1-1-2012

# Endothelial progenitor cells in mothers of low birthweight infants : a link between defective placentation and increased cardiovascular risk.

Thomas FJ King
*Royal College of Surgeons in Ireland*

# Endothelial Progenitor Cells in Mothers of Low Birthweight Infants: A link between defective placentation and increased cardiovascular risk?

Thomas F.J. King, MB, BCh, BAO, MRCP Edin

Thesis presented for the degree of MD in Medicine.

Submitted to:
The Royal College of Surgeons in Ireland,
123 St. Stephens Green, Dublin 2
October 2011

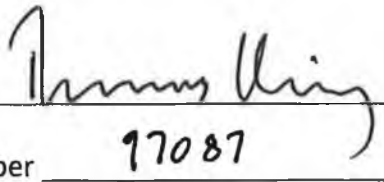
Research conducted at:
Department of Endocrinology, Royal College of Surgeons in Ireland, Connolly Hospital Blanchardstown, Dublin 15

Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9

Department of Obstetrics and Gynaecology, Royal College of Surgeons in Ireland, Rotunda Hospital, Dublin 1

Supervisor:
Dr. J. H. McDermott
Consultant Endocrinologist
Royal College of Surgeons in Ireland, Connolly Hospital Blanchardstown, Dublin 15

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree MD Doctor of Medicine is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed 

Student Number 97087

Date 6TH JAN 2012

Table of Contents

## Abstract

**Aims:**

This study tested the hypothesis that endothelial progenitor cell (EPC) pathology provides a unifying explanation for the tendency to carry low birthweight babies and subsequent increased maternal cardiovascular risk.

**Methods:**

We measured maternal EPC number and function, conventional cardiovascular risk markers, and cord blood adiponectin in 23 small for gestational age (SGA; birthweight <10th centile) and 23 appropriate for gestational age (AGA; birthweight ≥10th centile) pregnancies.

**Results:**

Median EPC count was lower (294 vs. 367, p=0.005), and EPC migratory capability was reduced (migration index 0.91 vs. 1.59, p<0.001) in SGA compared to AGA, but there was no significant difference in EPC adhesion (0.221 vs. 0.284 FU, p=0.257). Maternal triglyceride levels were higher in the SGA group compared to the AGA group (0.98 vs. 0.78 mmol/l, p=0.006), but there was no significant difference in cholesterol, glucose, insulin, HbA1c, adiponectin or blood pressure. Median umbilical cord blood adiponectin was lower in the SGA group (55.2 vs. 70.4 ng/ml, p=0.033), and there was a significant correlation between cord blood adiponectin and birthweight (r = 0.475, p=0.005).

**Conclusions:**

Giving birth to a SGA infant was associated with lower maternal EPC number and reduced migratory function in vitro. EPC pathology may explain both utero-placental insufficiency, leading to low birthweight, and future cardiovascular disease in the mother. SGA infants had lower cord blood adiponectin, potentially representing a mechanism for future cardiovascular disease in later life of the infant.

# Chapter 1. Introduction:

## 1.1 Discovery and significance of Endothelial Progenitor Cells (EPCs)

Blood vessels have a vital role in delivering blood and nutrients to every cell in the body. In addition to acting as a conduit for blood cells, the endothelial cells lining these vessels act to maintain vascular haemostasis, vessel wall permeability, appropriate blood flow, and lumen patency. Vascular endothelial cells regulate blood flow and organ perfusion via the release of autocrine and paracrine factors, they resist thrombus formation via suppression of platelet adhesion and aggregation and they maintain vessel patency by promoting fibrinolysis. The endothelium also has anti-oxidant and anti-inflammatory properties, regulates leucocyte adhesion and migration, smooth muscle proliferation and migration [1, 2].

The process of vasculogenesis, or new blood vessel formation, begins *in utero* with undifferentiated angioblasts sprouting vessels that invade developing organs [3]. In the adult, new blood vessel formation and repair are necessary after ischaemia and endothelial damage. It was previously thought that *de novo* vasculogenesis was confined to the developing fetus, and could only occur in the adult via pre-existing, fully differentiated endothelial cells *in situ* in the vessel wall.

In 1994, it was noticed that the endothelium that coats prosthetic arterial grafts arrived via haematogenous spread of circulating cells, rather than simply by local spread as was previously thought [4]. This discovery led to the concept that a circulating adult equivalent of the haemangioblast, an endothelial progenitor cell, could exist.

In 1997, Asahara *et al* [5] isolated CD34 positive mononuclear cells from human peripheral blood and cultured them on fibronectin coated plates. After 7 days in culture the resulting spindle shaped cells took up DiI-labelled acetylated low density lipoprotein (acLDL), and endothelial phenotype was confirmed by documenting expression of eNOS and KDR (the human homolog of VEGFR-2). To determine if the cultured cells were indeed putative adult haemangioblasts, the authors used a mouse model of hind limb ischaemia. CD34 positive or negative cells were injected into the ischaemic model, and on post mortem examination DiI-labelled cells were seen in the neovascularised hind limb. The cells had incorporated into the blood vessel walls, and no labelled cells were seen in the uninjured limb.

Asahara *et al* had therefore demonstrated that cells isolated from peripheral blood could differentiate into an endothelial phenotype *in vitro*, and that *in vivo* these cells were capable of selectively homing to areas of ischaemia where they mediate angiogenesis. Since their discovery, EPCs have been widely studied in many disease states, and many new methods of identification, isolation and indeed definition have emerged.

## 1.2 Definition of EPCs

The putative EPCs first described by Asahara *et al* are bone-marrow derived stem cells that proliferate in an immature state, can be mobilised into the blood stream, migrate to areas of endothelial damage or new blood vessel formation where they differentiate into a mature endothelial cell, and contribute and enhance the processes of endothelial repair and new blood vessel formation.

Therefore, in the laboratory, a 'true' EPC should be capable of being isolated from blood or bone marrow, should be capable of proliferation, should display an undifferentiated phenotype capable of developing into an endothelial phenotype in appropriate conditions, and when infused into the bloodstream it should incorporate into blood vessels improving endothelial function and ameliorating ischaemia.

The cells first described by Asahara *et al* possessed many of these features: they were isolated from the mononuclear layer of peripheral blood and expressed the stem cell antigen CD34. After 7 days in culture conditions favouring endothelial differentiation the cells expressed the endothelial markers eNOS and KDR. Infusion of the cells into an animal ischaemia model resulted in amelioration of the ischaemia and on post mortem examination the cells were detected at the site of neovascularisation.

Since their first description, there have been over 2000 EPC-related publications, but a variety of methodologies have been employed to define and measure them.

The two main techniques used to study EPCs are by cell culture and by flow cytometry:

### 1.2.1 Definition of EPCs by Flow Cytometry

Cell count by flow cytometry is based on labelling cells with antibodies directed against surface or intracellular antigens. Circulating EPCs are rare, and represent between 0.01% and 0.0001% of mononuclear cells, hence 1-2 million cells are generally needed to be analysed to obtain an accurate result [6]. This rarity imposes the use of only 2-3 surface antigens, and due to overlap with other cell lineages, the precise antigenic phenotype of EPCs is not known [7]. Commonly used markers include the stem cell antigens CD34 and CD133, and the endothelial antigens KDR/Flk-1 (Kinase-insert Domain Receptor in humans, and Fetal Liver Kinase-1 in mice, representing type 2 vascular endothelial growth factor receptor (VEGFR)-2, CD31, also known as platelet-endothelial cells adhesion molecule (PECAM)-1, and von Willebrand factor (vWf). Another limitation to flow cytometry is that one is basing the presumed complex function of a cell on a relatively simple antigenic phenotype. Despite these limitations, cell count by flow cytometry is rapid, sensitive, specific and reproducible, and is becoming an increasingly popular method of evaluating EPC number.

### 1.2.2 Definition of EPCs by Cell Culture

Asahara *et al* demonstrated that CD34+ cells and KDR+ cells give rise to endothelial-like cells as early as 3 days after plating onto human fibronectin in an

endothelial medium. These cells displayed typical functional properties of endothelial cells, such as uptake of acetylated low density lipoproteins (AcLDL) and binding of Ulex lectin, in addition to the expression of other endothelial lineage markers (such as CD31, Tie-2, KDR).

Since then, a number of variations of this protocol have been used with for example different culture media, or collagen instead of fibronectin coated plates. The majority of these studies have used the entire mononuclear layer rather than CD34+ cells selected by magnetic beads. In an attempt to overcome contamination with mature circulating endothelial cells, some groups have used a pre-plating procedure, whereby after 48 hours non-adherent cells are removed, re-plated and maintained in culture for counting at day 7. This method produces cells that form distinct colonies, giving this assay the name of Endothelial Cell-Colony Forming Units (CFU-EC), or Endothelial Colony Forming Cells (ECFC) assay [8]. It is these cells that are removed and discarded in Asahara's and our laboratory, yet they are still termed EPCs in other laboratories. In order to distinguish between the two methods, the cells derived by Asahara's and Dimmeler's [9] methods are also known as Circulating Angiogenic Cells (CAC). Although these two distinct sub-populations of EPCs are both involved in vessel formation, they have different phenotypes and functional capabilities.

CACs are a haemopoietic sub-population which have strong paracrine and hormonal activities, and stimulate cell migration and proliferation. The second sub-group, ECFCs, are highly proliferative and have more endothelial-like characteristics. Under the influence of CACs, they migrate to sites of vessel

formation where they differentiate into mature endothelial cells [10]. The term EPC is often applied to cells from either culture method, and although both sub-types have different properties, both have been linked to cardiovascular disease.

A recent Austrian study measured EPCs in patients with peripheral vascular disease using flow cytometry (CD34+/CD133+/KDR+ events), both culture methods for CAC and CFU-EC, and a modified Boyden chamber to assess EPC migratory function [11]. The authors conducted a prospective randomised control trial of 40 patients with symptomatic peripheral arterial disease, measuring EPC number and function using the above four methods before and after supervised exercise training. They found that after 6 months exercise training, there were statistically significant increases in EPC number and migratory function as measured by all four methods. It is interesting that despite the different properties of the 2 subgroups of EPCs, and the differences in methodology between flow cytometry and cell culture discussed above, all three methods showed parallel changes after the intervention.

### 1.2.3 Methods used in this paper

As *ex vivo* analysis of blood cells by flow cytometry can only count EPCs and quantify the expression of a limited number of surface or intracellular antigens, we elected to use cell culture techniques which allow both quantitative and qualitative properties to be defined. The cells cultures in our laboratory are isolated from the mononuclear layer of peripheral blood and are initially monocytic in morphology. After 7 days in culture favouring endothelial

differentiation they display an endothelial morphology, in that they are spindle shaped, adherent to fibronectin, stain positive for lectin and are capable of internalising low density lipoprotein (LDL). They also stain positive for the endothelial and stem cell markers KDR and CD34. In separate experiments not detailed in this thesis, infusion of these cells into animal models of hind-limb ischaemia promotes neovascularisation. We used a modified Boyden chamber with VEGF as a chemo-attractant, and a fibronectin adhesion assay to assess EPC function.

## 1.3 EPCs and Cardiovascular Disease

Since the role of EPCs in neovascularisation of mouse and rabbit ischaemic limbs was originally described in 1997, there has been much research into the role of EPCs in cardiovascular disease. There is now a large body of direct and indirect evidence to support their role in cardiovascular disease processes. These studies have demonstrated:

- A correlation between EPC number, endothelial function and cardiovascular risk factors

- Reduced number of EPCs in patients with risk factors for cardiovascular disease such as metabolic syndrome, diabetes and smoking

- Reduced EPC number and function in patients with critical limb ischaemia and coronary artery disease, and reduced EPC number is a

significant independent predictor of poor prognosis in patients with established coronary artery disease

- Interventions that reduce cardiovascular risk such as exercise and weight loss improve EPC number and function

- Reduced EPC number and function in obesity, which both normalise after weight loss, and EPCs from obese subjects show a reduced angiogenic response when injected into an animal ischaemia model

- Reduced EPC number in conditions that increase cardiovascular risk, and subsequent amelioration in EPC number with medical treatment, for example diabetes, hypertension and sub-clinical hypothyroidism

- Statins increase EPC number and function both *in vitro* and *in vivo*

- In animal models, exogenously administered EPCs migrate to areas of ischaemia where they mediate neovascularisation and improve endothelial function

- In human studies exogenous EPC infusions are beneficial in coronary and peripheral arterial disease

The accumulated evidence suggests that a balance between the damaging effects of conventional cardiovascular risk factors and the ability of circulating EPCs to effect endothelial repair determines cardiovascular risk.

### 1.3.1 Correlation between EPCs and other cardiovascular risk markers

Hill *et al* [8] cultured EPCs from 45 men with various degrees of cardiovascular risk, but without overt cardiovascular disease. They found a strong correlation between EPC number and combined Framingham risk factor score ($r=-0.47$, $P=0.001$). Measurement of flow-mediated brachial-artery reactivity also revealed a significant relation between endothelial function and the number of progenitor cells ($r=0.59$, $P<0.001$). They found that levels of circulating EPCs were actually a better predictor of vascular reactivity than the presence or absence of conventional risk factors. A β-galactosidase activity assay was used as a marker of cellular senescence on day 7 EPCs.

They found that a higher percentage of EPCs from patients with high Framingham risk scores displayed a senescent phenotype (72±15 percent of the cells derived from the high-risk subjects vs. 27±9 percent of the cells from the low-risk subjects had b-galactosidase staining ($P=0.005$)). The authors postulated that endothelial injury in the absence of sufficient circulating progenitor cells may affect the progression of cardiovascular disease.

Jialal *et al* [12] measured EPCs in subjects with and without metabolic syndrome using flow cytometry (CD34+ KDR+) and cell culture (CFU-EC method), and a

modified Boyden chamber was used to measure migratory activity. Smoking, atherosclerosis and diabetes were exclusion criteria and Metabolic Syndrome was defined as having 3 out of: central obesity, hypertension, dyslipidaemia or hypertension.

The subjects with metabolic syndrome had significantly lower (34%, $p<0.001$) CD34+KDR+ EPCs compared with their age-matched healthy volunteers. There were significantly lower numbers of cultured EPCs in the metabolic syndrome group (48%, $p<0.001$). There was also reduction in migratory capacity, but this difference did not reach statistical significance. Regression analysis revealed that CRP, triglycerides, age, and plasma glucose were the strongest predictors of reduced circulating CD34+KDR+ cells (adjusted R-squared = 0.147, model p = 0.01).

1.3.2 EPCs in patients with established cardiovascular disease

To assess the role of EPCs in coronary artery disease (CAD), Vasa *et al* [13] studied 45 subjects with angiographic evidence of coronary artery disease and 15 healthy controls. They measured EPC number with cell culture (number of cells adherent to fibronectin after 4 days culture in EBM with dual positive staining for LDL and lectin), flow cytometry (circulating CD34+/KDR+) and migratory function was assessed with a modified Boyden chamber (for 24 hours, with VEGF as a chemo attractant). To determine the influence of atherosclerotic risk factors, a risk factor score including age, sex, hypertension, diabetes, smoking, positive family history of CAD, and LDL cholesterol levels was used.

The number of risk factors was significantly correlated with a reduction of cultured EPC levels (R=-0.394, P=0.002) and CD34+/KDR+ cells (R=-0.537, P<0.001). Analysis of the individual risk factors demonstrated that smokers had significantly reduced levels of cultured EPCs (P<0.001) and CD34+/KDR+ cells (P=0.003). EPCs isolated from patients with CAD also revealed an impaired migratory response, which was inversely correlated with the number of risk factors (R=-0.484, P=0.002). By multivariate analysis, hypertension was identified as a major independent predictor for impaired EPC migration (P=0.043). The authors concluded that the correlation with risk factors, and the reduced levels and functional impairment of EPCs observed may contribute to impaired vascularisation in patients with CAD.

Yue *et al* [14] performed a cross sectional observational study of 174 patients with established CAD. They found significantly lower circulating log CD34/KDR(+) EPCs in smokers compared with non-smokers ($0.86 \pm 0.03$ vs $0.96 \pm 0.03 \times 10^{-3}$/ml, $p = 0.032$). Smokers with elevated pulmonary artery systolic pressure (PASP) also had significantly lower circulating EPCs, higher pulmonary vascular resistance, and larger right ventricular dimensions with impaired function. Log CD34/KDR+ and log CD133/KDR+ EPC counts were significantly and negatively correlated with PASP ($r = -0.30$, $p < 0.001$, and $r = -0.34$, $p < 0.001$, respectively) and pulmonary vascular resistance ($r = -0.29$, $p = 0.002$, and $r = -0.18$, $p = 0.013$, respectively). The reduced number of circulating EPCs and elevated PASP in smokers with CAD suggests that in smokers,

depletion of circulating EPCs might be linked to the occurrence of pulmonary vascular dysfunction.

Schmidt-Lucke *et al* [15] measured circulating EPCs (defined as CD34+ KDR+ on flow cytometry) in 120 patients with acute coronary syndrome, stable coronary artery disease and control subjects. Patients were followed up for a median 10 month period and the primary outcome was cardiovascular events (cardiovascular death, unstable angina, myocardial infarction, PTCA, CABG, or ischaemic stroke).

They found that patients suffering from cardiovascular events had significantly lower numbers of EPCs ($P<0.05$). Reduced numbers of EPCs were associated with a significantly higher incidence of cardiovascular events by Kaplan-Meier analysis ($P=0.0009$). By multivariate analysis, reduced EPC levels were a significant, independent predictor of poor prognosis, even after adjustment for traditional cardiovascular risk factors and disease activity (hazard ratio, 3.9; $P<0.05$).

Werner *et al* [16] measured CD34+ KDR+ cells with flow cytometry in 519 patients with coronary artery disease as confirmed on angiography, and patients were followed up for 12 months. After adjustment for age, sex, vascular risk factors, and other relevant variables, they found that increased levels of endothelial progenitor cells were associated with a reduced risk of death from

cardiovascular causes (hazard ratio, 0.31; 95 percent confidence interval, 0.16 to 0.63; P=0.001), a first major cardiovascular event (hazard ratio, 0.74; 95 percent confidence interval, 0.62 to 0.89; P=0.002), revascularization (hazard ratio, 0.77; 95 percent confidence interval, 0.62 to 0.95; P=0.02), and hospitalization (hazard ratio, 0.76; 95 percent confidence interval, 0.63 to 0.94; P=0.01). However, EPC levels were not predictive of myocardial infarction or of death from all causes. The authors concluded that the level of circulating EPCs predicts the occurrence of cardiovascular events and death from cardiovascular causes and may help to identify patients at increased cardiovascular risk.

Chen *et al* [17] cultured EPCs from 74 patients with Type 2 diabetes with and without critical leg ischaemia, and non-diabetic patients with and without lower extremity vascular disease. A modified Boyden chamber with VEGF as a chemo-attractant was used to assess cultured EPC migratory function *in vitro*. The migratory function was significantly impaired in diabetic patients without (48 count/view/well) and with (51 count/view/well) critical leg ischaemia and non-diabetic patients with critical leg ischaemia (49 count/view/well) compared with healthy subjects (63 count/view/well), $p < 0.0001$. As the migratory function of EPCs was impaired in patients with Type 2 diabetes, even in those without critical leg ischaemia, the authors postulate that Type 2 diabetes may alter EPC function and may account for the impaired neovascularisation and more aggressive clinical course in the development of critical limb ischaemia in patients with Type 2 diabetes compared with non-diabetic patients.

1.3.3 Reversible defects in EPC number and function in patients with an increased cardiovascular risk

The Austrian group mentioned above conducted a prospective randomised control trial of 40 patients with symptomatic peripheral arterial disease [11]. They measured EPC by flow cytometry (CD34+/KDR+/CD133+) and cell culture, and EPC migration was assessed using a modified Boyden Chamber. After 6 months of Supervised Exercise Training, they noted a significant increase in both measurements of EPC number, and migratory activity was also significantly increased.

Heida *et al* [18] studied EPC number and function in obese patients before and after weight loss in 49 obese (body mass index 42 +/- 7 kg/m2) and 49 age-matched lean volunteers. EPC number and function (migration through a modified Boyden chamber, adhesion to fibronectin and angiogenesis with HUVEC/Matrigel assay) were assessed at baseline, and in obese subjects who lost weight after 6 months (defined as current BMI <35 kg/m2 and/or >10% loss of body weight compared with baseline).

At baseline they found that obese subjects had lower EPC count, and that the cultivated EPCs displayed impaired adhesion, impaired migratory activity and an impaired ability to incorporate into network-like structures. The investigators went on to study the EPCs *in vivo* using a mouse hind limb ischaemia model. When injected into the mice, labelled EPCs from obese subjects were less

frequently detected within the ischaemic hind limb musculature after 10 days (28 +/- 25 vs. 88 +/- 85 chloromethylbenzamido-DiI–positive cells/mm2; p = 0.017; 11 mice per group). They also found that mice treated with EPCs from obese subjects revealed a reduced angiogenic response as assessed by the number of CD31-positive cells per square millimeter (p = 0.036 vs. lean).

After 6 months weight loss, 26 subjects had repeat sampling for EPC number and function. There was no change in medications over the period and mean weight loss was 26kg, and BMI dropped from 43 to 35 $Kg/m^2$, ($p < 0.001$ for weight and BMI differences from baseline). There was no significant difference in fasting glucose, total cholesterol or rates of hypertension, but serum triglycerides were lower (114 vs 149 mg/dl, p = 0.004) as was LDL cholesterol (115 vs 126 mg/dl, p = 0.024) when compared to pre-weight loss values.

Weight loss seemed to restore the number of acetylated low-density lipoprotein, lectin double-positive EPCs ($p < 0.05$ vs. initially obese, 10 per group). Weight loss also improved the adhesive properties of EPCs on fibronectin ($p < 0.05$ vs. initially obese), it normalised the migratory activity of EPCs ($p < 0.001$ vs. initially obese and $p > 0.05$ vs. lean; 8 per group), it and restored their angiogenic properties in the Matrigel assay ($p < 0.01$ and $p > 0.05$, respectively; n = 7).

In summary, when compared to lean controls, obese subjects have reduced EPC numbers, these cultivated EPCs are characterised by reduced adhesive and migratory capacity, and that these defects of EPCs are reversible after significant weight loss.

1.3.4 Statins and EPC biology

HMG-CoA reductase inhibitors (Statins) inhibit the rate limiting step in the formation of cholesterol, resulting in a reduction in serum cholesterol levels and a reduction in cardiovascular risk. In addition they have anti-inflammatory and anti-thrombotic properties, and are also thought to reduce cardiovascular risk independent of their lipid lowering properties [19]. Given the links between EPCs and cardiovascular disease, and the beneficial effects of statins in cardiovascular disease, there have been a number of studies on the effects of EPCs and statins.

Dimmeler *et al* [9] found that atorvastatin increases the number of differentiated adherent EPCs (dual positive Lectin/DiLDL cells) when incubated with human mononuclear cells at day 3 of culture. To test the in vivo relevance of their findings, mice were fed with simvastatin (20 mg/kg daily) for 3 weeks, and EPC numbers were determined. Statin treatment led to a more than twofold increase in DiLDL/lectin-positive cells, thus extending the in vitro data.

Published in the same edition of the Journal of Clinical investigation, Llevadot *et al* [20] used a modified Boyden chamber with simvastatin, VEGF, or vehicle as a chemoattractant. Simvastatin profoundly enhanced EPC migration (control versus 1 μM simvastatin, $5 \pm 4$ vs. $213 \pm 46$ cells per four high-powered [40×] fields; $p < 0.01$). Migration with 10 μM simvastatin was equal to VEGF, and the maximum migration was seen with 1 μM simvastatin. To investigate the effects of simvastatin on EPC mobilization, a chemotactic transwell assay was used.

Chemotactic activity was increased by simvastatin, with maximum chemotactic activity observed in the group treated with 1 μM simvastatin (control versus 1 μM simvastatin: 1,137 ± 148 vs. 4,681 ± 598 cells per 50 μl of lower chamber media; $P < 0.01$).

To evaluate EPC mobilization *in vivo*, a murine EPC culture assay was used as a functional index of circulating EPCs. After 4 days in culture, there were significantly more circulating EPCs in the peripheral blood of simvastatin-treated versus control mice (205 ± 5 vs. 147 ± 7 cells/mm2; $P < 0.05$) There was no statistically significant difference in the levels of serum cholesterol between treated and control mice.

To establish whether the in vitro and in vivo findings suggesting pro-vasculogenic effects of simvastatin on EPCs were associated with augmented neovascularisation, simvastatin therapy was studied in a murine model of corneal injury after bone marrow transplant. Simvastatin treatment resulted in augmented corneal neovascularisation and in more X-gal–positive cells than in the control group.

Sections from the simvastatin group documented more neovascularisation and more extensive incorporation of β-gal–positive cells compared with controls. Quantitative analysis of incorporated β-gal–positive cells revealed that simvastatin enhanced vasculogenesis in neovascular foci of corneas of simvastatin-treated versus control mice (25.7% ± 4.0% versus 7.3% ± 2.0% incorporation of β-gal–positive cells; $P < 0.05$). This study demonstrated that

simvastatin enhances EPC migration and chemotaxis *in vitro*, and that it increases EPC mobilisation from the bone marrow *in vivo*.

A recent trial by Tousoulis *et al* [21] evaluated EPCs (flow cytometry, CD34+/KDR+) in rosuvastatin treatment in patients with heart failure (21 patients assigned rosuvastatin 10mg/day, 18 assigned placebo). Rosuvastatin significantly increased circulating (CD34+/KDR+) EPCs, from 230 (170–380) to 390 (230–520), $p = 0.004$. This increase in the number of EPCs did not correlate with the decrease in LDL ($r=-0.12$, $p = 0.375$). No differences were observed regarding the origin of heart failure, and subgroup analysis showed that the increase in EPC numbers was similar in subjects with ischaemic and non-ischaemic disease (46% and 36%, respectively, $p = 0.63$).

### 1.3.5 The effect on EPC number of other interventions known to reduce cardiovascular risk

#### 1.3.5.1 EPCs and treatment of diabetes

Liao *et al* [22] quantified EPC number by flow cytometry (CD 45- / CD34+ / VEGFR2+) and endothelial function was assessed by flow-mediated brachial artery dilatation (FMD) in 46 newly diagnosed type 2 diabetic patients and 51 healthy subjects. Metformin was then administered to all patients for 16 weeks.

