

Endothelial damage/dysfunction and hypertension in pregnancy

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1. ABSTRACT

Hypertension is one of the most common medical conditions complicating pregnancy, with significant implications on maternal and perinatal morbidity and mortality. Abnormalities in placental function 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 being implicated in the development of cardiovascular disorders in later life in these women. Circulating endothelial cells (CECs) are a novel means of assessing endothelial dysfunction as 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 review article 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.

2. INTRODUCTION

Hypertensive disorders occur in 6-8% of pregnancies and contribute significantly to maternal as well as neonatal morbidity and mortality. (1) Progression to pre-eclampsia not only elevates this risk but also that of 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. (2)

Endothelial integrity and function has been described to be paramount to maintenance of vascular haemostasis and blood pressure control. (3) 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. (4) Circulating endothelial cells (CECs) are a novel means of assessing endothelial dysfunction as mature cells detached from the vascular intimal layer in response to a variety of insults.

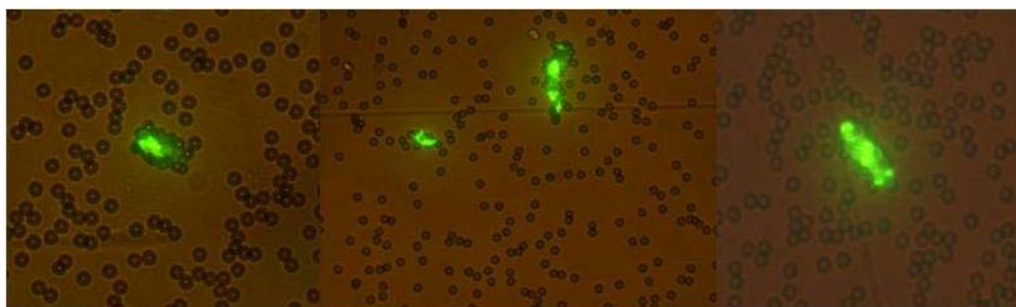


Figure 1. Circulating endothelial cells.

Endothelial progenitor cells (EPCs) are non-leukocyte cells derived from the bone marrow with proliferative potential that may be important in vascular regeneration.

This review article 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.

2.1. Search Strategy

We performed an on-line search of publication databases Cochrane Reviews, PubMed, MEDLINE and EMBASE, using the key words circulating endothelial cells, endothelial progenitor cells, hypertension and pregnancy. In order to identify any unpublished studies, abstracts from national (British Cardiac Society, Medical Research Society) and international (American Heart Association, American College of Cardiology, European Society of Cardiology), cardiology conferences in 2007 through 2010 were inspected. The reference lists of all papers yielded by the electronic database were scrutinized to identify any other potentially relevant articles.

3. CIRCULATING ENDOTHELIAL CELLS

3.1. 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 resulting in endothelial cell detachment, hence increasing CEC numbers in the blood stream. (5-7)

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. (8-13)

3.2. 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). 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 fluorescein isothiocyanate-stained endothelial specific Ulex europeus lectin or using flow cytometry. (14)

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°C. The anti-CD146 coated beads and blood/buffer mixture are placed in front of a magnet. The anti-CD146 coated beads (typically 5×10^7 /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 resuspended 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 Europaeus 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. (14) Subsequently, CECs are defined, on fluorescent microscopy, as cells 10 to 50 μ m in size with four or more immunobeads attached and staining positive for fluorescein isothiocyanate-stained Ulex Europeus (Figure 2).

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 multiparametric 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

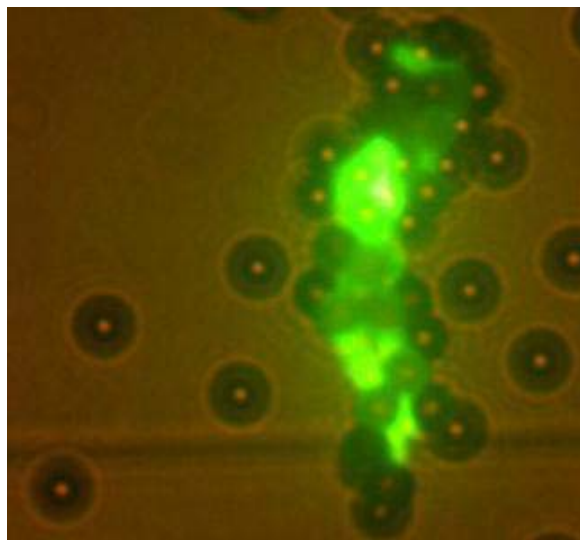


Figure 2. A typical circulating endothelial cell isolated using the immunobead method.

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. (5)

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. (15)

3.3. 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 cerebro vascular disease. (14- 33) 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, (29, 30) as also a strong correlation between CECs and several plasma markers of endothelial damage (vWf, tissue plasminogen activator, soluble E-selectin). (29-33)

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. (14)

3.4. 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. (34)

Data on CECs in hypertensive disorders of pregnancy are limited. (Table 1) For example, Canbakan *et al* (35) 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. (36)

4. ENDOTHELIAL PROGENITOR CELLS

4.1. 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. (12, 13, 37-39).

