitro-L-arginine methyl ester (L-NAME, which competes with L-arginine and inhibits NO synthesis) prolonged the duration of delivery and decreased the cervical extensibility. These findings not only suggest the importance of NO synthesis in the uterus and cervix during labor and pregnancy, but also point towards the roles of the various isoforms of *NOS* in regulating these effects[51].

Indirect biochemical assays support the finding of a gestational increase in NO activity. For instance, concentrations of cGMP, the second messenger for NO, are increased in plasma and urine from pregnant animals and humans, and a stable oxidation product of NO, nitrate, is found in elevated concentrations in the urine and plasma of pregnant rats and possibly in humans. However, interpretation of such measures is not straightforward, since cGMP can be elevated by atria1 natriuretic peptide and nitrate is present in the diet[56].

2.11. Nitric oxide regulation by progesterone

The earlypregnancy failures can be detected as random findings duringultrasonographic examination before bleeding or other signsof abortion have occurred. Uterine quiescence can thuspersist, at least for some time, in these conditions even thoughthe circulating levels of human chorionic gonadotropin (hCG)and P_4 are low. The combination of nonviablepregnancy and uterine quiescence may be associated with changesin NO in the uterus and/or cervix in humans, because in animals,a fall in P_4 inhibits the release of NO in the uterusand stimulates it in the cervix. These opposing changesin NO production should result in the start in of uterine contractions and cervical ripening[58].

It is possible that increased cervical NO release is a specificphenomenon in abortion, perhaps triggered by a fall in serumP_4 concentrations. This is supported by the resultsof animal experiments showing that P_4 has opposingeffects on NO release in the endomyometrium and cervix; it up-regulatesNO release in the former, but down-regulates it in the latter[58].

Progesterone can increase uterine quiescence by stimulating the relaxation mechanisms, mainly the uterine NO system[59].

Tommiska et al. (2004) has shown that women experiencing RPL haveincreased cervical NO release before the onset of clinical abortion.Moreover, cervical NO release was higher the lower the circulatingP_4 level, which suggests a causal relationship betweencervical NO release and P_4 deficiency[58].

Progesterone prevents vasoconstriction by increasing levels of NO, which causes vasodilation allowing blood vessels to relax, and so widens them allowing more blood to flow through and it inhibits platelet aggregation[60].

The fact that preterm labor can be induced by the inhibition of NO together with an antiprogesterone suggests that the NO relaxation system and the relaxation system controlled by P_4 may act jointly to maintain pregnancy[18].

Inhibition of nitric oxide synthesis together with blockade of P_4 action somewhat parallel the events that occur with the initiation of normal labor. In fact, a precipitous decrease in NO production together with the well-documented fall in P_4 levels accompanies the initiation of spontaneous labor. Both NO production and relaxation responsiveness to NO are increased during pregnancy, when P_4 levels are elevated, and decreased during labor at term, when P_4 levels fall. In experimental animals studies, preterm labor observed in the antiprogesterone + L NAME groups may be due to a combination of a reduction of NO production by L-NAME and multiple effects of antiprogesterone on (1) the cGMP relaxation system, (2) endogenous nitric oxide synthesis, and (3) receptors and excitability. Antiprogesterone at low doses may partially reduce the effects of NO on uterine relaxation but not completely enough to

produce preterm labor. On the other hand, a high-dose antiprogesterone may completely negate the NO-cGMP effects on relaxation and thus produce preterm labor without a necessary reduction in NO production. Previous studies propose that in the rat a reduction in the NO production together with P_4 withdrawal may be required to achieve labor and delivery. However, L-NAME infusion was unable to trigger initiation of labor toward the end of gestation, when P_4 levels are decreased, the reasons are not known;however, it may be due to a combination of insufficient withdrawal of P_4 (action) and incomplete inhibition of NO effects[18].

Mechanism(s) for the potentiation of antiprogesterone action by L-NAME are not known. The possibilities may include (1) an interaction between the NO system and P_4, which is more prominent before term, (2) effects related to decreased blood flow such as an effect of NO on steroid hormone production through actions on blood vessels to the placenta or ovary or altered metabolism of steroid hormones or antiprogesterone, or (3) other actions. Further studies are needed to define this interaction[18].

The uterine NO production and *NOS* expression are gestationally regulated and P_4-dependent. NO production, reflected in total nitrite produced by uterine tissue, was low in non pregnant rats, substantially elevated during the mid stage of gestation, and markedly lower at the time of spontaneous delivery and the first day postpartum. Likewise, there was a decrease in NO synthesis in the uterus and an increase in cervix during both term and onapristone-induced preterm birth[61].

The study by *Garfield et al. (1998)* in rats provided ample evidence that *iNOS* is the dominant isoform of *NOS* in the myometrium. In rats, myometrial *iNOS* expression seems to be regulated by P_4, since *iNOS* declines prior to normal parturition when serum P_4 concentrations are low. *iNOS* expression decreased during onapristone-induced preterm labor, an effect which can be reversed by P_4 agonist[61].

Due to P_4 action, uterine NO production is increased by *iNOS* during pregnancy, prior to parturition at a term, or after anti-progestin treatment at preterm, there is a decline in uterine NO production and a consequential decrease in relaxation. Hence, the NO system may contribute to the maintenance of uterine quiescence during pregnancy

when P_4 concentrations are eleveated, but not during delivery. Conversely, during term and pretem labor there is an up-regulation of the NO system in uterine cervix as a result of the inflammation cascade being activated, thereby contributing to the remodeling of the extracellular matrix. However the mechanism responsible for differential regulation of the *NOS* system in the uterus and the cervix remains to be established[61].

In summary, inhibition of NO by L-NAME together with low dose antiprogesterone administration produces preterm labor, indicating that uterine quiescence during pregnancy may be maintained by the synergistic effects of the NO and P_4. Thus, the interaction of NO and P_4 may be required to maintain pregnancy[18].

2.12. *eNOS* Gene polymorphisms

The endothelial nitric oxide synthase gene has been extensively screened for variation. Variants detected include numerous single nucleotide polymorphisms (SNPs), *a variable-number tandem repeat in intron 4,* and a *CA repeatmicrosatellite marker in intron 13.* Much attention has been focused on three putatively functional variants; *promoter -786T>C,exon 7 (894 G>T)* and *intron 4 (4a/4b) VNTR* polymorphisms (Figure 2.3), but little information has been available as to how these variants associate with one another[9]. Importantly, the level of NO_x metabolites appear to be associated with *eNOS* gene polymorphisms[7, 8, 48, 62].

The effects of these polymorphisms on *in-vivo* NO generation cannot be measured directly because most endogenous NO rapidly oxidizes to nitrite (NO_2^-) and is eventually converted to nitrate (NO_3^-), the predominant stable form of NO. Collectively, these inactive metabolites (NOx) have been used to reflect endogenous NO production, and in turn, their levels appear to be associated with *eNOS* polymorphisms[9].

The 5̀ flanking region, *promoter -786T>C and intron 4 (4a/4b) VNTR* polymorphisms have been associated with alterations in promoter activity. *Exon 7* (894G>T) polymorphism, which predicts a Glu298Asp amino acid substitution in the mature protein could also alter enzyme activity[63], and render the enzyme more susceptible to proteolytic cleavage[9].

A 27 bp of *the variable nucleotide tandem repeat (VNTR)* polymorphism in *intron 4* and the *Glu298Asp* polymorphism in *exon 7* were shown to influence the plasma NO level and were associated with clinical phenotypes in preeclampsia (PE) and cardiopulmonary disease[64].

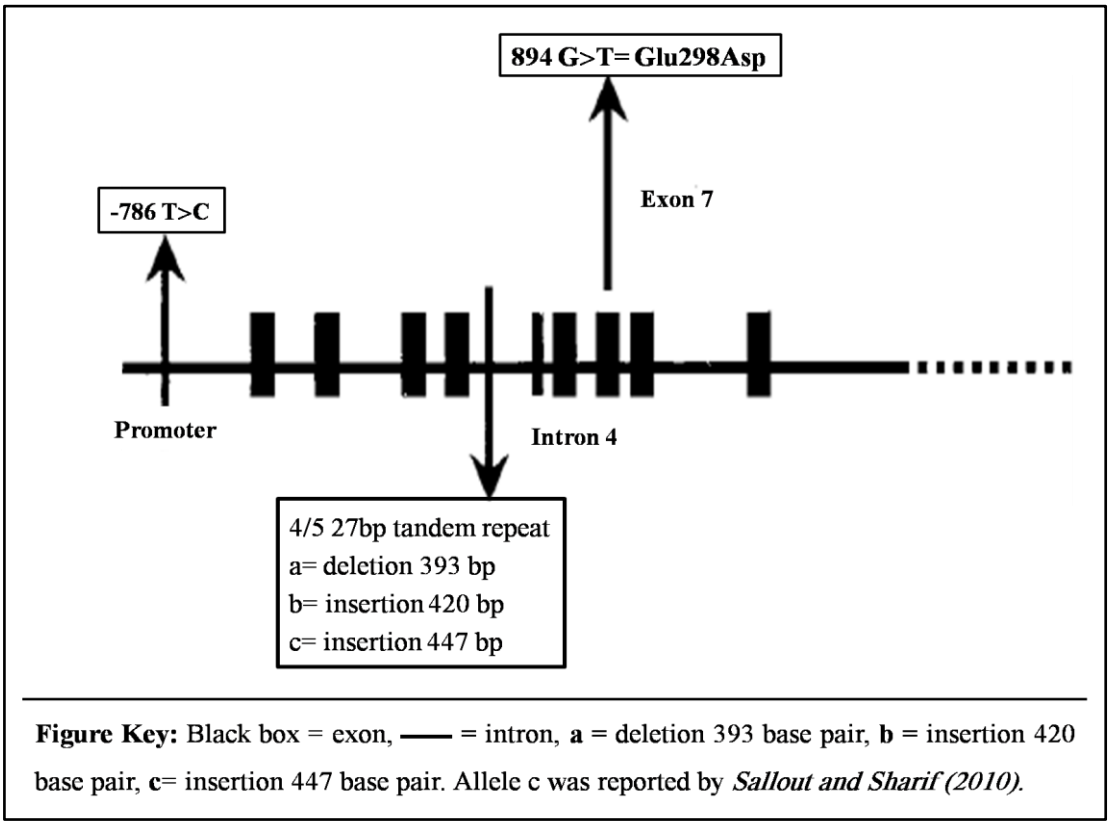


Figure Key: Black box = exon, —— = intron, **a** = deletion 393 base pair, **b** = insertion 420 base pair, **c**= insertion 447 base pair. Allele c was reported by *Sallout and Sharif (2010).*

Figure 2.3. **Organization of common *eNOS* gene polymorphisms (adapted from reference number 9).**

2.12.1.-*786T>C* polymorphisms in the promoter region

A single nucleotide polymorphism (SNP), -786T>C point mutation, was identified in the 5`-flanking region of the *eNOS* gene involving a substitution of thymine (T) to cytosine (C) at a locus 786bp upstream of *eNOS* gene[2, 65], results in a significant reduction in the *eNOS* gene promoter activity[66], this polymorphism has the potential to influence mRNA transcription[63], where it is associated with less placental mRNA, and lower serum nitrite/nitrate concentrations[67].

One manifestation of the T-7863C mutation is increased risk for coronary spasm[53].

2.12.2.*Glu298Asp or 894G>T* polymorphism in exon 7

The Glu298Asp missense mutation encoded by *exon 7* of the *eNOS* gene[20] is another common variant of *eNOS* that has a guanine (G) to thymine (T) transversion at nucleotide position 894[66], resulting in a replacement of glutamic acid by aspartic acid at codon 298 *(Glu298Asp)*[16]. The *exon 7 (894G>T)* is associated with reduced basal NO production. It has been linked to reduced flow-mediated dilatation of the brachial artery during pregnancy and impaired maternal–fetal circulation[67].

The polymorphism in *exon 7 of the eNOS* gene *(Glu298Asp)* has been reported to be associated with an increased risk for myocardial infarction[12, 28, 53], coronary artery spasm[28, 53], hypertension[53], placental abruption and preeclampsia but not with RPL[12, 20].

2.12.3.*Variable nucleotide tandem repeat (27 VNTR) of 4a4b* polymorphism in intron 4

A 27-bp repeat polymorphismin *intron 4 of the eNOS* gene, has been associated with variations in plasma levels of nitrite and nitrate (NOx)-stable metabolites of NO[66].

The intron 4 (4a/4b) VNTR polymorphisms of the gene encoding *eNOS)* has been shown to segregate with lower plasma NO metabolites in non pregnant Japanese[1, 12, 14, 20, 28], Also this polymorphism was found to be associated with RPL in Caucasians[2, 12, 20]. The heterozygosity for *4a* allele and *4b* allele of *eNOS(4a/4b)* was found to be associated with RPL in Austrian women, It is speculated that carriers of *4a/4b* and subsequently reduced NO serum levels are at increased risk for impaired placental perfusion and infarction[28].

Given the intronic location of the *intron 4 repeat* unit, it is perhaps less likely to be functional. Conflicting associations between the *intron 4 variant* and NO pathway activity have been described. Some reports indicate that carriers of this variant have lower NO plasma levels and decreased protein expression, but this finding is not supported by all studies. It is possible that the variant is in linkage disequilibrium with

other functional regulatory regions of the *eNOS* gene[9]. or associated with intronic micro-RNA (mi-RNA) expression[68, 69, 70, 71].

2.13. Previous Studies

2.13.1. Recureent pregnancy loss studies

In Turkey, Öztürk, et al. (2011) preformed a study to determine whether *intron 4 (4a4b) VNTR* or *exon 7 Glu298Asp (894 G>T)* polymorphisms of *eNOSgene* are associated with an increased risk for RPL in the Turkish population and to evaluate the association between NO levels and *eNOSgene* polymorphisms in women with RPL. A total of 120 women were enrolled in four groups. of these, 30 women were first trimester pregnant who had idiopathic RPL (Group I). 30 healthy multipara women were in the first trimester of pregnancy with no history of abortion (Group II), 30 women were non pregnant with a history of RPL (Group III). The remaining 30 subjects were healthy multipara non-pregnant women with no history of abortion (Group IV). The study observed that NO levels were significantly different between Group I and Group II. Therefore; the decreased NO levelsin the pregnant patient group were statistically significant from non-RPL. NO levels were also significantly different between Group III and Group IV. Therefore; the elevated NO levelsin the non pregnant patient group was statistically significant. The study also demonstrated that, there was no significant difference in the frequency of *intron 4 (4a4b) VNTR* genotype between the two groups. While, there was a marginally significant difference in the frequency of *exon 7 Glu298Asp (894 G>T)*genotype in patients with RPL in the Turkish population compared to controls. No association between NO levels and *intron 4 (4a4b) VNTR* or *exon 7 Glu298Asp (894 G>T)*genotypes was found in any of the groups. the authors concluded that the *exon 7 Glu298Asp (894 G>T)* polymorphism of *eNOS* could be an intriguing susceptibility factor that modulates an individual's risk of RPL in Turkish population. Further studies to explain the role of the NO pathway in the pathophysiology of RPL are needed[72].

In India, Parveen, et al. (2011) preformed a study on a total of 200 patients with unexplained recurrent pregnancy loss (URPL) and 300 controls, A 457-bp fragment

spanning from *intron 6* to *exon 8* of the *eNOSgene* was genotyped for six polymorphic regions of *eNOS* by PCR, re-sequencing and RFLP. This region included *intron 6 (12862A>G), exon 7 (12920C>T), exon 7 (12932C>T), exon 7 Glu298Asp (12965G>T), exon 8 (13222C>T)* and *intron 4 VNTR.* The study showed that The *GG genotype of 12862A>G,* the *G allele of exon 7* Glu298Asp *and the (4a4a) genotype* of *intron 4 VNTR* increased the risk of unexplained RPL by ~1.8-fold, ~3.5-fold and ~2-fold, respectively. the two *"AGbCCC"* wild-type allele and *"AGbTCC"* haplotypes were found to have a significant protective effect against RPL. Whereas the GGaCCC haplotype was found to increase the risk of URPL by ~2-fold. In conclusion, three common polymorphisms of *eNOSgene, intron 6 "12862A>G", exon 7 Glu298Asp* and *intron 4 VNTR* increase the risk of RPL in North Indian women. Risk of RPL may also be modified by the presence of particular haplotypes[73].

In Taiwan, Su, et al. (2011) preformed a systematic review and meta-analysis of the published literature from MEDLINE and EMBASE databases to investigate the role of angiogenesis- and vasoconstriction-related genes *(VEGF, p53 and eNOS)* in RPL. Aggregating data fromeligible studies were integrated into meta-analyses by means of random effects models. The meta-analyses of available data showed significant associations between the *promoter -1154G>A polymorphisms of VEGF, codon 72 Arg>Pro polymorphism of p53 gene,*and *exon 7 Glu298Asp* and *intron 4 (4a4b) VNTR polymorphisms of eNOS gene*and idiopathic RPL. The meta-analyses also showed that these angiogenesis- and vasoconstriction-related genes jointly confer higher susceptibility to idiopathic RPL[64].

In Korea, Shin, et al. (2010) carried out a study to investigate the association of *eNOS[promoter -786T>C, intron 4 (4a4b) VNTR,* and *exon 7 (894G>T)]* polymorphisms and haplotypes on a sample of 340 patients with RPL. They found that the *eNOS894 GT+TT* genotype of *exon 7* and the *-786T-4b-894T* haplotype are significantly associated with RPL in Korean women, but no significant differences in *promoter -786T>Cand intron 4 (VNTR) 4a4b* frequencies were observed between the control and the RPL patients[2].

In another Korean study, Shim, et al. (2010) preformed a study on 99 spontaneously aborted fetuses <20 weeks of gestational age and 103 child controls and 282 adult controls to evaluate the genotype frequency of three *eNOS [promoter -786T>C, intron 4 (4a/4b) VNTR, and exon 7 (894G>T)]* polymorphisms. The study showed that the frequency of -786TC and CC genotypes in aborted embryos were significantly higher than in both child and adult controls. The frequencies of *4a4a homozygote* of *VNTR* polymorphism in *intron 4* and *TT homozygote of 894G>T* polymorphisms were also higher in aborted embryos than in adult controls. Haplotype analysis suggests that *promoter -786T>C* polymorphism was a possible risk factor for spontaneously aborted embryos. The study concluded that *eNOSgene* studied polymorphisms are associated with risk of spontaneously aborted fetuses[74].

In Gaza Strip, Al-Sallout and Sharif (2010) conducted a study on 100 women who had at least 3 constitutive abortions using molecular biological techniques to investigate the correlation between RPL and common polymorphisms in *angiotensin-converting enzyme (ACE), plasminogen activator inhibitor 1 (PAI-1) 4G/5G* and *intron 4 (4a4b) VNTR polymorphism of eNOS gene*. They found that there is no significant association between *ACE I/D, PAI-1 or intron 4 (4a4b) VNTR of eNOS gene* and the occurrence of first-trimester RPL. Their study recommended an in-depth investigation on the association of *eNOS4a/4a* with RPL[22].

In Greece, karvela, et al. (2008) preformed a study on a total of 126 women who had at least three unexplained pregnancy losses before 20 weeks of gestation and 130 control group women with at least two live childbirths and without history of abortions, to determine whether the *intron 4 (4a/4b) VNTR* and *exon 7 Glu298Asp (894G>T)* missense mutation of the *eNOSgene* are associated with an increased risk for RPL, in the Greek population. The study did not show any influence of the two studied *eNOSgene* polymorphisms on early pregnancy[20].

In China, Fan, et al. (2007) investigated the association of *eNOSgene[intron 4 (4a/4b) VNTR* and *exon 7 (894G>T)]* polymorphisms with RPL on 140 patients. They found that the *intron 4 VNTR* polymorphism was associated with RPL. These results

support that the *"4a"* allele of the *intron 4* e*NOSgene* may be considered an RPL allele[21].

