C-786T (Promoter) ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA

Dissertation submitted for

M.D. BIOCHEMISTRY - Branch XIII

DEGREE EXAMINATION

DEPARTMENT OF BIOCHEMISTRY

GOVT KILPAUK MEDICAL COLLEGE

CHENNAI - 600 010

THE TAMILNADU Dr.MGR MEDICAL UNIVERSITY

CHENNAI - 600 032

MAY 2019

CERTIFICATE

This to certify that the dissertation entitled **"C-786T (Promoter) ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA"** is the bonafide original work done by **Dr.S.Arunadevi**, post graduate in Biochemistry**,** under overall supervision and guidance in the Department of Biochemistry, Kilpauk Medical College, Chennai, in partial fulfilment of the regulations of The Tamilnadu Dr. M.G.R. Medical University for the award of M.D. Degree in Biochemistry (Branch XIII) – during the academic period of May 2016 – May 2019.

Dr. V. MEERA M.D., DGO,
PROFESSOR AND HOD,
Department of Biochemistry,
Govt. Kilpauk Medical College,
Chennai – 600010.

Dr.P.VASANTHAMANI, M.D, DGO,
MNAMS, DCPSY, MBA,
DEAN,
Govt. Kilpauk Medical College,
Chennai – 600010.

CERTIFICATE BY THE GUIDE

This to certify that the dissertation entitled **"C-786T (promoter) ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA."** is the bonafide work done by Dr. S.Aruna Devi, Post Graduate, in the Department of Biochemistry, Kilpauk Medical College, Chennai, under the guidance and supervision of me **Dr. V.MEERA, M.D., DGO**., Professor and Head of the Department, Department of Biochemistry , Kilpauk Medical College, Chennai – 600010.

This dissertation is submitted to THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY, Chennai, in partial fulfilment of the university regulations for the award of DEGREE OF M.D. BIOCHEMISTRY (BRANCH – XIII) examinations to be held in May 2019.

GUIDE: **Dr .V.MEERA ,M.D., DGO.,**
Professor and HOD,
Department of Biochemistry,
Govt .Kilpauk Medical College,
Chennai-10.

CERTIFICATE

This is to certify that this dissertation work titled C – 786 T (Promoter) "ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA" of the candidate Dr.S.ARUNADEVI with registration number 201623101 for the award of M.D Degree in Bio-Chemistry (Branch XIII). I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows 8 percentage of plagiarism in the dissertation.

Dr.V.MEERA, M.D., D.G.O.,
Professor & H.O.D
Department of Biochemistry
Govt. Kilpauk Medical College
Chennai-600 010.

DECLARATION

I, Dr. S.Arunadevi, Post Graduate, Department of Biochemistry, Govt.Kilpauk Medical College, solemnly declare that this dissertation entitled **"C-786T (promoter) ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA."** is the bonafide work done by me in the Department of Biochemistry, Kilpauk Medical College, Chennai, under the guidance and supervision of **Dr.V.MEERA, M.D., DGO**., Professor and HOD, Department of Biochemistry , Kilpauk Medical College, Chennai – 600010.

This dissertation is submitted to THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY, Chennai, in partial fulfilment of the university regulations for the award of DEGREE OF M.D. BIOCHEMISTRY (BRANCH – XIII) examinations to be held in May 2019.

Date:

Place: Chennai

Dr.S.ArunaDevi,
Post Graduate student,
Department of Biochemistry.

ACKNOWLEDGEMENT

”**Gratitude is the humble gift I can give to my beloved Teachers”.** The author expresses her profound gratitude to the **Dean , Dr. P. Vasanthamani, M.D, DGO, MNAMS, DCPSY, MBA,** Kilpauk Medical College and Hospital, Chennai, for granting me permission to utilize the facilities and conduct the study at the Department of Biochemistry, Kilpak Medial College and Hospital .

The author expresses her heartfelt and respectful gratitude to **Dr. V. Meera, M.D., DGO**., The Professor and Head of the Department, Department of Biochemistry, Kilpauk Medical College & Hospital, for her invaluable help and constant encouragement during the course of the study. The author again wishes to express her sincere thanks and special gratitude to Head of the Department for the valuable guidance, supervision, suggestion and great support throughout the study.

The author wishes a sincere gratitude to **Dr.K.L.Malarvizhi, MD., DGO., DNB., Professor & HOD, Department of Obstetrics & Gynaecology,** Govt. Kilpauk Medical College Hospital**,** Chennai**,** for granting permission to obtain blood samples from the patients.

The author is extremely thankful to **Associate Professor, Dr.Panimathi, M.D., DCH, Assistant Professors Dr.K.Geetha M.D, Dr.K.Rekha M.D., Dr.R.Bhuvaneswari, M.D., DCP, Dr.G.Udayakumari M.D., DCP, Dr.J.Arulmoorthy DCH., and Dr.R.Preethi M.D., Department of Biochemistry** for their guidance, immense help, constructive ideas and continuous support throughout the study.

The author expresses her special thanks to her Co-PG Dr.P.Ponmalar, other PGs and lab technicians, DMLT students and other staff of Biochemistry department for their timely help, constant encouragement and unconditional support throughout the study.

The author expresses her thanks to Mr. Ravanan M.Sc., M.Phil., Ph.D, HOD of Statistics, Presidency College, Chennai-05 for the statistical analysis of the study.

The author is indebted to those patients and the persons from whom blood samples were collected for conducting the study.

Finally, the author expresses her special thanks to her husband Mr.R.Komahan and her children A.K.Tharagan , A.K.Aniruthan and her parents and sister in law, nephews S.Keerthi Varman and S.Ravi Varman for the moral support and encouragement extended by them throughout her study.

Above all, the author is grateful to the **ALMIGHTY** for providing this opportunity, without whose grace nothing could be accomplished.

ABBREVATIONS

ADMA - Asymmetric dimethyl Arginine

CAS - Candidate-gene Association Studies

CaMs – Calmodulins

DNA - Deoxyribonucleic Acid

NO - Nitric Oxide

eNOS -Endothelial Nitric Oxide Synthase

ELISA - Enzyme-linked Immunosorbent Assay

FAD - Flavin Adenine Dinucleotide

FMN - Flavin mononucleotide

GAS - Genetic Association Study

iNOS - Inducible NOS

GLDH - L-glutamate dehydrogenase

mRNA - Messenger RNA

NER - National Eclampsia Registry

NBT - Nitro Blue Tetrazolium

nNOS - Neural NOS

FOGSI - Federation of Obstetric and Gynaecological Societies of India

ICOG - Indian College of Obstetricians & Gynaecologists

HELLP - Haemolysis, Elevated Liver enzymes, Low Platelet Count

cGMP - Cyclic Guanosine Monophosphate

MLCK - Myosine Light Chain Kinase

sGC – Soluble Guanylyl Cyclase

PDEs – Phosphodiesterases

PCR – Polymerase Chain Reaction

GSNOR - S-nitrosoglutathione reductase

MPO –Myeloperoxidase

LDL - Low-Density Lipoproteins

LDH - Lactate dehydrogenase

TCA – Tricholoro Acetic Acid

TNF - Tumor Necrosing Factor

CONTENTS

INTRODUCTION

INTRODUCTION

Pregnancy is a hypercoagulable state with an increased tendency of thrombus formation. In normal pregnancy, successful implantation depends on the maternal endometrium receptivity. It is influenced by the synergistic actions of progesterone and nitric oxide. Pregnancy is accompanied by considerable physiological changes in the maternal cardio vascular system which includes increased uterine blood flow, altered response to vasopressor agents and reduced peripheral resistance and blood pressure. These changes confirm the adequacy of oxygen and nutrient delivery to the fetus.

As per the estimates of World Health Organisation, the global incidence of preeclampsia is 5% to 7% and in India it's reported in 8-10% of all pregnancies. In developing and developed countries, preeclampsia is one of the leading causes of maternal mortality and morbidity. It is associated with increased vascular reactivity and vasoconstriction and is characterized by placental abnormalities and maternal vascular endothelial dysfunction. The maternal syndrome of preeclampsia is characterized by hypertension, proteinuria, edema, abnormal clotting and impaired liver and renal function. These are explained by generalized vascular endothelial cell dysfunction.

Preeclampsia is defined as the presence of blood pressure higher than 140/90mmHg on two occasions more than six hours apart and proteinuria of 300 mg/24hr after the twentieth (20^{th} week of pregnancy. Genetic involvement in the preeclampsia, has long been recognized but the mode of inheritance and the genes involved has not been straightforward. The association of nitric oxide 3 gene polymorphisms and preeclampsia-risk states that it is a clinical syndrome characterized by new-onset of hypertension and proteinuria after 20 weeks of gestation[1]. It afflicts 3–5% of pregnancies and is a major cause of maternal and prenatal morbidity and mortality worldwide [2]. Essential in the pathogenesis of preeclampsia is endothelial dysfunction due to impaired trophoblast invasion and spiral artery remodelling, resulting in abnormal implantation and placental hypo-perfusion[3].

Although some dietary, environmental and genetic factors of preeclampsia have been identified, its mechanism is still not clear; therefore, its prevention remains a challenge[4]. Family-based studies have shown that genetic factors may play a role in preeclampsia [5]. In addition, candidate-gene association studies (CAS) on preeclampsia have not produced conclusive results so far [6].

As a potent vasodilator, circulating nitric oxide (NO) plays a crucial role in endothelial function regulation, blood pressure control and cardiovascular homeostasis[7].

Endothelial nitric oxide synthase (eNOS) is an enzyme which synthesizes NO constitutively via catalyzing the conversion of L-arginine to L-citrulline. Endothelial NO availability is largely regulated by its synthesis by eNOS. The gene that encodes eNOS, NOS3, is therefore considered as a candidate gene for preeclampsia [8].

However, the pathogenesis of preeclampsia is poorly understood and the search for low penetrant genes by hypothesis driven candidate-gene studies (genetic association study-GAS) and hypothesis-free genome-wide association studies is ongoing [9].

The leading hypotheses, concerning the pathogenesis of preeclampsia, are based on disturbed placental function and impaired remodelling of the spiral arteries [10].Endothelial nitric oxide synthase (NOS3) is an important regulator of vascular tone and contributes to the reduction of the uteroplacental resistance seen in normal pregnancy[11]. Therefore, the endothelial nitric oxide synthase gene (NOS3), located at the 7q35-q36 region, has emerged as a logical candidate gene in the development of preeclampsia. Variants (polymorphisms) of the NOS3 gene have been investigated for their association with preeclampsia and other disorders such as hypertension.

The three most common variants examined for clinical relevance, based on their potential functional effects are (i) a G894T substitution in exon 7 resulting in a Glu to Asp substitution at codon 298 (rs1799983), (ii) an insertion-deletion in intron 4 (4a/b) consisting of two alleles (the a*-deletion which has four tandem 27-bp repeats and the b*-insertion having five repeats), and (iii) a T786C substitution in the promoter region (rs2070744). A whole genome-scan meta-analysis for preeclampsia has already identified the locus of NOS3 gene as a promising candidate for preeclampsia susceptibility.

GAS that investigated the association between NOS3 variants and preeclampsia has produced controversial or in conclusive results and the replication record of these studies is relatively poor. Therefore, the status of association for the NOS3 variants remains ambiguous.

The significance of - 786T > C polymorphism of endothelial NO synthase (eNOS) gene in severe preeclampsia is believed to be induced by endothelial cell dysfunction in placenta. Highly polymorphic endothelial nitric oxide synthase (eNOS) activity belongs to the factors significantly influencing vasomotor tone in placenta and PE susceptibility. The presence of mutated homozygous CC genotype and C allele of -786T/C polymorphism of eNOS gene influences the higher susceptibility to develop severe Preeclampsia development[12]. Thus the topic for this research was decided.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Epidemiology

A standard evaluation by the World Health Organization indicates that hypertensive disorders account for 16% of all maternal deaths in developed countries, 9% in Africa and Asia, and as high as 26% in Latin America and the Caribbean [13].

Context India

Occurrence of hypertensive disorders in India is found to be 10.08 % as noticed through the data collected by the National Eclampsia Registry (NER) (11,266 out of 1,11,725 deliveries). Over the past 3 years, 2,554 patients out of 11,266 are presented with eclampsia. The FOGSI-ICOG NER has conducted forth some revealing trends. Eclampsia generality among registry patients was 1.9 %. National sample surveys in the past have shown prevalence to be 1–5 %. This is out of the 1,11,725 deliveries examined from the cases reported by 175 reporting centers. Number of eclampsia-cases is more than imminent eclampsia-cases. This points to the lost opportunities of prevention.

As per the NER data, preeclampsia was mostly diagnosed among asymptomatic (57 %) patients; 22 % had headache and very few had vomiting, epigastric pain, giddiness, etc. Antenatal eclampsia is noted to be common (76.78 %); however, post-partum convulsions (13.72 %) are also significant [14].

Fetal and neonatal effects

Fetal and neonatal outcomes related to preeclampsia vary around the world. Approximately 12–25% of fetal growth reduction and small-for-gestational-age infants and 15–20% of all preterm births are attributable to preeclampsia[15].

Recurrence in subsequent pregnancies

Studies have reported that there is a 7–20% chance of preeclampsia recurrence in an upcoming pregnancy[16].This risk is further increased if a woman has had two prior preeclamptic pregnancies and is also pretended by the gestational age of onset[17].

The American College of Obstetricians and Gynecologists (the College) Task Force on Hypertension in Pregnancy had been using the classification schema first initiated in 1972 by the College and modified in 1990 and 2000 and also incorporating the reports of the National High Blood Pressure Education Program Working Group[18]. Similar classifications can be found in the American Society of Hypertension guidelines, as well as College Practice Bulletins [19]. Although the task force has adjusted a few of the parts of the groups, it continues with this fundamental, accurate, and practical classification, which considers hyper- tension during pregnancy in only four categories:

1) Preeclampsia–eclampsia,
2) Chronic hypertension (of any cause),
3) Chronic hypertension with superimposed preeclampsia
4) Gestational hypertension.

Preeclampsia–Eclampsia

Preeclampsia is a pregnancy-specific hypertensive disease, diagnosed only after 20 weeks of gestation, with multitask involvement. It is characterized by hypertension and proteinuria. Preeclampsia is thought to grow as a result of poor placentation, causing endothelial dysfunction, disordered angiogenic balance and resultant hypertension, glomerular lesions and hepatic failure. However, some women present with hypertension and multi systemic signs usually indicative of disease severity in the absence of proteinuria.

Hypertension is defined as either a systolic BP of 140 mm Hg or greater, a diastolic BP of 90 mm Hg or greater, or both. Proteinuria is diagnosed when 24-hour excretion equals or exceeds 300 mg in 24 hours or the ratio of measured protein to creatinine in a single voided urine measures or exceeds 3.0 (each measured as mg/dL), termed as protein/creatinine ratio.

Eclampsia is the convulsive phase of the disorder and more severe indication of the disease. It is frequently preceded by precursory events, such as serious headaches and hyper-reflexia, but it can also occur in the absence of warning signs or symptoms.

Chronic Hypertension

Chronic hypertension in pregnancy is defined as high BP known to predate category or detected before 20 weeks of gestation. Previously, it was suggested that chronic hypertension indicated high BP diagnosed in the first half of pregnancy and modified postpartum.

Chronic Hypertension with Superimposed Preeclampsia

Preeclampsia may complicate all other hypertensive disorders, and in fact the incidence is forty five times higher than in non-hypertensive pregnant women[20]. In such cases, prognosis for the woman and her fetus is worse, while evidence from renal biopsy studies recommend that the diagnosis of superimposed preeclampsia may be often erroneous[21].

Gestational Hypertension

Gestational hypertension is characterized by new-onset elevations of BP after 20 weeks of gestation, often near term, in the absence of accompanying proteinuria. The failure of BP to normalize postpartum requires changing the diagnosis to chronic hypertension[22].

Postpartum Hypertension

It is important to remember that preeclampsia, including preeclampsia with severe systemic organ action and seizures, can first develop in the postpartum period. Since early hospital discharge is the current practice, this mandates to instruct the women discharged from the hospital to be aware of symptoms (eg. severe headache, visual disturbances, or epigastric pain) that should be detailed to a health care provider.

Risk factors for preeclampsia

The epidemiology of preeclampsia reflects a broad range of risk factors as well as the complexity and heterogeneity of the disease. Risk factors can be classified into pregnancy-certain features and maternal pre-existing features. The incidence of preeclampsia is increasing and may be associated to the higher prevalence of predisposing disorders such as hypertension, diabetes, obesity, delay in child-bearing, and the use of artificial reproductive technologies with associated increase in multi-fetal gestation[23].

Common risk factors for preeclampsia

(i) Pregnancy-specific issues

- Nuiparity
- Partner-related factors (new paternity, limited sperm exposure (e.g., barrier contraception)
- Multiple pregnancy
- Hydatidiform mole

(ii) Maternal pre-existing conditions

- Older age
- African-American race
- Higher body mass index
- Pregestational diabetes
- Chronic hypertension
- Renal disease
- Antiphospholipid antibody syndrome
- Connective tissue disorder (e.g. systemic lupus erythematosus)
- Family history or preeclampsia
- Lack of smoking

HELLP syndrome:

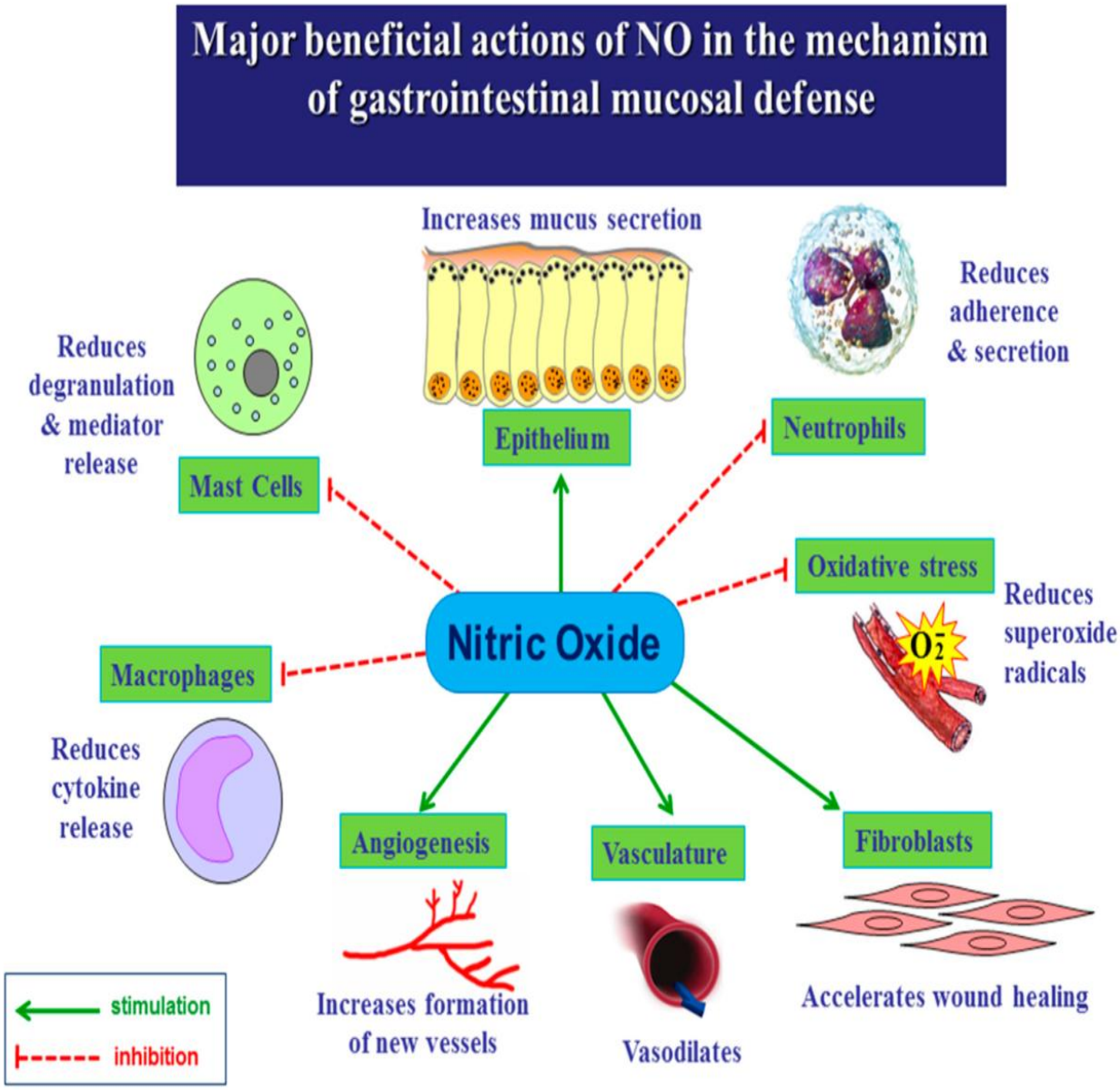
The HELLP syndrome is a major problem in pregnancy characterized by hemolysis, elevated liver enzymes and low platelet count, occurring in 0.5 to 0.9% of all pregnancies and in 10 – 20% of cases with severe preeclampsia. The present evaluation highlights occurrence, diagnosis, complications, surveillance and corticosteroid treatment, mode of delivery and risk of recurrence [24].

NITRIC OXIDE

The endothelium plays a central role in the regulation of vascular tone and blood flow, by the secretion and capture of paracrine vasoactive substances, which includes vasodilator substances (NO, prostacyclin & endothelium-derived hyperpolarising factor) and vasoconstrictor substances (endothelin-1, thromboxane A2 & platelet-activating factor).

NO is the main mediator of vasomotor tone regulation in physiological circumstances, small amount being continuously secreted by the endothelial cells [25]. To maintain a reduced arterial tone in the systemic and pulmonary circulation [26]. The vasodilator activity of NO is due to its interaction with the iron atom of the heme- prosthetic group of cyclic guanosine monophosphate (cGMP) [27]. In smooth muscle cells, this decreases intracellular calcium concentration, causing vascular relaxation [28].

Figure 1:

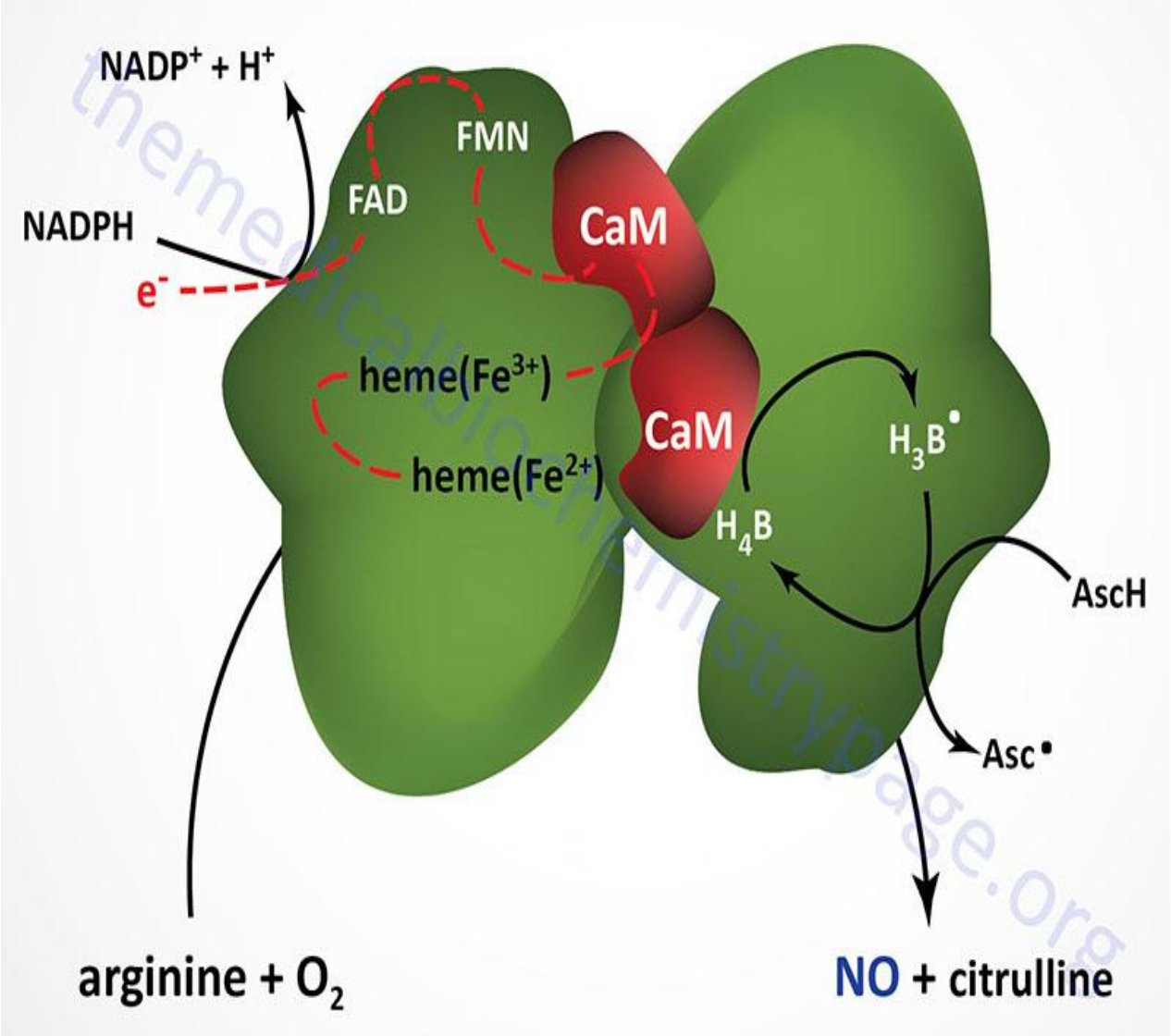

Major beneficial actions of NO in the mechanism of gastrointestinal mucosal defense
Increases mucus secretion
Epithelium
Reduces adherence & secretion
Neutrophils
Reduces degranulation & mediator release
Mast Cells
Oxidative stress
Reduces superoxide radicals
O_2^-
Nitric Oxide
Macrophages
Reduces cytokine release
Angiogenesis
Vasculature
Fibroblasts
Increases formation of new vessels
Vasodilates
Accelerates wound healing
stimulation
inhibition

Endothelial dysfunction is characterized by the reduction of the endothelium-derived vasodilators, by local increases in antagonists to these substances or by an association of two factors. Endothelial dysfunction appears to play a pathogenic role in the initial development of atherosclerosis and of unstable coronary syndromes, and their diverse risk factors viz hypercholesterolemia smoking, hypertension, diabetes mellitus, family history of premature coronary disease, hyperhomocysteinemia and aging.

