Vascular Biology of Pregnancy

A study of endothelial markers in hypertension in pregnancy

By

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Contents

i. Abstract

Hypertension is one of the most common medical conditions complicating pregnancy, with significant implications on maternal and perinatal morbidity and mortality. Abnormalities in placentation have been implicated as the primary pathology responsible for the development of hypertension during pregnancy and its effects such as pre-eclampsia and eclampsia. With advancing research, the focus is now gradually shifting towards abnormalities in the maternal vasculature, including endothelial damage/dysfunction and impaired repair as a probable cause for this, with the latter also being implicated in the development of cardiovascular disorders in later life in these women.

Hypertensive disorders occur in 6-8% of pregnancies. They also determine and influence the development of cardiovascular disease (CVD) in the mother in later life. Hypertension, obesity, metabolic syndrome and CVD are commoner in women with pre-eclampsia and preterm deliveries, whereas the risk of cerebrovascular disease is much higher in those with recurrent spontaneous abortions.

This research thesis is a study of the various processes occurring in the maternal vasculature, including angiogenesis, apoptosis, endothelial damage and regeneration/repair, the extra-cellular matrix and the haem oxygenase systems, the abnormalities that occur in them and their associations with hypertensive disorders of pregnancy and their complications.

ii. Declaration form

Filed in the original University copy of the thesis.

iii. Acknowledgements

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This thesis is dedicated to my mother, Rajalakshmi.

iv. List of illustrations

v. List of tables

vi. List of abbreviations used (in alphabetical order)

ACE inhibitors: Angiotensin converting enzyme inhibitors

ARB: Angiotensin receptor blockers

CEC: Circulating endothelial cells

CPC: Circulating progenitor cells

DBP: Diastolic blood pressure

HC: Non-pregnant healthy controls

HO-1: Haem oxygenase -1

HBP: Hypertensive pregnant women

MMP-9: Matrix metalloproteinase-9

NBP: Normotensive pregnant women

SBP: Systolic blood pressure

sFas: soluble Fas

sFasL: soluble Fas ligand

sFlt1: soluble fms-like tyrosine kinase

TIMP-1: tissue inhibitor of metalloproteinase-1

VEGF: vascular endothelial growth factor

vWf: von Willebrand Factor

Section 1: Literature Review

1.1. Hypertension in pregnancy: an overview

Hypertension is the most common medical condition that occurs in pregnancy, and a leading cause of maternal mortality, as well as other serious effects on pregnancy outcomes. (1)

The following chapter is a synopsis of hypertension in pregnancy and its evaluation and management.

Classification of Hypertension in Pregnancy

Hypertensive disorders are classified into 4 categories according to the Working Group Report on Hypertension in Pregnancy as chronic hypertension, preeclampsia-eclampsia, preeclampsia superimposed upon chronic hypertension and gestational hypertension (Table 1.1.1).(1)

Chronic hypertension is hypertension (blood pressure ≥140 mm Hg systolic and/ or ≥90 mm Hg diastolic) that is present and observable before pregnancy or diagnosed before the 20th week of gestation as well as that diagnosed for the first time during pregnancy and persistent postpartum.

Preeclampsia-eclampsia is a pregnancy-specific syndrome that usually occurs after 20 weeks' gestation, or earlier in trophoblastic diseases (hydatidiform mole or hydrops). The increased blood pressure is accompanied by proteinuria in this syndrome.

Preeclampsia is a syndrome characterized by hypertension, proteinuria and symptoms of headache, visual changes, epigastric or right upper quadrant pain and dyspnea. Several factors have been identified to be associated with an increased risk of preeclampsia such as age, parity, previous preeclampsia, family history, multiple

pregnancy, pre-existing medical conditions (insulin dependent diabetes mellitus (IDDM), obesity and insulin resistance, chronic hypertension, renal disease, auto-immune disease, anti-phospholipid syndrome, rheumatic disease), smoking, increased body mass index (BMI), raised blood pressure and proteinuria. In addition, couple-related factors including limited sperm exposure, primipaternity, pregnancies after donor insemination/ oocyte/ embryo donation have been found to play an important role too.

Eclampsia is the occurrence of seizures in a woman with preeclampsia that cannot be attributed to other causes.

Gestational hypertension or pregnancy induced hypertension (PIH) occurs with a blood pressure >140/90 mm Hg in a woman who was normotensive before 20 weeks' gestation. Severe gestational hypertension is a condition of sustained elevation in blood pressure of >160/110 mm Hg for 6 hours. Blood pressure normalizes in the postpartum period usually within 10 days. Patients may experience headache, blurred vision, abdominal pain and are noted to have abnormal lab tests, including low platelet counts and abnormal liver function tests.

Preeclampsia may occur in women with chronic hypertension, with a much worse prognosis than with either condition alone. (2) Superimposed preeclampsia is difficult to distinguish from worsening chronic hypertension, with possible findings that include new onset proteinuria in women with hypertension and no proteinuria in early pregnancy (<20 weeks' gestation), sudden increase in proteinuria or blood pressure in a woman with previously well controlled hypertension, thrombocytopenia (<100,000 platelets/mm3) or abnormal elevation in liver enzymes (alanine aminotransferase or aspartate aminotransferase). (2)

Measurement of blood pressure

Measurement of diastolic blood pressure during pregnancy has been a topic of debate. The National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (NHBPEP) recommends the use of Korotkoff phase V.

Pathophysiology

Preeclampsia is caused by the presence of the placenta or the maternal response to placentation. Poor placentation is a strong predisposing factor that leads to the maternal syndrome, the extent of which is related to the inflammatory signals (dependent on foetal genes) as well as the nature of the maternal responses (dependent on maternal genes).

Two diverse theories, vascular (ischemia-reperfusion resulting in oxidative stress and vascular disease) and immune (maternal-paternal immune maladaptation, i.e. a maternal alloimmune reaction triggered by a rejection of foetal allograft) are hypothesized to be responsible for preeclampsia. The aetio-pathophysiology of preeclampsia is complex and involves diverse factors such as genetic predisposition, disturbances in the renin-angiotensin-aldosterone axis, dysfunction of the maternal endothelium, maternal coagulopathies, cytokines, growth factors etc. (3, 4)

Is preeclampsia predictable?

Though various clinical and laboratory tests [Table 1.1.2] are currently used to predict the risk of women developing preeclampsia, no test reliably predicts preeclampsia.

The importance of clinical history cannot be over-emphasized here.

Prevention of preeclampsia

Several measures such as calcium supplementation, low dose aspirin, fish oils, etc. have been suggested to help prevent preeclampsia with varied results.

Management of hypertension in pregnancy

Baseline laboratory investigations (haematocrit, haemoglobin concentration, and platelet count and serum creatinine and uric acid levels) early during gestation, with monitoring of levels are particularly useful in the early diagnosis of preeclampsia.

Proteinuria of 1+ by routine urinalysis is an indication for a 24-hour collection for protein and creatinine estimation, to determine accuracy of collection and estimation of creatinine clearance. Accurate dating (with ultrasonographic methods, if required) and assessment of foetal growth in high-risk patients is important, as is a baseline sonogram at 25 to 28 weeks' gestation to evaluate foetal growth.

In women presenting with hypertension after mid-pregnancy, laboratory tests are recommended, with biweekly monitoring to distinguish preeclampsia from chronic and transient hypertension as well as to assess disease progression and severity. One or more abnormalities may be present in women with preeclampsia despite relatively minimal blood pressure elevations. A life threatening abnormality such as coagulopathy or abnormal hepatic and/or renal function may necessitate termination of pregnancy despite only mild hypertension.

Chronic hypertension in pregnancy

Women with hypertension require a full assessment of the severity of hypertension and advice on lifestyle changes (restriction of daily activities, refraining from vigorous exercise, restriction of daily sodium intake, stopping smoking and alcohol intake). Discontinuation of drugs known to be harmful to the foetus is particularly important, with a switch to safer alternatives. Those with target organ damage should be warned regarding the higher risk for adverse neonatal outcomes particularly in cases with early onset proteinuria.

Treatment of chronic hypertension

Careful evaluation and monitoring of blood pressures is all that may be required in most patients, particularly in the context of a physiological drop in blood pressure during the first half of pregnancy.

However, treatment may need to be continued in those with long-standing hypertension and target organ damage.

Management of preeclampsia

Delivery of the baby is the definitive treatment. However, the decision to deliver would need to take into account both maternal as well as foetal well-being and would depend on foetal gestational age, foetal status and severity of the maternal condition at assessment.

Some indications for delivery include severe overt clinical symptoms in the mother, grossly abnormal lab tests and foetal ill-health. (5)

Pharmacologic treatment

Antihypertensive treatment essentially prevent potential cerebrovascular and cardiovascular complications, the most common cause of maternal morbidity and mortality and do not prevent or alter the natural course of the disease in women with mild preeclampsia.

The commonly used drugs in the treatment of hypertension in pregnancy are methyldopa, labetalol, nifedipine. Magnesium sulphate is used in the management of preeclamptic patients to prevent eclamptic seizures. Sodium nitroprusside is the drug of choice in hypertensive crisis. Nitroglycerine is a mixed arterio-venous dilator and the drug of choice in preeclampsia associated with pulmonary oedema and control of hypertension associated with tracheal manipulation, though it is contraindicated in hypertensive encephalopathy due to its effects on cerebral perfusion and intracranial pressure. Recent guidelines issued by the National Institute of Health and Clinical Excellence (NICE) are comprehensive and stress the importance of a multi-disciplinary approach in the management of hypertensive disorders during pregnancy.(6)

Hypertensive Emergencies

Hypertensive encephalopathy, acute left ventricular failure, acute aortic dissection or increased circulating catecholamines (phaeochromocytoma, clonidine withdrawal) are acute emergencies that require urgent blood pressure lowering due to their potentially life-threatening consequences, particularly in women with underlying heart disease, chronic renal disease, multiple drug therapy to control hypertension, superimposed

preeclampsia in the second trimester and abruptio placentae with disseminated intravascular coagulation (DIC). (7)

Conclusions

Hypertension in pregnancy is an important medical condition with profound effects on the health of the mother and the foetus, the care of which requires a multi-disciplinary approach towards a safe and uneventful pregnancy and delivery.

TABLE 1.1.1 HYPERTENSIVE DISORDERS OF PREGNANCY

FINDINGS	CHRONIC HYPERTENSION	GESTATIONAL HYPERTENSION	PREECLAMPSIA-ECLAMPSIA
Time of onset of hypertension	< 20 weeks of gestation	After mid pregnancy	≥ 20 weeks of gestation
Proteinuria	Absent	Absent	Present
Haemo-concentration	Absent	Absent	Present
Thrombocytopenia	Absent	Absent	Present
Hepatic dysfunction	Absent	Absent	Present
Serum creatinine > 1.2mg/dL	Absent	Absent	Present
Raised serum uric acid	Absent	Absent	Present
Clinical Symptoms	Absent	Absent	Present

TABLE 1.1.2. TESTS FOR PREECLAMPSIA

Clinical Tests

Average second trimester Mean Arterial Pressure (MAP) ≥ 90 mm Hg

Angiotensin infusion test

Roll-over test

Uterine artery Doppler waveforms

Laboratory tests

Urinary calcium

Urine kallikrein to creatinine ratio

Plasma fibronectin

Serum inhibin

Serum Alpha feto protein (AFP)/ hCG (human chorionic gonadotrophin)

Serum urate

Haematocrit

Antithrombin III

Plasminogen activator inhibitors (1 and 2)

1.2. Endothelial dysfunction and hypertension in pregnancy

Endothelial integrity and function has been described to be paramount to maintenance of vascular haemostasis and blood pressure control. (8) Furthermore, it has been suggested that either endothelial dysfunction is present before pregnancy and predisposes women to hypertension in pregnancy and pre-eclampsia, or that the latter induces long-term changes in endothelial function, which could have implications for development of cardiovascular disease in later life. (9) Circulating endothelial cells (CECs), a novel means of assessing endothelial dysfunction, are mature cells detached from the vascular intimal layer in response to a variety of insults. Endothelial progenitor cells (EPCs) are non-leukocyte cells derived from the bone marrow with proliferative potential that may be important in vascular regeneration.

This chapter aims to provide an overview of current literature and concepts relating endothelial damage/dysfunction and impaired repair and the hypertensive disorders in pregnancy, with particular focus on CECs and EPCs.

Circulating Endothelial Cells (CECs)

Endothelial cells, as the name suggests, form the inner lining of the vascular tree and adhere to the basement, with little cell loss and subsequent clearance by the reticulo-endothelial system in healthy individuals. Complex pathological mechanisms such as mechanical injury, atherosclerotic processes, abnormalities in endothelial cellular adhesion molecules, matrix proteins and various apoptotic processes cause damage to the endothelial lining resulting in endothelial cell detachment, hence increasing CEC numbers in the blood stream. (10-12)

CECs are defined phenotypically by the expression of endothelial markers (e.g. von Willebrand factor, VE-cadherin, CD146) together with the absence of the expression of leukocyte (CD45) and immaturity markers (CD133). Amongst these, CD146 has evolved as the most popular marker for their identification, being concentrated at the endothelial junction where it plays a key role in the control of cell-cell cohesion, permeability and signalization. (13-18)

Isolation and quantification of CECs

The precise quantification of CECs has been difficult in view of their low numbers in circulation as well as differing morphological appearances (Figure 1.2.1). However, developments in cell enrichment and labelling techniques have improved their detection. CECs are counted in whole blood using either immunomagnetic separation technique (with CD146-coated immunomagnetic beads) and cellular counter staining using fluoroscein isothiocyanate-stained endothelial specific Ulex Europeus lectin or using flow cytometry. (19)

The immunobead method involves the use of 4.5 micrometre ferrous beads bound to an anti-CD146 monoclonal antibody. These coated beads are mixed with venous blood in a head over head mixer for 30 minutes at 4^{o}C. The anti-CD146 coated beads and blood/buffer mixture are placed in front of a magnet. The anti-CD146 coated beads (typically 5 x 10^{7}/ml of blood) bind to the CD146 epitope on the CECs and the magnet is then used to separate the bead-coated CECs from the other blood constituents. The unbound cells are washed away with buffer, and the bound cells are retained on the magnet. Following additional wash cycles, the cells are re-suspended in buffer and labelled (e.g. with acridine orange) before manual counting in a glass counting chamber under a fluorescent microscope. The use of an Fc blocking agent

(to prevent non-specific leukocyte binding) and relatively endothelial specific Ulex Europeus lectin 1 has improved the specificity of this technique. The endothelial (and non-leukocyte) origin of CD146-defined CECs has been amply demonstrated by co-marking with, for example, vWf, endothelial nitric oxide synthase and E-selectin. (19) Subsequently, CECs are defined, on fluorescent microscopy, as cells 10 to 50 μm in size with four or more immunobeads attached and staining positive for fluoroscein isothiocyanate-stained Ulex Europeus (Figures 1.2.2 & 1.2.3a).

CECs can also be isolated using flow cytometry, where whole blood is labelled with monoclonal antibodies tagged with fluorochromes; of note, this is also used to isolate EPCs and is discussed in detail later in this paper. Although this technique permits rapid multi-parametric analysis and the ability to detect sub-populations, there is potential for error in measurement as a result of inadequate standardization of flow conditions. For instance, the gating of the forward and side scatter as well as the threshold may collect measurements not only for intact CECs but also aggregates of leukocyte-endothelial cells as well as endothelial cell micro particles. The fluorescence measurements will be difficult to interpret since a consequence of the choice of gating is that measurements include many non-CECs. (10)

Although the immunobead method is a very specific method of CEC identification (with values in the order of 10 cells/ml), flow cytometry is more sensitive, often reporting greater numbers, up to 1000-fold higher than the former method.

CECs, endothelial damage/dysfunction and disease

Endothelial damage/dysfunction has been shown to be associated with a wide range of cardiovascular disease manifestations including hypertension, diabetes mellitus, atrial

fibrillation, heart failure, peripheral vascular disease, coronary artery disease and cerebrovascular disease. (19-38) Endothelial damage/dysfunction is measured using a variety of markers such as von Willebrand factor (vWf), soluble E-selectin, soluble thrombomodulin, [sTM]), reduced flow mediated dilatation (FMD) and impaired skin blood flow response using laser Doppler flowmetry.

Evidence to support an association between CECs and endothelial dysfunction has been mounting. An inverse correlation between CECs and FMD, a surrogate physiological marker of a perturbed endothelium has been previously demonstrated, (34, 35) as also a strong correlation between CECs and several plasma markers of endothelial damage (vWf, tissue plasminogen activator, soluble E-selectin). (34-38)

CECs are rarely found in healthy individuals, with typical counts being <3 cells/ml. Elevated numbers of CEC have been identified in a wide range of disease states, including those with underlying auto-immune, neoplastic, infective, haematological and thrombotic aetiologies. Further, longitudinal quantification of CECs in different diseases has shown variable levels according to the clinical condition/severity, suggesting its usefulness to monitor stable state, disease flare ups and response to treatment. (19)

CECs, endothelial damage/dysfunction and hypertensive disorders in pregnancy

Endothelial dysfunction has been described to play an important role in the pathogenesis of preeclampsia. Dysfunctional endothelial cells produce altered quantities of vasoactive mediators, which lead to a tip in the balance towards vasoconstriction. An imbalance in circulating angiogenic factors is emerging as a

prominent mechanism that mediates the endothelial dysfunction and the clinical signs and symptoms of preeclampsia. (39)

Data on CECs in hypertensive disorders of pregnancy are limited. (Table 1.2.1) For example, Canbakan et al (40) reported an increase in the number of circulating endothelial cells in women with preeclampsia (n=20) compared with healthy pregnant women, hypertensive women and non-pregnant controls (n=15 each). Preeclamptic patients had elevated numbers of CECs (13.2±5.2 cells/ml) compared with hypertensive patients (6.9±0.8 cells/ml), healthy pregnant women (5.2±1.4 cells/ml) and non-pregnant controls (4.0±1.8 cells/ml), ($P<0.0001$).

There is further evidence of an association between other indices of endothelial damage/dysfunction and hypertensive disorders in pregnancy, with other markers such as von Willebrand Factor (vWF), thrombomodulin and E-selectin, which are also noted to be raised in hypertension in pregnancy. (41)

Endothelial Progenitor Cells (EPCs)

Endothelial Progenitor Cells are a heterologous population of largely bone marrow-derived large non-leucocyte cells with properties similar to embryonal angioblasts, at different stages of maturation, from early (vascular endothelial growth factor receptor [VEGFR]/CD133+) to a more mature (VEGFR/CD34+) phenotype. EPCs are viable, can form colonies in vitro, have the capacity to differentiate into mature endothelial cells, and line the internal elastic membrane of the blood vessel. Hence, EPCs represent a subset of cells at varying stages of development present in the peripheral blood stream. (17, 18, 42-44)

However, more recent studies have challenged the above theories and have examined the origin as well as function of endothelial progenitor cells, although in the context of atherosclerosis and in animal models. (45, 46)

Bentzon et al (45) studied the origin of plaque smooth muscle cells in apoE$^{-/-}$ mice by a series of bone marrow transplantations and in models of atherosclerosis induced by surgically transferred arterial segments. Their experiments confirmed that smooth muscle cells in atherosclerotic plaques are exclusively derived from local vessel wall. (45)

In a subsequent study, Hagensen et al (46) investigated whether endothelial progenitor cells in the circulating blood are a source of plaque endothelial cells during atherogenesis. Their experiments also looked at plaques in lethally irradiated apoE$^{-/-}$ mice reconstituted with bone marrow cells from enhanced green fluorescent protein (eGFP) transgenic apoE$^{-/-}$ mice and plaques induced in segments of common carotid artery transplanted from apoE$^{-/-}$ mice into eGFP^{+} apoE$^{-/-}$ mice. The findings of their study also suggested that circulating endothelial progenitor cells rarely contribute to plaque endothelium. (46)*Isolation and quantification of EPCs*

EPCs can be isolated and quantified using Flow Cytometry. Red blood cells from a fresh sample of K3 EDTA anti-coagulated blood are lysed with BD lysing solution. The sample is gently inverted continually for 10 minutes following by centrifugation at 700G for 5 minutes. The obtained pellet is washed with a buffer solution [phosphate buffered saline (PBS), 5% bovine serum albumin BSA)] and then centrifuged again and washed twice. The resulting pellet is re-suspended and blocked with the Fc-receptor blocking antibody Octagam and 10% mouse blocking serum followed by incubation with CD133-PE (PhycoErythrin), CD45-FITC (Fluorescein

IsoThioCyanate) and CD34-PECy5 (PhycoErythrin Cy5) fluorochrome-labelled monoclonal antibodies for 20 minutes in the dark at 4ºC. The sample is then washed and centrifuged. The resulting cell pellet is re-suspended and fixed in 2% paraformaldehyde solution, before making it up to a 1mL sample with PBS-BSA buffer solution, ready for immediate flow cytometric analysis. Analysis is performed using a flow cytometer. CPCs are enumerated as a count of CD34+, CD133+, CD45- events per 1,000,000 collected events (Figure 1.2.3b).

Another method for the characterisation and quantification of EPCs is based on the culture of endothelial cells from circulating mononuclear cells. (47) This involves the isolation of peripheral blood mononuclear cells by density centrifugation of blood and subsequent culture on fibronectin coated plates. After 5–7 days in culture, adherent colonies are seen, where spindle shaped cells emerge from a cluster of round cells (EPC colony forming units, EPC-CFUs). These adherent cells display a variety of endothelial-like properties including the uptake of acetylated low density lipoprotein (AcLDL) and staining with UEA-1, a lectin of Ulex Europeus, specific for endothelial cells in a variety of tissues binding to the carbohydrate moiety al-fucose. (48) Whilst counting EPC-CFUs measures the capacity of circulating mononuclear cells to form endothelial cells, the colonies may not directly arise from the CD34C stem cells. The exact phenotype of EPC-CFUs remains a matter of debate in part because the purity of CD34C cells used in the initial study was only 15%. (44) Peripheral blood contains several cell types that can differentiate into endothelial-like cells in vitro, including haematopoietic stem cells, mononuclear phagocytes (monocyte-macrophages), and mature endothelial cells. (49)

EPCs and hypertensive disorders in pregnancy

Pregnancy involves adaptive changes in the maternal vasculature to ensure effective and adequate supply of nutrients to meet the increasing needs of the growing foetus. An up-regulation of endothelial function has been reported in pregnancy, resulting in vasodilatation as a result of increased release of vasodilators such as nitric oxide or a fall in the release of vasoconstrictors. EPCs have been detected among circulating mononuclear cells (MNCs) and in cord blood, and are thought to play an important role in vascular homeostasis. Bone marrow derived EPCs contribute to neovascularisation by vasculogenesis (de novo formation of blood vessels from precursors). The recruitment, mobilization and incorporation of bone marrow-derived EPCs have been shown to restore an intact endothelial lining. (50-53)

Our knowledge regarding the mechanisms of adaptive endothelial changes of normal pregnancy their attenuation of failure in women who develop preeclampsia is rather incomplete. Populations of bone-marrow derived EPCs exist in the adult that are mobilized into the circulation by stimuli such as oestrogen and vascular endothelial growth factor, which can then differentiate into endothelial cells lining the lumen of blood vessels and/or release growth factors that act in a paracrine fashion to support the endothelium. EPCs are thus thought to function as a cellular reservoir to replace dysfunctional or senescent endothelial cells, and therefore may be critical to the overall health of the vascular endothelium. Data are emerging to suggest that the number of EPCs in the maternal circulation increases with normal pregnancy and that this change fails to occur in women with preeclampsia. Although speculative, it has been hypothesised that an excess of anti-angiogenic factors [including soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin] interfere with nitric oxide-driven

mobilization or activity of EPCs in the maternal circulation, contributing to the widespread endothelial dysfunction underlying the clinical manifestations of preeclampsia. (54)

As with CECs, the data on EPCs in hypertensive disorders of pregnancy are limited. (50, 53, 55-59) For example, Sugawara et al (50) examined the level of circulating EPCs throughout uncomplicated pregnancies (n=20) and assessed the correlation between serum oestradiol levels and the number of EPCs. The number of circulating EPCs was noted to increase gradually, paralleling the progression of gestational age. In addition, the number of EPCs correlates significantly with the level of serum oestradiol, suggesting their role in the regulation and maintenance of the placental development and vascular integrity during pregnancy.