In Tunisia, Zammiti, et al. (2007) examined 350 patients with RPL and 200 healthy women to determine the association between *eNOS [intron 4 (4a/4b) VNTR, exon 7 894G>T,* and *promoter -786T>C]* polymorphisms, and homocysteine levels and RPL in Tunisian women. The study showed that neither *eNOSgene* polymorphisms nor homocysteine levelwas associated with RPL[23].

InIndia, Suryanarayana, et al. (2006) performed a study to investigate therelationship between idiopathic recurrent early pregnancy loss (REPL) and *intron 4 (4a4b) VNTR, exon 7 Glu298Asp* and *intron 6 (140A>G)* polymorphisms of *eNOSgene* among South Indian women on a total of 145 females with recurrent early pregnancy loss. The study identified and validated that the *intron 6 (140A>G)* novel polymorphism in the *eNOSgene* is associated with the risk of idiopathic RPL. However, neither genotype nor allele frequencies of *intron 4 (4a4b) VNTR* or *exon 7 (894G>T)* was found significantly different between RPL case and control groups[1].

In Germany, Buchholz, et al. (2004) preformed a study to investigate, whether two polymorphisms in the *angiotensinogen II type 1 receptor gene (AT1R C/C genotype)* and *intron 4 (4a4b) VNTR* of *eNOSgene* affect maternal vasoconstriction and RPL on a sample of 179 women with at least two unexplained consecutive spontaneous abortions before 25 weeks of gestation, and 126 healthy women with one or more normal term deliveries after uneventful pregnancies and no history of miscarriages. The study indicated that the vasoconstrictively acting genotypes *AT1R C/C* of the *angiotensinogen II type 1 receptor gene* and *eNOS 4a4b VNTR* of *eNOSgene* are of similar prevalence in RPL patients and in controls. The authors concluded that their results do not show any influence of the polymorphisms studied on early pregnancy development[75].

In USA, Hefler, et al. (2002) preformeda study to investigate the correlation between idiopathic RPL and common polymorphisms in *exon 2 (235M>T)* of the *angiotensinogen (Agt)gene,exon 7 Glu298Asp* of the *eNOSgene* and the *promoter -511C>T* polymorphism of the *interleukin-1beta (IL1β) genes* on 130 Caucasians women

with at least three spontaneous, consecutive miscarriages before 20 weeks of gestation and 67 healthy, post-menopausal white Caucasians women with at least two live births and no history of miscarriage. The study showed that the allele and genotype frequencies of all studied polymorphisms were similar among women with RPL and controls. Between women with primary and secondary recurrent pregnancy loss, no statistically significant differences between allele and genotype frequencies were observed. The authors concluded that the polymorphisms studied should not be included in further studies involving panels of various polymorphisms. However, they cannot exclude the possibility that other polymorphisms of *Agt, eNOS,* and *IL1βgenes* are associated with the disease and might be clinically potential markers to assess the woman's risk for RPL[15].

In Austria, Tempfer, et al. (2001) carried out a study on *intron 4 (4a4b) VNTR* polymorphism of *eNOSgene* in a sample of 105 women with RPL to investigate the relationship between RPL and a polymorphism of the gene encoding *eNOS*. The study supports a role for the *eNOSgene* as a genetic determinant of the risk of RPL, and demonstrates that the *intron 4 VNTR* polymorphism of the *eNOSgene* is associated with RPL, The study also showed that the heterozygous carriers of the e*NOS* polymorphism *(4a4b)* have a 1.6- fold increased risk of RPL compared to a control population[14].

2.13.2. Pregnancy complication studies

Previous published studies concerning the association between commonly studied *eNOS* polymorphisms and pregnancy complications [preeclampsia, intrauterine fetal death (IUFD), and placental abruption] has shown a conflicting results among different ethnic populations. Some of these studies reported that *promoter -786T>C* polymorphism is predisposing to preeclampsia[76, 77]. On the other hand, others reported that *promoter -786T>C* polymorphism is not a risk factor for preeclampsia[78, 79]. Several studied found that *intron 4 (4a/4b) VNTR* polymorphism is not associated with preeclampsia[78, 79, 80, 81] but it might modulate timing of IUFD in affected pregnancies[82]. Regarding *exon 7 Glu298Asp* polymorphism, some studies reported that this polymorphism could be a marker for developing both preeclampsia[80, 83, 84] and

placental abruption[85, 86]. Conversely, others found that *exon 7 Glu298Asp* polymorphism was neither associated with preeclampsia[87, 79, 88] nor with IUFD[82].

Several published meta-analyses and multicenter case control studies have been also concerned with the association between the commonly studied *eNOS* polymorphisms and pregnancy complications in different population e.g.,

In China, Chen, et al. (2011) performed a meta-analysis of 18 case-control association studies that examined the relationship between preeclampsia and the *exon 7 Glu298Asp, intron 4 VNTR and promoter -786T>C* polymorphisms of the *eNOSgene*. Subgroup analysis by ethnicity and potential sources of heterogeneity and bias were explored. The meta-analysis showed that for the allelic analysis of the *exon 7 Glu298Asp* variant, all studies showed no significant association, For the genotypic analysis, the combined studies of the *G allele* showed negative significance. All the studies showed positive significance when the T allele was combined, and results were also positively significant in non-Asian populations. For the allelic analysis of the *intron 4 VNTR variant,* all studies showed no significant association, but results were negatively significant in non-Asian populations, for the genotype analysis, combined studies of the b allele showed negative significance. Moreover, non-Asian studies showed negatively significant results. For the analysis of the *promoter -786T>C variant,* none of the studies showed significant results. This meta-analysis supports the fact that *intron 4a allele,* homozygosity for the *exon 7 894T* and *intron 4a* of *eNOS* are positively associated with preeclampsia. The study also found that genetic heterogeneity exists among ethnicities[89].

In the Uk, Yu, et al. (2006) carried out a meta-analysis on healthy women with singleton pregnancies recruited from 7 district general hospitals in London. Women at high risk of preeclampsia were genotyped for *exon 7 Glu298Asp* polymorphism of *eNOS* to examine its association with preeclampsia. The meta-analysis showed that the *exon 7 Glu298Asp* polymorphism in a recessive model was not significantly associated with preeclampsia. In the meta-analysis, under a recessive genetic model (1129 cases and 2384 controls) women homozygous for the *Asp298* allele were not at significantly

increased risk of preeclampsia. A dominant model (1334 cases and 2894 controls) was associated with no increase of risk of preeclampsia for women carriers of the Asp298 allele. The authors concluded that the *eNOS exon 7 Glu298Asp* polymorphism of the *eNOSgene* is not associated with a significantly increased risk of preeclampsia[90].

In Colombia, Serrano et al. (2004) performed a multicenter case-control study to assesse whether *exon 7 Glu298Asp, intron 4 VNTR* and *promoter -786T>C* genotypes in the *eNOSgene* alter the risk of preeclampsia in a population in which the incidence of this disorder is high. The study indicated that there is no increase in the risk of preeclampsia for the *intron 4 VNTR* or *promoter -786T>C* polymorphisms was observed under any model of inheritance. In contrast, *exon 7 Asp298 allele*, was associated with increased risk of preeclampsia as compared to carriers of the Glu298 allele. After a multivariate analysis, carriage of the "Asp298-786C-4b" haplotype was also associated with increased risk of preeclampsia when compared to carriers of the "Glu298-786T-4b" haplotype. The *eNOS* Glu298Asp polymorphism and the Asp298-786C-4b haplotype are risk factors for preeclampsia. In conclusion, the study suggests that the young Colombian women homozygous for the Asp298 allele are at increased risk of developing preeclampsia, but very large studies or meta-analysis will be required to confirm these findings and refine estimates of the effect size[91].

2.13.3. Human *eNOS,* cytokines and sex hormones studies

In India, Sharma, et al. (2011) conducted a cross-sectional study to evaluate cytokines pattern in preeclampsia and whether there is any relationship between gene and cytokines production and cytokine with disease severity. The sample included 100 women with preeclampsia and 100 healthy pregnant women. Their blood samples were analyzed for NO, inflammatory cytokines, and *eNOS(894 G>T) gene* polymorphism. The study showed that decreased NO and increased cytokine (tumor necrosis factor α, interleukin-2, and interferon γ) levels were found in preeclampsia. Significant differences were found in genotype/allele distribution between the two groups. A significant negative correlation was observed between NO and cytokine levels (tumor necrosis factor α, interleukin-2, and interferon γ) in the preeclamptic group. The authors concluded that preeclampsia is associated with decreased levels of NO and increased

levels of circulating inflammatory cytokines due to *(894 G>T)* single nucleotide polymorphisms, pointing toward the role of endothelial and inflammatory components[92].

In Iraq, Baban (2010) conducted a case control study, to investigate biochemical changes in lipid peroxidation, NO, and vitamin E in recurrent pregnancy loss women, and compared these with healthy pregnant, and non-pregnant women.In total 96 subjects categorized as 32 patients with RPL, and 32 pregnant women in their third trimester, and another 32 non-pregnant women were enrolled. were the blood samples collected from each patient at the time of pregnancy loss, and serum from patients and controls were then used to estimate malondialdehyde (MDA), NO, and vitamin E levels. The study showed that there was a significant elevation in patient serum MDA compared with third trimester pregnant women and non-pregnant women. Both serum vitamin E and NO levels in RPL patients also showed a highly significant decrease compared with third trimester pregnant, and non-pregnant women. A highly significant difference was found in the MDA/vitamin E ratio between RPL and control groups, while no significance was found between RPL and control groups NO/vitamin E ratio. The author concluded that the decrease in NO production and vitamin E is a result of RPL and not a causative factor, as the RPL was without pathological cause, medication, or fibroid presence, and no significant difference was found between the NO/vitamin E ratio in RPL and control groups[93].

In Brazil, Sandrim, et al. (2010) conducted a study to examine how three *eNOS[promoter -786T>C, intron 4 (4a4b) VNTR,* and *exon 7 Glu298Asp (894G>T)]* polymorphisms affect plasma nitrite concentration in 205 pregnant women [107 healthy pregnant (HP) and 98 preeclampsia (PE)]. The study showed that the *exon 7 Glu298Asp* polymorphism had no effects on the plasma nitrite concentrations. Higher nitrite levels were found in HP women with the *CC versus TT* genotype for the *promoter -786T>C* polymorphism. Lower nitrite levels were found in healthy women with the 4a4a versus 4b4b genotype for the *intron 4 VNTR* polymorphism. No effects of genotypes were found in PE women. The *"C Glu b"* haplotype was more frequent in the HP group than in the PE group. This haplotype was associated with higher nitrite concentrations than

the other haplotypes in healthy pregnancies. No differences in nitrite concentrations were found among PE women with different *eNOS* haplotypes. These findings indicate that *eNOS* polymorphisms affect endogenous NO formation in normal pregnancy, but not in PE, and that the "C Glu b" haplotype may protect against the development of PE by increasing endogenous NO formation[94].

In Italy, Rafaelli, et al. (2010) performed a study on a sample of thirty singleton pregnant women who experienced RPL, nine singleton pregnant women who presented with RPL, and 30 singleton healthy pregnant women matched for age, parity, and gestational age, to investigate the role played by platelet NO metabolism in patients with RPL compared with healthy pregnant women. The study reported that a modified NO pathway might play a key role in the physiological changes of advancing gestation but may also contribute to the pathophysiology of RPL. The study recommended that balancing NO metabolism might be useful in the treatment of RPL[11].

In Poland, Urban, *et al. (2007)* performed a studyto determine homocysteine and NO plasma concentrations in pregnancies complicated with intrauterine growth restriction (IUGR) on a total of 68 subjects. Non-fasting blood samples were collected from 36 patients with IUGR and 32 subjectss with normal pregnancy. Serum total homocysteine (tHcy) levels and NO concentrations were measured. The study revealed that serum homocysteine levels were higher in pregnancies complicated with IUGR, while serum total nitrite levels were lower in pregnancies complicated with IUGR. Both tHcy and NO are at the exponent of vessel endothelium function thus, simultaneous determination in IUGR is of great importance[95].

In Italy, Paradisi *et al. (2007)* performed a pilot study to evaluate the systemic production of NO in missed and threatened abortion and to define its role in the mechanisms regulating the first-trimester pregnancy evolution toward either positive continuation or negative termination on a sample of 4 groups categorized as the threatened abortion group (n=12), missed abortion group (n=14), pregnant control group (n=14), and non-pregnant control group (n=14). The study indicated that serum NO concentrations showed higher levels in the non pregnant versus the pregnant control

group. Serum NO levels in the missed abortion group were extremely significantly lower than both the non pregnant and the pregnant control groups. The threatened abortion group, too, presented NO levels frankly lower than the non pregnant control group. Furthermore, NO concentrations in the threatened abortion group were higher than in the missed abortion group. In conclusion, it is not yet clear whether the low levels of serum NO in patients with missed abortion is the result of altered immunologic activity within the peripheral circulation or the result of paracrine events in the uterus. However, the present findings support a functional role of the NO mediator in early embryonic development and confirms its importance in the uterus and cervix during abortion[96].

In Ukraine, Dosenko, et al. (2006) performed a study to investigate the mechanisms of phenotypic effect of allelic polymorphism of the *eNOSgene*. They identified the *promoter -786T>C, intron 4 (4a4b) VNTR,* and *exon 7 Glu298Asp (894G>T)* polymorphisms by reverse transcription-PCR of *eNOS* mRNA isolated from human platelets. They also measured *eNOS* enzyme activity by a fluorimetric assay. The study showed that the level of *eNOS* mRNA and activity of this enzyme in platelets depends on genotype. The level of *eNOS* mRNA is the lowest for the the CC genotype of *promoter -786T>C* polymorphism. In *exon 7 Glu298Asp (894G>T),* the level of RNA in the *homozygotes(894TT)*genotype was lower than its level of normal *homozygotes (894GG)*genotype, but higher than in *heterozygotes (894GT)*genotype. The *eNOS* activity in platelets was lower in carriers of *promoter (-786 CC)* than in normal *homozygotes (-786 TT)* or *heterozygotes (-786 CT).* The *eNOS* activity accompanying the *(894TT) variant ofexon 7* was also lower than in normal *homozygotes (894GG)*genotype. Regarding the polymorphism in *intron 4 (4a4b)* VNTR- the enzyme activity was lower in carriers of the (4a/4a) genotype as compared to normal homozygote's (4b/4b) and lower than in heterozygotes (4a/4b). These results allow one to conclude that the *promoter -786T>C* polymorphism of *eNOSgene* significantly affects the *gene* expression and *eNOS* activity[97].

In Torrance, California, Han, et al. (2005) performed a study to examine the influence of estrogen on the expression of *NOS* isoforms in human endometrial surface

epithelial cell line (HES) and primary endometrial cells. The expression of *NOS* isoform protein levels and mRNA were determined following estrogen/ P_4 stimulation. The study showed that Estradiol 17-β (E_2) induced a dose- and time-dependent increase in the expression of *eNOS* mRNA and protein and *iNOS* protein in HES cells which could be blocked by the estrogen receptor antagonist ICI 182,780. Estradiol also increased the expression of *eNOS* mRNA and protein in primary endometrial cells. Estrogen also induced phosphorylation of *eNOS* which could not be blocked by ICI 182,780. P_4 in physiologic concentrations augmented the effect of estrogen on the expression of both *eNOS* and phosphorylation of *eNOS(peNOS)* but not of *iNOS*. ICI 182,780 in high concentrations stimulated the expression of *iNOS* protein while inhibiting *eNOS*. In Conclusions: Estradiol through a genomic mechanism stimulates the expression of *NOS* isoforms in endometrial derived primary and HES cells. This effect is potentiated by P_4[98].

In Japan, Makino, et al. (2004) preformed a study on a sample of three groups to indicate whether 677C>T polymorphism of methylenetetrahydrofolate reductase *(MTHFR)*and *intron 4 (4a/4b) VNTR* polymorphism of *eNOSgene* are associated with recurrent pregnancy loss. As well as to determine whether the plasma levels of homocysteine and NO are associated with RPL. The first group consisted of 85 cases with a history of two or more unexplained first-trimester recurrent embryonal losses (before 10 weeks gestation). The second group consisted of 40 patients suffering fetal loss and the third group consisted 76 healthy women without obstetrical complications or any history of miscarriage. The study showed that the frequency of the MTHFR *gene* (T allele) was rather significantly lower than in controls whereas there was no difference in the frequency of the *eNOSgene(4a allele).* There were no differences in the plasma homocysteine levels among the three groups. However, the NO concentrations in the embryonal loss and fetal loss groups were significantly higher than that in controls. The authors concluded that the NO concentration but not *677C>T* polymorphism of *MTHFRgene*, *intron 4 VNTR*polymorphism of *eNOSgene*, or hyperhomocysteinemia is associated with RPL in Japanese[12].

In Finland, Väisänen-Tommiska et al. (2004) preformed a study on women with missed abortion (n = 56), blighted ovum (n = 36), or tubal pregnancy (n = 7); 140 women with amenorrhea-matched normal gestation were studied as controls, also cervical fluid samples were assessed for NO metabolites (NO_x) by means of Griess reaction. The study showed that, increased pre-abortal cervical NO release may contribute to cervical ripening and the onset of clinical pregnancy loss. The authors concluded that, spontaneous pregnancy loss is preceded by increasedrelease of cervical NO before the initiation of uterinecontractions. This may soften the cervix and facilitate theclinical onset and course of abortion[58].

In Sweden, Al-Hijji, et al. (2003) carried out a study to investigate the possible role of NO produced locally or intramurally in the quiescence of the pregnant myometrium. *NOS* activity was measured in samples from first trimester (villous, and non villous-trophoblast), term placenta and pregnant myometrium. *NOS* activity was measured in both cytosolic and particulate fractions by the formation of 14C-citrulline from 14C-arginine. Western immunoblotting was used to identify the *eNOS* and *nNOS* isoforms. The study showed that the activity of *NOS* in particulate fractions from all preparations was considerably higher than the cytosolic fractions. Activity in all fractions except the myometrium was highly Ca-dependent. More than 50% of particulate *NOS* from the myometrium was Ca-independent. *NOS* activity was highest in the villous trophoblast and there was a significant difference between the villous and non-villous trophoblast. In placenta and myometrium, *NOS* was 2–4 fold and 20–28-fold lower than the villous trophoblast, respectively. Western blot analysis showed clearly *eNOS* in the particulate fraction and a weak *eNOS* band in the cytosolic fractions, whereas *nNOS* was not detectable in any of the fractions. In view of the marginal activity of *NOS* in the myometrium, NO produced by the trophoblast and placenta could play a significant role in maintaining uterine quiescence by paracrine effect[49].

In Kuwait, Diejomaoh, et al. (2003) carried out a study to estimate the serum levels of nitrate and nitrite in women undergoing spontaneous preterm labor and induced labor. On a total of 39 patients before the onset of labor (control), 17 patients undergoing induction of labor who were in active labor (study group A), and 24 patients

in spontaneous preterm labor (study group B). Serum concentrations of nitrate and nitrite were estimated in the samples using HPLC. The study showed that there was no significant difference in the mean gestational age at delivery between the control and group-A patients; however, there was a significant difference between the control and group-B patients, and between study groups A and B. The mean serum levels of nitrite in groups A and B were significantly lower than the level in the control group. Although the serum nitrate levels in study groups A and B were lower than in the control group, this difference was not significant. They concluded that there is a drop in NO production in active preterm labor and induced labor[99].

In Korea, Choi et al. (2002) performed a study to investigate the changes in NO production during and after normal pregnancy and in pregnancies complicated by preeclampsia, They measured serum nitrates and nitrites (NOx) concentrations and serum iron markers in 347 subjects. The study showed that serum NOx concentrations were significantly higher in the first trimester than in non pregnant women. High NOx concentrations persisted throughout normal pregnancy, irrespective of serum ferritin concentrations, and returned to non pregnant levels by 9-12 wk postpartum. Mean NOx concentrations in preeclamptic women were significantly lower than those in the gestation age-matched normal pregnant women. In summary, NO production increases with advancing gestation during normal pregnancy and decreases in preeclampsia, regardless of serum ferritin concentrations, Elevated NOx concentrations during pregnancy return to normal within 12 weeks after delivery[100].

