Diabetes mellitus complicating pregnancy:
A study of maternal vascular endothelial dysfunction and of placental terminal villous ultrastructure.

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Declarations

1 Submission

This thesis has not been submitted in candidature for any other degree, diploma or professional qualification.

2 Statements of Originality

This thesis has been composed by myself. All the experimental work described in this thesis was carried out by myself except where stated in the acknowledgements section.

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Hypothesis

Insulin dependant diabetes is metabolic disorder associated with vascular dysfunction. Pregnancy, when occurs in a woman with diabetes, is associated with an increased risk of vascular complications. Maternal vascular dysfunction may alter the feto-placental environment and be associated with aberrant placental vascular development.

Aims

(1) To determine if pregnancy in the insulin dependant (type I) diabetic women is associated with increased maternal vascular endothelial dysfunction.

(2) To determine if the ultrastructure of the terminal placental villus, the functional exchange unit, is altered in pregnancies complicated by maternal diabetes mellitus.

Abstract

Insulin dependant diabetes is metabolic disorder associated with vascular endothelial dysfunction. Pregnancy, in a woman with diabetes, is associated with an increased risk of maternal vascular complications, such as pre-eclampsia and progression of microvascular disease. These vascular complications are in part mediated though inflammatory effects on the vessel wall. Insulin dependent diabetes is also associated with an increased risk of fetal complications including intrauterine hypoxia and stillbirth. Maternal vascular dysfunction may alter the feto-placental environment and be associated with aberrant placental vascular development. We therefore aimed to determine: (1) if pregnancy in the insulin dependent (type I) diabetic woman is associated with increased maternal endothelial dysfunction, and (2) if the ultrastructure of the terminal placental villus, the functional exchange unit, is altered in these pregnancies.

As an index of maternal vascular function circulating concentrations of defined endothelial-derived cell adhesion molecules were assessed. An ELISA was used to quantify the concentrations of these cell adhesion molecules, throughout diabetic and control pregnancies and in matched non-pregnant women. The circulating concentrations of the cell adhesion molecules: E-selectin and ICAM-1 were increased in non-pregnant diabetic subjects compared to non-pregnant controls. These cell adhesion molecules interact with neutrophils and provide evidence of endothelial dysfunction in our population of non-pregnant diabetic women. In contrast, during pregnancy there was no difference in the circulating concentrations of E-selectin or ICAM-1 between diabetic and control groups. The concentration of E-selectin was significantly reduced when measured in the pregnant compared to the non-pregnant diabetic cohorts. Circulating concentrations of von Willebrand factor, an established index of endothelial dysfunction, were also comparable in diabetic and control pregnant cohorts. These findings suggest that pregnancy, in our population of diabetic women, may actually be a time of vascular well-being. We hypothesize that this is a reflection of the improved glycaemic control achieved by these women during pregnancy.

The ultrastructure of the terminal placental villi from ten diabetic and control pregnancies was assessed by three techniques: transmission electron microscopic determination of cross-sectional architecture, scanning electron microscopy of specially prepared placental vascular casts and immunohistochemical assessment of villous stromal composition and cell turnover. The terminal villi of diabetic placentae were highly comparable to those of control placentae. Specifically we demonstrated no significant difference on comparison of villous diameter, villous capillary diameter, cytotrophoblast or syncytiotrophoblast nuclei number, stromal matrix content or cell turnover. However we demonstrated some small differences in the diabetic cases; an increased diffusion distance across the vasculosyncytial membranes, increased thickness of the cytotrophoblast basal lamina and longer, more branched terminal capillary loops. The lack of gross placental terminal villous maldevelopment is likely to reflect the absence of significant maternal vascular dysfunction in concert with the good glycaemic control demonstrated by our

population of diabetic mothers. We concluded that in these glycaemically controlled diabetic pregnancies, subtle abnormalities still exist in placental components intimately involved in nutrient exchange. In combination these findings are not consistent with significant placental hypoxia, indeed their significance is unclear but has implications for the future management of diabetic pregnancies.

Abbreviations

AC	abdominal circumference
ACE	advanced glycosylation end products
ADP	adenosine diphosphate
AEC	3-amino-9-ethylcarbazole
AEDFV	absent end diastolic flow velocity
ANP	atrial natiuretic peptide
APAAP	alkaline phosphatase: anti-alkaline phosphatase
APES	aminopropyltriethoxysilane
BSA	bovine serum albumin
CN	cytotrophobast nuclei
DAB	3,3'-diamino-benzidine
DCCT	Diabetes Control and Complications Trial
DDSA	dodecenyl succinic anhydride
DHO	disodium hydrogen orthophosphate
DSM	digital scanning microscope
EDRF	endothelium-derived growth factor
EGF	endothelial derived growth factor
ELISA	enzyme linked immunosorbant assay
et al.	and others
ET-1	endothelin 1
FGF	fibroblastic growth factor
g	grams
GMP	gyanoside monophosphate
HBA1c	haemoglobin A (adult)1c
HDL	high density lipoprotein
Hg	mercury
HLA	human leukocyte system
Hz	hertz
ICAM	intercellular adhesion molecule
ie	that is
IHC	immunohistochemistry
IL-1	interleukin-1
iu	international units
IUGR	intrauterine growth restriction
LDL	low density lipoprotein
m	metres
M	molar
mg	milligrams
mls	millilitres
mm	millimetres
n	number
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate-reduced
nm	nanometres
NO	nitric oxide
No.	number

NOS	nitric oxide synthase
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PAP	peroxidase: anti-peroxidase
PBS	phosphate buffered saline
PGH2	prostaglandin endoperoxide
PGI2	prostacyclin
PlGF	placenta growth factor
RAGE	receptor for advanced glycosylation end products
RI	resistance index
RNA	ribonucleic acid
SD	standard deviation
SE	standard error
SEM	scanning electron microscopy
SIGN	Scottish Intercollegiate Guidelines Network
SN	syncytial trophoblast nuclei
Sol.	solution
TEM	transmission electron microscopy
TNF	tumour necrosis factor
TPA	tissue plasminogen activator
TV	terminal villi
TXA2	thromboxane
u	units
μM	micrometres
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial derived growth factor
VLDL	very low density lipoprotein
vs.	versus

Contents

Tables and Figures

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Chapter 1

General Introduction

Type 1 Diabetes Mellitus is the most common medical condition complicating pregnancy in the United Kingdom, with an incidence of 3.5 per 1000 women. In Scotland it is estimated that approximately 235 diabetic women with type 1 diabetes are delivered each year (SIGN publication no.9, 1996). Despite recent advances in the medical management of diabetes and the obstetric care of pregnant diabetic women, they and their fetuses still remain at increase risk of complications.