NO and endothelin are the two major factors involved in endothelial dysfunction. High plasma concentrations of Endothelin-1 have been reported in myocardial infarction, cardiogenic shock, unstable angina pectoris, coronary artery disease in general, cardiac failure and essential hypertension [29]. Endothelin-1 action, unopposed by NO, tends to stimulate vasoconstriction and proliferation of vascular smooth muscle cells in conditons in endothelial dysfunction [30].

Reduction in synthesis or local availability of NO have been frequently considered the most important causes of endothelial dysfunction in various clinical conditions. NO release from the endothelium, is decreased in patients with established coronary atherosclerosis and hypertension [31.]

Figure 2:


NADP+ + H+
FMN
FAD
NADPH
e-
CaM
heme(Fe3+)
heme(Fe2+)
CaM
H3B•
H4B
AscH
Asc•
arginine + O2
NO + citrulline

NITRIC OXIDE PATHWAY:

NO is produced by the enzymenitric oxide synthase (NOS) [32] which catalyzes a five electron oxidation of a guanidino nitrogen of L - arginine (L - Arg). Oxidation of L-Arg to L–citrulline occurs via two successive mono - oxygenation reactions producing N^G hydroxy L – arginine as an intermediate. Two moles of O_2 and 1.5 moles of NADPH are consumed per mole of NO formed [33].

NO BASED CELL SIGNALING:

- **cGMP dependent signalling**

NO diffuses into the nearby target cells to interact with specific molecular targets. NO regulates protein activity by reversibly binding to the accessible acceptor functionalities, including heme iron and thiols [34]. The interaction between NO and the enzyme guanylyl cyclase, which mediates target cell responses such as vascular smooth muscle relaxation and platelet inhibition, has been well characterized [35]. After entering the target cell, NO binds to the heme moiety of guaynylyl cyclase and activates the enzyme by inducing a conformational reorder that displaces iron out of the plane of the phorphoryrin ring [36]. Guanylyl cyclase then catalyzes the production of cyclic GMP from GTP to evaluate cyclic GMP, which activates a cascade of intracellular events that brings about a reduction in calcium-dependent vascular smooth muscle tone, by inactivating myosine light chain kinase (MLCK)[37]. MLCK normally phosphorylates the regulatory set of myosin light chains. This event activates cross-bridge cycling and initiates

contraction [38]. cGMP modulates MLCK activity by activating a cGMP – dependent protein kinase that phosphorylates MLCK. Phosphorylation of MLCK diminishes its affinity for calmodulin and, as a consequence, decreases the phosphorylation of myosin light chain, which in revolve stabilizes the idleform of myosin. In this manner, cGMP may induce vasorelaxation by indirectly decreasing myosin light chain - dependent myosin activation.

GUANYLYL CYCLASE:

Soluble GC(sGC) is a heme - containing, heterodimeric NO receptor. sGC consists of two subunits, which makeup the active enzyme. sGC isoforms, products of four genes, have been identified so far as . Only and heterodimers are activated by NO [39]. The sGC is the most large iso form [40]. Vascular smooth muscle and endothelial cells indicate predominantly subunit[41]. The functional importance of sGC was demonstrated by the significantly decreased relaxing effects of major vasodilators such as acetylcholine, NO, YC – 1 and BAY 41 – 2272 in the sGC knockout mice [42].

Through the production of cGMP, sGC can exert many physiological results such as mediating vascular smooth muscle tone and motility, phototransduction, and maintaining fluid and electrolyte hemeostasis [43]. The sGC activity increases more than 200 fold in response to NO [44]. This signal is quickly removed by the action of phosphodiesterase 5A enzyme.

SPLICE FORMS AS NOVEL GENETIC REGULATORS OF sGC:

Recently, the necessary importance of sGC for mammalian physiology was directly confirmed by generation of sGC knockout mice [45]. The absence sGC protein resulted in a significant increase in blood pressure, complete loss of NO - dependent aortic relaxation and platelet aggregation in knockout animals, which died prematurely at the age of 4 weeks due to severe gastrointestinal disorder [46]. sGC function is affected not only by NO, but also by the regulation of the expression of sGC subunits at transcriptional levels. The steady state mRNA levels of subunits decrease with hypertension, ageing and vary during embryonic development [47]. The expression of sGC subunits is regulated by estrogen, cAMP – elevating compounds, cytokines and NO donors. Gene therapy with subunits may provide future therapeutic utility in hypertension.

ALLOSTERIC EFFE CTORS OF sGC:

There are many allosteric regulators of sGC which provide NO independent activation. Compounds that activate sGC in an NO – independent manner might therefore provide considerable therapeutic advantages [48]. NO - independent but heme - dependent stimulators of sGC are YC – 1, BAY 41 – 2272, BAY 41 – 8543, A-350619 and CFM-1571. NO as well as heme – independent sGC activators are BAY 58 – 2667 and HMR- 1766.

cGMP INDEPENDENT SIGNALLING:

Nitrite and Nitrate:

Nitrite is an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO formation [49]. Nitrite has recently moved to the forefront of NO biology [50] as, it represents an important storage from of NO in blood and tissues [51]. Much of the recent focus on nitrite physiology is due to its ability to be reduced to NO[52], during ischemic or hypoxic events [53]. They get converted to NO and exert their physiological events. Evidences for this include:

- Enriching dietary intake of nitrite and nitrate translates into significantly less injury from heart attack [54].
- Nitrite therapy given intravenously prior to reperfusion protects against myocardial injury [55] and cerebral vasospasm [56].
- Inhalation of nitrite selectively dilates the pulmonary circulation under hypoxicconditions in vivo in sheep [57].
- Topical application of nitrite improves skin infections and ulcerations [58].
- Oral nitrite has also been shown to reverse L-Nitro Arginine Methyl Ester (L-NAME) induced hypertension and serve as an alternate source of NO in vivo [59].
- Plasma nitrite levels progressively decrease with increasing cardiovascular risk [60].

In addition to the oxidation of NO, nitrite is also obtained from reduction of salivary nitrite by commensal bacteria in the mouth and gastrointestinal tract [61] as well as from nitrite rich diet. Since a substantial portion of nitrite in blood and tissue are derived from dietary sources[62], modulation of nitrite and/or nitrate intake may provide the first line of defense for conditions associated with NO insufficiency.

The primary dietary sources of nitrates and nitrites comprises plants, vegetables and few fruits, nuts, processed and cured meat, fish and poultry and drinking water, to which nitrites have been added. Plant foods are the primary sources of nitrate, while processed and cured meats are the primary sources of nitrites [63].

Since the NO radical is rapidly metabolized into the stable end-products, nitrite and nitrate. Determination of nitrite and nitrate does not demonstrate ongoing NO production. Considering that the half-life of nitrate in plasma is 5h [64], increased concentrations of nitrate in plasma after overnight fasting indicate recent NO production. The most commonly used nitrite and nitrite assay is based on the Griess diazotization reaction, which is specific for nitrite and does not detect nitrate. Therefore nitrate in samples must first be reduced to nitrite; subsequent nitrite determination thus yields the total nitrite + nitrate concentration of the sample (NOx).

Various study on plasma NOx assay:

(a) Recovery of nitrite and nitrate from plasma is near quantitative (87%) and reproducible.

(b) Nitrite and nitrate are stable in (frozen) plasma for at least 1 year.

(c) Nitrite in whole blood is very quickly (>95% in 1 h) oxidized to nitrate, and therefore plasma nitrite determination alone is unmeaning,

(d) Plasma nitrite and nitrate concentrations were not correlated (nitrite as % of total nitrite + nitrate varied from 3.9% to 88% in plasma samples).

(e) Plasma samples should be de-proteinized, and background controls should be included in the assay, to avoid measuring the falsely high nitrite and nitrate concentrations in plasma.

S-NITROSOTHIOLS:

S-nitrosothiols are thio-esters of nitrite, with the general structure R-S-N=O; naturally occurring examples include S-nitrosocysteine, S-nitrosoglutathione and nitrosoalbumin, in which R is an amino acid, polypeptide and protein respectively. Reactive protein thiols are regarded as crutial intracellular target of NO. Nitrite is in steady state of equilibrium with S-nitrosothiols.

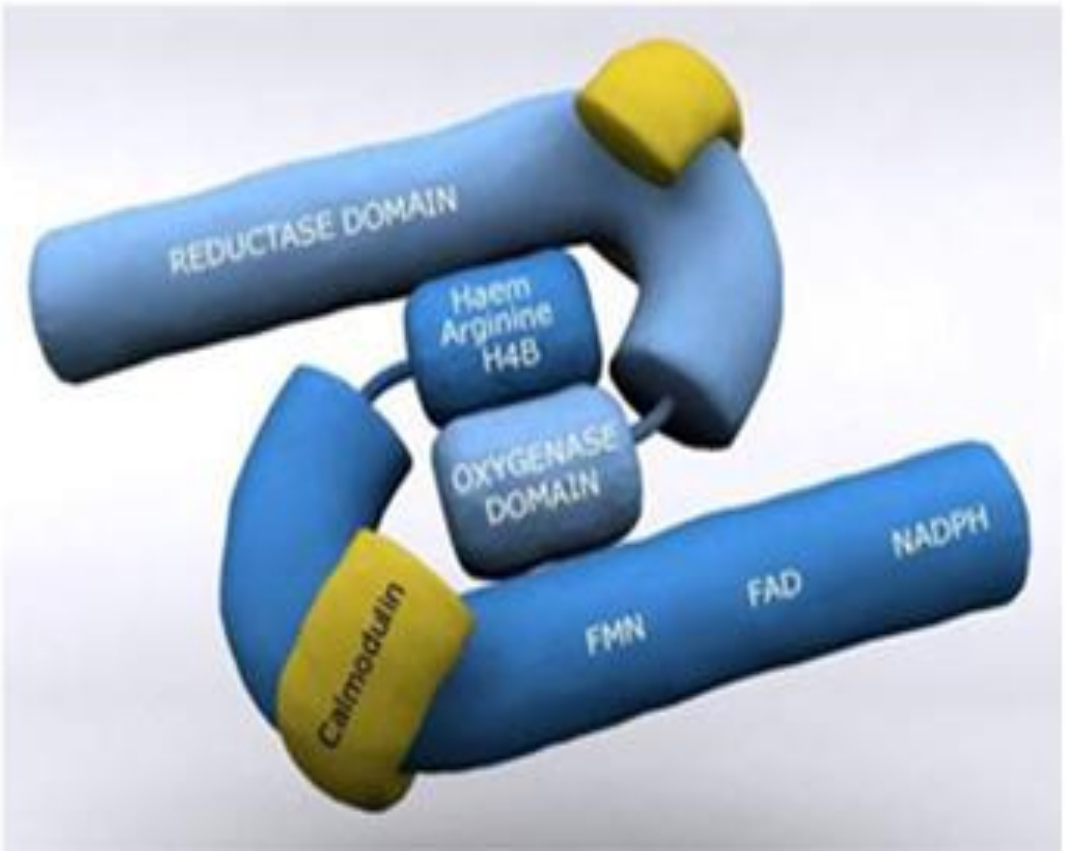
S-nitrosation has since been involved in the control of a broad array of protein functions and cell activities like, regulation of apoptosis, G-protein-coupled receptor based signalling, vascular tone and inflammatory responses [65]. Among the growing list of proteins whose activities are regulated by s-nitrosation some are, ion channel proteins, kinases, proteolytic enzymes, transcription factors and proteins involved in energy transduction [66]. Dysregulation of protein S-nitrosation is associated with a growing list of pathophysiological conditions and altered blood levels of RSNO have been associated with impaired clinical outcome in patients with cardiovascular disease [67].

S-nitrosoglutathione reductase (GSNOR), a member of alcohol dehydrogenase family, has been shown to be the primary pathway through which cells denitrosate intracellular proteins [68]. GSNOR has become an important target for developing agents that modulate NO bioactivity inside the cells.

NITROTRYOSINE:

NO reacts with superoxide anion to form peroxynitrite, that can further form peroxynitrous acid, a very unstable and reactive oxidizing species. Action of $ONOO^-$ is the most widely studied mechanism of protein nitration[69]. The formation of nitrotyrosine has been detected in various pathological conditions including atherosclerosis, myocardial infarction, hypertension, myocarditis heart failure, shock, diabetic complication and neurodegenerative and inflammatory disorders [70].

Figure 3:


REDUCTASE DOMAIN
Haem
Arginine
H4B
OXYGENASE
DOMAIN
NADPH
FAD
FMN
Calmodulin

Substantial evidence has emerged which reveal a very close association between the formation of nitro-tyrosine and the presence of activated granulocytes containing peroxidases, such as MPO [71]. MPO-generated reactive species participate in the induction of foam cell formation, endothelial dysfunction and development of plaque. Carr and Frei have revealed that, physiological concentrations of nitrite inhibit MPO mediated modification of LDL [72] providing a means to interrupt the process. These data also demonstrate the first line of evidence of nitrite acting as an "antioxidant" in atheroscleorosis. This may represent a novel mechanism by which metabolites of NO may exert an anti-atherogenic effect.

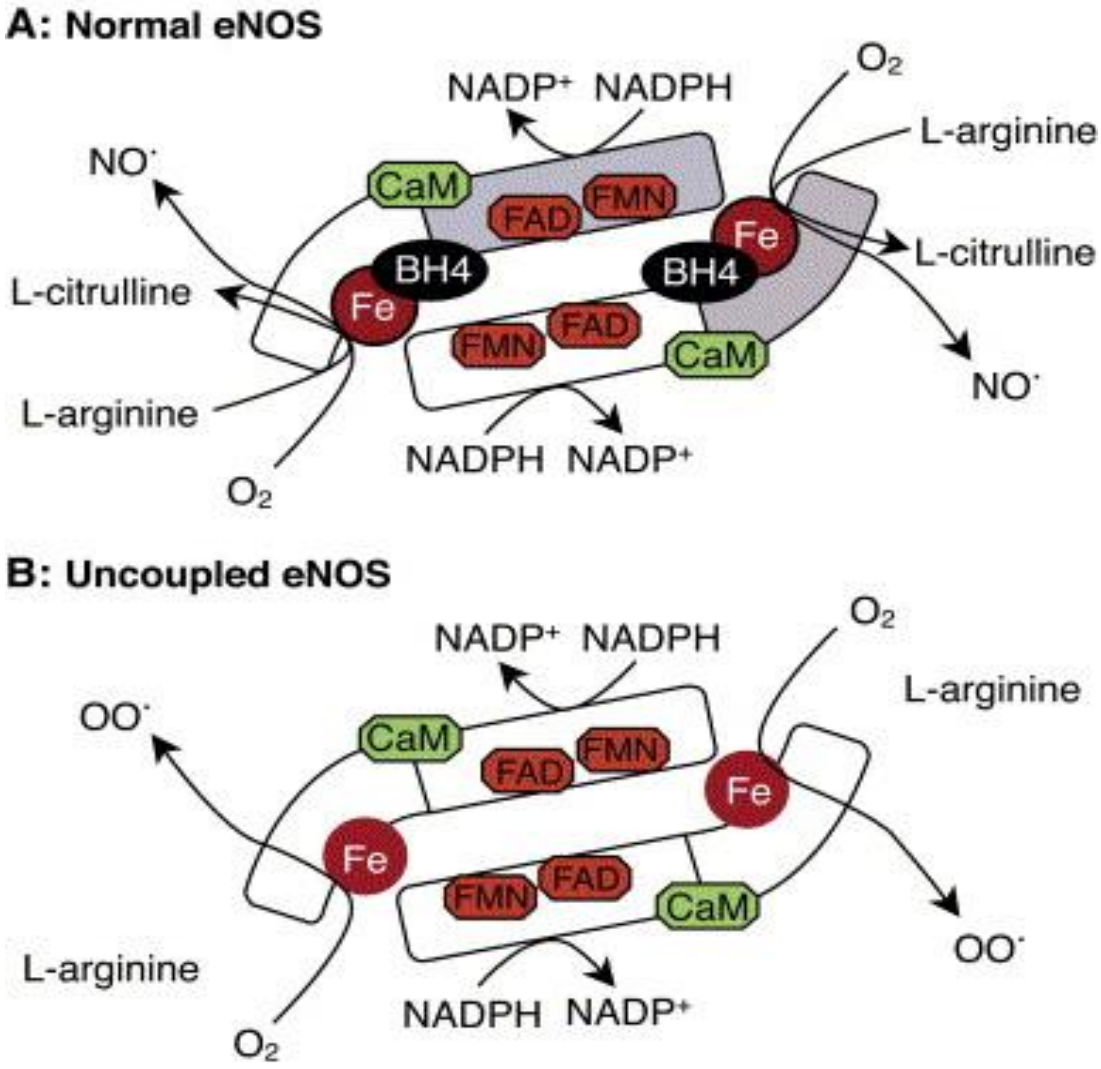
NITRIC OXIDE SYNTHASES:

The NOSs were first identified and described in 1989. The three major isoforms were cloned and purified between 1991 and 1994. The first X-ray crystal structures of NOS domains have been presented and published in 1998 and 1999.

Three quiet distinct isoforms of NOS have been identified as products of different genes, with different localization, regulation, catalytic properties, and inhibitor sensitivity, and with 51-57% homology between the human isoforms. These isoforms will be referred to, by the most common nomenclature, based on the order in which they were first purified and cloned.

Figure 4:

Regulation of Endothelial Nitric Oxide Synthase



nNOS (also known as Type 1, NOS-1 and NOS-**1**) being the isoform first found (predominating) in neuronal tissue.

iNOS (also known as Type 2, NOS-11 and NOS-2) being the isoform which is inducible in a wide range of cells and tissues.

eNOS (also known as Type 3, NOS-111 and NOS-3) being the isoform first found in vascular endothelial cells.

In the past, these isoforms have also been differentiated on the basis of their constitutive (eNOS and nNOS) versus inducible (iNOS) expression and their calcium – dependence (eNOS and nNOS) or independence (iNOS).

STRUCTURE:

NOS exhibits a bi-domain structure, in which an N-terminal oxygenase domain containing binding sites for heme, BH_4 and L-arginine, is linked by a calmodulin-recognition site to a C-terminal reductase domain, containing binding sites for FAD, FMN and NADPH[73.]. The known NOS enzymes are usually referred to as 'dimeric' in their active form, ignoring the required calmodulins (CaMs) which, strictly speaking, mean they are tetramers (of two NOS monomers associated with two CaMs). They contain relatively tightly bound cofactors BH4, FAD, FMN and heme.

NOS dimerization:

The association of the NOS into active dimers, involves a large interface in the oxygenase domain, involving two sections of the primary structure of NOS[74]. This interface includes the binding site for BH4 and helps to structure the active-site pocket containing the heme and the L-arginine binding site; it has two cysteine residues per monomer, which either forms a disulphide bridge between the monomers or ligates a zinc ion between the monomers[75]. Furthermore, there is an 'N-terminal hook domain', which swaps between the two monomers, to stabilize the dimer[76]. BH4 as well as heme and L-arginine promote and stabilize the active dimeric form of all the three isoforms.

The presence of heme appears to be mandatory, with BH4 and L-arginine promoting dimer formation and stabilization. The flow of electrons from reductase domain of one monomer, to the oxygenase domain of the other monomer forms NO.

Endothelial cell constitutive nitric oxide synthase can be activated by stimuli that include thrombin, adenosine, bradykinin, substance P, muscarinic agonists, catecholamines and shear stress[77].

REGULATION OF NOS:

CALMODULIN:

Calmodulin was the first protein shown to interact with NOS and is necessary for the enzymatic activity of the three isoforms. CaM binding increases the rate of electron transfer from the reductase domain to the heme centre[78].

PHOSPHORYLATION:

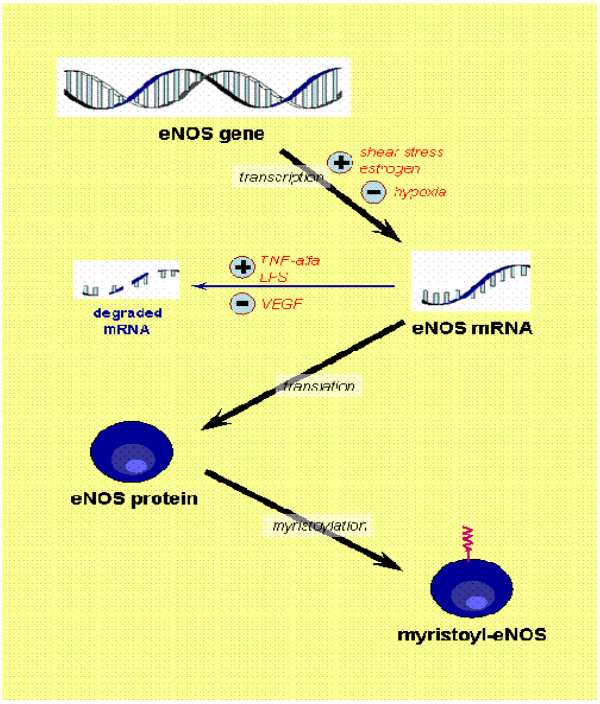
Phosphorylation of the nNOS and eNOS isoforms has an effect on NOS activity. Fluid shear stress elicits phosphorylation of eNOS and an increase in its activity[79].

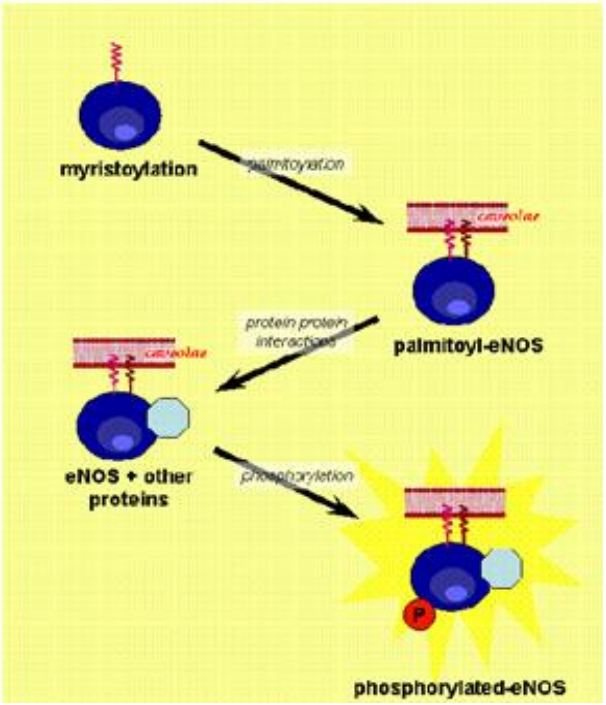
HEAT-SHOCK PROTEIN 90(hsp90):

The molecular chaperone hsp90 has been identified as a regulator of eNOS activity, possibly as an allosteric modulator[80]. Activation by vascular endothelial growth factor, histamine or fluid shear stess in human endothelial cells increases the interaction between eNOS and hsp90andincreaseseNOSactivity by approximately three-folds. Kallikrein appears to inhibit iNOS by preventing the formation of iNOS dimers[81] and may play a neuroprotective role during inflammation.

Figure 5:

REGULATION OF eNOS LOCALIZATION – MYRISTOYLATION & PALMYTOYLATION





REGULATION OF eNOS LOCALISATION:

MYRISTOYLATION and PALMITOYLATION:

Of the three NOS isoforms, only eNOS is acylated by both myristate and palmitate[82]. eNOS is co-translationally and irreversibly myristoylated at an N-terminal glycine residue while palmitoylation occurs post-translationally and reversibly at cysteine residues Dual acylation of eNOS is required for efficient localization to the plasmalemmal caveolae of endothelial cells[83]. Palmitoylation[84] is dynamically regulated by bradykinin – induced changes in intracellular Ca^{2+}.