The EPC number in the diabetic group was significantly lower than that in the control group ($p < 0.001$), and improved markedly after treatment ($p < 0.001$). The results of FMD were consistent with EPC variations among the three groups

($p < 0.001$). In multivariate regression analysis, the EPC number was an independent risk factor for FMD at baseline ($p < 0.05$). The absolute changes of EPC number showed significant correlation with the changes of FMD before and after treatment ($r = 0.63$, $p < 0.001$). The authors concluded that circulating EPC number was related to endothelial function and could be considered as a surrogate biological marker of endothelial function in Type 2 diabetes.

The same group [23] also evaluated treatment with gliclazide in type 2 diabetes. They quantified EPC number in 58 patients with newly diagnosed diabetes and control subjects. Baseline EPC count was lower in the diabetic group than in the control group at baseline (Cell count 1036±94 vs 1624±91, $p<0.05$), and improved significantly following 12 weeks of gliclazide treatment (Cell count after gliclazide 1411±106 vs 1036±94 at baseline, $p<0.05$).

1.3.5.2 EPCs and treatment of hypertension

Cacciatore *et al* [24] measured EPC number by cell culture and migratory function with a modified Boyden chamber (VEGF as chemoattractant) in hypertensive patients before and after treatment with an ACE-Inhibitor (enalapril 20 mg/day (n=18) or zofenopril 30 mg/day (n=18)). Carotid intimal medial thickness (IMT) was determined by ultrasonography at baseline and after 1 and 5 years of follow-up. There were no statistical differences between treatment groups during the follow up.

EPC number increased during the follow-up (69.6±12.0 vs. 80.6±8.4 for enalapril, 67.5±11.9 vs. 80.9±7.3 for zofenopril, $p<0.001$), but migrating capacity

of EPCs did not change. There was an inverse correlation between circulating EPCs and IMT increase over time. Multiple linear regression model demonstrated that carotid IMT was significantly inversely correlated with EPC ($p<0.001$) but not with migratory cells after adjusting for confounders.

1.3.5.3 EPCs and treatment of subclinical hypothyroidism

Subclinical hypothyroidism (SCH) has been associated with atherogenic lipid profile and endothelial dysfunction (assessed by flow-mediated dilatation), which improves with thyroid hormone replacement therapy [25]. Some studies have shown association of SCH and cardiovascular disease or mortality [26].

Shakoor *et al* [27] measured EPCs with cell culture and flow cytometry (CD34+/VEGFR-2+) and function (MMT assay) in 20 subjects with subclinical hypothyroidism (median TSH 6.5 mU/L) before and after 3 months thyroxine replacement (median TSH 2.26 mU/L), and 20 healthy controls (HC).

Flow cytometry EPC count was significantly reduced in SCH compared to HC (0.10 vs. 0.39, $P < 0.001$), and after treatment with thyroxine, EPC count increased to a similar level as HC (0.26 vs. 0.10, $P < 0.001$). EPC count as assessed by cell culture showed similar results (HC: 49 (46 –54) SCH at baseline: 47 (36 –54) p=0.06, SCH after treatment: 50 (32–79) p=0.08) but there was no difference in EPC function.

There was a significant positive correlation between EPCs with free T4 levels ($r = 0.38$; $P = 0.02$); high-density lipoprotein cholesterol levels ($r = 0.51$; $P =$

0.001); and negative correlation with TSH concentrations ($r = -0.64$; $P < 0.001$). SCH appeared to be the single, most important factor determining lower EPC count when corrected for other cardiovascular risk factors studied, which excludes the possibility that the reduction in EPC count could have been secondary to prevailing risk factors.

### 1.3.6 Beneficial effects of infusion of EPCs in animal models of ischaemia

Since Asahara's first demonstration of the role of the EPC in an animal model of ischaemia, there have been many subsequent studies confirming the same findings. A further study from the same group [28] transplanted *ex vivo* expanded human DiI-labelled EPCs to athymic nude mice with hind limb ischaemia. Blood flow recovery and capillary density in the ischaemic hind limb were markedly improved, and the rate of limb loss was significantly reduced. Time-course studies demonstrated that peak EPC incorporation was achieved within 3–7 days post administration of EPCs.

Review of sections retrieved from the ischaemic limbs animals identified labelled EPCs in up to 56 ± 4.7% (mean ± SEM, range 30–80%) of vessels in a ×10 field. Other than in ischaemic tissue and very rarely in the spleen, EPCs were detected neither in other organs nor the contra lateral non-ischaemic hind limb. This experiment confirmed the concept that *ex vivo* expanded human EPCs may have utility as a "supply-side" strategy for therapeutic neovascularisation.

An experiment from the same laboratory in Boston [29] sought to clarify the extent to which EPCs contribute to adult neovascularisation. The investigators studied the quantitative contribution of EPCs to newly formed vascular structures in an *in vivo* Matrigel plug assay and corneal micropocket assay. They transplanted irradiated mice with bone marrow mononuclear cells from transgenic mice constitutively expressing beta-galactosidase (beta-gal). After 4 weeks a subcutaneous matrigel plug containing fibroblast growth factor 2, or corneal pellet containing VEGF was injected, and mice were sacrificed 7 days later.

Bone marrow derived cells in the implants were identified by immunostaining for beta-gal, and 26.5% of all endothelial cells in the new blood vessels in the matrigel plug stained positive. In the corneal implant, 17.7% of the endothelial cells involved in neovascularisation were found to be bone marrow derived. Ki67 staining of the corneal tissue documented that the majority of EPC-derived cells were actively proliferating in situ. These findings suggest that bone marrow derived EPCs make a significant contribution to angiogenic growth factor-induced neovascularisation.

Kawamoto *et al* [30] from the same group investigated transplanted human EPCs in an athymic rat model of myocardial ischaemia. Labelled human expanded EPCs were injected intravenously 3 hours after ligation of the left anterior descending artery. After 7 days intravenous lectin was administered and the animals were immediately sacrificed. Fluorescence microscopy revealed that

transplanted EPCs accumulated in the ischaemic area and incorporated into foci of myocardial neovascularisation. To determine the impact on left ventricular function, 5 rats (EPC group) were injected intravenously with $10^6$ EPCs 3 hours after ischaemia; 5 other rats (control group) received culture media.

Echocardiography showed that the EPC group had ventricular dimensions that were significantly smaller and fractional shortening that was significantly greater in the than in the control group by day 28. Regional wall motion was also better preserved in the EPC group. Histological examination disclosed that capillary density was significantly greater in the EPC group than in the control group. Moreover, the extent of left ventricular scarring was significantly less in rats receiving EPCs than in controls. Immunohistochemistry revealed capillaries that were positive for human-specific endothelial cells. The authors therefore proved that *ex vivo* expanded EPCs incorporate into foci of myocardial neovascularisation and have a favourable impact on the preservation of left ventricular function.

Aicher *et al* [31] from the same German group mentioned above (Schmidt-Lucke, Vasa, Dimmeler) performed a similar experiment with transplanted human EPCs in an athymic rat model of myocardial ischaemia. However they labelled the EPCs with a radioactive Indium tracer to monitor tissue distribution of the transplanted EPCs. 8 rats had an induced myocardial infarction, 8 had a sham operation and scintigraphic images were acquired 1, 24, 48, and 96 hours after EPC injection. At 24 to 96 hours after intravenous injection of EPCs,

approximately 70% of the radioactivity was localized in the spleen and liver, with only approximately 1% of the radioactivity identified in the heart of sham-operated animals.

After myocardial infarction, the heart-to-muscle radioactivity ratio increased significantly, from 1.02+/-0.19 in sham-operated animals to 2.03+/-0.37 after intravenous administration of EPCs. Injection of EPCs into the left ventricular cavity increased this ratio profoundly, from 2.69+/-1.54 in sham-operated animals to 4.70+/-1.55 ($P<0.05$) in rats with myocardial infarction.

On pathological examination, immunostaining of infarcted hearts confirmed that EPCs homed predominantly to the infarct border zone. They concluded that although only a small proportion of radiolabelled EPCs were detected in nonischaemic myocardium, myocardial infarction profoundly increases homing of transplanted EPCs in vivo.

### 1.3.7 Human Studies of EPC administration

The same Frankfurt group published the Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial in 2002 [32]. This pilot study aimed to evaluate the safety and feasibility of autologous EPC transplantation in humans with ischaemic heart disease.

The authors randomly allocated 20 patients with reperfused acute myocardial infarction (AMI) to receive intracoronary infusion of either bone marrow-derived

(n=9) or circulating blood-derived progenitor cells (n=11) into the infarct artery 4 days after AMI.

EPCs were isolated from the patients' own blood and cultured on fibronectin coated plated prior to re-suspension, and bone marrow derived mononuclear cells (containing heterogeneous cell populations including hematopoietic progenitor cells) were isolated by density gradient centrifugation on the same day as infusion. Cells were administered in a 10ml direct infusion into the infarcted vessel during angiography.

Transplantation of progenitor cells was associated with a significant increase in global left ventricular ejection fraction from 51.6 to 60.1% (P=0.003), improved regional wall motion in the infarct zone, and profoundly reduced end-systolic left ventricular volumes at 4-month follow-up. In a non-randomized matched reference group, left ventricular ejection fraction only slightly increased from 51 to 53.5%, and end-systolic volumes remained unchanged. There were no differences for any measured parameter between blood-derived or bone marrow-derived progenitor cells and no signs of an inflammatory response or malignant arrhythmias were observed.

Despite the fact that there was no randomised control group, and the study was non-blinded, the authors showed that intracoronary infusion of autologous progenitor cells appears to be feasible and safe and may beneficially affect the post-infarction remodelling processes.

In 2003 a group in Japan [33] harvested CD34+ cells from peripheral blood and injected them into patients with critical limb ischaemia that were not suitable for surgical or percutaneous revascularisation. 2 patients were studied, both received granulocyte colony-stimulating factor (G-CSF) prior to the procedure, and cells were injected directly into the muscle of the ischaemic limb.

Transcutaneous oxygen pressure in the foot increased, clinical symptoms improved and newly visible collateral blood vessels were directly documented by angiography. Although this study was very small, and there was no control group to receive either G-CSF or placebo, the results add to the hitherto predominantly animal-derived evidence for a key role of the EPC in revascularisation.

In 2005 the first randomized placebo-controlled study of progenitor cells in coronary artery disease was published by Erbs *at al* [34]. The group recruited 26 patients with chronic total occlusion of a coronary artery and evidence of myocardial ischaemia or regional wall motion abnormality on cardiac imaging. GCSF was administered to increase the circulating number of EPCs, and after 4 days mononuclear cells were isolated from peripheral blood by density centrifugation. Cells were cultured for 4 days in endothelial medium in gelatin coated flasks, and 90% of the cells bound lectin and took up DiI-acLDL. After recanalization of the occlusion, patients were randomly assigned to receive intracoronary infusion of day 4 EPCs or control serum. Coronary flow reserve in response to adenosine was measured in the target vessel at the beginning of the

study and after 3 months. Left ventricular function and infarct size were assessed by MRI and metabolism by 18F deoxy-glucose positron emission tomography.

In patients administered day 4 EPCs there were statistically significant improvements in coronary flow reserve, and the number of hibernating segments in the target region had declined in the treatment group, whereas no significant changes were observed in the control group. MRI revealed a statistically significant reduction in infarct size by 16% and an increase in LV ejection fraction by 14% in the treatment group, because of an augmented wall motion in the target region. There were similar rates of re-stenosis in both groups (25% vs 27% in control).

This study proved that intracoronary transplantation of EPCs after recanalization results in an improvement of macro- and micro-vascular function and contributes to the recruitment of hibernating myocardium.

## 1.4 Summary

In summary, EPCs are bone-marrow derived stem cells that are postulated to contribute to post-natal vasculogenesis and to repair of damaged endothelium by incorporation into the vessel wall, secretion of paracrine hormones and stimulation of angiogenesis. Since their original description in 1997, EPCs have been widely studied in cardiovascular disease, and a lower level of circulating EPCs and reduced EPC function in vitro are associated with an increased cardiovascular risk. Medications that reduce an individual's cardiovascular risk have been shown to increase EPC number, and in animal models and human studies EPC infusions have been shown to increase blood flow to areas of ischaemia.

# Chapter 2. Pregnancy

## 2.1 EPCs in Pregnancy, and indirect evidence for the role of the EPC in low birth weight and future cardiovascular risk

Umbilical cord blood is a rich source of haemopoietic progenitor cells [35], and in 2001, Murohara *et al* [36] found that EPCs could be generated more efficiently from umbilical cord blood than from peripheral blood. They grew CD34+ cells from the mononuclear layer of human umbilical cord blood in an endothelial medium on fibronectin coated plates. After 7 days in culture endothelial phenotype was confirmed with uptake of Di-I-acLDL and formation of NO. When injected into an immunodeficient mouse ischaemia model, blood flow was augmented and the cells participated in capillary networks in ischaemic tissue. Subsequent studies have found that EPC numbers when cultured from cord blood are 15 times higher, and their population doubling time is 2.5 times faster than those cultured from adult peripheral blood [37]. Umbilical cord blood, therefore, is a very rich source of EPCs, and the expanded EPCs participate in neovascularisation *in vivo*.

Oestrogen levels are high during pregnancy, and oestrogen levels are thought to account for some of the differences in the incidence of cardiovascular disease between men and women [38]. Oestrogen alters serum lipid concentration and has a direct vasodilatory effect on blood vessels by affecting the bio-availability

of endothelial derived nitric oxide [39]. There is also emerging evidence of the role that oestradiol has on EPC mobilisation, proliferation and apoptosis:

Asahara's group [40] investigated the role of oestradiol in EPC mobilisation using a carotid injury model in oophorectamised mice receiving 17ß-oestradiol or placebo. They found that oestradiol accelerates re-endothelialisation and attenuates medial thickening after carotid injury in part by augmenting mobilisation and proliferation of bone marrow-derived EPCs and their incorporation into the recovering endothelium at the site of injury.

The same laboratory further investigated oestrogen regulation of physiological postnatal vasculogenesis by modulating the bioactivity of EPCs through the oestrogen receptor [41]. They used peripheral blood and bone marrow derived EPCs and measured neovascularisation in cyclic hormonally regulated mouse uterine endometrium. Under the physiological concentrations of estrogen (17ß-estradiol, E2), proliferation and migration were stimulated, whereas apoptosis was inhibited on day 7 cultured EPCs. These oestrogen-induced activities were blocked by a receptor antagonist. An *in vitro* assay for colony forming unit activity as well as flow cytometry using human EPCs at 5 different stages of the menstrual cycle revealed cycle-specific regulation of EPC kinetics. Their findings demonstrated that physiological postnatal vasculogenesis involves cyclical, oestrogen regulated bioactivity of BM-derived EPCs.

In order to further elucidate the role of oestrogen and EPCs during pregnancy, Sugawara *et al* [42] examined the level of serum oestradiol and circulating EPCs

in 20 uncomplicated pregnancies at various degrees of gestation. Peripheral blood mononuclear cells were cultured on fibronectin coated plates in endothelial medium and at day 7 cells stained dual positive for DiI-acLDL and Lectin. More than 80% of the adherent cells expressed von Willebrand factor, KDR/Flk-1, CD31, and ecNOS. The number of circulating EPCs increased gradually and paralleled the progression of gestational age. In addition, the number of EPCs correlated significantly with the level of serum oestradiol ($r=0.722$, $p=0.0024$). The fact that circulating EPCs increase with gestation in normal pregnancies, suggests that EPCs may play an important role in the regulation and maintenance of placental development and vascular integrity during pregnancy.

The authors of the study mentioned above also comment on the three adaptive mechanisms regulating maternal vascular development during pregnancy: vasodilatation, increased permeability, and neovascularisation. Up-regulated endothelial cells could contribute to these dynamic changes. In human pregnancy, flow-mediated vasodilatation of the radial artery has been shown to increase with gestational weeks [43]. Yoshida *et al* also found significantly less flow mediated dilatation in women with chronic hypertension and even less in women with pre-eclampsia ($p<0.001$ for normal vs. chronic hypertension vs. pre-eclampsia). As mentioned above, Hill *et al* [8] found a significant correlation between the number of circulating EPCs and degree of flow mediated vasodilatation of the brachial artery.

Putting these findings together with Sugawara's study, one can hypothesise that EPCs in the maternal circulation may contribute to the regulation and

maintenance of placental development, and endothelium-mediated vascular integrity during pregnancy. Conversely, defects in EPC number or function may contribute to placental dysfunction and subsequent fetal growth restriction

Indirect evidence further supports a role for EPCs in placental and fetal development and subsequent determination of birth weight. Babies of smoking mothers are smaller at birth, and EPC numbers have been found to be lower in smokers. EPC number and function are reduced in pregnant women with pre-eclampsia, an extreme manifestation of deficient placentation. Finally, a reduced degree of placentation has been found in patients with diabetes mellitus, another condition associated with EPC pathology:

2.1.1 Pre-eclampsia

Following their paper examining EPCs in normal pregnancy, Sugawara *et al* [44] cultured circulating EPCs from patients with pre-eclampsia (n=8) and normotensive pregnant women (n=7). They measured EPC number and EPC senescence by beta-galactosidase activity, and systemic inflammation was assessed with C-reactive protein measurement. They found that patients with pre-eclampsia had lower median EPC number (10 vs. 34; $P < 0.01$), and a higher rate of cellular senescence (33.9 vs. 22.9; $p<0.05$) than in controls.

Patients with pre-eclampsia were divided into two subgroups on the basis of serum CRP, either positive (CRP >0.1 mg/dl; n=4) or negative (CRP <0.1 mg/dl; n=4). EPC count was markedly decreased in the CRP-positive group (5 vs 25; $p<0.05$), and there was a non-significant increase in cellular senescence when compared to the CRP-negative group. Although the numbers are small, these

data suggest that the systemic inflammatory response observed in pre-eclampsia might be associated with the number and aging of circulating EPCs, which may lead to endothelial dysfunction and could be affected by systemic inflammation.

A subsequent similar Japanese study came to different conclusions. Matsubara *et al* [45] measured EPCs by flow cytometry (CD34+CD133+KDR+) and cell culture in 36 normal, 10 pre-eclamptic pregnancies and 20 non-pregnant control subjects. EPC proliferation was assessed with a commercially available ELISA kit, and angiogenesis was assessed by HUVEC/tubule formation. Of note median BMI was significantly higher in the pre-eclampsia group than in the normal pregnancy and control groups (28.4, 25.2 and 20.6 $Kg/m^2$ respectively, $p<0.001$ for pre-eclampsia vs. normal pregnancy). In contrast to Sugawara's findings, the authors found that EPC number declined during pregnancy, and there was no difference in EPC number in pre-eclampsia vs. controls as measured by flow cytometry. They found a significant increase in both number of cultured EPCs and EPC proliferation in the pre-eclampsia group compared to the control group. They did not report quantification of tubule formation in the HUVEC assay. Although there are a number of methodological uncertainties in this study, it does raise the possibility that EPCs are up-regulated in pre-eclampsia.

Hwang *et al.* [46] measured fetal EPC number (flow cytometry and cell culture) and function (beta-galactosidase activity) in umbilical cord blood from normal pregnancies(n=30) and from pregnancies complicated by pre-eclampsia but without IUGR (n=17). They found that EPC number was significantly lower and

cellular senescence was higher in the pre-eclampsia pregnancies when compared with healthy controls.

Kwon *et al.* [47] had similar findings when they measured umbilical cord blood EPCs and plasma VEGF in severe pre-eclampsia (n = 15) and normal pregnancy (n = 30). The severe pre-eclampsia group had significantly higher systolic blood pressure, lower birth weight, and higher rate of small for gestational age than the control group. Circulating EPCs in cord blood and umbilical cord plasma free VEGF were significantly decreased in severe pre-eclampsia compared to the control group (p = 0.009 and 0.04, respectively).

In conclusion abnormalities of EPC number and function seem to play a role in development of pre-eclampsia, but given some of the contrasting findings in different studies it is difficult to draw exact conclusions about the mechanisms involved until larger controlled studies are performed.

### 2.1.2 Smoking

Mothers who smoke during pregnancy are more likely to give birth to a smaller baby, and depending on the amount smoked the difference in birth weight compared to non-smokers ranges from 150-350g [48]. A study examining umbilical cord blood flow using doppler velocimetry showed a significant increase in the resistance index in the uterine arteries (P = 0.001) and umbilical artery (P = 0.001) in smoking compared to non-smoking pregnant women [49]. EPC number and function is reduced in subjects who smoke [13, 50], and in subjects who are exposed to second hand cigarette smoke [51]. The combination

of these findings is indirect evidence of the potential role of the EPC in birth weight.

### 2.1.3 Diabetes Mellitus

In a study of placental morphology of mothers with and without diabetes, Stoz *et al* [52] noted retarded maturation of the terminal villi and a decreased degree of vascularisation in the diabetic placentae when compared to the non-diabetic ones. Interestingly, they found that the degree of retardation ran in parallel with severity of diabetes, up until the most severe stage of diabetes, whose parameters were close to the normal placentae. The authors concluded that this was possibly due to a compensatory reaction of the fetal organ placenta to the reduction in utero placental blood flow in diabetes caused by diabetic angiopathy. It is well known that diabetes is associated with EPC pathology [53], and in patients with diabetes EPC proliferation correlates inversely with HbA1c [54]. These findings indirectly support the hypothesis that EPC pathology links defective placental vascularisation with future cardiovascular risk.

In summary, we hypothesise that EPC pathology results in defective maternal uterine spiral artery remodelling and reduced placental blood flow, which subsequently results in a low-birth weight baby, and that this EPC pathology is also responsible for the subsequent increased cardiovascular risk in the mother.

## 2.2 Early placental vascular changes

In order for healthy fetal development, an adequate blood supply through the placenta is required for the exchange of oxygen, nutrients and waste products between mother and fetus. This blood supply develops rapidly and within a few weeks of implantation, angiogenesis (the sprouting of new vessels or elongation of existing ones) as well as vascularisation (the *de novo* formation of blood vessels from progenitor cells) can be found in the placental villi [55]:

The embryo implants about one week after conception, and within a further week the chorion undergoes rapid proliferation and forms numerous villi, which sprout in order to give a maximum area of contact with maternal blood. Embryonic blood is carried to the villi by the branches of the umbilical arteries, and after circulating through the capillaries of the villi, is returned to the embryo by the umbilical veins.

These chorionic villi invade the uterine decidua during their 3 stages of development: Primary chorionic villi are initially small non-vascular structures consisting only of trophoblast, but at around 15-20 days post conception their cores become filled with mesenchymal cells thus generating secondary villi. At 21 days, progenitors of haemangiogenic cells differentiate to form *de novo* blood vessels inside the mesochyme, and at this vascularisation stage the villi are termed tertiary. The receptors of VEGF-R1 (Flt-1) and VEGFR-2 (Flk-1 or KDR) have been identified in the placenta [56], and knockout mouse experiments have shown that VEGFR-2 is vital for specification and early

differentiation of the haemangioblastic precursor cells into umbilical capillaries [57]. The villous trophoblast of this early stage of development is considered to be paramount in regulating the development of the placental vasculature [55].

Along with invasion of the maternal decidua by the extravillous trophoblast, there must be profound remodelling of the maternal vasculature in order to ensure normal development and function of the placenta. Uterine spiral arteries are remodelled from narrow vessels into highly dilated, inelastic tubes, a process called decidualization, or 'interstitial trophoblast-associated remodelling' [58]. This process of spiral artery remodelling reduces maternal blood flow resistance and increases uteroplacental perfusion to meet the requirements of the fetus. In addition, the loss of contractility and maternal vasomotor control guarantees maternal blood supply to the placenta, irrespective of maternal attempts to regulate the blood distribution within the body [59].

Remodelling takes place in three stages:

Very early in pregnancy, in the first phase of remodelling, there are changes in the utero-placental arteries. These changes include vacuolation, endothelial basophilia, disorganisation or hypertrophy of vascular smooth muscle and terminal luminal dilatation. This is thought to be a maternal response to pregnancy and occur independent of trophoblast invasion [60]. Prior to trophoblast invasion, in the second phase of remodelling there are further changes in the vessel wall including dilatation, a reduction in smooth muscle cells, deposition of fibrinoid material and accelerated nitric oxide production by the trophoblast [59]. The third phase involves infiltration of the vessel wall by

endovascular trophoblast cells. There is further reduction in the number of elastic fibres and smooth muscle cells and further dilatation, with an approximate threefold increase in original luminal diameter [55].

In summary, it is clear that an adequate blood supply is vital to supply nutrients and oxygen to the developing fetus. The development of this blood supply is two-part, from both the trophoblast and decidua, and involves complex processes of angiogenesis, vascularisation and vascular remodelling.

## 2.3 Pathology of Intra Uterine Growth Restriction and its associations

Intrauterine growth restriction (IUGR) is defined as fetal growth less than the 10th percentile for gestational age, although less than the 3rd percentile is probably a more reliable cut off for associated perinatal morbidity [61]. IUGR can be separated into symmetric and asymmetric types based on whether the head is spared or not. Symmetric IUGR is thought to be an early event, and is often either constitutional, or associated with a defined condition such as a chromosomal abnormality. The placenta in symmetrical IUGR is usually small, but free from pathological abnormalities. In contrast, asymmetrical IUGR is often associated with significant placental pathology, and most placental causes are due to maternal vascular compromise resulting in placental ischaemia [62]. This vascular compromise causing IUGR is often associated with hypertensive disorders in pregnancy, in particular the hyper-inflammatory condition of pre-eclampsia [63].

Since the first reports of the association between impaired maternal placental blood flow and hypertensive disorders of pregnancy in 1953 [64], many groups have examined the reasons for, and implications of inadequate placental vascular modification. There are still controversies as to the exact nature of the trophoblast–decidua interaction described above, and it is still an area of extensive research [58]. It is, however, clear that deficiencies in this complex process of vascular remodelling has consequences for both mother and fetus.