In a further study, Sugawara et al (53) found that the number of circulating EPCs was decreased in women with preeclampsia (n=8) compared with normotensive pregnant women (n=7) (median, 10.0 vs. 34.0 CFU; $P < 0.01$). The rate of cellular senescence was significantly increased in patients with preeclampsia (33.9%) compared with that in controls (22.9%; $P < 0.05$). Their patients with preeclampsia were divided into two subgroups: the CRP negative group (CRP <0.1 mg/dl; n=4) and the CRP-positive group (CRP >0.1 mg/dl; n=4). EPC CFU counts were markedly decreased in CRP-positive patients compared with those in CRP-negative patients (5.0 and 25.0 CFU, respectively; $P < 0.05$). They concluded that depletion and cellular aging of EPCs in patients with preeclampsia might be associated with endothelial dysfunction and could be affected by systemic inflammation.

A study by Buemi et al (55) analysing and comparing the concentrations of EPCs during the 3 trimesters of normal pregnancy, gestational diabetes and hypertension

found a progressive increase in EPC levels in normal pregnancy. On the contrary they noted a fall in levels of CD34+ cells in the third trimester of women with gestational diabetes compared with the other groups. Further, although they found no significant differences between the diabetic and hypertensive patients for the percentage of cells expressing CD133 and VEGFR2, in both groups the percentage of CD133+/VEGFR2+ elements was significantly higher than in the healthy control subjects. These findings suggest mechanisms regulating maternal vascular modifications during pregnancy as well as the different patterns of mobilization of endothelial progenitor cells during pathologic states in which endothelial disorders occur. Some of the other studies are summarised in Table 1.2.2.

Endothelial dysfunction, Virchow's triad & clinical implications

Endothelial dysfunction is an important component of the Virchow's triad. With its effects on the maternal vasculature during pregnancy as well as its long term effects on the cardiovascular health of women with gestational hypertension, a brief discussion on the relationship of endothelial dysfunction with the other components of the triad, i.e. abnormalities of haemorheology and turbulence at bifurcations and stenotic regions ('abnormal blood flow') and abnormalities in platelets as well as the coagulation and fibrinolytic pathways ('abnormal blood constituents') is noteworthy. (60)

Although hypertension, in general, involves blood flow under high pressures, its complications such as myocardial infarction and stroke are thrombotic and not haemorrhagic, further confirming that it fulfils the pre-requisites of Virchow's triad for thrombogenesis, leading to a pro-thrombotic or hypercoagulable state. Further, treatment of hypertension helps reverse some of these changes. (61, 62)

Figure 1.2.4 indicates a close relationship between thrombogenesis, atherogenesis and angiogenesis, the ‘vascular triad' (the Birmingham vascular triad), with the endothelium central to the processes.

What are the clinical implications of this?

The key adverse cardiovascular outcome clinically is myocardial infarction, particularly relevant in the current day, with many women having babies at an advanced age and being treated for risk factors for cardiovascular events, including hypertension, hypercholesterolemia, obesity, diabetes mellitus and family history of ischaemic heart disease. Although pregnancy is not typically a risk factor for acute myocardial infarction, it increases the risk 3- to 4-fold. (63, 64) Many risk factors are unique for pregnancy-related acute myocardial infarction, with super imposed hypertension being an important one. (65, 66)

The incidence of acute myocardial infarction has been reported to be 3 to 10 cases per 100 000 deliveries. (66, 67) Although rare, acute pregnancy-related myocardial infarction can be associated with significant morbidity and mortality, both to the mother and the foetus, with a case fatality rate as high as 37%. (68) Even a single death due to acute myocardial infarction makes a substantial contribution to maternal mortality in view of an overall low rate (fewer than 12 women per 100 000). (69)

Hence, a focussed approach by a multi-disciplinary team comprising of cardiologists, cardiology specialist nurses, obstetricians and mid wives is essential to managing pregnant women with hypertension in order to prevent cardiovascular morbidity and mortality and facilitate an uncomplicated and uneventful gestation and delivery.

Conclusions

Hypertension in pregnancy is a spectrum of disorders, with a range of pathogenetic processes that may contribute to the varied manifestations of the maternal and foetal syndrome as a consequence. There is mounting evidence that endothelial dysfunction may well be central to and responsible for other irregularities in the maternal vasculature, with implications not only on the current gestation, but may potentially determine the future cardiovascular health status of the woman.

Figure 1.2.1: Circulating Endothelial Cells

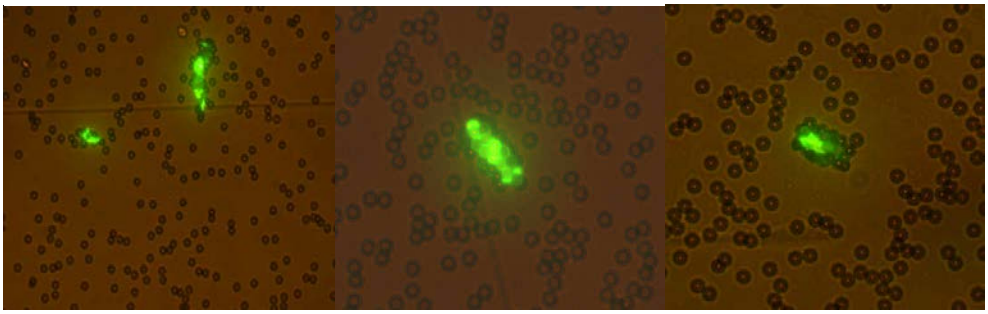


Figure 1.2.2: A typical Circulating Endothelial Cell isolated using the Immunobead Method

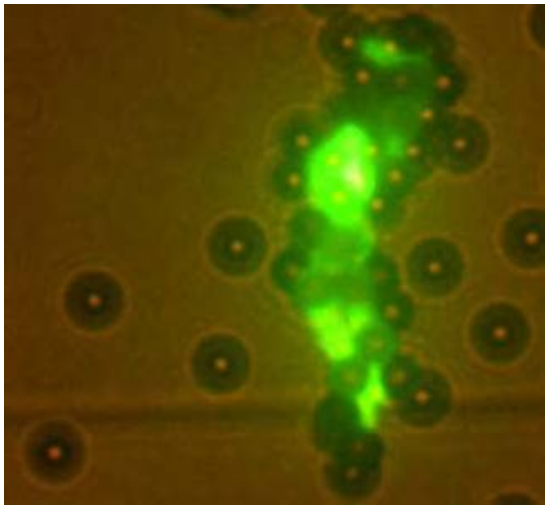
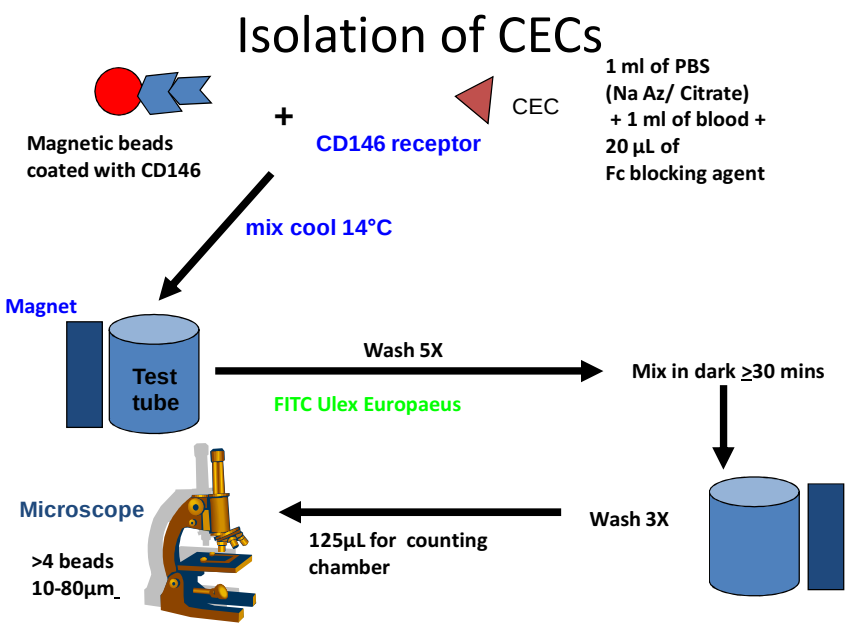
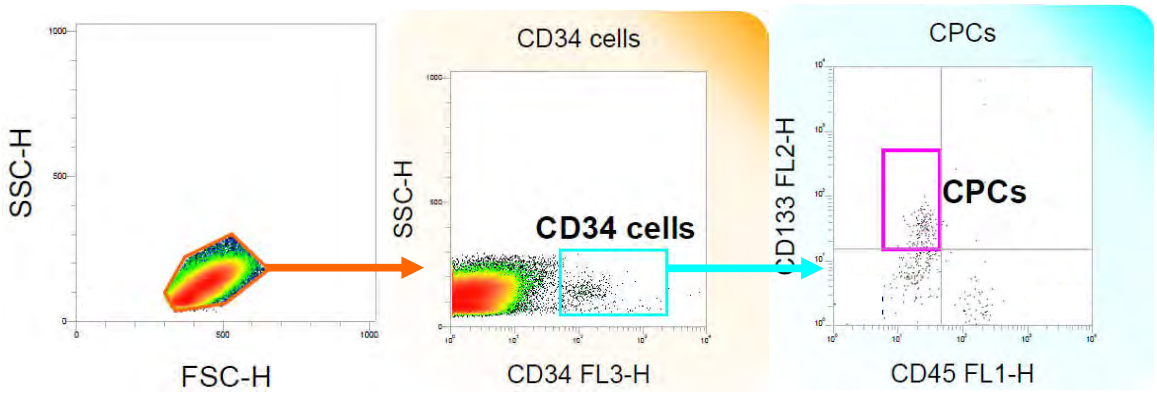


Figure 1.2.3a: Isolation of circulating endothelial cells by Flow Cytometry



CEC: Circulating Endothelial Cell

Figure 1.2.3b: Gating strategy for endothelial / circulating progenitor cell enumeration with flow cytometry [CPCs, circulating progenitor cells]



CPCs, circulating progenitor cells. The left hand FSC/SSC panel gates small lymphocyte-like cells, the centre panel identifies cells of low SSC but strongly expressing CD34, whilst the right hand panel show dual staining for CD133 and CD45. Thus CPCs are defined in the upper left quadrant as cells bearing CD34 and CD133, but not CD45.

Figure 1.2.4: Thrombogenesis, atherogenesis and angiogenesis in vascular disease: the Birmingham 'Vascular Triad'

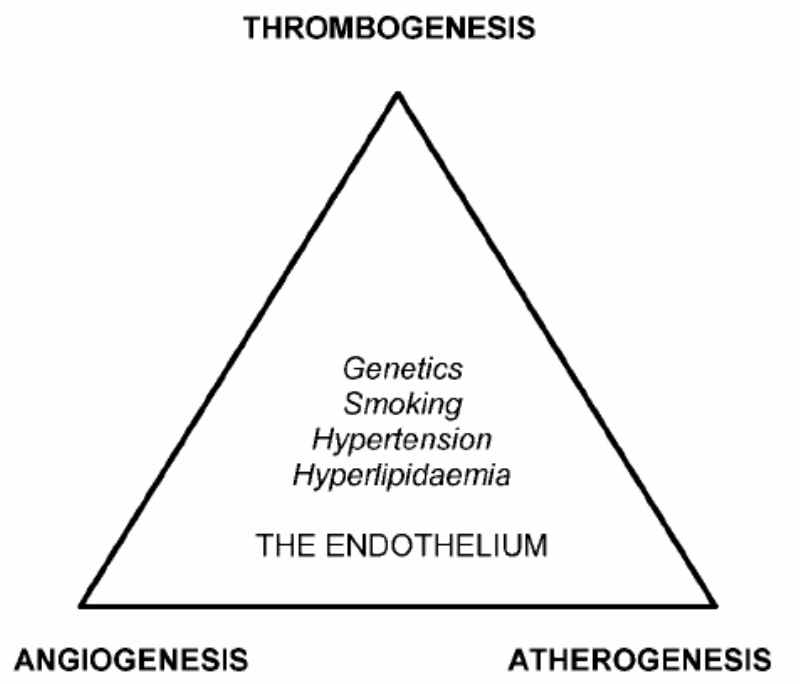


TABLE 1.2.1: ENDOTHELIAL DYSFUNCTION AND PREGNANCY

Study	Patients (n)	Markers studied	Findings
Canbakan et al (40)	Preeclampsia (20) Hypertensive pregnant women (15) Normotensive pregnant women (15) Healthy non-pregnant controls (15)	CECs, Homocysteine levels	CECs elevated in women with preeclampsia compared with other groups [$p < 0.0001$]
Nadar et al (41)	Pregnancy induced hypertension (36) Normotensive pregnant women (36)	Plasma vWf, E-selectin, thrombomodulin	Significantly higher levels of plasma vWf ($p=0.003$), E-selectin ($p=0.001$) and thrombomodulin ($p=0.01$) in women with pregnancy induced hypertension compared with normotensive controls

TABLE 1.2.2: ENDOTHELIAL PROGENITOR CELLS AND PREGNANCY

Study	Patients (n)	Findings
Sugawara et al (50)	Uncomplicated pregnancies (20)	Gradual increase in EPCs with progression of gestational age, with significant correlation with serum oestradiol levels
Sugawara et al (53)	Preeclampsia (8) Normotensive pregnant women (7)	Circulating EPCs decreased in preeclampsia ($p<0.01$) EPC Colony Forming Units (CFU) counts markedly reduced in C-reactive protein (CRP) positive patients ($p<0.05$)
Buemi et al (55)	Normal pregnancy (7) Gestational diabetes (7) Hypertensive pregnancy (7)	Progressive increase in EPCs with gestational age Significantly lower CD34+ cells in gestational diabetes Significantly higher CD133+/VEGFR2+ in the diabetic and hypertensive groups
Kwon et al (56)	Severe preeclampsia (15) Normotensive controls (30)	Significantly low EPCs in cord blood, umbilical cord plasma free vascular endothelial growth factor (VEGF) in severe preeclampsia compared to control group ($p=0.009$ & 0.04 respectively)
Xia et al (57)	Preeclampsia (14)	Significantly reduced placental/ foetal EPCs and in vitro cultivated

	Normotensive controls (10)	EPCs in preeclampsia ($p<0.001$); inverse correlation of EPCs with cord blood levels of soluble fms-like tyrosine kinase 1 (sFlt-1), suggesting a decrease and dysfunction of placental/foetal circulating EPCs in pre-eclampsia
Savvidou et al (58)	Normal singleton pregnancies (24) Normal twin pregnancies (21) Non-pregnant controls (8)	Lower levels of EPCs noted in both the pregnant groups compared with non-pregnant controls, with a fall in levels with progression of gestational age ($p=0.001$ and 0.002 respectively)
Lin et al (59)	Preeclampsia (12) Normal pregnancy (12)	EPC CFUs fourfold lower in preeclampsia compared with controls ($p<0.005$); elevated maternal plasma sFlt-1 ($p< 0.0001$) & reduced placental growth factor (PlGF) ($p< 0.01$) in preeclampsia, with no correlation with CFU-EPC counts.

1.3. Angiogenesis and apoptosis in hypertension in pregnancy

The causes of most cases of hypertension during pregnancy remain unknown, although one of the first hypotheses focused on abnormal vascular reactivity and the over-production of vasoactive substances such as angiotensin II by the foetal/placental system (1, 2). Subsequent hypotheses predicted a role for factors such as endothelial cell activation, nitric oxide and misaligned trophoblast invasion (5-8). More recently, it has been suggested that abnormalities in angiogenic growth factors (e.g. vascular endothelial growth factor [VEGF], angiopoietin, and more recently, angiogenin) and apoptosis may be important (9-13).

This chapter aims to provide an overview of current literature and concepts relating abnormalities in angiogenesis and apoptosis and the hypertensive disorders in pregnancy.

Angiogenesis in pregnancy

Normal intrauterine foetal development is dependent on adequate nutrient and substrate supply. Vasodilatation and development of new vessels both enable the adaptation of the uterine vasculature to the rising needs of the foetus. Angiogenesis is the process of neovascularisation from pre-existing blood vessels in response to hypoxia or substrate demands of tissues, and as such is a complex biological process comprising many steps that are precisely regulated by several molecules (15, 16). An initial step is the vasodilatation of the pre-existing vessel and formation of vesiculo-vacuolar organelles in the endothelial cells. The most important effector for this step is VEGF (70). Subsequently, vessel destabilization and matrix degradation occur, as perivascular stroma needs to be remodelled. Angiopoietin 2 and proteases (such as chymases and matrix metallo-proteinases) are involved in this step. Endothelial cell proliferation and migration along a gradient of chemotactic agents then

proceeds through the disintegrated basement membrane into the remodelled and softened perivascular space (18). Specific mitogens of endothelial cells in this step are VEGF, angiopoietins, and fibroblast growth factor, epidermal growth factor, CXC-chemokines and insulin-like growth factor type 1, which induce the proliferation of several types of cells. This leads to lumen formation and vessel stabilization, by the migrated endothelial cells first forming a monolayer and then tube-like structures with surrounding mesenchymal cells and vascular smooth muscle cells. Different forms of VEGF and integrins have been implicated in this step (71). In the pregnant uterus, the endometrium, decidua and placenta are sources rich of angiogenic growth factors. Angiogenic process is initiated by growth factors such as basic fibroblast growth factor (bFGF), VEGF, or placental growth factor (PlGF) (20, 21). Whilst the role of these growth factors is established, others, such as angiogenin, may also be important.

Angiogenin

Angiogenin is a member of the ribonuclease (RNase) super family. The RNases are enzymes of innate substrate specificity, but divergent functional capacities, whose distinct structure confers on angiogenin an endothelial binding motif, which it combines with its endonuclease enzyme activity and produces a potent stimulus for blood-vessel formation (22). Physiologically, angiogenin is also induced during inflammation, exhibiting wound healing properties as well as microbiocidal activity and conferring host immunity. Markedly high levels of angiogenin can be found in the circulation without proliferative impact. Angiogenin (or RNase 5) is a 14 kDa soluble protein, first isolated from the culture medium conditioned by colon carcinoma (HT-29) cells (72). The 123 amino acid chain and corresponding nucleotide sequences of angiogenin show 33% sequence identity and 65% homology with pancreatic RNase 1 (RNase A) (73). The tertiary structure of angiogenin still contains many

of the tertiary facets of peptide folding as seen in RNase 1, with the conservation of all key alpha helices and beta sheets (74).

Eight members have been identified in the RNase super family, and whilst each has distinct biological effects, all share a common enzymic ribonucleolytic activity on RNA (75). In the case of angiogenin, RNase activity is directed towards 28S and 18S rRNA with the major products being 100–500 nucleotides in length (76). Evolutionary analysis of the angiogenin lineage from non-mammalian species suggests that angiogenin and RNase 4 (closest family member to angiogenin) represent the most ancient forms of the RNase super family (77). Recently, it has been established that angiogenin acts as an endogenous microbicidal agent against systemic bacterial and fungal pathogens (78), extending a role for innate immunity for the rest of the RNase super family.

Angiogenin is present in plasma, in normal subjects, in a concentration of about 250–360 μg/L (77). During human development, angiogenin has been detected in organs such as heart (in a foetus at 19 weeks), spleen (foetus at 19 and 20 weeks), lung (foetus at 19 week and adults), liver (foetus at 20 week and adult), colon, prostate, breast, brain, retina, melanocyte and foreskin (only in adults)(73, 79-81). However, angiogenesis does not take place continuously in all tissues, at all stages, as angiogenin-mediated angiogenesis requires a catalytic substrate and a cell surface receptor. Angiogenin expression is stimulated by the RNA that is likely to be released during apoptotic and necrotic cell damage in wound healing. The source of plasma nucleic acids is likely to relate to the phagocytotic activity of macrophages on dead cells (82).

Angiogenin in pregnancy

The human placenta is one of the best in vivo models of physiological angiogenesis. Trophoblast cells invade the placental bed leading to remodelling of the spiral arterioles into maximally dilated low resistance vascular channels, which enables a high flow volume to the utero-placental bed (83). Hypoxia is a stimulus to increases the expression of mRNA and secretion of angiogenin in trophoblasts, at least in a human placenta experimental model. (83). Angiogenin levels increase from 150 to 250 ng/mL between weeks 10 and 40 in uncomplicated pregnancies (84), and during the first four postnatal days, there is a rapid increase in maternal serum angiogenin levels following healthy full-term pregnancies (85). Angiogenin probably has an important role in normal vascular development, as angiogenin levels are significantly decreased in patients with highly pathologic Doppler flow findings, compared with healthy pregnancies (84). Of note, pre-eclamptic patients who delivered low birth weight infants had higher levels of angiogenin than those who delivered infants with normal birth weight (85).

Pavlov et al (86) studied the expression of angiogenin in situ and in vitro, using the human term placenta as a model of physiological angiogenesis. Angiogenin was immunodetected by light and transmission electron microscopy, and its cellular distribution was established by double immunolabelling with a panel of endothelial cell markers. Angiogenin immunoreactivity was detected in villous and extravillous trophoblasts, the trophoblast basement membrane, the endothelial basal lamina, foetal blood vessels, foetal and maternal red blood cells, and amnionic cells. Villous cytotrophoblasts, isolated and differentiated in vitro into a functional syncytiotrophoblast, expressed and secreted angiogenin. Their data suggested that, in human placenta, angiogenin has a role not only in angiogenesis but also in vascular and tissue homeostasis, maternal immune tolerance of the foetus, and host defences.

Angiogenin has been studied alongside VEGF by three groups. Shaarawy et al (87) recruited 71 pregnant women with pre-eclampsia and 20 normotensive pregnant controls, finding maternal serum VEGF and angiogenin levels were significantly increased in cases of mild and severe pre-eclampsia compared to controls. This increase was positively correlated with elevated systolic and diastolic blood pressure, as well as abnormal Doppler velocimetry, and low birth weight. Their findings suggested a possibility of vascular reactivity and endothelial disturbance in pre-eclampsia, with measurement of these angiogenic factors in maternal serum as biomarkers for the assessment of the severity of the disease and of foetal outcomes. Lassus et al (88) measured VEGF and angiogenin in umbilical cord blood from 14 foetuses with erythroblastosis or alloimmune thrombocytopenia and at birth from 28 preterm foetuses, from 42 healthy term foetuses, and from 24 term foetuses born to mothers with insulin-treated diabetes, noting a significant correlation between VEGF and angiogenin levels. Gestational age correlated with both VEGF and with angiogenin, and VEGF levels were found to be lower in foetuses born to diabetic mothers than in the healthy term foetuses, although this difference was absent for angiogenin. Thus they found a developmental increase in concentrations of VEGF and angiogenin in umbilical cord plasma during the last trimester of gestation. The lower umbilical cord VEGF levels in term foetuses born to mothers with diabetes compared with those of healthy mothers may be secondary to abnormal foetal vascular development in diabetic pregnancies. Canbakan et al have (40) reported lower levels of angiogenin and VEGF in 38 normal and 38 hypertensive pregnancies compared to 50 non-pregnancy. In the hypertensive pregnancies, angiogenin was not significantly different at 15 weeks of pregnancy compared to 30 weeks implying low levels are present early and are fixed. However, although these studies are informative, it is clearly far too early to determine whether or not angiogenin has more to offer than does VEGF, in either clinical or pathophysiological matters. Table 1.3.1 summarises the major studies in this field.