In UK, Ledingham, et al. (2000) conducted a study to investigate the expression, using Western blotting, and localization, using immunohistochemistry, of the *NOS* enzymes, *iNOS*, *eNOS* and *nNOS* in the human cervix during pregnancy and parturition. Cervical biopsies were obtained from non-pregnant women, women in the first trimester of pregnancy, and pregnant women at term before and after the onset of labor. The study showed that each of the *NOS* isoforms was localized in the cervices of both non-pregnant and pregnant subjects. *iNOS* expression was significantly greater in early pregnancy compared with the non pregnant state. *iNOS* expression was up-regulated further in samples obtained in the third trimester compared with the first trimester.

nNOS expression was greater in samples from the first trimester of pregnancy than in non-pregnant samples, but showed no additional increase in late pregnancy or with the onset of labor. *eNOS* expression was increased in samples obtained in the third trimester both before and after the onset of labor when compared with non-pregnant samples. The increased expression of *NOS* isoforms in late pregnancy supports the hypothesis that NO is involved in the process of cervical ripening[25].

In Wisconsin (USA), Khorram, et al. (1999) performed a study to examine theexpression of *NOS* protein by Western immunoblot analysis and immunehistochemistry in the endometriumand myometrium in a total of 19 premenopausal and 18 postmenopausalwomen undergoing hysterectomy for benign gynecological reasons. The study demonstrated that the predominant isoform of*NOS* in the uterus is the endothelial isoform *(eNOS),*also they observed that there is a unique menstrual cycle-dependent expressionof *eNOS* that was different in the endometrium compared to themyometrium and was independent of uterine pathology. In the endometrium,there was 62% higher expression of *eNOS* during thesecretory phase compared to the proliferative phase,whereas in the myometrium, there was 74% greater expression of *eNOS* in the proliferative phase compared to the secretoryphase. Within the secretory phase, maximal endometrial *eNOS* expressionwas found in the mid-portion, whereas in the myometrium,highest *eNOS* expression occurred during the late secretory phase. Inpostmenopausal women not treated with hormones, a significant reductionin endometrial and myometrial expression of *eNOS* occurred,which was reversed by continuous hormone replacement therapy, the results of the study showed that both endogenous ovarian steroids and exogenous sex hormones influence uterine *eNOS* expression. The study also showed that estrogen may regulate myometrial *eNOS,* whereas P_4 or a combination of estrogen and P_4 may be more important in regulating endometrial *eNOS,* and NO may be a critical mediator of sex steroid actions in the human uterus[101].

In USA Conrad, et al. (1999) conducted a cross-sectional study on non pregnant women (*n* =15), normal pregnant women in the first (*n* = 9), second (*n* = 17) and third (*n* = 22) trimesters, as well as women with preeclampsia (*n* =15) and transient

hypertension of pregnancy (*n* = 7), following that they performed a serial study on the same women (*n* = 9) before, during, and after pregnancy. To test the hypothesis that NO biosynthesis increases during normal human pregnancy and decreases in preeclampsia. The major metabolites of NO, nitrate and nitrite (NOx), and cGMP were measured in both the plasma and 24-h urine of women subjected to a reduced NOx diet. The results of the investigation showed marked increases in cGMP production especially during the first trimester when the maternal circulation is rapidly vasodilating. In contrast, whole body NO production as estimated by the plasma level and urinary excretion of NOx was not elevated during the first trimester. These findings suggest *1*) another signal besides NO mediates augmented cGMP production and maternal vasodilatation during pregnancy, or *2*) body fluid NOx is an unreliable estimate of hemodynamically relevant NO. In preeclampsia, unequivocal support for reduced NO production was not demonstrated[102].

In UK, Wilson, et al. (1997) performed a study to test the hypothesis that parallel production of Interleukin 12 *(IL12)* and NO might occur in recurrent miscarriage. Serum levels of NO and Interleukin 12 *IL12* were measured on a total sample of healthy non-pregnant women (n=31); healthy pregnant women (n=18); women suffering spontaneous abortion (n=10); pregnant women with a history of recurrent miscarriage (n=29, of these 13 later aborted and 16 continued successfully to term); and non-pregnant women with a history of recurrent miscarriage (n= 20). The study showed that normal pregnancy was associated with a significant decrease in serum levels of nitrite. In women admitted with spontaneous abortion there was a significant increase in the levels of nitrite, but no change in *IL12* compared to normal pregnant women. In pregnant women with a history of recurrent miscarriage, levels of nitrite and *IL12* were significantly elevated compared to normal pregnancy. When these women were sampled prior to becoming pregnant the levels of NO were found to be significantly lower than those in the non-pregnant control group although levels of *IL12* were unchanged. No correlation was found between serum nitrite and *IL12* levels. This report further supports the idea that polarisation of the immune response during pregnancy may predispose to recurrent miscarriage[103].

In Australia, Wang, et al. (1997) performed a study using a combination of maximum-likelihood-based statistical genetic methods to explore the contributions of *intron 4 (4a4b) VNTR* polymorphisms of *eNOSgene* and other unmeasured genes to basal NO production measured by its metabolites (NO_x: nitrite and nitrate) in a total of 428 members of 108 nuclear families. The study showed that approximately 30% of the variance in fasting NO_x levels is due to genes. The plasma NO_x levels in those homozygous for the rare allele were found to be significantly higher than those homozygous for the common allele. The results of the variance component linkage analysis were consistent with linkage of a quantitative trait locus in or near the *eNOSgene* to variation in plasma NO_x levels. While many environmental factors have been shown to alter transiently plasma NO_x levels, The study pointed to a major gene effect on plasma NO_x levels, i.e, NO production. The authors concluded that because the reported *intron 4 (4a4b)VNTR* polymorphism accounts for over 25% of the basal plasma NO production, therefore the gene may contribute significantly to mechanisms mediating atherogenesis and other conditions[7].

InCanada, Haddad, et al. (1995) performed a study to examine the association between local production of NO by decidual mononuclear cells of pregnant mice and pregnancy loss. The study suggested a role for NO as an effector molecule in mediating early pregnancy loss and showed that the *in situ* activation of decidual macrophages was an early event preceding spontaneous pregnancy loss[104].

In Switzerland, Delacrétaz, et al. (1995) performed a study to measure the activity of nitric oxide-forming enzyme in normotensive pregnant and non-pregnant women, as well as in women who had developed preeclampsia. Nitric oxide synthase activity was measured in the platelets of 21 normotensive pregnant women, 16 non-pregnant women and seven pregnant women who had developed pre-eclampsia. The study showed that *NOS* activity was significantly higher in normotensive pregnant women than in non-pregnant control subjects and in women with preeclampsia. These data suggest that NO synthesis is increased during normal pregnancy, possibly contributing to the vasodilatation associated with this condition. NO generation, however, may be

inappropriately low in pregnant women developing preeclampsia, thus leading to an enhanced vasoconstriction[105].

2.13.4. Nitric oxide synthase gene expression and activity studies in experimental animals

In Poland, Andronowska, et al. (2008) performed a study to examine: 1) endometrial concentrations of nitrate/nitrite (NOx) in pregnant pigs, and 2) the influence of estradiol-17β (E_2) and/or P_4 on NOxproduction by porcine endometrium during the first half of pregnancy. Total NOxconcentrations were determined using a microplate assay method based on the Griess reaction. Evident fluctuations of endometrial NO*x* content were found during the examined time of pregnancy (days 5, 10, 15, 20, 25, 30, 35, 40 and 60 of pregnancy). The NOxconcentration was highest on days 10 and 15, andthen declined until day 60 of pregnancy. The study also demonstrated the stimulatory effect of E_2 and/or P_4 on NO *in vitro* production by porcine endometrial slices. The medium content of NOxdepended on the steroid type, treatment dose and day of pregnancy. P4 enhanced endometrial NO*x* production on days 5 to 35 of pregnancy, E2 inhibited NO production via reducing *iNOS* expression only in the absence of P4. Also the combination of E_2 and P_4 was sometimes more effective in the stimulation of NO production than the application of individual hormones. The authors demonstrated that endometrial NOxconcentrations changed dramatically during the first 60 days of pregnancyin pigs. and the differences in the strength of the stimulatory action of E_2 and/or P_4 onendometrial NOxproduction are associated with activation of differentisoforms of *NOS*[106].

In Canada, Lo and Kaufman (2001) performed a study to determine the effect of P_4 metabolite 5α-pregnan-3α-ol-20-one (pregnan) on NO biosynthesis and plasma volume in rats. Since the plasma 5α-pregnan-3α-ol-20-one levels and NO biosynthesis increase during pregnancy. The study sample consisted of a Virgin female Long-Evans rats that were implanted with indwelling cannulas and maintained on a low nitrate/ nitrite diet. After the rats recovered from surgery, 500 µg of pregnan or vehicle were given daily for 2 days. NO biosynthesis and plasma volume were measured in conscious animals before

and after treatment. The study showed that pregnan caused a significant increase in NO biosynthesis compared with the vehicle-treated control group. Similarly, there was a significant increase in plasma volume in the pregnan-treated group compared with the vehicle-treated control group. These results confirm that the pregnan can mimic pregnancy by its ability to increase both NO biosynthesis and plasma volume[107].

In USA, Chwalisz, et al. (1999) performed a study on rats to evaluate whether NO plays a role during the preimplantation [days 1–4 post coitum (p.c.)] and peri-implantation (days 6–8 p.c.) phases of pregnancy. Rats were treated with the non-specific *NOS* inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), and the *iNOS* inhibitor aminoguanidine in the presence and absence of low-dose antiprogestin, onapristone. The study demonstrated synergistic effects of *NOS* inhibitors and an antiprogestin in preventing pregnancy. The authors concluded that NOS, particularly the cytokine- and P_4-inducible *iNOS,* may represent a new target for novel therapeutic agents capable of promoting or inhibiting pregnancy[108].

In Japan, Thanda, *et al. (1996)* performed a study toassess the importance of NO generated in the placenta on pregnancy where, *NOS* activities where measured in the rat placentas of different gestational ages. The study showed that *NOS* activity distributed both in the soluble and particulate fractions. Inhibition of *NOS* activity by L-arginine analogs confirmed the substrate specificity. The requirement of calcium/calmodulin for the maximal activity indicated that the rat placenta *NOS* was of a constitutive calcium/calmodulin dependent isoform. The activities in both fractions were higher in the earlier gestational age placentas, decreasing with progression of gestation, and the lowest in the term placentas. The authors concluded that detection of *NOS* activity in the placenta throughout gestation and its highest activity in the early gestational age placenta, suggested a possible significant role of NO in early gestation[109].

*In Germany, Yallampalli, et al. (*1996*)* performed a study on pregnant rats to determine whether inhibition of NO synthesis would affect the action of an antiprogesterone to provoke preterm labor. Pregnant rats were continuously infused with N^G-nitro-L-arginine methyl ester starting on day 16 of gestation. On day 17 of

gestation groups of animals were injected subcutaneously with a single dose of either 3 or 30 mg/kg onapristone (progesterone receptor antagonist); Animals were monitored for preterm labor and delivery for up to 48 hours. The study showed that: (1) Combined treatment with N^G-nitro-L-arginine methyl ester (50 mg per day) and low dose onapristone (3 mg/kg) produced preterm labor; >70% of the fetuses were delivered within 27 hours of treatment, whereas, <5% of the fetuses were delivered in the animals receiving either of these compounds alone. (2) N^G-nitro-D-arginine methyl ester (50 mg per day) had no effect. (3) Inhibition of NO by N^G-nitro-L-arginine methyl ester also significantly increased the efficacy of high-dose onapristone (30 mg/kg) in preterm labor and delivery, The authors concluded that the treatment of pregnant rats with a combination of a NO inhibitor with onapristone significantly potentiated the ability of the antiprogesterone to induce preterm labor. The authors proposed that a decrease in NO synthesis together with the fall in P_4 levels at term could lead to the initiation of labor. The study concluded that the interaction of NO and P_4 may be required to maintain pregnancy[18].

Chapter (3)
Materials and Methods

3.1. Materials

3.1.1. Chemicals

- Agarose Molecular Biology grade (Promega, USA).
- Quick-load 100 bp DNA ladder (New England BioLabs, UK).
- EDTA disodium salt (Promega, USA).
- Ethidium bromide (Promega, USA).
- Ethanol 70% (Sigma, USA).
- Absolute Isopropanol (Sigma, USA).
- Tris base (hydroxymethyl aminomethane) (Promega, USA).
- Glacial Acetic acid (Sigma, USA).
- DNAse, RNAse free Water (Promega, USA).

3.1.2. Reagent Kits

- Nitric Oxide Colorimetric Kit (BioVision, USA).
- Immulite®/ Immulite® 1000 Progesterone (IMMULITE, USA).
- Wizard Genomic DNA purification Kit (Promega, USA).
- PCR mastermix (Promega, USA).

3.1.3. PCR Primers

- Primers were purchased from New England BioLabs, UK**.**

1. ***For 4a4b in Intron 4 VNTR polymorphism***
   - The sequence of the primers were as defined by ***(Shin, et al. 2010).***
     Forward 5`-AGG CCC TAT GGT AGT GCC TTT-3`
     Reverse 5`-TCT CTT TAG TGC TGT GGT CAC-3`

2. ***For - 786T>C polymorphism***
   - The sequence of the primers were as defined by ***(Shin, et al. 2010).***
     Forward 5`-ATG CTC CCA CCA GGG CAT CA-3`
     Reverse 5`-GTC CTT GAA TCT GAC ATT AGG G-3`

3. For 894G>T (Glu298Asp) polymorphism

- The sequence of the primers were as defined by ***(Shin, et al. 2010).***

  Forward 5`-CAT GAG GCT CAG CCC CAG AAC-3`

  Reverse 5`-AGT CAA TCC CTT TGG TGC TCA C-3`

3.1.4. Enzymes

- ***MboI*** Restriction enzyme (New England BioLabs).
- ***NgoMIV*** Restriction enzyme (New England BioLabs).

3.1.5. Ethidium bromide(stock solution)

- Ethidium bromide 10 mg/ml in water.

3.1.6. Buffers

1) 10X NEBuffer 4

- Each 1X NEBuffer 4 contains:
  - 50 mM potassium acetate.
  - 20 mM Tris-acetate.
  - 10 mM Magnesium Acetate.
  - 1 mM Dithiothreitol**.**
  - pH 7.9 at 25°C.

2) 50x TAE Buffer

- Composition:
  - Tris base ……... 242 g.
  - glacial acetic acid ……. 57.1 ml.
  - 0.5M EDTA …… 100ml.
  - H_2O to …….. 1000 ml.
  - pH 8.5

3) DNA loading buffer

- Composition:
  - bromophenol blue 0.25 g.
  - xylene cyanol 0.25 g.
  - glycerine 30 ml.
  - H_2O 70 ml.

3.1.7. Instruments and Disposables

- The following instruments and disposables were used in the present study:
  - Thermal Cycler (Biometra).
  - Centrifuge.
  - L.G. Microwave Oven .
  - Gel documentation system.
  - Vortex Mixer.
  - Electrophoresis tank (horizontal apparatus)
  - Power Supply.
  - Micro Centrifuge.
  - Freezer, Refrigerator.
  - Immulite 1000 Analyzer.
  - Semi autochemistry analyzer (Rayto).
  - Electronic Balance.
  - Microfuge tubes for PCR - thin wall 0.2 mL capacity.
  - Microfuge tubes - 1.5 mL capacity.
  - EDTA and plain tubes.
  - Microcuvettes.
  - Automatic pipettes.
  - Disposable tips.

3.2. **Study population**

3.2.1. Study Design

- Case control study.
- Association study.

3.2.2. Characteristics of the study population

The study population consisted of four groups as presented in Table 3.1**.**

Group 1: 30 non-pregnant women, between 18 – 35 years old from Gaza strip who had at least three unexplained RPLs ≤20 weeks of gestation.

Group 2: 30 non-pregnant women, between 18 – 35 years old from Gaza strip with at least two live births and without a previous history of abortion.

Group 3: 15 pregnant women, between 18 – 35 years old from Gaza strip who had at least three unexplained RPLs ≤20 weeks of gestation.

Group 2: 15 pregnant women, between 18 – 35 years old from Gaza strip with at least two live births and without a previous history of abortion.

Table 3.1. **Characteristics of study groups.**

Group	RPL	Control
Pregnant	15	15
Non pregnant	30	30
Total	45	45

3.2.3. Ethical considerations

- An authorization to carry out the study was obtained from the local ethics committee using an agreement letter prepared from the Islamic University of Gaza.
- The objective of the study was explained to all participants and their consent was taken.

3.3. Methods

3.3.1. Blood collection

Eight milliliters of venous blood were collected from each overnight fasting subject into one EDTA and one Plain tube, under quality control and safety procedure. Blood in Plain tubes was used on the same day for serum preparation, For each subject included in the study the serum was separated into two tubes, the first tube was used for P_4 level determination while the serum in the second tube was used for NO level determination. EDTA tube was used for genomic DNA extraction. Serum was stored at -80ºC while extracted DNA was stored at -20ºC till analyses.

3.3.2. DNA Extraction

After numerical coding of the patient's samples, DNA was extracted from the whole blood samples by using Wizard Genomic DNA Purification Kit (Promega, USA) which contains:

1) Cell Lysis Solution.
2) Nuclei Lysis Solution.
3) RNase Solution.
4) Protein Precipitation Solution.
5) DNA Dehydration Solution.

3.3.3. Procedure of Extracting DNA from Blood

1) Three hundred µl of whole blood were added to 900 µl of cell lysis solution in a 1.5 ml microcentrifuge tube. The tube was inverted 5-6 times to mix and then incubated at room temperature (RT) for 10 minutes to lyse RBCs.
2) The tube was centrifuged at 13000 rpm for 20 seconds at RT, then the supernatant was removed and discarded without disturbing the white pellet. The tube was then vortexed vigorously for 10-15 seconds until the white blood cells (WBCs) were resuspended.
3) Three hundred µl of nuclei lysis solution were added to the tube containing the resuspended cells. The solution was pipetted 5-6 times to lyse the WBCs.
4) One and a half µl of RNase solution were added to the nuclear lysate and the tube was mixed and then incubated at 37^{o}C for 15 minutes, and then the tube was cooled to RT.
5) A hundred µl of protein precipitation solution were added to the nuclear lysate and then the tube was vortexed vigorously for 10-20 seconds. The tube was then centrifuged at 13000 rpm for 3 minutes at RT.
6) The supernatant were transferred to a 1.5 ml microcentrifuge tube containing 300 µl of RT Isopropanol. The tube was gently mixed by inversion until white thread-like strands were visible.
7) Tube was then centrifuged at 13000 rpm for 1 minute at RT. The DNA was then visible as small white pellet.

8) The supernatant was then decanted and 300 µl of RT 70% ethanol were added to the DNA. The tube was inverted several times to wash the DNA pellet. Then the tube was centrifuged at 13000 rpm for 1 minute.
9) The ethanol was aspirated, and the tube was left to dry for 10-15 minutes.
10) A hundred µl of DNA rehydration solution were added to the DNA and the tube was incubated at 4^{o}C for overnight to rehydrate the DNA.
11) The DNA solution was stored at -20^{o}C.

3.3.4. Detection and quantition of extracted DNA

3.3.4.1. Agarose gel electrophoresis

- The quality of the isolated DNA was determined by running 5 µl of each sample on ethidium bromide stained 1.0% agarose gel. The DNA sample was then visualized on a Gel documentation system.

3.3.5. Genotyping

3.3.5.1. Polymerase Chain Reaction (PCR) for amplification of the three *eNOS* gene polymorphisms

Polymerase chain reaction (PCR) was carried out in a total volume of 20 µl, the reaction componentswere as described in Table 3.2.

Table 3.2. **Polymerase chain reaction components for amplification of the three *eNOS* gene polymorphisms.**

Reagent	Volume (µl)	Final concentration
Forward primer	2	20 pmol
Reverse primer	2	20 pmol
Nuclease free water	4	–
PCR mastermix	10	1X
DNA	2	100 ng

Microfuge tubes were then placed in a thermocycler and PCR amplification was started according to the program provided in Table 3.3.