1.1.1 Diabetes

Diabetes is the term given to a range of disorders that have in common a failure of normal glucose metabolism. This may occur either through a lack of pancreatic secretion of the hormone or a lack of response (peripheral resistance) to the action of circulating insulin. The American Diabetes Association (2000) has recently revised an aetiological classification of diabetes. This replaces an older classification based on treatment: insulin dependent or non-insulin dependent diabetes, which was inadequate to describe those patients whose treatment had changed over time to require insulin. The American classification of diabetes is summarised in table 1.1. Women with type 1 diabetes form the subject basis of this thesis. Women with other types of diabetes are not discussed further in detail.

Table 1.1 Aetiologic Classification of Diabetes	
Type 1 diabetes mellitus	β cell destruction, usually leading to absolute insulin deficiency. Immune mediated. Idiopathic.
Type 2 diabetes mellitus	Insulin resistance with or without varying degrees of insulin secretory deficiency.
Other specific types of diabetes	Genetic defects of β cell function. Genetic defects of insulin action. Disease of the exocrine pancreas. *(i.e. pancreatitis, trauma, neoplasia)* Endocrinopathies. *(i.e. Acromegally, Cushing's Syndrome)* Drug or chemical induced. Infections. *(congenital rubella, cytomegalovirus)* Unusual forms of immune-mediated diabetes. Genetic syndromes associated with diabetes. *(i.e. Down's syndrome, Turner's syndrome)*
Gestational diabetes mellitus (GDM)	Diabetes first detected during pregnancy. Placental hormones antagonise the effects of insulin. May represent subclinical type 1 or type 2 diabetes worsened by the pregnant state.

1.1.2 Type 1 diabetes mellitus

Type 1 diabetes mellitus is a chronic autoimmune disorder that results in the destruction of the pancreatic beta islet cells. It is manifest by a lack of insulin secretion and a disorder of carbohydrate, protein and fat metabolism. The classical symptoms of the disease, thirst, weight loss and polyuria develop over several weeks to months, whereas the asymptomatic destruction of the islet cells has occurred over many months to years.

There is strong evidence of a cell-mediated destruction of the islet cells. T-lymphocytes infiltrate the islets and cause an islitis. There is also a proposed humoral attack, which may be triggered by the T-cell attack. Autoantibodies to beta cell and islet antigens including insulin itself are present in the sera of patients at the time of diagnosis (Verge et at 1996). Some studies suggest the autoantibodies may inhibit insulin secretion or assist destruction of the islet cells.

The clinical incidence of diabetes is maximal in childhood with three-quarters of those affected being diagnosed by 15 years of age. The median age at diagnosis is 12 years. It is therefore interesting that decline in insulin secretion may be demonstrated for up to 12 years before the onset of clinical disease. At the clinical onset of the disease less than 10% of the B islet cell mass remains (Shatz 1995).

The diagnosis of diabetes rests upon the finding of elevated plasma glucose levels. The laboratory definition of diabetes is of a fasting venous plasma blood glucose greater than 7.8mmol/L and/or a plasma glucose greater than 11.0 mmol/L 2 hours after a glucose load (WHO Expert Committee 1980).

1.1.3 Glucose metabolism

Glucose metabolism is fundamental to life. Basic metabolic homeostasis requires that the body should be able to store, produce and expend energy. Intake of carbohydrate, fat, and protein provides sources of energy. The proportional intake of these substances and the level of energy expenditure by the body determine whether energy can be stored as carbohydrate or fat, and protein synthesis can occur (anabolism), or whether energy is required to be generated from these sources including protein degradation (catabolism).

The pancreatic hormones insulin and glucagon are the essential regulators of this process (Gernuth 1998). Insulin is synthesised by the pancreatic islet beta cells. It is stored in granules so that it can be released rapidly upon beta cell stimulation. Insulin facilitates the storage of glucose as glycogen in muscle and the liver. It also facilitates the storage of free fatty acids as triglycerides in adipose tissue, and amino acids in protein. Glucagon stimulates the mobilisation of glucose from glycogen and the breakdown of amino acids to form glucose and free fatty acid mobilisation and ketogenesis to further energy resources. The normal basal levels of insulin and glucagon maintain fasting glucose levels. After a meal insulin levels and hence insulin/glucagon ratio rises rapidly. When all the nutrients have been assimilated the plasma glucose returns to pre-prandial levels and the plasma insulin and insulin/glucagon ratio promptly return to basal levels, protecting against hypoglycaemia.

1.1.4 Acute complications of type 1 diabetes mellitus

Lack of insulin action impairs the uptake of glucose by muscle and adipose tissue. There is also a lack of suppression of hepatic glucose output. This results in severe hyperglycaemia (Genouth 1973). A high filtered load of glucose in the renal tubules leads to an osmotic diuresis and dehydration. A compensatory polydypsia occurs. A lack of insulin activity promotes lipolysis resulting in high levels of circulating free fatty acid, which promotes their increased uptake into the liver and ketogenesis resulting in a metabolic acidosis. Ketoacidosis is an acute life threatening complication of diabetes. Protein breakdown occurs in muscle to sustain gluconeogenesis. Weight loss therefore includes fat and lean body mass. Hyperglycaemia also predisposes to infection and can cause blurred vision from lens swelling.

1.1.5 Chronic complications of type 1 diabetes mellitus

Life span of people with type 1 diabetes is shortened by on average 10-20 years. This is the consequence of various vascular complications, which may be subdivided into microvascular and macrovascular pathologies. These complications are also associated with significant morbidity. There is evidence that these complications may arise solely due to chronic cellular exposure to hyperglycaemia, however other

complex metabolic disturbances such as of lipid metabolism are also likely to contribute, particularly in relation to macrovascular disease. The clinical onset of these complications is typically seen 10-15 years after the diagnosis of the disease.