Apoptosis

Apoptosis, the process of programmed cell death, has been implicated in the pathophysiology of pre-eclampsia (41). A working hypothesis states that inappropriate apoptosis of cytotrophoblast cells leading directly to poor vascularity and thus poor placentation, pre-eclampsia and foetal problems (42-44). The Fas/Fas ligand (Fas/FasL) system is one of the main apoptotic pathways and is expressed in immune as well as non-immune cells, including trophoblasts (45). Its expression and function respond to changes in the microenvironment and play a pivotal role in controlling cell proliferation and tissue remodelling. CD95 (Fas) is a type I membrane protein of 45 kDa that belongs to the TNF receptor family of proteins. FasL, a type II membrane protein of 37 kDa, belongs to the TNF and CD40 ligand family of proteins. Fas is widely expressed in many tissue types; in T and B cells, it is present constitutively in low levels on the surface of resting cells and its expression is enhanced following lymphocyte activation. In contrast to Fas, the expression of FasL is reported to be more restricted and often requires cell activation. The binding of the Fas receptor by FasL results in downstream activation of a cascade of intracellular proteolytic enzymes ending in apoptosis (89).

The Fas/FasL system and pregnancy

There is a considerable literature base demonstrating a role for Fas/FasL in pregnancy, many of which highlight regulated physiological apoptosis as part of an immunological response to the foetus (48-51). For example, some suggest a role for complex reversible molecular mechanisms including intra-decidual T cell tolerance that occur during and soon after implantation of the conceptus (89). There is also evidence for endothelial cell and smooth muscle cell apoptosis driven by Fas/FasL (53, 54).

Aberrations in the Fas/FasL system have also been implicated in disorders of pregnancy (55-58), including pre-eclampsia. The importance of the trophoblast in the pathophysiology of pre-eclampsia is clear, with reports of increased apoptosis of the extra villous trophoblast in the placental bed and villous trophoblast. The increased apoptosis may be secondary to an increase in trophoblast sensitivity to Fas-mediated apoptosis or an increase in material presented to a normally functioning cytotrophoblast/ syncytiotrophoblast system (59-63). The higher proliferation rate introduces more material into the syncytiotrophoblast via fusion and the multinucleated layer increases its apoptotic release of material to counterbalance this. Hence the increase in apoptosis is perhaps a sign of higher turnover of villous trophoblast secondary to an adaptive process. An increase in Fas expression coupled with decreased FasL expression in the villous trophoblast has been demonstrated (90). Serum from preeclamptic women has been shown to reduce trophoblast viability, and that effect seemed to be related to changes in trophoblast sensitivity to Fas-mediated apoptosis (91). Further, deportation of villous trophoblast debris directly into the maternal circulation is implicated in the genesis of an exaggerated intravascular maternal inflammatory response in pre-eclampsia (66). This may part-explain the link between the aberrant placentas and activated maternal endothelium seen in pre-eclampsia.

Whilst the Fas/FasL system operates between cells, soluble forms of both molecules may be found in the plasma (67, 68). Indeed, it has been suggested that soluble Fas protects from apoptosis (69). Whilst low soluble Fas is present in normal pregnancy compared to non-pregnancy, an increase in soluble Fas has been noted in pre-eclampsia (92) although Kuntz et al have been unable to confirm this increase (73). We have (93)found no differences in soluble Fas between 50 non-pregnant women, 20 normal pregnancies and 20 hypertensive pregnancies (free of pre-eclampsia) at early or late stages of the gestation. However, changes in soluble Fas are not specific for pre-eclampsia (75-77). Parallel data on soluble Fas ligand

are weak. Laskowska et al (72) reported no change in soluble Fas ligand in 11 cases of pre-eclampsia compared to 12 healthy normotensive pregnancies, whilst Kuntz et al (73) found raised levels in 20 pre-eclamptic hypertensive pregnancies compared to 18 controls. . The purpose of measuring the molecules in different disease groups of course hopes to identify a putative disease marker that would have predictive value. The experiments outlined above provide limited opportunity to do so, and far larger studies are required for the degree of sensitivity and specificity concerned. Nevertheless, Hsu et al have speculated that apoptosis triggered by the release of soluble Fas ligand may be an important pathophysiological process(92). Table 1.3.2 summarises major studies.

Conclusions

Angiogenesis and apoptosis are two important physiological processes that occur in the maternal vasculature, and angiogenin and the sFas/sFas ligand system are worthy of investigation. However, whether or not they provide fresh insights when compared to the VEGF (79-81), remains to be determined Significant abnormalities in both these processes are described in pregnancies complicated by diseases such as pre-eclampsia, but whether they are mere associations or causative in nature is unclear at present. Studies exploring their role in the pathogenesis of conditions including hypertension in pregnancy, before the development of pre-eclampsia/eclampsia and changes in their markers with progression of gestation would be helpful in understanding them better in the future.

TABLE 1.3.1: ANGIOGENESIS AND PREGNANCY

Study	Patients (n)	Markers studied	Findings
Shaarawy et al (87)	Pre-eclampsia (71) Normotensive pregnant women (20)	VEGF, angiogenin	• Significantly raised VEGF and angiogenin levels in mild and severe pre-eclampsia. • Positive correlation between their raised levels and poor bio-physical score, abnormal umbilical and uterine artery Doppler velocimetry and low birth weight.
Nadar et al (94)	Pregnancy induced hypertension (PIH) (69, pre-eclampsia, n=35) Normotensive pregnant women (64) Non-pregnant controls (30)	VEGF, angiopoietins (Ang-1, Ang-2), and their soluble receptors Flt-1 (sFlt-1) and Tie 2 (sTie-2)	• Significant differences in plasma VEGF, Ang-1, Ang2, sFlt-1 and Tie-2 between the study groups. • Ang-1 highest in the pre-eclampsia group ($p<0.001$) • Ang-2 highest in the normotensive pregnant group ($p=0.018$) • Plasma Tie-2 highest in the PIH group • VEGF levels significantly different between the pre-eclampsia group and the PIH group ($p<0.05$)
Kupferminc et al	Pre-eclampsia (19)	VEGF	• Significantly raised VEGF levels in pre-eclampsia

(95)	Normotensive pregnant women (19)		($p<0.001$); • Positive correlation between VEGF and systolic and diastolic blood pressures in the preeclamptic group ($r=0.56$; $p= 0.01$ and $r=0.48$; $p = 0.037$, respectively).
Baker et al (96)	Pre-eclampsia (27) Non-proteinuric PIH (15) Normotensive pregnant women (36)	VEGF	• VEGF levels below the lower limit of detection. • The proportion of detectable levels higher in the pre-eclampsia group (7/27) than in the normotensive group (1/ 36, $P < 0.05$).

TABLE 1.3.2: APOPTOSIS AND PREGNANCY

Study	Patients (n)	Markers studied	Findings
Laskowska et al (97)	Pre-eclampsia (11) Hypertensive pregnant women (12)	Maternal and umbilical serum soluble Fas and Fas ligand	• Increased maternal and umbilical vein serum sFas and increased umbilical vein serum sFasL levels in the study group in comparison with the control group ($p<0.001$).
Kuntz et al (98)	Pre-eclampsia (20) Normotensive pregnant women (18)	Paired maternal and umbilical cord blood serum soluble Fas and Fas ligand	• Soluble FasL levels higher in paired maternal and umbilical cord blood (CB) sera of hypertensive gestations ($p < 0.01$). • Soluble Fas levels similar between the groups. • Surface expression of FasL lower on maternal ($p < 0.01$) and CB ($p < 0.05$) neutrophils from affected gestations, whereas surface Fas expression lower on maternal ($p < 0.02$), but not CB, neutrophils and lymphocytes.
Hsu et al (92)	Pre-eclampsia (34) Normotensive pregnant women (34)	Serum soluble Fas levels	• Higher serum soluble Fas levels in preeclamptic women ($p < 0.001$).

1.4. The extra-cellular matrix in hypertension in pregnancy

Matrix metalloproteinases (MMPs) are a family of endopeptidases that degrade the components of the extracellular matrix (ECM) and contribute to the remodelling and physiological homeostasis of the ECM. They depend on the zinc and calcium for their activity and are regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). A balance between MMPs and TIMPs has been suggested to play an important role in vascular remodelling, angiogenesis and vasodilatation of both the uterine and systemic vasculature during normal pregnancy. Consequently, any imbalance between the two can result in alterations in the maternal vasculature and promote the development of the spectrum of hypertensive disorders of pregnancy. The altered balance can also cause other vascular diseases including abdominal aortic aneurysm, varicose veins, etc. Both MMP-9 and TIMP-1 levels have been shown to be altered in hypertension in pregnancy.(99, 100)

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of nine or more highly homologous Zn (++)-endopeptidases that collectively cleave most of the constituents of the extracellular matrix. They belong to a large family of proteases known as the metzincin super family and are involved in the cleavage of cell surface receptors, the release of apoptotic ligands (such as the FAS ligand), and chemokine/cytokine in/activation. (101) MMPs are also thought to play a major role on cell behaviours such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defence. They are distinguished from other endopeptidases by

their dependence on metal ions as cofactors, their ability to degrade extracellular matrix, and their specific evolutionary DNA sequence.(102)

MMPs are produced by many tissues and cell types including the vascular cells. They are either secreted from the cell or anchored to the plasma membrane and are often bound with heparin sulphate glycosaminoglycans on the cell surface. Metalloelastase (MMP-12) is expressed mainly in macrophages and is essential for macrophage migration (103). MMP-19 was identified by cDNA cloning from liver and as a T-cell–derived auto antigen from patients with rheumatoid arthritis (RASI) (104, 105).

The MMP family

The MMP family consists of 26 enzymes, 23 of which have been found in humans. They are divided into 6 groups as follows (106)

1. Collagenases [MMP-1, -8, -13, and -18 (Xenopus)] cleave interstitial collagens I, II, and III at a specific site three-fourths from the N-terminus, releasing ¼ and ¾ length fragments. They also cleave other ECM and non-ECM molecules.
2. Gelatinases, including gelatinase-A (MMP-2) and gelatinase-B (MMP-9) digest denatured collagens (gelatins).
3. Stromelysins, including stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10). MMP-3 has similar substrate specificity but higher proteolytic efficiency as compared to MMP-10.
4. Matrilysins [matrilysin-1 (MMP-7) and matrilysin-2 (MMP-26, endometase)].

5. Membrane-Type MMPs (MT-MMPs) [type-I transmembrane proteins MT1-, MT2-, MT3-, and MT4-MMP (MMP-14, -15, -16, and -24), and the glycosylphosphatidylinositol (GPI)-anchored proteins MT5-, and MT6-MMP (MMP-17 and -25)]. MT1-MMP can digest type-I, -II, and III-collagen and other components of ECM, and can activate proMMP to MMP.
6. Others, including MMP-11, -12, -19, -20, -22, -23, and -28.

Effects of MMPs

MMPs have been postulated to inhibit vascular smooth muscle contraction, although the exact mechanisms are unclear. They are also suggested to have a variable influence on angiogenesis. On one hand, MT1-MMP, MMP-2, -7, -9 and stromelysin-3 mRNA have been suggested to play an important role in angiogenesis, particularly in relation to tumor invasion and metastases. An augmentation in MMP activity is positively linked to an increase in metastatic and angiogenic potential of tumours. They can enhance angiogenesis by multiple mechanisms, including enabling the detachment of pericytes from vessels undergoing angiogenesis, releasing ECM-bound angiogenic growth factors, by exposing cryptic proangiogenic integrin binding sites in the ECM, by generating promigratory ECM component fragments and by cleaving endothelial cell-cell adhesions.(106)

MMPs during pregnancy

MMPs have been suggested to promote decidualization, an important prerequisite for successful implantation. Studies have shown endometrial production of proMMP-2, -3, -7, -9, and active MMP-2 (107). Further, MMP-26 mRNA is expressed in the mouse uterus during the oestrous

cycle and early pregnancy and may play a role in the cycling changes in the uterus during the estrous cycle and in embryo implantation (108).

MMPs are probably involved in placental remodeling throughout pregnancy. MMP-2 expression/activity in extravillous trophoblasts, and MMP-9 in villous cytotrophoblasts has been described in first trimester human placental tissue. The former has been observed in full term placental tissue, whereas the gelatinase activity was decreased or completely lost. The gelatinase activity was marked in early, but not term cytotrophoblasts. Invasive ability of early cytotrophoblasts was inhibited by TIMP-2 and anti-MMP-2 antibody. These data suggest that the invasive ability of trophoblasts may be regulated by gelatinases, especially MMP-2 (109). This is supported by reports of polarized release of MMP-2 and -9 from cultured human placental syncytiotrophoblasts (110). The studies emphasize the importance of temporal regulation of MMPs to perform specific functions during the gestation period.(111)

Increases in the plasma levels of MMPs have also been detected during normal pregnancy, suggesting their role in the pregnancy-associated changes in vascular function (111).

Further, MMPs probably play a role in the uterine artery remodeling during pregnancy and may help maintain uterine blood flow in late pregnancy. Continued elevated levels of some MMPs post-partum may contribute to vessel regression and return to a non-pregnant physiological state (112).

MMPs and hypertension

Hypertension is associated with vascular remodeling characterized by rearrangement of vascular wall components including ECM proteins. Recent studies have examined the role of MMPs and

TIMPs in the vascular remodeling associated with hypertension. A clinical study in 44 hypertensive patients and 44 controls demonstrated that the plasma levels and activities of MMP-2, MMP-9, and TIMP-1 are increased in hypertensive patients, which may reflect abnormal ECM metabolism (113). Other studies have shown different results and demonstrated that the plasma concentrations of active MMP-2 and MMP-9 are depressed in patients with essential hypertension. Also, a 6 month treatment with amlodipine normalized MMP-9 but not MMP-2 plasma concentrations (114). These studies suggested a role of abnormal ECM metabolism in hypertension, and raised the interesting possibility that antihypertensive treatment may modulate collagen metabolism. In addition, it demonstrates that MMPs from the same family can have significant and different effects in vascular function and disease processes.

Other studies examining the expression/activity of MMPs in internal mammary artery specimens obtained from normotensive and hypertensive patients undergoing coronary artery bypass surgery have shown that that not only MMP-1 and MMP-9, but MMP inducer and activator proteins are down regulated in the hypertensive state, which may result in enhanced collagen deposition in hypertension (115).

Experimental studies have also examined whether hypertension is associated with vascular remodeling and changes in the vascular tissue expression/activity of MMPs and the effects of MMP-9 during the progression of hypertension. It has been suggested that MMP-9 activation is associated with a beneficial role early on in hypertension by preserving vessel compliance and alleviating BP increase.

MMPs and preeclampsia

MMP-mediated vascular remodeling has been suggested to play a role in the pathogenesis of preeclampsia (111). MMP-2 is elevated in the plasma of women with preeclampsia (116) as well as in those who subsequently develop preeclampsia (117). Changes in circulating MMP-9 and TIMP-1 and -2 levels have also been observed in gestational hypertension (118). Increased net MMP-2 activity probably contributes to endothelial dysfunction that is central to the pathophysiology of preeclampsia (117). However, recent studies testing the hypothesis that proteases intrinsic to syncytiotrophoblast microvillous membranes (STBM) are the cause of the in vitro endothelial changes detected gelatinase activity and showed that it was due to MMP-9. Its presence has been confirmed by immunohistocytochemistry. Studies have, however, suggested that the effect of STBM on endothelial cells is unlikely to be caused by intrinsic proteases (119).The placenta has been suggested to be the source of the increased plasma MMPs. Low levels MMP-1, -9 and -3 were detected in extracts from the wall of human umbilical cord artery. MMP-2 is the main collagenolytic enzyme in umbilical cord artery (UCA) wall (both latent and active form). Preeclampsia is associated with a reduction in those MMPs content in comparison to control UCA. Hence the decrease of MMP content and activity in the umbilical cord artery may reduce the breakdown of collagen in the arterial wall. The accumulation of collagen with simultaneous reduction in elastin content in the UCA may reduce the elasticity of arterial wall and decrease the blood flow in the foetus of women with preeclampsia (120).Studies have examined the secretion of MMP by cultured human decidual endothelial cells from normal and preeclamptic pregnancies. MMP-9 and TIMP1 levels were similar between the two cell types; however, the basal and stimulated secretion of MMP-1 was markedly higher in normal compared with preeclamptic endothelial cells. It was suggested that the lower MMP1 expression

of decidual endothelial cells from preeclamptic women may inhibit endovascular invasion by cytotrophoblasts. These findings may, at least partly, explain the relative failure of trophoblasts to invade maternal decidual blood vessels in preeclamptic pregnancy (121).

MMPs and clinical disease

MMPs have been suggested to play a role in the pathogenesis of other related conditions including atherosclerosis and destabilisation of the atherosclerotic plaque, its rupture and coronary stenosis; formation of abdominal aortic aneurysm (AAA) and development of varicose veins. (106). There has been recent research looking into their role in left ventricular remodelling in hypertension as well as in the pathogenesis of end-organ damage secondary to hypertension (122-124).

Tissue inhibitors of metalloproteinases

The MMPs are inhibited by specific endogenous tissue inhibitor of metalloproteinases (TIMPs), which include four protease inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. They bind MMPs in a 1:1 stoichiometry and their expression is regulated during development and tissue remodelling.

A number of inhibitors of MMPs with potent anti-angiogenic activity are in early stages of clinical trials, primarily to treat cancer and cancer-associated angiogenesis. Initial clinical trials using MMP inhibitors as cancer treatments did not demonstrate efficacy in terms of reducing tumour progression partially because most trials were done in patients with advanced stage disease, when the tumour vasculature is already well-established, and also due to the fact that MMPs play multiple roles in both angiogenesis and tumour progression.

Experimental studies have suggested significant effects of doxycycline in reducing the progression of AAA. There is also widespread interest in developing MMP inhibitors for the prevention of atherosclerotic plaque rupture. The MMP inhibitors, EDTA and 1,10-phenanthroline, as well as recombinant TIMP-1, reduced the activities of MMP-1, -2, -9 and -3 which co-localized with regions of increased immunoreactive MMP expression, i.e., the shoulders, core, and microvasculature of the plaques (125).

MMPs and TIMPs in hypertension in pregnancy

Some of the studies looking at levels of MMP and TIMP are summarised in table 1.4.1.

Conclusions

The extracellular matrix is an important component of the vascular biology of the maternal vasculature and plays a significant role in the maintenance of the internal milieu in pregnancy.

Alterations in the relative concentrations of its constituent enzymes and their inhibitors have profound effects on the homeostatic mechanisms that control blood pressures in the mother.

TABLE 1.4.1: MMPs AND TIMPs IN PREGNANCY

Study	Patients (n)	Markers studied	Findings
Tayebjee et al (118)	Gestational Hypertension , GH (23) Normotensive pregnant women (30) Non pregnant healthy women (28)	MMP-9. TIMP-1, TIMP-2	• Significantly higher MMP-9 and lower TIMP-1 as well as MMP-9/TIMP-1 ratios in normotensive pregnant women compared with non-pregnant controls. • Higher TIMP-2 levels in the GH group. • Negative correlation between MMP-9 and MMP-9/TIMP-1 with age and diastolic blood pressure.
Palei et al (126)	Gestational Hypertension , GH (26) Preeclampsia (27) Normotensive pregnant women (30) Non pregnant healthy women (30)	MMP-2, MMP-9, TIMP-1, TIMP-2	• Higher pro-MMP-9 levels and higher pro-MMP-9/TIMP-1 ratios in women with GH. • No significant differences in pr-MMP-2 levels.
Montagnana et al (100)	Preeclampsia (14) Normotensive pregnant women (37) Non pregnant healthy women (21)	MMP-2, MMP-9, TIMP-1, TIMP-2	• Significantly raised MMP-2 and TIMP-1 levels in preeclampsia compared with other two groups. • MMP-9 and TIMP-2 levels higher in the normotensive

			pregnant group compared with non-pregnant controls. • Positive correlation between MMP-9 levels and gestational age in normotensive pregnant women.
Lavee et al (127)	Gestational Hypertension , GH (26) Normotensive pregnant women (30)	Amniotic MMP-2, TIMP-2	• Mean amniotic MMP-2 & TIMP-2 higher in women with GH; significantly higher MMP-2 levels in those who eventually developed preeclampsia.

Section 2: MD Proposal

The MD proposal was submitted at the time of registration to the University of Birmingham. The included proposal is an amended version appropriate to the thesis.

Aim:

To study the different aspects of vascular biology (angiogenesis, apoptosis, haem oxygenase system and extra cellular matrix turnover) in the aetiopathogenesis of hypertension in pregnancy, with particular emphasis on their relationship(s) to endothelial damage/dysfunction (circulating endothelial cells and progenitor cell pathophysiology).

Background:

Hypertension is a widely prevalent condition with an estimated 972 million affected worldwide.(128) It is the most common medical problem encountered during pregnancy, complicating 6-8% of pregnancies, with chronic hypertension being a primary disorder in most cases. Hypertensive disorders in pregnancy (defined in Appendix 1) are a leading cause of maternal mortality worldwide and increase perinatal mortality five-fold even in developed countries. (129) It is also one of the areas of clinical practice that has been studied extensively, yet remains less well understood.

The endothelium has many vital and diverse physiological roles, such as regulation of blood vessel tone, (130, 131) permeability, metabolism and haemostasis. Impairment of endothelial function manifests clinically as oedema, hypertension, abnormal vasoconstriction and hypercoagulability. Impaired endothelial function has also been found to be associated with hypertension, diabetes mellitus and heart failure (regardless of aetiology) (132-135), although whether it occurs as a cause or consequence is undetermined. Endothelial function is closely linked to inflammation and thrombosis as inflammatory cytokines induce expression of pro-

coagulants such as tissue factor. Hence, understanding endothelial function is likely to be a key to modifying risk factors of hypertensive disorders of pregnancy and their sequelae.

Circulating endothelial cells (CECs) are a novel way of determining endothelial cell damage. CECs are thought to be detached from the inside of vascular structures and enter the circulation as a result of vascular injury. Raised CECs have been shown in myocardial infarction, unstable angina and critical limb ischemia.(20, 37, 136) They are also increased in other diseases associated with endothelial injury, including septic shock, sickle cell crisis, ANCA (anti-neutrophil cytoplasmic antibody) small vessel vasculitis, thrombotic thrombocytopenic purpura (TTP), systemic lupus erythematosus (SLE) and Behcet's disease, with an increase in their numbers used for assessment of vascular damage and a marker of disease activity. (20) We are only aware of one study that has investigated CECs in pregnancy and pregnancy induced hypertension. In this small study, Wang et al(137) found a significant increase in CECs ($p<0.01$) in women with pregnancy induced hypertension compared to those with normal blood pressure. It is unclear if CECs are simply apoptotic or represent a stimulus to repair/angiogenesis, in relation to hypertension in pregnancy.