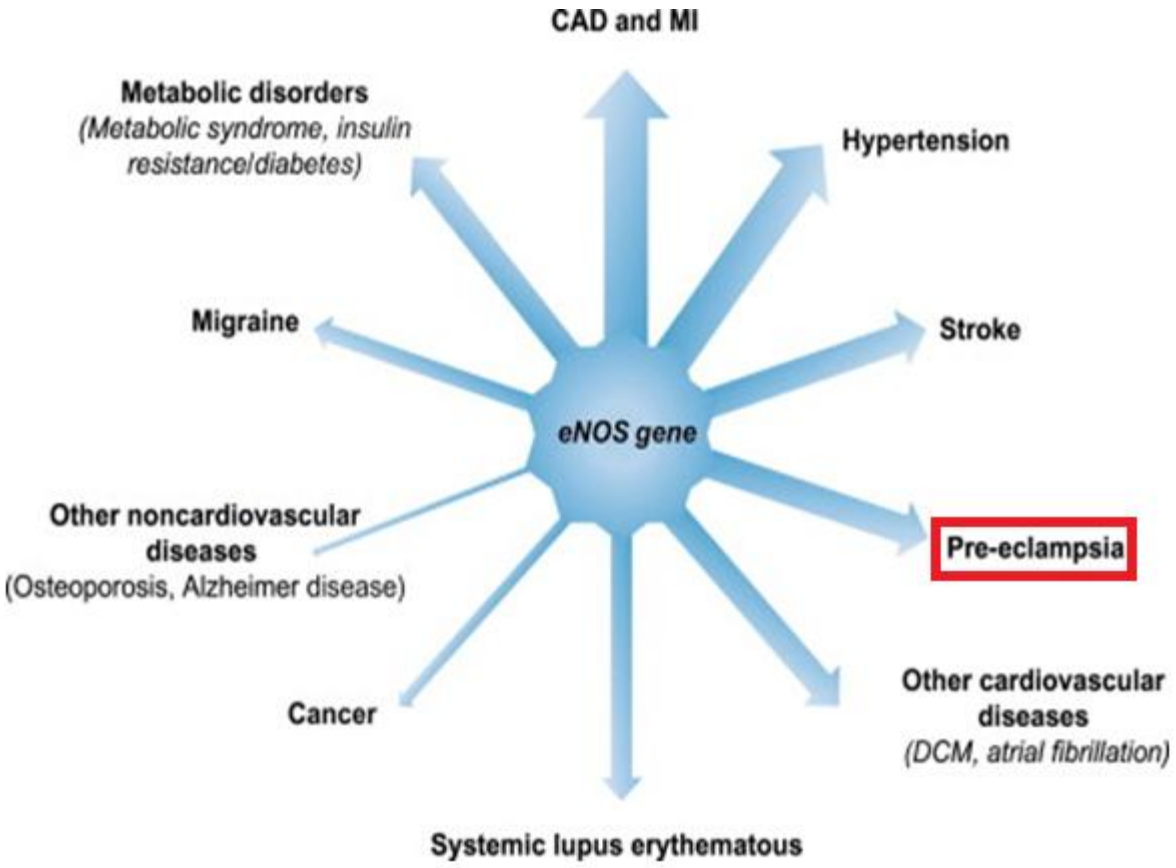
CAVEOLIN:

eNOS is localised to the caveolae[85], which are microdomains of the plasmalemmal membrane that are involved in a variety of cellular functions including signal transduction events. Vascular eNOS binds to caveolin-1, while in cardiac myocytes, eNOS is associated with caveolin-3[86]. Caveolin-1 and peptides from the 'caveolin-1 scaffold region' directly inhibit eNOS activity and this interaction is reversed by Ca^{2+}/CaM[87].Caveolin-3 binds to nNOS in the skeletal muscle, inhibiting NO synthesis and this inhibition is reversed by Ca^{2+}/CaM[88].

PHYSIOLOGICAL ROLE OF NOS:

Both eNOS and nNOS are constitutively expressed and are thought to contribute to the normal regulation of vasomotor tone and blood pressure[89]. eNOS derived NO was considered to be the most important regulator of vasomotor tone[90.]

Figure 6:


CAD and MI
Metabolic disorders
(Metabolic syndrome, insulin resistance/diabetes)
Hypertension
Migraine
Stroke
eNOS gene
Other noncardiovascular diseases
(Osteoporosis, Alzheimer disease)
Pre-eclampsia
Other cardiovascular diseases
(DCM, atrial fibrillation)
Cancer
Systemic lupus erythematous

eNOS AND VASOMOTOR TONE:

In the vascular endothelium, agonists such as acetylcholine and bradykinin stimulate inositol 1,4,5-triphosphate (IP_3) production the second messenger system. IP_3 binds to receptors on the endoplasmic reticulum and causes Ca^{2+} release from intracellular stores[91].

This transient elevation of intracellular Ca^{2+} promotes calcium binding to calmodulin, forming a complex that is a crucial cofactor for constitutive NOS activity[92].

eNOS GENE:

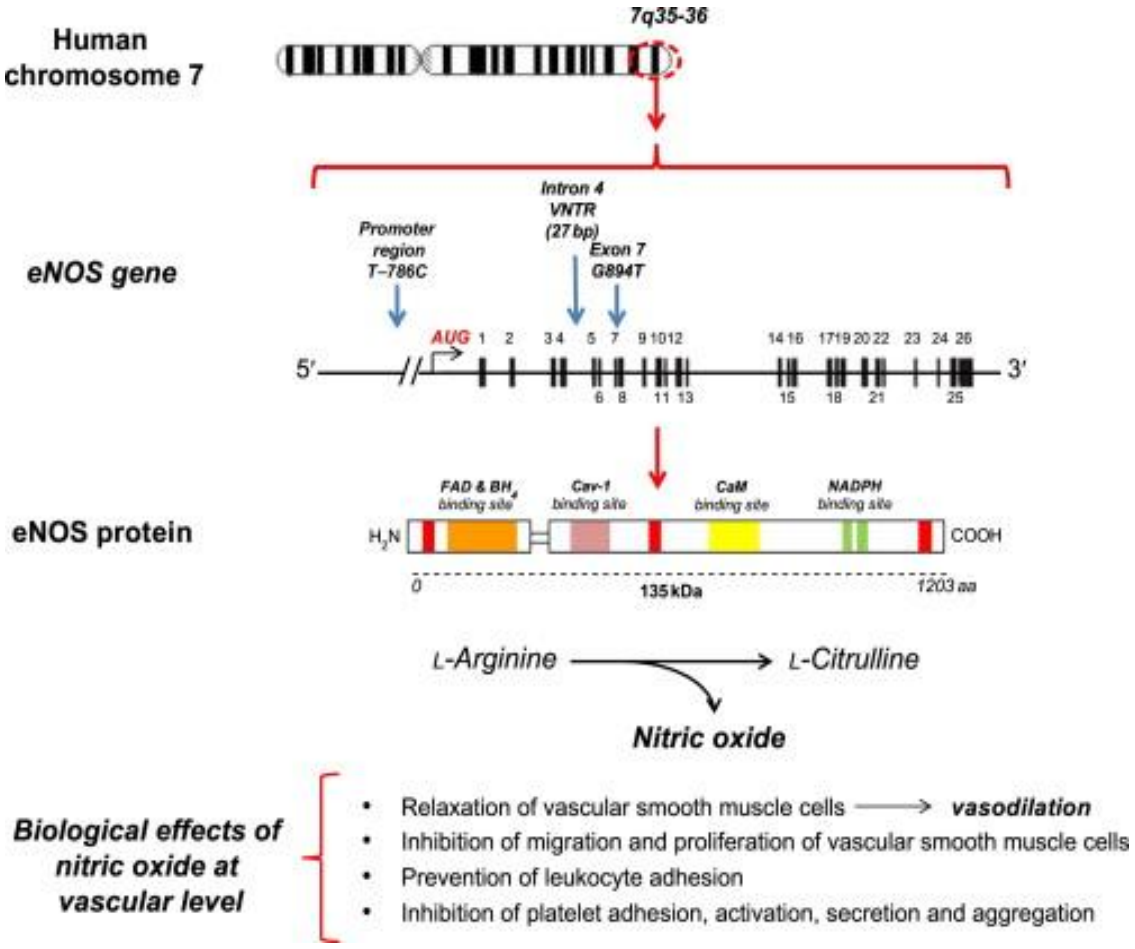
Given the pivotal role of eNOS in vascular homeostasis, the eNOS gene (NOS3) has emerged as a logical candidate gene in the investigation of hypertension genetics[93].

This down regulation of eNOS mRNA, undoubtedly, contributes to the reduced endothelial NO production and defective endothelium- dependant vaso relaxation[94] observed in diseased atherosclerotic vessel. It is now appreciated that decreased endothelial eNOS mRNA and protein can be observed in diseased human blood vessels.

NOS3 gene is located in the chromosome 7, corresponding to the 7q35-q36 region, contains 26 exons with an entire length of 21kb.

Figure 7:

REGULATION OF eNOS GENE EXPRESSION:



REGULATION OF eNOS GENE EXPRESSION:

Numerous exogenous stimuli and condition that are relevant to the pathology of vascular endothelium have been shown to alter eNOS expression through the modulation of steady-state eNOS mRNA[95]. This regulation happens at both the transcriptional and posttranscriptional levels.

TRANSCRIPTIONAL REGULATION:

eNOS CORE PROMOTER:

The eNOS gene encodes a mRNA of 4052 nucleotides and is present as a single copy in the haploid human genome. Sequence inspection of 5'-flanking regions revealed multiple potential *cis*-regulatory DNA sequences in the setting of a "TATA-less" promoter: Sp1, GATA, AP-1, NF-1, shear-stress response elements, and sterol-regulatory elements[96]. There are two tightly clustered *cis*-positive regulatory elements in the proximal promoter of the human eNOS gene.

Positive regulatory domain 1(PRD 1- 104 to -95 relating to transcription initiation) was mapped to a 10-bp *cis*-region corresponding to a high affinity Sp1 transcription factor recognition site. PRD 11 (-144 to -115) encompassed a 30-bp region of the core promoter and formed nucleoprotein complexes containing the transcription factors Elf-1, YY1,Spl and MAZ[185]. Mutating *cis*-elements in PRD 1 or PRD11 disrupted co-operative activation of the human promoter[97].

CELL SPECIFIC EXPRESSION:

eNOS gene expression is relatively restricted to the vascular endothelium. The existence of a distal enhancer element has the ability to direct the appropriate cell-specific expression pattern of the eNOS gene. The human eNOS proximal promoter was differentially methylated in expressing and non-expressing cell types[98]. In non-expressing cells, the core promoter CpG di-nucleotides were densely methylated, whereas in eNOS-expressing endothelial cells they were non-methylated. Differential promoter methylation was further implicated in the determination of cell-specific eNOS expression, by the successful induction of endogenous eNOS mRNA expression in non-expressing cell types. This may occur in response to de-methylation by 5-azacytidine, a DNA methyltransferase inhibitor. It has been suggested that promoter methylation down regulates transcription by recruitment of methyl-CpG binding proteins and histone de-acetylase activity, resulted in a close and transcriptionally repressive chromatin structure[99].

POST TRANSCRIPTIONAL REGULATION:

TUMOR NECROSING FACTOR (TNF):

TNF has been shown to decrease endothelium-dependant vaso-relaxation in vivo and ex vivo[100]. Some of the studies of eNOS gene regulation, identified modulation of mRNA stability as a regulatory target of pro-inflammatory cytokines[101]. Indeed, TNF the half- life of eNOS mRNA from 48 hours to 3 hours[102].

DUAL REGULATION:

TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL EFFECTS:

OXIDIZED LOW DENSITY LIPOPROTEINS:

Liao et al first reported that, oxidized low density lipoproteins (ox-LDL) caused a time and concentration-dependant decrease in steady-state eNOS mRNA and enzyme activity in human endothelial cells[103]. This down- regulation was found to occur principally at the post-transcriptional level, resulting in a reduction in eNOS mRNA half-life from 36 to 10 hours. Low concentrations are ox-LDL (≤10μg protein/ml) may be associated with a paradoxical increase in eNOS mRNA and protein expression[104]. Very high levels of native LDL were found to decrease eNOS mRNA expression[105]. Lysophosphatidyl choline a major component of ox-LDL, can increase eNOS mRNA in BAEC. Perhaps this provides a clarification for the biphasic effect of varying doses of ox-LDL.

eNOS GENE POLYMORPHISM:

Variants of the NOS3 gene located in the 7_q35-$_q36$ regions, contains 26 exons with an entire length of 21kb have been investigated for association with hypertension and other cardiovascular disorders. Of these, three polymorphisms have been broadly examined for clinical relevance, based on their potential functional effects and their relatively high minor allele frequency in various ethnic groups[106.]

(1) a G894T substitution in exon7 resulting in a Glu to Asp substitution at codon 298 (rs1799983).
(2) an insertion deletion in **intron4** (4a/b) consisting of two alleles (the a-deletion which has four tandem 27-bp repeats and the b-insertion having five repeats).
(3) a T786C substitution in the promoter region (rs2070744).

Several polymorphisms of eNOS gene are found to be associated with increased risk for cardiovascular disease. Of these 894 G greater than T variant in exon 7 is reported to be associated with cardiovascular disease [107] while 786 T greater than C polymorphism has been associated with hypertension and with coronary spasm. The transition markedly blunts the transcription of eNOS Gene and hence the nitric oxide production. The C allele creates the binding site for replication protein A1 (RPA1) that's acts as a suppressor for eNOS transcription. The transition markedly blunts the transcription of eNOS gene and hence the nitric oxide production. Replication protein A1 resides in endothelial cells and placenta. Hence Preeclampsia women are associated with a high frequency of promoter T-786C polymorphism presents with low serum levels of Nitric oxide.

The endothelial NOS isoform appears to be localized in the resistance vasculature of the placenta, but not in the capillary endothelium of terminal villi where there is no underlying smooth muscle. The pattern of staining with NBT is similar to that of endothelial NOS and suggests that other isoforms of NOS are not present in the placental unit[108].

A gene polymorphism is defined as the regular occurrence (>1%) in a population of two or more alleles at a particular chromosome location. For promoter -786 T>C polymorphism, the wild-type allele (-786T allele) is uncut upon Msp I digestion and is detected as a 178- bp band, whereas the polymorphic allele (-786C allele) is cut into two fragments detected as a 137- and a 41-bp bands. Therefore, wild-type homozygous individuals should generate a single 178-bp product, heterozygous individuals should generate three fragments 178,137-and a 41-bp, while mutant homozygous individuals should generate a two; 137- and a 41-bp fragments[109].

Nitric oxide (NO) is a potent vasodilator, considered to have major effects on gestational endothelial function[110]. NO is synthesized by nitric oxide synthases (NOS), namely endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS[111]. Studies investigating circulating levels of NO in preeclampsia have reported conflicting results[112]. This is in contrast to studies that have shown that plasma from women with preeclampsia elicits reduced endothelium-dependent vasodilatation in isolated vessels. NO availability may be decreased[113.]because of oxidative stress, vascular endothelial growth factor deficiency, or endogenous inhibitors, such as asymmetric dimethylarginine[114]. It is assumed that most of the circulating NO derives from maternal endothelium. But, the placenta may also contribute.

In the placenta, NOS is predominantly expressed in the syncytiotrophoblast, villous endothelium, and macrophages; the predominant isoform being eNOS[115]. The multinucleated syncytiotrophoblast layer lining the chorionic villi is the interface between the maternal and fetal vascular systems[116] and could contribute to circulating NO.

The gene for eNOS is located on chromosome 7 (7q35-q36), as a single copy of 26 exons with an entire length of 21 kb, and encodes an mRNA of 4052 nucleotides [117]. Disruption of the eNOS gene leads to hypertension in mice [118] and inhibition elevates blood pressure in healthy humans. eNOS is a constitutively expressed isoform whose activity is dependent on the intracellular changes in Ca2+ concentrations. eNOS expressed in the terminal villous vessels in the syncytiotrophoblast is the main enzyme required for vascular NO production and influences placental human chorionic gonadotrophin production during gestation. Trophoblast cells in the first trimester express high amounts of eNOS [119]. Studies undertaken to find eNOS gene variations have revealed that many cis- and trans-acting factors regulate the expression of eNOS and that its expression level in turn directly corresponds to the amount of NO in the blood [120].

It has been shown that polymorphisms in the coding and non-coding regions of eNOS may alter eNOS expression and/or activity and thus cause a decrease in NO synthesis [121], which may predispose patients to hypertension, vasospasm and atherosclerosis, renal failure and pre-eclampsia[122].

Nitric oxide (NO) plays a major role in vascular homeostasis. Produced in endothelial cells and platelets by endothelial NO synthase (eNOS) [123], NO mediates vascular dilation, inhibits platelet aggregation, posttranslational protein modification, cell migration and angiogenesis, and apoptosis [124]. Indeed, reduced bioavailability of NO has been linked to numerous important cardiovascular pathological processes [125] and there is now evidence of a genetic contribution to the variability in NO formation [126]. Due to the relevance of NO in the regulation of the cardiovascular system, eNOS gene polymorphisms have been associated with cardiovascular diseases [127].

The endothelial nitric oxide synthase (eNOS) gene has been enlisted by previous research as a candidate gene of preeclampsia predisposition. This study investigates the specific roles of 3 polymorphisms of the eNOS gene in a population of Chinese origin from mainland China. The 3 commonly studied polymorphisms of the eNOS gene, namely 4b/a, T786C and Glu298Asp, in a case-controlled sample of 220 patients diagnosed with preeclampsia and 200 healthy controls. The association between eNOS polymorphisms and preeclampsia was

evaluated by performing genotyping for the eNOS variants and calculating odds ratios (OR) and 95% confidence intervals. The plasma nitrite concentration in participants was determined to examine how 3 eNOS polymorphisms affect plasma nitric oxide (NO) concentrations in pregnant women. Polymorphisms of the Endothelial Nitric Oxide Synthase Gene in Preeclampsia in a Han Chinese Population[128]. As a potent vasodilator, circulating nitric oxide (NO) plays a crucial role in endothelial function regulation, blood pressure control, and cardiovascular homeostasis, and NO is essential for a predisposition to preeclampsia.

NO has been shown *in vitro* and *in vivo* to modulate placental circulations, and the inhibition of NO production has caused preeclampsia-like syndromes in pregnant rats. Endothelial nitric oxide synthase (eNOS) is an enzyme which synthesizes NO constitutively via catalyzing the conversion of L-arginine to L-citrulline. Because endothelial NO availability is largely regulated by its synthesis by eNOS, the gene that encodes eNOS, NOS3, is considered as a candidate gene for preeclampsia. Three NOS3 polymorphisms have been extensively studied: G894T (a guanine/thymine substitution at position 894 on exon 7 leading to a change from glutamate to aspartate at position 298; rs1799983); T-786C mutation (a thymine/cytosine substitution at position 786 in the 5'-flanking region of promoter; rs2070744); and a variable number of tandem repeats (VNTR) 4b/a polymorphism [the a* -deletion allele with 27 bp VNTR in intron 4 [129].

AIMS AND OBJECTIVES

AIM & OBJECTIVES

AIM OF THE STUDY:

- To find an association between eNOS gene polymorphism and preeclampsia.

OBJECTIVES:

- To find out the distribution of allelic variant in promoter T-786C of eNOS gene in our population.
- To look for any association between gene polymorphism and Preeclampsia.
- To assess the Nitric oxide level in study group.
- To correlate Nitric oxide level with eNOS gene polymorphism.

MATERIALS AND METHODS

MATERIALS AND METHODS

STUDY DESIGN : CASE-CONTROL STUDY

PLACE OF STUDY : Department of Biochemistry,

Department of Obstetrics and Gynaecology,

Govt.Kilpauk medical college,

Chennai – 10

DURATION OF STUDY: 6 Months

SAMPLE SIZE : 100

SAMPLE SELECTION:

Cases: 50 Preeclampsia women in the age group of 20-40 years.

Controls: 50 healthy pregnancy women.

(Age and Risk Factor matched).

No history or clinical evidence suggestive of Preeclampsia.

Confidence interval is taken at 95%

Power of the study: 75%

Ratio of cases and controls: 1:1

Prevalence of the disease: 8 %

INCLUSION CRITERIA:

Pregnant women age group of 20-40 years.

Gestational age of 24 to 36 weeks of pregnancy.

Blood pressure higher than 140/90 mmHg on two occasions more than 6hrs apart and proteinuria.300mg/24hrs, after the 20th week of pregnancy.

EXCLUSION CRITERIA

- Smokers
- Renal disease
- Chronic illness
- Diabetes mellitus
- Systemic hypertension
- Acute/Chronic infection
- Ischaemic stroke &Coronary artery disease
- Fever
- Previous H/O Recurrent pregnancy loss
- Previous H/O preeclampsia
- Anti Phospholipid Antibody Syndrome
- Multiple pregnancy
- Hypothyroidism

SAMPLE COLLECTION:

5 ml of Fasting venous blood sample: Collected in 2 tubes. 2ml in anticoagulant (1 part of 5 % EDTA for each parts of blood) and 3 ml in plane tube and mixed by gentle shaking. Whole blood used for DNA extraction and serum used for biochemical analysis.

Investigations:

The following investigations are to be done in Cobas C311 auto analyser:

1. Blood sugar – Hexokinase method

2. Urea – GLDH Method

3. Creatinine –Jaffe's Method

4. Uric Acid – Modified Trinder Method

5. Serum Nitric Oxide – Cadmium based reduction of nitrate to nitrate followed by griess Method

6. Urine Microprotein – TCA Method

7. Serum Calcium – Ortho-cresolphthalein Complexone Method

8. Genetic Polymorphic studies includes

I. **DNA EXTRACTION**:

- Double-Stranded DNA will be isolated from human body by lysis of leukocytes with Subsequent selective DNA precipitation with detergent.
- This DNA is concentrated and desalted by ethanol precipitation.
- Purity of DNA is to be ascertained by calculating the A260/A280 ratio.

II. GENOMIC DNA AMPLIFICATION BY PCR:

Using primers flanking the polymorphic region of the eNOS gene.

III. 2 % AGAROSE GEL RUN:

Following PCR, the presence of a 365bp Product will be ascertained by using 2 % agarose gel .Where it is visualized as a discrete band using a gel documentation system.

DNA EXTRACTION BY KIT METHOD:

DNA MINIPREPARATION KIT:

From helini biomolecules, Chennai.

PRINCIPLE :

On short incubation with proteinase K and in the presence of chaotropic salt, cells are lysed which immediately inactivates all nucleases. Nucleic acids in the cells, attach selectively to special glass fibres pre-packed in the Purefast purification filter tube. In a series of rapid 'wash and spin' steps it removes the contaminating cellular components, thus bound nucleic acids get purified. A special inhibitor removal buffer was added, this allows even the application of heparinised sample material with 100U/ml of heparin. Finally low salt elution buffer releases the nucleic acids from the glass fibre. This method eliminates the need for organic solvent extractions and DNA precipitation, allowing for rapid purification of many samples simultaneously.

COMPONENTS OF THE KIT

- Proteinase K solution
- Lysis buffer
- Wash buffer 1 & 2
- Isopropranolol
- Elution buffer
- Spin columns with collection tubes

PROTEINASE K SOLUTION STORAGE:

Solution were stored at -20 °C. It is stable for at -20°C for upto 6 months.

PROCEDURE FOR DNA PURIFICATION:

PRE-PROCEDURE STEPS:

- Set water bath to 56°C
- Warm elution buffer by keeping in water bath at 56°C
- Prepare fresh 1.2 ml of 70% ethanol per sample.

BLOOD / BUFFYCOAT:

200µl of blood or 200µl of buffy coat is added to a nuclease free 1.5ml microcentrifuge tube and the following steps are carried out.

1. 400µl of lysis buffer is added and
2. Immediately mixed well by inverting several times (or gently vortex)
3. 20µl of proteinase K was added.

4. Mixed by vortex for 10 seconds.
5. 300µl of isoproponalol was added and mixed well by inverting several times.
6. The sample was pipetted into the Purefast spin column and
7. Centrifuged at 12,000rpm for 1min.
8. The flow-through was discarded and the column is positioned back into the same collection tube.
9. 500µl of Wash buffer I was added to the Purefast spin column and
10. Centrifugation process for 1minute at 12,000 rpm and the flow-through is discarded and the column was placed back into the same collection tube.
11. 500µl wash buffer II was added to the Purefast spin column.
12. Centrifuged at 12,000rpm for one minute and the flow-through discarded the column was placed back into the same collection tube.
13. Do empty spin column centrifuge at 13000rpm for 2 minutes.
14. The flow-through was discarded and centrifuged for an additional. This step was essential to avoid residual ethanol.
15. The Purefast spin column is transferred into a fresh 1.5 ml microcentrifuge tube.
16. 60µl of the pre-warmed elution buffer was added to the centre of Purefast spin column membrane.

17. Take care not to contact the membrane with the pipette tip. Incubation pre warmed was done at room temperature for two minutes and is centrifuged for two minutes.
18. The column was discarded and the purified DNA was deep freezed at -20°C.

IDENTIFICATION

Extracted DNA was identified by 1% agarose gel electrophoresis and comparison with a known molecular weight 1kb DNA (Lambda DNA) ladder.

POLYMERASE CHAIN REACTION

HELINI rs 2070774 [C>T] Human SNP Genotyping PCR Kit

Kit components

No.of Reactions	50 tests
Red dye PCR Master Mix - 10µl/reaction	500 µl
Rs2010963 Primer Mix – 2.5µl/reaction	125 µl
PCR grade water	4 ml

C allele: PCR product size: 365 bp C

T allele: PCR Product size: 211 bp T

Control PCR Product size: 576 bp Control

C allele:

F:CCTCCACTGCTTTTCAGAGG

R:CTGAGGCAGGGTCAGACG

365bp

T allele:

F:CATCAAGCTCTTCCCTGTCT

R:TGACATTAGGGTATCCCTTCC

211bp

PRIMER RECONSTITUTION

Primers were supplied in lyophilized form. Millipore double distilled water was used to prepare 100× concentrations i.e. 10 times the molecular weight of primer is the volume of water required to prepare 100× concentrations which is 100µmolar solution.