Spontaneous miscarriage is often accompanied by a complete absence of trophoblast invasion, while less severe deficiencies are associated with IUGR and early onset pre-eclampsia. This raises the possibility that all three syndromes are part of a spectrum of placental pathologies somehow linked to impaired conversion of the spiral arteries [55].

In pre-eclampsia, the cytotrophoblasts fail to differentiate from an epithelial phenotype to an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow and they remain small calibre, resistant vessels [65]. Common pathological findings of pre-eclamptic placentas include medial disorganisation and hyperplasia in the myometrial arteries and acute atherosis in the decidual arteries [66].

The term 'maternal placental syndrome' has been applied to the presence of pre-eclampsia, gestational hypertension, placental abruption, or placental infarction. This is because all of these conditions share some common pathological

mechanisms, including diseased spiral arteries, placental ischaemia, and endothelial dysfunction [67].

Evidence of the significance of placental vascular compromise comes from a study published in The Lancet in 2005: A retrospective cohort study in Canada of 1·03 million women aimed to determine the future risk to the mother after maternal placental syndromes [68]. 7% of all pregnancies were complicated by maternal placental syndrome, and the mean age at delivery was 28 years for both those who were and were not affected. The average age at the time of the first cardiovascular event was 38 years. Compared with those not affected, those affected were more frequently diagnosed with traditional risk factors for cardiovascular disease before delivery: hypertension, diabetes, obesity and dyslipidaemia; but there was no difference in smoking rates. They found a hazard ratio of 2.0 (95 CI 1·7–2·2) for future maternal premature cardiovascular disease following one of the maternal placental syndromes. This risk was higher in the combined presence of a maternal placental syndrome and poor fetal growth (HR 3·1, 95 CI 2·2–4·5) or a maternal placental syndrome and intrauterine fetal death (HR 4·4, 95 CI 2·4–7·9). The gradient effect, with pre-eclampsia and intra-uterine death having a higher hazard ratio than isolated gestational hypertension, suggests that women with more severe placental disease are at the highest risk of future cardiovascular disease.

The authors comment that their findings do not imply a causal relationship, and that the relationship is likely due to a woman's abnormal metabolic milieu that predates her pregnancy and continues after delivery. They postulate that this chronic state of dysmetabolism might create an inhospitable environment during

the development of the placental spiral arteries, which can adversely affect fetal health, while negatively affecting the large arteries of a woman's heart, brain, and extremities over a broader period of time.

In summary, adequate vascular remodelling is vital to the development of a healthy placenta and normal fetal growth. Defective placental development with reduced vascularisation and endothelial dysfunction are thought to play a role in the pathogenesis of intra-uterine growth restriction and maternal placental syndromes. The presence of disease in pregnancy is a strong predictor for future maternal cardiovascular disease, and the two may share common pathological mechanisms.

## 2.4 Birthweight and cardiovascular disease

Birthweight is influenced by a wide range of environmental and genetic factors, including sex of infant, maternal weight gain during pregnancy, maternal weight before pregnancy, maternal smoking, and socioeconomic conditions [69].

In his seminal paper in 1989, Barker published the results of his analysis of a birth cohort of 5,654 men born from 1911-1930 in Hertfordshire, UK [70]. Weight at birth and at 1 year of age was correlated with future death from ischaemic heart disease. Hazard ratios for death from ischaemic heart disease fell with increasing birth weight. Men with the lowest weights at birth and at one year had the highest death rates from ischaemic heart disease (hazard ratios for death from ischaemic heart disease before 65 years of age: 1.50 (95 CI: 0.98-

2.31) and 2.22 (95 CI: 1.33-3.73) respectively. Similar epidemiological findings have subsequently been shown for women [71], and different ethnic populations [72].

This was the first conclusive evidence confirming Barker's theory that coronary heart disease may be linked to impaired fetal growth [73]. In this paper, published 3 years prior to the study mentioned above, he first examined the differences in mortality in different socio-economic areas in the UK in the 1960's. It had been noted that northern industrial towns and poor rural areas had much higher death rates from coronary heart disease than the more affluent southern and eastern areas of the country. These differences were in parallel to previous geographical differences in perinatal mortality rates. This association between infant and adult mortality did not seem to be purely due to dietary factors or poor social conditions, because the geographical distribution of both dietary fat consumption and death from lung cancer were different to past infant mortality, and maternal smoking was very rare in the 1920s. This led Barker to look for adverse environmental effects both *in utero* and during infancy that increased susceptibility to cardiovascular disease. In the early 20$^{th}$ century the majority of neonatal deaths occurred within a week of birth, and were commonly associated with low birth weight. Hence the search for adverse intra-uterine factors, rather than post-natal influences in early infancy, and the evolution of the fetal origins hypothesis: That fetal under-nutrition in middle to late gestation, which leads to disproportionate fetal growth, programmes later coronary heart disease.

In 1995, Barker published a further article: Fetal origins of coronary heart disease [74], in which he explores some of the mechanisms; Trends in coronary heart disease with birth weight are paralleled by similar trends in two of its major risk factors, hypertension and non-insulin dependent (type 2) diabetes. The latter may be explained by the fact that thin neonates lack both fat and skeletal muscle, and one of the responses to this is to develop insulin resistance. Numerous studies have shown that low birth weight is associated with raised blood pressure in childhood and adult life, and possible mechanisms include persisting changes in vascular structure, including loss of elasticity in vessel walls, and the effects of elevated glucocorticoids. Disordered cholesterol metabolism and blood coagulation in adults have been linked to disproportionate size at birth, and this may be due to under-nutrition in late gestation, with ensuing elevated LDL cholesterol and fibrinogen levels. Failure of infant growth may be due to growth hormone resistance, which is associated with high circulating levels of the hormone, which may cause cardiac enlargement and atheroma in later life. Finally there may be placental factors, as a disproportionately-sized placenta has been associated with future hypertension, impaired glucose tolerance, disordered blood coagulation, and death from coronary heart disease.

It should be noted that others have put forward alternative hypotheses to the fetal under-nutrition theory to explain the birth weight–future cardiovascular disease correlation. Hattersley and Tooke [75] propose that low birth weight, insulin resistance, and ultimately glucose intolerance, diabetes, and hypertension could all be phenotypes of the same insulin-resistant genotype. Polygenic influences resulting in insulin resistance could explain abnormal vascular development

during fetal life and early childhood, and also the increased risk of hypertension and vascular disease in later life. They conclude that the predisposition to type 2 diabetes and vascular disease is likely to be the result of both genetic and fetal environmental factors.

Commenting on the origins of his theory, Barker uses the concept of developmental plasticity: a critical period when a system is plastic and sensitive to the environment, followed by loss of plasticity and a fixed functional capacity [76]. For most organs and systems the critical period occurs *in utero*. These early-life metabolic adaptations help the survival of the organism by selecting a growth trajectory appropriate to the environmental cues, in the case of an adverse intra-uterine environment a 'thrifty phenotype'. Those with a thrifty phenotype who later develop in an affluent environment may be more prone to metabolic disorders, such as obesity and type 2 diabetes, whereas those who have received a positive maternal forecast will be adapted to good conditions and therefore better able to cope with rich diets. This 'programming' of risk of future cardiovascular disease seen in fetal under-nutrition is particularly increased if there is over-nutrition and accelerated growth in the post-natal period.

## 2.5 Birthweight and future maternal cardiovascular risk

As discussed above, the inverse relationship between an individual's birth weight and his or her cardiovascular risk in later life is well established, but there is increasing evidence that the mothers of low birth weight babies also have an increased cardiovascular risk in later life.

There is a strong correlation between an infant's birth weight and its mother's and, to a lesser extent, its father's own birth weight. It was this inter-generational effect that first suggested that birth weight of offspring could possibly be associated with the risk of CVD mortality among parents [77]. Parents of heavier babies would be expected to have lower mortality from cardiovascular disease than parents of lighter babies. In order to explore whether intergenerational influences on birth weight are mirrored by mortality risk, Davey Smith *et al* performed a prospective observational study on married couples in the West of Scotland [78]. The authors contacted married subjects who had been recruited into the Refnew and Paisley cohort study in the 1970s. If one or both of the couple had died then information on offspring was obtained via the death certificate.Offspring birth weight was obtained from a centralised database (1134 birth weights of the offspring for 794 couples). Parents' mortality was taken from another central register, and deaths were coded as cardiovascular or other. They found that women who had heavier babies were taller, had higher body mass index and better lung function, and were less likely to be smokers than mothers of lighter babies. Fathers of heavier babies were taller and less likely to be smokers than fathers of lighter babies. All cause and cardiovascular mortality were inversely related to offspring's birth weight for both mothers (RR for a 1 kg lower birth weight 2.00 (95 CI: 1.18, 3.33)) and fathers (1.52 (1.03 to 2.17)) for cardiovascular mortality. Adjustment for blood pressure, cholesterol, height, BMI, social class and smoking had little effect on these risk estimates, although levels of statistical significance were reduced.

The authors comment that the strength of the association was greater than would have been expected by the degree of concordance of birth weights across generations, but an extensive range of potential confounding factors could not account for the association. They concluded that mortality is therefore influenced by an unknown factor related to birth weight that is transmissible across generations.

This was a relatively small study, but several subsequent studies confirm that offspring birth weight is associated with future parental mortality. Davey Smith *et al* recently performed a meta-analysis of the six published studies of the association [79]. This meta-analysis encompassed data on 1.73 million people followed up for 10 to 34 years after the birth of the offspring whose birth weight was used in the analysis. The authors found that among mothers, the pooled, adjusted hazard ratio of cardiovascular disease mortality for a 1-standard deviation (approximately 500g) increase in birth weight was 0.75 (95 CI: 0.67, 0.84) and, among fathers, the equivalent association was 0.93 (95 CI: 0.91, 0.95), with statistical evidence of a difference between these two effects ($p < 0.001$). It should be noted that only 2 out of the 6 studies used birth weight that had been adjusted for pre-term delivery and gestational age. However these two studies did make up the vast majority of the subjects studied (1.68 million), and therefore the association is likely to be related to genuine small for gestational age infants, and not just infants who happened to be born prematurely.

As mentioned above, the association may reflect maternal/fetal nutritional factors and intrauterine programming, as women who themselves had poor fetal growth

and low birth weight tend to have offspring who are small for their gestational age. This effect may be mediated via maternal pelvic restriction, poor placental growth, and hence a programming effect of intrauterine nutrition across generations. One can also not rule out the potential effect of shared environmental exposures such as cigarette smoking that would explain disease in both generations. Only two of the studies included in the meta-analysis made adjustments for parental smoking. However, in the original Refnew and Paisley study, adjustment for smoking had little effect on the risk estimates, although levels of statistical significance were reduced (Relative maternal mortality for 1 quintile increase in birth weight, unadjusted: 0.82 (95 CI: 0.72, 0.94) $p<0.01$, after adjustment for all risks: 0.84 (0.73 to 0.97) $p<0.05$). In addition, if smoking and socio-economic factors were the only reason for the association, then one would expect similar risks for the mother and the father, as they would both be exposed to the same environment. The slight, albeit statistically significant association seen in paternal mortality may well reflect socio-economic or environmental factors not captured by the studies. The fact that mothers have a higher risk than fathers implies that there are inherent maternal factors affecting intra-uterine growth over and above environmental ones.

In summary, the authors found that a reduction in birth weight of 1 standard deviation (approx 500g) was associated with a 25% increased risk of future maternal cardiovascular mortality. The reasons for this relationship are not yet understood but maternal factors which affect intrauterine growth seem to have important influences on long-term mortality risk.

## 2.6 The potential role of the EPC in growth restriction in utero and future cardiovascular disease in mother and infant

Considering the mechanisms of angiogenesis and neovascularisation involved in placental development, and the future risk of cardiovascular disease in the mother associated with their dysfunction, immediately raises the possibility that abnormalities of EPC number or function may play a role. In addition, EPC pathology could explain Barker's hypothesis, that sub-optimal intra-uterine growth is associated with future adult cardiovascular disease [76].

In a recent review in *Placenta*, Sipos *et al* discuss the potential role of the EPC in complications of placental vasculature and long term cardiovascular risk in the infant [80]. They hypothesise that anomalies in either EPC number or function may impair formation of normal placental vessels and influence the embryonic endothelium. On the maternal side, functional irregularities could affect uterine vessels, restricting or attenuating blood flow, thus perpetuating placental disease. Potential mechanisms put forward include hypoxia and oxidative stress, which are both proposed utero-placental features of pre-eclampsia and IUGR. In pathological conditions such as tissue hypoxia, EPC numbers and function may be impaired. In the setting of excessive reactive oxygen species (ROS), reduced bioavailability of nitric oxide could also influence EPC mobilisation and recruitment [80].

We further hypothesise that in addition to playing a role in future cardiovascular disease in the infant, EPCs may also be involved in future cardiovascular disease in the mother.

# Chapter 3. Adiponectin

## 3.1 Adiponectin as a biomarker for cardiovascular disease

The epidemic of obesity and type 2 diabetes in recent years has focused much research attention on adipose tissue. One of the more important results of such research has been the finding that adipocytes do not merely function as inert repositories for energy stores, as previously thought, but can be considered as endocrine organs in their own right, synthesizing and releasing a number of hormones, collectively known as adipokines, that have a diversity of biological functions including the regulation of energy homeostasis. Adiponectin is the most abundant adipokine secreted by adipose cells tissue that may couple regulation of insulin sensitivity with energy metabolism. Some studies have suggested that adiponectin may provide the link between obesity, type 2 diabetes, insulin resistance and cardiovascular disease. This section will briefly review adiponectin, and the role that it may play in particular in diabetes-related and non-diabetes-related cardiovascular disease.

### 3.1.1 Background

Adiponectin, leptin, plasminogen activator inhibitor-1, resistin and tumour necrosis factor alpha are examples of hormones released by adipose tissue. Adiponectin, the most abundant adipokine circulates in the blood in relatively large concentrations when compared to other hormones. A 30-kDa protein that consists of an N-terminal collagenous domain and a C-terminal globular domain,

adiponectin was first isolated in the mid 1990s. Under normal conditions, the adiponectin gene (AMP1) located on chromosome 3q27 is expressed exclusively in adipose tissue. Adiponectin circulates in the blood in higher-order multimeric structural forms including trimeric (low molecular weight, LMW), hexameric (middle molecular weight, MMW), and high molecular weight (HMW) complexes [81]; the majority of studies that have linked adiponectin with metabolic diseases have used assays for total adiponectin [82]. In healthy populations, adiponectin levels vary with race and gender; serum adiponectin is lower in Indo-Asians when compared with Caucasians (median 3.3 vs. 4.9 mcg/ml), and women have about 40% higher circulating levels of adiponectin than men [83].

### 3.1.2 Adiponectin in type 2 diabetes

Many of the initial adiponectin studies were performed in patients with obesity and Type 2 Diabetes (T2DM). Despite being a hormone secreted mainly by adipose tissue, adiponectin levels are paradoxically inversely correlated with body weight or body mass index, suggesting that a negative feedback loop exists between adiponectin and adipose tissue. Plasma adiponectin levels are reduced in obese individuals [84], particularly in those with visceral obesity, and levels of adiponectin correlate inversely with the degree of insulin resistance present in such individuals. Evidence also suggests that substantial weight loss can increase adiponectin levels [85].

Reduced plasma adiponectin levels have also been found in patients with T2DM, and non-diabetic individuals with lower adiponectin levels at baseline have a higher future risk of developing T2DM [86]. The converse is also true, with the Atherosclerosis Risk in Communities Study [87] reporting that higher adiponectin levels are associated with a lower risk of later development of T2DM. Low plasma adiponectin is also a predictor of the metabolic syndrome independent of body mass index [88].

Quite apart from the association between type 2 diabetes and adiponectin levels in many of the studies quoted, a potential causative role for adiponectin in the pathophysiology of T2DM has been suggested by a number of studies. Administration of adiponectin to insulin-resistant mice has an insulin sensitizing effect. Intra-peritoneal injection of adiponectin leads to a fall in blood glucose in wild-type and ob/ob diabetic mice compared to non-injected control mice, and adiponectin enhanced insulin-induced suppression of glucose production from isolated primary rat hepatocytes [89]. Over-expression of adiponectin in ob/ob mice (an animal model of obesity associated T2DM) protected against development of diabetes and improved insulin sensitivity [90]. Also suggesting a causative role for adiponectin in type 2 diabetes is the fact that single nucleotide polymorphisms (SNPs) in the adiponectin gene are associated with T2DM in humans [91].

The presence of abnormal adiponectin levels in the high cardiovascular risk state of T2DM, and the association of hypoadiponectinaemia with the components of the metabolic syndrome, such as insulin resistance, elevated triglycerides, and

low HDL cholesterol [92], led to suggestions that low adiponectin levels in T2DM may provide the link between T2DM and cardiovascular disease. A number of in vitro and animal studies, detailed below, appeared to support this contention by demonstrating a number of beneficial effects of adiponectin at a cellular level which could potentially reduce risk of atheroma formation and cardiovascular disease.

### 3.1.3 Anti-inflammatory, Vasodilator and Anti-oxidant Effects

At a cellular level, adiponectin has been shown to have a number of effects that could potentially reduce the risk of developing atherosclerosis and cardiovascular disease. Adiponectin directly stimulates the production of vasoprotective nitric oxide (NO) by endothelial cells, and enhances endothelial NO synthase (eNOS) activity. Adiponectin also improves the endothelium reduction-oxidation state by suppressing NADPH oxidase-derived superoxide generation [93], another action with potential benefits on endothelial function. Evidence also suggests that HMW adiponectin suppresses endothelial cell apoptosis [94] and promotes vascular healing and angiogenesis. Furthermore, adiponectin prevents macrophage transformation into foam cells, a crucial step in atherogenesis [95].

In animal studies, adiponectin-knockout mice have shown increased neo-intimal proliferation in response to vascular injury and administration of adiponectin prevents atherosclerosis in apolipoprotein-E-deficient mice *in vivo* [96]. Arita *et al* [97] also showed that adiponectin suppresses proliferation of human aortic smooth muscle cells, suggesting a possible influence on vascular remodelling.

Taken together, the above *in vitro* and *in vivo* animal studies suggest that by acting as an anti-inflammatory, antioxidant and vasodilator agent among other effects, adiponectin can potentially prevent endothelial dysfunction and the progression of atherosclerosis.

### 3.1.4 Beneficial effects in cardiovascular disease

A number of epidemiological studies appeared to support this hypothesis, by demonstrating that plasma adiponectin levels are significantly lower in patients with coronary artery disease.

In one such study male patients with adiponectin levels of less than 4 mcg/ml were found to have a 2-fold increase in coronary artery disease prevalence independent of other known risk factors [98]. The fact that the relationship between adiponectin levels and cardiovascular disease persists after adjustment for possible mediators of this effect such as diabetes, HDL and Body Mass Index suggested a direct effect of adiponectin in the vascular system.

A case control study from 2004 found that healthy subjects with adiponectin levels in the upper 20% range have a 2-fold reduced risk for myocardial infarction [99]. Also higher plasma adiponectin levels are independently associated with a lower risk of acute coronary syndrome [100].

In a population-based 10-year follow-up study in elderly men, Frystyk *et al* [101] found that a 1 standard deviation increase in adiponectin levels was associated with a 23% reduction in risk of coronary heart disease.

In the SAPHIR [102] study there was a negative association between common carotid artery intimal media thickness and adiponectin levels, again suggesting that hypoadiponectinaemia is a risk factor for the development of early atherosclerosis.

A 5-year follow-up study of men with type 1 diabetes found adiponectin levels were inversely associated with BMI and directly associated with age, duration of diabetes, and alcohol. Adiponectin was associated with a decreased risk for cardiac events, independent of BMI, diabetes duration, age, smoking and lipid levels. The multivariate relative risk for CHD for a doubling of adiponectin was 0.71 (95% CI 0.53-0.95) [103].

Despite the apparent initial consensus on the relationship between adiponectin, diabetes and CV disease prompted by the studies detailed above, studies in type 1 diabetes and some further cardiovascular studies have suggested that the relationship between adiponectin and cardiovascular disease/diabetes may not be as straightforward as initially suggested.

### 3.1.5 Type 1 diabetes

Although not a condition associated with obesity and insulin resistance, the strong associations found between low adiponectin levels and T2DM, the

potential role for adiponectin in the pathophysiology CV disease in type 2 diabetes, and the high cardiovascular risk in type 1 diabetes inevitably led to studies of adiponectin in patients with type 1 diabetes (T1DM). Studies to date have indeed found abnormal adiponectin levels in patients with T1DM, but, in contrast to the findings in subjects with T2DM and in subjects with obesity, paradoxical *elevated* adiponectin levels have been found in T1DM subjects. In pre-pubertal children with uncomplicated T1DM, adiponectin levels were found to be higher than in healthy controls, and a significant positive correlation was found between adiponectin levels and HbA1c [104]. The Finnish Diabetic Nephropathy Study [105] found that increased serum adiponectin levels predict the progression from macroalbuminuria to end-stage renal disease in type 1 diabetic patients.

### 3.1.6 Association of adiponectin with increased cardiovascular risk

In a sub-study of the Hoorn Study [106], a population-based cohort study of diabetes and diabetes complications, the authors measured adiponectin levels from 2484 patients aged 50-75yrs from the cohort and studied the association with cardiovascular disease and mortality data from 15 years of follow up. At baseline, high adiponectin levels were strongly associated with a more favourable CVD risk profile, with lower weight, smaller waist, higher HDL cholesterol and lower triglycerides, lower insulin and glucose levels, and lower ALT.

On follow up, higher adiponectin reduced the risk of nonfatal CVD in women, but there was no statistically significant reduction in men, and there was no difference in men or women in the risk of all-cause or CVD mortality. A subgroup analysis found that high adiponectin levels were associated with *increased* mortality risk in both sexes with prevalent CVD at baseline, and with reduced risk in people without prevalent CVD at baseline. After adjustment for conventional cardiovascular risk factors, the hazard ratios for CVD mortality were 1.60 (1.14 –2.23) for patients with and 1.38 (1.06 –1.80) for patients without prevalent CVD. (Hazard ratios with 95% confidence interval per SD change in log-adiponectin)

The underlying mechanisms for these contradictory results in the Hoorn study and in type 1 diabetes are still unclear. It has been speculated that low adiponectin predicts cardiovascular events in low-risk populations for CVD, whereas in high-risk populations, a counter-regulatory increase of adiponectin occurs that represents an attempted defence mechanism against the cardiovascular alterations and the pro-inflammatory state associated with CVD, hence the paradoxical finding of an elevated cardiovascular risk associated with high adiponectin levels [107, 108]. Alternatively, resistance to adiponectin due to down-regulation of adiponectin receptors may play a role. It is of course possible, as the authors of the Hoorn study warn, that adiponectin also exerts harmful effects that contribute to the increased mortality risk associated with high adiponectin. It is possible that initially beneficial effects of adiponectin on CVD might become deleterious in advanced CVD states, in particular when the compensatory increase of adiponectin is overwhelming [106].

### 3.1.7 Summary

In summary, adiponectin is a key adipokine with multiple actions in the human body. At a cellular level it has anti-inflammatory, anti-apoptotic, anti-atherogenic and vasodilator effects. High plasma adiponectin level is associated with a favourable lipid and glucose metabolism, and with a reduced risk of CVD events in women. However, higher adiponectin levels have been associated with adverse outcomes, in particular in people with type 1 diabetes, advanced kidney disease or a history of CVD. This may be due to a compensatory up-regulation of adiponectin in advanced disease stages, but a more precise explanation remains elusive.

It seems, therefore, that despite initial promise adiponectin will not be a useful biomarker of disease or cardiovascular risk in all patient groups. Further research will be needed to elucidate the complex underlying molecular mechanisms involved before adiponectin can be used as a therapeutic target in cardiovascular disease. In the meantime, we can modify adiponectin levels and reduce cardiovascular risk in clinical practice by modifying the conventional and non-conventional risk factors for cardiovascular disease which have been associated with altered adiponectin levels, such as low HDL, obesity, insulin resistance, and poor glycaemic control.

3.2 Adiponectin, birthweight and cardiovascular risk

As discussed above, adiponectin is the most abundant hormone released by adipocytes, and is thought to couple regulation of insulin sensitivity with energy metabolism[109]. It has anti-inflammatory, anti-apoptotic and anti-atherogenic properties [93-95]. Some studies have suggested that adiponectin may provide the link between obesity, type 2 diabetes, insulin resistance and cardiovascular disease [82, 88, 89]. High levels are associated with favourable lipid and glucose metabolism, and a reduced risk of cardiovascular events in women [106]. Altered maternal adiponectin levels have been found in women with gestational diabetes [110, 111] and pre-eclampsia [112].

The discrepancies in adiponectin levels in established cardiovascular disease or diabetes described above would not be expected to interfere with measurement in our cohort. In the Hoorn study [106], adiponectin correlated with non-fatal cardiovascular disease in women, and the increase in mortality associated with high adiponectin levels was only seen in the subgroup that had pre-existing cardiovascular disease. Our cohort was made up of women with a mean age of 32 years and we excluded subjects with diabetes, significant medical illness or pre-existing cardiovascular disease. Hence adiponectin levels in our cohort could provide important information about future risk of cardiovascular disease.

Low serum adiponectin has been found in adults and children born small for gestational age, and cord blood adiponectin is independently associated with birth weight, with decreased levels characterising growth-restricted fetuses[113].

It was this twin study by Mazaki-Tovi et al [113] that gave weight to the argument that the lower cord blood adiponectin levels were due to abnormal placentation and not attributable to genetic disparity. The authors postulate that the observed reduced adiponectin levels in SGA neonates could be due to decreased fetal adipose tissue depots and increased fetal exposure to hypoxia, both of which are features of growth restriction.

It therefore seems that adiponectin has an important role in the regulation of major metabolic pathways and fetal growth and it may form part of the link between low birth weight and future metabolic complications in later life [113]. We planned to measure maternal and umbilical cord adiponectin levels in small for gestational age and appropriate gestational age control pregnancies.

# Chapter 4. Methods

**Aims:**

We aimed to investigate the link between EPCs, delivery of a low birth weight baby, and risk of future cardiovascular disease in the mother by measuring conventional cardiovascular risk markers and EPC number and function in mothers of Small for Gestational Age (SGA) and Appropriate for Gestational Age (AGA) infants. We also aimed to measure maternal and cord blood adiponectin to further investigate the role of adiponectin in birth weight and cardiovascular disease.