Generation of vessels is classified into angiogenesis and vasculogenesis, the former being the sprouting of capillaries from pre-existing vessels (e.g. tumours and embryos) and the latter being the development of blood vessels from in situ differentiating endothelial cells and is essential for the growth of blood islands and differentiation of the systemic vascular network in the embryonic yolk sac and foetus. Endothelial cells are generated in the periphery of blood islands from bone marrow-derived endothelial progenitor cells (EPCs). (138, 139) Indeed, EPCs are found in pregnant women and are thought to play important roles in vascularisation of the uterine

endometrium at embryo implantation and placentation during pregnancy, as angiogenesis is increased in the endometrium from implantation through early pregnancy.(140, 141) Abnormalities of EPCs may be involved in the pathogenesis of preeclampsia, with previous studies revealing that primitive EPCs are present in most pregnant women during the second trimester.(140) Their numbers increase gradually and parallel gestational age in normal uncomplicated pregnancies,(50) but decrease in women with preeclampsia, with increase in the rate of cellular senescence. (53)

Further, serum/plasma markers associated with neo-vascularisation and vascular growth (angiogenic factors), apoptosis and extra cellular matrix turnover are hypothesized to play an important role in endothelial dysfunction. New vessel formation during human placental development occurs by vasculogenesis and angiogenesis, the latter by elongation in the term placenta. Many angiogenic factors are available; the most commonly studied being vascular endothelial growth factor (VEGF) and more recently, angiogenin.

Angiogenin is a 14-kDa secreted protein with angiogenic and ribonucleolytic activities, and is one of the most potent inducers of neovascularisation in experimental models in vivo. Angiogenin is present throughout the foetal vascular tree and is expressed not only by these cells, but by all trophoblastic cells. The physiological role of angiogenin expression is believed to be related to endothelial-like functions assumed by the syncytiotrophoblast in direct contact with maternal blood. Successful pregnancy requires a harmonious and co-ordinated development of the vascular and trophoblastic components of the villi to form a functional area of exchange between the maternal and foetal blood, this is probably supported by angiogenin expression.(86) Shaarawy et al (87) studied 91 pregnant women (20 normotensive healthy pregnant controls, 55

women with mild preeclampsia and 16 with severe preeclampsia) in the late third trimester (34-40 weeks) and compared angiogenin and vascular endothelial growth factor (VEGF) in these three groups. Serum angiogenin levels were significantly elevated along with serum VEGF levels in women with mild and severe preeclampsia ($p<0.001$). These elevations were significantly associated with foetal poor biophysical profile scores and negatively correlated with infant birth weight in preeclamptic patients ($p<0.01$).

The haem oxygenase system is involved in vascular tone, regulating anti-inflammatory and anti-apoptotic responses as well as reducing oxidative stress and subsequent tissue damage in several organ systems. Haem oxygenase -1 (HO-1) is a 32-kDa inducible form of the haem oxygenase enzyme, found in most tissues of the body and expressed in high concentrations in the liver and spleen, areas of high erythrocyte turnover; HO-2 is a 36-kDa constitutive form and HO-3 the least characterized and least active isoform. The haem oxygenase system and their catalytic products are linked to several vital tasks of pregnancy such as placentation, placental hemodynamic control and antioxidant protection and hence are thought to play an important role in the progression of a healthy pregnancy to term. (142) HO-1 has been found to be reduced significantly in placentae from pregnancies complicated with preeclampsia that probably predisposes to placental injury, maternal endothelial cell activation and contributes to the development of preeclampsia and its complications.(143) Further, HO-1 is suggested to have a complex role in angiogenesis by mechanisms that are less understood presently (144, 145) and reduced HO-2 has been suggested to account for increased vascular resistance and oxidative injury to the trophoblast, resulting in endothelial dysfunction and apoptosis in pregnancy induced hypertension.(146)

The Fas-Fas ligand (FasL) system is one of the major pathways for the induction of apoptosis in cells and tissues. (147) Fas (CD95) is a type I membrane protein of 45 kDa that belongs to the tumour necrosis factor (TNF) super family and Fas L, a type II membrane protein of 42 kDa belongs to the TNF and CD40 ligand family. Fas is expressed widely in many tissues, T and B cells and in human trophoblast throughout gestation.(148) It is abnormally activated in diseases associated with impaired immune tolerance or chronic inflammation.(98) Previous studies have shown that maternal serum soluble Fas/ Fas ligand levels are elevated in pregnancies complicated by hypertension, preeclampsia and the HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome, a severe variant of preeclampsia.(97, 98, 149) The increased expression of Fas in trophoblasts is hypothesized to promote apoptosis of placenta in preeclampsia.(148)

The matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are also hypothesized to be responsible for the development of endothelial dysfunction, contributing to the pathophysiology of hypertension in pregnancy and preeclampsia.(117) Matrix metalloproteinases (MMPs) are zinc-based catalytic enzymes responsible for maintaining tissue allostasis. They are active at neutral pH and can catalyse the normal turnover of extra cellular matrix (ECM) macromolecules such as the interstitial and basement membrane collagens, proteoglycans as well as accessory ECM proteins such as fibronectin. Members of the MMP family include the "classical" MMPs, the membrane-bound MMPs (MT-MMPs) the ADAMs (a disintegrin and metalloproteinase; adamlysins) and the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif). There are more than 20 members in the MMP and ADAMTS family including the collagenases, gelatinases, stromelysins, some elastases and aggrecanases. MMP activity is regulated by a group of endogenous proteins, called, tissue

inhibitor of metalloproteinases (TIMPs) that bind to active and alternative sites of the activated MMP.(118, 150) However, previous studies investigating MMP-2, MMP-9 and TIMP-1 and TIMP-2 levels in pregnancy and preeclampsia have obtained contradictory results.(111, 118, 151) For example, a comparison of the levels of MMP-9, TIMP-1 and TIMP-2 in pregnant women revealed a significantly higher circulating MMP-9 and lower TIMP-1 levels in normotensive pregnant women compared to the non-pregnant. TIMP-2 was found to be higher in those with gestational hypertension. Consequently, MMP-9/TIMP-1 ratio was highest in the normotensive pregnant women and lowest in the non-pregnant control group. MMP-9/TIMP-2 ratio was highest in the normotensive pregnant group and lowest in the gestational hypertension group. (118)

In summary, there is as yet no report of a comprehensive assessment of endothelial dysfunction in pregnancy. The aim of this study is to assess indices suggestive of endothelial activation-dysfunction-damage and their relationship to angiogenesis, apoptosis and extra cellular matrix turnover.

Hypotheses to be tested:

This thesis aims to address three specific, focussed hypotheses pertaining to the vascular biology of hypertension in pregnancy.

1. When compared to normotensive pregnant women and healthy, non-pregnant women, hypertensive pregnant women have significant abnormalities of circulating endothelial cells and endothelial progenitor cells.
    a. EPCs are positively correlated to indices of angiogenesis (angiogenin, haem oxygenase) and negatively correlated to indices of apoptosis (Fas/ Fas Ligand), endothelial damage/dysfunction (CECs, von Willebrand factor (vWf)) and MMP/TIMP levels (as indices of extra cellular matrix turnover).
    b. CECs are positively correlated with indices of apoptosis, endothelial damage/ dysfunction and MMP/TIMP, and negatively correlated with indices of angiogenesis.
2. Sequential changes of these indices with progression of pregnancy (gestation weeks 12-14, 28-32) and post-partum are significantly different between hypertensive pregnant women and normotensive pregnant women.
3. Abnormalities of these indices can be related to pregnancy outcomes (birth weight, Ponderal index, placental weight).

Study Design:

1. Cross Sectional Study:

Pregnant women with pregnancies complicated by high blood pressure and its effects presenting to the antenatal hypertension clinic/ day assessment unit or the maternity wards M1/ M2 at City Hospital between 28 and 34 weeks, were compared with matched normotensive pregnant women and normal healthy non-pregnant controls. All patients had demographic details including age, sex, race, weight, blood pressure, co-morbid cardiovascular conditions recorded.

Inclusion criteria:

- Women aged between 18 and 45 years
- Pregnant women with normal (< 140/ 90 mm Hg and not on anti-hypertensive treatment) and high blood pressure (≥ 140/90 mm Hg or < 140/90 mmHg whilst on anti-hypertensive agents)
- Women able and willing to provide informed, written consent

Exclusion criteria:

- Women >45 years
- Presence of potential confounders- significant co-existing medical conditions, such as heart disease, renal disease, malignancy, vasculitis, connective tissue disease, recent major surgery or trauma
- Unable or unwilling to provide informed, written consent

2. Prospective Study:

Pregnant women attending the antenatal hypertension clinic had blood samples taken at booking [12-14 weeks of gestation], 28-34 weeks gestation and at approximately 6-8 weeks post-partum. Blood samples were measured on each occasion to assess their relation with gestational age and determine their prognostic value.

Endpoints for this study

One or more of the following:

- Development of pre-eclampsia/eclampsia
- Complications of pregnancy and/ or labour as consequence of high blood pressure
- All cause maternal and/or foetal mortality
- Adverse maternal or foetal outcome as a consequence of the high blood pressure or its effects (intra-uterine growth retardation, congenital anomalies, spontaneous miscarriages, intra-uterine deaths, oligohydramnios)

The design of the study was made by me with the support and advice from my supervisors. Any modifications during the course of the project were also made by me.

The recruitment of the study patients as well as healthy pregnant and non-pregnant controls was wholly made by me.

Laboratory assays:

1. 15 to 20 ml of blood was taken from subjects into 1 citrate, 2 EDTA and 1 serum tubes, using a 21 G butterfly needle®. Blood was venesected into 1 citrate, 2 EDTA and 1 serum Vacuette ® tubes. 1 EDTA bottle (usually the last tube filled) provided the whole blood samples needed for CEC analysis using immunomagnetic beads (1 ml) and EPC analysis using flow cytometry (1 ml). All other samples were centrifuged at 3000rpm for 20 minutes to obtain plasma/serum which was aliquoted and frozen at -80ºC for batch analysis.
2. CECs and progenitor cells were measured using whole blood collected in a fluoridated tube.
    a. CECs were quantified using an established Immunobead method.[41] These cells were separated using magnetic beads coated with monoclonal antibodies against CD146 and repeated washing with PBS buffer.
    b. Progenitor cells were quantified by Flow Cytometry. Peripheral mononuclear cells were obtained and stained for FACS analysis with antibodies to CD34, CD 45, and CD 133.
3. Citrated plasma was then aliquoted and frozen at -70°C for batch analyses using ELISA for the following parameters:
    a. Angiogenin, HO-1
    b. soluble Fas, soluble Fas ligand
    c. von Willebrand Factor, sFlt-1
    d. MMP-9 and TIMP-1

All laboratory methods are established in the University of Birmingham Centre for Cardiovascular Sciences laboratory.

All the lab work, including storing of samples, preparation of samples for analysis of CECs and CPCs and the ELISAs were performed by me, with the appropriate training on the above techniques provided by Dr A Blann and Mr B Balakrishnan.

The analysis of samples for CECs and CPCs was performed by blinding the samples before preparing them and then subsequent unblinding. Most of this was by obtaining a mix of samples from the three study groups on the days of recruitment and blinding the subject details using appropriate coding/ marking of samples. Further confirmation and validation of CEC numbers was made by seeking a second count by Dr S Jessani, my co-researcher, who was blinded to the source of the sample (the study groups).

Data analysis and statistics

For a power of 80% and 5% level of significance, based on the studies above and the limited data currently available, we aimed to recruit at least 40 patients in each group, for our cross-sectional analysis and at least 20 patients for the longitudinal study.

Following a test of statistical normality, data was expressed as mean (± standard deviation, SD) or median (inter-quartile range, IQR), as appropriate. Non-categorical data distributed normally have been analysed by one way ANOVA, and paired samples analysed using *t* test. Non-parametric data were analysed by Kruskal Wallis or by the Mann-Whitney *U* test. Correlations were sought by Spearman's rank method. Categorical data (sex, age) were analysed by the chi-squared test. A probability of ≤ 0.05 was considered as statistically significant.

Data collection, storage and analysis as well as statistical tests were performed by me, using SPSS 15 software, after obtaining training on the statistical methods relevant to the study from Dr. A Blann and Dr. D A Lane. The graphical representation of the data was made using Graph Pad Prism 4 software.

Ethical Considerations

The study was conducted in accordance with the declaration of Helsinki. Ethical approval was obtained from the Sandwell and West Birmingham Local Research Ethics Committee. Written informed consent was obtained from all patients.

The application process for approval of the study as well as appropriate amendments to the study was made by me (with guidance from my supervisors) with both the local Research and Development department as well as from Sandwell and West Birmingham Local Research Ethics Committee.

Section 3: Data chapters

3.1 Pharmacovigilance: Angiotensin converting enzyme inhibitors and angiotensin receptor blockers in pregnancy

Introduction

Chronic hypertension (blood pressure > 140/90mmHg) in women of childbearing age is usually mild hypertension, with the 10-year cardiovascular risk being very small, and cardiovascular events such as strokes and myocardial infarctions are extremely uncommon. In such low-risk women, there is little information on the level of blood pressure at which antihypertensive therapy should be prescribed, that is, in which the benefits of treatment outweigh the hazards. Current guidelines stress the importance of considering long-term cardiovascular risk rather than the absolute level of blood pressure alone prior to considering treatment and recommend treating patients with blood pressures that persistently exceed 160/100 mmHg (152-154). The British Hypertension Society guidelines suggest the use of angiotensin-converting enzyme inhibitors (ACE-Is) as first choice in patients younger than 55 years, with angiotensin receptor blockers (ARBs) reserved for patients intolerant of them (154). All guidelines, as well as national formularies and pharmaceutical company data sheets, however, advise that these agents should not be prescribed in pregnant women or women of child bearing age. Some women may, have an unplanned pregnancy while taking ACE-Is or ARBs, and in older women of childbearing age the coexistence of type 2 diabetes may prompt specialists in diabetes or general practitioners to prescribe these agents because of their reported benefits in reducing diabetic nephropathy.

There have been sporadic reports of developmental anomalies, particularly of the heart, skull and central nervous system in babies whose mothers were taking ACE-I at the time of conception

(155-159). One epidemiological study from Tennessee involving 29 507 infants reported 'major' congenital malformations in 7.1% of infants in which the mother had been taking an ACE-I in the first trimester of pregnancy. This contrasted with major anomalies in 2.0% of infants exposed to other antihypertensive drugs in pregnancy and 2.6% in which the mother took no antihypertensive drugs (160). The possibility that the ARBs may also be associated with developmental malformations has been raised by sporadic reports, and an analysis of 64 published cases in which unfavourable outcomes were reported in 42% (161, 162). To take things further, we investigated the possible hazards of ACE-Is and ARBs in pregnancy and analysed pregnancy outcomes in all mothers attending our antenatal hypertension clinic, who had conceived while taking these agents or who had had them prescribed in early pregnancy.

Methods

The City Hospital, Birmingham, serves about one-third of the population of the city of Birmingham, England with a catchment area comprising about 300 000 people. However, as the hospital has a large obstetric service and Neonatal Intensive Care Unit, mothers are also referred in from surrounding areas. Between 1 June 1977 and 30 April 2009, all women attending the weekly specialist antenatal clinic who had hypertension in early pregnancy were referred to a special weekly hypertension clinic to be seen jointly by a physician and an obstetrician. Data were collected prospectively in all 1078 hypertensive mothers referred to the clinic, with reference to their hypertension, the drugs they received and the obstetrical outcomes. After delivery, all babies are examined by a paediatrician before being discharged home. A total of 94 pregnancies were identified in which the mother had been or was taking either an ACE-I or an ARB. We have outcome data in 91 (96.8%) of these.

Results

Ninety-four pregnancies (including one twin pregnancy) were encountered in women who were receiving angiotensin-blocking drugs at the time of conception, and there were a further two women who had been prescribed an ACE-I in early pregnancy, one by their family doctor and one by a junior doctor in the diabetic clinic because of microproteinuria. In all women, these drugs were discontinued at the first visit to the clinic and in 48 (50%) these it was not considered necessary to substitute an alternative antihypertensive drug in the short term. Three women, all on ACE-Is, were lost to follow-up, having been delivered elsewhere (two in Pakistan). Outcome data are therefore available in 91 pregnancies.

Angiotensin-converting enzyme inhibitor pregnancies

Outcome data were available in 71 pregnancies in 66 women. Their mean (SD) age was 34.9 (4.9) years (range 19–44 years); 34 were South Asian, 22 were African Caribbean, 14 were European and one was Hispanic. Twenty mothers also had type 2 diabetes mellitus. The median interquartile range (IQR) duration of ACE-I use in pregnancy was 8.0 (6.0–13.0) weeks (range 6–28 weeks). The drugs were lisinopril (39 pregnancies; mean dose 10.4 mg), ramipril (14; 6.7 mg), enalapril (11; 18.8 mg), perindopril (4; 2 mg) and trandolapril, quinapril and captopril (one each; 2, 10 and 15 mg, respectively).

Angiotensin receptor blocker pregnancies

Outcome data were available in all 20 women, one of which had a twin pregnancy. Their mean (SD) age was 35.8 (5.3) years (range 23–46 years); eight were South Asians, 11 African Caribbeans and two Europeans. Four mothers also had type 2 diabetes mellitus. The mean

duration (IQR) of ARB use in pregnancy was 8.0 (6.0–10.0) weeks (range 5–18 weeks). The drugs were valsartan (7 pregnancies; mean dose 80 mg), losartan (5; 100mg), candesartan (4; 6mg), irbesartan (2; 150mg), telmisartan (2; 80 mg) and eprosartan (one patient; dose not recorded).

Outcomes

In the 71 pregnancies in women taking an ACE-I, there were 59 (83.1%) live births, eight miscarriages before 20 weeks gestation, one termination of pregnancy and three intrauterine or early neonatal deaths in which no developmental malformations were detected (Table 3.1.1). Six babies (10.2%) were found to have developmental abnormalities. These were one small ventricular septal defect which had closed spontaneously by the age of 6 months, one mild sensorineural deafness, one mild microcephaly (head circumference below the second centile) whose development up to the age of 2 years was normal, one hypospadias not requiring early surgery, one umbilical hernia, in an African Caribbean baby, not requiring surgery and one mild congenital hypotonia. In the 20 pregnancies (21 babies) in women who conceived while taking an ARB there were two miscarriages, one intrauterine death and one neonatal death due to trisomy 13 (Patau syndrome) (Table 3.1.1). Two developmental defects were identified in the remaining 17 babies (one inguinal hernia not requiring surgery and one craniosynostosis with tower skull. At follow-up this child did have delayed milestones and learning difficulties.

Discussion

The interpretation of these data must be done with great care and the following limitations need to be considered before drawing conclusions. Although the antenatal clinic data were collected

prospectively and represents all women receiving ACE-Is or ARBs in pregnancy in a single large hospital, the numbers are small. There were only eight developmental anomalies which might be attributed to these drugs, although this represents 8.8% of all live births. Many of the anomalies (e.g., the ventricular septal defect and the hernias) were very common, trivial or self-limiting. Only one defect, the craniosynostosis, can be considered to be major. Our major developmental anomaly rate was therefore 1.1%. Our results are, therefore, in sharp contradiction to those of Cooper et al. in the United States (160). Our results for the use of ARBs are also in contrast to the findings of Velazquez-Armenta et al. (162). Examining current literature they reported outcomes in 64 pregnancies. In 27 of these there were unfavourable outcomes including 14 (21.9%) developmental defects. This report may be affected by publication bias (i.e. the tendency of researchers to selectively publish adverse outcomes only). The patients attending our antenatal clinic were at high risk due to their age, ethnic origin and severity of hypertension. These characteristics would not be expected to cause developmental anomalies but might contribute to poor obstetrical outcomes. There were 10 miscarriages before 20 weeks gestation and five intrauterine or early neonatal deaths which were not due to structural anomalies. These numbers may reflect the high-risk status of the patients. The presence of concomitant diabetes might increase the risk of caudal regression syndrome. Major developmental defects were not encountered in the 24 diabetic patients in this study.

Although many of our patients were at high obstetrical risk, their short-term cardiovascular risk is low. We suspect that many of the women were started on their antihypertensive therapy after only cursory assessment of their cardiovascular risk status. Many may not have really needed blood pressure-lowering drugs in the first place. When we stopped the ACE-Is or ARBs, it was only necessary to substitute another agent (in the short term at least) in 47.8%. Among those

women who genuinely do need antihypertensive drugs in pregnancy there is a therapeutic dilemma. In the early British Hypertension Society guidelines the other class of drugs recommended in pregnancy was the β-receptor blockers (152). However, there is a body of evidence that strongly suggests that these drugs, or at least atenolol, when used in early pregnancy, may cause intrauterine growth retardation (163, 164). Labetolol is licensed for the treatment of hypertension in pregnancy, but the need to take this drug multiple (two or three) times per day raises issues with compliance and limits its usefulness except in very high-risk pregnancies (165). Methyldopa is known to be safe in pregnancy but many clinicians are reluctant to use this drug for fear of causing postnatal depression, although, there are no reports of this adverse effect in the world literature. It is possible that some clinicians are prescribing antihypertensive drugs in pregnancy in the hope that this will prevent the development of superimposed preeclampsia. There is, however, no evidence that the treatment of hypertension in early pregnancy leads to any reduction in the incidence of preeclampsia (165, 166). The benefits of antihypertensive drug therapy in pregnancy are confined to the prevention of the development of more severe hypertension and to avoid hospital admission for preterm delivery (166). Another factor influencing the choice of antihypertensive drugs is the pressure to prescribe angiotensin-blocking agents for patients with incipient or overt diabetic nephropathy (167). Twenty-two (23.9%) of our patients had type 2 diabetes and three of these had persistent proteinuria in early pregnancy. As previously mentioned, the ACE-I was started in pregnancy by clinicians in the diabetic clinic in one patient. Although the total number of pregnancies studied here is considerably smaller than the report by Cooper et al. (160), our pregnancy series has a number of strengths. All mothers were cared for by one clinician and, therefore, we are confident that this series includes all mothers on angiotensin-modulating drugs seen at one single large centre. In

addition, all babies were examined by a paediatrician or a paediatric specialist nurse before discharge home. Hence, it is very unlikely that any developmental anomalies went undetected. Ascertainment bias therefore does not explain why we have found fewer anomalies.

In conclusion, while our data are largely reassuring, the use of angiotensin-blocking drugs in pregnancy and in women who are likely to conceive should be avoided until further large case series and case–control studies are reported. The fact that we have encountered 94 pregnancies in which the mother was receiving angiotensin blocking drugs strongly suggests that treatment guidelines and pharmaceutical company data sheets are frequently being ignored.

TABLE 3.1.1: OUTCOMES IN PREGNANT WOMEN TREATED WITH ACE-I OR ARB IN EARLY PREGNANCY

Outcomes [n (%)]	ACE-Is in early pregnancy	ARBs in early pregnancy
Miscarriage before 20 weeks gestation	8 (11.3)	2 (10.0)
Termination of pregnancy	1 (1.4)	0 (0)
Intrauterine or early neonatal death (no anomaly detected except trisomy 13)	3 (4.2)	1 (5.0)
Live births	59 (83.1)	19 (95.0)*
Developmental malformations		
• None	53 (74.6)	17 (85.0)*
• Small ventricular septal defect	1 (1.4)	0 (0)
• Mild sensorineural deafness	1 (1.4)	0 (0)
• Mild microcephaly	1 (1.4)	0 (0)
• Mild hypospadias	1 (1.4)	0 (0)
• Small umbilical hernia	1 (1.4)	0 (0)
• Small inguinal hernia	0 (0)	1 (5.0)
• Neonatal hypotonia	1 (1.4)	0 (0)
• Craniosynostosis with tower skull	0 (0)	1 (5.0)
Total	71 (100)	20 (100)

* Includes one twin pregnancy.