1.1.5.1 Microvascular complications

Microvascular diseases include retinopathy with potential loss of vision (Davis 1992) and nephropathy, which can lead to end stage renal disease (Breyer 1992).
Background retinopathy is the earliest form of retinopathy and occurs in almost all diabetic subjects with time. The initial pathology is loss of capillary pericytes, which causes capillary dilatations termed microaneurysms. Leakage of blood from microaneurysms is seen as dot haemorrhages on the retina and leakage of serum form hard exudates. These lesions are seen by fundoscopy. The lesions can occur anywhere on the retina but particularly threaten vision when they occur near the macula. The capillaries can, with progression of disease, become obstructed causing retinal ischaemia. Infarcted areas of the retina appear as soft (cotton wool) exudates. Ischaemia of the retina stimulates neovascularisation. This advanced retinopathy is termed proliferative retinopathy. In this sinister progression of the disease fragile new vessels, growing forward into the vitreous, are poorly supported by matrix and are at risk of rupture. The resulting haemorrhage and subsequent fibrosis, which can cause tractional retinal detachment, can both result in blindness.

Diabetic nephropathy complicates 35-45% of patients with type 1 diabetes and is associated with a high mortality. The vascular lesions are characterised by thickening of the capillary basement membrane, followed by accumulation of mesangial material throughout the glomerulus. These defects cause a reduction in glomerular filtration rate, loss of protein through the glomerulus and hypertension. Microalbinuria (30 – 300 mg albumin/day) may progress to clinical proteinuria (0.5g protein/day), nephrotic syndrome and eventually end stage renal failure. Hypertension aggravates progressive renal disease. The incidence of nephropathy peaks after approximately 15 years of clinical diabetes (Anderson et al 1983).

1.1.5.2 Macrovascular complications

Macrovascular disease can take the form of cardiovascular, peripheral vascular disease and cerebrovascular disease. Myocardial infarction is a major cause of death (Gernuth 1995). The histology of the macrovascular disease is similar to the atherosclerotic lesions seen in non-diabetic subjects. However the disease process is seen more often and appears to be accelerated in the presence of diabetes. Epidemiological studies indicate that diabetic subjects have a 3-8 fold increased risk of premature cardiovascular disease (Bierman 1992).

The vascular lesions demonstrate increased endothelium basement membrane thickness, also seen in microvascular disease. Proliferation of the basement membrane results in numerous irregular layers of basal lamina in which lipoprotein

derived vesicles are trapped (Simionescu 1986). The underlying extracellular matrix is also expanded (Simionescu 1996) and smooth muscle hypertrophy occurs. Increased fibronectin and type IV collagen accumulation reduce vascular compliance. (Heickendorff et al 1994). In the larger vessels adherence and diapedesis of blood monocytes occurs across the endothelium and lipid loaded macrophages (foam cells) appear in the hyperplastic matrix. With advancement of the process calcification occurs under the lipid laden endothelium, the endothelial layer thins and may be disrupted, exposing the underlying atheromatous plaque, and acutely increasing the risk of overlying thrombus formation and clinical symptoms.

1.1.6 The aetiology of type 1 diabetes mellitus

The aetiology of type 1 diabetes is yet to be fully determined, however both genetic and environmental factors pay a role. The prevalence of type 1 insulin dependent diabetes in the general population is 0.15%. Siblings of diabetics have a 5-15% risk of developing the disease, and offspring of diabetics have a 4.9% risk. Concordance in non-identical twins is 50%. This is higher in monozygotic twins but does not reach 100% (Winter et al 1993). This suggests that it is a susceptibility to diabetes and not diabetes itself that is inherited, and implicates an interacting aetiological role for environmental factors.

1.1.6.1 The genetic basis of diabetes

The family studies do not support a Mendelian inheritance of type 1 diabetes, nor has a single gene defect been implicated (Schatz 1995). It is proposed that genetic susceptibility to type 1 diabetes is linked to several genes. As type 1 diabetes is an autoimmune disease the genes studied first were those within the major histocompatibilty (HLA) complex located on the short arm of chromosome 6. Genetic association and linkage studies show siblings with diabetes share entire HLA haplotypes (Raffel 1997). DR3 and DR4 are susceptibility alleles that appear to operate synergistically (Schatz et al 1995). The DR2 allele decreases the risk of diabetes and dominates the susceptibility effect of DR3 and DR4. The HLA-DQ locus also is associated with an increased risk of diabetes. To date studies of other chromosomes have shown that type 1 diabetes is associated with at least 15 additional loci on nine other chromosomes including one near the insulin gene on chromosome 11 (Field et al 1997). It is estimated that the HLA region contributes about 50% of the genetic susceptibility to type 1 diabetes, and the area on chromosome 11 may contribute an additional 5-10% of total susceptibility.

1.1.6.2 The environmental basis of diabetes

The aetiological role of environmental factors is suggested by the major geographical variations in diabetic prevalence, and the observation that immigrants take on the risk of diabetes mellitus of their new country. Exposure to certain viruses i.e., the group

B coxsackie virus and the rubella virus has been implicated (Pearson 1991). These agents may provoke a cross reactive immunological response to the islet cells. There is demonstrated short sequence similarity between certain islet β cell antigens and a protein of the coxsackie B virus (Nepon 1995) in addition to a rubella virus protein (Schatz et al 1995). Thus if the immune system targets these viral proteins the islet cells may also be destroyed. A bovine serum albumin has also been found to have a similar peptide sequence to that expressed by human islet cells. This has been hypothesised to explain the association between lack of exclusive breast-feeding in the first 3 months of life with the subsequent increased incidence of diabetes (Schatz et al 1995). Environmental or dietary exposure to various toxins has also been implicated in the destruction of genetically vulnerable B cells. Nitrosamines found in smoked and cured meats and coffee have proposed diabetogenic effects (Pearson 1991).

1.1.7 Medical treatment of diabetes

1.1.7.1 Exogenous insulin therapy

The fundamental basis of treatment of diabetes mellitus is to replace the lack of endogenous insulin secretion and action, by parenteral administration of an exogenous supply. The first insulin preparations were purified from bovine and porcine sources and administered in once or multiple daily doses. This allowed control of severe hyperglycemia but was wholly inadequate at mimicking endogenous release of insulin. In non-diabetic subjects a sharp rise in insulin occurs after meals, which accounts for approximately 50% of insulin release from the pancreas each day. These peaks in insulin secretion are superimposed on a more constant background secretion. The onset of action of standard (soluble) insulin can be delayed, and duration of action extended, by the addition of protamine or zinc to form crystals in the preparation. Combinations of different insulin preparations have allowed replacement regimes to be developed with the aim of mimicking physiological insulin secretion more effectively.