From this stock solution 10× concentration was prepared as the working solution for PCR.

Both forward and reverse primer were spinned.

For each polymorphic site TEA buffer was taken at a particular volume corresponding to the respective forward & reverse primer of 100 pmol/µl.

Inverted & Vortexed &mixed for 5 minutes & were centrifuged

Eppendorf were named for the polymorphic sites.

180 µl of sterile distilled water were taken in the labelled Eppendorf.

10 µl forward primer + 10 µl reverse primer were added

200 µl of reconstituted primers containing both forward and reverse primers for the respective polymorphic sites.

MASTER MIX:

2×PCR Master mix was used in the following composition

Master Mix consists of basic components necessary for PCR.

Reaction buffer consisted of Tris Hcl - pH 8.5, $(NH_4)_2SO_4$, MgCl2 – 3 mM acts as catalyst & 0.2% Tween 20.

dNTP' s were used in a concentration of 0.4 mM each.

Taq polymerase in a concentration of 0.2 U/ µl.

Primers were used in a concentration of 10 pmol and DNA was used in a concentration of 200ng.

PCR was carried out in a reaction in volume of 20µL with the following components in the following manner,

PCR master mix (contains gel loading dye)	– 10 µL
Reconstituted primers (for the site+83)	– 5.0 µL
DNA	– 5.0µL

Total	- 20.0µL

POLYMERASE CHAIN REACTION (PCR)

Amplification of the extracted DNA was carried out in CYBERLAB SMART PCR-PRO, thermal cycler with the following cycling conditions.

Negative Control

Use 7.5µl of nuclease free water

Centrifuge PCR vials briefly before placing before into thermal cycler.

STEPS INC-786T (PROMOTER) OF NITRIC OXIDE SYNTHASE GENE PCR POLYMORPHIC SITE:

- Initial denaturation - 95° C /5min

  34cycles of

- Cycle Denaturation at 94°C for 30 seconds
- Cycle Annealing at 58°C for 30 seconds
- Cycle Extension at 72°C for 30 seconds
- Final Extension at 72°C for 10min

Amplified product–amplicons of 365 bp was identified by 2.5% agarose gel electrophoresis by comparison with a known 100bp DNA ladder.

AGAROSE GEL ELECTROPHORESIS

- Agarose gel preparation
- Gel casting
- Sample preparation
- Electrophoresis

Material Required

Micro centrifuge

UV transilluminator (optional) (Cat.no.09-01)

Mini submarine electrophoresis unit with power pack (Cat.No.03-01P)

Micro oven or water bath

Micropipettes & fresh tips (10µl - 200µl, 200µl-1000µl)

Conical flask, ice bath or crushed ice

Micro centrifuge tubes, measuring cylinder

Distilled water

Material Required

Materials	**15 Exp**	**Storage**
Agarose	5 gm	Room temperature
50X TAE	100 ml	Room temperature
6X gel loading dye	500µl	-20°C
Ethidium bromide	100µl	-20°C

Figure 8:

GEL DOCUMENTATION OF EXTRACTED DNA:

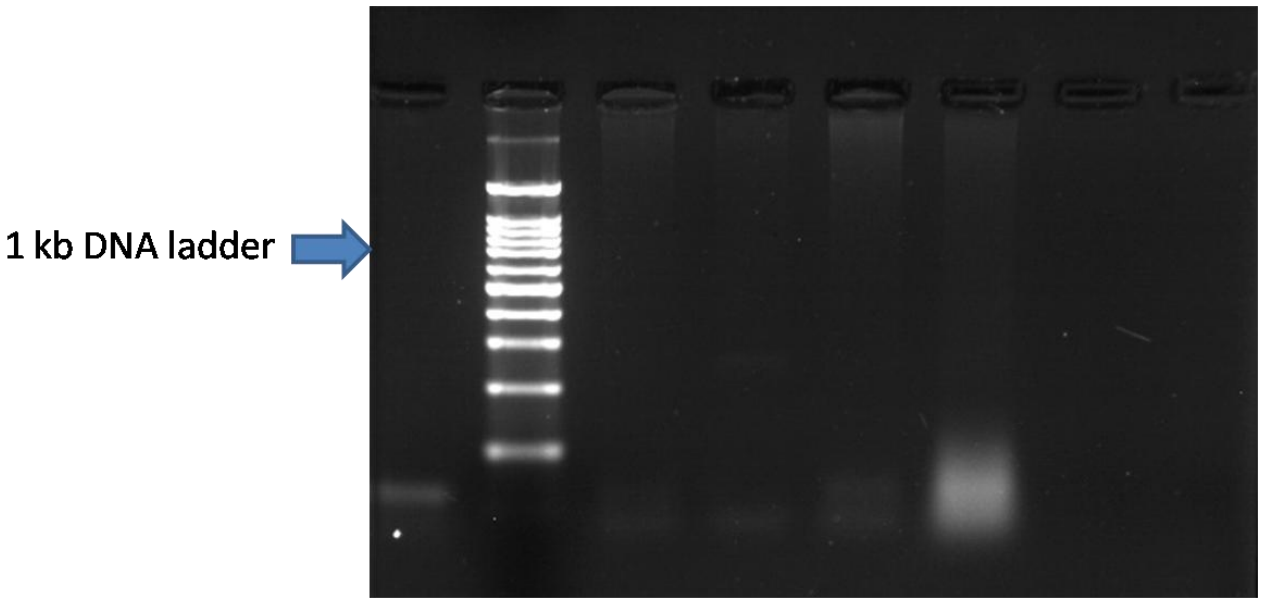
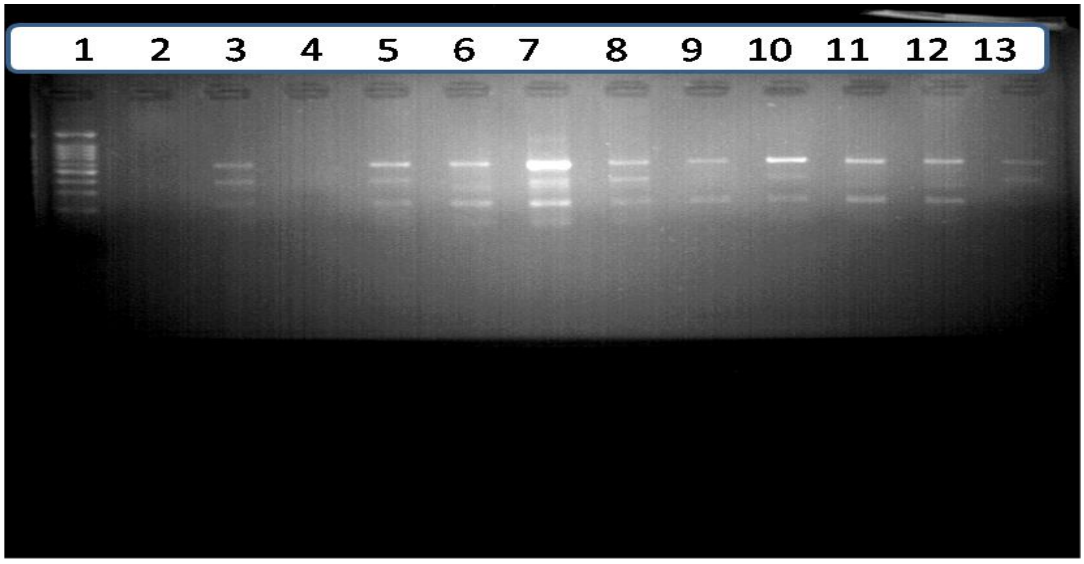


Figure 9:

ELECTROPHORETOGRAM OF PCR PRODUCTS



Lane 1 – 100 bp DNA ladder
Lane 2,4 – Negative control
Lane 3,5,6,7,8,10 – CT genotype
Lane 9,11,12-TT genotype
Lane 13 – CC genotype

- PCR product was run on 2.5% agarose gel in a 25 ml agarose cast as follows: 0.625g of agarose was weighed and dissolved in 25 ml of TAE buffer with a pH of 8.0.
- It was microwaved for 60 secs, cooled and ethidium bromide was added in a concentration of 0.5 µg/ml from the stock of 10mg/ml added. It is poured into a cast and allowed to solidify for 45 min before it was kept in the electrophoresis tank.
- 10µL of PCR product was loaded onto wells and 4µL of 100bpDNA ladder was loaded onto single well as a marker. It is run in an electrophoresis tank for 30min and visualized under UV illumination in e gel imager from life technologies.
    - C allele : PCR product size : 365 bp C
    - T allele: PCR Product size: 211 bp T
    - Control PCR Product size : 576 bp Control

C ALLELE-PCR product size 365bp C

T ALLELE-PCR product size 211bpT

Control PCR Product size : 576 bp Control

CC (homozygous individuals) - will yield 365bp, 576bp

CT (heterozygous individuals) - will yield 365bp, 576bp, 211bp

TT (homozygous individuals) - will yield 211bp, 576bp

MEASUREMENT OF SERUM NOx:

CADMIUM BASED REDUCTION OF NITRATE TO NITRATE FOLLOWED BY GRIESS METHOD:

Principle:

In this method, cadmium, exposed to copper sulphate solution reduces Cu^{2+} form a porous metallic copper "coat". This copper facilities electron transfers from cadmium to nitrate, thereby reducing nitrate to nitrate. Cadmium has a reduction potential of -0.403 V. On the other hand, redox potential of nitrate/nitrate is dependent on pH ranging from 0.94 V in acidic solution to 0.015V in basic solution. Therefore, reduction reaction of nitrate to nitrate by cadmium is thermodynamically favourable.

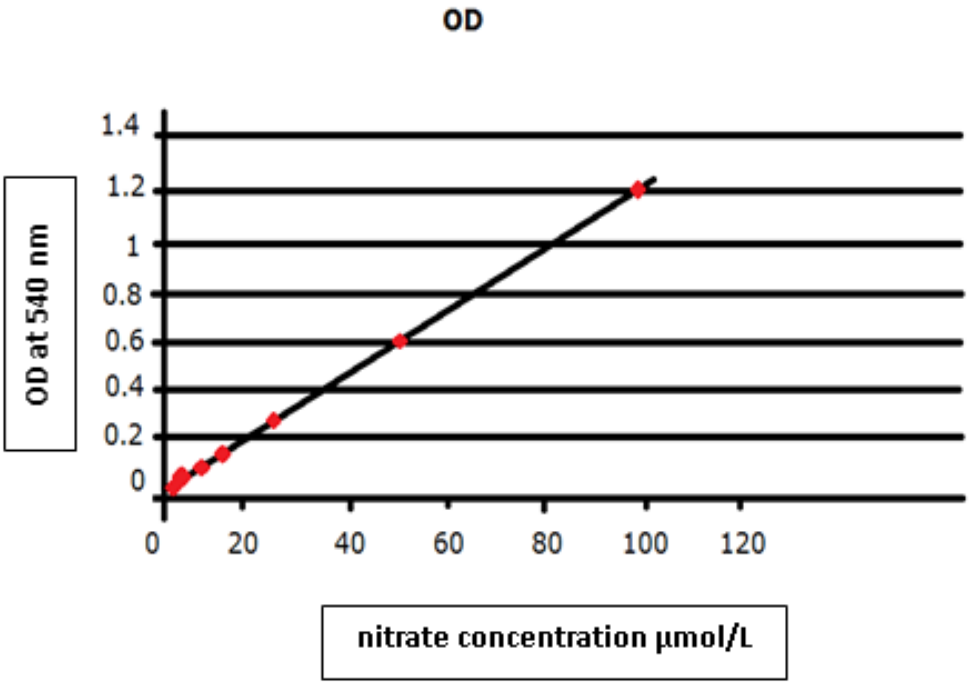
Nitrate reacts with sulfanilamide in acidic media to form transient diazonium salt. N-naphthyl-ethylenediamine (NED) converts this salt to a stable azo compound with intense purple color, measured at 540 nm.

Procedure:

Step 1: De-proteinisation:

300 µl of serum by adding 250 µl of 75 mmol/L $ZnSO_4$ solution, stirring, and centrifuging at 10000_g for 1 minute at room temperature, after which 350 µl of 55 mmol/L NaOH was added.

Graph 1:


OD
OD at 540 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
nitrate concentration µmol/L

Again, the solution was stirred and centrifuged at $10{,}000_g$ for 3 minutes and the supernatant was recovered (free of turbidity). Then diluted by mixing 750µl of supernatant with 250 µl of glycine buffer (45g/L, pH 9.7).

Step 2:

Cadmium granules were rinsed three times, with deionized distilled water and mixed in a shaker gently in a 200 mmol/L copper sulfate^{231}solution in glycine-NaOH buffer (15 g/L, pH 9.7) for 5 minutes till the color of the solution fades. The solution was drained off and the step was repeated. The copper-coated granules dried in tissue paper, should be used within 10 minutes. After use, the granules were rinsed and stored in 100 mmol/L H_2SO_4 solution: they can be regenerated by repeating the steps.

Step 3:

The nitrite and nitrate calibrators were diluted with glycine buffer. Calibration curves were made over a linear range of nitrite between 0 and 100 umol/L. Freshly activated cadmium granules (2-2.5g) were added to 1 ml of pre-treated de-proteinized serum and calibrator. After continuous stirring for 10 minutes, the samples were transferred to appropriately labeled tubes for nitrate determination.

Step 4: Nitrite Assay:

Nitrite was estimated by Griess method.

Reagent 1:5 mg of ***N***-naphthyl ethylenediamine dissolved in 250 ml of distilled water.

Reagent 2: 5 g of sulfanilic acid in 500 ml of 3 mol/L HC1.

Both solutions are stable for one year at 4 ^{o}C.

From the above tubes 200 µl of sample were placed into fresh glass tubes. Then 800 µl sulfanilic acid solution was mixed, followed by 750 µl NED solution.

After an incubation period of 10 minutes at temperature, pink color was developed and its absorbance read at 545 nm within 60 min. The measured OD was plotted on the standardization graph and concentration found out. (Graph: 1)

Estimation of Glucose in Blood (Hexokinase Method).

Principle:

Hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate by ATP. Glucose-6-phosphate dehydrogenase oxidizes glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and is measured photometrically.

hexokinase

Glucose + ATP ----------> G-6-P + ADP

Glucose 6 phosphate dehydrogenase

G-6-P + NADP+----------> Gluconate-6-P + NADPH + H^+

Reagents:

R1

- MES buffer: 5.0 mmol/L, pH 6
- Mg2+: 24 mmol/L
- ATP:> 4.5 mmol/L
- NADP: > 7.0 mmol/L
- Preservative

R2

- HEPES buffer: 200 mmol/L, pH 8.0
- Mg2+: 4 mmol/L
- HK (yeast): > 300 µkat/L
- G-6-PDH (E. coli): > µkat/L
- Preservative.

Reagent preparation

Reagent are provided ready to use.

Procedure

The assay was carried out using Cobas C311 autoanalyser.

Reference Range

Normal serum level: FBS: 70 – 109 mg/dl and PPBS – 80-139 mg/dl.

Estimation of serum urea (Kinetic test with urease and glutamate dehydrogenase method)

Principle:

The urea is hydrolysed by urease to produce ammonia and carbonate. In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD+ for each mole of urea hydrolyzed.

The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen and is measured photometrically.

$$\text{Urea} + 2\,H_2O \xrightarrow{\text{Urease}} 2\,NH_4^+ + CO_3^{2-}$$

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{Glutamate dehydrogenase}} \text{L-glutamate} + NAD^+ + H_2O$$

Reagent

R1

- NaCl 9%

R2

- TRIS buffer: 220 mmol/L, pH 8.6;
- 2-oxoglutarate: 73 mmol/L;
- NADH: 2.5 mmol/L;
- ADP: 6.5 mmol/L;
- Urease (jack bean): > 300 µkat/L;
- GLDH (bovine liver): > 80 µkat/L;
- Preservative;
- Nonreactive stabilizers

Procedure

The estimation was carried out by Cobas C 311 autoanalyser.

Reference range

Normal serum level: 15 – 40mg/dl.

Estimation of serum Creatinine (Buffered Kinetic Jaffe's Reaction without deproteinisation)

Principle

In alkaline solution creatinine reacts with picrate to form a yellow-red adduct. The rate of the dye formation (color intensity) is directly proportional to the creatinine concentration in the specimen and is measured photometrically.

Creatinine + picric acid --------> yellow-red complex.

Reagents

R1

- Potassium hydroxide: 900 mmol/L;
- Phosphate: 135 mmol/L; pH> 13.5;
- Preservative;
- Stabilizer

R2/R3

- Picric acid: 38 mmol/L; pH 6.5;
- Non reactive buffer

Procedure

The estimation was carried out using Cobas C 311 autoanalyser.

Reference range

Normal serum level: Men: 0.9-1.3mg/dl.

Women: 0.6-1.1mg/dl.

ESTIMATION PF CALCIUM BY ORTHO-CRESOLPHTHALEIN COMPLEXONE METHOD (OCPC METHOD)[232]

Principle:

Ortho-cresolphthalein reacts with calcium in alkaline solution to form a purple coloured complex. The intensity of the purple colour formed is proportional to the calcium concentration and is measured photometrically at 578nm. Interface from magnesium is overcome by the presence of 8-hydroxyl quinoline in reagent 2 which binds free magnesium ions.

Reagents:

Reagent 1: AMP Reagent

2-Amino-2-methyl-1 propanol	505mmol/L
Surfactant	-

Reagent 1: OCPC Reagent

OCPC	0.06mmol/L
8-Hydroxy Quinoline	6. 9 mmol/L
HCl	45 mmol/L
Surfactant	-

Calcium Standard:

Calcium Standard	10.0mg/dl (2.5 mmol/L)

Working reagent preparation:

Allow the Reagent '1' and Reagent '2' to attain room temperature. Just prior to use, prepare working reagent by mixing equal quantities to Reagent 1 and Reagent 2.

Specimen:

Fasting serum or heparinized plasma can be used, other anticoagulants such as citrate, oxalate and EDTA are unsuitable.

Assay Parameters:

Mode	**End Point**
Wavelength 1 (nm)	578
Wavelength 2 (nm)	630
Sample Volume (µl)	5/10
Reagent Volume (µl)	500/100
Incubation Time (min)	1
Incubation Temp. (^{o}C)	37
Normal Low (mg/dl)	8.4
Normal High (mg/dl)	10.4

Mode	End Point
Linearity Low(mg/dl)	0
Linearity High(mg/dl)	20
Concentration of Standard (mg/dl)	10
Blank with	Reagent
Absorbance Limit (Max)	0.500
Units	(mg/dl)

Procedure:

Pipette into tubes marked	Blank	Standard	Test
Working Calcium Reagent	1000µl	1000µl	1000µl
Distilled Water	10µl	-	-
Standard	-	10µl	-
Test	-	-	10µl

Standardization:

Pipette the following into appropriately labeled test tubes. The contents of the tubes are mixed well. The spectrophotometer is set to zero with blank at 578 nm.

Pipette into tubes	Blank	S1	S2	S3	S4	S5	S6	Test
Standard (µl)	0	2	4	6	8	10	12	-
Distilled water (µl)	12	10	8	6	4	2	0	-
Sample	-	-	-	-	-	-	-	10
Reagent	1000	1000	1000	1000	1000	1000	1000	1000
Conc.Of Calcium (mg%)	0	2	4	6	8	10	12	9.28
Absorbances	0		0.09	0.20	0.36	0.45	0.52	0.40

Mix well and at 578 nm against reagent blank.

Calculation:

Calcium (mg/dl) =$\frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}}$ x 10

Normal Values:

Normal serum calcium: 9-11 mg/dl.

ESTIMATION OF URIC ACID BY MODIFIED TRINDER METHOD[233]

Principle:

$$\text{Uric acid} + O2 + H_2O \xrightarrow[\text{Uricase}]{} \text{Allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{TBHB} \xrightarrow[\text{Peroxidase}]{} \text{Quinoneimine} + H_2O$$

The intensity of chromogen (Quinoneimine) formed is proportional to the uric acid concentration in the sample when measured at 546 nm.

Reagents:

Uric Acid Reagent:

Active Ingredient	**Concentration**
4-Aminoantipyrine	0.5mmol/L
TBHB	1.75.mmol/L
Uricase	>120U/L
Peroxidase	>500 U/L
Tris Buffer (pH 8.25± 0.1 at 20°C)	50 mmol/l

Uric Acid Standard:

Uric Acid Standard	6 mg/dl (0.36 mmol)

Specimen:

Unhemolysed serum or plasma should be separated from the cells as soon as possible. Recommended anticoagulants are heparin and EDTA.

Assay Parameters:

Mode	**End Point**
Wavelength 1 (nm)	505
Wavelength 2 (nm)	670
Sample Volume (µl)	10/20
Reagent Volume (µl)	500/1000
Incubation Time (min)	5
Incubation Temp. (^{o}C)	37
Normal Low (mg/dl)	2.5
Normal High (mg/dl)	7.2
Linearity Low (mg/dl)	0
Linearity High (mg/dl)	25
Mode	**End Point**
Concentration of Standard (mg/dl)	6
Blank with	Reagent
Absorbance Limit (Max)	0.3
Units	(mg/dl)

Procedure:

Pipette into tubes marked	Blank	Standard	Test
Working Reagent	1000µl	1000µl	1000µl
Distilled Water	20µl	-	-
Standard	-	20µl	-
Test	-	-	20µl

Standardization:

Pipette the following into appropriately labelled test tubes. The contents of the tubes are mixed well and incubated for 15 min. The spectrophotometer is set to zero with blank at 546 nm.

Pipette into tubes	Blank	S1	S2	S3	S4	S5	Test
Standard (µl)	0	5	10	15	20	25	-
Distilled water (µl)	20	20	15	10	5	0	-
Sample	-	-	-	-	-	-	20
Reagent	1000	1000	1000	1000	1000	1000	1000
Conc.Of Calcium (mg %)	0	1.5	3	4.5	6	7.5	3.6
Absorbances	0	0.36	0.72	1.05	1.44	1.80	0.86

Mix well and incubate for 15 minutes at 37^{o}C. Read the absorbance of standard and each test at 546/670 nm against reagent blank.

Calculation:

$$\text{Uric Acid (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 6$$

Normal Values:

Males: 3.5 -7.2 mg/dl or 0.21-0.43 mmol/L; Females: 2.5 – 6.2 mg/dl or 0.15-0.37 mmol/l

24 HOUR URINARY PROTEIN ESTIMATION

Method:

TCA precipitation method.

Principle:

Proteins in urine are denatured and precipitated as particles with TCA and are quantitated by measuring turbidity at 620nm.

Reagents:

Sulfo Salicylic Acid 3%

Normal saline

Protein standard – bovine albumin 1g%

Working standard 1mg/dl to 100 mg/dl was prepared from the stock.

Procedure:

Three clean dry test tubes are taken and marked as 'B' for blank 'S' for standard and 'T' for test. 1ml of distilled water is taken in B tube, 1 ml of working standard in S tube and 1ml of urine in T test tube. 3 ml of 3% sulfosalicylic acid is added to all the three test tubes.

All the test tubes are mixed thoroughly and kept at room temperature for 10 min. Turbidity is measured against blank at 620nm.

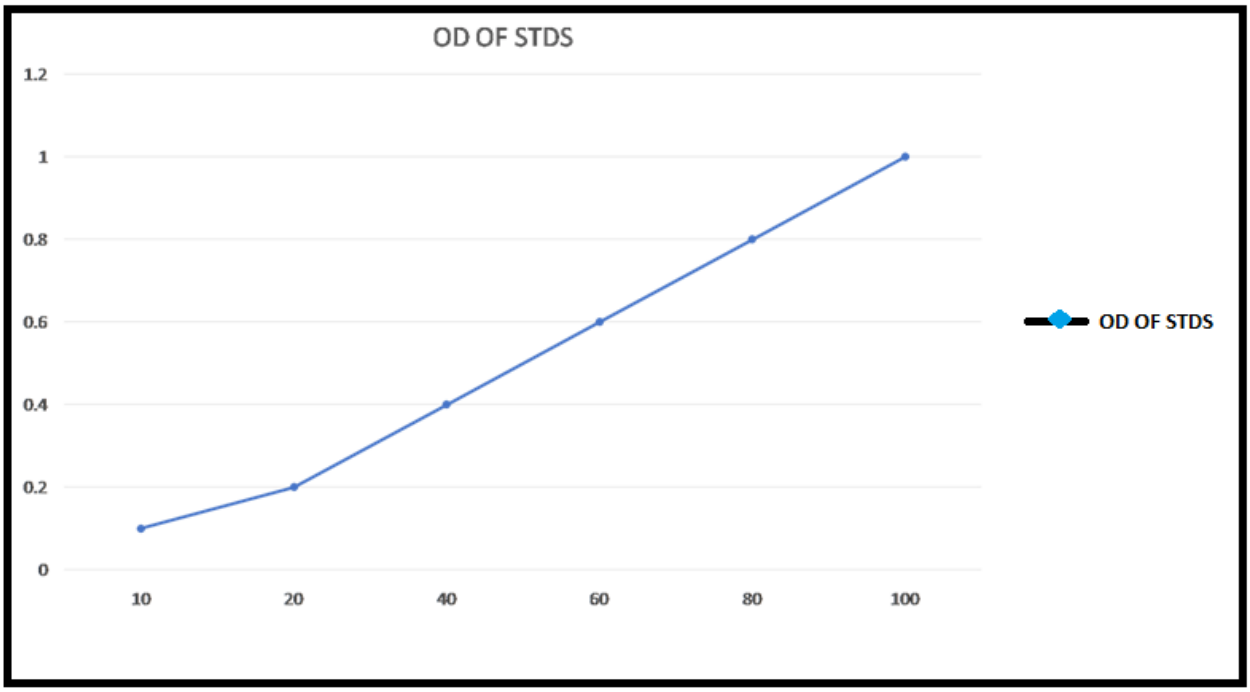
Standard graph is prepared using working standard from stock.