3.2 Endothelial dysfunction and hypertension in pregnancy: Associations between circulating endothelial cells, circulating progenitor cells and plasma von Willebrand factor

Introduction

Hypertension in pregnancy, preeclampsia and eclampsia has been studied extensively, with abnormalities in placentation being implicated as a cause of these disorders. Current focus has been directed towards abnormalities in the maternal vasculature, including endothelial dysfunction and impaired repair as a probable cause for preeclampsia, with the latter also being implicated in the development of cardiovascular disorders in later life in these women. (168)

This chapter explores the relationship between indices of endothelial dysfunction and hypertension in pregnancy.

Circulating endothelial cells (CECs) are mature cells detached from the vascular intimal layer in response to a variety of insults and are defined phenotypically by the expression of endothelial markers (e.g. von Willebrand factor, VE-cadherin, CD146) together with the absence of the expression of leukocyte (CD45) and immaturity markers (CD133). Amongst these, CD146 has evolved as the most popular marker for their identification, being concentrated at the endothelial junction where it plays a plays a key role in the control of cell-cell cohesion, permeability and signalization (13, 15). Increased numbers of CECs are taken to imply vascular damage (19).

Circulating progenitor cells (CPCs) are non-leukocytes derived from the bone marrow with proliferative potential that may be important in vascular regeneration with properties similar to embryonal angioblasts, at different stages of maturation, from early (vascular endothelial growth

factor receptor [VEGFR]/CD133+) to a more mature (VEGFR/CD34+) phenotype. CPCs are viable, can form colonies in vitro, have the capacity to differentiate into mature endothelial cells, and line the internal elastic membrane of the blood vessel. Hence, CPCs represent a subset of cells at varying stages of development present in the peripheral blood stream. (18, 42-44). It follows that as CECs reflect endothelial damage, and CPCs are mechanism to repair that damage, then the ratio of CECs to CPCs may have pathophysiological significance. Their number has been established as a biomarker of cardiovascular risk and is known to decrease with age.(169)

Increased levels of von Willebrand factor (vWf), as are present in many cardiovascular, connective tissue and neoplastic diseases, are indicative of endothelial cell damage/dysfunction (170), although an increase in vWf during pregnancy is physiological (171). Nevertheless, raised vWf (41, 172, 173) and CEC levels (40, 174) have been previously demonstrated in pregnancy induced hypertension and preeclampsia, while altered endothelial progenitor cell levels have been shown in pregnancies complicated with diabetes and hypertension. (50, 53, 55, 58, 175)

We hypothesised abnormal levels of circulating endothelial cells (CECs), circulating progenitor cells (CPCs) and plasma vWf in pregnant women with hypertension, when compared to non-pregnant hypertensives and normotensive controls. We also hypothesised an altered balance between the degenerative and restorative processes, as determined by an altered CEC:CPC ratio. We tested this hypothesis on a cross-section study of women attending our antenatal hypertension clinic.

Methods

The study group included pregnant women between 18 and 45 years with raised blood pressure (≥140/90 mm Hg) or normal blood pressure (<140/90 mm Hg) and on anti-hypertensive drugs.

Collection of Blood Samples and Laboratory Methods, isolation of CECs and CPCs, and estimation of vWf levels has already been described.

Power calculations and statistical analyses

For a power of 80% and 5% level of significance, based on the limited data currently available, we aimed to recruit 30 patients in each group. Following a test of statistical normality, data were expressed as mean (± standard deviation, SD) or median (inter-quartile range, IQR), as appropriate. Non-categorical data distributed normally were analysed by one way ANOVA, and paired samples using t test. Non-parametric data were analysed by Kruskal Wallis or by the Mann-Whitney U test. Correlations were sought by Spearman's rank method. Categorical data (sex, age) were analysed by the chi-squared test. A probability of ≤0.05 was considered as statistically significant. A multiple regression analysis sought those indices likely to predict levels of CECs, CPCs and vWf.

Results

We recruited 38 hypertensive pregnant women, 40 normotensive pregnant women and 50 non-pregnant healthy women as controls. The subject groups and controls were matched for age. The gestational age and parity were higher in the hypertensive pregnant women group (Table 3.2.1). As expected, both systolic and diastolic blood pressures were significantly higher in the

hypertensive pregnant group. Hypertensive pregnant women had significantly high CEC levels (2-fold compared with normotensive pregnant women and 4-fold compared with non-pregnant healthy controls). CPCs were raised in normotensive pregnant group compared with hypertensive pregnant and non-pregnant healthy controls; the CPC counts were lower in hypertensive compared with normotensive pregnant groups, but this did not reach statistical significance. CPC: CEC ratio was lower in hypertensive pregnant women compared with non-pregnant healthy controls (Table 3.2.1) (Figure 3.2.1).

A negative correlation was noted between CPC: CEC ratio and BP (Spearman $r = -0.30$, $p<0.005$ and $r = -0.26$, $p<0.05$, respectively). Both the pregnant women groups had significantly higher vWf levels than the non-pregnant controls. vWf correlated significantly with CEC levels (Spearman $r = 0.39$, $p<0.0001$) and CEC levels correlated with both systolic and diastolic BP (Spearman $r = 0.28$, $p<0.005$ and $r = 0.31$, $p<0.001$, respectively). Multiple linear regression analysis revealed hypertension in pregnancy as an independent predictor of CEC levels ($p<0.0001$).

Discussion

This study demonstrates a significant difference in the levels of CECs, CPCs and their ratios as well as vWf in hypertension in pregnancy prior to the development of pre-eclampsia. CECs were noted to be doubled in hypertensive pregnant women compared with normotensive pregnant women and four times that in non-pregnant controls. This is perhaps the first study looking at relationship between CECs and CPCs, and with vWf, and determining their ratios as a potential marker of imbalance between endothelial damage and repair in hypertension in pregnancy in the absence of preeclampsia.

Previous studies (40, 50, 53, 55, 58, 174, 175) have investigated CECs and CPCs in preeclampsia. An increase in CECs, confirming endothelial dysfunction in preeclampsia has been described, and our data confirm this finding. However, variable results have been obtained with CPCs. Some studies have described a reduction in CPCs, with higher levels in normal pregnancies that parallel the progression of gestation, suggesting an important role they may play in regulating and maintaining placental development and vascular integrity during pregnancy. Matsubara et al noted that CPC number in peripheral blood did not differ significantly between preeclampsia and normal pregnancy, although their proliferation was significantly increased in preeclampsia (175). In contrast to the above findings, Savvidou et al noted an actual decrease in CPC levels in normal pregnancy (58). Our finding of increased CPCs in normal pregnancy adds to the uncertainty as to the role of these cells. We suggest that the raised CPCs in the healthy pregnant women in our study may reflect a general mobilisation from the bone marrow, perhaps under the influence of vascular endothelial growth factor (176), known to be increased in pregnancy (177). Alternatively, raised CPCs may be associated with increased placental angiogenesis (178).

vWf, a robust and well-established marker of endothelial damage/dysfunction, has been previously shown to be raised in pregnancy induced hypertension and preeclampsia (41, 170-173) . Of these, Nadar et al (41) noted that plasma vWf was significantly raised in pregnancy induced hypertension along with other markers of endothelial activation. The study by Molvarec et al (172) demonstrated significantly higher vWf in preeclampsia compared to healthy pregnant and non-pregnant controls. We are unaware of any other study correlating vWf and CEC levels in hypertension in pregnancy, and their correlation underlines their value as markers of vascular damage. In addition, hypertension in pregnancy and preeclampsia has been recognised as a risk

factor for the development of hypertension in later life (1). Endothelial dysfunction and its markers (including CECs and vWf) are associated with a variety of inflammatory states as well as hypertension and cardiovascular disease states. (170, 179) Our data therefore confirms and extends other work on vascular dysfunction in pre-eclampsia.(174)

The above findings suggest the presence of a dynamic process of endothelial damage due to the shearing effects of a hyper dynamic circulation and attempts at regeneration towards restoring endothelial integrity and preventing the development of complications as a result of failure in these mechanisms and raised blood pressures. A corollary to this would be to determine who amongst both the hypertensive group and the normotensive group develop preeclampsia-eclampsia and adverse pregnancy outcomes and hypertension and cardiovascular disease in later life.

The clinical implications of the alterations in CEC: CPC ratio remains to be seen in larger studies. Further, the role of CEC: CPC ratio as a biomarker to predict endothelial dysfunction would require the development of a single test that is reliable, validated and easily reproducible in clinic or at the bedside.

Limitations

This study is limited by its cross-sectional design and relatively small sample size. Although the subject groups were matched with respect to age, the gestational ages and parities of the hypertensive pregnant women was higher. However, whether this has had any influence on our findings is rather unclear. Further, the antigenic definition of CPCs is highly variable across literature, with no universally accepted profile as yet (18, 40, 42-44, 50, 53, 55, 58, 174, 175,

180). Thus, it is not possible to definitively extrapolate current data and apply this to all progenitor cell populations. Finally, our study has analysed CPCs using only one laboratory technique looking at cell counts and has not taken into account cell function – i.e. the potential for CPCs to reproduce and integrate into the endothelium. Such assessment is usually based on cell culture experiments, but is an important characteristic to define CPCs. Further studies, longitudinal in design and involving larger sample sizes are required to clearly establish a possible aetiological association between an imbalance in endothelial damage and repair and the development of complications and adverse outcomes in hypertension in pregnancy.

In conclusion, hypertension in pregnancy is characterised by abnormalities in the vascular endothelium, with abnormal CECs and vWf that correlate with blood pressure. This may reflect dysfunctional processes that are counteracted with reparative attempts at restoring endothelial integrity.

TABLE 3.2.1: LEVELS OF BLOOD PRESSURE, vWF, CECs, CPCs AND CEC:CPC RATIOS BY GROUPS

Mean (SD)	Hypertensive Pregnant (n=30)	Normotensive Pregnant (n=39)	Non-pregnant healthy controls (n=43)	p-value
Age (years)	31.9 (5.5)	28.8 (5.8)	30.6 (8)	0.146
Gestational Age (weeks)	30.3 (2.9)	26.7 (3.4)	NA	< 0.0001
Median (IQR) Parity	2 (0-3)	1 (0-2)	0 (0-1)	< 0.0001
SBP (mm Hg)	133 (17)	112 (9)	118 (9)	< 0.0001
DBP (mm Hg)	87 (11)	66 (6)	75 (9)	< 0.0001
vWf (IU/dL) [§]	157 (34) [*]	156 (27) [*]	139 (37)	< 0.0001
CEC levels (/ ml) [§]	4 (2-5) [*]	2 (1-2)	1 (0-2)	< 0.0001
CPC levels (/ ml)	296 (145-548)	370 (250-494) [*]	254 (144-378)	< 0.05
CPC:CEC ratio	78 (41-172) [†]	194 (101-370)	120 (78-230)	< 0.05

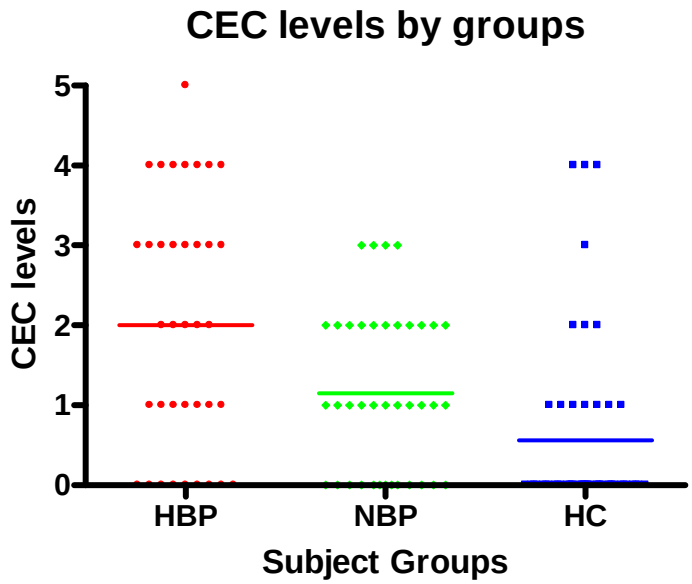
Between group analyses by ANOVA with Tukey post hoc test and Kruskal Wallis test with Dunn's post hoc test, as appropriate

[*]Significantly higher than healthy controls [†] Significantly lower than healthy controls

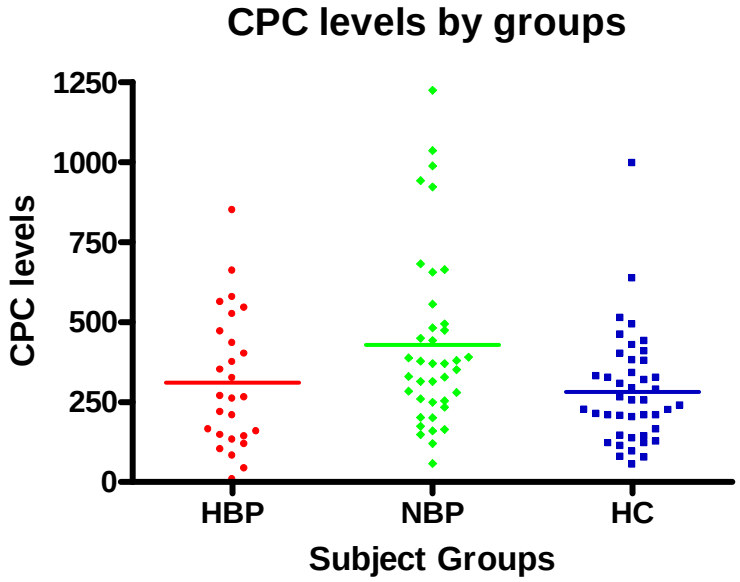
[§] Hypertensive pregnant women (n=38); Normotensive pregnant women (n=40); Non-pregnant healthy controls (n=50). Data are presented as mean (SD), median (IQR) or absolute numbers.

Figure 3.2.1: Circulating Endothelial Cell levels (CECs), Circulating Progenitor Cell levels (CPCs), Circulating CEC:CPC ratios and von Willebrand Factor levels by groups

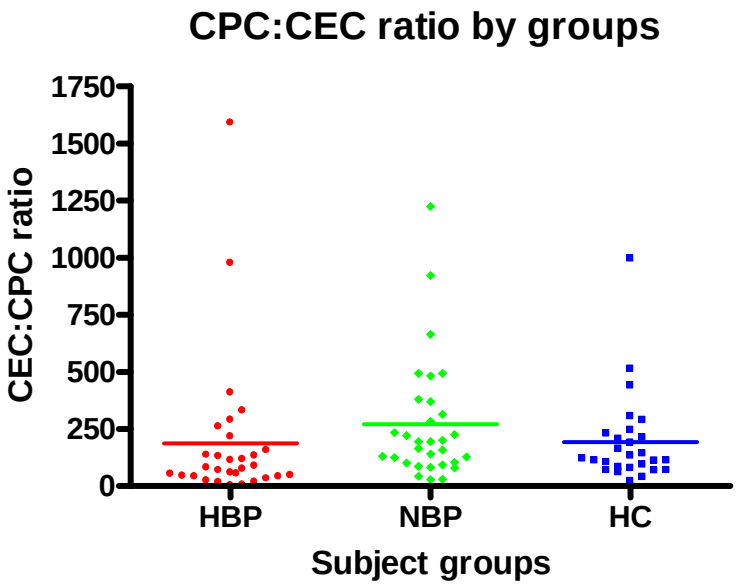
(a) Circulating Endothelial Cells



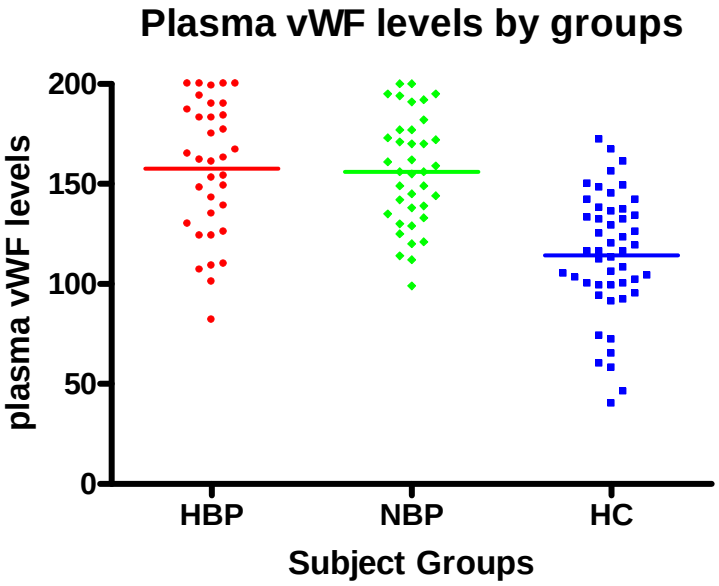
(b) Circulating Progenitor Cells



(c) Circulating Progenitor Cell : Circulating Endothelial Cell ratios



(d) Plasma von Willebrand factor



HBP: Hypertensive pregnant women; NBP: Normotensive pregnant women; HC: Non-pregnant healthy controls

3.3 Angiogenin, Haem oxygenase 1, soluble Flt, VEGF in hypertension in pregnancy

Introduction

Key aspects of the development of various pathologies in pregnancy, including hypertension in pregnancy, pre-eclampsia and eclampsia, are changes in the release of various growth factors and their cell bound receptors. These include vascular endothelial growth factor (VEGF), its receptor Flt-1 on endothelial cells {a soluble form (sFlt-1) of which can be found in plasma (96, 181-183)} and angiogenin. (84, 87, 184) Whilst VEGF and sFlt-1 have been extensively studied in clinical hypertension, and also in women with hypertension in pregnancy (94, 185, 186), there are no reports of angiogenin in women whose pregnancy is complicated by hypertension. Haem oxygenase-1 (HO-1) is an enzyme involved in the breakdown of haem to biliverdin, iron and carbon monoxide. As the latter is, like nitric oxide, a vasodilator, HO-1 may have a role in regulating blood vessel tone and thus hypertension. (187, 188) Notably, there are data suggesting a link between HO-1, sFlt-1 and VEGF, and thus, angiogenesis. (189-192) This has generated a hypothesis that impaired regulation of HO-1 may be important in the pathophysiology of pre-eclampsia. (142, 193) However, there are no data on HO-1 in the hypertension of pregnancy.

We hypothesised altered levels of angiogenin and HO-1 in the plasma of women whose pregnancy is complicated by hypertension. As comparator molecules, we chose VEGF and sFlt-1, (189-193) but also von Willebrand factor (vWf). This pro-coagulant was included because it is a product of the endothelium, is known to be raised in normal pregnancy, and also in hypertension (41, 170, 171) as well as in pre-eclampsia. (172, 173) We tested our hypothesis on a cross-sectional study of women attending a specialist antenatal hypertension clinic, a standard

antenatal clinic, and in healthy non-pregnant women. Samples were obtained from hypertensive women on two occasions to determine whether or not there were changes as their pregnancy progressed.

Methods

The study and control groups have been described earlier.

Collection of Blood Samples and Laboratory Methods

Blood samples were collected into sodium citrate and were centrifuged within 30 min at 1,500g for 20 min at 4°C. Plasma was collected and stored at -70°C until batch processing by ELISA for vWf (Dako-Cytomation, Ely, UK), VEGF, sFlt-1, angiogenin (R&D Systems, Abingdon, UK) and haem oxygenase-1 (HO-1, Stressgen, Ann Arbour, USA). The intra and inter-assay co-efficient of variation was <5% and <10%, respectively for all assays. Lower limits of detection were vWf 10 IU/dL, VEGF 0.05 ng/mL, sFlt 1.0 ng/mL, angiogenin 20 ng/mL and HO-1 0.3 ng/mL.

Power calculations and statistical analyses

Based on existing literature on growth factors in normal and pre-eclamptic pregnancy (84, 87, 94, 183, 184, 186) we hypothesised a change in VEGF (our test statistic) of 50% in normal pregnancy and of 66% in hypertensive pregnancy compared to levels in non-pregnancy (modelled median 100 units, interquartile range [IQR] 67-176 units). For a 1-beta power of 80% and alpha 5% giving ANOVA p=0.001, we needed 35 patients in each pregnant group and 50 controls. In hypothesising a change of 33% at 2 time points, paired samples from 20 women are

required for p=0.005 if levels are increased in 12 women, are unchanged in 3, and fall in 5 women. For extra confidence, we recruited slightly in excess in the pregnancy groups. Following a test of normality, data were expressed as mean (standard deviation, SD) or median (IQR) and analysed by one way ANOVA or the Kruskal Wallis test respectively. Intergroup differences were sought by Tukey's post hoc test. Data 2 time points was analysed by paired t test or Wilcoxon's test. Correlations were sought by Spearman's rank method. Categorical data were analysed by chi-squared tests. A two-tailed probability of <0.05 was considered significant.

Results

We recruited 38 hypertensive pregnant women (of whom 23 were studied at 2 time points), 38 normotensive pregnant women and 50 non-pregnant healthy women as controls.

VEGF was lower in the hypertensive pregnant women compared with the non-pregnant women, with no difference in sFlt-1, but the sFlt-1/VEGF ratio was higher in both pregnant groups compared with the non-pregnant group. Angiogenin was lower in normal pregnancy compared with non-pregnancy and hypertension-pregnancy, whilst HO-1 was also lower in normal pregnancy compared with non-pregnant controls. There was no difference in HO-1 between the pregnant hypertensive women and the non-pregnant women (Table 3.3.1) (Figure 3.3.1). With the exception of vWf and VEGF, there were no differences in any marker or blood pressure at the 2 presentations on average 15 weeks apart (Table 3.3.2).

Angiogenin and vWf correlated significantly in the hypertensive and normotensive pregnant women (r=0.35, p=0.03 and r=0.34, p=0.04), respectively, but in the non-pregnant women the

correlation was not significant (r=0.13, p=0.367). None of the plasma indices correlated with age, duration of pregnancy or blood pressure.

Discussion

The contribution of the current study is to examine the role of plasma angiogenin and HO-1 in the hypertension of pregnancy, focussing on blood pressure, VEGF, and its receptor sFlt-1. We found low angiogenin in normal pregnancy, and that these low levels were partially restored in hypertension compared with levels in healthy non-pregnant women. Low levels of HO-1 in normal pregnancy were also reinstated by the hypertension of pregnancy. There were no changes in angiogenin or HO-1 at weeks 15 or 30 of 23 hypertensive pregnancies. Neither angiogenin nor HO-1 correlated with any other index, except that angiogenin and vWf correlated significantly in the hypertensive and normotensive pregnant women but not in the non-pregnant women.

There is evidence of the involvement of angiogenin and HO-1 in angiogenesis and in hypertension, both conditions where the endothelium is crucial. These 2 conditions combine in the hypertension of pregnancy, the most severe form of which is pre-eclampsia, which also implicates growth factor dysregulation (6-26, (194-196). Data on angiogenin and VEGF in pre-eclampsia are conflicting. For example, Reuvekamp et al (184) reported low VEGF but no difference in angiogenin in 30 women with pre-eclampsia (mean 524 ng/ml) compared with 30 non-pregnant controls (mean 670 ng/mL), although p=0.058 implies a type 1 (false negative due to small number) artefact. However, they suggest that deficient angiogenic growth factors may in part explain the shallow placentation found in pre-eclampsia. Shaarawy et al (87) reported raised angiogenin and VEGF that correlated with systolic and diastolic blood pressure in 71 pre-eclamptics vs. 20 normotensive pregnancies. They hypothesise that raised growth factors could

confirm the existence of vascular reactivity and endothelial disturbance in pre-eclampsia. With modest power we found that in normal pregnancy there was a significant reduction of angiogenin to 64% of non-pregnant levels, but that in hypertensive pregnancy this reduction was to a significant 87%.