In the 1980s human insulin became available with the advent of genetic engineering and recombinant DNA technology. Human insulins are now the standard preparations of use when initiating therapy as they have low risks of immunogenicity, which can lead to allergy or drug resistance. They are also manufactured more efficiently. Currently there are around 20 preparations of human insulins almost all of which are produced by two manufacturers Eli Lilly and Co and Novo/Nordisk Phar. Inc (Sengewald 1999). In a modern intensive regime of insulin replacement a once daily long acting insulin is typically given to provide the background insulin action, in combination with pre-meal injections of a short acting insulin (Figure 1.1) (Skyler 1998, Hirsch 1999).

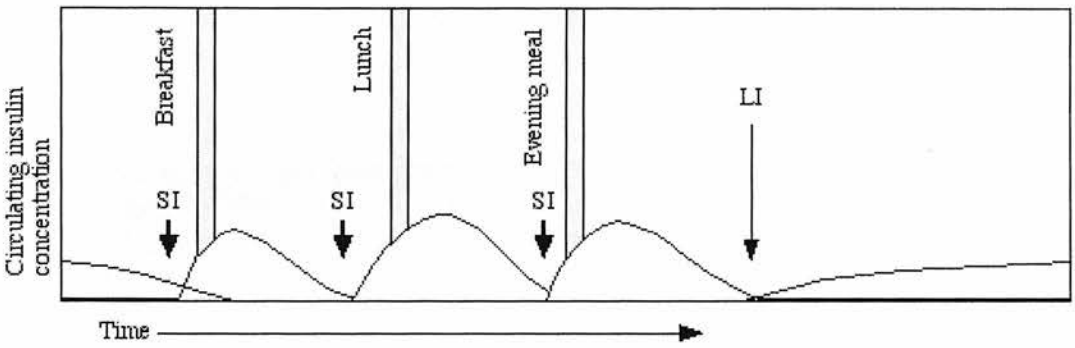


Figure 1.1 A typical Insulin replacement regime

Short acting soluble insulin (SI) and long acting insulin (LI) are given in an intensive (multiple injection) regime to mimic the lost physiological endogenous insulin response to meals.

Insulin requirements are increased principally by increased carbohydrate intake but also by weight gain of both lean body mass and fat mass. Increased insulin is also required during infections and other medical or surgical stresses and during pregnancy. In an individual the pharmacokinetics of replaced insulin can vary from day to day. Absorption from the skin can vary with the injection site, depth and angle of injection, ambient temperature and exercise of an injected limb. Injection into the subcutaneous tissue of the abdomen produces the least variable results. Adequate professional support to guide insulin replacement should therefore always be available and is an important facilitator of glycaemic control.

1.1.7.2 Dietary therapy

Diet is another important factor in management. Post-prandial glucose concentration is directly dependent on the carbohydrate content of the meal (Peterson et al 1990). The aim of dietary advice is to regulate the carbohydrate intake throughout each day. Ideally the carbohydrate should be unrefined and therefore slowly absorbed from fibre rich foods to prevent rapid swings in circulating glucose. Carbohydrate should provide approximately 50% of calorie intake. The total dietary prescription should provide 24-30 kcal/kg current body weight, or in pregnancy 30-35 kcal/ kg pre-pregnancy ideal body weight (Mulford et al 1993, British Diabetic Association 1992) A stable diet allows tailored insulin regimes to be effective and modified to their best advantage.

1.8 Monitoring glycaemic control

Blood glucose levels provide the best assessment of daily glycaemic control. Regular capillary samples throughout the day (4 to 7 point profiles) provide a detailed assessment of pre- and post- meal glucose levels and aid tailoring of insulin replacement. The aim is to achieve normoglycaemia (preprandial glucose values of < 6 mmol/L). Glucose concentrations can be determined by reagent sticks and manually read against a scale, or determined and stored electronically via the combined use of a glucometer. The use of the latter aids objective assessment of treatment compliance.

Assessment of glycaemic control over a larger time period can be achieved by quantifying the percentage of haemoglobin that has undergone the physiological process of glycation (Hb A1c). This is a two-step reaction resulting in the formation of a covalent bond between the glucose molecule and the terminal valine of the B-chain of the haemoglobin molecule. The rate at which this reaction occurs is related to the glucose concentration, and the percentage of glycated haemoglobin determined by electrophoresis reflects this and the life span of the red blood cell (Goldstein et al 1986). HbA1c determination therefore reflects the average level of glycaemic control over a period of several weeks. This objective assessment of glycaemic control has proven valuable in clinical studies of diabetes, as its value is not readily manipulated.

1.2 Diabetes and Pregnancy

1.2.1 Effect of pregnancy on glucose metabolism.

During normal pregnancy pancreatic β-cell hyperplasia occurs with increasing oestrogen and progesterone levels. Increased secretion of insulin and peripheral utilisation of glucose results in a 10% reduction in maternal glucose levels in the first trimester. There is reduced availability of gluconeogenic amino acids and a state of accelerated starvation occurs during fasting. This is marked by increased fat breakdown and increased levels of triglycerides and ketones (Kuhl 1995).

Placental hormones, which include human placental lactogen, glucagon and cortisol, impair glucose uptake into insulin sensitive cells resulting in a state if insulin resistance (Ryan and Erns 1988). In non-diabetic pregnancies there is a documented 44% decline in insulin sensitivity by the late third trimester. Overall glucose homeostasis is maintained by an exaggerated rate and amount of insulin release from the β cells (Yen 1973). However the altered metabolic state results in increased prostprandial circulating concentrations of glucose which serves the fetus by increasing glucose supply to the placenta.

In women with diabetes the altered glucose metabolism of pregnancy predisposes to hypoglycaemia particularly in the first trimester. Hyperemesis and nausea result in an exaggerated starvation state and increase the risk of ketoacidosis. Due to the insulin resistance of pregnancy women can expect to almost double their pre-pregnancy doses by 30 weeks gestation.

1.2.2 Diabetes and pregnancy - historical aspects

Prior to the discovery of insulin pregnancy was an extremely rare event for women with type 1 insulin dependent diabetes. The majority of affected women were ammenorrheic and infertile. Pregnancy when observed was associated with a greater than 50% maternal and fetal mortality rate (Williams 1909) and therefore most pregnancies were terminated by obstetricians. With the discovery of insulin fertility was returned to diabetic women and maternal mortality approached that seen in the non-diabetic population. However increasing numbers of women with more severe disease became pregnant and the perinatal mortality rate remained high. Late intrauterine death at 36-40 weeks gestation accounted for two thirds of all stillbirths.