Calculation:

$$\text{Urine protein in mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times \frac{\text{Conc. of S}}{\text{Vol. of S}} \times 100$$

$$\text{24hr urine protein in mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times \frac{\text{Conc. of S}}{\text{Vol. of S}} \times 100 \quad \frac{\text{24hr vol. in ml}}{100}$$

Graph 2:



Standard curve for 24 hour urinary protein

STATISTICS AND RESULTS

STATISTICAL ANALYSIS

- Data were analysed using IBM SPSS 22.5, Microsoft word and Excel have been used to generate graphs, tables.
- The mean and standard deviation for all parameters.
- The student *t test* – to analyse clinical and laboratory data, and the χ2 test wherever required.
- The frequency of genotypes (TT, CT, CC) at -786 promoter region of the eNOS gene by using the χ2 test.
- Allele frequency by using the equation p + q = 1 where p and q are the frequencies of each allele at the particular locus.
- Hardy Weinberg equation equilibrium as shown by p2 + q2 + 2pq = 1.
- The unpaired Student t *test* and analysis of variance to analyse the significance of difference in values of nitric acid in different genotypes at 786 promoter region.

Concept of P value

- If the P value is 0.000 to 0.010, it implies Significant at 1 level (Highly Significant).
- If the P value is 0.011 to 0.050, it implies Significant at 5 level (Significant).
- If the P value is 0.051 to 1.000, it implies Not Significant at 5 level (Not Significant).

Baseline characteristics

Table 1: Comparison of age, gravida and gestational age

Variable		**Cases** **Mean (SD)**	**Control** **Mean (SD)**	**P value**
Age		26.00 (0.48)	25.04 (0.51)	0.17 ^
Gestational age		32.86 (0.61)	33.80 (0.42)	0.21^
Variable	Category	Cases No (%)	Control No (%)	P value
Gravida	1	28 (48.3%)	30 (51.7%)	0.42* (NS)
	2	22 (52.4%)	20 (47.6%)	
Genotype	CC genotype	12 (75.0%)	4 (25.0%)	**0.06*** (NS)
	CT genotype	23 (59.0%)	16 (41.0%)	
	TT genotype	15 (33.3%)	30 66.7%)	

^ - independent t test * - Chi square test

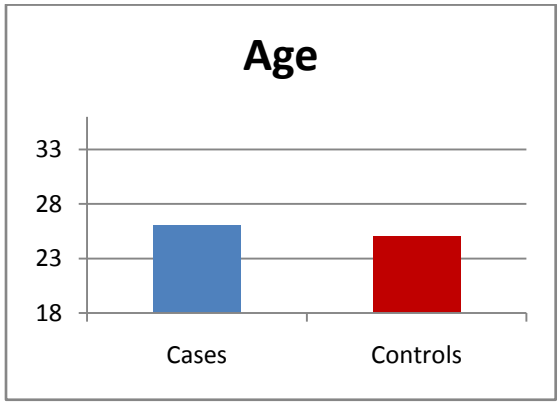
NS – P value not significant

Table 2: Comparison of biochemical parameters in cases and controls

	group	N	Mean	Std. Deviation	P value
SR NO	Cases	50	12.44	4.73	<0.001*** (S)
	Controls	50	18.40	6.85	
SR CA	Cases	50	8.44	0.79	<0.001*** (S)
	Controls	50	9.67	0.83	
UA	Cases	50	6.300	1.2206	<0.001*** (S)
	Controls	50	4.224	.6690	
RBS	Cases	50	90.36	11.376	0.178 (NS)
	Controls	50	87.58	8.960	
UREA	Cases	50	25.86	6.446	0.694 (NS)
	Controls	50	25.38	5.689	
SR CR	Cases	50	.724	.1170	0.683 (NS)
	Controls	50	.734	.1272	

S – Significant, NS – Non Significant

Bar Diagram 1: Age Distribution in cases and controls



The mean (SD) of age among the cases was 26.00(3.36) while the mean (SD) among the controls was 25.04(3.59). This difference was not statistically significant (p =0.170).

Table 3: UR ALB * Group
Crosstab

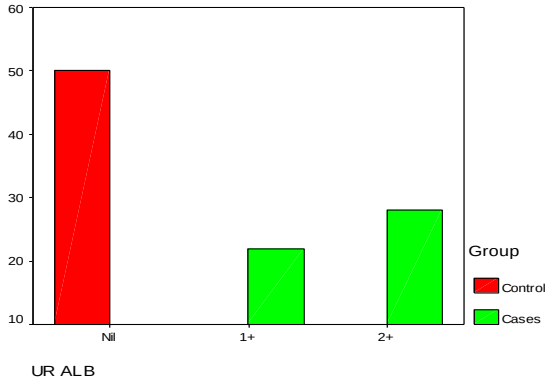
			Group		Total
			Control	Cases	
UR ALB	Nil	Count	50	0	50
		% within UR ALB	100.0%	.0%	100.0%
		% within Group	100.0%	.0%	50.0%
	1+	Count	0	22	22
		% within UR ALB	.0%	100.0%	100.0%
		% within Group	.0%	44.0%	22.0%
	2+	Count	0	28	28
		% within UR ALB	.0%	100.0%	100.0%
		% within Group	.0%	56.0%	28.0%
Total		Count	50	50	100
		% within UR ALB	50.0%	50.0%	100.0%
		% within Group	100.0%	100.0%	100.0%

Table 4: Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	100.000(a)	2	.000
Likelihood Ratio	138.629	2	.000
Linear-by-Linear Association	82.329	1	.000
N of Valid Cases	100		

a 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.00.

Bar Diagram 2: Urine Albumin



Urine Albumin level among cases and controls were statistically significant by Chi-square test (P value < 0.001).

Table 5: T-Test – SBP / DBP

Group Statistics

	Group	N	Mean	Std. Deviation	Std. Error Mean
SBP	Control	50	104.60	9.521	1.346
	Cases	50	146.00	7.559	1.069
DBP	Control	50	69.40	7.117	1.007
	Cases	50	95.20	8.862	1.253

Table 6: Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
SBP	Equal variances assumed	3.666	.058	-24.080	98	.000	-41.40	1.719	-44.812	-37.988
	Equal variances not assumed			-24.080	93.208	.000	-41.40	1.719	-44.814	-37.986
DBP	Equal variances assumed	1.127	.291	-16.051	98	.000	-25.80	1.607	-28.990	-22.610
	Equal variances not assumed			-16.051	93.639	.000	-25.80	1.607	-28.992	-22.608

The systolic and diastolic Blood Pressure among cases and controls was statistically significant by Chi-square test (P value < 0.001).

Table 7: Genotype

	Cases	Control	Odds ratio	P value
CC + CT	35 (63.6%)	20 (36.4%)	3.5 (1.529 – 8.012)	0.003 (S)
TT	15 (33.3%)	30 (66.6%)		

The presence of C gene in cases is 3.5 times more than that of the controls. The 95% confidence interval is 1.529 – 8.012 ($p<0.01$).

Bar Diagram 3: Genotype

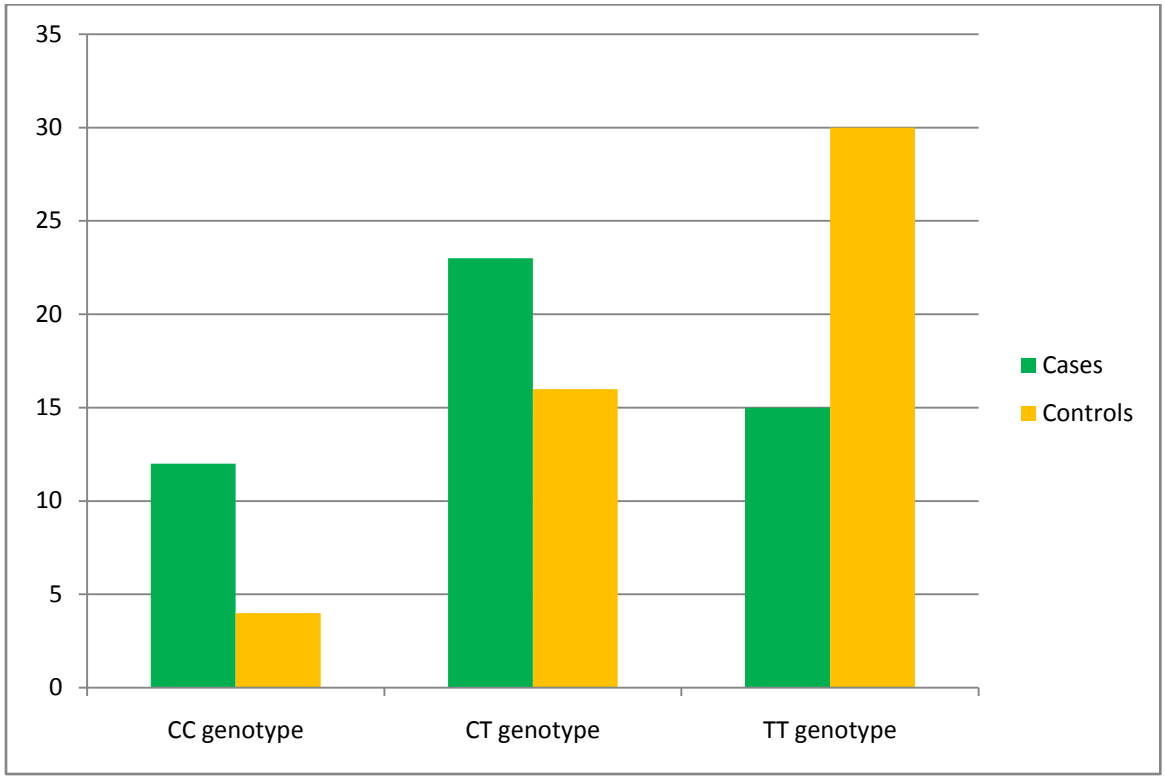


Table 8: Frequency of the promoter C-786 T polymorphism among Preeclampsia patients and controls

					Total	X^2 test	Odd's ratio (95% C.I.)
			Pre-Eclampsia	Controls		p value	
Genotype	TT		15	30	45		
			33.3%	66.7%	100.0%	0.003** (S)	0.286 (0.125 – 0.654)
	CC + CT		35	20	55		
			63.6%	36.4%	100.0%		
Total			50	50	100		
			50.0%	50.0%	100.0%		

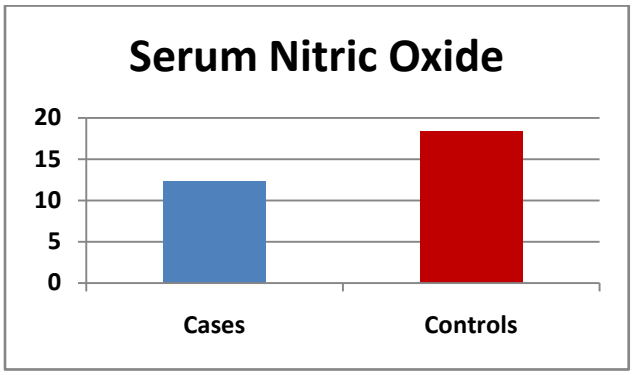
The frequency of the polymorphic C allele carrier which represented by (CC+CTG) genotypes was 63.6% in Preeclampsia patients and 36.4% in Controls. The frequency of wild-type TT genotype was 33.3% in Preeclampsia patients and 66.7% in controls. The statistical analysis of frequency of C-786 T polymorphism among the preeclampsia patients and controls by Chi-square test showed that a statistical difference was evident between two groups (P-value <0.01, P=0.003).

Table 9: Allele frequencies of the eNOS gene C-786 T polymorphism among Preeclampsia patients and controls

					Total	X^2 test	Odd's ratio
			Pre-eclampsia	Control		p value	(95% C.I.)
Allele	T allele		53	76	129	0.001**	0.356 (0.195 – 0.651)
			41.1%	58.9%	100.0%		
	C allele		47	24	71		
			66.2%	33.8%	100.0%		
Total			100	100	200		
			50.0%	50.0%	100.0%		

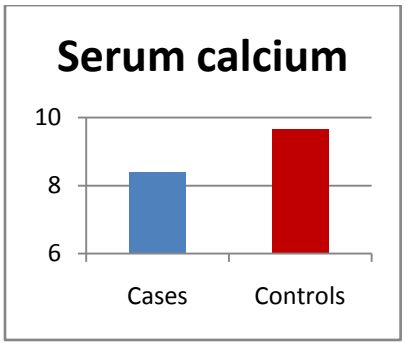
The frequency of the polymorphic C allele was 66.2% in Preeclampsia patients and 33.8% in controls. The frequency of wild type T allele was 41.1% in preeclampsia patients and 58.9% in controls**.** The statistical analysis of allele frequencies of the eNOS gene C-786 T polymorphism among Preeclampsia patients and controls by Chi-square test showed that there was a statistical significance between two groups (P value<0.001).

Bar Diagram 4: Serum nitric oxide



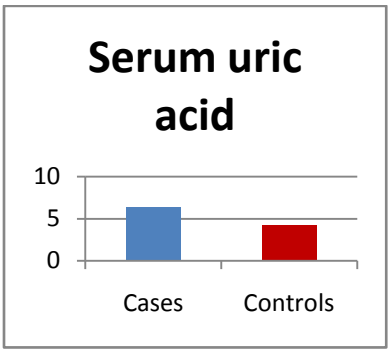
The mean (SD) levels of Serum nitric oxide among the cases was 12.32(4.79) which was lower than the mean (SD) Serum nitric oxide levels among the controls, 18.40(6.85). This difference was found to be statistically significant by t test ($p < 0.001$).

Bar Diagram 5: Serum calcium



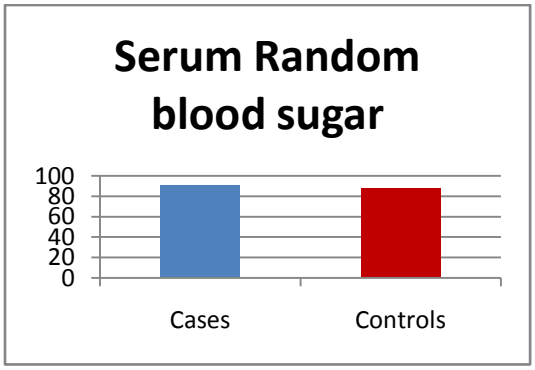
The mean (SD) levels of Serum calcium among the cases was 8.40(0.80) which was lower than the mean (SD) Serum calcium levels among the controls, 9.67(0.83). This difference was found to be statistically significant by t test ($p < 0.001$).

Bar Diagram 6: Serum uric acid



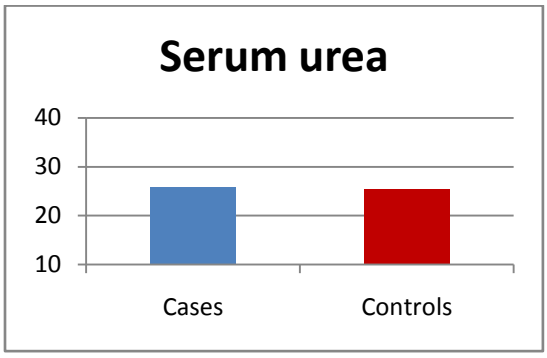
The mean (SD) levels of Serum uric acid among the cases was 6.3(1.22) which was higher than the mean (SD) Serum uric acid levels among the controls, 4.2(0.67). This difference was found to be statistically significant by t test ($p < 0.001$).

Bar Diagram 7: Serum Random blood sugar



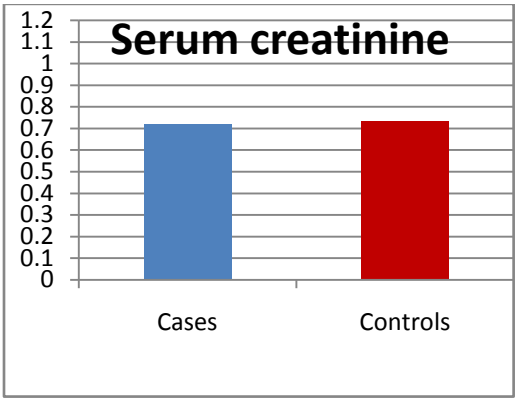
The mean (SD) levels of Serum Random blood sugar among the cases was 90.36(11.38) while in the controls it was 87.58(8.96). This difference was not statistically significant by t test ($p = 0.178$).

Bar Diagram 8: Serum urea



The mean (SD) levels of Serum urea among the cases was 25.86(6.45) while in the controls it was 25.38(5.69). This difference was not statistically significant by t test (p = 0.694).

Bar Diagram 9: Serum creatinine



The mean (SD) levels of Serum creatinine among the cases was 0.72(0.12) while in the controls it was 0.73(0.13). This difference was not statistically significant by t test (p =0.683).

Master Chart 1: Cases

S.NO	Group	NAME	AGE	GRAVIDA	GESTAGE	SBP	DBP	URALB	GENOTYPE	SrNO	RBS	UREA	SRCR	CA	UA
1	1	RADHIKA.M	24	2	35	170	100	2+	2	17.2	108	40	0.8	7.9	7.0
2	1	CHANDRA.N	31	1	36	160	110	2+	1	12.8	96	35	0.9	7.3	6.8
3	1	ANGEL JENNET.J	32	2	34	150	100	2+	2	13.1	74	18	0.6	9.4	5.3
4	1	ASMA.M	21	1	36	140	100	2+	2	9.8	80	18	0.9	8.0	6.2
5	1	KAVITHA.R	28	2	36	140	90	1+	2	21.1	85	26	0.7	9.0	5.0
6	1	MAHESHWARI.A	23	1	34	140	90	1+	1	6.1	82	24	0.6	7.3	6.5
7	1	MOHANA.R	27	1	36	140	90	1+	2	8.9	111	29	0.7	8.1	6.3
8	1	BHARATHI.K	27	1	28	140	90	1+	3	14.0	88	19	0.7	8.8	5.7
9	1	RADHA.V	21	1	36	140	90	1+	3	6.7	87	31	0.6	8.5	5.5
10	1	AMUDHAVALLI.R	26	2	36	140	90	1+	2	9.8	110	29	0.8	7.2	6.1
11	1	MARIAMMAL.R	20	1	36	140	80	1+	3	11.8	90	25	0.9	7.5	5.8
12	1	VADIVUKARASI.R	31	2	36	140	100	2+	2	23.7	106	22	0.6	8.3	6.4
13	1	SARALA.S	23	1	36	140	90	1+	3	10.2	79	18	0.7	9.0	5.2
14	1	MUTHULAKSHMI.S	28	1	36	150	90	2+	3	13.0	95	19	0.8	8.3	5.8
15	1	SHEELARANI.V	26	1	36	150	100	2+	2	6.0	70	20	0.8	8.2	4.7
16	1	ARUNA.B	28	1	36	150	100	2+	3	17.8	105	21	0.6	7.4	5.7
17	1	SARANYA.K	27	1	32	140	90	1+	2	8.3	83	18	0.8	8.8	6.7
18	1	SIVAGAMI.G	26	1	24	150	100	2+	3	9.8	110	36	0.6	9.3	7.8
19	1	BRINDHAVANAM.A	25	2	36	140	90	1+	2	9.7	98	20	0.8	8.5	5.3
20	1	GOWRI.A	23	1	36	140	100	2+	2	11.3	113	27	0.5	8.3	4.3
21	1	JEYANTHI.R	32	2	36	150	100	2+	2	10.9	109	20	0.6	9.0	6.1
22	1	RESHMA.A	27	2	36	140	90	1+	1	17.9	93	18	0.6	8.2	4.6
23	1	MANJULA.M	21	1	36	140	100	2+	1	7.9	89	21	0.8	9.0	5.2
24	1	NANDHINI.M	22	1	36	150	90	2+	2	9.5	67	19	0.6	8.9	5.5
25	1	PREETHADEVI	25	2	24	150	100	2+	1	21.6	98	22	0.7	8.4	5.7
26	1	SAVEETHA.R	21	1	24	160	80	2+	2	10.3	100	25	0.8	7.8	6.6
27	1	CAROLINA.N	22	1	20	150	100	2+	1	6.9	107	24	0.7	7.2	9.5
28	1	KALPANA.E	24	1	24	140	90	1+	1	13.8	90	30	0.6	8.4	5.5
29	1	MUTHULAKSHMI.S	28	1	36	150	100	2+	2	6.8	85	17	0.8	7.9	6.3
30	1	SHAKTHI.J	29	2	36	140	100	2+	2	11.1	78	18	0.7	8.9	5.4
31	1	SHRILEKHA.S	24	1	36	160	140	2+	3	5.8	92	24	0.7	10.2	5.9
32	1	SARANYA.S	28	2	30	150	100	2+	3	18.9	92	37	0.9	10.0	4.9
33	1	SHANTHI.V	30	1	28	140	90	1+	1	11.7	87	29	0.7	9.5	4.3
34	1	ESWARI.R	28	2	28	140	90	1+	2	21.9	85	23	0.7	8.3	9.1
35	1	SRIPRIYA.R	30	1	32	160	100	2+	1	10.8	93	35	0.9	8.8	7.3
36	1	PREMA.S	25	1	28	140	90	1+	2	9.8	80	33	0.8	7.7	8.4
37	1	NITHYA.R	27	2	36	150	100	2+	3	15.7	89	40	1.0	8.3	8.9
38	1	KAUSALYA.A	29	2	32	140	90	1+	3	11.3	93	27	0.6	9.3	6.7
39	1	SUDHA.S	30	2	34	140	90	1+	1	17.5	95	23	0.8	9.7	8.6
40	1	PADMAVATHI.M	29	2	30	150	100	2+	3	11.4	80	25	0.7	8.9	6.3
41	1	SHARMILABHANU.S	23	1	36	140	90	1+	2	17.9	83	21	0.8	9.6	7.4
42	1	SHAHINA NISHA.T	23	2	36	150	100	2+	2	12.1	95	30	0.8	8.7	7.3
43	1	AMIRTHAM.V	24	1	30	140	90	1+	3	9.7	78	32	0.6	8.0	6.9
44	1	AZHAGU MEENA.K	22	2	28	140	90	1+	1	9.9	82	29	0.9	7.5	5.7
45	1	SRIPRIYA.R	29	2	36	150	100	2+	2	16.9	93	34	0.6	9.3	7.6
46	1	JENIFER.R	20	1	36	150	90	2+	1	9.4	75	28	0.7	7.0	5.2
47	1	CHITRA.V	28	2	32	140	90	1+	2	7.3	78	20	0.6	8.8	6.8
48	1	MADHUMITHA.A	24	1	30	140	90	1+	3	20.1	86	35	0.8	7.9	6.5
49	1	ANUSHA.D	29	2	32	150	90	2+	3	18.4	80	27	0.5	7.1	5.4
50	1	SUGANTHI.K	30	2	34	160	100	2+	2	7.5	96	32	0.8	8.5	7.3