## 4.1 Inclusion and Exclusion Criteria, Patient Recruitment

Ethical approval for our study was obtained from the Research Ethics Committee in the Rotunda Hospital, Dublin (Chairman: Professor T. Clarke). The Rotunda Hospital is a large University Maternity Hospital with approximately 8,700 deliveries per year. All patients and controls were recruited in the antenatal clinic and fetal assessment unit.

We attempted to exclude patients confounding factors that could influence EPC number or function; therefore subjects with fetal and maternal conditions were excluded, such as:

- Multiple gestation (more than one fetus in current pregnancy), fetal structural or genetic anomaly

- Pre-existing or gestational diabetes mellitus
- Pre-existing cardiovascular disease or hypertension
- Renal, hepatic or autoimmune disease
- Malignancy, anaemia, haemolysis or severe infection
- Current cigarette smoking, drug or alcohol use.

Patients and controls studied satisfied the following criteria:

- Twenty-four to thirty-two weeks gestational age at enrolment.
- Gestational age confirmed by date of last menstrual period, and/or first trimester ultrasound if dates unknown, or menstrual cycle irregular.
- Able to comprehend the information sheet and consent form.
- Attending antenatal services in the Rotunda Hospital.
- Willingness to participate in study, and attend for fasting blood tests six weeks post-partum.

**Recruitment of patients and controls was as follows:**

Prior to each antenatal clinic, the charts of all patients scheduled to attend were reviewed by the author, in order to find patients fulfilling the inclusion and exclusion criteria for the study. Appropriate patients were then approached at the time of their clinic visit. The study was explained in detail and the patient information leaflet (information sheet, appendix 1) was given. After they had at least 24 hours to think about participation, if the patients wished to volunteer for the study then written informed consent was obtained (consent form, appendix 2).

Mothers of potential small for gestational age (SGA) babies were identified by ultrasound scanning and estimation of fetal weight by bi-parietal diameter, head circumference, abdominal circumference and fetal length, using Hadlock's method [114]. Controls matched for age, BMI and ethnicity were recruited in the same manner. Subjects were classified into 2 groups based on their EFW: AGA group (EFW >$10^{th}$ centile), and SGA group (EFW<$10^{th}$ centile).

Medical history and family history were obtained, blood pressure was checked, patients were weighed and height was measured. The details of booking weight, blood pressure, urinalysis and laboratory investigations were recorded from the medical notes.

**Patients were screened for diabetes as follows:**

In our Hospital, routine oral glucose tolerance testing (OGTT) is only performed in patients who are obese, over 40 years of age, from an ethnic group with a high prevalence of diabetes, have a history of polycystic ovarian syndrome (PCOS), have had previous macrosomia, have a first degree relative with type 2 diabetes or in those with glycosuria. All patients were screened for glycosuria at every visit. If patients in the study had not had an OGTT performed, or if it was not scheduled to be performed, then they were screened with a blood test for glucose and HbA1c. The time of the last meal was noted and if the result was abnormal then a formal OGTT was performed, and patients were excluded from the study if diabetes was confirmed. Diagnosis of diabetes was based on the current consensus guidelines of the American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD)[115].

Gestational hypertension was defined as BP > 140/90mmHg, and pre-eclampsia was defined as hypertension and proteinuria (>300 mg of protein in a 24-hour urine sample).

At the time of delivery, 10ml of umbilical cord blood was collected in EDTA coated bottles, stored at 4 degrees centigrade and transported to the laboratory. Mode of delivery was recorded, and placental examination was performed by the local pathology department.

At delivery the gestational age and actual birthweight were used to confirm position on a normal distribution birthweight centile chart with $<10^{th}$ centile defined as SGA. Subjects were classified into 2 groups based on birthweight, 23 mothers with SGA infants (birthweight <10th centile) and 23 mothers with AGA infants (birthweight ≥10th centile).

Six weeks after delivery, patients attended for a follow up visit, and were instructed to fast for at least 12 hours. Weight and blood pressure were measured, and the method of infant feeding was documented. Blood was then drawn in the supine position from a vein in the antecubital fossa. Sixty ml of blood was drawn into lithium-heparin coated bottles for EPC culture. Blood was also drawn for conventional cardiovascular risk markers and adiponectin. Patients were observed until well enough to leave the unit.

**Ultrasonography**

All subjects underwent detailed high resolution doppler ultrasonography at 28weeks and at least every 4 weeks after that until delivery. Fetal well-being and biometry were measured, and blood flow through the umbilical cord was assessed with colour flow doppler. All scans were performed in the department, by a doctor or specialist midwife ultrasonographer.

## 4.2 Laboratory Methods

Unless stated otherwise, cell culture reagents were obtained from Gibco BRL (Karlsruhe, Germany). All other chemical reagents were purchased from Sigma-Aldrich (Dublin, Ireland) and were of the highest purity available.

### 4.2.1 EPC isolation and culture

Blood was brought to the laboratory as soon as possible after sampling meaning that all samples were processed within 45 minutes of blood draw.

Sixty millilitres of blood for EPC isolation was decanted into 50 ml tubes, and diluted 1:1 in HBSS (Hanks Balanced Salt Solution). 30 mls of the blood/HBSS mixture was then gently layered onto 20mls of Ficoll-Paque Plus (GE Healthcare Life Sciences, UK) for density gradient centrifugation. After centrifuging at 1800 rpm for 30 minutes the mononuclear/buffy coat layer was removed with Pasteur pipettes and transferred into 50 ml tubes. The solution was made up to 50 mls with Phosphate Buffered Saline (PBS), and centrifuged for 10 minutes at 1600 rpm. The supernatant was then removed and discarded, and the pellet re-

suspended in 5 mls of Red Cell Lysis buffer, with pipette mixing for 30 seconds to lyse contaminating red blood cells. The tube was then centrifuged for 10 minutes at 1600 rpm. The supernatant was removed, the pellet was re-suspended in 5 mls Phosphate Buffered Saline, and spun again for 10 minutes. This step was repeated twice. The final pellet was then resuspended in Endothelial Basal Medium-2 (EBM-2, Lonza Walkersville, Inc. MD, USA) supplemented with 5% fetal-bovine serum, human VEGF-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, hydrocortisone and heparin (EGM-2 bulletkit, Lonza Walkersville, Inc. MD, USA). 10 x $10^6$ cells were plated in each well of a fibronectin-coated 6 well plate in 2 mls of EGM-2.

The plates had been pre-coated with fibronectin as follows: Each well was covered in 600 microlitres of HBSS. 25 microlitres of Human Fibronectin 0.1% solution was added to the HBSS and the mixture allowed to dry. The supernatant was decanted to aid drying.

Cells were placed in an incubator at 37°C in humidified 5% $CO_2$. After 4 days in culture non-adherent cells were removed, new media applied, and the culture maintained through day 7. Adherent cells at day 7 underwent analysis.

For EPC characterisation at day 7, 10 microlitres of 1,19-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine labelled acetylated low-density lipoprotein (DiI-acLDL, Invitrogen Corporation, CA, USA) was added to 1 ml of media, and cells replaced in the incubator for 5 hours. Cells were then washed with PBS, media

replaced, and 10 microlitres of fluorescein isothiocyanate-labelled lectin from *Ulex Europaeus* (FITC-Lectin, Sigma-Aldrich, MO, USA) was added, and cells replaced in the incubator for a further 4 hours. Cells were then washed with PBS, and viewed under fluorescent microscopy. Cells dual-staining for dil-acLDL and lectin were considered EPCs.

The number of EPCs was determined by counting 12 random high-power (400x) microscope fields per subject's day 7 cells. The counting was performed by a single investigator, who was blinded to the identity and status of the EPC donor.

4.2.2 EPC functional studies

After counting on day 7, EPCs were carefully detached using EDTA (Enzyme-Free Cell Dissociation Buffer, Invitrogen Corporation, CA, USA) and re-suspended in serum free media (EBM-2). These cells then underwent adhesion and migration studies as below.

4.2.2.1 EPC Migration Study

Adapted from Choi et al [116] and Soncin et al [117]. EPC migratory function, which is essential for angiogenesis, was examined using a modified Boyden chamber technique (BD Biocoat Growth Factor Reduced Matrigel Invasion Chamber, BD Biosciences, Oxford, UK). The chamber consists of a 24-well cell culture insert with an 8 micrometre pore size PET membrane, uniformly coated with Matrigel matrix (Figure 1). The matrix provides a barrier to non-invasive

cells while presenting an appropriate protein structure for invading cells to penetrate before passing through the membrane. EPCs at a concentration of 5 x $10^4$ in 500 microlitres VEGF-free EGM-2 were added to upper part of the chamber. 750 microlitres of media was added to the lower part of the chamber, and VEGF was used as a chemoattractant. The stimulated wells contained EGM-2 (which contains VEGF) and the control wells contained VEGF-free EGM-2. After 24 hours incubation at 37˚C in humidified 5% $CO_2$, the upper side of the filter was scrubbed and the filter was fixed in 100% methanol. For quantification, cells were stained with an eosin and methylene blue stain (Speedy-Diff Complete Kit, Clin-Tech Ltd, Guilford, UK). The membrane was removed and mounted on a slide. Cells migrating into the lower chamber were counted manually in 12 random high power (400x) microscope fields by a single investigator, who was blinded to the identity and status of the EPC donor. A migration coefficient was calculated by dividing the number of migrated cells in the stimulated chambers by the number migrated in the control chambers (for example a coefficient of 1 means that there were the same number of cells in both chambers, and that there was no increase in migration towards VEGF). Assays were run in duplicates.

4.2.2.2 EPC Adhesion Study

EPC adhesion to a matrix molecule was assessed using fibronectin-coated plates, adapted from Tepper et al [54]. 96 well plates (Nunc Denmark) were coated with fibronectin as follows: Each well was covered in 50 microlitres of HBSS. 2 microlitres of Human Fibronectin 0.1% solution was added to the HBSS and the mixture was allowed to dry. Wells were blocked for 2 hours with 1% Bovine

Serum Albumin (BSA) in PBS. Wells were then rinsed with 300 microlitres of PBS. 5 x $10^4$ cells in 100 microlitres Serum Free Media were added to each well and allowed to attach for 1 hour at 37°C in humidified 5% $CO_2$. After incubation, non-adherent cells were washed off with 300 microlitres PBS. Cells were fixed with 200 microlitres 10% formaldehyde for 5 minutes. The centre of each well was photographed at 100x magnification for future counting. Adherent cells were stained with 0.1% Crystal Violet for 5 minutes. Wells were rinsed twice with 300 microlitres PBS and allowed to dry briefly. 100 microlitres 100% methanol was added to elute the stain from the cells. Attached cells were quantified by analysing the optical density of the solution at a wavelength of 595nm with a microtitre plate reader (Wallac Victor 2 1420 Multi-label counter). The background optical density was subtracted and each data point was the average of 5 wells. The optical density is expressed in Fluorescence Units (FU), with a lower reading indicating a lower number of adherent cells. (Figure 2)

### 4.2.3 Additional Confirmation of EPC Phenotype

In addition to dual staining with FITC-Lectin and DiI-acLDL under fluorescent microscopy, we performed other experiments to confirm EPC phenotype in our culture conditions. Expression of the stem cell and endothelial antibodies CD34 and anti-human kinase insert domain receptor-1 (KDR, otherwise known as VEGFR-2, Vascular Endothelial Growth Factor Receptor-2), typical of EPC, cells were analysed with western blot and laser scanning cytometry.

#### 4.2.3.1 Western Blot

Cells were taken from EPC culture described above at days 0, 4 and 7, and expression of CD34 and KDR were analysed using a western blot.

4.2.3.1.1 Sample Collection

Cells (1 x $10^7$) were taken at day zero (ie cells purified from the mononuclear layer prior to plating), and at days 4 and 7 one well was scraped with a cell scraper and the contents collected. Samples were centrifuged at 1500rpm for 5min, the supernatant was removed and the pellet re-suspended in 100ul RIPA buffer (RIPA buffer: 400ul tris(hydroxy methyl)aminomethane (tris) (pH 7.4), 100ul EGDT, 200ul EDTA, 2ul Na3VO4, 25ul NaF, 0.106g sodium pyrophosphate, 100ul triton, 20ul NP-40, 4ul PMSF in 20ml dH2O). After 10min the solution was centrifuged for 10min at 13000rpm, 4°C, the debris was removed and the supernatant was frozen at -20°C.

4.2.3.1.2 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

As the proteins for detection were 150 kDa (KDR) and 109 kDa (CD34), a 10% separating gel was prepared. 9.6ml deionised water (dH2O), 6ml 1.5M Tris-HCl (pH 8.9), 8ml protogel (10% acrylamide, 0.9% bis-acrylamide stock solution), 0.24ml 10% SDS, 0.15ml 10% ammonium persulphate (APS), 0.006ml Tetramethylethylenediamine (TEMED) was added to a 50ml tube. The chambers were prepared, filled with separating gel and overlayed with 200 microlitres isopropanol to ensure and even setting of the gel. Chambers were left to set for 30 minutes and the isopropanol was washed out with distilled water. Stacking gel was prepared (3.4ml H2O, 0.83ml 30% protogel, 0.63ml 0.5M Tris-HCl (pH

6.8), 0.05ml 10% SDS, 0.05ml APS, 0.005ml TEMED) and overlayed onto separating gel with a comb. After chamber had set the silicone gasket and comb were removed and the chamber was inserted into the electrophoresis chamber.

Sample buffer was prepared (2g SDS, 1.54g DDT, 2ml 0.2% bromophenol blue and 50% sucrose, 0.5ml EDTA (pH 7.0), 2.1ml tris-HCl (pH 6.7) in 10ml dH2O) and 5ul sample buffer was added to both 5ul and 10ul samples from day 0, 4 and 7. Eppendorf tubes were heated at 99°C for 10min and then centrifuged for 10sec.

Samples were added to the electrophoresis chamber, along with myosin blue marker (250kDa) and phosphorylase orange marker (148kDa), and 100v was applied until elecrophoresis was complete (approx 75min). The gel was then cut off the glass and placed in coomassie stain overnight. The gel was then placed in a de-stain solution (25% methanol, 10% acetic acid and 65% dH2O) and the intensity of the bands was measured. The intensity of the bands was 29000, 22000 and 7000 for days 0, 4 and 7 respectively. In order to standardise the protein levels in each of the samples, the SDS-PAGE was repeated with 3.8ul of day 0, 5ul of day 4 and 15.7ul of day 7 samples.

The gel was transferred onto nitrocellulose using transfer buffer (80ml 1x running buffer and 20ml methanol) for 90min at 150mA, 7 volts. Equal bands were visualised with a temporary Ponceau S stain. The nitrocellulose gel was placed in blocking solution for 1hr (3% powdered milk, 1% BSA in TBS) (TBS = 11.69g NaCl, 6.06g trizma base in 1l dH2O at pH 7.0).

100ul KDR goat polyclonal antibody added to 20ml blocking solution, and the blot was left in this on rocking tray at 4°C overnight. The blot was rinsed for 30min with PBS tween, and this step was repeated. The blot was then placed in 25ul donkey anti-goat IgG-HRP in 25ml blocking solution for 1hr. After washing in PBS tween, PICO fluorescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford IL, USA) was used for the western blot.

The blot was washed in PBS tween and the above steps were repeated using monoclonal CD34 mouse primary antibody, and anti-mouse IgG-HRP secondary antibody. After washing, PICO fluorescence and Femto maximum sensitivity substrate (SuperSignal West Femto Substrate, Thermo Scientific, Rockford IL, USA) was added.

To ensure equal loading, GAPDH rabbit polyclonal IgG primary antibodies, and anti-rabbit IgG-HRP secondary antibodies were used in the same manner described for CD34 and KDR antibodies.

4.2.3.2 Laser-scanning Cytometry

Cells were grown directly on chamber slides to allow quantification of expression of CD34 and KDR with laser scanning cytometry.

Chamber slides were pre-coated with fibronectin as follows: Each well was covered in 160 microlitres of HBSS. 10 microlitres of Human Fibronectin 0.1%

solution was added to the HBSS and the mixture allowed to dry. 1 x $10^5$ cells in 1ml EGM-2 were added to each well, and cells were cultured up to day 7, with a change of media on day 4, in an identical manner to the method described above.

On day 7 non-adherent cells were washed off with PBS. Adherent cells were fixed with methanol 100% for 10 minutes and then rinsed with PBS. Wells were then blocked with 2% BSA in PBS for 15 minutes at room temperature. Primary antibodies used were goat anti-human KDR and mouse anti-human CD34. 200microlitres of each primary antibody was added to designated wells for 30min at 4°C in a humidified chamber. The blocking solution was left on control wells. All wells were then rinsed with PBS and labelled with secondary antibody for 30min at 4°C in a humidified chamber. Secondary antibodies were fluorescein isothiocyanate (FITC) conjugated donkey anti-goat and bovine anti-mouse. Wells were washed with PBS and a 1:1 mix of counter-stain (PI, propidium iodide 1:1000 in PBS) and permeabilising solution (0.1% sodium citrate and 0.1% triton x-100 in PBS) was added to each well for 10 minutes at room temperature in the dark. Wells were washed x3 with PBS, allowed to dry and then chambers were removed from the slides. Fluorescence was measured at 588nm for nucleolus (PI) and 530nm for FITC labelled antibodies and quantified with CompuCyte software (Westwood, MA, USA). Six areas of each slide were analysed, and 3000 cells were counted in each area. Mean FITC expression was calculated and the control wells were compared to those that had been treated with the primary antibodies.

### 4.2.4 Measurement of cardiovascular risk factors

At the same time as sampling for EPC culture, peripheral blood was drawn from the patients after a 12 hour fast. Lipids, glucose, insulin and glycosylated haemoglobin were measured in the local laboratory (Fusion 51 (Johnson & Johnson, USA) and HA/8160 (Menarini Diagnostics, UK)).

Blood in Li-heparin bottles for adiponectin levels was immediately separated by centrifugation for 10 minutes at 1500 rpm 4°C. The plasma supernatant was removed, placed in cryovials using a transfer pipette, and stored at -80°C until assay.

The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was used to estimate steady state beta cell function (%B) and insulin sensitivity (%S) as a percentage of a normal reference population [118]. HOMA-IR was calculated using fasting serum insulin and fasting plasma glucose using the HOMA2 calculator (Diabetes Trials Unit, University of Oxford).

### 4.2.5 Measurement of adiponectin

#### 4.2.5.1 Introduction

The assays were performed at the same time using a commercially available enzyme-linked immunosorbent assay (ELISA), (Millipore, MA, USA). This assay is a Sandwich ELISA based on the following sequence:

1) Concurrent capture of Human Adiponectin molecules from samples to the wells of a microtiter plate coated with a monoclonal anti-human adiponectin antibodies, and binding of a second biotinylated monoclonal anti-human antibody to the captured molecules.

2) Washing of unbound materials from samples.

3) Binding of streptavidin-horseradish peroxidise conjugate to the immobilised biotinylated antibodies.

4) Washing of excess of free enzyme conjugates.

5) Quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine.

The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm – 590nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured Human Adiponectin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of Human Adiponectin.

4.2.5.2 Methods

At the time of delivery, 10ml cord blood was collected into EDTA bottles from attached umbilical cords of fresh placenta by gravity flow. Cord blood was immediately separated by centrifugation for 10 minutes at 1500rpm and the resulting plasma was frozen at -80°C until assay. At the time of the 6 week visit, 2 mls of maternal plasma was immediately separated and stored at -80°C until

assay for Adiponectin. The assays were all performed at the same time, stored plasma samples were allowed to come to room temperature and assays were performed according to the manufacturer's instructions (see Figure 3).

Human adiponectin standard was prepared and serial dilutions were performed, giving 7 concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56 ng/mL). 2 quality control vials were also prepared, and stored samples were allowed to come to room temperature prior to assay, and all assays were performed in duplicate.

The microtitre assay plate was washed 3 times with wash buffer, and residual wash buffer was removed. 60ul assay buffer was added to all wells, and 20ul to blank wells. 20 ul sample (standard, quality control or sample) was then added to appropriate wells. Then 20ul detection antibody was added to all wells and the plate was incubated at room temperature for 2 hours on a plate shaker at 500rpm. Residual solutions were decanted and wells were washed 3 times with 300ul wash buffer. 100ul enzyme solution was then added to each well, the plates were covered and incubated for 30 minutes as before. Solutions were decanted and wells were washed 5 times with 300ul wash buffer. 100ul substrate solution was added to each well and incubated for 5-10 minutes until blue colour was seen forming in the wells. 100ul stop solution was added and plates were shaken to ensure adequate mixing. Any air bubbles were removed and plates were read on a microtitre plate reader at 450nm and 590nm. A standard curve was calculated for each plate with the results from the human adiponectin standard. The results of unknown samples were then calculated using a sigmoidal 5 parameter logistic equation. Quality control samples were used to check the quality control range.

## 4.3 Sample size and statistical analysis

The primary analysis was to compare EPC number and function, adiponectin and conventional cardiovascular risk markers in mothers of AGA and SGA infants. Based on a sample size of 46 participants, the study had sufficient power (80%) to detect a difference in EPC count of at least 25 in AGA and SGA infants at the 5% level of significance.

Continuous variables were described using the median and interquartile range while categorical variables were presented as frequency tabulations. The SGA and AGA groups were compared using non-parametric statistical methods (Fisher's Exact test for binary data, Wilcoxon rank sum test for continuous data, and Spearman's rank correlation coefficient) in order to alleviate the influence of possible non-normality or outlying values. Continuous variables among the three groups were analysed by Jonckheere-Terpstra test for ordered hypothesis.

In order to assess the impact of possible confounding variables, a multivariate analysis was performed using a forward stepwise logistic regression, as implemented in SPSS (using the default 5% level of significance for variable entry and 10% probability for variable removal). The Bonferroni adjustment was used to adjust p-values for multiple comparisons with statistical significance set at the nominal 5% level. The statistical software programme SPSS Version 18 was used for statistical analysis.

# Chapter 5. Results

## 5.1 Confirmation of EPC phenotype and measurement of adiponectin

In our culture conditions all adherent cells at day 7 were dual-positive for diI-acLDL and lectin, therefore staining was not performed routinely. (Figure 4)

### 5.1.1 Western Blot

On GAPDH staining there was equal loading of protein in all three samples at 37kDa. On staining for KDR, protein bands were seen at 150 kDa, increasing in intensity from day 0 to 4 to 7. On staining for CD34, no band was seen using PICO fluorescence, so Femto maximum sensitivity substrate was added and a band was seen at 110kDa on the day 7 sample only. These findings correspond with an increasing expression of KDR as cells progressed through culture, and at day 7 cells expressed both KDR and CD34 (Western Blot Images, figure 5).

### 5.1.2 Laser-scanning Cytometry

We found significantly higher expression of both CD34 and KDR than control in day 7 cells; mean CD34 vs control (141164 vs 118608, $p < 0.01$), mean KDR vs control (348448 vs 186198, $p<0.01$), Table 1. The results confirm dual expression of CD34 and KDR in day 7 EPCs in our culture conditions.

### 5.1.3 Adiponectin ELISA

A standard curve was calculated for each plate with the results from the human adiponectin standard (Figure 6). The results of unknown samples were then

calculated using a sigmoidal 5 parameter logistic equation. Quality control values were confirmed to fall within the quality control range.

5.2 Patient characteristics

There were 23 mothers in the Appropriate for Gestational Age (AGA) group who delivered babies with birth weights between the 10th and 90th centile, and 23 mothers in the Small for Gestational Age (SGA) group with babies under the 10th centile for birth weight. Table 2 details the baseline characteristics for the two groups.

There was a trend towards taller and heavier mothers in the control group but the difference was not statistically significant and there was no difference in BMI. Both maternal ages and paternal ages were marginally lower in the SGA group as compared to the AGA group but not statistically significant. In addition, the SGA and AGA groups had comparable parity and ethnicity.

There were five patients with pregnancy induced hypertension or preeclampsia in the study population (2 vs. 3 patients in the SGA and AGA groups, respectively, p=0.639.) Two patients had a family history of ischaemic heart disease, both appearing in the SGA group but too few to be statistically significant. There were significantly more patients with an abnormal umbilical artery doppler ultrasound in the SGA group compared to the AGA group (4 vs. 0, p=0.038).

There was a borderline non-significant higher median diastolic blood pressure at 30 weeks gestation in the SGA group compared to the control group (70 [68-79] vs. 70 [60-70] mmHg, p = 0.052), and when patients returned for the post-partum visit (table 4) there was no difference in blood pressure between the two groups.

As expected, birth weight was lower in the SGA group compared to AGA group (2.34 [2.13-2.65] vs. 3.6 [3.17-3.85] Kg, p<0.001), and when adjusted for gestational age the median birth centiles in SGA versus AGA groups were 2nd [0.5-3.5] vs. 58th [33.5-76] (p<0.001, Table 3). The rate of caesarean section and the gender of the infant were similar in the two groups.

Table 4 details results of testing at 6 weeks post partum. Fasting triglyceride levels were higher in the SGA group than the control group (0.98 [0.81-1.38] vs 0.78 [0.66-0.91] mmol/l, p=0.006), but there were no other significant differences in the lipid profile or cardiovascular risk markers.

5.3 EPC number and function

Median EPC count was significantly lower (294 [236-338] vs. 367 [331-415], p=0.005), Table 5 and Figures7-9, and EPC migratory capability was reduced (migration index 0.91 [0.86-1] vs. 1.59 [1.22-1.81], p<0.001) in SGA compared to AGA, but there was no statistically significant difference in EPC adhesion (0.221 [0.179-0.296] vs. 0.284 [0.214-0.4] FU, p=0.257). Using spearman's rank correlation coefficient (r) we found moderate correlation between birth centile and EPC number (r=0.493, p=0.001) and EPC migration (r=0.682, p<0.001), but

no correlation with EPC adhesion (r=0.281, p=0.088). Correlations were also seen between birth weight and EPC number and migration, but these were slightly less strong than those seen with birth centile (r=0.486 and 0.638 for EPC number and migration respectively), Figures 10-12. There was a moderate negative correlation between triglycerides and EPC migration (r=-0.475, p=0.001), Figure 13.