Table 3.3. **Thermocycler program for PCR amplification of the three *eNOS* gene polymorphisms.**

No. of cycles	Temperature (ºC)	Time
1	94	5 min
35	94	1 min
	58	45 sec
	72	45 sec
1	72	7 min

For *intron 4 VNTR 4a4b polymorphism,* PCR products were *electrophoresed* on 2.0% agarose gel and was visualized by ethidium bromide staining. The wild-type allele (allele 4b) was detected as a 420-bp band (five copies of a 27-bp repeat). The polymorphic allele (allele 4a) was detected as a 393-bp band (four copies of the same repeat). While *RFLP* of *promoter -786 T>C* and *exon 7 Glu298Asp (894G>T)* were carried out by mixing PCR product with 10X NEBuffer 4 and the restriction enonuclease; *MboI* for *exon 7 Glu298Asp (894G>T)* or *NgoMIV* for *promoter -786 T>C.* The quantities and volumes were as shown in Table 3.4.

Table 3.4. **The enzymatic digestion components of amplified *eNOS gene* for detection of *exon 7 Glu298Asp (894G>T)* and *promoter -786 T>CRFLPs*.**

Reagent	Volume (µl)	Final concentration
PCR product	17.5	–
10X NEBuffer 4	2.0	1X
Restriction enonuclease	0.5	

Microfuge tubes were then placed in a thermocycler at 37ºC for 16 hrs to allow the restriction endonuclease to digest the PCR product. Digested PCR product was *then electrophoresed* on 2.0% agarose gel and was visualized by ethidium bromide staining.

For *exon 7 Glu298Asp (894G>T),* the wild-type allele *(894G allele)* remained uncut upon *MboI* digestion and was detected as a 206-bp band, whereas the polymorphic allele *(894T allele)* was cut into two fragments detected as a 119- and a 87-bp bands. Therefore, wild-type homozygous individuals should generate a single 206-bp product,

heterozygous individuals should generate three fragments 206-, a 119- and a 87-bp bands, while mutant homozygous individuals should generate a two; 119- and a 87-bp fragments. For *promoter -786 T>C polymorphism*, The wild-type allele *(-786T allele)* remained uncut upon *NgoMIV* digestion and was detected as a 236-bp band, whereas the polymorphic allele *(-786C allele)* was cut into two fragments detected as a 203- and a 33-bp bands. Therefore, wild-type homozygous individuals should generate a single 236-bp product, heterozygous individuals should generate three fragments 236-, 203- and a 33-bp, while mutant homozygous individuals should generate a two; 203- and a 33-bp fragments.

3.3.6. Serum Nitric Oxide level Determination

❖ **Principle**

The total nitrate/nitrite concentration was measured in a two-step process. The first step is to convert nitrate to nitrite utilizing nitrate reductase. The second step involves addition of the Griess reagents which convert nitrite into a deep purple azo-compound. Measurement of the absorbance of the azo-chromophore accurately determines the total NO production.

❖ **Method**

- Seum NO level was determined by a colorimetric assay according to Griess reaction.

❖ **Components**

- The component of the kit are as shown in Table 3.5.

Table 3.5. **Components of biovision Nitric Oxide colorimetric assay kit.**

Component	Volume
Assay Buffer	30 ml
Enzyme cofactor	Lyophilized
Enhancer	Lyophilized
Nitrate Reductase	Lyophilized
Nitrate Standard	Lyophilized
Griess Reagent R1	10 ml
Griess Reagent R2	10 ml

❖ **Reconstitution of Reagents**

- **Enzyme Cofactor:** the enzyme cofactor was reconstituted with 1.1 ml of Assay Buffer. The mixture was aliquoted and stored at -20°C.
- **Enhancer:** the enhancer was reconstituted with 1.1 ml distilled water and stored at 4°C.
- **Nitrate Reductase:** the Nitrate Reductase was reconstituted to 1.1 ml with Assay Buffer, aliquoted and stored at -20°C.
- **Nitrate Standard:** the Nitrate Standard was reconstituted with 100µl of Assay Buffer to generate 100 mM standard. The mixture was stored at 4°C when not in use.

❖ **Standard Curve Preparation**

1) Five µl of the 100 mM reconstituted Nitrate standard was mixed with 495 µl of Assay Buffer to generate 1 mM standard working solution.
2) Zero, 2, 4, 6, 8, 10 µl of standard working solution were added into a series of Microcuvettes. The volume was adjusted to 85 µl with Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/Microcuvette of Nitrate Standard as shown in Tables 3.6A, 3.6B, and 3.6C.

Table 3.6A. **Components of standard curve preparation for serum NO determination.**

Microcuvette	Nitrate Standard (µl)	Assay Buffer (µl)
A1	0	85
B1	2	83
C1	4	81
D1	6	79
E1	8	77
F1	10	75

3) To each of the standard working solution contained in Microcuvettes, the next steps were carried out by mixing the components shown in Table 3.6B.

Table 3.6B. **Components and volumes of standard curve preparation for serum NO test.**

Reagent	Volume (µl)
Nitrate Reductase	5
Enzyme cofactor	5

4) Microcuvettes were covered and incubated at RT for 1hr to convert nitrate to nitrite. The next reactions were performed by mixing the components shown in Table 3.6C.

Table 3.6C. **Components of standard curve preparation for serum NO test.**

Component	Volume (µl)
Enhancer	5
• Incubated at RT for10 minutes	
Griess Reagent (R1)	50
Griess Reagent (R2)	50

5) the color was allowed to develop for 10 minutes at RT.
6) The absorbance was read within 1 hour at wavelength 546 nm by using a spectrophotometer.

❖ **Assay procedure**

- The reagents and samples were brought to RT**.**
- Enzyme Cofactor and Nitrate Reductase were Kept on ice during use.

a) Sample blank assay procedure

1) The sample blank was prepared by mixing the components shown in Table 3.7.

Table 3.7. **Components and volumes of sample blanks for NO test.**

Component	Volume (µl)
Sample	85
Assay Buffer	115

2) The sample blank absorbance was read by a spectrophotometer at wavelength 546 nm.

b) Unknown Samples assay procedure

1) The samples were prepared by mixing the components shown in Tables 3.8A and 3.8B in a reaction microcuvette.

Table 3.8A. **Components and volumes for serum NO test.**

Component	Volume (µl)
Sample	85
Assay Buffer	115
Nitrate Reductase	5
Enzyme cofactor	5

2) The Microcuvettes were covered and incubated at room temperature for 1hr to convert nitrate to nitrite.
3) The next steps were carried out by mixing the components shown in table 3.8B.

Table 3.8B. **Components and volumes for serum NO test.**

Component	Volume (µl)
Enhancer	5
• Incubated at RT for10 minutes	
Griess Reagent (R1)	50
Griess Reagent (R2)	50

4) The color was allowed to develop for 10 minutes at RT.
5) The absorbance was read within 1 hour by a spectrophotometer at wavelength 546 nm.

❖ **Calculation**

$$Nitrate/nitrite\ concentartion\ (\mu M) = \left[\frac{sampleAbs. - blankAbs.}{Slope\ of\ std\ curve \times \mu l\ of\ sample}\right] \times 1000$$

- The serum NO reference level according to Biovision Nitric Oxide colorimetric assay kit is ~20µM for nitrate and ~2µM for nitrite.

3.3.7. Serum Progesterone Level Determination

❖ Serum progesterone level was carried out by the Immulite 1000 analyzer.
❖ **Principle:** sequential competitive immunoassay.
❖ The pregnant serum progesterone reference level according to the Immulite®/ Immulite® 1000 Progesterone assay kit are:
  - **First trimester:** 9.3 – 33.2 ng/ml.
  - **Second trimester:** 29.5 – 50.0 ng/ml.
  - **Third trimester:** 83.1 – 160 ng/ml.

3.3.8. Statistical analysis

Genetic power calculation has been determined to estimate the representative sample size for each of the three polymorphisms included in the current study, they summarized as shown in in table 3.9.

Table 3.9. **Genetic power calculation of eNOS gene polymymorphis.**

Polymorphism	Promoter -786 T>C	Exon 7 (894 G>T)	Intron 4 (4a4b) VNTR
Frequency of risk allele	0.44	0.20	0.25
Odds ratio (OD)	1.36	1.39	1.37
N cases for 80% power	398	588	575

Statistical analysis were carried out using Chi (X^2) square test and independent samples t-test test of the *Statistical Package for Social Sciences (SPSS) version 13* for Windows.

Chi (X^2) square test was used to assess the frequencies of genotypes and alleles. While independent samples t-test was used to compare the difference in the mean levels of NO. Odds Ratio (OR) and odds ratio (95% CI) were analyzed by Fisher's exact test using the *StatsDirect softwareVersion 2.7.2* to measure the strength of association between *eNOS* genotypes, NO and RPL. The results were presented through histograms, tables and charts. For normally distributed data, means and standard deviations were calculated. The Hardy-Weinberg equilibrium (HWE) was used to calculate estimated genotype frequency and experienced genotype frequency. *P-value* less than 0.05 was considered statistically significant. Pearson's correlation was used to analyze the relation between NO and Progesterone in the study population.

Chapter (4)

Results

4.1. PCR Genotyping results

The following figures are representative examples of the *eNOS* gene polymorphisms investigated in the study.

Figures 4.1, 4.2 (a and b), and 4.3 represent the PCR results for the genotyping of *promoter -786T>C, exon 7 (894 G>T)* and *intron 4 (4a4b) VNTR* polymorphisms, respectively.

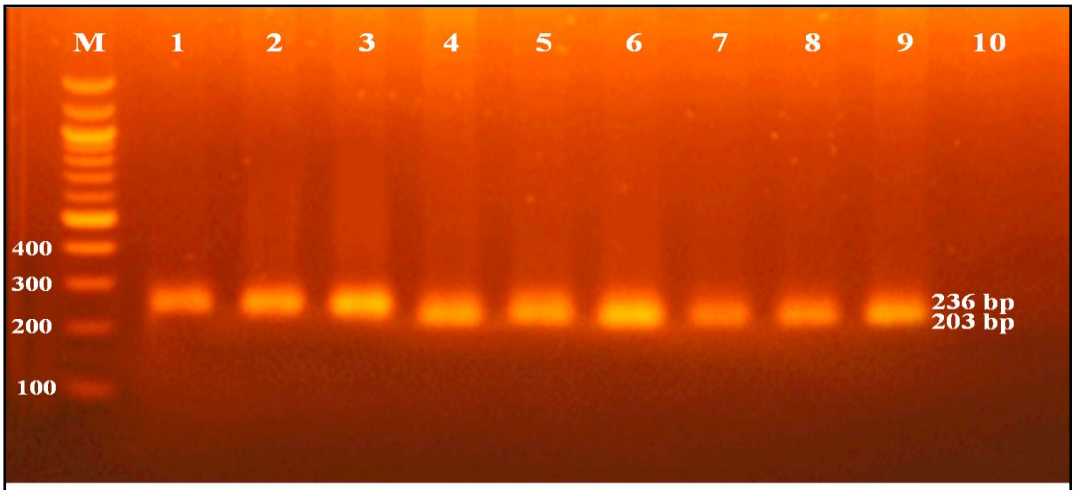


Figure 4.1. A photograph of ethidium bromide stained 2% agarose gel showing the RFLP-PCR product for the promoter -786 T>C polymorphism. M= 100 bp DNA ladder, lanes 1,2 and 3 indicate homozygous sample for the wild type allele (TT); lanes 4, 5, 6 and 9 indicate heterozygous samples (CT); lanes 7 and 8 indicate homozygous samples for the mutant allele (CC); lane 10 is a negative control.

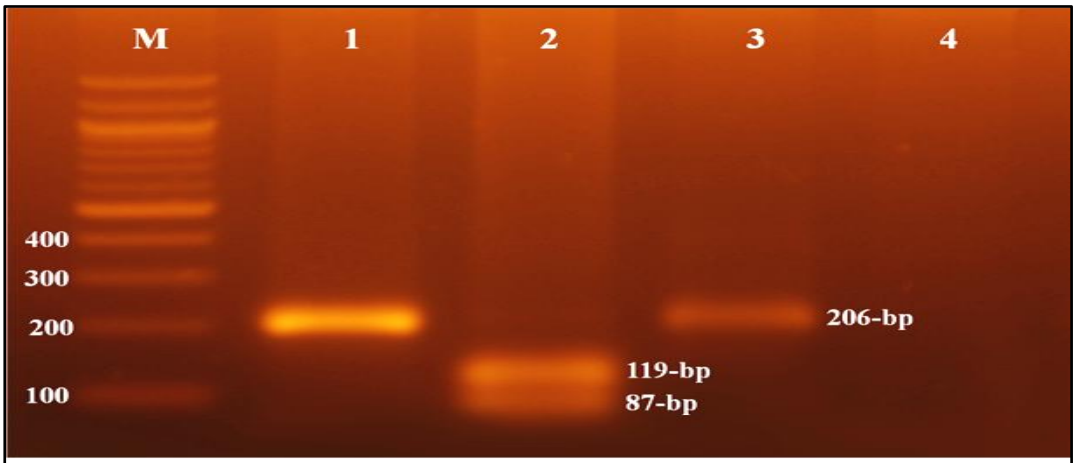


Figure 4.2a. A photograph of ethidium bromide stained 2% agarose gel showing the RFLP-PCR product for exon 7 (894G>T) polymorphism. M= 100 bp DNA ladder, lane 1 and 3 indicates homozygous samples for the wild type allele (GG); lanes 2 indicates homozygous samples for the mutant allele (TT); lane 4 is a negative control.

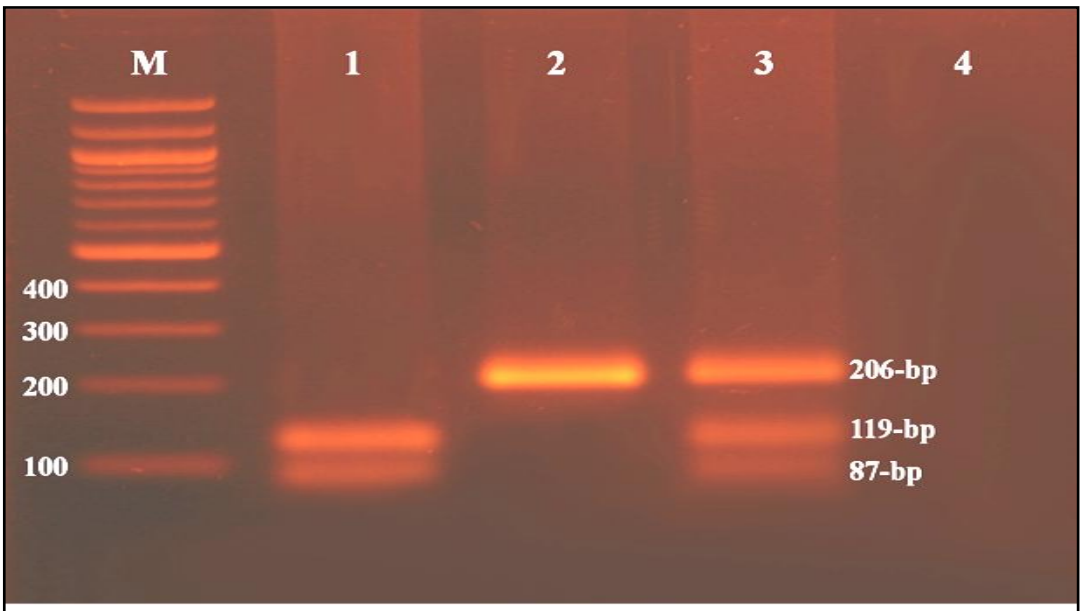


Figure 4.2b. A photograph of ethidium bromide stained 2% agarose gel showing the RFLP-PCR product for exon 7 (894G>T) polymorphism. M= 100 bp DNA ladder, lane 1 represents homozygous sample for the mutant allele (TT); lanes 2 indicates homozygous sample for the wild type allele (GG); lane 3 represents heterozygous samples (GT); lane 4 is a negative control.

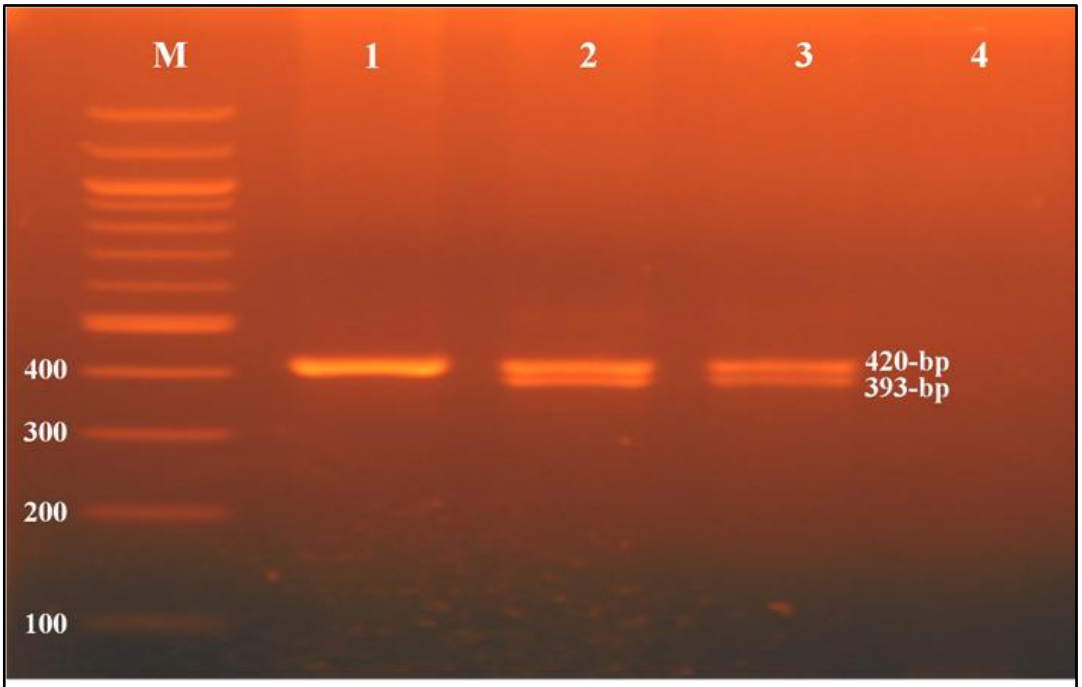


Figure 4.3. A photograph of ethidium bromide stained 2% agarose gel showing the VNTR product for intron 4 (4a4b) polymorphisms. M= 100 bp DNA ladder, lanes 1 indicates homozygous samples for the wild type allele (4b4b); lanes 2 and 3 indicates heterozygous samples (4a4b); lane 4 is a negative control.

4.2. The *eNOS* gene *promoter -786T>C* polymorphism

4.2.1. Frequency of the *promoter -786T>C* polymorphism among RPL patients and control subjects

Table 4.1. illustrates the frequencies of the *eNOS* gene *promoter -786T>C* polymorphism among RPL patients and control subjects. The frequency of thepolymorphic*C*allelecarrier which represented by*(CC + CT)* genotypeswas 46.7% in RPL patients and 13.3% in controls, while the frequency of wild-type*TT* genotype was 53.3% in RPL patients and 86.7% in controls. The statistical analysis of frequency of the *promoter -786T>C* polymorphism among the RPL patients and controls by Chi (X^2)square test showed that a statistical significance was evident between the two groups (P-value <0.001). Fisher's exact test was used to assess the odds ratio (95% CI) and indicated a significant difference between the frequency of *(CC + CT)* genotypesand the frequency of the wild-type *TT* genotype (P-value <0.001), Odds Ratio (95% CI) for *(CC + CT)* genotypes= 5.57 (1.8 -19.4).