URALB	GENOTYPE
1 = 1+	1 = CC
2 = 2+	2 = CT
3 = 3+	3 = TT
0 = Nil	

Master Chart 2: Controls

S.NO	Group	NAME	AGE	GRAVIDA	GESTAGE	SBP	DBP	URALB	GENOTYPE	SrNO	RBS	UREA	SRCR	CA	UA
1	2	ANNAPOORANI.S	25	2	36	110	70	NIL	3	17.2	70	18	0.6	8.6	3.6
2	2	SARANYA.G	27	1	32	100	60	NIL	2	18.5	84	22	0.8	9.0	3.9
3	2	VISHALI .G	23	1	36	100	70	NIL	3	17.0	85	24	0.7	9.2	4.7
4	2	SHOBANA.P	27	2	34	100	60	NIL	2	37.0	80	18	0.6	8.8	4.4
5	2	ANANDHI.R	21	1	30	100	80	NIL	2	15.5	92	24	0.8	10.0	4.0
6	2	GOMATHY.A	26	1	36	110	70	NIL	3	34.8	78	18	0.6	7.8	4.2
7	2	MAHALAKSHMI.A	24	2	28	100	60	NIL	2	14.6	95	30	0.9	9.8	5.0
8	2	JOTHI SHREE.P	30	2	36	120	80	NIL	2	38.1	86	22	0.7	9.4	3.8
9	2	KOWSALYA.G	19	1	36	110	70	NIL	3	23.3	78	19	0.8	10.2	5.4
10	2	SARANYA.E	27	1	34	120	80	NIL	2	12.2	97	26	0.9	10.5	4.8
11	2	UMA.M	21	1	28	110	80	NIL	3	14.2	103	34	0.6	9.4	3.0
12	2	TAMILSELVI.G	22	1	36	110	60	NIL	2	21.1	87	18	0.6	8.9	3.7
13	2	SHARMILA.M	20	1	32	90	60	NIL	3	13.5	75	20	0.8	9.5	4.3
14	2	AGALYA.M	21	1	32	100	60	NIL	3	8.0	87	22	0.7	10.3	4.1
15	2	MOHANAVALLI.A	26	2	36	100	60	NIL	1	17.3	75	26	0.8	9.7	3.8
16	2	CHITRA.U	25	1	28	100	70	NIL	3	14.5	84	32	0.9	9.4	5.0
17	2	BAKIALAKSHMI.R	23	1	36	90	60	NIL	1	14.6	76	20	0.6	10.0	4.5
18	2	MEENAKSHI.D	20	1	28	100	70	NIL	3	23.5	80	30	0.8	9.8	4.4
19	2	NANDHINI.M	27	2	28	100	60	NIL	1	11.5	94	25	0.6	10.8	3.9
20	2	ILAKIA.T	29	2	30	110	70	NIL	3	23.6	100	32	0.8	8.5	3.5
21	2	DIVIYA BHARATHI.D	28	2	32	90	60	NIL	3	15.1	86	30	0.9	11.0	5.0
22	2	LAKSHMI DEVI.M	28	2	34	120	70	NIL	3	28.5	80	25	0.7	10.3	4.9
23	2	AMMU.M	28	2	36	110	70	NIL	3	21.1	87	29	0.9	10.7	4.8
24	2	JOTHI LAKSHMI.A	23	1	36	120	80	NIL	2	21.8	91	18	0.8	9.5	3.8
25	2	UDHAYAKUMARI.T	25	2	36	100	70	NIL	3	31.6	92	33	0.7	10.2	3.4
26	2	NITHIYA.R	31	2	36	90	60	NIL	1	9.6	90	28	0.9	11.4	5.5
27	2	SATHYAKUMARI.M	28	1	36	100	70	NIL	3	24.9	86	27	0.6	10.1	4.7
28	2	HEMALATHA.A	22	1	28	100	60	NIL	2	17.5	79	37	0.6	9.3	3.5
29	2	KANIMOZHI.K	28	2	36	100	70	NIL	3	21.4	86	31	1.0	8.8	3.0
30	2	SUMITHRA.S	26	1	32	120	80	NIL	3	19.8	94	27	0.8	8.4	4.6
31	2	JANCY RANI.B	21	1	36	90	60	NIL	3	14.1	102	23	0.7	9.6	4.1
32	2	NANDHINI.S	21	1	36	100	80	NIL	2	11.1	105	19	0.9	10.6	3.8
33	2	KALAVATHI.E	31	2	36	100	70	NIL	3	14.7	93	20	0.7	11.3	4.7
34	2	BASMATH LIMCY.V	24	1	32	120	80	NIL	3	18.4	80	28	0.7	9.0	3.6
35	2	SATHYA.S	28	2	36	110	70	NIL	2	21.7	89	24	0.5	10.7	5.0
36	2	DIVYA.P	23	1	36	110	70	NIL	3	18.0	90	18	0.8	10.0	3.1
37	2	BENNY.S	27	2	36	100	70	NIL	3	17.4	96	21	0.7	9.5	4.6
38	2	USHARANI.N	25	1	36	110	80	NIL	2	10.5	76	25	0.7	9.1	4.9
39	2	ANURADHA.S	34	1	36	120	70	NIL	3	23.2	87	36	0.9	8.6	4.0
40	2	AISHWARYA.V	26	2	36	110	70	NIL	2	8.2	70	24	0.6	8.0	5.3
41	2	SUGAPRIYA.N	20	1	34	100	70	NIL	3	21.5	106	19	0.9	9.9	3.8
42	2	KALAIVANI.P	29	1	36	100	70	NIL	2	19.6	98	32	0.7	9.6	3.6
43	2	SANGEETHA.R	22	1	34	110	70	NIL	3	10.1	83	20	0.5	9.1	4.9
44	2	SANDHYA.J	26	1	36	100	70	NIL	2	18.2	80	35	0.8	8.9	4.2
45	2	SUNITHA.T	28	2	36	120	80	NIL	2	16.4	95	31	0.8	9.3	4.0
46	2	JANANI.T	19	1	30	100	70	NIL	3	17.2	79	18	0.7	9.8	3.8
47	2	RATHNEASHWARI.K	19	1	28	90	70	NIL	3	14.3	100	26	0.9	10.0	3.1
48	2	JEELANIBEE.S	29	2	36	100	70	NIL	3	18.7	96	20	0.6	10.2	5.5
49	2	SHEELA.S	27	2	34	120	80	NIL	3	16.0	92	34	0.6	11.3	4.4
50	2	LATHA.E	23	1	36	90	60	NIL	3	9.2	85	31	0.5	9.5	3.6

URALB	GENOTYPE
1 = 1+	1 = CC
2 = 2+	2 = CT
3 = 3+	3 = TT
0 = Nil	

RESULTS

Master Charts 1 & 2 give the various data about the case and control groups respectively. Table 1 shows the baseline characters of variables such as age, gestational age, gravida and genotype. Table 2 shows the bio chemical parameters in cases and controls.

Bar Diagram 1 is related to Age which reveals that the mean (SD) of age among the cases was 26.00(3.36) while the mean (SD) among the controls was 25.04 (3.59). This difference was not statistically significant ($p = 0.170$).

Table 3 & 4 and Bar Diagram 2 is related to Urine Albumin level from which it is found that Urine Albumin level among cases and controls was statistically significant by Chi-square test (P value < 0.001).

Table 5 & 6: T-Test is related to SBP / DBP from which it is found that the systolic and diastolic Blood Pressure among cases and controls was statistically significant by Chi-square test (P value < 0.001).

Table 7 and Bar Diagram 3 are related to Genotype from which it is found that the presence of C gene in cases is 3.5 times more than that of the controls. The 95% confidence interval is 1.529 – 8.012 (P < 0.01).

Table 8 shows the frequency of the promoter C-786 T polymorphism among Preeclampsia patients and controls

Cases:

TT: 33.3% CC+CT: 63.6%

Controls:

TT: 66.7% CC+CT: 36.4% P value 0.003 (P<0.01) (S)

Table 9 shows Allele frequencies of the eNOS gene C-786 T polymorphism among Preeclampsia patients and controls

Cases:

T Allele: 41.1% C Allele: 66.2%

Controls:

T Allele: 58.9% C Allele: 33.8% P value < 0.001 (S)

Bar Diagram 4 is related to Serum Nitric Oxide from which it is found that the mean (SD) levels of Serum nitric oxide among the cases was 12.32(4.79) which was lower than the mean (SD) Serum nitric oxide levels among the controls, 18.40(6.85). This difference was found to be statistically significant by t test ($P < 0.001$).

Bar Diagram 5 is related to Serum Calcium from which it is found that the mean (SD) levels of Serum calcium among the cases was 8.40(0.80) which was lower than the mean (SD) Serum calcium levels among the controls, 9.67(0.83). This difference was found to be statistically significant by t test ($p < 0.001$).

Bar Diagram 6 is related to Serum Uric Acid level from which it is found that the mean (SD) levels of Serum uric acid among the cases was 6.3(1.22) which was higher than the mean (SD) Serum uric acid levels among the controls, 4.2(0.67). This difference was found to be statistically significant by t test ($p<0.001$).

Bar Diagram 7 is related to Serum RBS from which it is found that the mean (SD) levels of Serum Random blood sugar among the cases was 90.36(11.38) while in the controls it was 87.58(8.96). This difference was not statistically significant by t test ($p =0.178$).

Bar Diagram 8 is related to Serum Urea level from which it is found that the mean (SD) levels of Serum urea among the cases was 25.86(6.45) while in the controls it was 25.38(5.69). This difference was not statistically significant by t test ($p = 0.694$).

Bar Diagram 9 is related to Serum Creatinine from which it is found that the mean (SD) levels of Serum creatinine among the cases was 0.72(0.12) while in the controls it was 0.73(0.13). This difference was not statistically significant by t test ($p =0.683$).

DISCUSSION

DISCUSSION

Preeclampsia is an important clinical and stressful problem that has been studied tremendously but the causes and treatment have not been fully resolved [130]. Preeclampsia affects about 1-5% of women who conceive [131] and accounts for about 20% of clinically recognized pregnancy losses [132]. Despite extensive researches to explain the causative effects of Preeclampsia, about 50% - 60% of Preeclampsia is still idiopathic. Endothelial damage, impaired placental vascularization and resultant oxidative stress have been proposed to play a role in the pathophysiology of Preeclampsia.

In case of normal pregnancy, the Nitric Oxide 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 [133]. eNOS has been regarded as the source of endothelial NO, which has a critical role in vascular physiology and impaired placental vascularization [134]. In recent years much attention was paid to determine the association between eNOS gene 786 T (Promoter) allelic variants and preeclampsia. However, the results of the studies have been controversial among different ethnic groups [135].

Few studies have investigated the relation between eNOS promoter C-786 T polymorphism and the development of Preeclampsia and other reproductive complications in women from various populations. Our results for the promoter C-786 T polymorphisms are in agreement with those published by Shim, et al (2010) who showed that the promoter C-786 T polymorphism is associated with the risk of spontaneously aborted foetuses. This result is also consistent with those recorded for Indian women origin where a significant association between the C-786 T promoter and preeclamptic pregnancy complication was observed.

In contrast, our results do not support the previously published results for women from Korean and Tunisian populations[136] which indicated that C-786 T promoter polymorphisms is not significantly associated with Preeclampsia.

Frequency of the promoter C-786 T polymorphism among Preeclampsia patients and controls:

The table 8 reveals that the frequency of the polymorphic C allele carrier which represented by (CC+CTG) genotypes was 63.6% in Preeclampsia patients and 36.4% in Controls. The frequency of wild-type TT genotype was 33.3% in Preeclampsia patients and 66.7% in controls. The statistical analysis of frequency of C-786 T polymorphism among the preeclampsia patients and controls by Chi-square test showed that a statistical difference was evident between two groups

(P-value <0.01, P=0.003). The Odds ratio with 95% confidence interval is 3.5 which indicate the presence of polymorphic C allele in cases 3.5 times more than that of controls.

Allele frequencies of the eNOS gene C-786 T polymorphism among Preeclampsia patients and controls:

The table 9 reveals that the frequency of the polymorphic C allele was 66.2% in Preeclampsia patients and 33.8% in controls. The frequency of wild type T allele was 41.1% in preeclampsia patients and 58.9% in controls**.** The statistical analysis of allele frequencies of the eNOS gene C-786 T polymorphism among Preeclampsia patients and controls by Chi-square test showed that there was a statistical significance between two groups (P value<0.001).

The findings regarding eNOS polymorphism and its association with Preeclampsia clearly showed that eNOS gene C-786 T polymorphism, namely "allele-786C"is associated with Preeclampsia in our study population.

Association of eNOS C-786 T polymorphism and Nitric Oxide Synthesis:

As per Bar Diagram 4, the results of present study showed that C-786 T polymorphism is associated with low Serum Nitric Oxide level. The mean (SD) level of Serum Nitric Oxide among the cases was 12.32 (4.79) lower than the mean (SD) Serum Nitric Oxide level among the controls which was 18.40 (6.85). This difference was found to be statistically significant by t test (P<0.001).

The study showed that promoter C-786 T substitution markedly blunts the transcription rate of eNOS gene and hence the Nitric Oxide production by creating a binding site for a Replication Protein A1 (RPA1) by C allele that acts as a suppressor of eNOS transcription.

The RPA1 protein is not only in endothelial cells but also in placenta which is rich in vasculature and the level of eNOS mRNA in placenta with promoter C-786 T substitution mutation is significantly lower than in placenta without mutation.

These findings confirm our results that preeclampsia women are associated with high frequency of promoter C-786 T polymorphism. It explains the association between the polymorphism and Serum Nitric Oxide level.

Since Nitric Oxide pathway plays an important role in the pathophysiology of Preeclampsia, any factors balancing Nitric Oxide metabolism could be useful in the treatment of Preeclampsia, thus reducing maternal and foetal morbidity and mortality.

These findings confirm our results that Preeclampsia women are associated with a high frequency of promoter-786T (Promoter) allelic variants might explain why this polymorphism is associated with a low serum NO levels.

The table 4 and 5 show the significant statistical difference between cases and controls (P value < 0.001).

The Bar Diagrams 5 and 6 show that the mean (SD) levels of Serum calcium and Uric Acid among the cases were lower than the controls. These were found to be statistically significant by t test ($p < 0.001$).

The Bar Diagrams 7, 8 and 9 show that the mean (SD) levels of Serum Random blood sugar, Urea and Creatinine among the cases were lower than the controls. These differences were not statistically significant by t test ($p = 0.178$, 0.694 and 0.683 respectively).

The present data add to the importance of ethnic as well as intraregional variability in such studies concerning multifactorial disorders including preeclampsia. Our findings regarding the eNOS polymorphisms and its association with preeclampsia clearly showed that the promoter C-786T polymorphism of the eNOS gene, namely "allele -786C" is associated with preeclampsia in our study population.

We recommend for testing the promoter -786T (Promoter) allelic variants polymorphism of eNOS gene in all Indian women experiencing preeclampsia or unexplained hypertension during pregnancy. Since NO pathway plays an important role in the pathophysiology of preeclampsia, thus, any factors balancing NO metabolism could be useful in the treatment of preeclampsia, consequently, reducing the substantial morbidity and associated maternal and fetal mortality.

The rapid accumulation of advanced knowledge in genetics and molecular biology as well as fast progress in diagnostic methods enabled to distinguish a new research field called molecular medicine. Nowadays molecular biology methods are the core applications used in investigations concerning etiology of numerous complex human diseases and contribute to gene therapy development. A key method used in that kind of diagnostics are polymerase chain reaction (PCR) – based applications which are characterized by much higher sensitivity and specificity than classical diagnostics [137].

Many issues of molecular biology consider problems from obstetrical and gynecological field. There have been revealed numerous gene polymorphisms which are favourable to clear some pregnancy complications and gynecological diseases [138].

It is also suggested that some genetic variants may be the markers of increased risk of several diseases in pregnant women as well as in gynaecologic disturbances. Typical example of the practice of molecular biology methods are investigations on the osteoporosis field.

Nowadays, the analysis of candidate genes is one of the study ways of genetic background of etiology in preeclampsia (PE). The candidate gene is a gene of documented biological activity involved in the PE pathways, with polymorphic

activity. This process is one of the conditions of inter-individual variability. At present there are more than sixty candidate genes to PE development. It was distinguished some groups of candidate genes in PE pathophysiology.

Family-based studies have shown that genetic factors may play a role in preeclampsia [139]. In addition, candidate-gene association studies (GAS) on preeclampsia have not produced conclusive results so far [140]. However, the pathogenesis of preeclampsia is poorly understood and the search for low-penetrance genes by hypothesis-driven candidate gene studies (genetic association study-GAS) and hypothesis-free genome-wide association studies is ongoing [141].

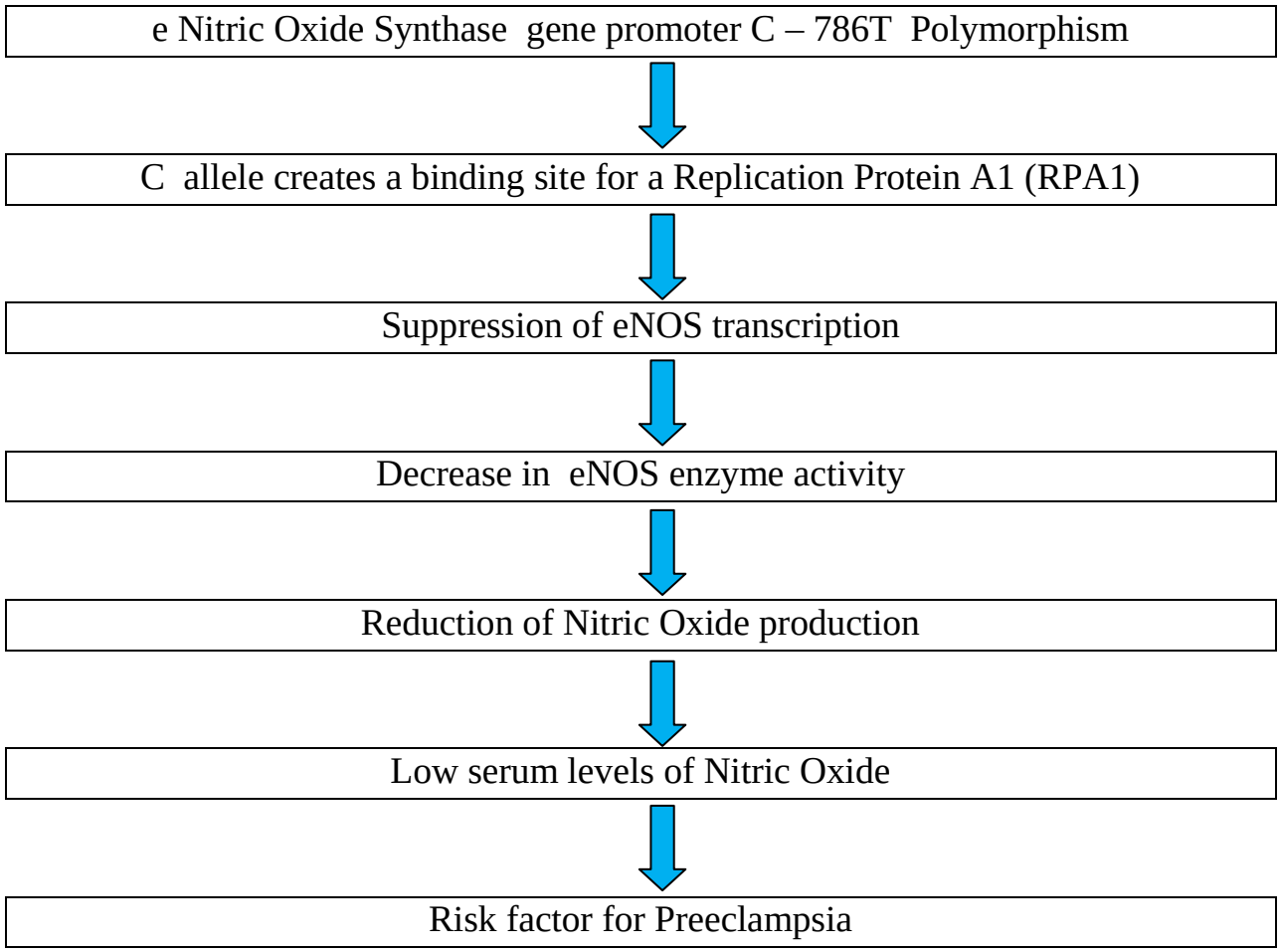
The leading hypotheses, concerning the pathogenesis of preeclampsia, are based on disturbed placental function and impaired remodelling of the spiral arteries [142].

Endothelial nitric oxide synthase (NOS3) is an important regulator of vascular tone and contributes to the reduction of the uteroplacental resistance seen in normal pregnancy [143]. Therefore, the endothelial nitric oxide synthase gene (NOS3), located at the 7q35-q36 region, has emerged as a logical candidate gene in the development of preeclampsia. Variants (polymorphisms) of the NOS3 gene have been investigated for association with preeclampsia and other disorders such as hypertension.

Endothelial dysfunction, as a syndrome, is defined as the absence of antithrombotic, angiogenic, inflammatory and vasodilator functions of endothelium. Evidences from documented cases have revealed that the bioactivity or bioavailability of nitric oxide (NO) is reduced in this condition, and, in turn, vasodilator capacity and vascular protection against harmful agents is impaired. The NO is a powerful endogenous vasodilator, which performs key roles in blood pressure regulation, vascular dilation, vascular smooth-muscle proliferation and inhibition of platelet aggregation. These abilities recommend this molecule as an attractive candidate for predisposition to Preeclampsia.

SUMMARY

Association of eNOS gene polymorphism, Nitric Oxide Synthesis & Preeclampsia:



CONCLUSION

CONCLUSION

- In this study, C allele was significantly increased in Cases compared to Controls which implies that the presence of C allele in C-786 T promoter polymorphism of eNOS gene found to be the risk factor for Preeclampsia.
- The presence of polymorphic C allele in C-786 T promoter polymorphism of eNOS gene was found to have an effect on Serum Nitric Oxide levels by altering the transcriptional efficiency which led to decrease in endothelial Nitric Oxide synthase enzyme activity thereby reducing Nitric Oxide production.
- Hence, the Nitric Oxide level in serum was low which may be an independent risk factor for preeclampsia.
- So, it may be concluded that there is an association between eNOS gene polymorphism (C-786 T promoter), low Nitric Oxide level and Preeclampsia.

FUTURE SCOPE OF STUDY

FUTURE SCOPE OF STUDY

- Genetic differences across different ethnic groups for this polymorphic site (C-T786 promoter of eNOS gene) have to be established.
- The Nitric Oxide level may be estimated in routine antenatal investigations for Pregnancy Induced Hypertensive (PIH) patients.
- By taking large sample size for genetic polymorphic studies in PIH women, we can segregate high risk group of patients to focus on their diet and life style modification for safe confinement.
- Further research may focus upon gene – gene interactions and gene environmental interactions and its relationship with genotypic variation of eNOS gene in patients with Preeclampsia so as to provide efficient preventive measures in genetically susceptible population in future.
- The whole genome sequence analysis is needed to reveal extensive level of variation and heterogeneity between individuals and populations.
- Genome- Wide Association Studies (GWAS) has to be carried out, as these analyses eliminate biases in the selection of candidate genes.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Schoofs, K. et al. The importance of repeated measurements of the sFlt-1/PlGF ratio for the prediction of preeclampsia and intrauterine growth restriction. J Perinat Med 42, 61–68 (2014).

2. MacKay, A. P., Berg, C. J. & Atrash, H. K. Pregnancy-related mortality from preeclampsia and eclampsia. Obstet Gynecol 97, 533–538 (2001)

3. Steegers, E. A., von Dadelszen, P., Duvekot, J. J. & Pijnenborg, R.Pre-eclampsia. Lancet 376, 631–644 (2010).

4.Zintzaras E, Kitsios G, Harrison GA, Laivuori H, Kivinen K, Kere J, Messinis I, Stefanidis I, Ioannidis JP: Heterogeneity-based genome search meta-analysis for preeclampsia. Hum Genet. 2006, 120: 360-370.10.1007/s00439-006-0214-1.

5. Ward K: Genetic factors in common obstetric disorders. Clin Obstet Gynecol. 2008, 51: 74-83. 10.1097/GRF.0b013e3181616545.

6. Roten LT, Johnson MP, Forsmo S, Fitzpatrick E, Dyer TD, Brennecke SP, Blangero J, Moses EK, Austgulen R: Association between the candidate susceptibility gene ACVR2A on chromosome 2q22 and pre-eclampsia in a large Norwegian population-based study (the HUNT study). Eur J Hum Genet.2009, 17: 250-257. 10.1038/ejhg.2008.158.

7. Moncada, S. & Higgs, A. The L-arginine-nitric oxide pathway.N Engl J Med 329, 2002–2012 (1993).

8. Hingorani, A. D. Polymorphisms in endothelial nitric oxide synthase and atherogenesis: John French Lecture 2000. Atherosclerosis 154, 521–527 (2001).

9. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R: Pre-eclampsia. Lancet.2010, 376: 631-644.10.1016/S0140-6736(10)60279-6.

10.Norris LA, Higgins JR, Darling MR, Walshe JJ, Bonnar J: Nitric oxide in the uteroplacental, fetoplacental, and peripheral circulations in preeclampsia. Obstet Gynecol. 1999, 93: 958-963. 10.1016/S0029-7844(99)00007-1.

11. Sladek SM, Magness RR, Conrad KP: Nitric oxide and pregnancy. Am J Physiol. 1997, 272: R441-463.

12. The journal of maternal-fetal & neonatal medicine; 24(3):432-6 • March 2011

13. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. The Lancet. 2006 Apr 8; 367(9516):1066–1074.

14.(Gupte Sanjay,Wagh Girija, Preeclampsia–Eclampsia. The Journal of Obstetrics and Gynecology of India (January–February 2014) 64(1):4–13.)

15.Goldenberg RL, Rouse DJ.Prevention of premature birth. N Engl J Med. 1998;339(5):313–320.

16. Hernandez-Diaz S, Toh S, Cnattingius S. Risk of pre-eclampsia in first and subsequent pregnancies: prospective cohort study. BMJ. 2009;338:b2255.

17. Sibai BM, Mercer B, Sarinoglu C. Severe preeclampsia in the second trimester: recurrence risk and long-term prognosis. Am J Obstet Gynecol. 1991; 165(5 Pt 1):1408–1412.

18.Sauvé N, Powrie RO, Larson L, Phipps MG, Weitzen S, Fitzpatrick D, et al. The impact of an educational pam- phlet on knowledge and anxiety in women with pre- eclampsia. Obstet Med 2008;111–7.

19.You WB, Wolf M, Bailey SC, Pandit AU, Waite KR, Sobel RM, et al. Factors associated with patient understanding of preeclampsia. Hypertens Pregnancy 2012;31:341–9.

20.Saftlas AF, Olson DR, Franks AL, Atrash HK, Pokras R. Epidemiology of preeclampsia and eclampsia in the Unit- ed States, 1979–1986. Am J Obstet Gynecol 1990; 163: 460–5.

21.Goldenberg RL, McClure EM, Macguire ER, Kamath BD, Jobe AH.Lessons for low-income regions following the reduction in hypertension-related maternal mortality in high-income countries. Int J Gynaecol Obstet 2011;113: 91–5.

22.Ogunyemi D, Benae JL, Ukatu C. Is eclampsia prevent- able? A case control review of consecutive cases from an urban underserved region. South Med J 2004;97:440–5.