## 5.4 Adiponectin

There was no significant difference in maternal serum adiponectin levels between the two groups, however median umbilical cord blood adiponectin was lower in the SGA group (55.2 [47-65] vs. 70.4 [54.2-76.6] ng/ml, p=0.033), Table 6 and Figure 14. Analysis using Spearman's rank correlation coefficient indicated a linear relationship between birth weight and umbilical cord blood adiponectin, with a moderate correlation (r = 0.475, p=0.005), Figure 15. There was a weak borderline non-significant correlation between maternal and cord blood adiponectin (r = 0.34, p=0.049).

## 5.5 Multivariate model

We performed a multivariate analysis to assess possible confounding variables (Age, Parity, Breast feeding, BMI, BP (during and after pregnancy), Glucose, HbA1c, Cholesterol, HDL, LDL, Triglycerides, Adiponectin), Table 7. A stepwise logistic regression analysis showed that only EPC measures (count and migration) remain in the models. Therefore other markers such as triglycerides

and blood pressure are not as strong predictors, nor do they improve the prediction of SGA/AGA status when added to EPC measures.

## 5.6 Physiologically small vs. pathologically small

We aimed to differentiate between infants that were physiologically or constitutionally small and those that had a pathological cause of growth restriction.

The American Society for The American College of Obstetricians and Gynaecologists defines intra-uterine growth restriction (IUGR) as a fetus with an estimated weight below the 10th percentile for gestational age [119]. However not all fetuses weighing less than the $10^{th}$ centile for gestational age are at risk for adverse outcomes, and may just be constitutionally small. One study identified a false positive rate of 74% for the diagnosis of IUGR using $<10^{th}$ centile alone [120] - in other words the majority of those labelled as IUGR may in fact be normal or constitutionally SGA and therefore not at risk of poor fetal or neonatal outcomes. Some groups have suggested using a cut-off of $<3^{rd}$ centile to identify genuine IUGR fetuses [121], and others have suggested the use of growth charts that are customised to physiological pregnancy variables such as maternal height, weight, parity and ethnic group [122].

Then pattern of growth restriction can also give clues as to the significance of any deficiencies identified, and IUGR can be classified as symmetric and

asymmetric. Symmetric growth restriction implies a fetus whose entire body is proportionally small. Asymmetric growth restriction implies a fetus who is undernourished and is directing most of its energy to maintaining growth of vital organs, such as the brain and heart, at the expense of the liver, muscle and fat. This type of growth restriction is usually the result of placental insufficiency [123].

For the purpose of identifying genuine pathologically small babies, the SGA group was further divided into 2 groups: Constitutionally Small for Gestational Age (CSGA) and IUGR. IUGR was defined as having at least 2 out of the following 3 parameters:

- 1. Asymmetric growth or abnormal progression through EFW centile chart
- 2. Abnormal doppler ultrasound of placental artery (absent or reversed end-diastolic flow)
- 3. Histological analysis of placenta consistent with utero-placental insufficiency.

These three parameters were assessed by independent investigators who were blinded to the status of the patients as follows: Anonymised ultrasound doppler images and measurements (performed by midwife ultrasonographers in a single unit) were presented to an independent consultant obstetrician who determined if there was abnormal placental artery blood flow. In a similar way, anonymised growth centile charts were examined for normality by an independent consultant

obstetrician. A consultant pathologist who was blinded to the status of the patient assessed the placentae for features of placental insufficiency.

There were 18 subjects in the CSGA subgroup and 5 in the IUGR subgroup, and the baseline characteristics of these two subgroups compared to the control group are shown in Table 8. Categorical variables are presented as n (%) and continuous variables median [IQR]. The Jonckheere-Terpstra test for ordered hypothesis was used to compare the three groups.

There was no statistically significant difference in BMI, age, paternal age, parity, ethnicity or family history of ischaemic heart disease between groups. There was a significantly higher median diastolic blood pressure at 30 weeks gestation in the IUGR group compared to the AGA and CSGA groups (80 [77-82] vs. 70 [60-70] and 70 [66-72] mmHg respectively, p=0.012), but when patients returned for the post-partum visit there was no difference in blood pressure between the two groups.

Table 9 shows the delivery details of the three groups. Birth weight and birth centile were even lower in the IUGR group compared to the AGA and CSGA groups (Birth weight 2.1 [1-4.75] vs. 3.6 [3.17-3.85] and 2.45 [2.25-2.7] Kg respectively, p<0.001, and birth weight centile <1$^{st}$ [0.1-1] vs. 58$^{th}$ [34-76] and 2$^{nd}$ [2.02-2.16] respectively, p<0.001).

Table 10 details results of testing at 6 weeks post partum. Although the differences were not statistically significant, the IUGR subgroup had higher

insulin levels and were more insulin resistant then the AGA and CSGA groups (Insulin 9.0 [8.8-14.4] vs. 6.3 [5.0-7.8] and 6.9 [5.7-7.8] mU/l respectively, p=0.056, and HOMA-IR 1.2 [1.1-1.8] vs. 0.8 [0.7-1.0] and 0.9 [0.7-1.0] respectively, p=0.053). Similarly, there was a significantly higher triglyceride level (1.6 [1.2-1.8] vs. 0.8 [0.7-0.9] and 0.9[0.8-1.2] mmol/l respectively, p=0.001). There were no other significant differences in the lipid profile or cardiovascular risk markers.

When comparing EPC number and function between the three groups, we found a progressive decline in EPC count from the AGA to CSGA to IUGR groups (367 [331-415] vs. 316 [244-349] vs. 276 [206-297], p=0.003). The IUGR group had similar defects in EPC migration as the CSGA group (Migration coefficients for AGA, CSGA and IUGR: 1.59 [1.22-1.81], 0.90 [0.85-1.0] and 0.91 [0.88-0.99] respectively, p<0.001). There was no significant difference in EPC adhesion between the three groups (Table 11).

There was no significant difference in umbilical cord blood or maternal adiponectin between the three groups (Table 12 and Figure 15).

# Chapter 6. Discussion

## 6.1 EPC number and function

The link between maternal cardiovascular disease and offspring birth weight is a fascinating one, and to date, remains unexplained. EPCs are thought to play a key role in vasculogenesis. Since their discovery in 1997 [5] EPCs have been found to be associated with a wide variety of disease states and are thought to accurately reflect cardiovascular risk [8]. Our study produced a number of interesting results and supports our hypothesis that EPC pathology is an important determinant of deficient placentation leading to low birth weight and increased maternal cardiovascular risk.

We report a 20% decline in EPC number in mothers of babies born small for gestational age compared to a control group of mothers giving birth to normal size babies. The migratory capacity of isolated EPCs was also significantly impaired in the SGA group, with the EPCs from this group showing no migratory response to VEGF, with a net reduction in migration of 68%. Although there was a trend towards a reduced adhesive capacity of the cells in the SGA group, there was no statistically significant decline in the ability of EPCs from the SGA group to adhere to fibronectin *in vitro*.

The decline in EPC number and function which we have demonstrated may account for the expected increase in cardiovascular risk between the two groups studied. The median birth weight difference in our groups was 1260g, and the

median birth weight centile was over 2 standard deviations below normal in our SGA group. The meta-analysis by Davey Smith reported that a 1SD (approx 500g) decline in birth weight is associated with a 25% increase in future maternal cardiovascular mortality [79]. Extrapolating these data to our subjects would suggest a 50% increase in future cardiovascular mortality in our SGA group. Previous studies have shown a 10% reduction in EPC number in smokers [14], a 34% reduction in patients with metabolic syndrome [12], a 69% reduction in type 2 diabetes, [124] and a 47% reduction in haemodialysis patients [125]. The 20% decline in EPC numbers along with the 68% decline in EPC migratory capacity that we discovered in our SGA subjects, therefore, could account for an increased cardiovascular risk approximating that predicted.

There was no significant difference in maternal age, body mass index or family history of cardiovascular disease in the two groups at baseline, and we found no significant difference in maternal fasting glucose, insulin, glycosylated haemoglobin, low and high density lipoprotein or HOMA-IR at 6 weeks post-partum. Fasting triglyceride levels were significantly higher in the SGA group ($p=0.006$), however, and we found a borderline non-significant difference in diastolic blood pressure at 30 weeks gestation, although the latter difference had resolved by the time of the 6 week post-partum visit. When adjusting for multiple comparisons the difference in triglycerides was not statistically significant (Bonferroni p value = 0.06) and therefore the observed difference could be due to a chance finding in our study population.

Hypertriglyceridaemia is an independent risk factor for coronary heart disease, and epidemiological data suggest that a 1mmol/l increase in triglycerides is associated with a relative risk of cardiovascular disease of 1.37 (95% CI, 1.13 to 1.66) [126]. Extrapolating this to our data, the median difference of 0.2mmol/l in triglyceride levels between the SGA and AGA groups could account for a 7.4% difference in cardiovascular risk. Although this cannot be ignored, it is unlikely to be the sole explanation for the predicted increase in cardiovascular risk in mothers of low-birth-weight babies. Similarly, the difference in diastolic blood pressure during pregnancy was not statistically significant, and there was no difference in rates of pre-eclampsia or gestational hypertension between the two groups.

The hazard ratio for future cardiovascular disease in women who have had a maternal placental syndrome (pre-eclampsia, gestational hypertension, placental abruption, or placental infarction) is 2.0 (CI 1.7-2.2) [68]. Therefore a difference in the incidence of these conditions, or indeed a difference in blood pressure could potentially explain a difference in predicted cardiovascular mortality between the two groups. However, the difference in diastolic blood pressure during pregnancy in our groups was not statistically significant, had resolved completely by the time of the 6 week visit, and there were similar rates of maternal placental syndrome and true hypertensive disorders in our 2 groups, making it unlikely that the blood pressure difference found could contribute to an increase in cardiovascular risk.

Hill *et al* [8] found that when compared with subjects with high EPC numbers, those with low EPC numbers had higher triglyceride levels (2.04 +/- 0.41 vs. 1.26 +/- 0.18, p=0.09) and were more likely to have hypertension (53 vs 7% of subjects diagnosed with hypertension, p=0.01). In regression analysis, Vasa *et al* [13] found that hypertension was an independent predictor for impaired EPC migration (p=0.043), but not reduced EPC number (p=0.174). In a similar regression analysis of their data on EPCs in metabolic syndrome, Jialal *et al* [12] found that elevated serum triglyceride predicted reduced circulating EPC number. However, opposite findings were shown in a larger study examining EPC number in 571 subjects, by Xiao *et al* [127]. Subjects with low EPC numbers had lower median serum triglycerides than subjects with high EPC numbers (1.26 (1.03-1.70) vs. 1.40 (1.04-2.04) mmol/l, p=0.001), and after removing subjects that were taking medications the positive correlation remained. The authors also found a surprising positive correlation between Framingham risk score and EPC number. The evidence is clearly conflicting and, to our knowledge, there have been no studies examining EPC number and function in selected patients with hypertriglyceridaemia.

We found a moderate negative correlation between serum triglycerides and EPC migration (r=-0.475) in our cohort, however after adjustment for triglycerides and blood pressure by stepwise multivariate logistic regression analysis, EPC migration remains a strongly significant predictor of SGA/AGA status (p<0.001). Therefore triglycerides could explain some of the EPC differences, but the adjustment for multiple comparisons and adjustment in logistic regression imply

that they do not explain all of the differences in EPC number or function, or predicted future cardiovascular risk between the two groups.

The higher number of subjects with abnormal umbilical cord blood flow on ultrasound doppler in the SGA group supports our contention that the defects in EPC number and migratory ability which we have identified in our SGA group may be responsible for deficient development of the placental vasculature leading to low birthweight. Larger studies will be required to determine if mothers with abnormal umbilical artery blood flow have reduced EPC number or function and a greater future cardiovascular risk than those with normal ultrasound doppler.

## 6.2 IUGR vs. SGA

We had aimed to recruit 20 subjects in each group, but despite 18 months of active recruiting, we only managed to recruit 5 non-smoking healthy mothers of IUGR babies that did not have growth restriction due to disease or chromosomal abnormality. However, the analysis of the IUGR group revealed some interesting findings, despite the small numbers.

There was a trend toward being younger and having a lower BMI, although the differences did not reach statistical significance. Despite this, the IUGR subgroup appears to have a much higher cardiovascular risk, in that diastolic blood pressure during pregnancy was significantly higher, and there was a non-significant higher blood pressure at the follow up visit. The difference in

triglycerides observed between the original AGA and SGA groups was now more pronounced in the IUGR group vs. the AGA group.
Again, despite the fact that the IUGR group trended to be younger and thinner, we found a trend towards higher levels in insulin resistance and LDL cholesterol, although the differences did not reach statistical significance. The lack of statistical significance observed may be due to our small sample size.

The mothers in the IUGR subgroup delivered even smaller babies even earlier than both the AGA and CSGA groups. As regards EPC number and function between the three groups, we found a progressive step-wise decline in EPC count from the AGA to CSGA to IUGR groups, the IUGR had similar defects in migration as the CSGA group, and there were no differences in adhesion. As with the differences in blood pressure and triglycerides, the differences in EPC number and migratory ability are interesting. Given the fact that the IUGR group trended to be younger with lower BMIs, if anything one could have postulated that they would have improved EPC number and function.

The IUGR group by definition were growth restricted due to placental insufficiency, and the fact that we found reduced EPC number and function in this group supports our hypothesis that EPCs may play a role in reduced placental angiogenesis and vascularisation, and furthermore that EPC pathology may be linked to the increase in future maternal cardiovascular risk.

## 6.3 Significance of our findings

In their review article, Sipos *et al* [80] consider the role of the EPC in intra-uterine growth restriction and future cardiovascular risk in the offspring. They postulate that anomalies in either EPC number or EPC recruitment to the early placenta may impair formation of normal placental vessels and influence the embryonic endothelium. They also postulate that EPC functional irregularities could impact on the maternal side, affecting uterine vessels, restricting or attenuating intrauterine blood flow and thus perpetuating placental disease. These functional irregularities (namely EPC number, mobilisation and recruitment) could possibly result from hypoxia or oxidative stress, and the authors comment that given these possibilities, further research is warranted. We have shown a defect in both EPC number and migratory capacity, with no difference in EPC adhesion. These EPC anomalies may explain the increase in predicted cardiovascular risk in not only the child, but also in the mother.

When one considers the maternal long term implications after maternal placental syndrome, IUGR and gestational diabetes, it becomes clear that pregnancy represents a unique opportunity to identify women who may be at increased risk of chronic diseases later in life [128]. After identification of one of these conditions in pregnancy, clinicians should not miss the opportunity to screen for cardiovascular risk factors, and to institute lifestyle changes or pharmacological intervention to prevent complications in the future. Manten *et al* [129] followed up women with a history of IUGR (defined as a birth weight $<5^{th}$ centile and a delivery because of signs of fetal distress before 34 weeks of gestation). They

found that women with a history of IUGR had higher concentrations of cholesterol and showed a tendency to higher BMI, higher triglyceride concentrations, and increased insulin resistance as compared with women with a history of normal pregnancy. We found similar findings in both our SGA and IUGR groups. Therefore, irrespective of our findings in relation to EPCs, our study has highlighted the presence of hypertension and dyslipidaemia in mothers of both SGA and IUGR infants. Both of these are modifiable risk factors that increase the risk of future cardiovascular morbidity and mortality.

## 6.4 Adiponectin

Adiponectin is the most abundant hormone released by adipocytes, and is thought to couple regulation of insulin sensitivity with energy metabolism[109]. It has anti-inflammatory, anti-apoptotic and anti-atherogenic properties [93-95]. Some studies have suggested that adiponectin may provide the link between obesity, type 2 diabetes, insulin resistance and cardiovascular disease [82, 88, 89]. High levels are associated with favourable lipid and glucose metabolism, and a reduced risk of cardiovascular events in women [106].

Altered maternal adiponectin levels have been found in women with gestational diabetes [110, 111] and preeclampsia [112]. Children who were born SGA have been shown to have lower serum adiponectin and levels are even lower in those with postnatal catch-up growth [130]. Results of cord blood adiponectin levels have been varying, with some authors reporting lower levels in SGA fetuses (defined as >1.5 SD below normal) [131], and others reporting no difference in

SGA (defined as <2500g) [132]. Mazaki-Tovi *et al* [113] performed an elegant study measuring cord blood in normal and discordant growth restricted (<10$^{th}$ centile with abnormal umbilical cord doppler ulstrasound) twins. They found that cord blood adiponectin was independently associated with birth weight, with decreased levels characterising growth-restricted fetuses [113]. The nature of the twin study eliminated confounding maternal factors and gave weight to the argument that the lower cord blood adiponectin levels were due to abnormal placentation and not attributable to genetic disparity. The authors postulate that the observed reduced adiponectin levels in SGA neonates could be due to decreased fetal adipose tissue depots and increased fetal exposure to hypoxia, both of which are features of growth restriction.

We found a significantly lower umbilical cord blood adiponectin in our SGA group, with a moderate correlation between birth weight and adiponectin in all neonates (r=0.475), Figure 2. There was no difference in maternal adiponectin levels between the groups, and there was only a weak correlation between maternal and cord blood adiponectin (r=0.34) This confirms the findings of previous studies, and provides further evidence that fetal adiponectin levels are unlikely to be related to maternal levels.

When analysed in the three groups of AGA, CSGA and IUGR, the differences cease to have statistical significance, Table 12 and Figure 14. Although the numbers in the IUGR subgroup are small, and the differences are not statistically significant, the adiponectin levels appear to increase to a near normal level, leading to the possibility that levels may in fact be up-regulated in IUGR.

However, unlike the other studies examining cord blood adiponectin in SGA fetuses mentioned above, the discordant twin study that found a lower level did examine pathologically small fetuses with abnormal umbilical cord blood flow. Therefore the differences observed in our subgroup may be due to our small sample size.

In summary, adiponectin has an important role in the regulation of major metabolic pathways and fetal growth, and the data from our two groups provides additional evidence to the hypothesis that it may form part of the link between low birth weight and future metabolic and cardiovascular complications in later life.

## 6.5 Conclusion

EPCs are crucial to post-natal vasculogenesis and intimately associated with cardiovascular risk. Placental insufficiency as a result of deficient placental vascularisation may be responsible for maternal placental syndromes including low birth weight babies. In this thesis I tested the hypothesis that reduced maternal EPC number and/or function may play a role in the processes that lead to defective placental development and hence low birthweight in babies, and may also provide a unifying explanation for the associated future increased risk of cardiovascular disease in mothers of low birthweight babies.

I aimed to investigate the link between EPCs, delivery of a low birthweight baby, and risk of future cardiovascular disease in the mother by measuring EPC

number and function, conventional cardiovascular risk markers, and levels of the adipokine adiponectin in mothers of SGA and AGA infants. In addition, maternal and cord blood adiponectin measurements were used to further investigate the role of adiponectin in birthweight and cardiovascular disease.

I found that giving birth to an SGA infant was associated with lower maternal EPC number and reduced EPC migratory function *in vitro*, and that the IUGR subgroup had even lower EPC number and similar defects in EPC migration. These results support the hypothesis that EPC pathology may represent the hitherto elusive link between utero-placental insufficiency (leading to low birth weight) and future risk of cardiovascular disease in the mother.

I also report significantly lower cord blood adiponectin levels in SGA infants compared to normal birth weight infants, and a correlation between cord blood adiponectin and birth weight, confirming previous reports and potentially representing a further mechanism for future cardiovascular disease and insulin resistance in later life of the infant.

## Chapter 7. References


1. Ramcharan, K.S., et al., *The endotheliome: A new concept in vascular biology.* Thromb Res, 2010.
2. Wu, K.K. and P. Thiagarajan, *Role of endothelium in thrombosis and hemostasis.* Annu Rev Med, 1996. **47**: p. 315-31.
3. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-4.
4. Scott, S.M., et al., *The role of circulating cells in the healing of vascular prostheses.* J Vasc Surg, 1994. **19**(4): p. 585-93.
5. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.
6. Khan, S.S., M.A. Solomon, and J.P. McCoy, Jr., *Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry.* Cytometry B Clin Cytom, 2005. **64**(1): p. 1-8.
7. Fadini, G.P., et al., *Technical notes on endothelial progenitor cells: ways to escape from the knowledge plateau.* Atherosclerosis, 2008. **197**(2): p. 496-503.
8. Hill, J.M., et al., *Circulating endothelial progenitor cells, vascular function, and cardiovascular risk.* N Engl J Med, 2003. **348**(7): p. 593-600.
9. Dimmeler, S., et al., *HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway.* J Clin Invest, 2001. **108**(3): p. 391-7.

10. Sieveking, D.P., et al., *Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay.* J Am Coll Cardiol, 2008. **51**(6): p. 660-8.

11. Schlager, O., et al., *Exercise training increases endothelial progenitor cells and decreases asymmetric dimethylarginine in peripheral arterial disease: A randomized controlled trial.* Atherosclerosis, 2011.

12. Jialal, I., et al., *Decreased number and impaired functionality of endothelial progenitor cells in subjects with metabolic syndrome: implications for increased cardiovascular risk.* Atherosclerosis, 2010. **211**(1): p. 297-302.

13. Vasa, M., et al., *Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease.* Circ Res, 2001. **89**(1): p. E1-7.

14. Yue, W.S., et al., *Smoking is associated with depletion of circulating endothelial progenitor cells and elevated pulmonary artery systolic pressure in patients with coronary artery disease.* Am J Cardiol, 2010. **106**(9): p. 1248-54.

15. Schmidt-Lucke, C., et al., *Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair.* Circulation, 2005. **111**(22): p. 2981-7.

16. Werner, N., et al., *Circulating endothelial progenitor cells and cardiovascular outcomes.* N Engl J Med, 2005. **353**(10): p. 999-1007.

17. Chen, M., et al., *Complications impaired endothelial progenitor cell function in Type 2 diabetic patients with or without critical leg*

*ischaemia: implication for impaired neovascularization in diabetes.* Diabet Med, 2009. **26**(2): p. 134-41.

18. Heida, N.M., et al., *Effects of obesity and weight loss on the functional properties of early outgrowth endothelial progenitor cells.* J Am Coll Cardiol, 2010. **55**(4): p. 357-67.
19. Quist-Paulsen, P., *Statins and inflammation: an update.* Curr Opin Cardiol, 2010. **25**(4): p. 399-405.
20. Llevadot, J., et al., *HMG-CoA reductase inhibitor mobilizes bone marrow--derived endothelial progenitor cells.* J Clin Invest, 2001. **108**(3): p. 399-405.
21. Tousoulis, D., et al., *Effects of rosuvastatin and allopurinol on circulating endothelial progenitor cells in patients with congestive heart failure: the impact of inflammatory process and oxidative stress.* Atherosclerosis, 2011. **214**(1): p. 151-7.
22. Liao, Y.F., et al., *Number of circulating endothelial progenitor cells as a marker of vascular endothelial function for type 2 diabetes.* Vasc Med, 2010. **15**(4): p. 279-85.
23. Chen, L.L., et al., *Effects of gliclazide on endothelial function in patients with newly diagnosed type 2 diabetes.* Eur J Pharmacol, 2011. **659**(2-3): p. 296-301.
24. Cacciatore, F., et al., *Effects of ACE inhibition on circulating endothelial progenitor cells, vascular damage, and oxidative stress in hypertensive patients.* Eur J Clin Pharmacol, 2011.
25. Razvi, S., et al., *The beneficial effect of L-thyroxine on cardiovascular risk factors, endothelial function, and quality of life in subclinical*

*hypothyroidism: randomized, crossover trial.* J Clin Endocrinol Metab, 2007. **92**(5): p. 1715-23.

26. Hak, A.E., et al., *Subclinical hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women: the Rotterdam Study.* Ann Intern Med, 2000. **132**(4): p. 270-8.

27. Shakoor, S.K., et al., *Endothelial progenitor cells in subclinical hypothyroidism: the effect of thyroid hormone replacement therapy.* J Clin Endocrinol Metab, 2010. **95**(1): p. 319-22.

28. Kalka, C., et al., *Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3422-7.

29. Murayama, T., et al., *Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo.* Exp Hematol, 2002. **30**(8): p. 967-72.

30. Kawamoto, A., et al., *Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia.* Circulation, 2001. **103**(5): p. 634-7.

31. Aicher, A., et al., *Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling.* Circulation, 2003. **107**(16): p. 2134-9.

32. Assmus, B., et al., *Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI).* Circulation, 2002. **106**(24): p. 3009-17.

33. Kudo, F.A., et al., *Autologous transplantation of peripheral blood endothelial progenitor cells (CD34+) for therapeutic angiogenesis in patients with critical limb ischemia.* Int Angiol, 2003. **22**(4): p. 344-8.

34. Erbs, S., et al., *Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study.* Circ Res, 2005. **97**(8): p. 756-62.

35. Nakahata, T. and M. Ogawa, *Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors.* J Clin Invest, 1982. **70**(6): p. 1324-8.

36. Murohara, T., et al., *Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization.* J Clin Invest, 2000. **105**(11): p. 1527-36.

37. Ingram, D.A., et al., *Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood.* Blood, 2004. **104**(9): p. 2752-60.

38. Knopp, R.H., *Cardiovascular effects of endogenous and exogenous sex hormones over a woman's lifetime.* Am J Obstet Gynecol, 1988. **158**(6 Pt 2): p. 1630-43.

39. Mendelsohn, M.E. and R.H. Karas, *The protective effects of estrogen on the cardiovascular system.* N Engl J Med, 1999. **340**(23): p. 1801-11.

40. Iwakura, A., et al., *Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury.* Circulation, 2003. **108**(25): p. 3115-21.

41. Masuda, H., et al., *Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis.* Circ Res, 2007. **101**(6): p. 598-606.

42. Sugawara, J., et al., *Circulating endothelial progenitor cells during human pregnancy.* J Clin Endocrinol Metab, 2005. **90**(3): p. 1845-8.

43. Yoshida, A., et al., *Flow-mediated vasodilation and plasma fibronectin levels in preeclampsia.* Hypertension, 2000. **36**(3): p. 400-4.