Table 4.1. **Frequency of the *eNOS* gene *promoter -786T>C* polymorphism among RPL patients and control subjects.**

Polymorphism	Frequency		X^2 Test	Fisher's exact test	
	RPL	Control	P-value	Odds Ratio (95% CI)	P-value
TT	24 (53.3%)	39 (86.7%)	<0.001	**1.00 (wild-type)**	
CC + CT	21 (46.7%)	6 (13.3%)		5.57 (1.8 – 19.4)	<0.001
Total	**45**	**45**			

4.2.2. Allele frequencies of the *eNOS* gene *promoter -786T>C* polymorphism among RPL patients and control subjects

Table 4.2. illustrates the allele frequencies of the *eNOS* gene *promoter -786T>C* polymorphism among RPL patients and controls. The frequency of the polymorphic *C*allele was 26.7% in RPL patients and 6.7% in controls, while the frequency of the wild-type *T* allele was 73.3% in RPL patients and 93.3% in controls. The statistical

analysis of allele frequencies of the *promoter -786T>C* polymorphism among the RPL patients and controls by Chi (X^2)square test showed that a statistical significance was evident between the two groups (P-value <0.001). Fisher's exact test indicated a significant difference between the frequency of polymorphic *C* allele and the frequency of the wild-type *T* allele (P-value < 0.001), Odds Ratio (95% CI) for *C* allele= 5.09 (1.87 - 15.99).

Table 4.2. **Allele frequencies of the *eNOS* gene *promoter -786T>C* polymorphism among RPL patients and control subjects.**

Polymorphism	Frequency		X^2 Test	Fisher's exact test	
	RPL	Control	P-value	Odds Ratio (95% CI)	P-value
T allele	66 (73.3%)	84 (93.3%)	< 0.001	**1.00 (wild-type)**	
C allele	24 (26.7%)	6 (6.7%)		5.09 (1.87 - 15.99)	<0.001
Total	**90**	**90**			

4.2.3. Difference in the mean level of nitric oxide with respect to the *promoter -786T>C* polymorphism

Table 4.3. illustrates the difference in the mean NO levels with respect to the *promoter -786T>C* polymorphism in the study population. The mean NO level in women who had *(CC + CT)* genotypes (*C* allele carriers)was 9.22 ± 1.62 µM while, the mean NO level of women who had the wild-type*TT* genotype was 19.30 ± 6.39 µM. Statistical analysis by independent samples t-test showed that there is a significant difference between the two means of NO level and the *promoter -786T>C* polymorphism (P-value < 0.001).

Table 4.3. **Difference in the mean level of nitric oxide with respect to the *promoter -786T>C* polymorphism in the study population.**

Polymorphism	Mean ± SD (µM)	p-value
TT	19.30 ± 6.39	<0.001
CC + CT	9.22 ± 1.62	

4.3. The *exon 7 (894 G>T)* polymorphism

4.3.1. Frequency of the *exon 7 (894 G>T)* polymorphism among RPL patients and control subjects

Table 4.4. illustrates the frequencies of the *eNOS* gene *exon 7 (894 G>T)* polymorphism among RPL patients and control subjects. The frequency of polymorphic *T* allele carrier which represented by*(TT + GT)* genotypeswas 42.2% in RPL patients and 51.1% in controls, while the frequency of wild-type *GG* genotype was 57.8% in RPL patients and 48.9% in controls. The statistical analysis of frequency of the *exon 7 (894 G>T)* polymorphism among the RPL patients and controls by Chi (X^2)square test showed that there is no statistically significant difference between the two groups (P-value= 0.398). Fisher's exact test was used to assess the odds ratio (95% CI) and indicated that there is no statistically significant difference between the frequency of *(TT + GT)* genotypesand the frequency of the of wild-type *GG* genotype (P-value= 0.526), Odds Ratio (95% CI) for *(TT + GT)* genotypes= 0.699 (0.28 - 1.74).

Table 4.4. **Frequency of the *eNOS* gene *exon 7 (894 G>T)* polymorphism among RPL patients and control subjects.**

Polymorphism	Frequency		X^2 Test	Fisher's exact test	
	RPL	Control	P-value	Odds Ratio (95% CI)	P-value
GG	26 (57.8%)	22 (48.9%)	0.398	**1.00 (wild-type)**	
TT + GT	19 (42.2%)	23 (51.1%)		0.699 (0.28 – 1.74)	0.526
Total	**45**	**45**			

4.3.2. Allele frequencies of the *eNOS* gene *exon 7 (894 G>T)* polymorphism among RPL patients and control subjects

Table 4.5. illustrates the allele frequencies of the *eNOS* gene *exon 7 (894 G>T)* polymorphism among RPL patients and control subjects. The frequency of the polymorphic *T* allele was 24.4% in RPL patients and 30.0% in controls. While the frequency of the wild-type*G* allelewas 75.6% in RPL patients and 70.0% in controls.

The statistical analysis of allele frequencies of the *exon 7 (894 G>T)* polymorphism among the RPL patients and controls by Chi (X^2)square test showed that there is no statistically significant difference between the two groups (P-value= 0.402). Fisher's exact test indicated that there is no statistically significant difference between the frequency of polymorphic *T* allele and the frequency of the wild-type *G* allele (P-value= 0.503), Odds Ratio (95% CI) for *T* allele= 0.754 (0.37- 1.54).

Table 4.5. **Allele frequencies of the *eNOS* gene *exon 7 (894 G>T)* polymorphism among RPL patient and control subjects.**

Polymorphism	Frequency		X^2 Test	Fisher's exact test	
	RPL	Control	P-value	Odds Ratio (95% CI)	P-value
G allele	68 (75.6%)	63 (70.0%)	0.402	**1.00 (wild-type)**	
T allele	22 (24.4%)	27 (30.0%)		0.754 (0.37 – 1.54)	0.503
Total	**90**	**90**			

4.3.3. Difference in the mean levels of nitric oxide with respect to *exon 7 (894 G>T)* polymorphism in the study population

Table 4.6. illustrates the difference in the mean NO levels with respect to *exon 7 (894 G>T)* polymorphism in the study population. The mean NO level in women who had *(TT + GT)* genotypes (*T* allele carriers) was 14.94 ± 5.49µM while, the mean NO level of women who had the wild-type *GG* genotype was 17.44 ± 8.17 µM. Statistical analysis by independent samples t-test showed that there is no significant difference between the two means of NO level and the *exon 7 (894 G>T)* polymorphism (P-value= 0.096).

Table 4.6. **Difference in the mean nitric oxide level with respect to *exon 7 (894 G>T)* polymorphism in the study population.**

Polymorphism	Mean ± SD (µM)	p-value
GG	17.44 ± 8.17	0.096
TT + GT	14.94 ± 5.49	

4.4. *The intron 4 (4a4b) VNTR* polymorphism

4.4.1. Frequency of the *intron 4 (4a4b) VNTR* polymorphism among RPL patients and control subjects

Table 4.7. illustrates the frequencies of the *eNOS* gene *intron 4 (4a4b) VNTR* polymorphism among RPL patients and controls. The frequency of the wild-type *4b4b* genotype was 95.56% in RPL patients and 100% in controls. While, the frequency of *4a4b* genotype was 4.44% in RPL patients group but was not encountered in the control subjects. The *4a4a* genotype, however, was not found in either group. The statistical analysis of frequency of *intron 4 (4a4b) VNTR* polymorphism among the RPL patients and controls by Chi (X^2)square test showed that a statistical significance is not evident between the two groups (P-value= 0.153). Fisher's exact test was used to assess the odds ratio (95% CI) and indicated that there is no statistically significant difference between the frequency of heterozygous *4a4b* genotype and the frequency of the wild-type *4b4b* genotype (P-value= 0.49), Odds Ratio (95% CI) for *4a4b* genotype= ∞ (0.19 - ∞).

Table 4.7. **Frequency of the *intron 4 (4a4b) VNTR* polymorphism among RPL patient and control subjects.**

Polymorphism	Frequency		X^2 Test	Fisher's exact test	
	RPL	Control	P-value	Odds Ratio (95% CI)	P-value
4b4b	43 (95.56%)	45 (100%)	0.153	**1.00 (wild-type)**	
4a4b	2 (4.44%)	0 (0.0%)		∞ (0.19 - ∞)	0.49
Total	**45**	**45**			

4.4.2. Allele frequencies of the *eNOS* gene *intron 4 (4a4b) VNTR* polymorphism among RPL patients and control subjects

Table 4.8. illustrates the allele frequencies of the *eNOS* gene *intron 4 (4a4b) VNTR* polymorphism among RPL patients and control subjects. The frequency of the wild-type *4b* allele was 97.8% in RPL patients and 100.0% in controls. While, the frequency

of *4a* allele was 2.2% in RPL patient but was not present in the control subjects. The statistical analysis of allele frequencies of the *eNOS* gene *intron 4 (4a4b) VNTR* polymorphism among the RPL patients and controls by Chi (X^2)square test showed that there is no statistically significant difference between the two groups (P-value= 0.155). Fisher's exact test indicated that there is no statistically significant difference between the frequency of polymorphic *4a* allele and the frequency of the wild-type *4b* allele (P-value= 0.50), Odds Ratio (95% CI) for *4a* allele= ∞ (0.19 - ∞).

Table 4.8. **Allele frequencies of the *eNOS* gene *intron 4 (4a4b) VNTR* polymorphism among RPL patients and control subjects**.

Polymorphism	Frequency		X^2 Test	Fisher's exact test	
	RPL	Control	P-value	Odds Ratio (95% CI)	P-value
4b allele	88 (97.8%)	90 (100.0%)	0.155	**1.00 (wild-type)**	
4a allele	2 (2.2%)	0 (0.0%)		∞ (0.19 - ∞)	0.50
Total	**90**	**90**			

4.4.3. Difference in the mean levels of nitric oxide with respect to *intron 4 (4a4b) VNTR* polymorphism

Table 4.9. illustrates the mean NO levels and *intron 4 (4a4b) VNTR* polymorphism. The mean NO level of the women who had *4b4b* genotypes was 16.21 ± 0.75 µM. The *4a4b* genotype was encountered in only two RPL patients, one in the non-pregnant RPL patients group and the other in the pregnant RPL patients group, Their NO levels were 30.41and 8.85 µM, respectively and their mean NO level was 19.22 ± 11.20.

Table 4.9. **The mean levels of NO with respect to *intron 4 (4a4b)* VNTR polymorphism.**

Polymorphism	N	Mean ± SD
4b4b	88	16.21 ± 0.75
4a4b	2	19.22 ± 11.20

4.5. Serum Nitric Oxide and Progesterone Levels

4.5.1. Difference in the mean levels of nitric oxide between RPL patients and controls

Table 4.10. illustrates the difference in the mean levels of NO between RPL patients and controls. The mean NO level of RPL women was 14.17 ± 6.95 µM. whereas, the mean NO level of control women was 18.40 ± 6.73 µM. Statistical analysis by independent sample t-test showed that a significant difference was evident between the two groups (P-value= 0.004).

Table 4.10. **Difference in mean levels of nitric oxide between RPL and control groups.**

Group		Mean ± SD (µM)	P-value
RPL (N= 45)	pregnant = 15 / non-pregnant = 30	14.17 ± 6.95	0.004
Control (N= 45)	pregnant = 15 / non-pregnant = 30	18.40 ± 6.73	

4.5.2. Difference in the mean level of nitric oxide between non-pregnant RPL patient and non-pregnant control women

Table 4.11. illustrates the difference in the mean level of NO between non-pregnant RPL patients and non-pregnant control women. The mean NO level of non-pregnant RPL patient women was 11.54 ± 4.08 µM. while, the mean NO level of non-pregnant control women was 15.52 ± 4.47 µM. Statistical analysis by independent sample t-test indicated that a significant difference was evident between the two groups (P-value <0.001).

Table 4.11. **Difference in the mean level of nitric oxide between non-pregnant RPL patient and non-pregnant control women.**

Group	N	Mean ± SD (µM)	P-value
non-pregnant RPL	30	11.54 ± 4.08	< 0.001
non-pregnant Control	30	15.52 ± 4.47	

4.5.3. Difference in the mean level of nitric oxide between pregnant RPL patient and pregnant control women

Table 4.12. illustrates the difference in the mean level of NO between pregnant RPL patient and pregnant control women. The mean NO level of pregnant RPL women was 19.42 ± 8.56 µM. While, the mean NO level of pregnant control women was 24.13 ± 6.94 µM. Statistical analysis by independent sample t-test showed the absence of significant difference between the two groups (P-value= 0.11).

Table 4.12. **Difference in the mean level of nitric oxide between pregnant RPL patient and pregnant control women.**

Group	N	Mean ± SD (µM)	P-value
Pregnant RPL	15	19.42 ± 8.56	0.11
Pregnant Control	15	24.13 ± 6.94	

4.5.4. Difference in the mean level of nitric oxide between non-pregnant control and pregnant control women

Table 4.13. illustrates the difference in the mean level of NO between non-pregnant control and pregnant controlwomen. The mean NO level of non-pregnant control women was 15.52 ± 4.47 µM. While, the mean NO level of pregnant control women was 24.13 ± 6.94 µM. Statistical analysis by independent sample t-test indicated that a significant difference was evident between the two groups (P-value <0.001).

Table 4.13. **Difference in the mean level of nitric oxide between non-pregnant control and pregnant control women.**

Group	N	Mean ± SD (µM)	P-value
Non-pregnant Control	30	15.52 ± 4.47	<0.001
Pregnant Control	15	24.13 ± 6.94	

4.5.5. Correlation between serum levels of nitric oxide and progesterone in the study population

Table 4.14 and Figure 4.4 illustrate the correlation between serum levels of NO and P_4 in the study population. Correlation analysis showed a significant correlation between serum NO and P_4 levels in the study population (P-value= 0.002).

Table 4.14. **Correlation between serum levels of nitric oxide and progesterone in the study population.**

Mean level of serum		N	Correlation analysis	
NO (µM)	Progesterone (ng/ml)		Correlation coefficient	P-value
16.28	10.34	90	0.319	0.002

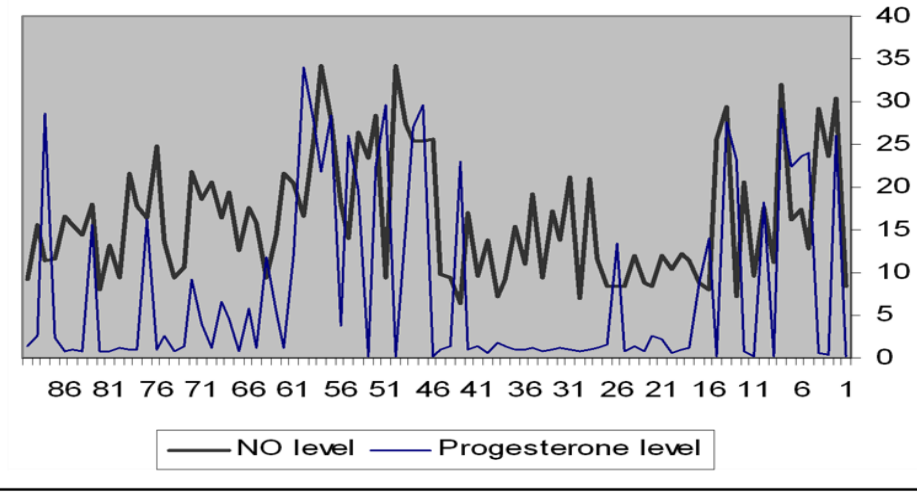


Figure 4.4. **Correlation between serum levels of nitric oxide and progesterone in the study population.**

4.6.Difference in the mean levels of progesterone level with respect to *promoter -786T>C* polymorphism in the study population

Table 4.15. illustrates the difference in the mean P_4 levels with respect to the *promoter -786T>C* polymorphism in the study population. The mean P_4 level in women

who had *(CC + CT)* genotypes (*C* allele carriers) was 6.56 ± 9.31 ng/ml while, the mean P_4 level of women who had the wild-type *TT* genotype was 8.58 ± 10.77 ng/ml. Statistical analysis by independent samples t-test showed that there is no significant difference between the two means of P_4 level and the *promoter -786T>C* polymorphism (P-value= 0.401).

Table 4.15. **Difference in the mean level of progesterone with respect to the *promoter -786T>C* polymorphism in the study population.**

Polymorphism	Mean ± SD (ng/ml)	p-value
TT	8.58 ± 10.77	0.401
CC + CT	6.56 ± 9.31	

Chapter (5)
Discussion

Recurrent pregnancy loss (RPL) is an important clinical and stressful problem that has been studied tremendously but the causes and treatment have not been fully resolved[12]. RPL affects about 1-5% of women who conceive[64] and accounts for about 20% of clinically recognized pregnancy losses[1]. Despite extensive researches to explain the causative effects of RPL, about 50%-60% of RPLs are still idiopathic. Endothelial damage, impaired placental vascularization and resultant oxidative stress have been proposed to play a role in the pathophysiology of RPL. *eNOS* has been regarded as the source of endothelial NO, which has a critical role in vascular physiology and impaired placental vascularization[72].

A normal pregnancy is dependent on adequate placental circulation and fetal vasculature. The development of a normal functioning vascular network requires complicated cooperation between different cell types and various growth factors in the processes of implantation, embryo development and placentation[64]. In case of normal pregnancy, the NO pathway is activated and leads to increased NO availability and level which is further responsible for maternal vasodilation required to accommodate the increase in circulating volume during pregnancy without a rise in blood pressure[73, 76, 89]. Contrary to PE in which very low NO level production exclude vasodilatation[76].

Nitric oxide (NO) has come to prominence in recent years as a major mediator of numerous biological processes, including vascular, immune, and reproductive functions. During pregnancy, NO is involved in three crucial physiological adaptations of mammalian gestation: vasodilatation of the maternal systemic circulation, increased uterine and feto-placental blood flow, and quiescence of the uterus before parturition. In addition, recent experimental evidence in rodents has suggested that NO may play a role very early in pregnancy contributing not only to maternal vasodilatation but also to immune suppression and acting as a regulator of embryonic development. As recently reviewed, however, while the biosynthesis of NO increases in gravid rats and sheep, the status of NO biosynthesis during normal pregnancy in women is controversial. In the study by *Seligman et al.(1994)* serum concentrations of NO were slightly, but significantly, increased compared with non-pregnant women. In contrast, other

investigators failed to observe increases in circulating levels of NO during human pregnancy[110].

It has been found that reduced NO production can lead to impaired placental perfusion and compromised oxygen and nutrient supply to the fetus[1, 2, 73] which might affect the ability of the embryo to resist maternal rejection early in pregnancy[15, 73].

The clinical utility of NO has been challenged by interference of other confounding factors such as bacterial nitrate synthesis in the bowels, denitrifying liver enzymes, saliva formation, environmental nitrogen oxides, diet, sex differences, ethnicity, clinical conditions, medications, and smoking, On the other hand, measuring nitrite concentrations in plasma has consistently been shown to reflect NO synthase activity. Moreover, either activation or inhibition of NO synthase activity was associated with corresponding increases or decreases in circulating nitrite concentrations. Approximately 70% of plasma nitrite has been shown to be derived from NO synthase activity in the endothelium[111].

In humans, abnormal NO levels as well as the polymorphic variants have been shown to play a role in preeclampsia[1, 2, 23, 73], and vascular disorders in women[23]. Abnormalities of placental vasculature may result in several gestational complications, including pregnancy loss, IUFD, IUGR and preeclampsia[64].

In recent years much attention was paid to determine the association between *eNOS* gene *[promoter -786 T>C, exon 7 Glu298Asp (894 G>T)* and *intron 4 (4a4b) VNTR)]* polymorphisms and RPL. However, the results of these studies have been controversial among different ethnic groups[2]. Moreover, the present study, is the first to evaluate these three commonly studied *eNOS* gene polymorphisms, and serum NO and P_4 levels in RPL Palestinian women residing in Gaza strip. In which we investigated 45 women with RPL as compared to 45 normal females.

5.1. Genetic power calculation and sample size

According to genetic power calculation, the estimated number of RPL subjects to detect the association between*Promoter -786 T>C, Exon 7 Glu298Asp (894 G>T),* and

Intron 4 (4a4b) VNTR polymorphisms of *eNOS* gene and RPL were 398, 588, 575, respectively. However, our population is a small and has a high frequency of consanguineous marriage. Thus, we expect that our population gene pool is homogenous, we also expect that the alleles frequency are low. Therefore, the small sample size recruited in the current study reflect the genotype and allele frequency. Moreover, we found some difficulties in sample collection, we also met limitations and difficulties in obtaining material and kits due to the siege imposed on Gaza, in addition to their arrival delay, their high cost, and the lower financial possibilities, made it impossible to increase the sample size. Thus, the study was carried out on 90 subjects (45 RPL and 45 controls).