23.Berg CJ, MacKay AP, Qin C, Callaghan WM. Overview of Maternal Morbidity During Hospitalization for Labor and Delivery in the United States: 1993-1997 and 2001-2005.Obstet Gynecol. 2009; 113(5):1075–1081.

24.Kjell Haram, Einar Svendsen and Ulrich Abildgaard.The HELLP syndrome: Clinical issues and management. A Review .BMC Pregnancy Childbirth. 2009 Feb 26;9:8.

25.Palmer RM, Ferrige AG, Moncada S.Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327: 524-6.

26.Stamler JS, Loh E, Roddy MA, Currie KE, Creager MA. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation 1994; 89:2035-40.

27.Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci USA 1977; 74: 3203-7.

28.Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. Prog Cardiovasc Dis 1995;38:87-104.

29.Shichiri M, Hirata Y, Ando K, et al. Plasma endothelin levels in hypertension and chronic real filure. Hypertension 1990; 15: 493-6.

30.Lopez JA, Armstrong ML, Piegors DJ, Heistad DD. Vascular responses to endothelin -1 in atherosclerotic primates. Arterisclerosis 1990;10: 1113-8.

31.Ludmer PL, Selwyn AP, Shook TL, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 1986; 315: 1046-51.

32. Moncada S, Higgs A. The L-arginine-nitric oxide pathway.N Engl J Med. 1993; 329: 2002-2012.

33. Arnold, W.P.; Mittal C.K.; Katsuki, S.; Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proceedings of the National Academy of Sciences of the United States of America 74: 3203-3207; 1977

34. Thomsen, L.L., Scott, J.M., Topley, P., Knowles, R.G., Keerie, A.J. and Frend, A.J. (1997) Selective inhibition of inducible nitric oxide synthase inhibits tumor growth *in vivo*: studies with 1400W, a novel inhibitor. Cancer Res.57, 3300-3304

35. Wray, G.M., Millar, C.G., Hinds, C.J. and Thiemermann, C. (1998) Selective inhibition of the activity of inducible nitric oxide synthase prevents the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin. Shock 9, 329-335.

36. Koarai, A., Ichinose,M., Sugiura, H., Yamagata, S., Hattori, T. and Shirato, K. (2000) Allergic airway hyperresponsiveness and eosinophil infiltration is reduced by a selective iNOS inhibitor, 1400W, in mice. Pulmon.Pharmacol.Ther. 13, 267-275

37. Kankuri, E., Vaali, K., Knowles, R., Lahde, M., Korpela, R., Vapaatalo, H. and Moilanen, E. (2001) Suppression of acute experimental colitis by an iNOS-selective inhibitor N-3-(aminomethyl)benzyl] acetamidine (1400W). J.Pharmacol, Exp.Ther., in the press

38. Dawson, J. and Knowles, R.G. (1998) A microtiter-plate assay of human NOS isoforms. Methods Mol.Biol.100, 237-242.

39. Russwurm, M.; Behrends, S.; Harteneck, C.; Koesling, D. Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. The Biochemical Journal 335 (Pt1): 125-130; 1998.

40. Budworth, J.; Meillerais, S.; Charles.I.; Powell, K. Tissue distribution of the human soluble guanylate cyclases. Biochemical and biophysical research communications 263:696-701; 1999.

41. Mergia, E.; Russwurm, M.; Zoidl, G.; Koesling, D. Major occurrence of the new alpha2betal isoform of NO-sensitive guanylyl cyclase in brain. Cellular signalling 15: 189-195; 2003

42. Nimmegeers, S.; Sips, P.; Buys, E.; Brouckaert, P.; Van de Voorde, J. Functional role of the soluble guanylyl cyclase alpha(1) subunit in vascular smooth muscle relaxation. Cardiovascular research 76: 149-159; 2007.

43.Lucas, K.A.; Pitari G.M.; Kazerounian, S.; Ruiz-Stewart, I.; Park, J.; Schulz, S.; Chepenik, K.P.; Waldman, S.A. Guanylyl cyclases and signalling by cyclic GMP. Pharmacol Rev 52:375-414; 2000.

44.Humbert, P.; Niroomand, F.; Fischer, G.; Mayer, B.; Koesdling, D.; Hinsch, K.D.; Gayusapohl, H.; Frank, R.; Schultz, G.; Bohme, E. Purification of guanylyl cyclase from bovine lung by a new immunoaffinity chromatographic method. European journal of biochemistry/FEBS 190:273-278; 1990.

45.Mergia, E.; Friebe, A.; Dangel, O.; Russwurm, M.; Koesling, D. Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. The journal of clinical investigation 116:1731-1737; 2006.

46.Friebe, A.; Mergia, E.; Dangel, O.; Lange, A.; Koesling, D. Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide sensitive guanylyl cyclase. Proceedings of the National Academy of Sciences of the United States of America 104:7699-7704

47. Pyriochou, A.; Papapetropoulos, A. Soluble guanylyl cyclase: more secrets revealed. Cellular signalling 17:407-413; 2005

48. Evgenov, O.V.; Pacher, P.; Schmidt, P.M.; Hasko, G.; Schmidt, H.H.; Stasch, J.P. NO-independent simulators and activators of soluble guanylate cyclase: discovery and therapeutic potential. Nat Rev Drug Discov 5:755-768; 2006.

49. Kleinbongard, P.; Dejam, A,; Lauer, T.; Rassaf, T.; Schindler, A.; Picker, O.; Scheeren, T.; Godecke, A.; Schrader, J.; Schulz, R.; Heusch, G.; Schaub, G.A,; Bryan, N.S.; Feelisch, M.; Kelm, M. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med 35:790-796; 2003.

50. Gladwin, M.T.; Schechter, A.N.; Kim-Shapiro, D.B.; Patel, R.P.; Hogg, N.; Shiva, S.; Cannon, R.O., 3rd; Kelm, M.; Wink, D.A.; Espey, M.G.; Oldfield, E.H.; Pluta, R.M.; Freeman, B.A.; Lancaster, J.R., Jr.; Feelish, M.; Lundberg, J.O. The emerging biology of the nitrite anion. Nat Chem Biol 1:308-314;2005.

51. Bryan, N.S. Nitrite in nitric oxide biology: Cause or consequence? A systems-based review. Free Radic Biol Med 41: 691-701; 2006.

52.Bryan, N.S.; Calvert, J.W.; Elrod, J.W.; Gundewar, S.; Ji, S.Y.; Lefer, D.J. Dietary nitrite supplementation protects against myocardial ischemiareperfusion injury.Proceedings of the National Academy os Sciences of the United States of America 104:19144-19149; 2007.

53.Lundberg, J.O.; Weitzberg, E.; Lunberg, J.M.; Alving, K. Intragastric nitric oxide production in humans: measurements in expelled air. Gut 35: 1543-1546; 1994.

54.Webb, A.; Bond, R.; Mclean, P.; Uppal, R.; Benjamin, N.; Ahluwalia, A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proc Natl Acad Sci U S A 101: 13683-13688; 2004.

55. Duranski, M.R.; Greer, J.J.; Dejam, A.; Jaganmohan, S.; Hogg.; Langston, W.; Patel, R.P.; Yet, S.F.; Wang, X.; Kevil, C.G.; Gladwin, M.T.; Lefer, D.J. Cytoprotective effects of nitrite during vivo ischemia-reoerfusion of the heart and liver. J Clin Invest 115: 1232-1240; 2005.

56. Pluta, R.M.; Dejam, A.; Grimes, G.; Gladwin, M.T.; Oldfield, E,H. Nitrite infusions to prevent delayed cerebral vasospasm in a primate model of subarachnoid haemorrhage. Jama 293: 1477-1484; 2005.

57. Hunter, C.J.; Dejam, A.; Blood, A.B.; Shields, H.; Kim-Shapiro, D.B.; Machado, R.; Tarekegn, S.; Mulla, N.; Hooper, A.O.; Schechter, A.N.; Power, G.G.; Gladwin M.T. Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. Nat Med 10:1122-1127; 2004.

58. Hardwick, J.B.; Tucker, A.T.; Wilks, M.; Johnston, A.; Benjamin, N. A novel method for the delivery of nitric oxide theraphy to the skin of human subjects using a semi-permeable membrane. Clin Sci (Lond) 100:3

59. Tsuchia, K.; Kanematsu, Y.; Yoshizumi, M.; Ohnishi, H.; Kirima, K.; Izawa, Y.; Shikishima, M.; Ishida, T.; Kondo. S.; Kagami, S.; Takiguchi, Y.; Tamaki, T. Nitrite is an alternative source of NO in vivo. Am J Physiol Heart Circ Physiol 288:H2163-2170; 2005.

60. Kleinbongard, P.; Dejam, A.; Lauer, T.; Jax, T.; Kerber, S.; Gharini, P.; Balzer, J.; Zotz, R.B.; Scharf, R.E.; Willers, R.; Schechter, A.N.; Feelisch, M.; Kelm, M.

Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans. Free Redic Biol Med 40:295-302;2006.

61. Tannenbaum, S.R.; Sinskey, A.J.; Weisman, M.; Bishop, W. Nitrite in human saliva. Its possible relationships to nitrosamine formation. J Natl Cancer Inst 59:79-84; 1974.

62.Bryan, N.S.; Fernandez, B.O.; Bauer, S.M.; Garcia-Saura, M.F.; Milsom, A.B.; Rassaf, T.; Maloney, R.E.; Bharti, A.; Rodriguez, J.; Feelish, M. Nitrite is a signalling molecule and regulator of gene expression in mammalian tissues. Nat Chem Biol 1:290-297; 2005.

63. Pennington, J.A. T. Dietary exposure models for nitrates and nitrites. Food Control 9:385-395; 1998.

64. Veszelovsky E, Holford NHG, Thomsen LL, Knowles RG, Baguley, BC. Plasma nitrate clearance in mice: modelling of the systemic production of nitrate following the induction of nitric oxide synthesis. Cancer Chemother Pharmacol 1995;36:155-159.

65.Hess, D.T.; Matsumoto, A.; Kim, S.O.; Marshall, H.E.; Stamler, J.S. Protein S-nitrosylation: purview and parameters. Nature reviews 6:150-166; 2005.

66. Foster, M.W.; McMahon, T.J.; Stamler, J.S. S-nitrosylation in health and disease. Trends in molecular medicine 9:160-168; 2003.

67.Massy, Z.A.; Fumeron, C.; Borderie, D.; Tuppin, P.; Nguyrn-Khoa, T.; Benoit, M.O.; Jacquot, C.; Buisson, C.; Drueke, T.B.; Ekindjian, O.G.; Lacour, B.; Iliou, M.C. Increased plasma S-nitrosothiol concentrations predict cardiovascular outcomes among patients with end-stage renal disease: a prospective study. J Am Soc Nephrol 15:470-476; 2004.

68. Liu, L.; Hausladen, A.; Zeng, M.; Que, L; Heitman, J.; Stamler, J.S.; A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. Nature 410:490-494; 2001.

69. Pacher, P.; Bekman J.S.; Liaudet, L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315-424; 2007.

70. Turko, I.V.; Murad, F. Protein nitration in cardiovascular diseases. Pharmacol Rev 54:619-624; 2002.

71.Souza, J.M.; Daikhin, E.; Yudkoff, M.; Raman, C.S.; Ischiropoulous, H. Factors determining the selectivity of protein tyrosine nitration. Arc Biochem Biophys 371:169-178; 1999.

72.Carr, A.C.; Frie, B. The nitric oxide congener nitrite inhibits myeloperoxidase/H2O2/C1- -mediated modification of low density lipoprotein. J Boil Chem 276:1822-1828; 2001.

73.McMillan K, and Masters, B.S. (1995) Prokaryotic expression of the heme and flavin-binding domains of rat neuronal nitric oxide synthase as distinct

polypeptides: identification of the heme-binding proximal thiolate ligand as cysteine-415. Biochemistry 34, 3686-3693.

74.Crane, B.R., Arvai, A.S., Ghosh, D.K., Wu, C., Getzoff, E.D., Stuehr, D.J. and Tainer, J.A. (1998) Structure of nitric oxide synthase oxygenase dimer with pterine and substrate. Science 279, 2121-2126.

75.Raman, C.S., Li, H., Martasek, P., Kral, V., Masters, B.S., and poulos, T.L (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function of involving a novel metal center. Cell (Cambridge, Mass.) 95, 939-950

76.Crane, B.R., Rosenfield, R.J., Arvai, A.S., Ghosh, D.K., Ghosh, S., Tainer, J.A., Stuehr, D.J. and Getzoff, E.D. (1999) N-terminal domain swapping and metal ion binding in nitric oxide synthase dimerization. EMBO J. 18, 6271-6281.

77. Bryk, R. and Wolf, D.J. (1998) Mechanism of inducible nitric oxide synthase inactivation by aminoguanidine and L-N^6-(1-iminoethyl)lysine. Biochemistry 37, 4844-4852.

78. Abu-Soud, H.M., Yoho, L.L. and Stuehr, D.J. (1994) Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron tyransfer. J.Biol.Chem. 269, 32047-32050.

79.Corson, M.A., James, N.L, Latta, S.E., Nerem, R.M., Berk, B.C. and Harrison D.G. (1996) Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circ.Res. 79, 984-991

80.Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulous, A. and Sessa, W.C. (1998) Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature (London) 392, 821-824.

81.Ratovitski, E.A., Alam, M.R., Quick, R.A., McMillan, A., Bao, C, Kazlovsky, C., Hand, T.A., Johnson, R.C., Mains, R.E., Eipper, B.A. and Lowenstein, C.J. (1999) Kalirin inhibition of inducible nitric- oxide synthase. J.Biol.Chem. 274, 993-999.

82. Michel, T. (1999) Targrting and translocation of endothelial nitric oxide synthase. Braz.J.Med.Biol.Res.32, 1361-1366.

83.Shaul, P.W., Smart, E.J., Robinson, L.J., German, Z., Yuhanna, I.S., Ying, Y., Anderson, R.G. and Michel, T. (1996) Acylation targets endothelial nitric oxide synthase to plasmalemmal caveolae. J.Biol.Chem. 271, 6518-6522

84.Prabhakar, P., Thatte, H.S., Goetz, R.M., Cho, M.R., Golan, D.E. and Michel, T. (1998) Receptor-regulated translocation of endothelial-nitric oxide synthase. J.Biol.Chem. 273, 27383-27388.

85. Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J.E. and Sessa, W.C. (1996) Targeting of nitric oxide synthase to endothelial cell caveolae via polmitoylation: implications for nitric oxide signaling. Proc.Natl.Acad.Sci. U.S.A. 93, 6448-6453.

86.Feron, O., Belhassen, L., Kobzik, L., Smith, T.W., Kelly, R.A. and Michel, T. (1996) Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocites and endothelial cells. J.Biol.Chem. 271, 22810-22814.

87.Ju, H., Zou, R., Venema, V,J. and Venema, R.C. (1997) Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. J.Biol.Chem. 272, 18522-18525

88. Venema, V.J., Ju, H., Zou, R. and Venema, R.C. (1997) Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. J.biol.chem. 272, 28187-28190.

89.Garvey, E.P., Oplinger, J.A., Furfine, E.S., Kiff, R.J., Laszlo, F., Whittle, B.J.R. and Knowles, R.G. (1997) 1400W is a slow, tight binding and highly selective inhibitor of inducible nitric-oxide synthase in *vitro* and *invivo*. J.Biol.Chem. 272, 4959-4963.

90. Knowles, R., Dawson, J., Waslidge, N., Russell, R., Angell, A., Craig, C., Schwartz, S., Evans, S., Whittle, B., Garvey, E. (2000) GW273629 is a highly selective, short-acting iNOS (NOS-2) inhibitor both *invitro* and *invivo*: beneficial

effects in endotoxin shock. In biology of nitric oxide, vol. 7 (Moncada, s., gustaffson, l.e., wiklund, n.p. and higgs, e.a., eds.), pp. 21, Portland press, London

91. Hagen, T.J., Bergmains, A.A., Kramer, S.W., Fok, K.F., Schmelzer, A.E., Pitzele, B.S., Swenton, L., Jerome, G.M., Kornmeier, C.M., Moore, W.M. (1998) 2-Iminopyrrolidines as potent and selective inhibitors of human inducible nitric oxide synthase. J. Med. Chem. 41, 3675-3683.

92. Hansen, D.W., Peterson, K.B., Trivedi, M., Kramer, S.W., Webber, R.K., Tjoing, F.S., Moore, W.M., Jerome, G.M., Kornmeier, C.M., Manning, P.T. (1998) 2-Iminohomopiperidinium salts as selective inhibitors of inducible nitric oxide synthase (iNOS). J. Med .Chem. 41, 1361-1366.

93.Fagan KA, Tyler RC, Sato K, Fouty BW, Morris KG Jr, Huang PL, McMurty IF, Rodman, DM. Relative contributions of endothelial, inducible, and neuronal NOS to tone in the murine pulmonary circulation. Am J Physiol. 1999; 277: L472-L478.

94. Bosaller C, Habib GB, Yamamoto H, Williams C, Wells S, Henry PD. Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5'-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. *JClinInvest*. 1987; 79: 659-670.

95. Forsterman U, Boissel JP, Kleinert H. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS 1 and NOS 111). *FasebJ*. 1998; 12: 773-790.

96. Marsden PA, Heng HH, Schrer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC, Schappert KT. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *JBiolChem*. 1993; 268: 17478-17488.

97. Karantzoulis-Fegaras F, Antoniou H, Lai S, Kulkarni G, D' Abreo C, Wong GKT, Miller TL, Chan y, Atkins J, Wang y, Marsden PA. Characterization of the human endothelial nitric oxide synthase promoter.*JBiolChem*. 1999; 274: 3076-3093.

98. Teichert A-M, Karantzoulis-Fegaras F, Chan Y, Marsden P. Cell-specific regulation of the human eNOS gene: a unique model [abstract]. *ActaPhysiologicaScandinavica*.1999; 167: 75.

99. Robertson KD, Wolffe AP. DNA methylation in health and disease. *Nature Reviews Genetics.*2000; 1: 11-19.

100.Marsden PA, Schappert KT, Chen HS, Flowers M, Sundel CL, Wilcox JN, Lamas S, Michel T. Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBSlett*. 1992; 307: 287-293.

101. Rosencranz-Weiss P, Sessa WC, Milstein S, Kaufman S, Watson CA, Pober JS. Regulation of nitric oxide synthesis by proinflammatory cytokines in human

umbilical vein endothelial cells. Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *JClinInvest*. 1994; 93: 2236-2243.

102. Yoshizumi M, Perella MA, Burnett JC, Jr., Lee ME. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life.*CircRes*. 1993; 73: 205-209.

103. Liao JK, Shin WS, Lee WY, Clark SL. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *JBiolChem*. 1995; 270: 319-324.

104.Hirata K, Miki N, Sakoda T, Kawashima S, Yokoyama M. Low concentration of oxidized low-density lipoprotein and lysophosphatidylcholine upregulate constitutive nitric oxide synthase mRNA expression in bovine aortic endothelial cells. *CircRes*. 1995; 76: 958-962.

105. Vidal F, Colome C, Mortenez-Gonzalez J, Badimon L. Atherogenic concentrations of native low-density lipoproteins down-regulate nitric-oxide synthase mRNA and protein levels in endothelial cells. *EurJBiochem*. 1998; 252: 378-384.

106. Casas JP, Cavalleri GL, Bautista LE, Smeeth L, Humphries SE, Hingorani AD. Endothelial nitric oxide synthase gene polymorphisms and cardiovascular disease: a HuGE review. American Journal of Epidemiology. 2006; 164(10): 921-

935. Cooke GE, Doshi A. Binkey PF. Endothelial nitric oxide synthase gene: prospects for treatment of heart disease. Pharmacogenomics. 2007;8(12):1723-1734.

107. Hingorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, et al. A common variant of endothelial nitric oxide synthase (Glu 298-Asp) is a major risk factor for coronary artery disease in UK. Circulation 1999;100:1515-20.

108. Myatt L, Brockman DE, Eis AL, Pollock JS. Immunohistochemical localization of nitric oxide synthase in the human placenta.Placenta. 1993 Sep-Oct; 14(5):487-95.

109. Madhavi Puppala, Lakshmi Kalpana Veerathu, Anuradha A, Sudhakar Godi Endothelial Nitric Oxide Synthase "eNOS" Gene Polymorphisms in Preeclampsia in South Indian Population.

110. Sladek SM, Magness RR, Conrad KP Nitric oxide and pregnancy. Am J Physiol. 1997 Feb; 272(2 Pt 2):R441-63.

111. Ignarro LJ Nitric oxide. A novel signal transduction mechanism for transcellular communication. Hypertension. 1990 Nov; 16(5):477-83.

112. Shah DA, Khalil RA Bioactive factors in uteroplacental and systemic circulation link placental ischemia to generalized vascular dysfunction in hypertensive pregnancy and preeclampsia. Biochem Pharmacol. 2015 Jun 15; 95(4):211-26.

113. Ashworth JR, Warren AY, Johnson IR, Baker PN. Plasma from pre-eclamptic women and functional change in myometrial resistance arteries. Br J Obstet Gynaecol. 1998;105:459–461.

114.Khalil A, Hardman L, O Brien P The role of arginine, homoarginine and nitric oxide in pregnancy. Amino Acids. 2015 Sep; 47(9):1715-27.

115.Myatt L, Brockman DE, Eis AL, Pollock JS Immunohistochemical localization of nitric oxide synthase in the human placenta. Placenta. 1993 Sep-Oct; 14(5):487-95.

116.Redman CW Current topic: pre-eclampsia and the placenta. Placenta. 1991 Jul-Aug; 12(4):301-8. Maltepe E, Fisher SJ Placenta: the forgotten organ. Annu Rev Cell Dev Biol. 2015; 31():523-52.

117.Marsden, P.A., Heng, H.H., Scherer, S.W., Stewart, R.J., Hall, A.V., Shi, X.M., Tsui, L.C., Schappert, K.T., 1993. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J. Biol. Chem. 268, 17478–17488.

118.Van Vliet, B.N., Chafe, L.L., 2007. Maternal endothelial nitric oxide synthase genotype influences offspring blood pressure and activity in mice. Hypertension 49, 556–562

119.Al-Hijji, J., Andolf, E., Laurini, R., Batra, S., 2003. Nitric oxide synthase activity in trophoblast, term placenta ad pregnant myometrium. Reprod. Biol. Endocrinol. 1, 51.

120.Karantzoulis-Fegaras, F., Antoniou, H., Lai, S.L., Kulkarni, G., D'Abreo, C., Wong, G.K., Miller, T.L., Chan, Y., Atkins, J., Wang, Y., Marsden, P.A., 1999. Characterization of the human endothelial nitric-oxide synthase promoter.J. Biol. Chem. 274, 3076–3093.

121.Logan, J.F., Chakravarthy, U., Hughes, A.E., Patterson, C.C., Jackson, J.A., Rankin, S.J., 2005. Evidence for association of endothelial nitric oxide synthase gene in subjects with glaucoma and a history of migraine. Invest. Ophthalmol.Vis. Sci. 46, 3221–3226.

122. Akcay, A., Sezer, S., Ozdemir, F.N., Arat, Z., Atac, F.B., Verdi, H., Colak, T., Haberal, M., 2004. Association of the genetic polymorphisms of the renin-angiotensin system and endothelial nitric oxide synthase with chronic renal transplant dysfunction. Transplantation 78, 892–898.

123. Tanus-Santos, J.E., Desai, M., Deak, L.R., Pezzullo, J.C., Abernethy, D.R., Flockhart, D.A., and Freedman, J.E. (2002). Effects of endothelial nitric oxide synthase gene polymorphisms on platelet function, nitric oxide release, and interactions with estradiol. Pharmacogenetics 12, 407–413

124. Cooke, G.E., Doshi, A., and Binkley, P.F. (2007). Endothelial nitric oxide synthase gene: prospects for treatment of heart disease. Pharmacogenomics 8, 1723–1734.

125. Yetik-Anacak, G., and Catravas, J.D. (2006). Nitric oxide and the endothelium: history and impact on cardiovascular disease. Vascul Pharmacol 45, 268–276.

126. Metzger, I.F., Sertorio, J.T., and Tanus-Santos, J.E. (2007). Modulation of nitric oxide formation by endothelial nitric oxide synthase gene haplotypes. Free Radic Biol Med 43, 987–992.

127. Hingorani, A.D. (2001). Polymorphisms in endothelial nitric oxide synthase and atherogenesis: John French Lecture 2000. Atherosclerosis 154, 521–527.

128. Chen Y. · Wang D. · Zhou M. · Chen X. Chen J. Polymorphisms of the Endothelial Nitric Oxide Synthase Gene in Preeclampsia in a Han Chinese Population. Gynecol Obstet Invest. 2014;77(3):150-5.

129.Fangfang Zeng, Sui Zhu, Martin Chi-Sang Wong, Zuyao Yang, Jinling Tang, Keshen Li,and Xuefen Su.Associations between nitric oxide synthase 3 gene polymorphisms and preeclampsia risk: a meta-analysis Sci Rep. 2016; 6: 23407.

130. 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.

131. 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.

132. 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.

133. 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.

134. 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.

135. 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.

136. 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.

137. Ward K. (2008) Genetic factors in common obstetric disorders. Clin. Obstet. Gynecol. 51: 74-83.

138. Varga E. (2007) Inherited thrombophilia: key points for genetic counseling. J. Genet. Couns. 16: 261-277.

139.Zintzaras E, Kitsios G, Harrison GA, Laivuori H, Kivinen K, Kere J, Messinis I,Stefanidis I, Ioannidis JP: Heterogeneity-based genome search metaanalysis for preeclampsia. Hum Genet 2006, 120:360-370.