44. Sugawara, J., et al., *Decrease and senescence of endothelial progenitor cells in patients with preeclampsia.* J Clin Endocrinol Metab, 2005. **90**(9): p. 5329-32.

45. Matsubara, K., et al., *Circulating endothelial progenitor cells during normal pregnancy and pre-eclampsia.* Am J Reprod Immunol, 2006. **56**(2): p. 79-85.

46. Hwang, H.S., et al., *Increased senescence and reduced functional ability of fetal endothelial progenitor cells in pregnancies complicated by preeclampsia without intrauterine growth restriction.* Am J Obstet Gynecol, 2008. **199**(3): p. 259 e1-7.

47. Kwon, J.Y., et al., *Decreased endothelial progenitor cells in umbilical cord blood in severe preeclampsia.* Gynecol Obstet Invest, 2007. **64**(2): p. 103-8.

48. Bailey, B.A., et al., *Infant Birth Outcomes Among Substance Using Women: Why Quitting Smoking During Pregnancy is Just as Important as Quitting Illicit Drug Use.* Matern Child Health J, 2011.

49. de, B.M.J., et al., *Quantitative effects of tobacco smoking exposure on the maternal-fetal circulation.* BMC Pregnancy Childbirth, 2011. **11**: p. 24.

50. Michaud, S.E., et al., *Circulating endothelial progenitor cells from healthy smokers exhibit impaired functional activities.* Atherosclerosis, 2006. **187**(2): p. 423-32.

51. Heiss, C., et al., *Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production.* J Am Coll Cardiol, 2008. **51**(18): p. 1760-71.

52. Stoz, F., R.A. Schuhmann, and A. Schmid, *Morphometric investigations of terminal villi of diabetic placentas in relation to the White classification of diabetes mellitus.* J Perinat Med, 1987. **15**(2): p. 193-8.

53. Liew, A., et al., *Endothelial progenitor cells for the treatment of diabetic vasculopathy: panacea or Pandora's box?* Diabetes Obes Metab, 2008. **10**(5): p. 353-66.

54. Tepper, O., et al., *Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures.* Circulation, 2002. **106**(22): p. 2781-6.

55. Huppertz, B. and L.L. Peeters, *Vascular biology in implantation and placentation.* Angiogenesis, 2005. **8**(2): p. 157-67.

56. Clark, D.E., et al., *Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy.* Hum Reprod, 1996. **11**(5): p. 1090-8.

57. Shalaby, F., et al., *Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice.* Nature, 1995. **376**(6535): p. 62-6.

58. Pijnenborg, R., L. Vercruysse, and M. Hanssens, *The uterine spiral arteries in human pregnancy: facts and controversies.* Placenta, 2006. **27**(9-10): p. 939-58.

59. Kaufmann, P., S. Black, and B. Huppertz, *Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia.* Biol Reprod, 2003. **69**(1): p. 1-7.

60. Craven, C.M., T. Morgan, and K. Ward, *Decidual spiral artery remodelling begins before cellular interaction with cytotrophoblasts.* Placenta, 1998. **19**(4): p. 241-52.

61. *ACOG practice bulletin. Antepartum fetal surveillance. Number 9, October 1999 (replaces Technical Bulletin Number 188, January 1994). Clinical management guidelines for obstetrician-gynecologists.* Int J Gynaecol Obstet, 2000. **68**(2): p. 175-85.

62. Roberts, D.J. and M.D. Post, *The placenta in pre-eclampsia and intrauterine growth restriction.* J Clin Pathol, 2008. **61**(12): p. 1254-60.

63. Redman, C.W., G.P. Sacks, and I.L. Sargent, *Preeclampsia: an excessive maternal inflammatory response to pregnancy.* Am J Obstet Gynecol, 1999. **180**(2 Pt 1): p. 499-506.

64. Browne, J.C. and N. Veall, *The maternal placental blood flow in normotensive and hypertensive women.* J Obstet Gynaecol Br Emp, 1953. **60**(2): p. 141-7.

65. Lam, C., K.H. Lim, and S.A. Karumanchi, *Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia.* Hypertension, 2005. **46**(5): p. 1077-85.

66. Meekins, J.W., et al., *A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies.* Br J Obstet Gynaecol, 1994. **101**(8): p. 669-74.

67. Granger, J.P., et al., *Pathophysiology of hypertension during preeclampsia linking placental ischemia with endothelial dysfunction.* Hypertension, 2001. **38**(3 Pt 2): p. 718-22.

68. Ray, J.G., et al., *Cardiovascular health after maternal placental syndromes (CHAMPS): population-based retrospective cohort study.* Lancet, 2005. **366**(9499): p. 1797-803.

69. Kramer, M.S., *Determinants of low birth weight: methodological assessment and meta-analysis.* Bull World Health Organ, 1987. **65**(5): p. 663-737.

70. Barker, D.J., et al., *Weight in infancy and death from ischaemic heart disease.* Lancet, 1989. **2**(8663): p. 577-80.

71. Osmond, C., et al., *Early growth and death from cardiovascular disease in women.* BMJ, 1993. **307**(6918): p. 1519-24.

72. Valdez, R., et al., *Birthweight and adult health outcomes in a biethnic population in the USA.* Diabetologia, 1994. **37**(6): p. 624-31.

73. Barker, D.J. and C. Osmond, *Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales.* Lancet, 1986. **1**(8489): p. 1077-81.

74. Barker, D.J., *Fetal origins of coronary heart disease.* BMJ, 1995. **311**(6998): p. 171-4.

75. Hattersley, A.T. and J.E. Tooke, *The fetal insulin hypothesis: an alternative explanation of the association of low birthweight with diabetes and vascular disease.* Lancet, 1999. **353**(9166): p. 1789-92.

76. Barker, D.J., *The origins of the developmental origins theory.* J Intern Med, 2007. **261**(5): p. 412-7.

77. Smith, G.D., et al., *Birth dimensions of offspring, premature birth, and the mortality of mothers.* Lancet, 2000. **356**(9247): p. 2066-7.

78. Davey Smith, G., et al., *Birth weight of offspring and mortality in the Renfrew and Paisley study: prospective observational study.* BMJ, 1997. **315**(7117): p. 1189-93.

79. Davey Smith, G., et al., *Offspring birth weight and parental mortality: prospective observational study and meta-analysis.* Am J Epidemiol, 2007. **166**(2): p. 160-9.

80. Sipos, P.I., et al., *Endothelial progenitor cells: their potential in the placental vasculature and related complications.* Placenta, 2010. **31**(1): p. 1-10.

81. Nakano, Y., et al., *Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma.* J Biochem, 1996. **120**(4): p. 803-12.

82. Chang, L.C., et al., *The clinical implications of blood adiponectin in cardiometabolic disorders.* J Formos Med Assoc, 2009. **108**(5): p. 353-66.

83. Han, S.H., et al., *Adiponectin and cardiovascular disease: response to therapeutic interventions.* J Am Coll Cardiol, 2007. **49**(5): p. 531-8.

84. Arita, Y., et al., *Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity.* Biochem Biophys Res Commun, 1999. **257**(1): p. 79-83.

85. Antoniades, C., et al., *Adiponectin: from obesity to cardiovascular disease.* Obes Rev, 2009. **10**(3): p. 269-79.

86. Spranger, J., et al., *Adiponectin and protection against type 2 diabetes mellitus.* Lancet, 2003. **361**(9353): p. 226-8.

87. Duncan, B.B., et al., *Adiponectin and the development of type 2 diabetes: the atherosclerosis risk in communities study.* Diabetes, 2004. **53**(9): p. 2473-8.

88. Santaniemi, M., Y.A. Kesaniemi, and O. Ukkola, *Low plasma adiponectin concentration is an indicator of the metabolic syndrome.* Eur J Endocrinol, 2006. **155**(5): p. 745-50.

89. Berg, A.H., et al., *The adipocyte-secreted protein Acrp30 enhances hepatic insulin action.* Nat Med, 2001. **7**(8): p. 947-53.

90. Yamauchi, T., et al., *Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis.* J Biol Chem, 2003. **278**(4): p. 2461-8.

91. Hara, K., et al., *Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population.* Diabetes, 2002. **51**(2): p. 536-40.

92. Rothenbacher, D., et al., *Adiponectin, risk of coronary heart disease and correlations with cardiovascular risk markers.* Eur Heart J, 2005. **26**(16): p. 1640-6.

93. Motoshima, H., et al., *Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL.* Biochem Biophys Res Commun, 2004. **315**(2): p. 264-71.

94. Kobayashi, H., et al., *Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin.* Circ Res, 2004. **94**(4): p. e27-31.

95. Ouchi, N., et al., *Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages.* Circulation, 2001. **103**(8): p. 1057-63.

96. Okamoto, Y., et al., *Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice.* Circulation, 2002. **106**(22): p. 2767-70.

97. Arita, Y., et al., *Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell.* Circulation, 2002. **105**(24): p. 2893-8.

98. Kumada, M., et al., *Association of hypoadiponectinemia with coronary artery disease in men.* Arterioscler Thromb Vasc Biol, 2003. **23**(1): p. 85-9.

99. Pischon, T., et al., *Plasma adiponectin levels and risk of myocardial infarction in men.* JAMA, 2004. **291**(14): p. 1730-7.

100. Wolk, R., et al., *Association between plasma adiponectin levels and unstable coronary syndromes.* Eur Heart J, 2007. **28**(3): p. 292-8.

101. Frystyk, J., et al., *Serum adiponectin is a predictor of coronary heart disease: a population-based 10-year follow-up study in elderly men.* J Clin Endocrinol Metab, 2007. **92**(2): p. 571-6.

102. Iglseder, B., et al., *Plasma adiponectin levels and sonographic phenotypes of subclinical carotid artery atherosclerosis: data from the SAPHIR Study.* Stroke, 2005. **36**(12): p. 2577-82.

103. Schulze, M.B., et al., *Adiponectin and future coronary heart disease events among men with type 2 diabetes.* Diabetes, 2005. **54**(2): p. 534-9.

104. Celi, F., et al., *Circulating adipocytokines in non-diabetic and Type 1 diabetic children: relationship to insulin therapy, glycaemic control and pubertal development.* Diabet Med, 2006. **23**(6): p. 660-5.

105. Saraheimo, M., et al., *Serum adiponectin and progression of diabetic nephropathy in patients with type 1 diabetes.* Diabetes Care, 2008. **31**(6): p. 1165-9.

106. Dekker, J.M., et al., *Prognostic value of adiponectin for cardiovascular disease and mortality.* J Clin Endocrinol Metab, 2008. **93**(4): p. 1489-96.

107. Teoh, H., et al., *Adiponectin and myocardial infarction: A paradox or a paradigm?* Eur Heart J, 2006. **27**(19): p. 2266-8.

108. Rathmann, W. and C. Herder, *Adiponectin and cardiovascular mortality: evidence for "reverse epidemiology".* Horm Metab Res, 2007. **39**(1): p. 1-2.

109. Kadowaki, T., et al., *Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome.* J Clin Invest, 2006. **116**(7): p. 1784-92.

110. Ategbo, J.M., et al., *Modulation of adipokines and cytokines in gestational diabetes and macrosomia.* J Clin Endocrinol Metab, 2006. **91**(10): p. 4137-43.

111. Lowe, L.P., et al., *Inflammatory mediators and glucose in pregnancy: results from a subset of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study.* J Clin Endocrinol Metab, 2010. **95**(12): p. 5427-34.

112. Masuyama, H., et al., *Different profiles of circulating angiogenic factors and adipocytokines between early- and late-onset pre-eclampsia.* BJOG, 2010. **117**(3): p. 314-20.

113. Mazaki-Tovi, S., et al., *Adiponectin and leptin concentrations in dichorionic twins with discordant and concordant growth.* J Clin Endocrinol Metab, 2009. **94**(3): p. 892-8.

114. Hadlock, F., R. Harrist, and J. Martinez-Poyer, *In utero analysis of fetal growth: a sonographic weight standard.* Radiology, 1991. **181**(1): p. 129-33.

115. *Standards of medical care in diabetes--2010.* Diabetes Care, 2010. **33 Suppl 1**: p. S11-61.

116. Choi, J., et al., *Decreased number and impaired angiogenic function of endothelial progenitor cells in patients with chronic renal failure.* Arterioscler Thromb Vasc Biol, 2004. **24**(7): p. 1246-52.

117. Soncin, S., et al., *A practical approach for the validation of sterility, endotoxin and potency testing of bone marrow mononucleated cells used in cardiac regeneration in compliance with good manufacturing practice.* Journal of Translational Medicine, 2009. **7**(1): p. 78.

118. Wallace, T.M., J.C. Levy, and D.R. Matthews, *Use and abuse of HOMA modeling.* Diabetes Care, 2004. **27**(6): p. 1487-95.

119. *American College of Obstetricians and Gynecologists, Intrauterine growth restriction: ACOG Practice Bulletin no. 12*, in *American College of Obstetricians and Gynecologists, Washington DC* 2000.

120. Manning, F.A., L.M. Hill, and L.D. Platt, *Qualitative amniotic fluid volume determination by ultrasound: antepartum detection of intrauterine growth retardation.* Am J Obstet Gynecol, 1981. **139**(3): p. 254-8.

121. Platz, E. and R. Newman, *Diagnosis of IUGR: traditional biometry.* Semin Perinatol, 2008. **32**(3): p. 140-7.

122. Gardosi J, F.A., *Customised Weight Centile Calculator – GROW-Centile v.5.12*, G. Network, Editor. 2009.

123. Peleg, D., C.M. Kennedy, and S.K. Hunter, *Intrauterine growth restriction: identification and management.* Am Fam Physician, 1998. **58**(2): p. 453-60, 466-7.

124. Fadini, G.P., et al., *Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus.* J Am Coll Cardiol, 2005. **45**(9): p. 1449-57.

125. Jie, K.E., et al., *Reduced endothelial progenitor cells in children with hemodialysis but not predialysis chronic kidney disease.* Pediatrics, 2010. **126**(4): p. e990-3.

126. Hokanson, J.E. and M.A. Austin, *Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein*

*cholesterol level: a meta-analysis of population-based prospective studies.* J Cardiovasc Risk, 1996. **3**(2): p. 213-9.

127. Xiao, Q., et al., *Endothelial progenitor cells, cardiovascular risk factors, cytokine levels and atherosclerosis--results from a large population-based study.* PLoS One, 2007. **2**(10): p. e975.

128. Saade, G.R., *Pregnancy as a window to future health.* Obstet Gynecol, 2009. **114**(5): p. 958-60.

129. Manten, G.T., et al., *Risk factors for cardiovascular disease in women with a history of pregnancy complicated by preeclampsia or intrauterine growth restriction.* Hypertens Pregnancy, 2007. **26**(1): p. 39-50.

130. Cianfarani, S., et al., *Adiponectin levels are reduced in children born small for gestational age and are inversely related to postnatal catch-up growth.* J Clin Endocrinol Metab, 2004. **89**(3): p. 1346-51.

131. Takaya, J., et al., *Intracellular magnesium and adipokines in umbilical cord plasma and infant birth size.* Pediatr Res, 2007. **62**(6): p. 700-3.

132. Martinez-Cordero, C., et al., *Body fat at birth and cord blood levels of insulin, adiponectin, leptin, and insulin-like growth factor-I in small-for-gestational-age infants.* Arch Med Res, 2006. **37**(4): p. 490-4.

133. Barker, D.J., *Fetal nutrition and cardiovascular disease in later life.* Br Med Bull, 1997. **53**(1): p. 96-108.

Appendix 1: Patient Information Sheet

RCSI

The
Rotunda Hospital
DUBLIN

**Patient Information Leaflet:**
**Endothelial Progenitor Cells in Mothers of Low-birth-weight babies**

The blood vessels in the placenta (after-birth) supply nutrition to the growing baby in the womb. In some cases, when babies are born small-for-dates, it can be as a result of reduced formation of these blood vessels. Endothelial progenitor cells (EPCs) are cells which circulate in the blood and are responsible for forming new blood vessels in adults.

We plan to measure numbers of EPCs in the blood of mothers of small-for-dates babies, and also numbers of EPCs in umbilical cord blood, to see if a low EPC number may influence the formation of blood vessels in the placenta. We also hope to measure cholesterol, blood sugar level, hormone levels such as adiponectin, and blood pressure, to further evaluate the link between all of these factors and blood vessel formation. We hope that the results of the study will improve our understanding of the reasons for low-birth weight babies and may allow for development of EPC-based treatment in the future to prevent this condition. In addition to mothers of small-for-dates babies, we also need to study mothers of normal birth weight babies (the control group) in order to compare the numbers of EPCs between the two. We therefore plan to perform all of the above tests on both groups of mothers and babies.

If you agree to take part in our study, we will perform a detailed ultrasound scan of the placenta and placental blood vessels. At delivery a sample of blood will be drawn from the umbilical cord. This cord blood shall be analysed for EPC number and adiponectin level (a hormone that is associated with low birth weight), and shall be discarded after the analysis is complete. We will arrange to perform a brief physical examination (checking blood pressure, height and weight) at your post-natal visit, and then draw some blood for testing. All of this should not take much more than 20 minutes. All of your details will be kept confidential. There will be no need for any further visits apart from your usual hospital visits.

Participation in this study is entirely voluntary and you may withdraw at any time, without giving reason, and without this decision effecting any future treatment or medical care.

**Our responsibilities as investigators:**
If you experience any ill effects as the result of your participation, then we will take care of your medical needs. If we discover any medical problems such as high blood pressure or cholesterol, this would be discussed with you in detail, and you would be offered appropriate treatment and follow up in a specialist clinic or with your GP. However we will not be in a position to pay for any prescription medication, should it be required.

If there are any new findings during the course of the study that may affect the validity of the study or your participation in it, then the findings will be made available to you and you will receive the appropriate medical care and advice depending on the test results.

**Confidentiality:**
Only anonymous data, ie. data that do not identify you by name, will be collected. Only authorised representatives of the study team will have direct access to your personal medical records. All study team representatives and medical staff are obliged to maintain confidentiality at all times. The data shall be destroyed by shredding and deletion of computer files at the end of the study

**For additional information now or any future time please contact:**

Dr. Thomas King, Research Registrar
Connolly Hospital, Blanchardstown, Dublin 15. Phone: 01 646 5756

Dr. John McDermott, Consultant Endocrinologist
Connolly Hospital, Blanchardstown, Dublin 15. Phone: 01 646 5758

Prof. Fergal Malone, Consultant in Obstetrics and Gynaecology
RCSI Unit, Rotunda Hospital, Dublin 1. Phone: 01 878 6070

14th April 2009

Appendix 2: Consent Form

RCSI

The
Rotunda Hospital
DUBLIN
the maternity hospital of choice

**CONSENT FORM**

**Protocol Title:** Endothelial Progenitor Cells in Mothers of Low-birth-weight babies

**Please tick the appropriate answer.**

- I consent to take part in this study. ☐**Yes** ☐**No**

- I confirm that I have read and understood the Patient Information Leaflet attached (version 2, 14/4/9), and that I have had ample opportunity to ask questions all of which have been satisfactorily answered.
  ☐**Yes** ☐**No**

- I have been given a copy of the Patient Information Leaflet and this Consent form for my records.
  ☐**Yes** ☐**No**

- I understand that my participation in this study is entirely **voluntary** and that I may withdraw at any time, without giving reason, and without this decision affecting my future treatment or medical care.
  ☐**Yes** ☐**No**

- I understand that my medical records may be viewed by members of the research team
  ☐**Yes** ☐**No**

- I understand that my identity will remain confidential at all times.
  ☐**Yes** ☐**No**
- I am aware of the potential risks of this study.
  ☐**Yes** ☐**No**
- I consent for umbilical cord blood to be taken at the time of delivery, and for blood tests (from myself) at my six week post-natal visit.
  ☐**Yes** ☐**No**

- I agree that I will not restrict the use to which the results of this study may be put. I give my approval that anonymous data concerning my person may be stored or electronically processed for the purpose of scientific research and may be used in related or other studies in the future. (This would be subject to approval by an independent body which safeguards the welfare and rights of people in biomedical research studies - the Rotunda Hospital Ethics (Medical Research) Committee.)

  **☐Yes ☐No**

- I consent for further follow-up studies

  **☐Yes ☐No**

Patient _____

_____

Signature and dated Name in block capitals

**To be completed by the Principal Investigator or his nominee.**

I the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a manner that he/she could understand. I have explained the risks involved, as well as the possible benefits and have invited him/her to ask questions on any aspect of the study.

_____

Signature, qualifications and date Name in block capitals

**Figure 1: Manufacturer's illustration of Matrigel insert (Modified Boyden Chamber)**

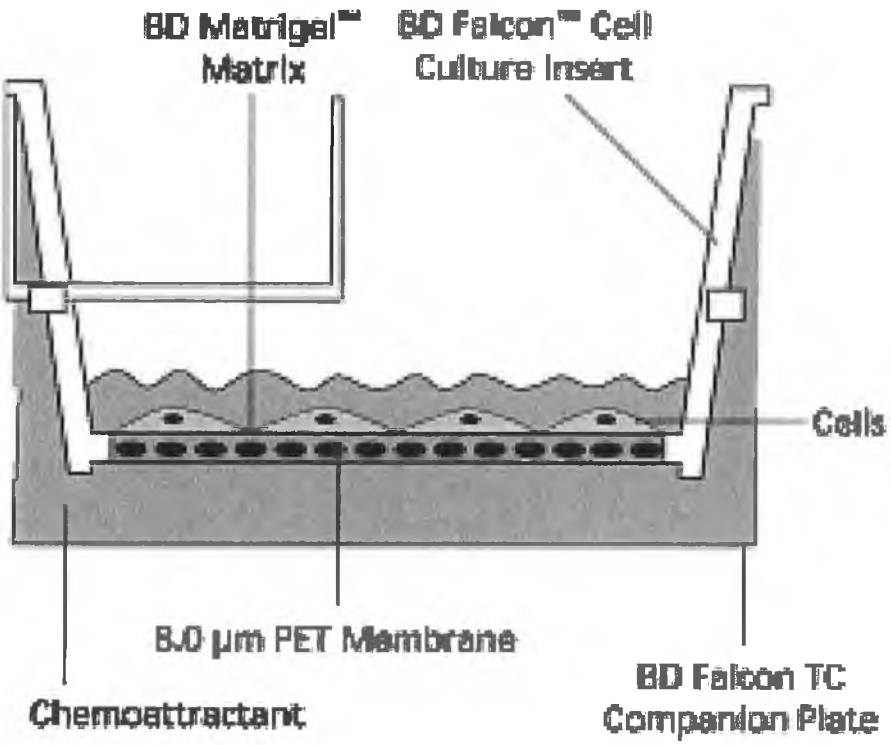


**Figure 2: EPC Adhesion at 1 hour, light microscopy 400x**

**Fig 2a: Before staining**

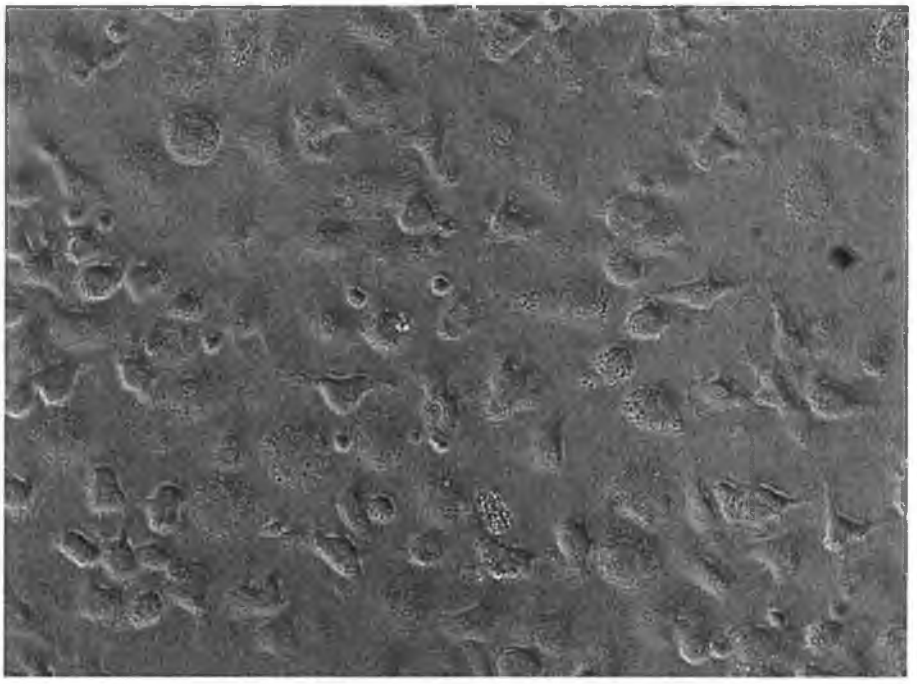
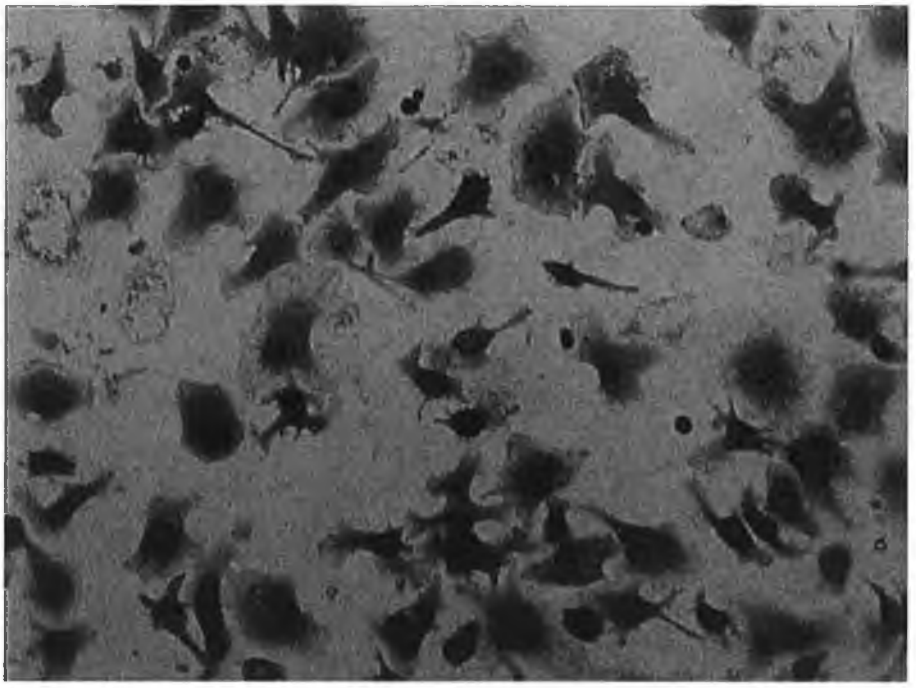