The care of the pregnant diabetic women was pioneered by Priscilla White at the Joslin clinic, (Boston, USA), and Jorgen Pedersen in Copenhagen. They, like the physicians, recognised the importance of achieving good glycaemic control with insulin in reducing the complications of this condition and were the first to advocate the regular review of diabetic pregnant women by both an obstetrician and a physician (White 1949, Pedersen 1954). Under their influence the perinatal mortality rate fell. Fetal monitoring when introduced in the 1960's and 1970's, initially as biochemical screening of placental function and then as fetal heart rate and ultrasound assessment, aided a lower perinatal mortality rate despite the lack of rigid

glycaemic control in many patients. The leading causes of perinatal mortality became congenital abnormalities, severe intrauterine growth restriction due to maternal vascular disease and preterm birth. Unexplained intrauterine death remained more prevalent in pregnancies to women with diabetes compared to pregnancies to non-diabetic women.

With the improvement in perinatal mortality rate reducing the incidence of perinatal morbidity (due to macrosomia, fetal distress in labour, birth trauma, neonatal jaundice, hypoglycaemia and respiratory distress syndromes) and maternal morbidity (due to progressive retinopathy, pre-eclampsia, infection, polyhydramnios and caesarean section) have become the increasing aims of diabetic care (Coustan, Berkowitz and Hobbins 1980).

White class	Description of diabetes/complications
A	Diabetes treated with diet or drugs
B	Age of onset ≥ 20 years, and duration <10 years
C	Age of onset 10 -19 years, or duration 10-19 years
D	Age of onset < 10years, or duration >20 years
E	Calcification of pelvic vessels
F	Nephropathy
R	Proliferative retinopathy
FR	Nephropathy and proliferative retinopathy
G	Poor obstetric history

Table 1.2 The White classification of diabetic pregnancy.
Modified White classes based on the original classification (White 1949)

1.2.3 The St Vincent Declaration

In 1989 it was recognised by the European Office of the World Health Organisation and the European Regional Committee of the International Diabetes Federation that despite the previous efforts to improve diabetic pregnancy outcome, there remained an unacceptable increased risk of perinatal death, neonatal morbidity and congenital malformations in the infant of the diabetic mother. In response to this they issued the St Vincent Declaration (WHO 1990), the aim of which was "to achieve pregnancy outcome in the diabetic woman that approximates that of the non-diabetic woman" and set a five year time objective to achieve this aim. In response to this document a guideline of diabetic pregnancy management has been drawn up by the Scottish Intercollegiate Guidelines Network (SIGN) to improve the care of diabetic women when planning and during pregnancy (SIGN guideline No.9, 1996). Key areas

recognised in this report include the use of pre-pregnancy care and access to a specialised team including both an obstetrician and a diabetologist.

1.3 Maternal complications associated with insulin dependent diabetes

Pregnancy occurring in an insulin dependent diabetic woman remains associated with a range of maternal complications. Several classifications of diabetic pregnancies have made use of these complications as predictive indicators of poor pregnancy outcome (White 1949, Pedersen 1965). The incidence of these complications with the exception of pre-eclampsia, can be reduced by tight metabolic control.

1.3.1 Pre-eclampsia

Pre-eclampsia is a specific vascular disorder of pregnancy. It is characterised by hypertension, proteinuria and oedema. The aetiology of this condition is yet to be clearly determined but endothelial dysfunction is established as the pivotal intermediate pathology. An abnormal maternal response to placentation, which may occur as a result of genetic or immunological factors, is believed to release an undefined mediator into the maternal circulation. This factor is postulated to trigger the vascular dysfunction.

The clinical disorder of pre-eclampsia may progress to eclampsia, where cerebral manifestations of the disease, vasospasm and cerebral oedema cause convulsions. Neurological sequelae of eclampsia include coma and cerebral haemorrhage. Vascular dysfunction may provoke significant platelet consumption, a coagulation disturbance and haemolytic anaemia. Vascular damage may also affect liver function resulting in increased circulating concentrations of transaminases. The altered placentation is associated with fetal complications such as intrauterine growth restriction, hypoxia and in-utero demise.

Pre-eclampsia is more commonly observed in pregnancies complicated by maternal diabetes. The incidence of this condition was described in the Joslin Clinic records as approximating 50% before 1935 (White 1953). The incidence subsequently declined due to improved maternal and fetal management and surveillance. However it has recently been demonstrated that the incidence of pre-eclampsia remains raised in diabetic (9.9%) compared to non-diabetic pregnancies (4.3%) (Garner et al 1990). Diabetic pregnancies complicated with pre-eclampsia are associated with high perinatal mortality and morbidity rates due to their association with premature delivery (largely iatrogenic in maternal interests), and intrauterine growth retardation. Differentiation of pre-eclamspsia from acute deterioration of nephropathy with associated hypertension can be difficult. Thrombocytopenia, hyperuricaemia and fetal growth restriction suggest preeclampsia.

1.3.2 Diabetic retinopathy

Many clinical studies have assessed the short-term effect of pregnancy on diabetic retinopathy. These studies have been relatively small and have varied greatly in their methodology but have on the whole consistently demonstrated that pregnancy is associated with increased incidence and progression of retinopathy in diabetic women.

Moloney and Drury (1982) studied 53 pregnancies and found that background retinopathy developed during pregnancy in 15% of women and that 29% of women with background retinopathy prior to conception experienced progression. Both rates were significantly increased above that in non-pregnant diabetic women over a similar time course. Similar results were found by the Diabetes in Early Pregnancy Study (Chew et al 1995). This was a prospective cohort study of 155 diabetic women followed from pregnancy diagnosis to one-month post-partum. They demonstrated that progression of retinopathy occurred in pregnancy and that this was more likely to occur in those with more advanced initial disease; 6.8% of women with mild background retinopathy (n = 32) progressed to develop proliferative retinopathy compared to 30% of those with severe background retinopathy (n = 31). Progression of retinopathy is additionally more likely to occur in patients with hypertensive disorders, both hypertension preceding pregnancy and preeclampsia (Rosenn et al 1992, Lovestam-Adrian et al 1997). Regression of retinal changes has been documented during the immediate post partum period (Maloney and Drury 1982, Rosenn et al 1992).