140.Ward K: Genetic factors in common obstetric disorders. Clin Obstet Gynecol 2008, 51:74-83.

141.Roten LT, Johnson MP, Forsmo S, Fitzpatrick E, Dyer TD, Brennecke SP,Blangero J, Moses EK, Austgulen R: Association between the candidate susceptibility gene ACVR2A on chromosome 2q22 and pre-eclampsia ina large Norwegian population-based study (the HUNT study). Eur J Hum Genet 2009, 17:250-257.

142.Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R: Pre-eclampsia. Lancet 2010, 376:631-644.

143. Norris LA, Higgins JR, Darling MR, Walshe JJ, Bonnar J: Nitric oxide in the uteroplacental, fetoplacental, and peripheral circulations in preeclampsia. Obstet Gynecol 1999, 93:958-963.

ANNEXURES

PROFORMA

Date:

Name: **OP/IP No:**

Age: **Sex :**

Address: **Occupation:**

Presenting Complaints:

Past H/O:

Smokers, Renal disease, Chronic illness, Diabetes mellitus, Systemic hypertension, Acute/Chronic infection

Treatment H/O:

Previous hospitalization:

Personal H/O:

SMOKING / ALCOHOL INTAKE

O/E:

Built -

Height-

Weight-

Pedal edema/Anemia/Clubbing /Lymphadenopathy

VITALS:

BP:

Pulse Rate:

SYSTEMIC EXAMINATION :

CVS: Abdomen:

RS : CNS:

Diagnosis:

Investigations:

1. Blood sugar

2. Urea

3. Creatinine

4.Uric acid

5.Serum Nitric oxide

6.Urine microprotein

7.Serum calcium

7.Genetic polymorphic studies

- DNA EXTRACTION
- GENOMIC DNA AMPLIFICATION BY PCR
- 2% AGAROSE GEL RUN
- RESTRICTION DIGESTION OF THE PCR PRODUCT
- Genotype :
- TT/CT/CC:

Master Chart - Cases

S.NO	Group	NAME	AGE	GRAVIDA	GESTAGE	SBP	DBP	URALB	GENOTYPE	SrNO	RBS	UREA	SRCR	CA	UA
1	1	RADHIKA.M	24	2	35	170	100	2+	2	17.2	108	40	0.8	7.9	7.0
2	1	CHANDRA.N	31	1	36	160	110	2+	1	12.8	96	35	0.9	7.3	6.8
3	1	ANGEL JENNET.J	32	2	34	150	100	2+	2	13.1	74	18	0.6	9.4	5.3
4	1	ASMA.M	21	1	36	140	100	2+	2	9.8	80	18	0.9	8.0	6.2
5	1	KAVITHA.R	28	2	36	140	90	1+	2	21.1	85	26	0.7	9.0	5.0
6	1	MAHESHWARI.A	23	1	34	140	90	1+	1	6.1	82	24	0.6	7.3	6.5
7	1	MOHANA.R	27	1	36	140	90	1+	2	8.9	111	29	0.7	8.1	6.3
8	1	BHARATHI.K	27	1	28	140	90	1+	3	14.0	88	19	0.7	8.8	5.7
9	1	RADHA.V	21	1	36	140	90	1+	3	6.7	87	31	0.6	8.5	5.5
10	1	AMUDHAVALLI.R	26	2	36	140	90	1+	2	9.8	110	29	0.8	7.2	6.1
11	1	MARIAMMAL.R	20	1	36	140	80	1+	3	11.8	90	25	0.9	7.5	5.8
12	1	VADIVUKARASI.R	31	2	36	140	100	2+	2	23.7	106	22	0.6	8.3	6.4
13	1	SARALA.S	23	1	36	140	90	1+	3	10.2	79	18	0.7	9.0	5.2
14	1	MUTHULAKSHMI.S	28	1	36	150	90	2+	3	13.0	95	19	0.8	8.3	5.8
15	1	SHEELARANI.V	26	1	36	150	100	2+	2	6.0	70	20	0.8	8.2	4.7
16	1	ARUNA.B	28	1	36	150	100	2+	3	17.8	105	21	0.6	7.4	5.7
17	1	SARANYA.K	27	1	32	140	90	1+	2	8.3	83	18	0.8	8.8	6.7
18	1	SIVAGAMI.G	26	1	24	150	100	2+	3	9.8	110	36	0.6	9.3	7.8
19	1	BRINDHAVANAM.A	25	2	36	140	90	1+	2	9.7	98	20	0.8	8.5	5.3
20	1	GOWRI.A	23	1	36	140	100	2+	2	11.3	113	27	0.5	8.3	4.3
21	1	JEYANTHI.R	32	2	36	150	100	2+	2	10.9	109	20	0.6	9.0	6.1
22	1	RESHMA.A	27	2	36	140	90	1+	1	17.9	93	18	0.6	8.2	4.6
23	1	MANJULA.M	21	1	36	140	100	2+	1	7.9	89	21	0.8	9.0	5.2
24	1	NANDHINI.M	22	1	36	150	90	2+	2	9.5	67	19	0.6	8.9	5.5
25	1	PREETHADEVI	25	2	24	150	100	2+	1	21.6	98	22	0.7	8.4	5.7
26	1	SAVEETHA.R	21	1	24	160	80	2+	2	10.3	100	25	0.8	7.8	6.6
27	1	CAROLINA.N	22	1	20	150	100	2+	1	6.9	107	24	0.7	7.2	9.5
28	1	KALPANA.E	24	1	24	140	90	1+	1	13.8	90	30	0.6	8.4	5.5
29	1	MUTHULAKSHMI.S	28	1	36	150	100	2+	2	6.8	85	17	0.8	7.9	6.3
30	1	SHAKTHI.J	29	2	36	140	100	2+	2	11.1	78	18	0.7	8.9	5.4
31	1	SHRILEKHA.S	24	1	36	160	140	2+	3	5.8	92	24	0.7	10.2	5.9
32	1	SARANYA.S	28	2	30	150	100	2+	3	18.9	92	37	0.9	10.0	4.9
33	1	SHANTHI.V	30	1	28	140	90	1+	1	11.7	87	29	0.7	9.5	4.3
34	1	ESWARI.R	28	2	28	140	90	1+	2	21.9	85	23	0.7	8.3	9.1
35	1	SRIPRIYA.R	30	1	32	160	100	2+	1	10.8	93	35	0.9	8.8	7.3
36	1	PREMA.S	25	1	28	140	90	1+	2	9.8	80	33	0.8	7.7	8.4
37	1	NITHYA.R	27	2	36	150	100	2+	3	15.7	89	40	1.0	8.3	8.9
38	1	KAUSALYA.A	29	2	32	140	90	1+	3	11.3	93	27	0.6	9.3	6.7
39	1	SUDHA.S	30	2	34	140	90	1+	1	17.5	95	23	0.8	9.7	8.6
40	1	PADMAVATHI.M	29	2	30	150	100	2+	3	11.4	80	25	0.7	8.9	6.3
41	1	SHARMILABHANU.S	23	1	36	140	90	1+	2	17.9	83	21	0.8	9.6	7.4
42	1	SHAHINA NISHA.T	23	2	36	150	100	2+	2	12.1	95	30	0.8	8.7	7.3
43	1	AMIRTHAM.V	24	1	30	140	90	1+	3	9.7	78	32	0.6	8.0	6.9
44	1	AZHAGU MEENA.K	22	2	28	140	90	1+	1	9.9	82	29	0.9	7.5	5.7
45	1	SRIPRIYA.R	29	2	36	150	100	2+	2	16.9	93	34	0.6	9.3	7.6
46	1	JENIFER.R	20	1	36	150	90	2+	1	9.4	75	28	0.7	7.0	5.2
47	1	CHITRA.V	28	2	32	140	90	1+	2	7.3	78	20	0.6	8.8	6.8
48	1	MADHUMITHA.A	24	1	30	140	90	1+	3	20.1	86	35	0.8	7.9	6.5
49	1	ANUSHA.D	29	2	32	150	90	2+	3	18.4	80	27	0.5	7.1	5.4
50	1	SUGANTHI.K	30	2	34	160	100	2+	2	7.5	96	32	0.8	8.5	7.3

URALB	GENOTYPE
1 = 1+	1 = CC
2 = 2+	2 = CT
3 = 3+	3 = TT
0 = Nil	

Master Chart - Controls

S.NO	Group	NAME	AGE	GRAVIDA	GESTAGE	SBP	DBP	URALB	GENOTYPE	SrNO	RBS	UREA	SRCR	CA	UA
1	2	ANNAPOORANI.S	25	2	36	110	70	NIL	3	17.2	70	18	0.6	8.6	3.6
2	2	SARANYA.G	27	1	32	100	60	NIL	2	18.5	84	22	0.8	9.0	3.9
3	2	VISHALI .G	23	1	36	100	70	NIL	3	17.0	85	24	0.7	9.2	4.7
4	2	SHOBANA.P	27	2	34	100	60	NIL	2	37.0	80	18	0.6	8.8	4.4
5	2	ANANDHI.R	21	1	30	100	80	NIL	2	15.5	92	24	0.8	10.0	4.0
6	2	GOMATHY.A	26	1	36	110	70	NIL	3	34.8	78	18	0.6	7.8	4.2
7	2	MAHALAKSHMI.A	24	2	28	100	60	NIL	2	14.6	95	30	0.9	9.8	5.0
8	2	JOTHI SHREE.P	30	2	36	120	80	NIL	2	38.1	86	22	0.7	9.4	3.8
9	2	KOWSALYA.G	19	1	36	110	70	NIL	3	23.3	78	19	0.8	10.2	5.4
10	2	SARANYA.E	27	1	34	120	80	NIL	2	12.2	97	26	0.9	10.5	4.8
11	2	UMA.M	21	1	28	110	80	NIL	3	14.2	103	34	0.6	9.4	3.0
12	2	TAMILSELVI.G	22	1	36	110	60	NIL	2	21.1	87	18	0.6	8.9	3.7
13	2	SHARMILA.M	20	1	32	90	60	NIL	3	13.5	75	20	0.8	9.5	4.3
14	2	AGALYA.M	21	1	32	100	60	NIL	3	8.0	87	22	0.7	10.3	4.1
15	2	MOHANAVALLI.A	26	2	36	100	60	NIL	1	17.3	75	26	0.8	9.7	3.8
16	2	CHITRA.U	25	1	28	100	70	NIL	3	14.5	84	32	0.9	9.4	5.0
17	2	BAKIALAKSHMI.R	23	1	36	90	60	NIL	1	14.6	76	20	0.6	10.0	4.5
18	2	MEENAKSHI.D	20	1	28	100	70	NIL	3	23.5	80	30	0.8	9.8	4.4
19	2	NANDHINI.M	27	2	28	100	60	NIL	1	11.5	94	25	0.6	10.8	3.9
20	2	ILAKIA.T	29	2	30	110	70	NIL	3	23.6	100	32	0.8	8.5	3.5
21	2	DIVIYA BHARATHI.D	28	2	32	90	60	NIL	3	15.1	86	30	0.9	11.0	5.0
22	2	LAKSHMI DEVI.M	28	2	34	120	70	NIL	3	28.5	80	25	0.7	10.3	4.9
23	2	AMMU.M	28	2	36	110	70	NIL	3	21.1	87	29	0.9	10.7	4.8
24	2	JOTHI LAKSHMI.A	23	1	36	120	80	NIL	2	21.8	91	18	0.8	9.5	3.8
25	2	UDHAYAKUMARI.T	25	2	36	100	70	NIL	3	31.6	92	33	0.7	10.2	3.4
26	2	NITHIYA.R	31	2	36	90	60	NIL	1	9.6	90	28	0.9	11.4	5.5
27	2	SATHYAKUMARI.M	28	1	36	100	70	NIL	3	24.9	86	27	0.6	10.1	4.7
28	2	HEMALATHA.A	22	1	28	100	60	NIL	2	17.5	79	37	0.6	9.3	3.5
29	2	KANIMOZHI.K	28	2	36	100	70	NIL	3	21.4	86	31	1.0	8.8	3.0
30	2	SUMITHRA.S	26	1	32	120	80	NIL	3	19.8	94	27	0.8	8.4	4.6
31	2	JANCY RANI.B	21	1	36	90	60	NIL	3	14.1	102	23	0.7	9.6	4.1
32	2	NANDHINI.S	21	1	36	100	80	NIL	2	11.1	105	19	0.9	10.6	3.8
33	2	KALAVATHI.E	31	2	36	100	70	NIL	3	14.7	93	20	0.7	11.3	4.7
34	2	BASMATH LIMCY.V	24	1	32	120	80	NIL	3	18.4	80	28	0.7	9.0	3.6
35	2	SATHYA.S	28	2	36	110	70	NIL	2	21.7	89	24	0.5	10.7	5.0
36	2	DIVYA.P	23	1	36	110	70	NIL	3	18.0	90	18	0.8	10.0	3.1
37	2	BENNY.S	27	2	36	100	70	NIL	3	17.4	96	21	0.7	9.5	4.6
38	2	USHARANI.N	25	1	36	110	80	NIL	2	10.5	76	25	0.7	9.1	4.9
39	2	ANURADHA.S	34	1	36	120	70	NIL	3	23.2	87	36	0.9	8.6	4.0
40	2	AISHWARYA.V	26	2	36	110	70	NIL	2	8.2	70	24	0.6	8.0	5.3
41	2	SUGAPRIYA.N	20	1	34	100	70	NIL	3	21.5	106	19	0.9	9.9	3.8
42	2	KALAIVANI.P	29	1	36	100	70	NIL	2	19.6	98	32	0.7	9.6	3.6
43	2	SANGEETHA.R	22	1	34	110	70	NIL	3	10.1	83	20	0.5	9.1	4.9
44	2	SANDHYA.J	26	1	36	100	70	NIL	2	18.2	80	35	0.8	8.9	4.2
45	2	SUNITHA.T	28	2	36	120	80	NIL	2	16.4	95	31	0.8	9.3	4.0
46	2	JANANI.T	19	1	30	100	70	NIL	3	17.2	79	18	0.7	9.8	3.8
47	2	RATHNEASHWARI.K	19	1	28	90	70	NIL	3	14.3	100	26	0.9	10.0	3.1
48	2	JEELANIBEE.S	29	2	36	100	70	NIL	3	18.7	96	20	0.6	10.2	5.5
49	2	SHEELA.S	27	2	34	120	80	NIL	3	16.0	92	34	0.6	11.3	4.4
50	2	LATHA.E	23	1	36	90	60	NIL	3	9.2	85	31	0.5	9.5	3.6

URALB	GENOTYPE
1 = 1+	1 = CC
2 = 2+	2 = CT
3 = 3+	3 = TT
0 = Nil	

INSTITUTIONAL ETHICS COMMITTEE
GOVT. KILPAUK MEDICAL COLLEGE,
CHENNAI-10
Protocol ID. No. 02/2017 Meeting held on 14.11.2017

The Institutional Ethical Committee of Govt. Kilpauk Medical College, Chennai reviewed and discussed the application for approval **"C-786T (promoter) ALLEIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA"** submitted by Dr.S.ARUNA DEVI , Post Graduate in Bio-Chemistry, Govt. Kilpauk Medical College, Chennai-10.

The Proposal is **APPROVED.**

The Institutional Ethical Committee expects to be informed about the progress of the study any Adverse Drug Reaction Occurring in the Course of the study any change in the protocol and patient information /informed consent and asks to be provided a copy of the final report.

15.11.2017

DEAN
Govt. Kilpauk Medical College,
Chennai-10.

15/11/17

ME 1 Sec> Ethical Committee

PATIENT CONSENT FORM

STUDY TITLE: **"C-786T(promoter) ALLELIC VARIANTS OF *NITRIC OXIDE SYNTHASE* GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA."**

STUDY CENTRE: GOVT. KILPAUK MEDICAL COLLEGE HOSPITAL, CHENNAI-10

PATIENT'S NAME:

PATIENT'S AGE:

IDENTIFICATION NUMBER:

I confirm that I have understood the purpose and procedure of the above study. I have the opportunity to ask any questions and all my questions and doubts have been answered to my complete satisfaction.

I understand that my participation in this study is voluntary and that I am free to withdraw at any time without giving reason, without my legal rights being affected.

I understand that the sponsor of clinical study, working on sponsor's behalf, the ethical committee and the regulatory authorities will not need my permission to look at my health records, both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the study I agree to this access. However I understand that my identity would not be revealed in any information released to third parties unless as required under the law. I agree not to restrict the use of any data or results that arise from the study.

I hereby consent to participate in this study.

I hereby give permission to undergo complete clinical examination and diagnostic tests including hematological, biochemical and radiological tests.

Signature/thumb impression

Signature of the investigator:

Patient's name and address:

Name of the investigator:

Place:

Date:

நோயாளி ஒப்புதல் படிவம்

ஆராய்ச்சியைப் பற்றிய விவரம்: முன்சூல் வலிப்பு மற்றும் இதர சாதாரண கர்ப்பிணி பெண்களில் உள்ள கைப்பிடி அகலவீடு திசுத்தேல் மரபணு பல்லுருத் தோற்றத்

ஆராய்ச்சி மையம்: தினால் உண்மான தொடர்பு.

அரசு கீழ்ப்பாக்கம் மருத்துவக் கல்லூரி மருத்துவமனை

நோயாளியின் பெயர்: நோயாளியின் வயது:

பதிவு எண்:

நோயாளி கீழ்கண்டவற்றுள் கட்டங்களை (✓) செய்யவும்

1. மேற்குறிப்பிட்டுள்ள ஆராய்ச்சியின் நோக்கத்தையும் பயனையும் முழுவதுமாக புரிந்துகொண்டேன். மேலும் எனது அனைத்து சந்தேகங்களையும் கேட்டு அதற்கான விளக்கங்களையும் தெளிவுபடுத்திக் கொண்டேன். ☐

2. மேலும் இந்த ஆராய்ச்சிக்கு எனது சொந்த விருப்பத்தின் பேரில் பங்கேற்கிறேன் என்றும், மேலும் எந்த நேரத்திலும் எவ்வித முன்னறிவிப்புமின்றி இந்த ஆராய்ச்சியிலிருந்து விலக முழுமையான உரிமை உள்ளதையும், இதற்கு எவ்வித சட்ட பிணைப்பும் இல்லை என்பதையும் அறிவேன். ☐

3. ஆராய்ச்சியாளரோ, ஆராய்ச்சி உதவியாளரோ, ஆராய்ச்சி உபயத்தாரோ, ஆராய்ச்சி பேராசிரியரோ, ஒழுங்குநெறி செயற்குழு உறுப்பினர்களோ எப்போது வேண்டுமானாலும் எனது அனுமதியின்றி எனது உள்நோயாளி பதிவுகளை இந்த ஆராய்ச்சிக்காகவோ அல்லது எதிர்கால பிற ஆராய்ச்சிகளுக்காகவோ பயன்படுத்திக்கொள்ளலாம் என்றும், மேலும் இந்த நிபந்தனை நான் இவ்வாராய்ச்சியிலிருந்து விலகினாலும் தகும் என்றும் ஒப்புக்கொள்கிறேன். ஆயினும் எனது அடையாளம் சம்பந்தப்பட்ட எந்த பதிவுகளும் (சட்டபூர்வமான தேவைகள் தவிர) வெளியிடப்படமாட்டாது என்ற உறுதிமொழியின் பெயரில் இந்த ஆராய்ச்சியிலிருந்து கிடைக்கப்பெறும் முடிவுகளை வெளியிட மறுப்பு தெரிவிக்கமாட்டேன் என்று உறுதியளிக்கின்றேன். ☐

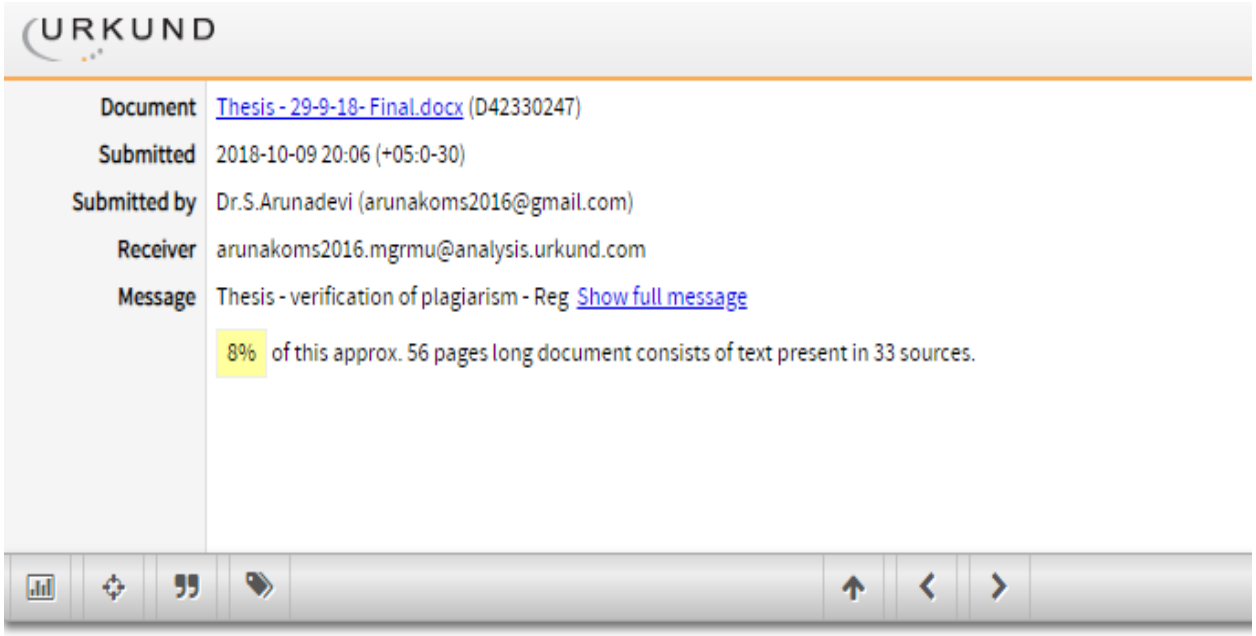
4. இந்த ஆராய்ச்சிக்கு நான் முழுமனதுடன் சம்மதிக்கின்றேன் என்றும் மேலும் ஆராய்ச்சிக் குழுவினர் எனக்கு அளிக்கும் அறிவுரைகளை தவறாது பின்பற்றுவேன் என்றும் இந்த ஆராய்ச்சி காலம் முழுவதும் எனது உடல் நிலையில் ஏதேனும் மாற்றமோ அல்லது எதிர்பாராத பாதகமான விளைவோ எற்படுமாயின் உடனடியாக ஆராய்ச்சி குழுவினரை அணுகுவேன் என்றும் உறுதியளிக்கின்றேன். ☐

5. இந்த ஆராய்ச்சிக்குத் தேவைப்படும் அனைத்து மருத்துவப் பரிசோதனைகளுக்கும் ஒத்துழைப்பு தருவேன் என்று உறுதியளிக்கின்றேன். ☐

6. இந்த ஆராய்ச்சிக்கு யாருடைய வற்புருத்தலுமின்றி எனது சொந்த விருப்பத்தின் பேரிலும் சுயஅறிவுடனும் முழுமனதுடனும் சம்மத்திக்கின்றேன் என்று இதன் மூலம் ஒப்புக்கொள்கிறேன். ☐

நோயாளியின் கையொப்பம் / பெருவிரல் கைரேகை ஆராய்ச்சியாளரின் கையொப்பம்

இடம்: தேதி:



C-786T (Promoter) ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA

Dissertation submitted for M.D. BIOCHEMISTRY - Branch XIII DEGREE EXAMINATION

DEPARTMENT OF BIOCHEMISTRY GOVT .KILPAUK MEDICAL COLLEGE CHENNAI- 600010 THE TAMILNADU DR.MGR MEDICAL UNIVERSITY CHENNAI-600032 MAY 2019

CERTIFICATE

This to certify that the dissertation entitled "C-786T (Promoter) ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA." is the bonafide original work done by Dr.S.Arunadevi, post graduate in Biochemistry , Underoverall supervision and guidance in the Department of Biochemistry, kilpauk Medical College, Chennai, in partial fulfilment of the regulations of The TamilnaduDr. M.G.R.. Medical University for the award of M.D.Degree in Biochemistry (Branch XIII) – during the academic period of May 2016 – May 2019.

Dr. V. MEERA M.D., DGO, Dr.P.VASANTHAMANI.M.D,DGO, PROFESSOR AND HOD ,DEAN, Department of Biochemistry, Govt. Kilpauk Medical College , Govt. Kilpauk Medical College ,Chennai – 600010. Chennai – 600010.

CERTIFICATE BY THE GUIDE

This to certify that the dissertation entitled "C-786T(promoter) ALLELIC VARIANTS OF NITRIC OXIDE SYNTHASE GENE AND ITS ASSOCIATION WITH NITRIC OXIDE IN PREECLAMPSIA." is the bonafide work done by DR. S.Aruna Devi , Post graduate , in the Department of Biochemistry, Kilpauk Medical College , Chennai, under the guidance and supervision of me ,Dr. V.MEERA, M.D., DGO., Professor and HOD, Department of Biochemistry , Kilpauk Medical College, Chennai – 600010. This dissertation is submitted to THE TAMIL NADU Dr. M.G.R