5.2. Association between *eNOS* gene polymorphisms and RPL

Our results showed that the genotype and the allele frequencies of *promoter -786 T>C* were significantly different between RPL patients and the controls (all P-values were <0.001). On the other hand, neither genotype nor allele frequencies of *exon 7 Glu298Asp (894 G>T)* and *intron 4 (4a4b) VNTR* were significantly different between RPL patients and the controls (P-values for genotype and allele frequency of *exon 7 Glu298Asp* were 0.398 and 0.402, respectively, while for *intron 4 (4a4b) VNTR* they were 0.153 and 0.155, respectively). It can be inferred that the mutant *C* allele of the *promoter -786 T>C* variant of the *eNOS* gene is related to an increased risk for RPL. The rare allele *"4a"* of the *intron 4 (4a4b) VNTR* polymorphism and the mutant *T* allele of the *exon 7 Glu298Asp* missense variant, however, do not seem to contribute to an increased risk for RPL.

Lack of association between RPL and *exon 7 Glu298Asp* polymorphism observed in this study is in agreement with the findings recorded for women from Indian[1], Austrian[15], Greek[20], Chinese[21] and Tunisian[23] populations. In the contrary, results for women from Korea[2] Turkey[72] and North India [73] indicated that *exon 7 Glu298Asp*polymorphisms is significantly associated with RPL.

Absence of association between RPL and *intron 4 (4a4b) VNTR* polymorphism found in our study population is compatible with results documented for women from

Indian[1], Korean[2], Japanese[12], Greek[20], Tunisian[23], Turkish[72] and German[75] populations. However, our results do not support the previously published results for women from Austria[14], China[21] and North India[73] where they all indicated that *intron 4 (4a4b) VNTR* polymorphism is significantly associated with RPL. Regarding this polymorphism, our results showed that both the *4a/4b* genotype and the *4a* allele were evident in only 2 cases which belonged to the RPL group. This result indicates that the *4a* allele is not common in our population and may explain why the *4a/4a* genotype was not encountered in any of the subjects enrolled in the study. This finding supports the earlier results of *Sallout and Sharif (2010)* where they also found that the *4a* allele is not common in our population[22]. A very low frequency of *"4a"* allele was also observed in Iranian, Spanish, Turkish, Japanese, Caucasians of Australia, Koreans and South Indian Tamil populations[112].

Still, the role of the *"4a"* allele in RPL should not be neglected since this allele was found only in RPL subjects both in the current study and in the study of *Sallout and Sharif (2010)*[22]. Therefore, studies on larger samples are needed in order to verify this point.

Few studies have investigated the relation between *eNOSpromoter -786T>C* polymorphism and the development of RPL and other reproductive complications in women from various populations[2]. Our results for the *promoter -786 T>C*polymorphism are in agreement with those published by *Shim, et al.(2010)* who showed that *the promoter -786 T>C* polymorphism is associated with the risk of spontaneously aborted fetuses[74]. This result is also consistent with those recorded for Caucasians women of Polish origin where a significant association between the *promoter -786 T>C* and preeclamptic pregnancy complication was observed. In contrast, our results do not support the previously published results for women from Korean[2] and Tunisian[23] populations which indicated that *promoter -786 T>C*polymorphisms is not significantly associated with RPL.

Contradictory results in associating an allele, genotype and RPL in different populations can be attributed to the variation in the genetic background, in particular linkage disequilibirium to varying genetic elements.

5.3. Association between *eNOS* polymorphisms and serum NO levels

The results of the current study exclude significant association between *exon 7 (894 G>T*) and/or *intron 4 (4a4b) VNTR)* polymorphisms and serum NO levels in any of the examined subjects (all P-values >0.05). On the other hand, the *promoter -786 T>C* polymorphism was found to be associated with lower NO level in the RPL women. The mean NO level in women who had the *(CC + CT)*genotypes was significantly lower than in those who had the *TT* genotype the study groups (P-value <0.001).

Our findings regarding the association between *promoter -786 T>C* polymorphism and serum NO levels is compatible with the previous studies which reported that*promoter -786 T>C* polymorphism was associated with low *eNOS* gene promoter activity in platelets[97], reduced placental *eNOS* mRNA levels, and low serum nitrite/nitrate levels[113]. Some studies reported that -786 T>C polymorphism resulted in reduced *eNOS* gene promoter activity[114, 115], while, others reported that *promoter -786 T>C* polymorphism inhibited *eNOS* promoter activity, leading to reduced NO production in blood vessels and endothelial dysfunction[66, 116, 117]. In contrast, our results are different from those which reported a higher nitrite levels in healthy pregnant women with the polymorphic *CC* versus wild-type *TT* genotype for the *promoter -786T>C* polymorphism[94].

The association between the *-786C* allele in the untranscribed promoter region and reduced NO level should point to the presence of a binding site for a yet to be discovered transcription factor in this upstreram promoter region and the critical role of this particular nucleotide in this context.

Our results concerning the association between *exon 7 (894 G>T)* polymorphism and serum NO levels are compatible with those of the Turkish women which indicated the lack of any significant association between *exon 7 (894G>T)* or *intron 4 (4a4b)*

VNTR polymorphism and NO levels in both the RPL and control groups[72]. Conversely, our results are different from several of the previously published literature which focused on the effect of *exon 7 (894 G>T)* polymorphism on NO levels and *eNOS* activity. However, their results were conflicting since some of these studies found that *exon 7 (894 G>T)* polymorphism was associated with reduced NO levels[118], reduced NO generation[119, 120], lower *eNOS* activity and lower *eNOS* mRNA level[97]. while others indicated that *exon 7 (894 G>T)* polymorphism was associated with increased NO levels[48].

In case of *intron 4 (4a4b) VNTR*polymorphism, the *4a/4b* genotype was encountered in only 2 women, and the means NO level of the *4a/4b* genotype was accompanied with an elevated standard deviation, These findings did not help performing any further statistical analysis to determine the association between *intron 4 (4a4b) VNTR* polymorphism and serum NO levels in the RPL patients. Previous studies also pointed to their analysis limitations of the 4a4b polymorphism and NO level due to the low frequency of the *"4a"* allele[111, 121, 122]. In contrast, other studies demonstrated a role of *intron 4 (4a4b) VNTR* polymorphisms on NO levels including those which reported that *intron 4 (4a4b) VNTR* polymorphisms segregated with lower plasma NO metabolites[8, 97], altered plasma NO concentrations[123], and even overproduction of NO[7]. Moreover, *Yoon et al. (2000)* reported both positive and negative associations between a rare allele *"4a"* of *intron 4 VNTR* polymorphism and plasma NO concentration. That later study indicated that there was a substantial effect of *intron 4 (4a4b) VNTR* polymorphisms on the variance of plasma NO concentrations in Korean population and that this effect was dependent on smoking status[112]. With regard to RPL, a recent study on Turkish women indicated the lack of any significant association between *intron 4 (4a4b) VNTR* polymorphism and NO levels in both the RPL patients and the control group[72].

5.4. Association between serum NO levels and RPL regardless of the *eNOS*polymorphisms

Statistical analysis of the mean NO level in pregnant/non-pregnant RPL patients versus pregnant/non-pregnant controls, indicated the presence of significant statistical differences between the RPL patients versus controls (P-value= 0.004), between the mean NO level of non-pregnant RPL patients versus non-pregnant controls (P-value <0.001), and between the mean NO level of non-pregnant control versus pregnant controls (P-value <0.001), However, we did not find a significant difference between the mean NO level of pregnant RPL patients as compared to pregnant controls women (P-value= 0.11).

The lack of a significant difference in the mean NO level between pregnant RPL patients versus pregnant controls, may be attributed to the small number of the two groups which were compared of 15 subjects each. Though, not significant, it should be emphasized here that the mean NO level (19.42 µM) in the pregnant RPL group was clearly lower than its level in the pregnant control subjects (24.13 µM).

Several studies have been concerned with the role of NO in RPL, but their findings were contradictory. Our result is in agreement with those of *Baban et al (2010)* where they found that serum NO levels in RPL patients showed a highly significant decrease compared with third trimester pregnant, and non-pregnant control women. They also reported that the decrease in NO production is a result of RPL and not a causative factor[93]. *Paradisi et al (2007)*reported that serum NO levels in the missed abortion group were extremely significantly lower than both the non-pregnant and the pregnant control groups. They also showed that threatened abortion group, too, presented NO levels frankly lower than the non-pregnant control group. Conversely, our results are different from the finding of that serum NO levels was significantly higher in non-pregnant control group than pregnant control group [96]. *Diejomaoh et al. (2003)*showed that the mean serum levels of nitrite in active labor and preterm labor were significantly lower than the level in the control group, and that there is a drop in NO production in active preterm labor and induced labor[99]. *Delacretaz et al. (2005)*suggested that NO

synthesis is increased significantly during normal pregnancy, possibly contributing to the vasodilatation. While, NO generation, may be inappropriately low in pregnant women developing preeclampsia, thus leading to an enhanced vasoconstriction[105]. *Thanda et al. (1996)* showed that the NOS activity is highest in the early gestational age placenta, suggesting a possible significant role of NO in early gestation[109]. Our results also support the findings of *Wilson et al (1997)* where they reported that serum NO levels are significantly lower in the non-pregnant RPL group than those in the non-pregnant control group. Conversely, our results are different from their finding that serum NO levels are increased significantly in women with spontaneous abortion and in pregnant RPL compared with normal pregnancy women[103].

Other investigators, however, reported contradictory results e.g., *Öztürk et al. (2011)*where they observed that elevated NO levels are evident in the non-pregnant RPL patients when compared to non-pregnant controls. But in accordance with our results they recorded statistically significant decreased NO levels in the pregnant RPL patients[72]. *Raffaelli et al. (2010)* showed a significant increase in platelet NO in RPL pregnant women as compared to healthy controls[11]. *Makino et al. (2004)* found that plasma NO concentrations in the embryonal loss and fetal loss groups were significantly higher than that in controls[12]. *Mandach et al (2003)* found that NO metabolite (NOx) levels increased significantly through normal and particularly abnormal pregnancy, predominantly in the fetal compartments, suggesting that NO production is an additional instrument in the fetal control of the intrauterine environment[124], *Conrad et al. (1999)* found that during normal human pregnancy, the stable NO metabolites (NOx), nitrite and nitrate, were either unchanged or reduced[102].

Given the vasodilation nature of NO we believe that normal pregnancy phases should be associated with particular levels of NO and that imbalances in those levels can lead to adverse outcomes such as preeclampsia and fetal loss. But whether NO level is the cause of complication or a consequence of it is still an open question.

5.5. Correlation between serum levels of NO and progesterone

In the current study, we found that there is a significant correlation between serum NO and P_4 levels in the study population (P-value= 0.002, correlation coefficient= 0.319). Our results are compatible with those reported by *Yallampalli et al. (1996)* where they found that the treatment of pregnant rats with a combination of a NO inhibitor and onapristone (progesterone receptor antagonist) significantly potentiated the ability of the antiprogesterone to induce preterm labor. They also proposed that a decrease in NO synthesis together with the fall in P_4 levels at term could lead to the initiation of labor, and the interaction of NO and P_4 may be required to maintain pregnancy[18]. Our results are also consistent with those of *Khorram et al. (1999)*who suggested that estrogen may regulate myometrial *eNOS*, whereas P_4 or a combination of estrogen and P_4 may be more important in regulating endometrial *eNOS*, and NO may be a critical mediatorof sex steroid actions in the human uterus[101].Additionally, our results are congruent with those reported by *Han et al. (2005)*who found that P_4 potentiated the effect of E_2 through a genomic mechanism that stimulates the expression of *NOS* isoforms in endometrial surface epithelial cell line (HES) and primary endometrial cells[98]. Moreover, *Andronowska et al. (2008)*found that P_4 enhanced endometrial NO*x* production on days 5 to 35 of pregnancy, and that the combination of E_2 and P_4 was sometimes more effective in the stimulation of NO production than the application of individual hormones[106]. In the same context *Lo F and Kaufman S (2001)* confirmed that P_4 metabolite 5α-pregnan-3α-ol-20-one (pregnan) can mimic pregnancy by its ability to increase both NO biosynthesis and plasma volume[107]. Our results also are in agreement with those of *Chwalisz et al. (1999)* who demonstrated that there was a synergistic effects of *NOS* inhibitors and an antiprogestin in preventing pregnancy. Thus, *NOS*, particularly the cytokine- and progesterone-inducible *iNOS*, may represent a new target for novel therapeutic agents capable of promoting or inhibiting pregnancy[108].

Conversely, our results are different from those reported by *Fabregues et al (2000)* where they found that there is no correlation between nitrite/nitrate serum

concentration and E_2 or P_4 serum levels after *in vitro* fertilization and embryo transfer[110].

5.6. Association between *eNOSpromoter -786 T>C* polymorphisms and serum progesterone levels

The results of the current study exclude significant association between *-786 T>C*polymorphism and serum P_4 levels in the study population(P-values=0.401), it may be attributed to the small number of the pregnant group which were 30 subjects (15 RPL and 15 control), Since, pregnancy is a stateexperiencing a weekly changes and increase in progesterone levels particularly in the first trimesters. it's also may be due to the cyclic changes of sex hormone levels which control the menstrual cycle of non-pregnant subjects, on the other hand, the means P_4 level of the *promoter -786T>C* polymorphism was accompanied with an elevated standard deviation. Though, not significant, it should be emphasized here that the mean P_4 level in women who had *(CC + CT)* genotypes (6.56 ng/ml) was clearly lower than its level in the women who had the wild-type *TT* genotype was (8.58 ng/ml).

5.7. Discussion summary

The conflicting outcomes of RPL genetic association studies may be attributed to differences in genetic background and gene environment interactions among various populations. Moreover, the small number of patients or controls enrolled in some studies might lead to unreliable results [20, 64]. Therefore, the present results cannot be considered contradictory to some of the previous studies as there is considerable ethnic variability in each of the studied polymorphic loci. The present data add to the importance of ethnic as well as intra-regional variability in such studies concerning multifactorial disorders including RPL. Our findings regarding the three investigated *eNOS* polymorphisms and their associations with RPL clearly showed that the *promoter -786T>C* polymorphism of the *eNOS* gene, namely *"allele -786C"* is associated with RPL in Palestinian women residing in Gaza strip.

Results of the present study showed that the *-786C*-allele of the *promoter -786T>C* polymorphism is associated with lowering NO level. Reporter gene studies have shown that *promoter -786T>C* substitution markedly blunts the transcription rate of the *eNOS* gene, and hence NO production, likely because the *C* allele creates a binding site for a replication protein A1 (RPA1) that acts as a suppressor of *eNOS* transcription. Furthermore, it has been shown that RPA1 protein is present not only in endothelial cells but also in placenta, which is rich in vasculature, and that the level of *eNOS* mRNA in placentas with *promoter -786T>C* substitution mutation is significantly lower than in placentas without the mutation [125, 126]. These findings confirm our results that RPL women are associated with a high frequency of *promoter-786T>C* polymorphism *C* allele and might explain why this polymorphism is associated with a low serum NO levels.

Defective placentation and resultant oxidative stress are believed to be largely responsible for preeclampsia and RPL[72]. In literature, although some studies have demonstrated elevated NO levels in RPL cases, others have shown that decreased NO levels are associated with RPL [11, 12, 93, 96, 99, 105]. This difference could be due to the complexity of NO pathways, and that a balanced level of NO is needed for maintenance of a healthy pregnancy.

The correlation between NO level and P_4 observed in this study is in harmony with many earlier studies, which all proposed that P_4 can up-regulate *eNOS* protein expression in the myometrium[127, 128, 129, 130] and in turn stimulate NO production [128, 131, 132], both by non genomic and genomic mechanisms[133].The non-genomic mechanism is executed through a rapid signaling mechanism involving activation of a membrane bound receptor and subsequent activation of mitogen-activated protein kinase *(MAPK)* and *PI 3-kinase/Akt* pathways resulting in *eNOS* phosphorylation and increased *eNOS* activity[129, 133, 134]. The genomic mechanism is assumed to be through increase in *eNOS* mRNA and subsequent NOS production[133]. By *in silico* analysis of the eNOS gene sequence we detected five possible progesterone receptor binding sites, which all have the canonical "5`-TGTTCT-3`"[135]. Two of these putative binding sites are located at (4138 bp) and (2246 bp) up-stream the translation start site (TSS). The remaining 3 sites

are located in intron 8, intron 11, and (21765 bp) down-stream the TSS. We therefore assume that these sites could explain the correlation observed between P_4 and NO levels. Further investigation, however, is necessary in order to confirm this point.

Finally, we assume two different pathophysiologic models to explain the role of NO in RPL. First, the reduced NO levels may lead to endothelial dysfunction, vasoconstriction, infarction, impaired placental perfusion, and then to RPL. Second, reduced NO levels could reduce smooth muscle relaxation and cervical extensibility and ripening, leading to RPL. Whether these two models operate separately or, more likely, together still needs to be further investigated. However, the present findings support a functional role of the NO as a mediator in early embryonic development and confirm its importance in the uterus and cervix during pregnancy loss.

Chapter (6)
Conclusion and Recommendations

6.1. Conclusion

1) The study showed that the *C* allele carriers which represented by *(CC + CT)* genotypes and the *C* allele of the promoter *-786T>C* polymorphism are a possible risk factor for RPL. Where they presented with a high frequency in RPL women and were associated with decreased serum NO levels in this group.
2) The present study confirmed that neither *exon7 Glu298Asp (894G>T)* nor *intron 4 (4a4b) VNTR* polymorphism is associated with the risk of RPL in Palestinian women.
3) Both *exon 7 Glu298Asp (894G>T)* and *intron 4 (4a4b) VNTR* polymorphisms did not show a significant effect on the serum NO level in the study population.
4) Regardless of the *eNOS* polymorphisms, the study showed that serum NO levels were lower in RPL patients as compared to their representative controls.
5) Our findings showed that there is a positive proportional correlation between serum NO and P_4 levels in the study population.
6) The present study polymorphisms did not show a significant association between the *eNOS* promoter *-786T>C* polymorphisms serum and serum P_4 level in the study population.
7) Our findings showed that the level of NO is critical for maintenance of a healthy pregnancy, and might play an important role in the pathophysiology of RPL.

6.2. Recommendations

1) We recommend for testing the promoter *-786T>C* polymorphism of *eNOS* gene in all Palestinian women experiencing RPL, preeclampsia, other pregnancy related complications. it's also of unexplained cases.
2) Our results are in agreement with the previous studies which suggested that there is a possible correlation between the P_4 inhibitors and NOS inhibitors, such results, may open the way to balancing *eNOS* gene expression and NO metabolism.
3) Since NO pathway plays an important role in the pathophysiology of RPL, thus, any factors balancing NO metabolism could be useful in the treatment of RPL, consequently, reducing the substantial morbidity and associated mortality.

4) It's recommended to perform larger studies and perhaps mrta-analysis in order to refine the frequency *intron 4 (4a4b) VNTR* polymorphisms among RPL Palestinian women, since the 4a4a genotype of *intron 4 (4a4b) VNTR* polymorphisms was not encountered any subject enrolled in the current study.
5) It's also recommended to perform a further study to investigate the association with the *intron 4 (4a4b) VNTR* polymorphism and serum nitric oxide levels.
6) Further studies are recommended in order scrutinize the molecular basis of the correlation between NO and progesterone/estradiol and the possibly of finding a sequence represents a putative progesterone receptor binding element in the promoter region of *eNOS.*
7) It's recommended to perform a larger studies to investigate the association between the *promoter -786T>C* polymorphism and serum P_4 level in pregnant RPL matched to pregnant control women and non-pregnant RPL matched to non-pregnant control women.
8) As cGMP might be a more stable metabolite in the signaling pathway of NO, further studies are needed in order to verify the utility of cGMP in idiopathic RPL cases.