Notably, in both pregnant groups, angiogenin correlated with vWf, the gold standard index of endothelial damage/dysfunction (170), known to be raised in normal pregnancy (171) and hypertension, (197) but there was no correlation in non-pregnant women. Interestingly, vWf was not further raised in hypertensive pregnant women compare with pregnant women, despite the presence of an addition stimulus to increase vWf in the former. The vWf/angiogenin relationship does not necessarily imply that raised angiogenin is related to endothelial damage/dysfunction, but it does clearly imply some relationship, the pathophysiological significance of which is unknown. Although angiogenin is present in human umbilical vein endothelial cell tissue culture supernatants, but not platelets, it may also arise from other cells. (198)

Blood obtained at 2 time points in 23 hypertensive women showed no change in angiogenin or HO-1, implying that the abnormalities in these markers develop at an early stage of pregnancy and then persist. vWf increased during pregnancy as expected, (171) and although VEGF also increased, the statistical significance of this rise is small and so at risk of bias. However, interpretation is marred by the lack of data at 2 time points in normal pregnancies.

HO-1 is an enzyme involved in iron metabolism, but whose product, carbon monoxide, may be important in blood pressure regulation (187, 188) and possibly in angiogenesis. (189-191) These and other findings have led to the hypothesis that HO-1 has a role in pre-eclampsia. (142, 193) We are unable to find data on plasma HO-1 in either hypertension or pregnancy, although Bao et

al (199) imply a relationship with diabetes and hypertension. Plasma HO-1 may theoretically raise from the endothelium, (198) but although there is a growing body of tissue culture and animal model data on this molecule, (200, 201) at present we are unable to speculate further on its role in pregnancy induced hypertension. Nevertheless, it is clear that HO-1 is being viewed with increased interest, as it has been hypothesised that there is a protective role of HO-1 in pregnancy and that HO-1 may be a target for the treatment of preeclampsia. (202) The cardiovascular drugs, statins, stimulate HO-1 expression and inhibit sFlt-1 release in vivo and in vitro, thus, they have the potential to ameliorate early onset preeclampsia. This is being tested in the StAmP trial.

Although limited by its cross-sectional design and relatively small sample size, the subject groups were matched with respect to age, and we therefore conclude that normal pregnancy is characterised by low angiogenin, low HO-1 and a high sFlt-1/VEGF ratio, but in hypertensive pregnancy, angiogenin and HO-1 levels remain similar to those in normal pregnancy. Further studies, longitudinal in design and involving larger sample sizes are required to clearly establish a possible aetiological association between an imbalance in growth factor regulation and HO-1 and the development of complications and adverse outcomes in hypertension in pregnancy. This may reflect differences in endothelial cell biology in the differing physiological and pathological states.

TABLE 3.3.1: LEVELS OF vWF, VEGF, sFlt-1, sFlt-1:VEGF RATIO, ANGIOGENIN AND HO-1 LEVELS BY GROUPS

	Non-pregnant controls (n=50)	Normotensive Pregnant (n=38)	Hypertensive Pregnant (n=38)	P value
Age (years)	30 (8)	29.5 (6)	33 (6)	0.113
vWf (IU/dl)	115 (30)	156 (27)[a]	157 (32)[a]	<0.001
VEGF (ng/mL)	0.59 (0.24-1.35)	0.26 (0.1-1.67)	0.18 (0.1-0.68)[a]	0.013
sFlt-1 (ng/mL)	13.5 (1.0-121)	17.5 (9.5-270)	12.5 (7.2 – 26.3)	0.195
sFlt-1/VEGF ratio	23 (8-132)	135 (54-272)[a]	74 (21-147)[a]	<0.001
Angiogenin (ng/mL)	225 (134-331)	145 (91-230)[a]	195 (145-320)[b]	0.027
Haem oxygenase-1 (ng/mL)	2.4 (1.6-4.0)	1.47 (0.8-2.4)[a]	1.6 (1.4-3.2)	0.026

Data are presented as mean with (SD) or median with (IQR). Analyses by ANOVA or the Kruskal Wallis test as appropriate. Tukey's post hoc test for inter-group differences – a: Significantly different from non-pregnant controls ($p<0.05$), b: Significantly different from normal pregnancy ($p<0.05$).

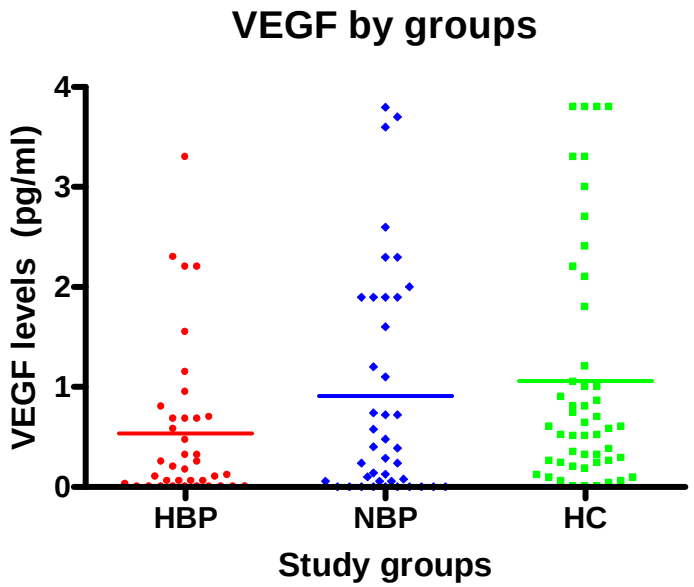
TABLE 3.3.2: CHANGES IN BLOOD PRESSURE AND PLASMA MARKERS IN 23 HYPERTENSIVE PREGNANCIES

	Early pregnancy: 14.9 (2.9) weeks	Late pregnancy: 30.1 (3.3) weeks	P value
SBP (mmHg)	133 (14)	133 (17)	0.943
DBP (mmHg)	88 (10)	86 (10)	0.569
vWf (IU/dl)	127 (46)	162 (29)	0.008
VEGF (ng/mL)	0.11 (0.05-0.46)	0.21 (0.05-0.70)	0.022
sFlt-1 (ng/mL)	11.0 (6.2-16.1)	12.0 (6.4-24.0)	0.601
Angiogenin (ng/mL)	220 (125-370)	165 (125-260)	0.126
Haem oxygenase-1 (ng/mL)	1.56 (0.8-3.0)	1.60 (1.4-3.2)	0.351

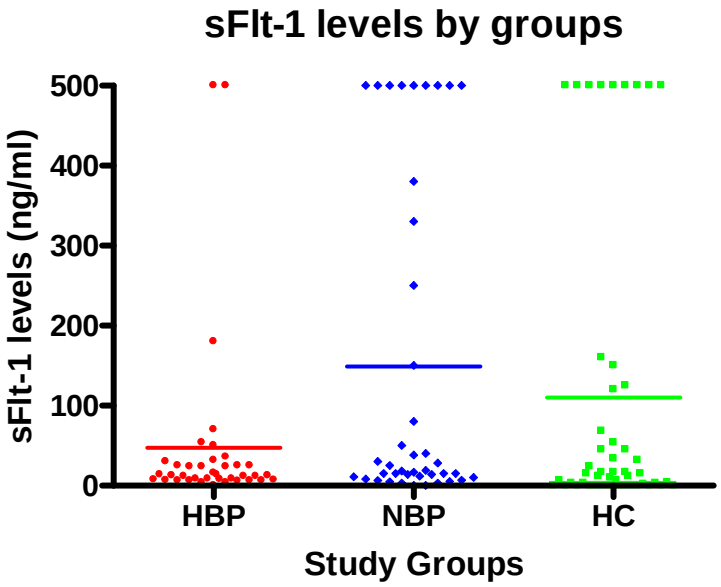
Data are presented as mean with (SD) or median with (IQR). Analyses by paired t test or Wilcoxon's test as appropriate.

Figure 3.3.1: Levels of Vascular Endothelial Growth Factor (VEGF), soluble Flt-1, VEGF: sFlt-1 ratio, angiogenin and Haem oxygenase-1 (HO-1) by groups

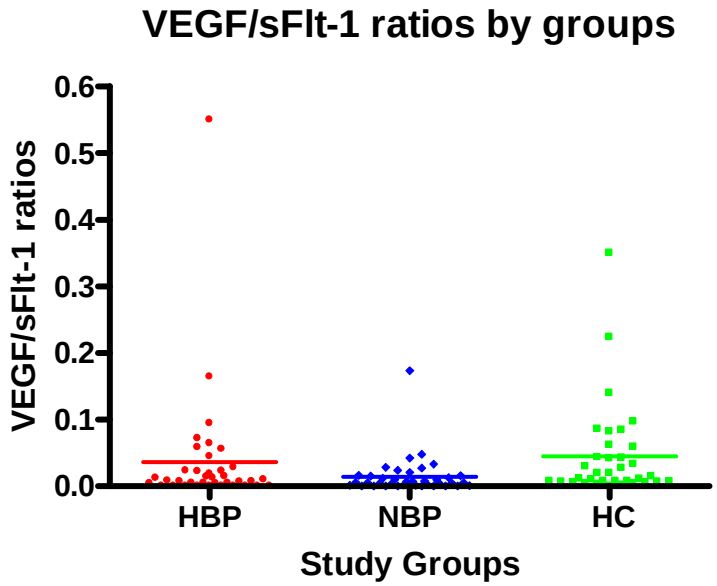
(a) Vascular Endothelial Growth Factor



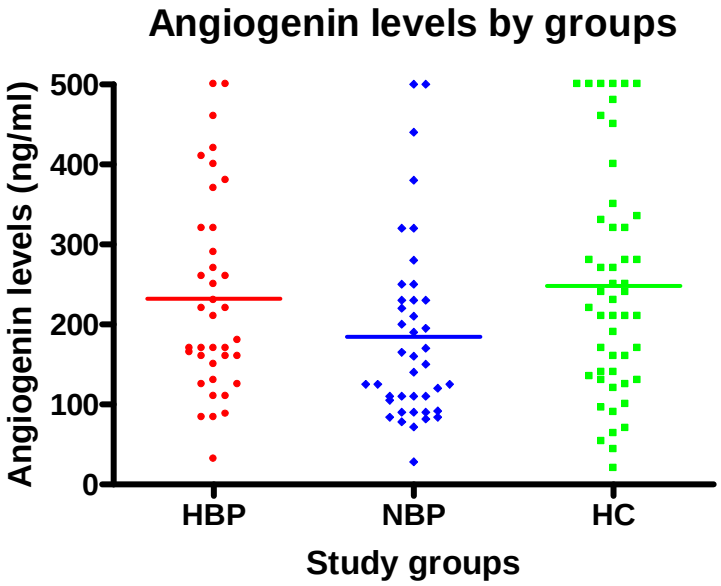
(b) Soluble Flt-1



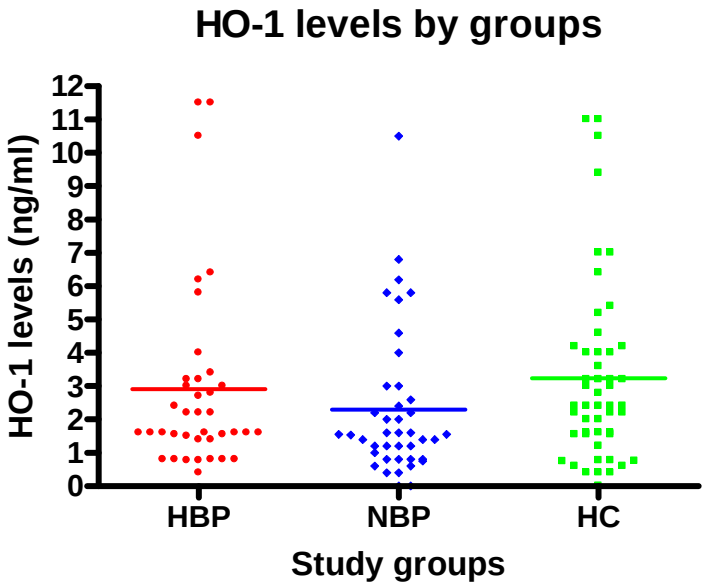
(c) VEGF: sFlt-1 ratios



(d) Angiogenin



(e) Haem oxygenase-1



3.4 MMP-9/ TIMP-1 in hypertension in pregnancy

Introduction

A normally functioning extra cellular matrix is essential for successful trophoblast invasion. The matrix metalloproteinases (MMP) and their tissue inhibitors (TIMP) play a vital role and enable the restructuring of the extracellular matrix and hence tissue composition. This is important for processes including angiogenesis, vasculogenesis and normal endothelial function.

The TIMP are a group of endogenous MMP inhibitors that can both modulate and inhibit MMP activity. It has been hypothesised that a balance between their respective activities determines the dynamics of the extra cellular matrix. Their levels are considered to reflect extra cellular matrix turnover. (203-205)

We hypothesized that hypertension in pregnancy is characterised by abnormalities in the extra cellular matrix as indicated by altered levels of MMP-9 and TIMP-1 as well as MMP-9/ TIMP-1 ratios. We tested our hypothesis on a cross-section of women attending our antenatal hypertension clinic.

Methods

Subjects

The study compared markers of extra cellular matrix (MMP-9 and TIMP-1) in hypertensive pregnant women and in controls (normotensive pregnant women and matched non pregnant controls).

Collection of Blood Samples and Laboratory Methods

Blood samples were collected from a large ante cubital vein using a 21-gauge needle directly into Vacutainer® tubes (Becton Dickinson, UK) containing 7.2mg tri-potassium (K3) Ethylene Diamine Triacetic Acid (EDTA), 0.5mL 3.2% sodium citrate or Z serum clot activator. For enzyme-linked immunosorbent assay (ELISA) blood samples were centrifuged within 30 min from collection at 1,500g for 20 minutes at 4°C. The resultant supernatant (plasma/serum) was then collected and stored at -70°C until batch processing by ELISA to measure MMP-9 and TIMP-1 [R & D Systems, Europe, Oxon, UK]. All assays were performed according to the manufacturers' guideline and locally developed protocols. The intra and inter-assay co-efficient of variation was <5% and <9% respectively for all assays.

Power calculations and statistical analyses

These have been described in previous chapters.

Results

We recruited 38 hypertensive pregnant women, 40 normotensive pregnant women and 50 non-pregnant healthy women as controls.

Both hypertensive pregnant women as well as normotensive pregnant women had raised MMP-9 levels compared to non-pregnant healthy controls. TIMP-1 levels were relatively similar in the three groups. The MMP-9: TIMP-1 ratios were however significantly altered between the groups, with raised ratios in the hypertensive pregnant groups ($p < 0.05$). (Figure 3.4.1)

Discussion

The role of the extra cellular matrix in the pathogenesis of hypertension in pregnancy and complications including preeclampsia has been examined previously. (116, 118, 126)

The circulating levels of MMP-9 and TIMP-1 have been shown to be raised in hypertension. (206) This trend is noted in our current study, although the raised levels in hypertensive pregnancy are not statistically significant, perhaps related to small numbers of subjects.

However, the altered ratio between MMP-9 and TIMP-1 suggests a possible abnormality in the extra cellular matrix turn over. The raised MMP-9/TIMP-1 ratio in both the pregnant groups and more so in the hypertensive pregnant group compared with the non-pregnant control group suggests enhanced metalloproteinase activity and extra cellular matrix breakdown. This finding is similar to that in the study by Palei et al, (126) who noted a higher net MMP-9 activity in their cohort with gestational hypertension. The suggestion of higher MMP-9 activity leading to accelerated cleaving of big endothelin-1 to yield medium endothelin-1, a more potent vasoconstrictor than endothelin-1 itself is worth consideration.

Studies have shown that MMPs can activate vasoconstrictors (e.g. endothelin), inactivate vasodilators (e.g. calcitonin gene related peptide) and transactivate cell surface receptors responsible for vasoconstriction (e.g. epidermal growth factor receptor). (207)

Study Limitations

The limitations discussed previously remain so with this study. The study findings and the lack of significant differences in the levels of MMP-9 and TIMP-1 are different from those of a

previous study in our department. (206) It is difficult to explain this and relates probably to differences in the study cohort, maternal age, gestational age etc.

Conclusions

Our findings confirm significant alterations in matrix metalloproteinase activity in pregnancies, particularly those complicated with hypertension. Further studies involving larger sample sizes and longitudinal in design are required to clearly establish a possible aetiological association between abnormalities in extra cellular matrix breakdown and hypertension in pregnancy.

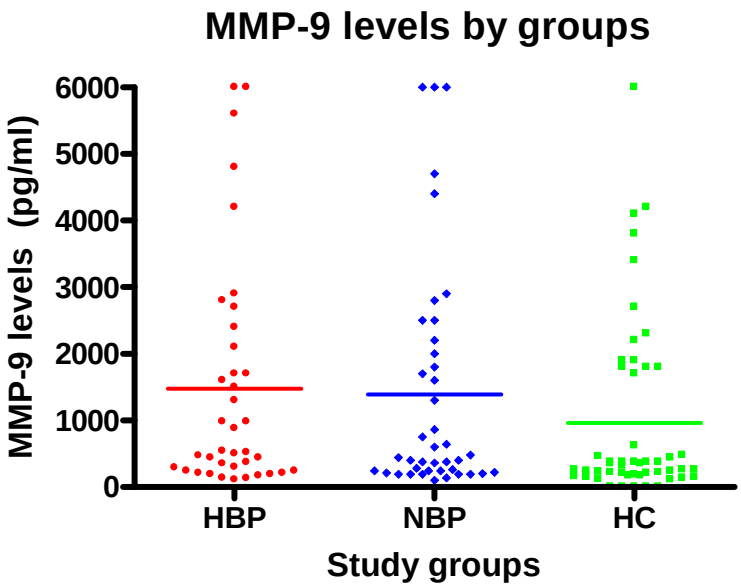
TABLE 3.4.1: LEVELS OF MMP-9, TIMP-1 and MMP-9: TIMP-1 RATIOS BY GROUPS

Mean (SD)	Hypertensive Pregnant (n=38)	Normotensive Pregnant (n=41)	Non-pregnant healthy controls (n=50)	p-value
Age (years)	32.61 (5.93)	29.32 (6.11)	30.46 (7.72)	0.0935
Gestational Age (weeks)	30.00 (3.07)	26.70 (3.40)	NA	< 0.0001
Median (IQR) Parity	2 (0-5)	1 (0-6)	0 (0-4)	< 0.0001
Median (IQR) MMP-9 (pg/ml)	530 (100-6000)	440 (100-6000)	335 (100-6000)	0.0748
Median (IQR) TIMP-1 (pg/ml)	2000 (400-6000)	2000 (500-6000)	1900 (400-6000)	0.3122
MMP-9: TIMP-1 ratio	0.45	0.34	0.19	< 0.05 (0.0414)

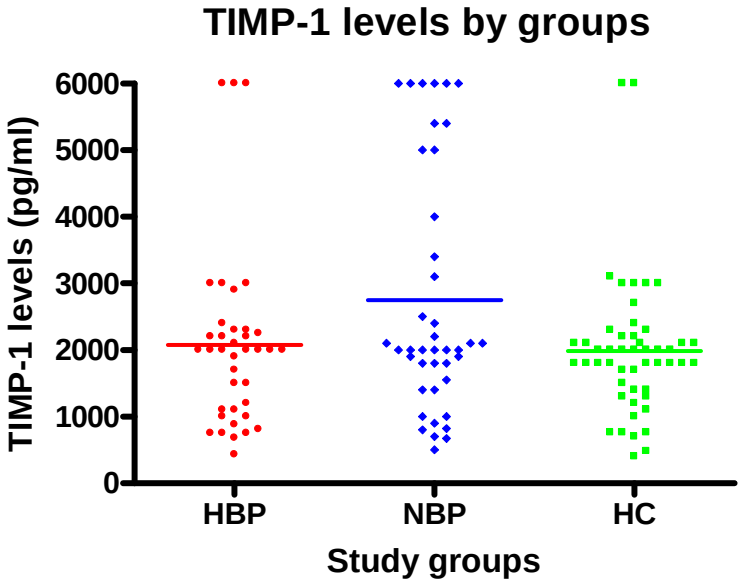
Between group analyses by ANOVA with Tukey post hoc test and Kruskal Wallis test with Dunn's post hoc test, as appropriate.

Figure 3.4.1: MMP-9, TIMP-1 and MMP-9: TIMP-1 ratios by groups

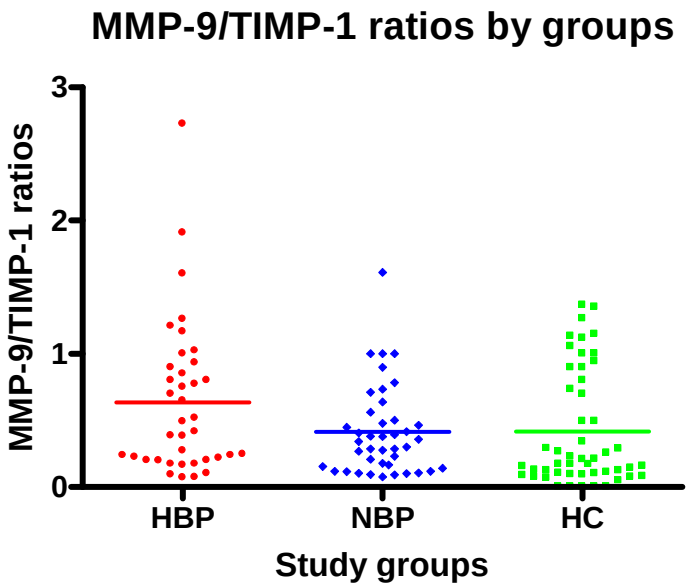
(a) MMP-9



(b) TIMP-1



(c) MMP-9: TIMP-1 ratios



3.5 Soluble Fas, soluble Fas-ligand in hypertension in pregnancy

Introduction

This study explores the role of apoptosis in hypertension in pregnancy and alterations in the levels of markers of apoptosis compared with healthy controls.

Although abnormalities in placentation may be the primary pathology responsible for the development of pre-eclampsia and its effects, and a possible cause for this may be irregularities in the crucial relationship between Fas (a member of the tumour necrosis factor receptor superfamily [TNFRSF]) and its ligand (i.e. FasL), which in part regulate apoptosis, (208-210) on key endothelial cells. Fas (CD95, but also known as Apo-1 and TNFRSF6) is a 45-kDa type I membrane receptor containing an 80-amino acid intra-cellular 'death' domain in the intracytoplasmic tail. Engagement of membrane bound Fas by its ligand (CD95L, also known as TNFSF6), a 36-40 kDa type II membrane TNFSF member) triggers apoptosis by inducing the caspase series of enzymes. Current thought suggests apoptosis has a central role in the villous trophoblast turnover, as controlled apoptosis, mediated mainly through Fas-FasL signalling, and may be a defence mechanism against rejection of the foetal allograft by maternal immune system. (211)

The Fas/FasL system may also be important in small-for-gestational age babies, although this is controversial. (97, 212, 213) Nevertheless, there is evidence of changes in Fas and FasL that imply a role in pre-eclampsia. (89, 97, 98) Furthermore, Fas and FasL may also be involved in the hypertension of pregnancy as Koenig and Chegini reported differential expression of Fas and FasL in foetal membranes, decidua and placentas from gestations with hypertension than in normal controls. (214) Matrix metalloproteinases are able to cleave FasL, leading to a soluble

form measurable in plasma, as reported in pre-eclampsia, pregnancy and in the syndrome of haemolysis, elevated liver enzymes, and low platelets. (98, 149, 215, 216) However, there are no reports of plasma Fas and FasL in the hypertension of pregnancy or any of the effects of parity.