Very few studies have addressed the long-term effect of pregnancy on retinopathy. Kaaja et al (1996) studied 26 women 7 years after delivery and compared them to 16 matched controls and found no difference in degree of retinopathy, suggesting no long-term effect of pregnancy on progression. A small prospective study (Miodovnik et al 1998) supports this finding. Twenty-three pregnant women were compared to 23 non- pregnant women similarly treated for a nine-month "sham pregnancy" period. The women were followed for 14 – 43 months post pregnancy. Progression of retinopathy was found in four women who had been pregnant compared to progression in three of the " sham pregnancy" group.

1.3.3 Diabetic nephropathy

Diabetic nephropathy is estimated to be present in up to 5% of type 1 diabetic pregnancies. In addition to hyperglycaemia, factors implicated in the development of nephropathy; increased glomerular filtration rate; hypertension and increased protein excretion are all increased or can occur more commonly in pregnancy. Pregnancy is therefore likely to be associated with progression of nephropathy. In women with advanced nephropathy (raised serum creatinine / reduced creatinine clearance) there is evidence from a few small studies that this does occur. Biesenbach et al (1992) followed 5 women through pregnancy and demonstrated a greater than expected fall in creatinine clearance over the time course of pregnancy and the postpartum period. All these women had developed end stage renal disease by 42 weeks postpartum. A

similar study (Purdy et al 1996) suggested a more modest but still significant 40% risk of accelerated nephropathy leading to end stage renal disease. In this study pregnant women with moderate or advanced nephropathy were followed over as similar length of time to non-pregnant diabetic women.

Cross sectional studies of parous and non-parous women have however not shown a difference in prevalence or severity of nephropathy between these groups (Kaaja et al 1996, Hemachandra et al 1995). These studies included a large proportion of diabetic women with no nephropathy or mild nephropathy at the time of conception. This suggests that pregnancy itself may not be associated with development of nephropathy or acceleration of mild disease. In the small prospective controlled study by Modovnik et al (1998) none of the pregnant diabetic women or the "sham" pregnant diabetic women had nephropathy prior to pregnancy and none developed the complication when followed to 14-43 weeks post partum. This supports the implications drawn from the cross sectional studies.

Advanced nephropathy therefore has a poor prognosis when combined with pregnancy. Hypertension is a frequent associated factor occurring in 30% of those affected at booking and in 75% by delivery (Lavoie et al 1988) and further drives advancement of nephropathy. Women with milder forms of nephropathy may be counselled more optimistically with regard to pregnancy outcome. Surveillance for peeclampsia is required, as this additional vascular pathology may lower the threshold for disease progression.

1.3.4 Macrovascular disease

Symptomatic macrovascular disease although commoner in diabetic women is rarely seen during childbearing age because of its later clinical onset. Rosenn et al (2000) detected 20 published case reports of myocardial infarction in diabetic women prior, during or shortly after pregnancy between 1953 and 1998. Mortality was observed only in the cases occurring during pregnancy or the puerperium (7/13), and only in the cases documented prior to 1980 (7/10). The selection bias of these reported cases is likely to be significant. Pregnancy may adversely affect coronary artery disease by a number of mechanisms most notably increased cardiac output, oxygen consumption and cardiac work. Particularly hazardous events in women with underlying ischaemic heart disease include hypoglycaemia and the altered haemodynamics occurring at delivery. Hypoglycaemia precipitates the release of catecholamines with resultant tachycardia. In the immediate postpartum period there is acute release of veno-caval obstruction, autotransfusion of uteroplacental blood and rapid mobilisation of extracellular fluid. All increase venous return and may demand an acute 60 – 80% increase in cardiac output.

1.3.5 Other maternal complications.

Approximately 80% of diabetic pregnancies are complicated by at least one episode of maternal infection compared to 26% of non-diabetic pregnancies (Stamler et al

1990). There is an increased incidence of urogenital, respiratory, wound and endometrial infections. Pyelonephritis occurs in 4% if diabetic pregnancies compared to 1% of non-diabetic pregnancies and is associated with increased incidence of premature rupture of membranes, preterm labour, and perinatal morbidity (Cousins 1987).

Diabetes is associated with an increased maternal morbidity due to polyhydramnios. The mechanism for the increased incidence of this condition is thought to arise from an osmotic diuresis by a hyperglycaemic fetus (Naeye et al 1970). The condition may also arise in association with a congenital malformation (Moya et al 1960). Polyhydramnios contributes to increased perinatal morbidity and mortality due to its association with premature labour.

Maternal ketoacidosis remains a serious acute maternal complication, associated with a 20% fetal loss rate (Hogay 1994). It occurs more frequently during pregnancy due to the relative insulin resistance of pregnancy and the increased incidence of predisposing factors; infections, hyperemesis gravidum and the use of tocolytic therapy (B-sympathomimetic agents).

Hypoglycaemic episodes are also commoner during pregnancy reflecting altered glucose metabolism. The incidence of this complication has risen due to the improved aims of glycaemic control during pregnancy. If severe, hypoglycaemia may precipitate seizures or loss of consciousness. Rosenn et al (1995) prospectively followed 84 women from 9 weeks of pregnancy who had their intensive insulin regimes adjusted weekly following glucometer readings. Clinical hypoglycaemia requiring assistance from another person occurred in 71% of women. It is imperative that the women and their partners are taught to recognise and treat this complication. Animal studies have indicated that chronic maternal hypoglycaemia may have a deleterious effect on the embryo during development (Buchanan et al 1986), however there are no human clinical trials to support this.

1.4 Fetal complications of maternal diabetes

Improved maternal glycaemic control, fetal surveillance and neonatal intensive care have reduced fetal/ neonatal loss in diabetic pregnancies (Reece 1994). However the incidence of fetal complications remains significantly raised over that observed in non-diabetic pregnancies largely from an increased spontaneous abortion rate, increased incidence of congenital malformations and "unexplained" late fetal death.