**Fig 2b: After staining with 0.1% Crystal violet**



| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blank | 12.5 ng/mL | QC 1 | | | | | | | | | |
| B | Blank | 12.5 ng/mL | QC 1 | | | | | | | | | |
| C | 1.56 ng/mL | 25 ng/mL | QC 2 | | | | | | | | | |
| D | 1.56 ng/mL | 25 ng/mL | QC 2 | | | | | | | | | |
| E | 3.125 ng/mL | 50 ng/mL | Sample 1 | | | | | | | | | |
| F | 3.125 ng/mL | 50 ng/mL | Sample 1 | | | | | | | | | |
| G | 6.25 ng/mL | 100 ng/mL | Sample 2 | | | | | | | | | |
| H | 6.25 ng/mL | 100 ng/mL | Sample 2 | | | | | | | | | |

## Figure 3: Assay Procedure for Human Adiponectin ELISA kit

| | Step 1 | Step 2 | Step 3-4 | Step 5 | Step 6 | Step 6-8 | Step 9 | Step 10-11 | Step 12 | Step 12 | Step 13 | Step 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Well # | Dilute each bottle of 10X Wash Buffer with 450mL Deionized Water. | Wash plate 3X with 300 µl Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels | Assay Buffer A | Standards/Controls/ | Detection Ab | Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µl Wash Buffer | Enzyme Solution | Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 5X with 300 µl Wash Buffer | Substrate | Seal, Agitate, incubate 5 - 20 minutes at Room Temperature. | Stop Solution | Read Absorbance at 450 nm and 590 nm. |
| A1, B1 | | | 80 µl | | 20 µl | | 100 µl | | 100 µl | | 100 µl | |
| C1, D1 | | | 60 µl | 20 µl of 1.56 ng/mL Standard | | | | | | | | |
| E1, F1 | | | 60 µl | 20 µl of 3.125 ng/mL Standard | | | | | | | | |
| G1, H1 | | | 60 µl | 20 µl of 6.25 ng/mL Standard | | | | | | | | |
| A2, B2 | | | 60 µl | 20 µl of 12.5 ng/mL Standard | | | | | | | | |
| C2, D2 | | | 60 µl | 20 µl of 25 ng/mL Standard | | | | | | | | |
| E2, F2 | | | 60 µl | 20 µl of 50 ng/mL Standard | | | | | | | | |
| G2, H2 | | | 60 µl | 20 µl of 100 ng/mL Standard | | | | | | | | |
| A3, B3 | | | 60 µl | 20 µl of QC I | | | | | | | | |
| C3, D3 | | | 60 µl | 20 µl of QC II | | | | | | | | |
| E3, F3 | | | 60 µl | 20 µl of Sample | | | | | | | | |
| G3, H3 | | | 60 µl | 20 µl of Sample | | | | | | | | |
| A4, B4 ↓ | | | 60 µl | 20 µl of Sample | | | | | | | | |

### XI. MICROTITER PLATE ARRANGEMENT

Human Adiponectin ELISA

**Figure 4: Dual staining of EPCs (400x)**

**Fig 4a: Light microscopy**

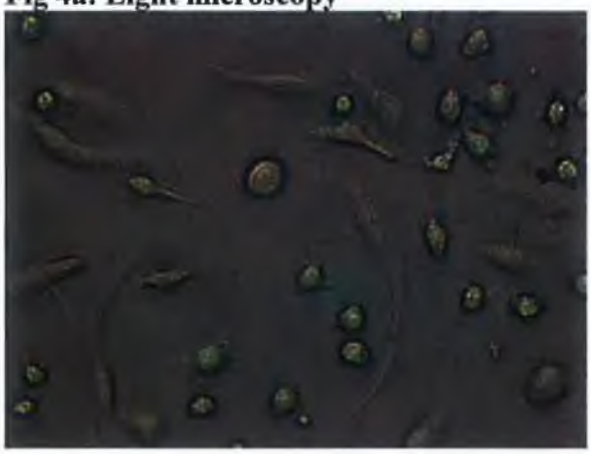


**Fig 4b: Red fluorescence with DiI ac-LDL**

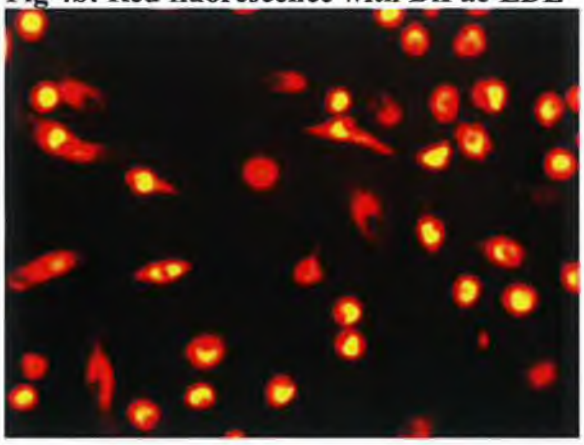


**Fig 4c: Green fluorescence with FITC-Lectin**

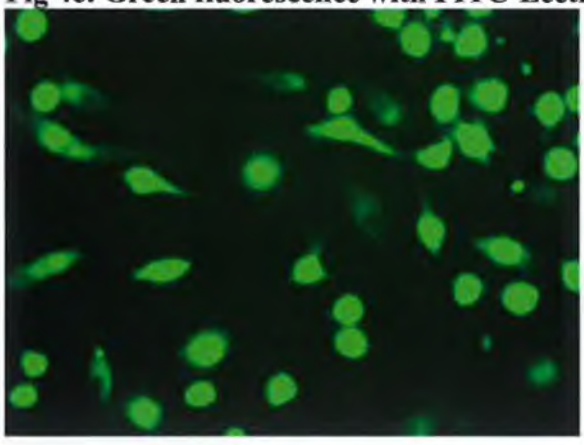


**Figure 5: Western Blot Images of cultured cells from Day 0, 4 and 7**

**Fig 5a: GAPDH, KDR and CD34**

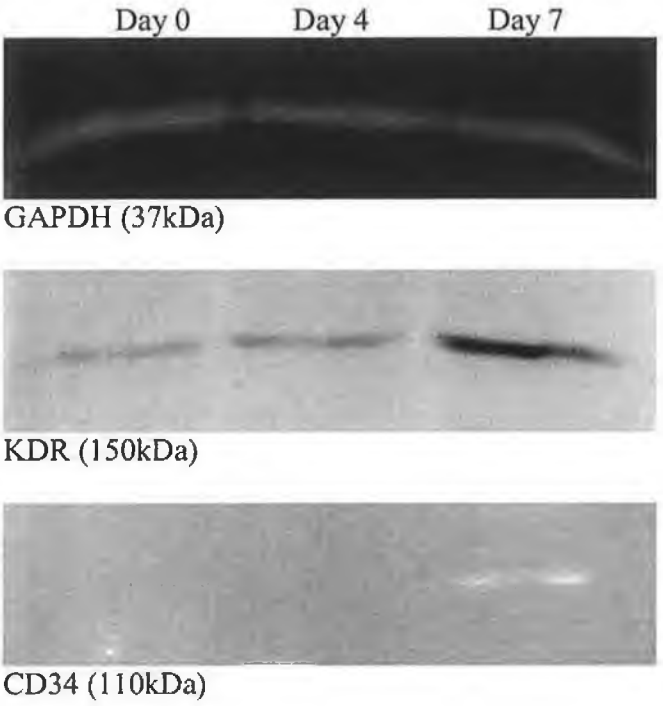


**Figure 5b: Coomassie stain**

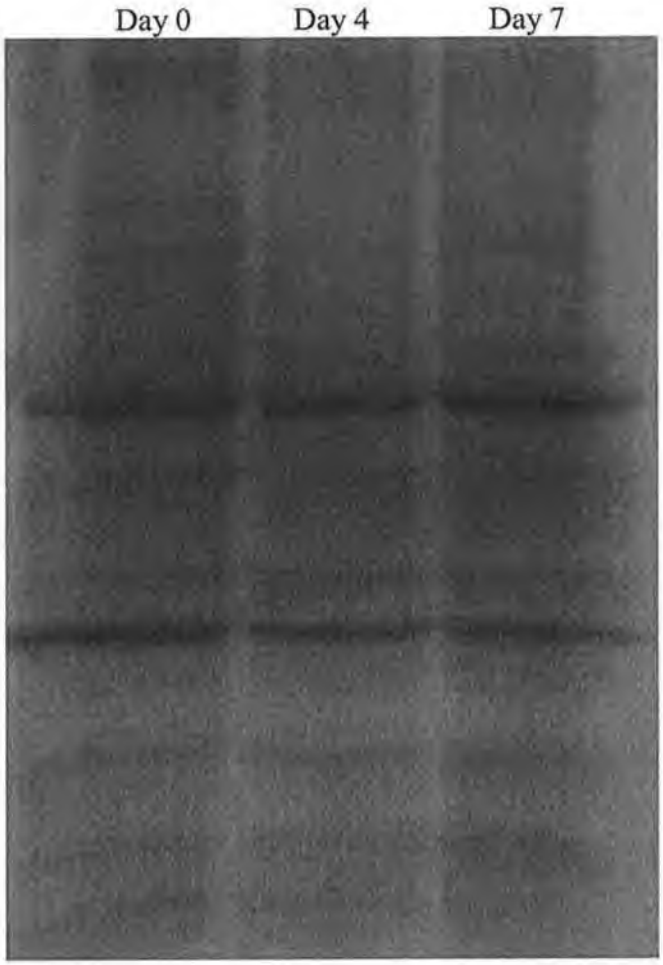


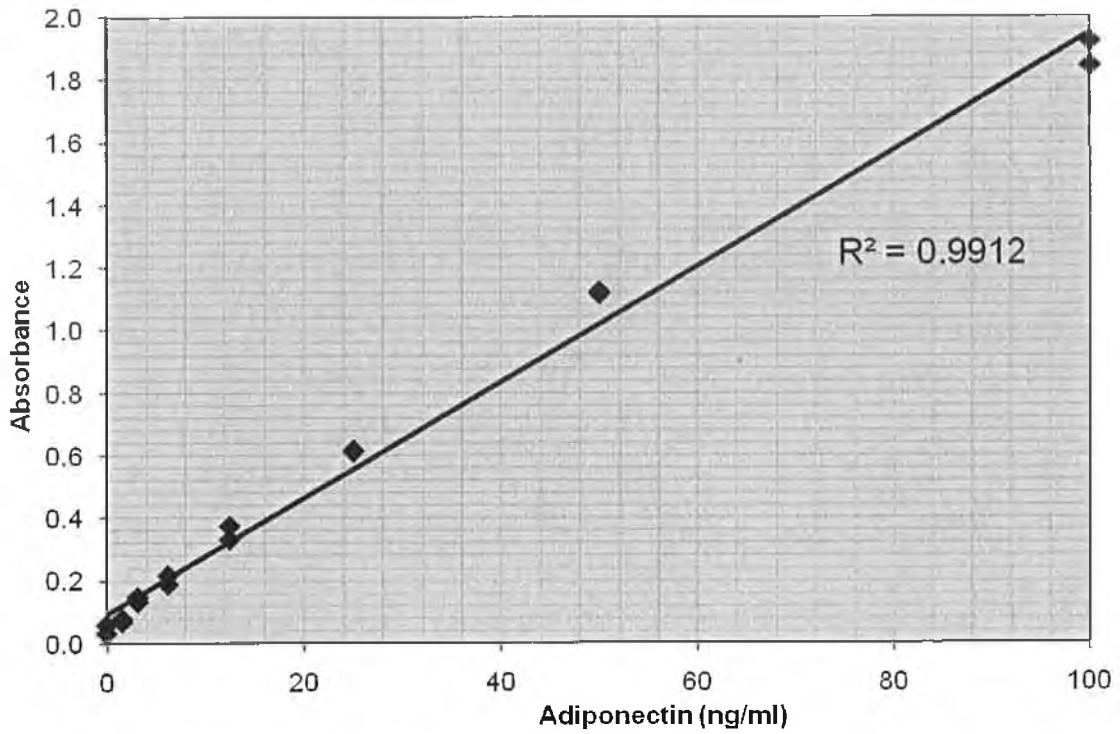
**Table 1: Laser scanning cytometry results**

**Mean Expression of CD34 and KDR in Day 7 Cells vs. Control Wells**

| Sample | CD34 | Control (CD34) | KDR | Control (KDR) |
|---|---|---|---|---|
| **1** | 154992 | 129178 | 384719 | 191879 |
| **2** | 155291 | 124152 | 349454 | 193639 |
| **3** | 137226 | 125175 | 351302 | 191558 |
| **4** | 139650 | 117696 | 323579 | 176894 |
| **5** | 140773 | 104759 | 369705 | 181981 |
| **6** | 119052 | 110690 | 311931 | 181237 |
| **Mean** | **141164*** | **118608** | **348448*** | **186198** |

*p-value <0.01 for CD34 vs. Control and for KDR vs. Control.

**Figure 6: Standard Curves for Human Adiponectin ELISA**



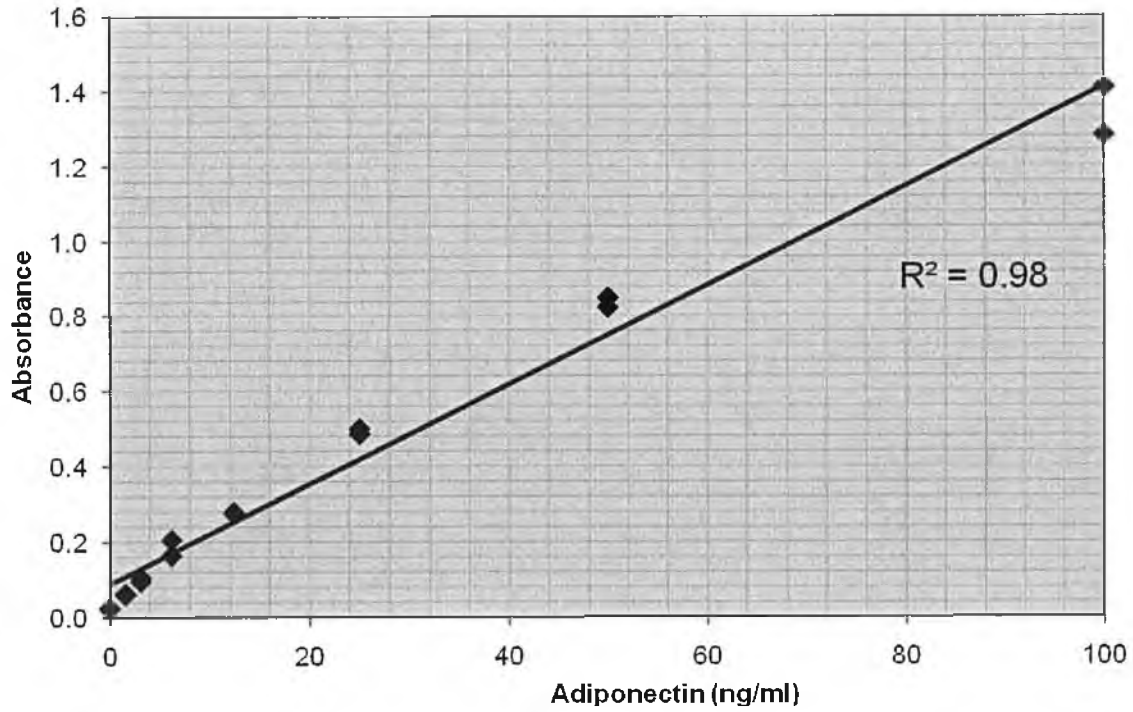



**Table 2: Baseline Characteristics for Control (AGA) and Small (SGA) groups (n (%) and Median[IQR])**

| | **SGA** (n=23) | **AGA** (n=23) | **p-value** |
|---|---|---|---|
| Age (yrs) | 31 [27-36] | 34 [32-37] | 0.146 |
| Father's Age (yrs) | 32 [27-37] | 34 [29-38] | 0.507 |
| Nulliparous | 15 (65%) | 14 (61%) | 0.763 |
| Caucasian | 19 (82%) | 21 (91%) | 0.386 |
| Family History of IHD | 2 (9%) | 0 (0%) | 0.153 |
| Booking Weight (kg) | 58 [54-67] | 61.7 [59-72] | 0.099 |
| Height (m) | 1.60 [1.55-1.65] | 1.64 [1.6-1.67] | 0.054 |
| BMI ($kg/m^2$) | 23.2 [20.4-25.5] | 23.1 [20.8-26.4] | 0.750 |
| Systolic BP at 30wks (mmHg) | 118 [110-123] | 120 [110-122] | 0.765 |
| Diastolic BP at 30wks (mmHg) | 70 [68-79] | 70 [60-70] | 0.052 |
| Pregnancy induced hypertension / Preeclampsia | 3 (13%) | 2 (9%) | 0.639 |
| Abnormal umbilical artery doppler ultrasound | 4 (17%) | 0 (0%) | 0.038 |

Abbreviations; IHD: ischaemic heart disease, BMI: body mass index, BP: blood pressure

**Table 3: Delivery details**

| | AGA (n=23) | SGA (n=23) | P-value |
|---|---|---|---|
| Birth weight (kg) | 3.6 [3.17-3.85] | 2.34 [2.13-2.65] | - |
| Birth weight centile | 58 [34-76] | 2 [0.5-3.5] | - |
| Gestation (days) | 278 [270-286] | 271 [260-278] | 0.023 |
| Caesarean section (n) | 15 (65%) | 13 (57%) | 0.665 |
| Male infant | 15 (65%) | 9 (39%) | 0.080 |

Categorical variables are presented as n (%) and continuous variables median [IQR]. P-values are not presented for birthweights or centiles since birthweight defines SGA/AGA status.

**Table 4: Results at 6 week visit, (Median[IQR])**

| | AGA (n=23) | SGA (n=23) | P-value |
|---|---|---|---|
| Systolic BP (mmHg) | 110 [105-117] | 110 [110-117] | 0.772 |
| Diastolic BP (mmHg) | 70 [70-75] | 72 [65-79] | 0.477 |
| Glucose (mmol/l) | 4.3 [3.95-4.5] | 4.4 [4.15-4.65] | 0.256 |
| HbA1c (%) | 5.0 [4.9-5.3] | 5.2 [5.1-5.4] | 0.108 |
| Insulin (mU/l) | 6.3 [5.05-7.8] | 7.2 [5.75-9.35] | 0.124 |
| HOMA-IR | 0.8 [0.65-0.95] | 0.9 [0.7-1.2] | 0.116 |
| Cholesterol (mmol/l) | 5.1 [4.5-5.4] | 5.5 [4.45-5.85] | 0.333 |
| LDL (mmol/l) | 1.76 [1.57-2.02] | 1.63 [1.41-1.93] | 0.368 |
| HDL (mmol/l) | 2.8 [2.29-3.19] | 3.08 [2.24-3.51] | 0.231 |
| Triglycerides (mmol/l) | 0.78 [0.66-0.91] | 0.98 [0.81-1.38] | **0.006** |
| Breast feeding (n(%)) | 11 (48%) | 7 (30%) | 0.195 |

Abbreviations; BP: blood pressure, HbA1c: glycosylated haemoglobin, HOMA-IR: homeostasis model assessment of insulin resistance, LDL: low density lipoprotein, HDL: low density lipoprotein.

**Table 5: EPC number and function, (Median[IQR])**

| | **AGA** (n=23) | **SGA** (n=23) | p-value |
|---|---|---|---|
| **EPC Count** (cells in 12x high power fields) | **367** [331-415] | **294** [236-338] | 0.005 |
| **Migration coefficient** (fractional migration towards VEGF) | **1.59** [1.22-1.81] | **0.91** [0.86-1] | <0.001 |
| **Adhesion** (FU, Fluorescence Units) | **0.284** [0.214-0.4] | **0.221** [0.179-0.296] | 0.121 |