We hypothesised altered levels of Fas and FasL in the plasma of women whose pregnancy is complicated by hypertension compared with women with normotensive pregnancies and normotensive non-pregnant women that levels would change as pregnancy proceeds. We tested our hypotheses on a cross-sectional study of women attending a specialist antenatal hypertension clinic, a standard antenatal clinic, and in healthy non-pregnant women.

Methods

Twenty women were recruited early in their pregnancy (around week 14), and 29 later in their pregnancy, at approximately at week 28. Eighteen women were studied at both stages. A parallel group of 20 pregnant women with normal blood pressures were recruited at week 14, and another 29 women were recruited at around week 28. The study was completed by the recruitment of 50 women who were not pregnant.

Collection of Blood Samples and Laboratory Methods

Blood samples were collected using a 21-gauge needle directly into Vacutainer® tubes (Becton Dickinson, UK) containing sodium citrate. Blood samples were centrifuged within 30 min from collection at 1,500g for 20 min at 4°C. The resultant supernatant plasma was collected and stored at -70°C until batch processing by ELISA for Fas and sFas ligand (R&D Systems, Abingdon, UK). Lower limited of detection were 100 pg/mL for sFas and 20 pg/mL for sFasL. The intra and inter-assay co-efficient of variation was <5% and <10%, respectively for all assays.

Power calculations and statistical analyses

There are no reports of sFas or sFasL in the hypertension of pregnancy on which to base a power calculation. However, as other sample sizes in other sFas/sFasL cross sectional studies range from n=20 to n=30 per group, (149, 214-216) we recruited to this target for the pregnant groups, and to approximately twice as many non-pregnant women. Following a test of statistical normality, data were expressed as mean (standard deviation, SD) or median (inter-quartile range, IQR), as appropriate. Non-categorical data distributed normally were analysed by one way ANOVA, non-parametric data were analysed by Kruskal Wallis. Intergroup differences were sought by Tukey's post hoc test. Correlations were sought by Spearman's rank method. Categorical data (parity) were analysed by the chi-squared test, paired data (18 hypertensive women at week 20 and again at week 29) by paired t test or Wilcoxon's test. A two-tailed $p<0.05$ was considered as significant.

Results

Clinical, demographic and sFas/sFasL data of the subjects are presented in table 3.5.1. The subject groups and controls were matched for age but not parity, which increased from the non-pregnant women (median no children), to the normotensive women (median one child), to the hypertensive women (median 2 children).

There were no differences in sFas between the groups at either time point (early or late pregnancy). sFasL was lower, and the sFas/sFasL ratio was higher, in hypertensive pregnancy at both time points compared to both other groups. In the early stages of pregnancy (approximately week 14), sFasL was unrelated to parity ($p=0.058$). In the late stages of pregnancy

(approximately week 28), sFasL was also unrelated to parity (p=0.065). (Table 3.5.1) (Figure 3.5.1).

sFas and sFasL were measured in 18 women both early and late in their pregnancy. There was no difference in levels of sFas at the two time points: 1550 (1200-1750) pg/mL at week 20 and 1550 (1175-1825) pg/mL at week 29 (p=0.955). However, sFasL fell from 130 (85-213) pg/mL at week 20 to 125 (98-735) pg/mL at week 29 (p=0.028). The sFas/sFasL ratio also fell from 10.3 (6.0-18.1) to 9.9 (4.1-14.1) (p=0.021). sFas, sFasL and their ratio failed to correlate significantly with gestational age, maternal age, SBP or DBP at either time point in the pregnant women, or in the pregnant women. sFas correlated with sFasL in all groups at all time points (r 0.45 - 0.74, p=0.002 - <0.001) except in the hypertensive women early in their pregnancy (r=0.39, p=0.092). (Table 3.5.2)

Discussion

We examined the role of sFas and sFasL in normal pregnancy and in the hypertension of pregnancy, focussing on both early and late stages. We found no difference in sFas, sFasL or their ratio in normal pregnancy compared with no pregnancy, and no change over the duration of the pregnancy, thus failing to confirm others who found low sFas in similar numbers of women with a normal pregnancy. (216) Harirah et al found serum levels of sFas, but not sFasL, to be significantly higher in women with the syndrome of haemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome than healthy gravidas. (149) Although Koenig and Chegini observed greater expression of Fas and FasL in amnion and decidual tissue from hypertensive pregnancy gestations than in normal control pregnancies, they did not report plasma levels. (214) We complement their data by reporting low sFasL and high sFas/sFasL ratio in hypertensive

pregnancy compared to non-pregnancy and normal pregnancy at both early and late stages of pregnancy. However, Kuntz et al (98) also found higher sFasL in pre-eclamptic pregnancy compared with control gestations but no difference in sFas. Thus, although comparison between research reports may be flawed, we and Kuntz et al both found measurement of sFas to be unhelpful. Our finding of low sFasL in hypertensive pregnancy is the reverse of their finding of high levels in pre-eclamptic pregnancy.

The relationship between Fas and FasL within the pregnant uterus may be important in minimising the maternal allogeneic response (208-211, 217-219), and failure of this system may be important in pre-eclampsia and other conditions. (89, 220, 221) This has led to interest in potential roles for the measurement of these molecules in maternal blood as indicators of actual or potential complication such as intra-uterine growth retardation. (97, 98, 149, 212, 213, 216)

Although a recent report failed to find a role for sFas and sFasL in intra-uterine growth retardation, (213) these molecules may be important in the neonatal period. (222) Although limited by its cross-sectional design and relatively small sample size (which is comparable to, or exceeds, similar studies, (97, 98, 149, 213, 214, 216, 222) the subject groups were matched with respect to age. An additional caveat is the marked difference in parity.

We therefore conclude that in normal pregnancy there is no change in sFas or sFasL. However, we found that sFas is low in hypertensive pregnancy, although this may be an effect of the increased parity in this group. Although sFasL and the sFas/sFasL ratio fell in 18 hypertensive women between weeks 20 and 29, this ‘mathematical’ decrease of 4% is unlikely to have clinical value. Further studies, longitudinal in design and involving larger sample sizes are required to

clearly establish a possible aetiological association between an imbalance in sFas and sFasL and the development of complications and adverse outcomes in hypertension in pregnancy.

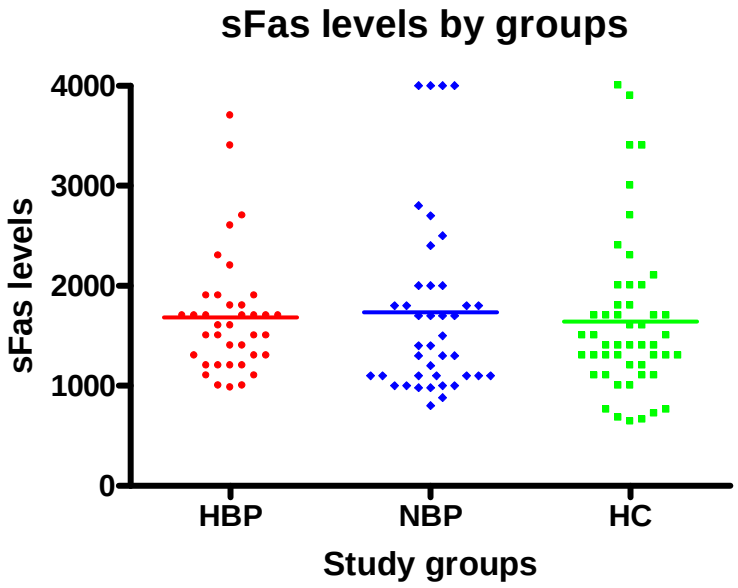
TABLE 3.5.1: SERUM FAS/FAS LIGAND DATA BY GROUPS

	Non-preg women (n=50)	Early normotensive pregnancy (n=20)	Early hypertensive pregnancy (n=20)	P value (early)[a]	Late normotensive pregnancy (n=29)	Late hypertensive pregnancy (n=29)	P value (late)[b]
Age (years)	30.5 (7.7)	28.9 (6.3)	33.0 (3.2)	0.143	29.5 (6.1)	32.5 (5.8)	0.232
Gestation age (weeks)	-	13.7 (2.5)	14.8 (3.1)	0.224	27.8 (3.5)	29.0 (2.6)	0.150
sFas (pg/mL)	1400 (1175-1850)	1300 (985-1700)	1450 (1200-1700)	0.350	1700 (110-2200)	1500 (1200-1850)	0.702
sFas Ligand (pg/mL)	985 (198-2000)	730 (113-1650)	157** (98-438)	0.005	1050 (148-1925)	180** (100-1525)	0.038
sFas/sFas Ligand ratio	1.9 (0.9-6.5)	1.95 (1.1-8.5)	8.45** (3.0 – 16.8)	0.005	2.0 (1.4-9.7)	9.8** (1.6-13.4)	0.007

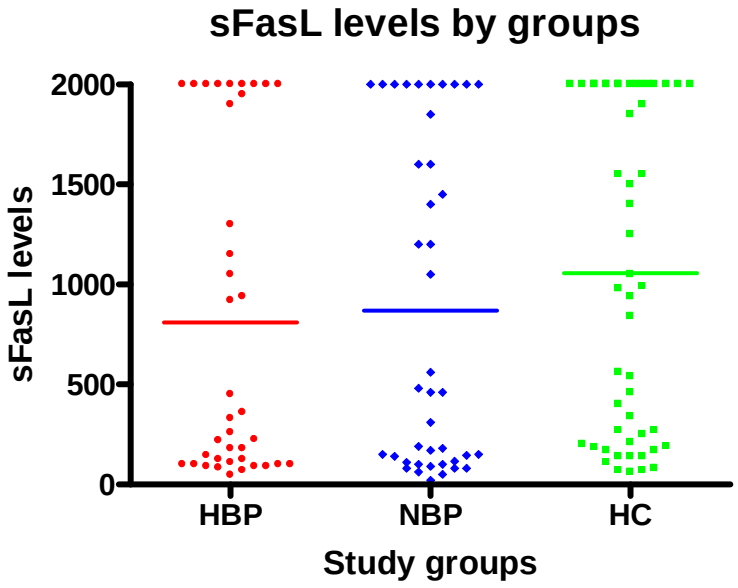
Data presented as mean (SD) or median (IQR). P values by ANOVA or the Kruskal-Wallis test. [a]Between non pregnant women, early normotensive pregnancy and early hypertensive pregnancy. [b]Between non pregnant women, late normotensive pregnancy and late hypertensive pregnancy. Sub-analyses by Tukey's test: **Significantly different ($p<0.05$) in hypertensive pregnancy than in the other 2 groups.

Figure 3.5.1: soluble Fas, soluble Fas ligand and sFas: sFasL ratios by groups

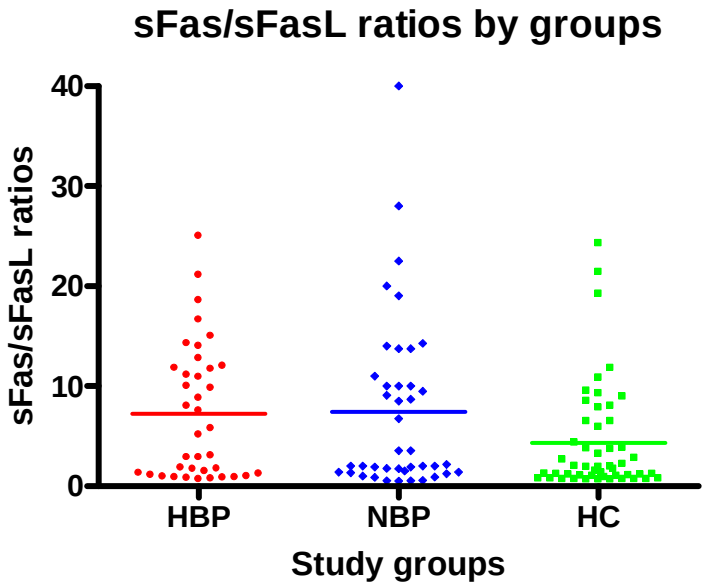
(a) sFas levels



(b) sFasL levels



(c) sFas: sFasL ratios



3.6 Pregnancy Outcomes and correlation between biomarkers

Introduction

The Vascular Biology of Pregnancy explored the role of various processes occurring in the maternal vasculature and their role in the pathogenesis of hypertensive disorders of pregnancy and its effects on pregnancy.

This chapter aims at examining the differences in the pregnancy outcomes observed in the two pregnant groups and seeks to establish correlations between the biomarkers studied.

Methods

Subjects

The study compared pregnancy outcomes in hypertensive pregnant women and in the normotensive pregnant controls.

The inclusion and exclusion criteria of study subjects have been discussed as before, as also the collection of blood samples and various laboratory methods.

Results

We recruited 40 hypertensive pregnant women, 60 normotensive pregnant women and 50 non-pregnant healthy women as controls. The gestational age and parity were higher in the hypertensive pregnant women group. (Table 3.6.1)

We report the pregnancy outcome data on 34 hypertensive and 38 normotensive pregnant women. As expected, both systolic and diastolic blood pressures were significantly higher in the hypertensive pregnant group ($p<0.001$).

Women in the hypertensive group were older in age (mean age 33 years) compared with those in the normotensive group (mean age 27 years) and delivered earlier (median gestational age 38.6 weeks, compared with 39.8 weeks in the normotensive group). Of the 34 hypertensive women, 16 delivered by Caesarean section compared with only 10 normotensive women who required a Caesarean section. The earlier delivery in the hypertensive group could perhaps be related to elective Caesarean sections in view of potential risks anticipated with the progression of gestation.

Although the babies born to the hypertensive group were smaller than those born to the normotensive group, there was no significant difference in length, weight, head circumference and APGAR scores between the babies in the two groups. No significant morphological abnormalities were noted in the babies born to both groups of women. This is easily explained by the relatively small numbers of subjects in both groups and lack of power to demonstrate a significant difference in outcomes.

We estimated the correlations between the biological markers [circulating endothelial cells (CECs), circulating progenitor cells (CPCs), von Willebrand factor (vWf), vascular endothelial growth factor (VEGF), soluble fms like tyrosine kinase -1 (sFlt-1), serum fas (sFas), serum fas ligand (sFasL), matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), and haem oxygenase-1 (HO-1).

A negative correlation was noted between gestational age at delivery and systolic as well as diastolic blood pressures (Spearman correlations -0.312, $p<0.001$). Similarly, birth length, weight and head circumference of the babies born also negatively correlated with the systolic as well as diastolic blood pressures of the mothers (systolic blood pressures: Pearman correlations, -0.501, -0.379 and -0.435 respectively, $p<0.05$; diastolic blood pressures: Pearman correlations, -0.401, -0.312 and -0.324 respectively, $p<0.05$). Amongst the biomarkers, circulating endothelial cells correlated positively with both systolic and diastolic blood pressures (Spearman correlation rho 0.279 and 0.309, $p<0.001$ respectively).

An analysis of correlations between the biomarkers revealed a few positive correlations, which are summarised in table 3.6.2. It is noteworthy that CEC and CPC levels both correlate well with vWF levels. In addition, CEC levels correlate positively with sFlt1 and MMP 9 levels. vWF and VEGF levels correlate well with sFlt 1. Further, VEGF levels also correlate with the markers of apoptosis and HO-1.

We have demonstrated a positive correlation between angiogenin and HO-1 levels. HO-1, in turn correlates well with VEGF, sFlt1 as well as sFas and sFasL levels.

Discussion

This study is probably one of the first to explore a range of biomarkers and their association with clinically relevant pregnancy outcomes.

The findings confirm a relationship between raised blood pressures in pregnant women and adverse outcomes, including relatively low birth weight of the new born babies.

Also notable is the association between the different processes occurring in the maternal vasculature, including endothelial dysfunction and its repair, new blood vessel formation (angiogenesis), the break down processes (apoptosis), and the extracellular matrix and the haem oxygenase systems.

The correlations described above generate many a question and raise one's inquisitiveness to further explore the maternal vasculature with a view to evolving novel, but safe therapies targeting these specific pathways so as to prevent the development of hypertensive disorders in pregnancy and hence preventing any adverse outcomes. It may be noted that the study design does not allow for a detailed exploration of the underlying mechanisms and causes for the observed correlations between the biomarkers and the above theories are merely speculative.

Study Limitations

The limitations of the study have been extensively discussed in the individual data chapters and pertain mainly to the cross-sectional design and relatively small numbers of subjects.

We were unable to demonstrate clear alterations in the biomarkers with progression of gestation, particularly in the normotensive pregnant group due to very small numbers being followed up in the hospital clinics, compared with the hypertensive pregnant women, who were followed up more closely both before and after delivery.

Conclusions

Our findings confirm the association between hypertension in pregnancy and adverse outcomes as well as the complex interplay and relationship between the dynamic processes that occur in

the maternal vasculature. Whether these are merely associated with hypertension or whether they have a cause-effect relationship needs to be explored in larger clinical trials.

Further, large longitudinal studies would need to be conducted to demonstrate a significant difference in clinical outcomes in pregnant women affected by hypertensive disorders compared with those whose blood pressures remain normal throughout their pregnancies.

TABLE 3.6.1: BASELINE CHARACTERISTICS OF THE STUDY GROUPS

Mean (SD)	Hypertensive Pregnant (n=34)	Normotensive Pregnant (n=38)	p-value
Age (years)	32.78 (4.82)	28.11 (5.9)	0.007
Gestational Age (weeks)	37.85 (3.94)	39.48 (1.46)	0.05
Median (IQR) Parity	2 (0-5)	1 (0-7)	< 0.0001
SBP (mm Hg)	133(17)	112 (9)	< 0.0001
DBP (mm Hg)	87 (11)	66 (6)	< 0.0001
Birth weight (kg)	3.03 (0.81)	3.29 (0.50)	0.180
Birth length (m)	0.50 (0.06)	0.51 (0.03)	0.181
Ponderal Index	23.99 (3.83)	24.25 (2.97)	0.794
Head Circumference (m)	0.33 (0.04)	0.34 (0.01)	0.214
APGAR1	9 (0-9)	9 (6-9)	0.455
APGAR2	9 (0-10)	9 (8-10)	0.275

Between group analyses by ANOVA with Tukey post hoc test and Kruskal Wallis test with Dunn's post hoc test, as appropriate

TABLE 3.6.2: CORRELATIONS BETWEEN THE BIOMARKERS

			CEC	CPC	vWF	VEGF	sFlt1	Angiogenin	sFas	sFasL	MMP9	TIMP1	HO1
Spearman's rho	CEC	Correlation Coefficient	1.000	-.091	.402**	.024	.204*	-.092	.094	.077	.277**	.014	-.011
		Sig. (2-tailed)	.	.337	.000	.785	.021	.303	.292	.388	.002	.872	.898
		N	128	112	128	128	128	128	128	128	128	128	128
	CPC	Correlation Coefficient	-.091	1.000	.230*	-.083	.044	.071	-.114	-.120	.173	.115	.015
		Sig. (2-tailed)	.337	.	.015	.384	.644	.458	.230	.208	.068	.226	.876
		N	112	112	112	112	112	112	112	112	112	112	112
	vWF	Correlation Coefficient	.402**	.230*	1.000	.000	.208*	.107	-.068	.034	.408**	.159	-.086
		Sig. (2-tailed)	.000	.015	.	.994	.019	.229	.445	.701	.000	.074	.336
		N	128	112	128	128	128	128	128	128	128	128	128
	VEGF	Correlation Coefficient	.024	-.083	.000	1.000	.531**	.051	.485**	.790**	.018	.060	.182*
		Sig. (2-tailed)	.785	.384	.994	.	.000	.564	.000	.000	.841	.499	.039
		N	128	112	128	129	129	129	129	129	129	129	129
	sFlt1	Correlation Coefficient	.204*	.044	.208*	.531**	1.000	.011	.330**	.538**	.104	.094	.180*

		CEC	CPC	vWF	VEGF	sFlt1	Angiogenin	sFas	sFasL	MMP9	TIMP1	HO1
	Sig. (2-tailed)	.021	.644	.019	.000	.	.902	.000	.000	.240	.292	.041
	N	128	112	128	129	129	129	129	129	129	129	129
Angiogenin	Correlation Coefficient	-.092	.071	.107	.051	.011	1.000	.038	.027	-.142	-.161	.257**
	Sig. (2-tailed)	.303	.458	.229	.564	.902	.	.667	.764	.108	.069	.003
	N	128	112	128	129	129	129	129	129	129	129	129
sFas	Correlation Coefficient	.094	-.114	-.068	.485**	.330**	.038	1.000	.445**	-.118	.025	.324**
	Sig. (2-tailed)	.292	.230	.445	.000	.000	.667	.	.000	.182	.777	.000
	N	128	112	128	129	129	129	129	129	129	129	129
sFasL	Correlation Coefficient	.077	-.120	.034	.790**	.538**	.027	.445**	1.000	.051	.105	.156
	Sig. (2-tailed)	.388	.208	.701	.000	.000	.764	.000	.	.563	.236	.077
	N	128	112	128	129	129	129	129	129	129	129	129
MMP9	Correlation Coefficient	.277**	.173	.408**	.018	.104	-.142	-.118	.051	1.000	.576**	.051
	Sig. (2-tailed)	.002	.068	.000	.841	.240	.108	.182	.563	.	.000	.565
	N	128	112	128	129	129	129	129	129	129	129	129
TIMP1	Correlation Coefficient	.014	.115	.159	.060	.094	-.161	.025	.105	.576**	1.000	.118
	Sig. (2-tailed)	.872	.226	.074	.499	.292	.069	.777	.236	.000	.	.183

			CEC	CPC	vWF	VEGF	sFlt1	Angiogenin	sFas	sFasL	MMP9	TIMP1	HO1
		N	128	112	128	129	129	129	129	129	129	129	129
	HO1	Correlation Coefficient	-.011	.015	-.086	.182*	.180*	.257**	.324**	.156	.051	.118	1.000
		Sig. (2-tailed)	.898	.876	.336	.039	.041	.003	.000	.077	.565	.183	.
		N	128	112	128	129	129	129	129	129	129	129	129

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Section 4: Summary

4.1 Summary of Findings

The results of the Vascular Biology of Pregnancy project can be summarised as follows:

- Hypertension in pregnancy is associated with significant alterations in the internal milieu of the maternal vasculature.
- It is characterised by endothelial dysfunction, with a significant increase in circulating endothelial cells as well as von Willebrand factor compared with pregnant women with normal blood pressures and non-pregnant healthy controls.
- An active process of endothelial repair occurs in these women, suggested by a reduced number of circulating progenitor cells, possibly related to a consumptive state, compared with normotensive pregnant controls.
- Vascular endothelial growth factor levels are lower, further raising the possibility of their recruitment in the endothelial reparative processes.
- Our findings also suggest an increase in angiogenesis, evidenced by higher angiogenin levels; alterations in the matrix metalloproteinases (increase in MMP-9 levels);
- However, we were unable to demonstrate significant changes in the markers of apoptosis.