1.4.1 Spontaneous miscarriage

Maternal diabetes mellitus has long been associated with an increased risk of spontaneous miscarriage. Precise estimates of risk are difficult to obtain due variations in definition of miscarriage and the poor quantification of the non-diabetic background rate of pregnancy loss. A prospective uncontrolled trial by Miodovnik et al in 1984 demonstrated an overall 30% spontaneous miscarriage rate in diabetic

women (approximately 12 times the compared background rate). There was an increased incidence in diabetic women with advanced diabetic diseases as defined by White class and an association with poor glycaemic control in the first trimester. The relationship between the increased risk of miscarriage and poor maternal glycaemic control has recently been clearly demonstrated in the Diabetes in Early Pregnancy study (1995) and the Diabetes Control and Complications trial (1996). The latter demonstrated that diabetic women intensively treated to achieve good metabolic control (HbA1 = 7.4% at conception) were no more at risk of spontaneous miscarriage (13.3%) than non-diabetic women.

1.4.2 Congenital malformations

Maternal diabetes is associated with a two to four fold increased incidence of congenital malformation of their infant over the background incidence of 2% (Lowey, Beard and Goldschmidt 1986, Mills et al 1988, Hawthorne et al 1997). One recent population study of diabetic pregnancies (where there was no regional guidelines for the management of diabetic pregnancy) has documented a much higher (10-fold) prevalence (Casson et al 1997). As improved perinatal care has led to a general reduction in the perinatal mortality of diabetes, this complication now accounts for 40% of prenatal deaths. There is a high risk of neural tube abnormalities (19.5/1000, compared with 2-5/1000 for non-diabetic pregnancies), (Milunsky et al 1982). Cardiac abnormalities are found in 4% of diabetic pregnancies (five-fold increase) and include ventricular septal defects, transposition of the great vessels and coarctation of the aorta (Rowland, Hubbell and Naclas 1973). Renal and gastrointestinal anomalies are also more common. The caudal recession syndrome although rare, occurs an estimated 200 times more frequently in diabetic compared to non-diabetic pregnancies (Kucera 1971).

The association between poor maternal metabolic control and increased incidence of congenital malformations has been recognised for many years. Recent studies have stressed the particular importance of poor glycaemic control in the first trimester of pregnancy (i.e. embryogenesis) in the pathogenesis of this complication (Reid et al 1984, Stubbs et al 1986). The causal effect of hyperglycaemia has been clearly shown in animal studies (Eriksson et al 1982). In human studies support for this aetiology arises from the lower incidence (4.7%) of congenital anomalies when tight maternal metabolic control has been achieved (Mills et al 1988, The DCCT 1996).

1.4.3 Unexplained late intrauterine death

Unexplained stillbirth accounts for nearly 50% of the perinatal mortality rate of diabetic pregnancies (Beard and Lowey 1982). Two recent population studies of diabetic pregnancy outcome in Liverpool (Casson et al 1997) and the north of England (Hawthorne et al 1997) document a stillbirth rate of 2% compared to a background rate of approximately 0.5% in the non-diabetic pregnant population. The stillbirth incidence is greater after 36 weeks gestation especially in diabetic

pregnancies complicated by poor metabolic control, maternal vascular disease, ketoacidosis, or pre-eclampsia. Predisposing factors are thought to include chronic hypoxia, lactic acidaemia and hypertrophic cardiac myopathy. Chronic hypoxia may arise from the organomegally induced by fetal hyperinsulinaemia and hence increased fetal demand for oxygen (Carson 1980). Chronic hypoxia stimulates extramedullary haematopoiesis. Fetal polycythaemia has been demonstrated in diabetic pregnancies and may predispose to fetal intravascular thrombosis (Salvesen, Brudenell and Nicolaides 1992). Lactic acidosis may occur, especially in association with hypoxia, because the rate of maternal glucose transfer across the placenta is independent of maternal perfusion whereas the rate of oxygen transfer is not.

1.4.4 Altered fetal growth

1.4.4.1 Macrosomia

Fetuses of diabetic pregnancies are often large for gestational age. Typical series demonstrate that 25% of diabetic infants have a birth weight greater than the 90th percentile for gestational age (Beard and Maresh 1989, Persson and Hanson 1996). Infants weighing greater than 4.5kg at birth, irrespective of gestational age are termed macrosomic. The increase in birth weight is secondary to increase in fat, muscle and organ size. The growth promotion of the infant is believed to result from poor maternal metabolic control. Pedersen first postulated this in 1954. He hypothesised that maternal hyperglycaemia led to fetal hyperglycaemia, fetal pancreatic β-cell proliferation, and therefore fetal hyperinsulinaemia. Fetal insulin then acts as a growth hormone.

The Diabetes in Early Pregnancy Study (Jovanovic-Peterson and Peterson 1990) found that postprandial maternal blood glucose concentrations in the third trimester were strongly predictive of birth weight and fetal macrosomia. Johnstone et al (2000) demonstrated that the most predictive variable was glycated haemoglobin concentrations at 27-33 weeks gestation. This however was documented to explain only 6.3% of birth weight variance. This finding may help explain why despite improved maternal control, the incidence of macrosomia remains raised. It indicates possible aetiological roles for maternal weight; genetic factors and insulin like growth factors. The antenatal diagnosis of this condition is limited which increases further the risk of traumatic delivery of these infants. The macrosomic infant is also at increased risk of unexplained fetal death and neonatal morbidity.

1.4.4.2 Intrauterine growth restriction

Intrauterine growth restriction is used to describe a range of disorders in which the fetus fails to achieve its genetic growth potential. The condition may follow intrauterine infection or accompany congenital abnormalities. In these pathologies the fetus is usually symmetrically small. Asymmetrical growth restriction occurs in a range of conditions (see sections 4.3.1and 4.3.2) that are associated with inadequate transfer of nutrients from the mother to the fetus, which limits growth. The fetus preferentially channels glucose to the vital structures such as the brain at the expense

of laying down glycogen in the liver. Fetal head growth is therefore preserved whist liver and hence abdominal growth fall behind resulting in the asymmetrical picture.

Asymmetric intrauterine growth restriction of infants has been documented to occur more commonly in diabetic pregnancies (Reece and Homko 1994). Its increased incidence is limited to those mothers who have significant pre-pregnancy vascular disease, possibly by limiting uterine perfusion, and to those that develop preeclampsia during pregnancy.

1.4.5 Intra-partum fetal risks

The fetus of the diabetic mother is at increased risk of the two major complications of labour, trauma and asphyxia. As previously described the fetus may be suffering from a mild degree of asphyxia during the antenatal period and this will be worsened in labour by hypoxia during uterine contractions. Trauma of the infant during delivery almost exclusively occurs due to shoulder dystocia, as it is the body and not the head of the macrosomic infant that is significantly enlarged. Maternal diabetes increases the risk of shoulder dystocia 3-4 fold (Acker et al 1985).