4.2. Isolation and quantification of EPCs

EPCs can be isolated and quantified using Flow Cytometry. Red blood cells from a fresh sample of K3 EDTA anticoagulated 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

Table 1. Endothelial dysfunction and pregnancy

Study (reference)	Patients (n)	Markers studied	Findings
Canbakan <i>et al</i> (35)	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 <i>et al</i> (36)	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

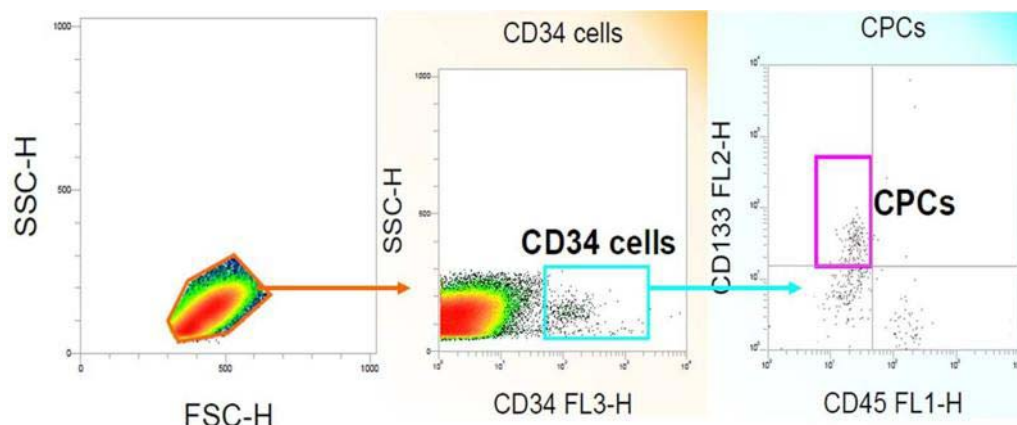


Figure 3. Gating strategy for endothelial / circulating progenitor cell enumeration with flow cytometry (CPCs, circulating progenitor cells).

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 4°C. 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 3).

Another method for the characterisation and quantification of EPCs is based on the culture of endothelial cells from circulating mononuclear cells. (40) 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 europaeus*, specific for endothelial cells in a variety of tissues binding to the carbohydrate moiety α -l-fucose. (41) 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%. (39) 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. (42)

4.3. 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. (43-46)

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 estrogen 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

Table 2. Endothelial progenitor cells and pregnancy

Study (reference)	Patients (n)	Findings
Sugawara <i>et al</i> (43)	Uncomplicated pregnancies (20)	Gradual increase in EPCs with progression of gestational age, with significant correlation with serum estradiol levels
Sugawara <i>et al</i> (46)	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 <i>et al</i> (48)	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 <i>et al</i> (49)	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 <i>et al</i> (50)	Preeclampsia (14) Normotensive controls (10)	Significantly reduced placental/ foetal EPCs and <i>in vitro</i> cultivated 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 <i>et al</i> (51)	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 <i>et al</i> (52)	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.

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 antiangiogenic 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. (47).

As with CECs, the data on EPCs in hypertensive disorders of pregnancy are limited. (43, 46, 48-52) For example, Sugawara *et al* (43) examined the level of circulating EPCs throughout uncomplicated pregnancies ($n=20$) and assessed the correlation between serum estradiol 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 estradiol, suggesting their role in the regulation and maintenance of the placental development and vascular integrity during pregnancy.

In a further study, Sugawara *et al* (46) 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* (48) 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 2.

5. ENDOTHELIAL DYSFUNCTION AND ITS CLINICAL IMPLICATIONS

5.1 Endothelial dysfunction and the Virchow's triad

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 haemorrhology 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. (53)

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

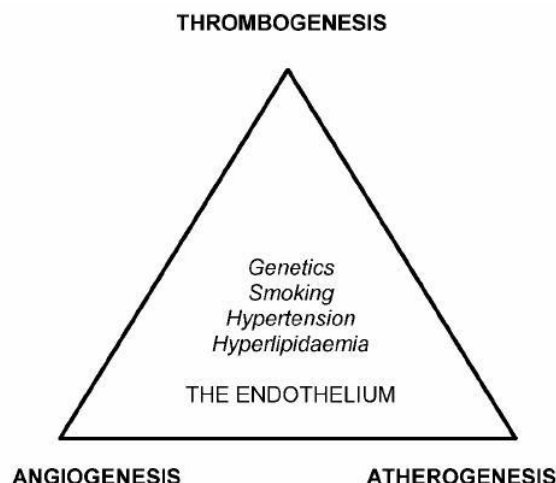


Figure 4. Thrombogenesis, atherogenesis and angiogenesis in vascular disease: the Birmingham 'Vascular Triad'.

a pro-thrombotic or hypercoagulable state. Further, treatment of hypertension helps reverse some of these changes. (54, 55)

Figure 4 indicates a close relationship between thrombogenesis, atherogenesis and angiogenesis, the 'vascular triad' (the Birmingham vascular triad), with the endothelium central to the processes.

5.2. 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 (56, 57). Many risk factors are unique for pregnancy-related acute myocardial infarction, with super imposed hypertension being an important one. (58)

The incidence of acute myocardial infarction has been reported to be 3 to 10 cases per 100 000 deliveries. (59, 60) 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%. (61) 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). (62).

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.

6. CONCLUSIONS

Hypertension in pregnancy is a spectrum of disorders including chronic hypertension, pregnancy induced hypertension and preeclampsia-eclampsia. A wide range of pathogenetic processes may contribute to the varied manifestations of the maternal and foetal syndrome as a consequence of hypertension. 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.

Further research aimed at establishing temporal trends of markers such as CECs and EPCs during the course of pregnancy and the effects of anti-hypertensive treatment on these markers would be beneficial towards optimising treatment, improving pregnancy outcomes and promoting long term maternal health. Indeed, there have been no previous studies that have examined a relationship between CECs and EPCs concurrently, in the context of hypertensive disorders in pregnancy. However, based on the findings of the individual studies looking at either of these two markers in hypertension in pregnancy, an association between the two is highly plausible, in pregnancies complicated with hypertension, compared with normotensive pregnancies.

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