Chapter (7)

References

References

1) Suryanarayana V, Rao L, Kanakavalli M, Padmalatha V, Deenadayal M, Singh L: Recurrent early pregnancy loss and endothelial nitric oxide synthase gene polymorphisms. Archives in Gynecology and Obstetrics. 2006; 274(2): 119-124.

2) Shin SJ, Lee HH, Cha SH, Kim JH, Shim SH, Choi DH, Kim NK: Endothelial nitric oxide synthase gene polymorphisms (-786T>C, 4a4b, 894G>T) and haplotypes in Korean patients with recurrent spontaneous abortion. European journal of Obstetrics and Gynceology and Reproductive Biology. 2010; 152(1): 64-67.

3) Evans M: Recurrent pregnancy loss. available from Huntington reproductive in California: URL: http://www.havingbabies.com/single-article/?tx_ttnews[tt_news]=36&cHash=84678d86e80ead0312160bfd5f6ac4b5. (Accessed on December, 2011).

4) Casas JP, Cavalleri GL, Bautista LE, Smeeth L, Steve E. Humphries SE, Hingorani AD: Endothelial Nitric Oxide Synthase Gene Polymorphisms and Cardiovascular Disease: American Journal of Epidemiology. 2006; 164(10): 921- 935.

5) Alderton WK, Cooper CE, Knowles RG: Nitric oxide synthases: structure, function and inhibition. The biochemical journal. 2001; 357(3): 593-615.

6) Vaisanen-Tommiska MR: Nitric oxide in the human uterine cervix: Endogenous ripening factor. Annals of Medicine. 2008; 40(1): 45–55.

7) Wang XL, Mahaney MC, Sim AS, Wang J, Wang J, Blangero J, Almasy L, Badenhop RB , Wilcken DE: Genetic Contribution of the Endothelial Constitutive Nitric Oxide Synthase Gene to Plasma Nitric Oxide Levels. *Arteriosclerosis, Thrombosis, and Vascular Biology.*1997;17:3147-3153.

8) Tsukada T, Yokoyama K, Arai T, Takemoto F, Hara S, Yamada A, Kawaguchi Y, Hosoya T, Igari J: Evidence of association of the ecNOS gene polymorphism

with plasma NO metabolite levels in human. Biochemical and Biophysical Research Communication. 1998; 245(1): 190-193.

9) Hassan A, Gormley K, O'Sullivan M, Knight J, Sham P, Vallance P, Bamford J: Endothelial Nitric Oxide Gene Haplotypes and Risk of Cerebral Small-Vessel Disease. Stroke. 2004; 35(3): 654-659.

10) Khorram O: Nitric oxide and its role in blastocyst implantation. Reviews in Endocrine and Metabolic Disorders. 2002; 3(2): 145-149.

11) Raffaelli F, Nanetti L, Vignini A, Mazzanti L, Giannubilo SR, Curzi CM, Turi A, Paola Vitali P, Tranquilli AL: Nitric oxide platelet production in spontaneous miscarriage in the first trimester. Fertility and Sterility. 2010; 93(6): 1976-1982.

12) Makino A, Nakanishi T, Sugiura-Ogasawara M, Ozaki Y, Suzumori N, Suzumori K: No association of C677T methylenetetrahydrofolate reductase and an endothelial nitric oxide synthase polymorphism with recurrent pregnancy loss. American Journal of Reproductive Immunology. 2004; 52(1): 60-66.

13) Bartlett S R, Bennett P R, Campa J S, Dennes W J, Slater D M, Mann G E, Poston L, Poston R: Expression of nitric oxide synthase isoforms in pregnant human myometrium. Journal of Physiology. 1999; 521(3): 705-716.

14) Tempfer C, Unfried G, Zeillinger R, Hefler L, Nagele F, Huber JC: Endothelial nitric oxide synthase gene polymorphism in women with idiopathic recurrent miscarriage. Human Reproduction. 2001a; 16(8): 1644-1647.

15) Hefler L, Termpfer C, Bashford MT, Unfried G, Zeillinger R, Scheeberger C, Koelpl H, Nagele F, Huber JC: Polymorphisms of the angiotensinogen gene, the endothelial nitric oxide synthase gene, and the interleukine-1beta gene promoter in women with idiopathic recurrent miscarriage. Molecular Human Reproduction. 2002; 8(1): 95-100.

16) Colomba D, Duro G, Corrao S, Argano C, Di Chiara T, Nuzzo D, Pizzo F, Parrinello G, Scaglione R, Licata G: Endothelial nitric oxide synthase gene polymorphisms and cardiovascular damage in hypertensive subjects: an Italian case-control study. Immunity & Ageing. 2008; 5(4): 1742-4933.

17) Young SL, Lessey BA: Progesterone Function in Human Endometrium: Clinical Perspectives. Seminars in reproductive medicine. 2010; 28(1): 5-16.

18) Yallampalli C, Buhimschi I, Chwalisz K, Garfield RE, Dong LY: Preterm birth in rats produced by the synergistic action of a nitric oxide inhibitor (N^G-nitro-L-arginine methyl ester) and an antiprogestin (onapristone). American Journal of Obstetrics and Gynecology. 1996; 175(1): 207-212.

19) Yallampalli C, Izumi H, Byam-Smith M, Garfield RE. An L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. American Journal of Obstetrics and Gynecology. 1994; 170(1): 175-185.

20) Karvela M, Papadopoulou S, Tsaliki E, Konstantakou E, Hatzaki A, Florentin-Arar L, Lamnissou K: Endothelial nitric oxide synthase gene polymorphisms in recurrent spontaneous abortions. Archives of Gynecology and Obstetrics. 2008; 278(4): 349-352.

21) Fan W, Li SW, Wang Y: Association of genetic polymorphisms in endothelial nitric oxide synthase 3 gene with recurrent early spontaneous abortion. Chinese journal of medical genetics. 2007; 24(1):23-26.

22) Al Sallout RJ and Sharif AF: Polymorphisms in NOS3, ACE and PAI-1 Genes and Risk of Spontaneous Recurrent Miscarriage in the Gaza Strip. Medical Principles and Practice. 2010; 19(2): 99-104.

23) Zammiti W, Mtiraoui N, Mahjoub T: Lack of Consistent Association Between Endothelial Nitric Oxide Synthase Gene Polymorphisms, Homocysteine Levels and

Recurrent Pregnancy Loss in Tunisian Women. American Journal of Reproductive Immunology. 2008; 59(2): 139-145.

24) Tanus-Santos JE, Desai M, Flockhart DA: Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. 2001; 11(8): 719-725.

25) Ledingham MA, Thomson AJ, Young A, Macara LM, Greer IA, Norman JE: Changes in the expression of nitric oxide synthase in the human uterine cervix during pregnancy and parturition. Molecular Human Reproduction. 2000; 6(11): 1041-1048.

26) Li TC, Makris M, Tomsu M, Tuckerman E, Laird S: Recurrent miscarriage: aetiology, management and prognosis. Human Reproduction Update. 2002;8(5): 463-481.

27) Coulam CB, Wallis D, Weinstein J,Dipankar S. DasGupta DS, Jeyendran RS: Comparison of Thrombophilic Gene Mutations Among Patients Experiencing Recurrent Miscarriage and Deep Vein Thrombosis. American Journal of Reproductive Immunology. 2008; 60(5): 426-431.

28) Yamada H, Sata F, Saijo Y, Kishi R, Hisanori Minakami H: Genetic Factors in Fetal Growth Restriction and Miscarriage. Seminars in Thrombosis and Hemostasis, 2005;31(3): 334-345.

29) Gris JC, Mercier E, Quéré I, Lavigne-Lissalde G, Cochery-Nouvellon E, Hoffet M, Ripart-Neveu S, Tailland ML, Dauzat M, Pierre M: Low-molecular-weight heparin versus low-dose aspirin in women with one fetal loss and a constitutional thrombophilic disorder. Blood. 2004; 103(10): 3695-3699.

30) Clark P, Walker ID, Langhorne P, Crichton L, Thomson A, Greaves M, Whyte S, Greer I: SPIN (Scottish Pregnancy Intervention) study: a multicenter, randomized controlled trial of low-molecular-weight heparin and low-dose aspirin in women with recurrent miscarriage. Blood. 2010; 115(21): 4162-4167.

31) D'Uva M, Di Micco P, Strina I, Ranieri A, Alviggi C, Mollo A, Fabozzi F, Cacciapuoti L, di Frega MT, Iannuzzo M, De Placido G: Etiology of hypercoagulable state in women with recurrent fetal loss without other causes of miscarriage from Southern Italy: new clinical target for antithrombotic therapy. Biologics: Targets & Therapy. 2008: 2(4): 897-902.

32) Dolitzky M, Inbal A, Segal Y, Weiss A, Brenner B, Carp H: A randomized study of thromboprophylaxis in women with unexplained consecutive recurrent miscarriages. Fertility and Sterility. 2006; 86(2): 362-366.

33) Brenner B, Hoffman R, Carp H, Dulitsky M, Younis J: Efficacy and safety of two doses of enoxaparin in women with thrombophilia and recurrent pregnancy loss (the live- Enox study). Journal of Thrombosis and Haemostasis. 2005; 3(2): 227-229.

34) Kutteh WH, Stephenson MD: Recurrent Pregnancy Loss. in: Bieber EJ, Sanfilippo JS, Horowitz IR (eds.): Clinical Gynecology. Churchill Livingstone, Elsevier Inc. 2006; 797-802.

35) Moshage H: Simple and Reliable Measurement of Nitric Oxide Metabolites in Plasma. Clinical Chemistry. 2009; 55(10): 1881–1882.

36) Marin J, Rodriguez-Martinez MA: Nitric oxide, oxygen-derived free radicals and vascular endothelium. Journal of Autonomic Pharmacology. 1995; 15(4): 279-307.

37) Matter H, Kotsonis P: Biology and Chemistry of the Inhibition of Nitric Oxide Synthases by Pteridine-Derivatives as Therapeutic Agents. Medicinal Research Reviews. 2004; 24(5): 662-684.

38) Moshage H: Nitric Oxide Determinations: Much Ado About NO-Thing?. Clinical Chemistry. 1997; 43(4): 553-555.

39) Kone BC: Molecular biology of natriuretic peptides and nitric oxide synthases. Cardiovascular Research. 2001; 51(3) :429-441.

40) Mayer B, Hernmens B: Biosynthesis and action of nitric oxide in mammalian cells. Trends in Biochemical Sceinces. 1997; 22(12): 477-481.

41) Davies MG, Fulton GJ, Hagen PO: Clinical biology of nitric oxide. British Journal of Surgery. 1995; 82(12): 1598-1610.

42) Morris NH, Eaton BM, and Dekker G: Nitric oxide, the endothelium, pregnancy and pre-eclampsia. British Journal of Obstetrics and Gynaecology. 1996; 103(1): 4-15.

43) Moncada S, Higgs A: The L-arginine-nitric oxide pathway. The new England journal of medicine. 1993; 329(27): 2002-2012.

44) Goyal P, Jain A, Aggarwal S, Nigam PK, Khanna S, Bhattacharjee J: Nitric oxide-cyclic GMP signal transudation pathway in pregnancy induced hypertension. International Journal of Biological and Medical Research. 2010; 1(4):165-167.

45) Ledingham MA, Thomson AJ, Greer IA, Norman JE: Nitric oxide in parturition. British Journal of Obstetrics and Gymecology. 2000; 107(5): 581-593.

46) Chwalisz K, Garfield RE: Role of nitric oxide in implantation and menstruation. Human Reproduction. 2000; 15(3): 96-111.

47) Baylis C, Vallance P: Measurement of nitrite and nitrate level in plasma and urine-what does this measure tell us about the activity of the endogenous nitric oxide system. Current Opinion in Nephrology & Hypertension. 1998; 7(1): 59-62.

48) Yoon Y, Song J, Hong SH, Kim JO: Plasma Nitric Oxide Concentrations and Nitric Oxide Synthase Gene Polymorphisms in Coronary Artery Disease. Clinical Chemistry. 2000; 46(10): 1626-1630.

49) Al-Hijji J, Andolf E, Laurini R, Batra S: Nitric oxide synthase activity in human trophoblast, term placenta ad pregnant myometrium. Reproductive Biology and Endocrinology. 2003;1(51): 1-7.

50) Savvidou MD, Vallance PJT, Nicolaidis KH, Hingorani AD: Endothelial Nitric Oxide Synthase Gene Polymorphism and Maternal Vascular Adaptation to Pregnancy. Hypertension. 2001; 38(6): 1289-1293.

51) Rosselli M: Nitric oxide and reproduction. Molecular Human Reproduction. 1997;3(8):639–641.

52) Athanassakis I, Aifantis I, Ranella A, Giouremou K, Vassiladis S: Inhibition of nitric oxide production rescues LPS-induced fetal abortion in mice. Nitric Oxide. 1999; 3(3): 216-224.

53) Hyndman ME, Parsons HG, Verma S, Bridge PJ, Edworthy S, Jones C, Lonn E, Charbonneau F, Anderson TJ: The T-786→C Mutation in Endothelial nitric oxide synthase is associated with hypertension. Hypertension. 2002; 39(4): 919-922.

54) Rossmanith WG, Hoffmeister U, Wolfahrt S, kleine B, Mclean M, Jacobs RA, Grossman AB: Expression and functional analysis of endothelial nitric oxide synthase (eNOS) in human placenta. Molecular Human Reproduction. 1999; 5(5): 487-494.

55) Norman JE, Thomson AJ, Telfer JF, Young A, Greer IA, Cameron IT: Myometrial constitutive nitric oxide synthase expression is increased during human pregnancy. Molecular Human Reproduction. 1999; 5(2): 175-181.

56) Williams DJ, Vallance PJ, Neild GH, Spencer JA, Imms FJ: Nitric oxide- mediated vasodilation in human pregnancy. The American Journal of Physiology. 1997; 272(2): 748-752.

57) Weiner CP, Lizasoain I, Ignazio S, Baylis SA, Knowles RG, Charles IG, Moncada S: Induction of calcium dependent nitric oxide synthases by sex hormones. Proceeding of the National Academy of Sciences of the united states of America. 1994; 91(11): 5212-5216.

58) Vaisanen-Tommiska M, Mikkola TS, Ylikorkala O: Increased Release of Cervical Nitric Oxide in Spontaneous Abortion before Clinical Symptoms: A Possible Mechanism for Preabortal Cervical Ripening. The journal of clinical endocrinology and metabolisms. 2004; 89(11): 5622-5626.

59) Chwalisz K, Garfield RE: Regulation of the uterus and cervix during pregnancy and labor. Role of progesterone and nitric oxide. Annals New York Academy of Science. 1997; 828: 238-253.

60) Progesterone Therapy. Effects of Progesterone. available from: URL: http://www.progesteronetherapy.com/effects-of-progesterone.html. (Accessed on January, 2011).

61) Garfield RE, Saade G, Buhimschi C, Buhimschi I, Shi I, Shi SQ, Chwalisz K: Control and assessment of the uterus and cervix during pregnancy and labour. human reproduction update. 1998; 4(5): 673-695.

62) Li R, Lyn D, Lapu-Bula R, Oduwole A, Igho-Pemu P, Lankford B, Morgan J, Nkemdechi S, Liu G, Pack C, Silvestrov N, Deutsch DA, Song Q, Abukhalaf IK, Ofili E: Relation of Endothelial Nitric Oxide Synthase Gene to Plasma Nitric Oxide Level, Endothelial Function, and Blood Pressure in African Americans. The American Journal of Hypertension. 2004; 17(7): 560-567.

63) Hingorani AD: Polymorphisms in endothelial nitric oxide synthase and atherogenesis: John French Lecture 2000. Atherosclerosis. 2000;154(3):521-527.

64) Su MT, Lin SH, Chen YC: Genetic association studies of angiogenesis- and vasoconstriction- related genes in women with recurrent pregnancy loss: a systematic review and meta-analysis. Human Reproduction Update. 2011; 17(6): 803-812.

65) Khurana VG, Sohni YR, Mangrum WI, McClelland RL, O'Kane DJ, Meyer FB, Meissner I: Endothelial Nitric Oxide Synthase T-786C Single Nucleotide

Polymorphism: A Putative Genetic Marker Differentiating Small Versus Large Ruptured Intracranial Aneurysms. *Stroke*. 2003; 34(11): 2555-2559.

66) Senthil D, Raveendran M, Shen YH, Utama B, Dudley D, Wang J, Wang XL: Genotype dependent expression of endothelial nitric oxide synthase (eNOS) and its regulatory proteins in cultured endothelial cells. DNA Cell Biology. 2005; 24(4): 218-224.

67) Hocher B, Chen YP, Hugle S, Repey J, Krause K, Slowinski T, Godes M, Schaeffeler E, Guthmann F, Wauer R, Halle H, Gossing G. Pfab T: Impact of maternal endothelial nitric oxide synthase gene polymorphisms on blood pressure, protein excretion and fetal outcome in pregnancy. Journal of Human Hypertension. 2008; 22(9): 641–647.

68) Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Chen L, Zhang Y, Coselli JS, Wang XL: Effect of 27nt Small RNA on Endothelial Nitric-Oxide Synthase Expression. Molecular Biology of the Cell. 2008; 19(9): 3997-4005.

69) Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Zhang Y, Coselli J, Wang XL: Biogenesis of Short Intronic Repeat 27-Nucleotide Small RNA From Endothelial Nitric-oxide Synthase Gene. The journal of biological chemistry. 2008; 283(21): 14685-14693.

70) Zhang MX, Ou H, Shen YH, Wang J, Wang J, Coselli J, Wang XL: Regulation of endothelial nitric oxide synthase by small RNA. Proceedings of the national academy of sciences of the united states of america. 2005; 102(47): 16967-16972.

71) Yan L, Hao H, Elton TS, Liu Z, Ou H: Intronic microRNA suppresses endothelial nitric oxide synthase expression and endothelial cell proliferation via inhibition of STAT3 signaling. Molecular and Cellular Biochemistry. 2011; 357(1-2): 9-19.

72) Öztürk E, BalatÖ, Pehlivan S, Uğur MG, Özkan Y, Sever T, Namıduru E, Kul S: Nitric oxide levels and endothelial nitric oxide synthase gene polymorphisms in

Turkish women with idiopathic recurrent miscarriage. Journal of the Turkish-German Gynecological Association. 2011; 12(4): 234-238.

73) Parveen F, Faridi RM, Alam S, Agrawal S: Genetic analysis of eNOS gene polymorphisms in association with recurrent miscarriage among North Indian women. Reproductive BioMedicine Online. 2011; 23(1): 124-131.

74) Shim SH, Yoon TK, Cha DH, Han WB, Choi DH, kim NK: Endothelial Nitric Oxide Synthase (eNOS) gene polymorphisms in spontaneously aborted embryos. Genes and Genomics. 2010; 32(3): 283-288.

75) Buchholz T, Lohse P, Kosian E, Thaler CJ. Vasoconstrictively acting AT1R A1166C and NOS3 4/5 polymorphisms in recurrent spontaneous abortions (RSA). American Journal of Reproductive Immunology. 2004; 51(5): 323-328.

76) Seremak-Mrozikiewicz A, Drews K, Barlik M, Sieroszewski P, Grzeskowiak E, Mrozikiewicz PM: The significance of -786T>C polymorphism of endothelial NO synthase (eNOS) gene in severe preeclampsia. The Journal of Maternal-Fetal and Neonatal Medicine. 2011; 24(3): 432-436.

77) Seremak-Mrozikiewicz A, Drews K, Mrozikiewicz PM: The -786T/C polymorphism of the endothelial nitric oxide synthase gene in preeclampsia. European Journal of Obstetrics, Gynecology, and Reproductive Biology. 2008; 138(1): 114-124.

78) Salimi S, Naghavi A, Mokhtari M, Noora M, Yaghmaei M: Lack of relationship between endothelial nitric oxide synthase gene 4b/a and T-786C polymorphisms with preeclampsia in southeast of Iran. Archives of Gynecology and Obstetrics [Epub ahead of print]. 2011.