4.2 Conclusions

The ‘Vascular Biology of Pregnancy’ is a comprehensive study of the maternal vasculature in pregnancies complicated by hypertension, both pre-existing as well as pregnancy-induced. The study has revealed significant associations between endothelial dysfunction and abnormalities in angiogenesis and apoptosis as well as the haem oxygenase system and the extra-cellular matrix in hypertensive pregnancies and is perhaps the first of its kind to demonstrate correlations between the different biomarkers that characterise these processes.

Progression of hypertensive pregnancies to the development of preeclampsia and its clinical manifestations are hypothesised to be associated with two potentially interrelated events: a relative placental hypoxia/ischemia linked to diffuse maternal endothelial cell activation. In a majority of women, the development of preeclampsia occurs at or close to term when prompt delivery of the foetus is associated with good maternal and foetal outcome. (223)

However, a proportion of women develop more severe early onset disease (<34 weeks gestation) associated with increased foetal and maternal morbidity and mortality. (224) It is, however, unclear whether these two phenotypes (mild late onset and severe early onset) represent two differing pathophysiologies (placental vs. maternal). (225) Changes in the maternal circulation, including increases in plasma volume, cardiac output and heart rate combined with decreasing blood pressure during pregnancy, together constitute the largest cardiovascular challenge that the maternal system ever faces (226). Further, pre-existing altered vascular function/reactivity resulting from a systemic response to inflammation and oxidative/nitrative stress in the mother arising from an inadequately perfused placenta is the underlying cause of preeclampsia. (225)

Significant changes occur in the maternal cardiovascular system throughout gestation beginning soon after conception with the objective of increasing blood flow and nutrient delivery to the foetal-placental unit. Blood volume increases from 6 to 8 weeks gestation onwards by 45% to reach approximately 5 litres at 32-weeks' gestation. (227) The increase in red blood cell mass (20–30%) leads to physiologic haemodilution, reduces blood viscosity, thus potentially protecting from the predisposition for thromboembolic events in pregnancy (228) and permitting placental perfusion. Cardiac output increases 30–50% in pregnancy. (229) Normal pregnancy is associated with increased endothelium-mediated relaxation, blunted response to vasoconstrictors and increased flow-mediated dilation. (230) Blood flow to the uterus increases tenfold in gestation (from 2% to 17% of cardiac output) reaching 500–800 mL/min at term, half of which goes to the placental intervillous space to sustain the developing placenta and foetus. (231). Animal model studies have shown that reductions in uteroplacental blood flow can lead to a hypertensive state that closely resembles preeclampsia. (232)

Arteries in the placental bed arteries are modified to reach a high-flow, low-resistance state to support this increased blood flow. This is achieved by extra villous trophoblast-mediated remodeling of spiral arteries with replacement of endothelium by trophoblasts. (233) Placental bed biopsies from women with preeclampsia have shown that physiological changes in the arteries found in normal pregnancies are restricted, being limited to decidual portions of the vessels or absent altogether. (234, 235) The mean external diameters of the spiral arteries in women with preeclampsia are less than half of similar vessels from uncomplicated pregnancies. The decreased diameter results in reduced placental perfusion.

Endothelial dysfunction appears to be the key and central pathological process related to various disease processes, including hypertension and cardiovascular diseases such as stroke and myocardial infarction. Some of the currently available drug therapies are hypothesised to influence and improve endothelial function and perhaps help prevent further events. However these therapies may be unsafe for use in women of child bearing age and during pregnancy. The other abnormalities described probably have more than a mere associative relationship to the disease processes.

Although predominantly cross-sectional, it offers insight into the change in levels of the biomarkers with progression of gestation. Some of the findings are consistent with those from previous studies, whereas others have revealed a reverse trend, probably related to the characteristics of the study groups and notably the absence of pre-eclampsia/ eclampsia. Understandably, the numbers involved were small to explore for any clinical relevance of the difference in the biomarkers in the study groups.

Hence we can conclude that abnormalities in the maternal vasculature as well as those affecting placentation and complex interactions between these two processes are related to hypertensive disorders in pregnancy and the resultant complications.

4.3 Suggestions for future studies

The findings from our study provide insight into the pathophysiology of the changes in the vascular tree of pregnant women with hypertension.

However, they also raise important questions that can perhaps be answered only by large multi-centre prospective trials involving hundreds or thousands of patients to look further at the role of these pathological processes in the development of clinically significant adverse outcomes in the mother and the foetus.

Further studies could aim at exploring the role and influence of the above biomarkers on clinical parameters including placental size, birth weight of the foetus, development of preeclampsia and eclampsia.

Also of relevance would be large randomised trials to assess the influence of drugs (currently used to treat hypertensive disorders in pregnancy) on these pathological processes and whether they can cause regression of the underlying abnormalities and improve clinical outcomes. This would mean a multi-factorial prospective randomised trial comparing outcomes in pregnant women treated with currently available anti-hypertensive medications deemed safe for use during pregnancy.

Another important question that needs to be addressed from future studies is to look for persistence in the described changes after delivery and following their trends in future pregnancies, perhaps even before conception, and whether they precede the clinical rise in blood

pressure., this would also be relevant with respect to any predisposition to the development of hypertension and cardiovascular disease in later life in the mother.

Of particular note, this would also help determine whether periodic review and monitoring as well as continued treatment (if relevant) will prevent or delay the onset of cardiovascular disease in later life. However, monitoring with biomarkers would perhaps require evolving novel (cost-effective) methods of testing that are easily reproducible, reliable and validated.

vii. Appendix

Appendix 1

Definition of Hypertension in pregnancy

Hypertensive disorders are classified into 4 categories according to the Working Group Report on Hypertension in Pregnancy.(1) They are chronic hypertension, preeclampsia-eclampsia, preeclampsia superimposed upon chronic hypertension and gestational hypertension.

Chronic Hypertension

Chronic hypertension is defined as hypertension (blood pressure ≥140 mm Hg systolic and/ or ≥90 mm Hg diastolic) that is present and observable before pregnancy or diagnosed before the 20^{th} week of gestation. Hypertension diagnosed for the first time during pregnancy and that does not resolve postpartum is also classified as chronic hypertension.(1)

Preeclampsia-eclampsia

Preeclampsia-eclampsia is a pregnancy-specific syndrome that usually occurs after 20 weeks' gestation, or earlier in trophoblastic diseases (hydatidiform mole or hydrops). The increased blood pressure is accompanied by proteinuria in this syndrome.(1)

Preeclampsia

Preeclampsia is a syndrome characterized by hypertension, proteinuria and symptoms of headache, visual changes, epigastric or right upper quadrant pain and dyspnea. It may be mild or

severe, depending on the degree of hypertension, proteinuria and other organ system involvement. (7)

Eclampsia

Eclampsia is defined as the occurrence in a woman with preeclampsia of seizures that cannot be attributed to other causes. (1)

Gestational Hypertension

Gestational hypertension or pregnancy induced hypertension (PIH) is defined as a blood pressure >140 mm Hg systolic and/ or >90 mm Hg diastolic in a woman who was normotensive before 20 weeks' gestation. These recordings are on at least two occasions 6 hours apart, with the BP recordings used to establish the diagnosis no more than 7 days apart. (151)Severe gestational hypertension is a condition of sustained elevation in systolic blood pressure of >160 mm Hg and/ or in diastolic blood pressure of >110 mm Hg for 6 hours.

Other clinical symptoms suggestive of this condition include headache, blurred vision, abdominal pain or abnormal lab tests, including low platelet counts and abnormal liver function tests. (1)

Preeclampsia superimposed on chronic hypertension

Preeclampsia may occur in women with chronic hypertension, with a much worse prognosis than with either condition alone.

Possible findings in superimposed preeclampsia include

- New onset proteinuria (≥0.3g/ 24 hours) in women with hypertension and no proteinuria in early pregnancy (<20 weeks' gestation).
- Sudden increase in proteinuria
- Sudden increase in blood pressure in a woman with previously well controlled hypertension
- Thrombocytopenia (<100,000 platelets/mm^3)

Abnormal elevation in liver enzymes (alanine aminotransferase or aspartate aminotransferase)(1)

viii. Publications from these studies

Original Research:

1. Watson T, Shantsila E, Karthikeyan VJ, Jessani S, Goon PK, Lip GY. The effects of exercise stress testing, diurnal variation and temporal decline on circulating progenitor cells. Thromb Haemost. 2010 Feb; 103(2):419-25.
2. Karthikeyan VJ, Ferner RE, Baghdadi S, Lane DA, Lip GY, Beevers DG. Are angiotensin-converting enzyme inhibitors and angiotensin receptor blockers safe in pregnancy: a report of ninety-one pregnancies. J Hypertens. 2011 Feb; 29(2):396-9.
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5. Karthikeyan VJ, Lip GY, Baghdadi S, Lane DA, Beevers DG, Blann AD. Angiogenin and Haem oxygenase in Pregnancy: Influence of Hypertension. Angiology. 2012 Apr; 63(3):194-8.
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Reviews and Editorials:

7. Karthikeyan VJ, Lip GY. Hypertension in pregnancy: patho-physiology and management strategies. Curr Pharm Des. 2007; 13(25):2567-79.
8. Karthikeyan VJ, Lip GY. Endothelial damage/dysfunction and hypertension in pregnancy. Front Biosci (Elite Ed). 2011 Jun 1;3:1100-8
9. Karthikeyan VJ, Lip GY. Matrix metalloproteinases and hypertension: a link between left ventricular hypertrophy and diastolic dysfunction? Tohoku J Exp Med.2006 Feb; 208(2):93-7.

Chapters:

10. Karthikeyan VJ. Hypertension in Pregnancy. Oxford: Hypertension: 2008.

Letter:

11. Karthikeyan VJ, Beevers DG, Lip GY. Ethnic disparities in malignant hypertension: further observations. J Hypertens. 2007; 25:895.

ix. Abstracts from these studies (International & National)

- **American College of Cardiology 59th Annual Scientific Session, Atlanta, USA, March 2010.**

1. Vellore J. Karthikeyan, Shahirose Jessani, Balu Balakrishnan, Deirdre A. Lane, Sabah Baghdadi, D. Gareth Beevers, Gregory Y H Lip. Serum soluble Fas and Fas Ligand in hypertension in pregnancy (poster).
2. Vellore J. Karthikeyan, Robin E. Ferner, Sabah Baghdadi, Deirdre A. Lane, Gregory Y H Lip, D. G. Beevers,Angiotensin Converting Enzyme inhibitors and Angiotensin Receptor Blockers in pregnancy: an increasing problem (poster).

- **American College of Cardiology 58th Annual Scientific Session, Orlando, USA, March 2009.**

3. Vellore J. Karthikeyan, Shahirose Jessani, Balu Balakrishnan, Deirdre A. Lane, Sabah Baghdadi, D Gareth Beevers, Gregory Y H Lip. Vascular Endothelial Growth Factor and soluble fms-like tyrosine kinase 1 in the pathogenesis of hypertension in pregnancy (oral).

- British Cardiovascular Society Annual Scientific Conference, Manchester, June 2008.

4. V J Karthikeyan, T Watson, D A Lane, S Baghdadi, D G Beevers, G Y H Lip. Circulating Endothelial Cells and Circulating Progenitor Cells in hypertension in pregnancy: A balance between endothelial damage and repair? (poster).

- **American College of Cardiology 57th Annual Scientific Session, Chicago, USA, March-April 2008.**

5. Vellore J. Karthikeyan, Shahirose Jessani, Christopher J. Boos, Deirdre A. Lane, Sabah Baghdadi, D Gareth Beevers, Gregory Y H Lip. Circulating Endothelial Cells and plasma

von Willebrand factor in hypertension in pregnancy: evidence for endothelial damage/dysfunction (poster).

6. Vellore J. Karthikeyan, Timothy Watson, Deirdre A. Lane, Sabah Baghdadi, D Gareth Beevers, Gregory Y H Lip. Circulating Endothelial Cells and Circulating Progenitor Cells in hypertension in pregnancy: A balance between endothelial damage and repair? (poster)

- **Medical Research Society / AMS / RCP Clinical Scientists in Training meeting, London, UK, February 2008.**

7. V J Karthikeyan, Shahirose Jessani, Timothy Watson, Christopher J. Boos, Deirdre A. Lane, Sabah Baghdadi2, D Gareth Beevers, Gregory Y H Lip. Hypertension in pregnancy: An altered balance between endothelial damage/dysfunction and repair? (poster).

- **European Society of Cardiology Congress, Vienna, Austria, September 2007.**

8. V J Karthikeyan, S Jessani, C Boos, D Lane, S Baghdadi, D G Beevers, G Y H Lip. Circulating Endothelial Cells as an index of endothelial damage/ dysfunction in hypertension in pregnancy (poster). [**awarded Young Investigator Travel Grant**]

- **British Pharmacological Society 75th Annual Winter meeting, Oxford, 20 December 2006 and Evans and Gaisford Research Prize, City Hospital, Birmingham, 11 December 2006.**

9. V J Karthikeyan, F Ali, S F Jewels, R E Ferner, S Baghdadi, & D G Beevers. Angiotensin converting enzyme inhibitors and angiotensin receptor blockers in pregnancy: a cause for concern (oral presentation).

x. Standard Operating Procedures

1. von Willebrand factor

Synopsis:

This is the standard ELISA for the measurement of von Willebrand factor (vWf) using commercial antisera from Danish company Dako. The following method is for two plate's worth of samples (about 80), taking about half a day.

Brief Method:

1. Coat microtitre plate with 100 µl of a dilution of primary antisera (35 µl in 20ml coating buffer) at room temperature (RT) for >60 minutes or overnight in the fridge.
2. Wash 3x, add 100 µl 1/40 serum or plasma in pbs/tween, or neat tissue culture fluid, incubate for >60 minutes at RT.
3. Wash 3x, add 100 µl peroxidase-labelled conjugate (35 µl in 20ml PBS) for >45 minutes at RT.
4. Wash and add 100 µl substrate (OPD, hydrogen peroxide, citrate buffer). The colour develops almost immediately.
5. Stop with 100 µl acid. Read at 492 nm.

Expected values in citrated normal plasma in the region of 100 $\pm$ 30 IU/dL stable atherosclerosis typically 130 $\pm$ 35 IU/dL, acute coronary syndromes often 150 $\pm$ 40. Data are generally of normal distribution.(236)

2. Vascular Endothelial Growth Factor (VEGF)

Synopsis:

The Vascular endothelial growth factor (VEGF) ELISA uses commercial antibody and requires about 4-6 hours to complete.

Brief Method:

1. Coat microtitre plate with 112 µl of primary antisera l in 20 ml PBS buffer for 2 plates overnight in the fridge.
2. Wash, block with 100 µl /well of 5% Marvel (1g in 20mls PBS-T for 2 plates) for 1 hour at RT.
3. Wash and add 100 µl of neat plasma, or neat tissue culture fluid, and recombinant standards for 2 hours at RT. Standards are double diluted down the plate. Use fresh tips for each sample.
4. Wash and add 112 µl of biotinylated anti-human VEGF antibody in 20 ml PBS tween for 90 minutes at RT.
5. Wash and add Streptavidin (100µl/well) for 45 minutes at RT.
6. Wash and add 100 µl substrate (Solutions A and B) .Blue colour develops well within 20 to 30 minutes
7. Stop with 50 µl/well acid. Colour goes yellow. Read at 450 nm.

Expected values:

Data usually not normally distributed. Controls generally have median values of about 30-50 pg/ml [but inter-quartile range (IQR) may be over 200pg/ml]. Patients' median values generally 100 to > 200 pg/ml. (186, 237).

3. Vascular Endothelial Growth Factor Receptor (sFlt-1)

Synopsis:

The soluble form of Flt-1 (sFlt-1) ELISA measures free, as opposed to complexed sFlt-1 and will require about 10 hours to complete.

Brief Method:

1. Coat microtitre plate with 100µl of primary antisera (40µl of 40µg/ml in 10ml coating buffer for 1 plate) overnight in the fridge.
2. Wash and block with 200µl /well of 5% Marvel (2.5g in 50mls PBS-T for 2 plates) for 2 hours.
3. Wash and add rhVEGF to saturate the plate. Incubate for 2 hours at RT.
4. Wash and add 100µl of neat plasma, or neat tissue culture fluid, and recombinant standards for 2 hours at RT
5. Wash and add 100µl of 500ng/ml of biotinylated goat anti-human Flt-1 antibody (100µl of 5µg/ml in 10ml PBS) for 2 hours at RT.
6. Wash and add extravidin peroxidase (100µl/well) for 45 minutes at RT.
7. Wash and add 100µl substrate (OPD, hydrogen peroxide, citrate buffer) for 45minutes at RT.
8. Stop with 50µl/well acid. Read at 492 nm

Expected values:

The values are typically not normally distributed data. Median plasma concentration 10-30ng/ml with no changes or even lower levels in patients with vascular disease has been reported.(238, 239).

4. Angiogenin

Brief Method:

1. Coat microtitre plate with 100µl of primary antisera/well at room temperature (RT) for 90 minutes or at 4^{o}C overnight.
2. Wash and add serum/plasma and recombinant standards diluted in PBS/tween for 90 minutes at RT.
3. Wash and add 100µl of biotinylated anti-human angiogenin antibody (one vial for 10ml PBS-T) to each well for 90 minutes at RT.
4. Wash and add 100ul/well of streptavidin-HRP (50ul strep-HRP in 10mls of PBS-T for 1 plate) and incubate for at least 20 minutes at RT (avoid direct light).
5. Wash and add 100µl WARM substrate solution (5mls A + 5mls B for 1 plate). Colour will develop in less than 5 minutes.
6. Stop with 50µl/well acid. Read at 450 nm

Expected values:

Data usually not normally distributed. Controls generally have median values of about 5mcg/ml. Patients' median values generally lower.

5. Soluble Fas

Brief Method:

1. Coat microtitre plate with 100µl of primary antisera (56µl of reconstituted primary Ab in 10ml PBS for 1 plate) room temperature (working concentration 1µg/ml).
2. Wash, block with 200µl /well of 5% Marvel (2.5g in 50mls PBS-T for 2 plates) for 1 hours at RT.
3. Wash and add 100µL serum (or EDTA plasma) diluted to 1 in 5 (by adding 20µL of sample to 80µL PBS-T) and recombinant standards for 2 hours at RT.
4. Wash with PBS-T, add 100µl 56µl of reconstituted secondary Ab in 10ml PBS-T for 2 hours at RT (working concentration 50ng/ml).
5. Wash and add 100ul/well of streptavidin-HRP (50ul strep-HRP in 10mls of PBS-T for 1 plate) and incubate for 20 minutes at RT (avoid direct light).
6. Wash and add 100µl substrate solution 5 mls Colour regent A + 5 mls B for 1 plate. Colour will develop in less than 5 minutes.
7. Stop with 50µl/well acid. Read at 450 nm.

Expected values:

Data are usually not normally distributed. Controls generally have median values of about 5mcg/ml. Patients' median values are generally lower.

6. Soluble Fas ligand

Brief Method:

1. Coat microtitre plate with 100µl of primary antisera (56µl of reconstituted primary Ab in 10ml PBS for 1 plate) room temperature (working concentration 2µg/ml).
2. Wash, block with 200µl /well of 5% Marvel (2.5g in 50mls PBS-T for 2 plates) for 1 hour at RT.
3. Wash and add 100µl serum and 100 µl recombinant standards for 2 hours at RT.
4. Wash with PBS-T and add 56µl of reconstituted secondary Ab in 10ml PBS-T, then incubate for 2 hours at RT (working concentration 50ng/ml).
5. Wash and add 100ul/well of streptavidin-HRP (50ul strep-HRP in 10mls of PBS-T for 1 plate) and incubate for 20 minutes at RT (avoid direct light).
6. Wash and add 100µl substrate solution 5 mls Colour regent A + 5 mls B for 1 plate. Colour will develop in less than 5 minutes.
7. Stop with 75µl/well acid. Read at 450 nm.

Expected values:

Data usually not normally distributed. Controls generally have median values of about 5mcg/ml. Patients' median values generally lower.

7. Matrix Metalloproteinase -9

Synopsis:

This is the standard ELISA for the measurement of Matrix Metalloproteinase-9 (MMP-9) using Duoset development kit from R & D Systems Europe. The following method is for two plate's worth of samples (about 70), taking about a day.

Brief Method:

1. Coat microtitre plate with 100 µl of a dilution of primary antisera (112 µl in 20ml coating buffer) overnight in the fridge.
2. Block with Marvel (1.25g in 25mls PBS-Tween).
3. Wash and add 100 µl 1/100 plasma, or neat tissue culture fluid, along with the standards for two hours at RT.
4. Wash and add 100 µl biotinylated conjugate (112 µl in 20ml PBS-Tween) for 2 hrs at RT.
5. Wash and add 100 µl substrate (Streptavidin - 100 µl in 20mls PBS-Tween) for 20 minutes in the dark.
6. Wash four times and add 100 µl colour reagent (A&B, 5mls each).
7. Stop with 100 µl acid. Read at 450 nm.

8. Tissue Inhibitor of Metalloproteinase-1

Synopsis:

This is the standard ELISA for the measurement of Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) using commercial antisera from R & D Systems Europe. The following method is for TWO plate's worth of samples (about 40), taking about a day.

Brief Method:

1. Coat microtitre plate with 100 µl of a dilution of primary antisera (112 µl in 20ml coating buffer) overnight in the fridge.
2. Block with Marvel (0.4g in 10mls PBS-Tween).
3. Wash and add 100 µl 1/00 plasma, or neat tissue culture fluid, for two hours at RT.
4. Wash and add 100 µl biotinylated conjugate (112µl in20 mls PBS-Tween) for 2 hrs at RT.
5. Wash and add 100 µl substrate (Streptavidin - 100 µl in 20mls PBS-Tween) for 20 minutes in the dark.
6. Wash four times and add 100 µl colour reagent (A&B, 5mls each).
7. Stop with 100 µl acid. Read at 450 nm.

9. Haem oxygenase-1

Synopsis:

This is the standard ELISA for the measurement of Haem oxygenase-1 (HO-1) using commercial antisera from Stressgen.

Brief Method:

Bring to room temperature: Anti-HO-1 Immunoassay Plate, 10X

1. Wash Buffer, Sample Diluent, Anti-Human HO-1 Diluent, Anti-Rabbit IgG:HRP Conjugate Diluent, TMB Substrate and Acid Stop Solution.

2. Prepare Recombinant HO-1 Standard and samples in Sample

Diluent:

3. Add 100µL prepared standards and samples in duplicate to wells of Anti-HO-1 Immunoassay Plate. Cover immunoassay plate.

4. Incubate plate at room temperature for 30 minutes.

5. Wash wells 6X with 1X Wash Buffer.

6. Add 100µL diluted Anti-Human HO-1 to each well. Cover immunoassay plate.

7. Incubate plate at room temperature for 1 hour.

8. Wash wells 6X with 1X Wash Buffer.

9. Add 100µL diluted Anti-Rabbit IgG:HRP Conjugate to each well. Cover immunoassay plate.

10. Incubate plate at room temperature for 30 minutes.

11. Wash wells 6X with 1X Wash Buffer.

12. Add 100µL TMB Substrate to each well.

13. Incubate at room temperature for 15 minutes (preferably in the dark).

14. Add 100µL Acid Stop Solution to each well.

15. Measure absorbance at 450nm.

16. Plot the HO-1 standard curve and calculate HO-1 sample concentrations.

xi. References:

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