**Fig. 7: EPC Number, Migration and Adhesion**

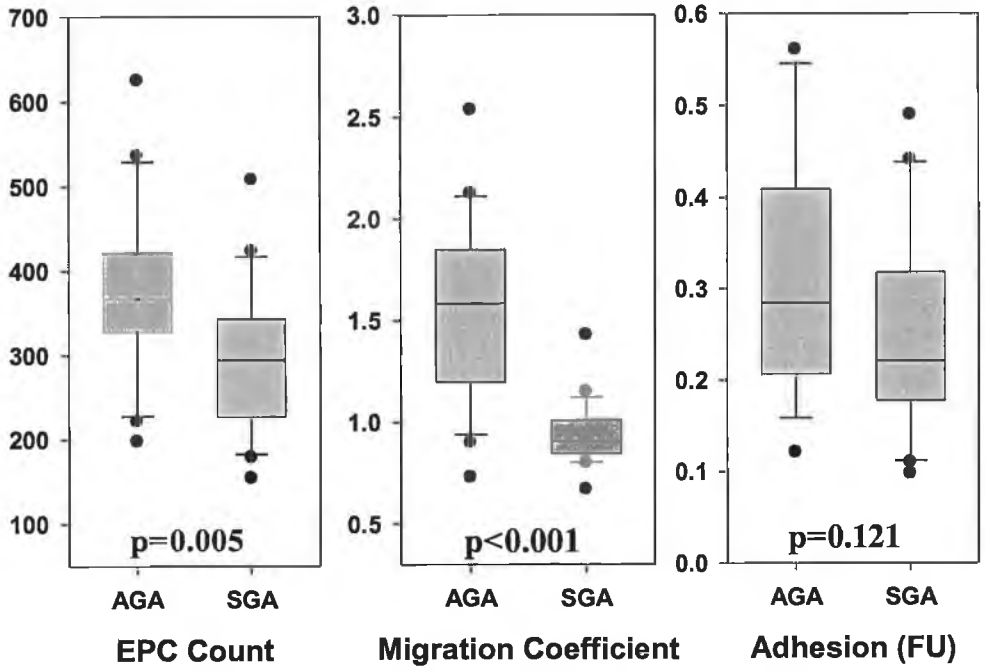


**Figure 8: Representative images of Day 7 Cells (Light microscopy 400x)**

**Fig 8a: Cells from Mother of AGA infant**

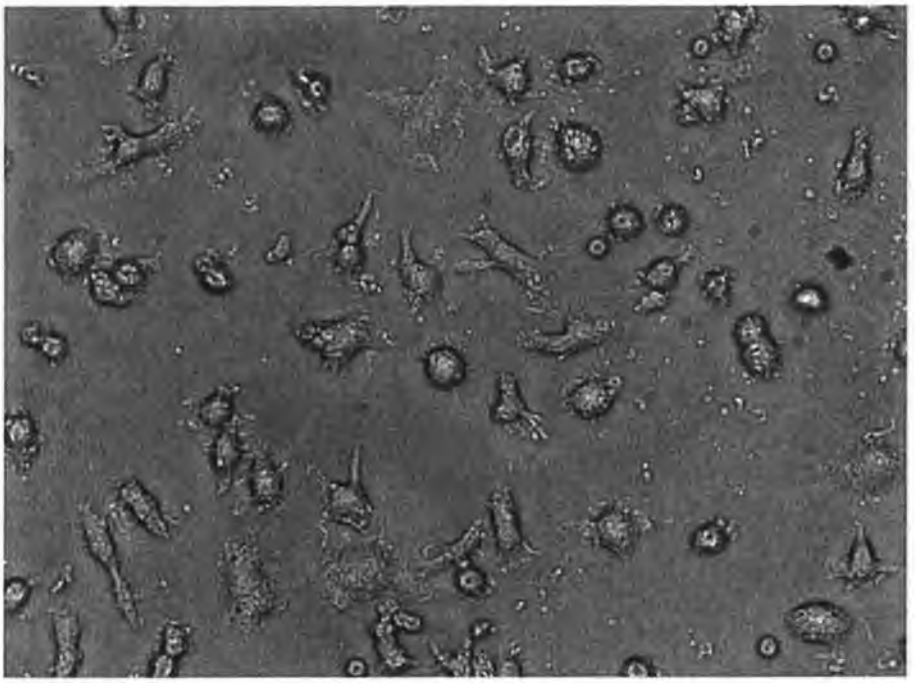


**Fig 8b: Cells from Mother of SGA infant**

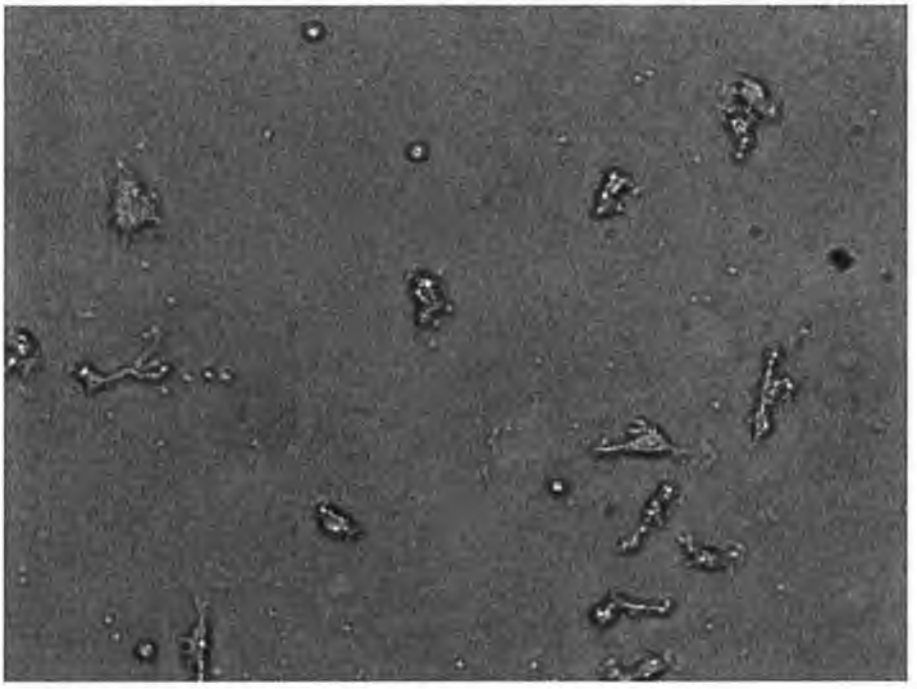


**Figure 9: EPC migration through modified Boyden Chamber**

**Fig 9a: Control chamber**

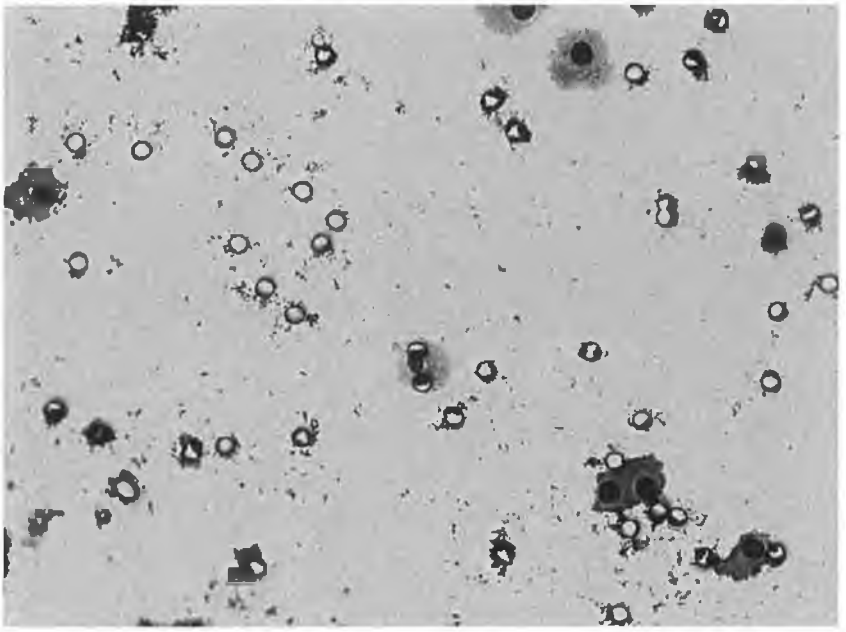


**Fig 9b: VEGF chamber**

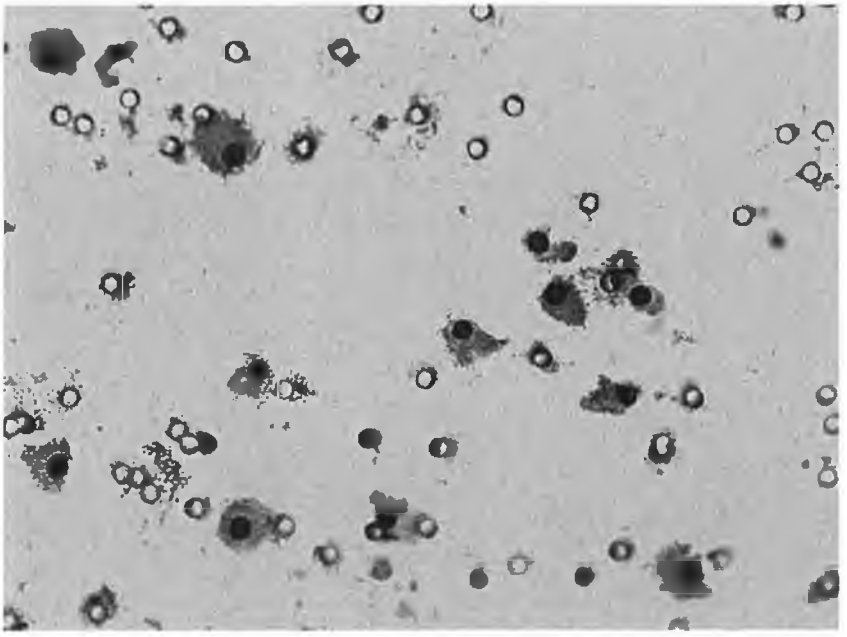


**Fig. 10: Relationship between birth weight and EPC number**

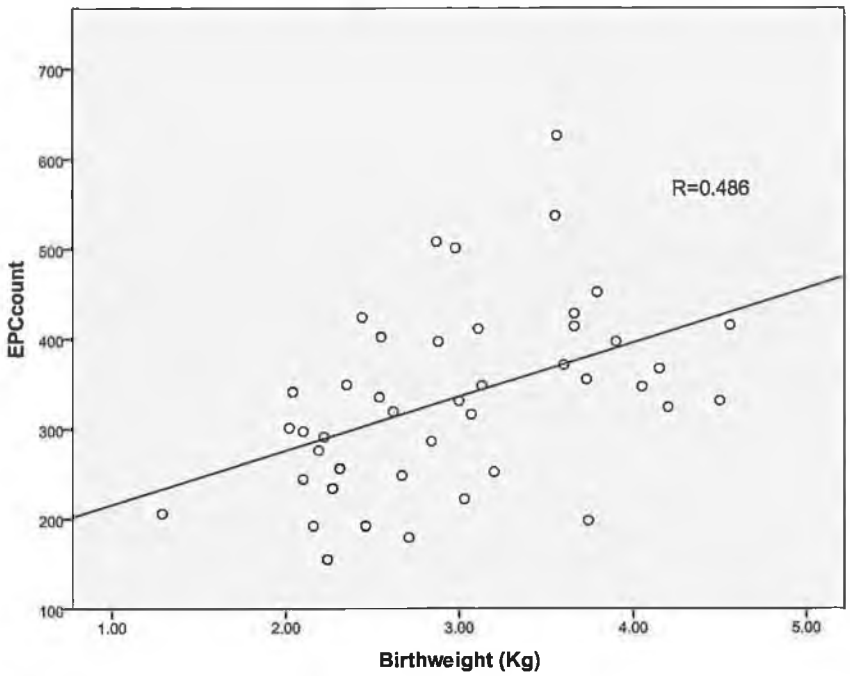



**Fig. 11: Relationship between birth weight and EPC migration**

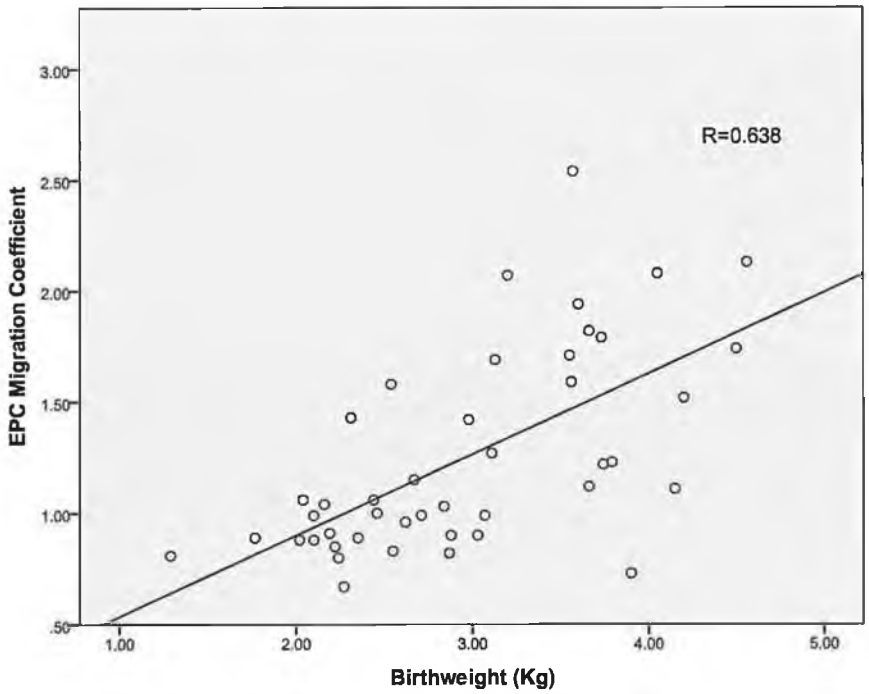


**Fig. 12: Relationship between birth weight and EPC adhesion**

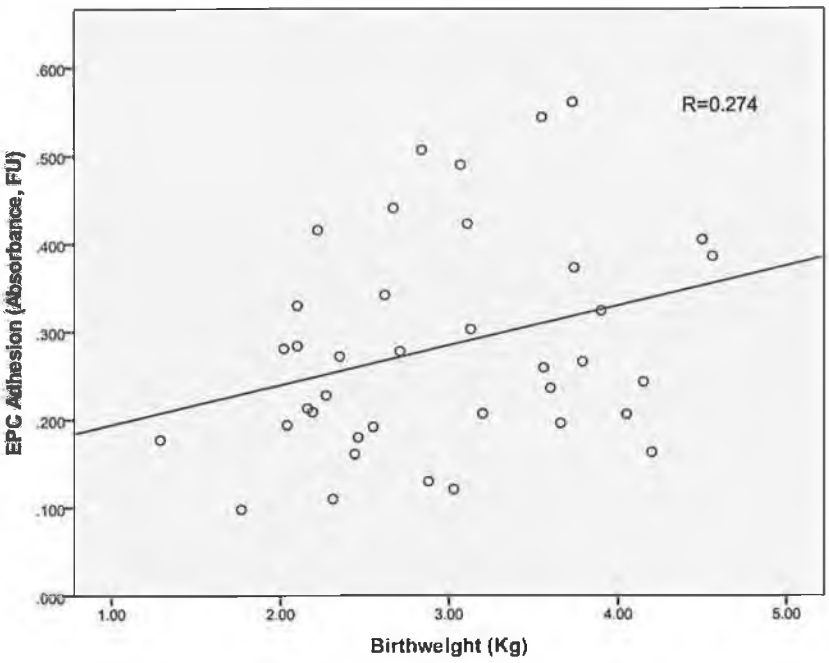



**Fig. 13: Relationship between EPC migration and serum triglycerides**

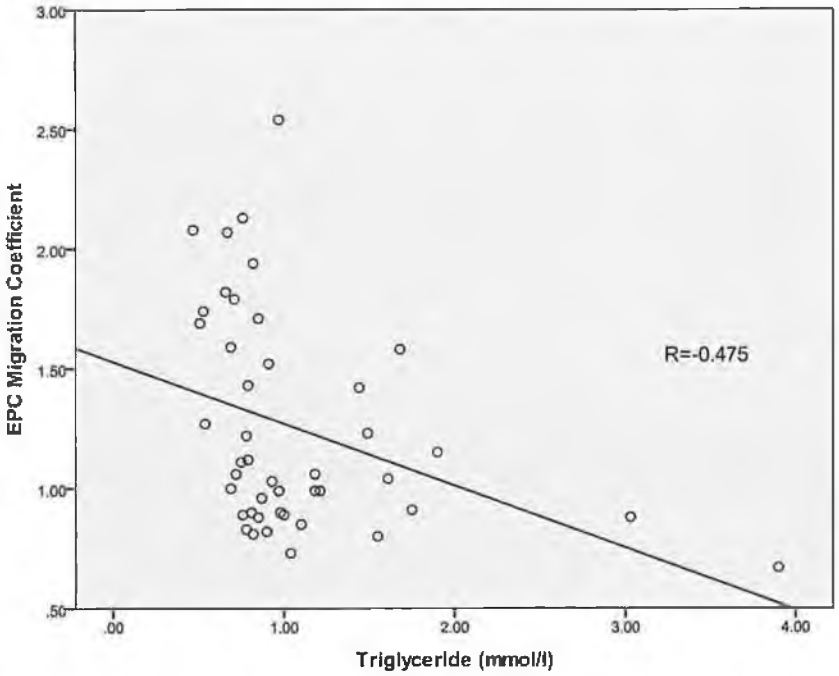


**Table 6: Adiponectin, (Median[IQR])**

| | AGA (n=23) | SGA (n=23) | P-value |
|---|---|---|---|
| Umbilical Cord Blood Adiponectin (ng/ml) | 70.4 [54.2-76.6] | 55.2 [47-65] | **0.033** |
| Maternal Plasma Adiponectin (ng/ml) | 17.7 [14.8-26] | 20.7 [15.3-25.6] | 0.8 |

**Figure 14: Cord Blood Adiponectin in AGA and SGA groups**

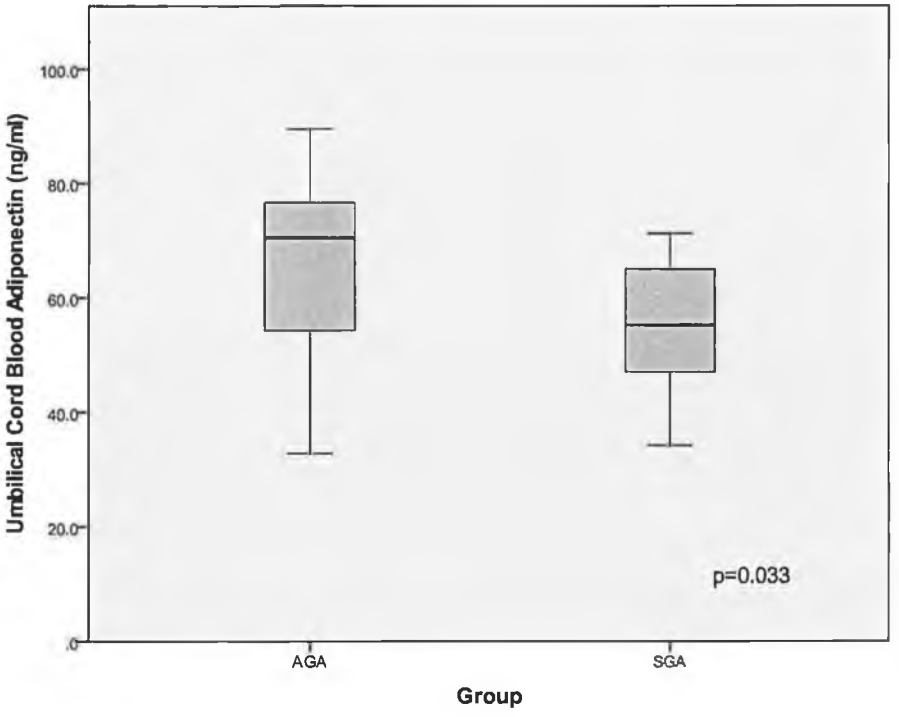


**Fig. 15: Relationship between birth weight and umbilical cord blood adiponectin**

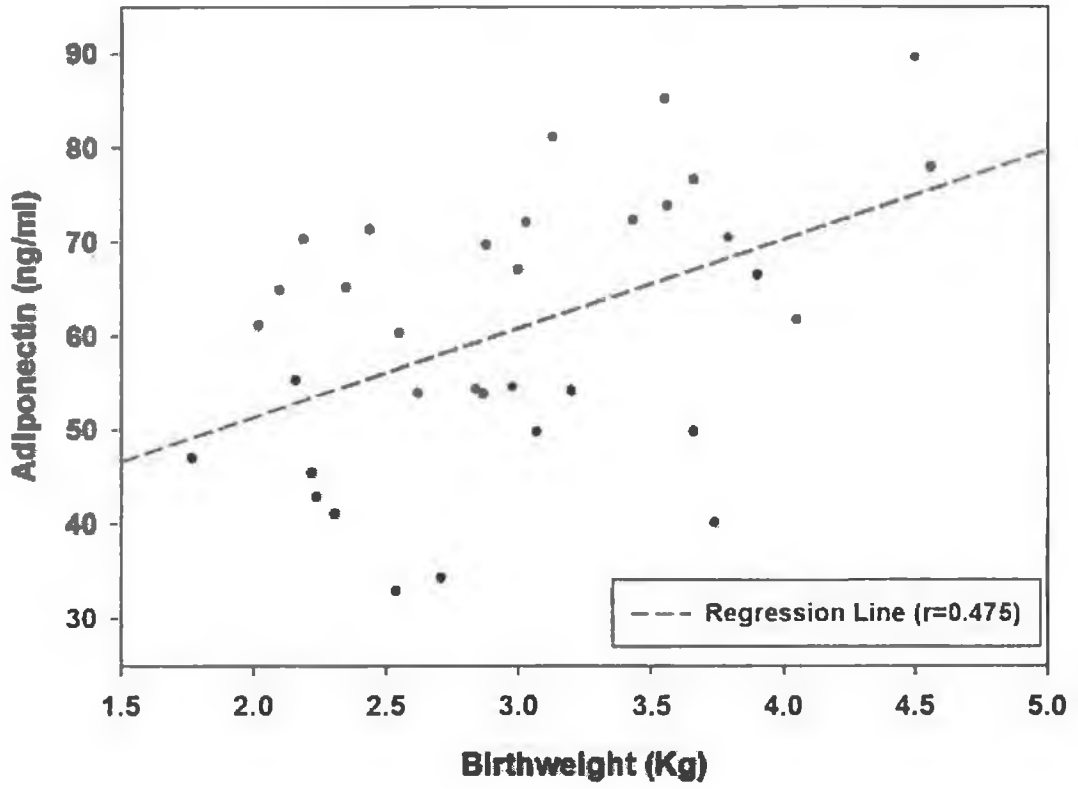


**Table 7: Multivariate Analysis for Confounding Factors**

Method = Forward Stepwise (Conditional)

**Table 7a: Omnibus Tests of Model Coefficients**

| | | Chi-square | df | Sig. |
|---|---|---|---|---|
| Step 1 | Step | 21.662 | 1 | .000 |
| | Block | 21.662 | 1 | .000 |
| | Model | 21.662 | 1 | .000 |

**Table 7b: Model Summary**

| Step | -2 Log likelihood | Cox & Snell R Square | Nagelkerke R Square |
|---|---|---|---|
| 1 | 29.604[a] | .443 | .591 |

a. Estimation terminated at iteration number 6 because parameter estimates changed by less than .001.

**Table 7c: Classification Table[a]**

| Observed | | | Predicted | | |
|---|---|---|---|---|---|
| | | | Group | | Percentage Correct |
| | | | Control | Small | |
| Step 1 | Group | Control | 14 | 4 | 77.8 |
| | | Small | 1 | 18 | 94.7 |
| | | Overall Percentage | | | 86.5 |

a. The cut value is .500

**Table 7d: Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) |
|---|---|---|---|---|---|---|---|
| Step 1[a] | Migration | -5.905 | 2.037 | 8.401 | 1 | .004 | .003 |
| | Constant | 6.942 | 2.258 | 9.456 | 1 | .002 | 1035.207 |

a. Variable(s) entered on step 1: EPC Migration.

**Table 7e: Model if Term Removed[a]**

| Variable | | Model Log Likelihood | Change in -2 Log Likelihood | df | Sig. of the Change |
|---|---|---|---|---|---|
| Step 1 | EPC Migration | -26.692 | 23.779 | 1 | .000 |

a. Based on conditional parameter estimates

**Table 7f: Variables not in the Equation**

| | | | Score | df | Sig. |
|---|---|---|---|---|---|
| Step 1 | Variables | Triglyceride | 1.314 | 1 | .252 |
| | | Age | 2.402 | 1 | .121 |
| | | Parity | 1.399 | 1 | .237 |
| | | Systolic BP | .027 | 1 | .869 |
| | | Diastolic BP | 3.501 | 1 | .061 |
| | | Breastfeeding | 1.079 | 1 | .299 |
| | | BMI | 1.013 | 1 | .314 |
| | | Glucose | .010 | 1 | .921 |
| | | Insulin | .049 | 1 | .825 |
| | | HOMAIR | .021 | 1 | .885 |
| | | HbA1c | .674 | 1 | .412 |
| | | Cholesterol | .051 | 1 | .822 |
| | | HDL | .298 | 1 | .585 |
| | | LDL | .016 | 1 | .899 |
| | | Adiponectin | .521 | 1 | .471 |
| | | EPC Absorbance | 1.319 | 1 | .251 |
| | | EPC Count | 1.632 | 1 | .201 |
| | Overall Statistics | | 16.419 | 17 | .494 |

**Table 8: Baseline Characteristics of 3 groups (n (%) and Median[IQR])**

| | AGA (n=23) | CSGA (n=18) | IUGR (n=5) | p-value* |
|---|---|---|---|---|
| Age (yrs) | 34 [32-37] | 33 [27-37] | 24 [26-30] | 0.6 |
| Father's Age (yrs) | 34 [29-38] | 35 [30-37] | 26 [24-30] | 0.338 |
| Nulliparous | 14 (61%) | 12 (67%) | 3 (60%) | 0.83 |
| Caucasian | 21 (91%) | 15 (83%) | 4 (80%) | 0.376 |
| Family History of IHD | 0 (0%) | 1 (6%) | 1 (20%) | 0.078 |
| Booking Weight (kg) | 61.7 [59-72] | 58 [54-68] | 61 [57-66] | 0.143 |
| Height (m) | 1.64 [1.6-1.67] | 1.59 [1.55-1.63] | 1.67 [1.6-1.68] | 0.131 |
| BMI ($kg/m^2$) | 23.1 [20.8-26.4] | 23.5 [20.7-25.2] | 21.6 [20.2-25.8] | 0.728 |
| Systolic BP at 30wks(mmHg) | 120 [110-122] | 117 [110-124] | 120 [116-120] | 0.872 |
| Diastolic BP at 30wks(mmHg) | 70 [60-70] | 70 [66-72] | 80 [77-82] | **0.012** |
| Pregnancy induced hypertension / Preeclampsia | 2 (9%) | 1 (6%) | 2 (40%) | 0.291 |
| Abnormal placental artery ultrasound doppler | 0 (0%) | 2 (11%) | 2 (40%) | **0.011** |

AGA: Appropriate for Gestational Age, CSGA: Constitutionally Small for Gestational Age, IUGR: Intra-Uterine Growth Restriction. *Jonckheere-Terpstra test

**Table 9: Delivery details of 3 groups**

| | AGA (n=23) | CSGA (n=18) | IUGR (n=5) | p-value* |
|---|---|---|---|---|
| Birth weight (kg) | 3.6 [3.17-3.85] | 2.45 [2.25-2.7] | 2.1 [1-4.75] | **<0.001** |
| Birth weight centile | 58 [34-76] | 2 [2.02-2.16] | <1 [0.1-1] | **<0.001** |
| Gestation (days) | 278 [270-286] | 272 [265-280] | 260 [257-262] | **0.007** |
| Caesarean section (n) | 15 (65%) | 13 (87%) | 3 (60%) | 0.234 |
| Male infant | 15 (65%) | 6 (33%) | 3 (60%) | 0.165 |

Categorical variables are presented as n (%) and continuous variables median [IQR] *Jonckheere-Terpstra test

**Table 10: Results at 6 week visit, (Median[IQR])**

| | AGA (n=23) | CSGA (n=18) | IUGR (n=5) | p-value* |
|---|---|---|---|---|
| Systolic BP (mmHg) | 110 [105-117] | 110 [110-116] | 113 [110-130] | 0.624 |
| Diastolic BP (mmHg) | 70 [70-75] | 70 [65-77] | 80 [76-84] | 0.263 |
| Glucose (mmol/l) | 4.3 [4.0-4.5] | 4.4 [4.1-4.6] | 4.6 [4.3-4.9] | 0.169 |
| HbA1c (%) | 5.0 [4.9-5.3] | 5.2 [5.1-5.4] | 5.1 [5.1-5.2] | 0.155 |
| Insulin (mU/l) | 6.3 [5.0-7.8] | 6.9 [5.7-7.8] | 9.0 [8.8-14.4] | 0.056 |
| HOMA-IR | 0.8 [0.7-1.0] | 0.9 [0.7-1.0] | 1.2 [1.1-1.8] | 0.053 |
| Cholesterol (mmol/l) | 5.1 [4.5-5.4] | 5.2 [4.4-5.6] | 5.9 [5.8-6.4] | 0.155 |
| LDL (mmol/l) | 1.8 [1.6-2.0] | 2.7 [2.1-3.5] | 3.4 [3.2-3.8] | 0.214 |
| HDL (mmol/l) | 2.8 [2.3-3.2] | 1.6 [1.4-1.9] | 1.8 [1.4-2.0] | 0.268 |
| Triglycerides (mmol/l) | 0.8 [0.7-0.9] | 0.9[0.8-1.2] | 1.6 [1.2-1.8] | **0.001** |
| Breast feeding (n(%)) | 11 (48%) | 6 (67%) | 1 (20%) | 0.195 |

Abbreviations; BP: blood pressure, HbA1c: glycosylated haemoglobin, HOMA-IR: homeostasis model assessment of insulin resistance, LDL: low density lipoprotein, HDL: low density lipoprotein. *Jonckheere-Terpstra test

**Table 11: EPC number and function, (Median[IQR])**

| | **AGA** (n=23) | **CSGA** (n=18) | **IUGR** (n=5) | p-value* |
|---|---|---|---|---|
| **EPC Count** (cells in 12x high power fields) | **367** [331-415] | **316** [244-349] | **276** [206-297] | **0.003** |
| **Migration coefficient** (fractional migration towards VEGF) | **1.59** [1.22-1.81] | **0.90** [0.85-1.0] | **0.91** [0.88-0.99] | **<0.001** |
| **Adhesion** (FU, Fluorescence Units) | **0.284** [0.214-0.4] | **0.228** [0.171-0.336] | **0.213** [0.209-0.281] | 0.146 |

*Jonckheere-Terpstra test

**Table 12: Adiponectin, (Median[IQR])**

| | AGA (n=23) | CSGA (n=18) | IUGR (n=5) | p-value* |
|---|---|---|---|---|
| Umbilical Cord Blood Adiponectin (ng/ml) | 70.4 [54.2-76.6] | 53.8 [45.3-65.0] | 62.9 [59.6-66.1] | 0.099 |
| Maternal Plasma Adiponectin (ng/ml) | 17.7 [14.8-26] | 20.8 [15.6-25.8] | 20.7 [15.3-21.9] | 0.899 |

*Jonckheere-Terpstra test

**Figure 15: Cord Blood Adiponectin in AGA, CSGA and IUGR groups**