79) Zdoukopoulos N, Doxani C, Messinis IE, Stefanidis I, Zintzaras E: Polymorphisms of the endothelial nitric oxide synthase (NOS3) gene in preeclampsia: a candidate-gene association study. BMC Pregnancy and Childbirth. 2011; 11(89): 1-5.

80) Ozturk E, Balat O, Pehlivan S, Ugur MG, Ozcan C, Sever T, Kul S: Endothelial nitric oxide synthase gene polymorphisms in preeclampsia with or without eclampsia in a Turkish population. Journal of Obstetrics and Gynaecology Research. 2011; 37(12): 1778–1783.

81) Zhou R, Yang Y, Xiong Q, Cao X: Detection of endothelial nitric oxide synthase gene polymorphism in preeclampsia. Journal of Sichuan University. Medical science. 2003; 34(4):671-673.

82) Huber A, Grimm C, Jirecek S, Heim K, Zeillinger R, Husslein P, Hefler L: Polymorphisms of the NOS3 gene and unexplained late intrauterine fetal death. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2005; 122(2): 151-155.

83) Yaghmaei M, Salimi S, Mokhtari M, Naghavi A, Saravani M, Farajian- Mashhadi F: Endothelial nitric oxide synthase gene Glu298Asp polymorphism and risk of preeclampsia in South East of Iran. African Journal of Biotechnology. 2011; 10(52): 10712-10717.

84) Yoshimura T, Yoshimura M, Tabata A, Shimasaki Y, Nakayama M, Miyamoto Y, Saito Y, Nakao K, Yasue H, Okamura H: Association of the missense Glu298Asp variant of the endothelial nitric oxide synthase gene with severe preeclamsia. Journal of the Society for Gynecologic Investigation. 2000; 7(4): 238-241.

85) Hillermann R, Carelse K, Gebhardt GS: The Glu298Asp variant of the endothelial nitric oxide synthase gene is associated with an increase risk for abruptio placentae in pre-eclampsia. Journal of Human Genetics. 2005; 50(8): 415-419.

86) Yoshimura T, Yoshimura M, Tabata A, Yasue H, Okamura H: The missense Glu298Asp variant of the endothelial nitric oxide synthase gene is strongly associated with placental abruption. Human Genetics. 2001; 108(3): 181-183.

87) Landau R, Xie HG, Dishy V, Wood AJ, Stein CM, Smiley RM: No Association of the Asp298 Variant of the Endothelial Nitric Oxide Synthase Gene With Preeclampsia. American journal of hypertension. 2004; 17(5): 391-394.

88) Hakli T, Romppanen EL ,Hiltunen M, Helisalmi S, Punnonen K, Heinonen S: Endothelial Nitric Oxide Synthase Polymorphism in Preeclampsia. Journal of the society of gynecologic investigation. 2003; 10(3): 154-157.

89) Chen H, Zhao G, Sun M, Wang H, Liu J, Gao W, Meng T: Endothelial Nitric Oxide Synthase Gene Polymorphisms (G894T, 4b/a and T-786C) and Preeclampsia: Meta-Analysis of 18 Case-Control Studies. DNA and cell biology [Epub ahead of print]. 2011.

90) Yu CK, Casas JP, Savvidou MD, Sahemey MK, Nicolaides KH, Hingorani AD. Endothelial nitric oxide synthase gene polymorphism (Glu298Asp) and development of pre-eclampsia: a case-control study and a meta-analysis. BMC Pregnancy Childbirth. 2006; 6(7): 1-9.

91) Serrano NC, Casas JP, Díaz LA, Páez C, Mesa CM, Cifuentes R, Monterrosa A, Bautista A, Hawe E, Hingorani AD, Vallance P, López-Jaramillo P: Endothelial NO Synthase Genotype and Risk of Preeclampsia: A Multicenter Case-Control Study. Hypertension. 2004; 44(5): 702-707.

92) Sharma D, Singh A, Trivedi SS, Bhattacharjee J: Intergenotypic variation of nitric oxide and inflammatory markers in preeclampsia: A pilot study in a North Indian population. Human Immunology.2011; 72(5): 436-439.

93) Baban RS: Oxidative stress in recurrent pregnancy loss women. Saudi medical journal. 2010; 31(7): 759-763.

94) Sandrim VC, Palei ACT, Sertorio JT, CavalliRC, Duarte G, Tanus-Santos JE:Effects of eNOS polymorphisms on nitric oxide formation in healthy pregnancy and in pre-eclampsia. Basic science of Reproductive Medicine. 2010; 16(7): 506-510.

95) Urban J, Jrochi S, Bielechi D, Urban R: Serum homocysteine and nitric oxide levels in pregnancy complicated with intrauterine fetal growth restriction. Archives of prenatal medicine. 2007; 13(3): 27-29.

96) Paradisi R, Fabbri R, Battaglia, Facchinetti F, Venturoli S. Nitric oxide levels in women with missed and threatened abortion: results of a pilot study. Fertility and Sterility. 2007; 88(3): 744-748.

97) Dosenko VE, Zagoriy VY, Haytovich NV, Gordok OA, Moibenko AA: Allelic polymorphism of endothelial NO-synthase gene and its functional manifestations. Acta Biochimica Polonica. 2006; 53(2): 299-302.

98) Han G, Magee T, Khorram O: Regulation of nitric oxide synthase isoforms by estrogen in the human endometrium. Fertility and Sterility. 2005; 84(2): 1220-1227.

99) Diejomaoh MF, Omu AE, Taher S, Taher S, Nasser Al-Busiri N, Tunde Fatinikun T, Sanjit Fernandes S, Saed Al-Othman S:Nitric oxide metabolites in preterm and induced labor. Gynecologic and obstetric investigation.2003*;* 56*:* 197-202.

100) Choi JW, Im MW, Pai SH: Nitric Oxide Production Increases during Normal Pregnancy and Decreases in Preeclampsia. Annals of Clinical & Laboratory Science. 2002; 32(3): 257-263.

101) Khorram O, Garthwaite M, Magness RR: Endometrial and Myometrial Expression of Nitric Oxide Synthase Isoforms in Pre- and Postmenopausal Women. Journal of Clinical Endocrinology & Metabolism. 1999; 84(6): 2226-2232.

102) Conrad KP, Laurie J. Kerchner LJ, Mosher MD: Plasma and 24-h NOx and cGMP during normal pregnancy and preeclampsia in women on a reduced NOx diet. American Journal of Physiology- Renal Physiology. 1999; 277(1): 48-57.

103) Wilson R, McInnesa I, Leunga B, McKillopa JH, Walkerb JJ: Altered interleukin 12 and nitric oxide levels in recurrrent miscarriage. European Journal of Obstetrics & Gynecology and Reproductive Biology. 1997; 75(2): 211-214.

104) Haddad EK, Duclos, AJ, Baines, MG: Early embryo loss is associated with local production of nitric oxide by decidual mononuclear cells. The journal of experimental medicine. 1995; 182(4): 1143-1152.

105) Delacretaz E, de Quay N, Waeber B, Vial Y, Schulz PE, Burnier M, Brunner HR, Bossart H, Schaad NC. Differential nitric oxide synthase activity in human platelets during normal pregnancy and pre-eclampsia. Clinical Science. 1995; 88(6): 607-610.

106) Andronowska A, Chrusciel M: Influence of estradiol-17β and progesteroneon nitric oxide (NO) production in the porcineendometrium during first half of pregnancy. Reproductive biology. 2008; 8(1): 43-55.

107) Lo F, Kaufman S: Effect of 5α-pregnan-3 α-ol-20-one on nitric oxide biosynthesis and plasma volume in rats. American Journal of Physiology- Regulatory, Itegrative and Comparative Physiology. 2001; 280(6): 1902-1905.

108) Chwalisz K, Winterhager E, Thienel T, Garfield RE: Synergistic role of nitric oxide and progesterone during the establishment of pregnancy in the rat. Human Reproduction. 1999; 14(2): 542-552.

109) ThandaM, Saheki S, KitagawaH, Yano J, MatsuuraS: Gestational Changes in Nitric Oxide Synthase Activity in the Rat Placenta. The journal of Obstetrics and Gynecology research. 1996; 22(3): 267-273.

110) Fábregues F, Balasch J, Manau D, Creus M, Jiménez W, Carmona F, Casamitjana R, Vanrell JA: Circulating levels of nitric oxide in successful and unsuccessful implantation after *in vitro* fertilization and embryo transfer. Relationship to estradiol and progesterone. Acta Obstetetricia et Gynecologica Scandinavica. 2000; 79(7): 564-569.

111) Metzger IF, Sertorio JT, Tanus-Santos JE: Modulation of nitric oxide formation by endothelial nitric oxide synthase gene haplotypes. Free Radical Biology & Medicine. 2007; 43(6): 987-992.

112) Angeline T, Isabel W, Tsongalis GJ: Endothelial nitric oxide gene polymorphisms, nitric oxide production and coronary artery disease risk in a South Indian population. Experimental and Molecular Pathology. 2010; 89(3): 205-208.

113) Miyamoto Y, Saito Y, Nakayama M, Shimasaki Y, Yoshimura T, Yoshimura M, Harada M, Kajiyama N, Kishimoto I, Kuwahara K, Hino J, Ogawa E, Hamanaka I, Kamitani S, Takahashi N, Kawakami R, Kangawa K, Yasue H, Nakao K: Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T→C mutation associated with coronary spastic angina. Human Molecular Genet. 2000; 9(18): 2629 -2637.

114) Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, Motoyama T, Saito Y, Ogawa Y, Miyamoto Y, Nakao K: T^{-786}→C mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation 1999; 99(22): 2864-2870.

115) Ghilardi G, Biondi ML, DeMonti M, Bernini M, Turri O, Massaro F, Guagnellini E, Scorza R: Independent risk factor for moderate to severe internal carotid artery stenosis: T786C mutation of the endothelial nitric oxide synthase gene. Clin Chem. 2002; 48 (7): 989–993.

116) Brown KS, Kluijtmans LA, Young IS, Woodside J, Yarnell JW, McMaster D, Murray L, Evans AE, Boreham CA, McNulty H, Strain JJ, Mitchell LE, Whitehead AS: Genetic evidence that nitric oxide modulates homocysteine: the NOS3 894TT genotype is a risk factor for hyperhomocysteinemia. Arteriosclerosis, Thrombosis and Vascular Biology. 2003; 23(6):1014-1020.

117) Kimura T, Yokoyama T, Matsumura, Yoshiike N, Date C, Muramatsu M, Tanaka H: NOS3 genotype dependent correlation between blood pressure and physical activity. Hypertension. 2003; 41(2): 355-360.

118) Veldman BA, Spiering W, Doevendans P A, Vervoort G, Kroon AA, de-Leeuw PW, Smits P: The Glu298Asp polymorphism of the NOS 3 gene as a determinant

of the baseline production of nitric oxide. Journal of Hypertension. 2002; 20(10): 2023-2027.

119) Tesauro M, Thompson WC, Rogliani P, Qi L, Chaudhary PP, Moss J: Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. Proceedings of the National Academy of Science of the united states of America. 2000; 97(6): 2832-2835.

120) Persu A, Stoenoiu MS, Messiaen T, Davila S, Robino C, El-Khattabi O, Mourad M, Horie S, Feron O, Balligand JL, Wattiez R, Pirson Y, Chauveau D, Lens XM, Devuyst O: Modifier effect of eNOS in autosomal dominant polycystic kidney disease. Human Molecular Genetics. 2002; 11(3): 229 -241.

121) Metzger IF, Souza-Costa DC, Marroni AS, Nagassaki S, Desta Z, Flockhart DA, Tanus-Santos JE: Endothelial nitric oxide synthase gene haplotypes associated with circulating concentrations of nitric oxide products in healthy men. Pharmacogenetics and Genomics. 2005; 15(8): 565-570.

122) Sandrim, VC, de Syllos RW, Lisboa HR, Tres GS, Tanus-Santos JE: Influence of eNOS haplotypes on the plasma nitric oxide products concentrations in hypertensive and type 2 diabetes mellitus patients. Nitric Oxide. 2007; 16(3): 348-355.

123) Wang XQ, Vaziri ND: Erythropoietin depresses nitric oxide synthase expression by human endothelial cells. Hypertension. 1999; 33(3):894-899.

124) von Mandach U, Lauth D, Huch R: Maternal and fetal nitric oxide production in normal and abnormal pregnancy. The Journal of Maternal–Fetal and Neonatal Medicine. 2003; 13(1): 22-27.

125) Miyamoto Y, Saito Y, Nakayama M, Shimasaki Y, Yoshimura T, Yoshimura M, Harada M, Kajiyama N, Kishimoto I, Kuwahara K, Hino J, Ogawa E, Hamanaka I, Kamitani S, Takahashi N, Kawakami R, Kanjawa K, Yasue H, Nakao K:

Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T→C mutation associated with coronary spastic angina. Human Molecular Genetics. 2000; 9(18): 2629 -2637.

126) Cattaruzza M, Guzik TJ, Slodowski W, Pelvan A, Becker J, Halle M, Buchwald AB, Channon KM, Hecker M: Shear stress insensitivity of endothelial nitric oxide synthase expression as a genetic risk factor for coronary heart disease. Circulation Research. 2004; 95(8): 841-847.

127) Kakui K, Itoh H, Sagawa N, Yura S, Korita D, Takemura M, Miyamaoto Y, Saito Y, Nakao K, Fujii S: Augmented endothelial nitric oxide synthase (eNOS) protein expression in human pregnant myometrium: possible involvement of eNOS promoter activation by estrogen via both estrogen receptor (ER)α and ERβ. Molecular Human Reproduction. 2004; 10(2): 115-122.

128) Shaamash AH, Zakhari MM: Increased serum levels of nitric oxide metabolites among users: a possible role in progestin-induced bleeding. Human Reproduction. 2005; 20(1): 302-306.

129) Ogando D, Farina M, Ribeiro ML, Martinez S, Cella M, Rettori V, Franchi A: Steroid hormones augment nitric oxide syntahse activity and expression in rat uterus. Reproduction, fertility and development. 2003; 15(5): 269-274.

130) Zervou S, klentzeris LD, Old RW: Nitric oxide synthase expression and steroid regulation in the uterus of women with menorrhagia. Molecular Human Reproduction. 1999; 5(11): 1048-1054.

131) Simoncini T, Mannella P, Fornari L, Caruso A, Willis MY, Garibaldi S, Baldacci C, Genazzani AR: Differential signal transduction of progesterone and medroxyprogesterone acetate in human endothelial cells. Endocrinology. 2004; 145(12): 5745-5756.

132) Rupnow HL, Phernetton TM, Shaw CE, Modrick ML, Bird IM, Magness RR: Endothelial vasodilator production by uterine and systemic arteries. VII. Estrogen

and progesterone effects on eNOS. American Journal of Physiology (Heart and Circulatory Physiology). 2001; 280(4): 1699-1705.

133) Duckles SP, Miller VM: Hormonal modulation of endothelial NO production. European journal of physiology. 2010; 459(6): 841-851.

134) Welter BH, Hansen EL, Saner KJ, Wei Y, Price TM: Membrane-bound progesterone receptor expression in human aortic endothelial cells. The Journal of Histochemistry and Cytochemistry. 2003; 51(8): 1049-1055.

135) Yin P, Roqueiro D, Huang L, Owen JK, Xie A, Navarro A, Monsivais D, Coon JS, Kim JJ, Dai Y, Bulun SE: Genome-Wide Progesterone Receptor Binding: Cell Type Specific and Shared Mechanisms in T47D Breast Cancer Cells and Primary Leiomyoma Cells. PloS one.2012; 7(1): e29021.

Appendices

Palestinian National Authority
Ministry of Health
Helsinki Committee

السلطة الوطنية الفلسطينية
وزارة الصحة
لجنة هلسنكي

Date: 07/03/2011

التاريخ: 07/03/2011

Name: Emad Mohammed El-Gharably

الاسم: عماد محمد الغرابلي

I would like to inform you that the committee has discussed your application about:

نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:-

Endothelial Nitric Oxide Synthase (eNOS) Gene Polymorphisms, Nitric Oxide and Progesterone levels in Idiopathic Recurrent Pregnancy Loss.

In its meeting on March 2011 3 2011

and decided the Following:-

و ذلك في جلستها المنعقدة لشهر

وقد قررت ما يلي:-

To approve the above mention research study.

الموافقة على البحث المذكور عاليه.

Signature

توقيع

Member

Member

عضو

Chairperson

Conditions:-

- Valid for 2 years from the date of approval to start.
- It is necessary to notify the committee in any change in the admitted study protocol.
- The committee appreciates receiving one copy of your final research when it is completed.

الجـــــــــامعة الإسلامية – غزة كلية العلوم

The Islamic University of Gaza

منسق برنامج ماجستير العلوم الحياتية

التاريخ/ ٢٠١١/٠٢/١٦

الأخ الدكتور/ رئيس مجلس إدارة جمعية بنك الدم حفظه الله ،،،

السلام عليكم ورحمة الله وبركاته ،،،

الموضوع / تسهيل مهمة باحث

تشهد ادارة برنامج ماجستير العلوم الحياتية بالجامعة الإسلامية أن الطالب : ***عماد محمد الغرابلي*** طالب في برنامج ماجستير العلوم الحياتية تخصص ***تحاليل طبية*** يقوم بإجراء البحث النهائي في برنامج الماجستير والذي بعنوان:

Association between endothelial nitric oxide synthase (eNOS) Gene Polymorphisms, Nitric Oxide and Progesterone Levels, and Idiopathic Recurrent Pregnancy Loss

لذا نرجو من سيادتكم تسهيل مهمة الباحث مع العلم بأن الباحث بحاجة لحفظ العينات وفصلها في الجمعية مع العلم بان الطالب سيحضر كل ما هو مطلوب للعمل.

ولكم منا جزيل الشكر والتقدير ،،،

منسق برنامج ماجستير العلوم الحياتية

د. طارق البشيتي

جمعية بنك الدم المركزي

د. زياد سليمان شعث

رئيس مجلس الإدارة

Islamic University- P. Box 108 - Al Rimal Gaza Palestine
Tel:(970-8)2860700 Fax:(970-8)2860700 2863552 e-mail:public@mail.iugaza.edu Web Site:www.iugaza.edu

الجامعة الإسلامية-غزة – الرمال ص.ب ١٠٨ فلسطين

بسم الله الرحمن الرحيم

الجامعة الإسلامية – غزة
The Islamic University - Gaza

كلية العلوم
FACULTY OF SCIENCE

الرقم: ج س غ/148/64 Ref
التاريخ: 2011/4/2 Date

الوارد
الرقم: 13all
التاريخ: 3.4.2011

الأخ الفاضل / د. عائد ياغي حفظه الله،،،
مدير الإغاثة الطبية

السلام عليكم ورحمة الله وبركاته،،

الموضوع / تسهيل مهمة

بدايةً تهديكم عمادة كلية العلوم بالجامعة الإسلامية بغزة عاطر تحياتها، ونرجو من سيادتكم التكرم بتسهيل مهمة **الطالب / عماد محمد سعيد الغرابلي** المسجل لدينا في برنامج ماجستير (العلوم الحياتية/تحاليل طبية)، وذلك بجمع عينات دم لدراسة بعض الجينات التي لها علاقة بحدوث حالات الإجهاض المتكرر بقطاع غزة؛ وذلك لإتمام بحث رسالة الماجستير.

شاكرين لكم حسن تعاونكم معنا،،،

عميد كلية العلوم

د. نظام محمود الأشقر

عمادة كلية العلوم
The Islamic University
FACULTY OF SCIENCE

- صورة للملف.

تمت المراقبة على جمع العينات بالتعاون مع مجلس الإغاثة الطبية الفلسطينية
Palestinian Medical Relief Society

أ/ محمد البرعصي
للتواصل
2011/4/3

ص.ب. 108 الرمال، غزة، فلسطين هاتف: +970 (8) 286 0700 فاكس: +970 (8) 286 0800
P.O. Box 108, Rimal, Gaza, Palestine Tel: +970 (8) 286 0700 fax: +970 (8) 286 0800
public@iugaza.edu.ps www.iugaza.edu.ps

fc:\Sabri\General\general.doc