1.4.6 Neonatal complications

The infant of the diabetic mother is at increased risk of a number of organ dysfunctions and biochemical disturbances. An increased incidence of respiratory dysfunction occur in part due to the increased incidence of preterm birth and caesarean section in this group of infants and in part to a hyperinsulinaemic inhibition of surfactant production (Bourbon and Farrell 1985). Hypertrophic cardiac myopathy may cause ventricular outflow obstruction. Hypoglycaemia occurs frequently and reflects poor maternal control during pregnancy and fetal hyperinsulinaemia. Polycythaemia occurs more commonly in these infants for the reasons previously described. Its associated hyperviscosity predisposes to necrotising enterocolitis, renal vein thrombosis, and in association with the relative liver immaturity described in these infants, neonatal hyperbilirubinaemia (Ylinen, Raivio and Termo 1981). Hypocalcaemia and hypomagnesaemia are two other complications described in diabetic neonates.

1.5 Management of pregnancy in women with type 1 diabetes

1.5.1 Pre-pregnancy clinics

Many of the maternal and fetal complications of pregnancy can be reduced by good maternal glycaemic control in pregnancy, however the fetal complications of spontaneous miscarriage and congenital malformation require good maternal glycaemic control at the time of conception. Significant maternal disease such as nephropathy and ischaemic cardiovascular disease may advance during pregnancy. For these reasons it is imperative that women optimise their diabetic management before pregnancy and be adequately counselled to the expected effect of pregnancy on their diabetes. Ideally all adolescent diabetic females should be given the relevant advice through their General Practitioner and diabetic clinic, however this is infrequently effective.

Steel and her colleagues in Edinburgh first demonstrated the need for specific pre-pregnancy clinics (Steel et al 1982). Pre-pregnancy clinics have been shown to be cost effective in human and financial terms (Scheffler et al 1992). Pre-pregnancy care allows evaluation of diabetic complications, communicates the advantages of good glycaemic control during pregnancy and provides support to establish this prior to conception by optimising diet, insulin therapy and glycaemic monitoring. Advice about management of ketonuria and hypoglycaemia is given. It is an ideal forum for smoking advice, Rubella immunisation and the commencement of folic acid supplementation to reduce the risk of fetal neural tube defects.

A highly successful pre-pregnancy clinic in the United Kingdom is that founded by Steel (1989). As the same physician runs the adolescent and pre-pregnancy service over 75% of women have specific pre-pregnancy counselling. Unfortunately it is recognised in other areas with less integrated access to pre-pregnancy care, only a third of diabetic women appear to attend for advice. Such a service may self-select the most motivated women who achieve good glycaemic control outwith pregnancy (Gregory and Tattersall 1992). Promoting better utilisation of pre-pregnancy care is fundamental if implementation of the St Vincent Declaration is to be achieved (Steel and Johnstone 1992).

1.5.2 Antenatal care of the diabetic woman

An experienced multidisciplinary team led by a named obstetrician and physician should provide comprehensive maternity care (SIGN publication no.9, 1996). This can only be achieved if the care of all diabetic women is centralised to one obstetrician within each hospital. Realistic individualised aims for each patient form the basis of a successful patient – staff relationship (Johnstone 1997).

Dietary advice should be available at all diabetic antenatal clinics. Folic acid supplementation should continue to 12 weeks gestation to reduce the risk of neural tube defects (MRC Vitamin Study Research Group 1991). Diabetes specialist nurses and midwifes have an important role in educating women on the need for home

blood glucose monitoring (4-6 times a day, aiming for a blood glucose concentration = 4-7 mmol/L) and intensive insulin regimes. They also counsel women how to cope with various pregnancy related complications such as hypoglycaemia and hyperemesis that may provoke ketoacidosis. Twenty-four hour access to emergency services should be provided and the accessed instructions should be explicit.

The diabetic physician should review glycaemic control at each visit and aid the specialist nurse/ midwife in altering intensive insulin regimes. Insulin regimes vary with the physician's preference; most prescribe an intensive regime of multiple preprandial doses of soluble insulin in combination to one dose of long acting insulin commonly administered at night. Pregnancy induced insulin resistance requires steady increases in insulin doses. Blood glucose profiles should be supplemented by glycated haemoglobin measurements performed at least monthly. If glycaemic control is being inadequately achieved intensive inpatient care may temporarily be required.

Fundoscopy during each trimester is advised but may be required more frequently in patients with poor glycaemic control, hypertension or advanced disease. Referral to an ophthalmologist is indicated when there is potential to rapid development of neovascularisation. The urine should be screened for protein at each visit. Twenty-four hour protein and creatinine excretion rates should be studied by the physician on a monthly basis if proteinuria develops. Treatment of established nephropathy might require the use of antihypertensive agents.

The obstetrician should provide standard antenatal maternal care. Including management of obstetric complications such as hyperemesis, ante-partum haemorrhage, pre-eclampsia and threatened preterm labour. Initiation of any therapy such as antihypertensives or steroid therapy for preterm delivery requires close liaison with the physician. Obstetrical input is however more specialised toward the fetus and the management and timing of delivery.

Visits to the antenatal clinic usually occur 2-4 weekly. More frequent review or inpatient care may be needed if concerns arise. The specialist nurse midwife should be able to provide telephone advice outwith clinic hours.

1.5.3 Fetal monitoring in diabetic pregnancy

Assessment of fetal wellbeing relies on an accurate estimation of gestational age. All pregnancies should have gestational age as determined by last menstrual period confirmed by first trimester ultrasound biometry. This booking scan also confirms fetal viability. The increased risk of fetal loss in diabetic pregnancies does not warrant repeated viability scans; however these should be performed if indicated clinically.

The increased incidence of structural abnormalities in diabetic pregnancies justifies the offer of routine fetal anomaly ultrasound screening to all women with diabetes during pregnancy. This is usually performed at 18 weeks gestation. This may detect

over 90% of major neurological abnormalities, but is effective in diagnosing only around 50% of cardiac abnormalities. To improve detection detailed structural assessment of the fetal heart may be repeated later in gestation.

The disorders of growth in diabetic pregnancies can be screened for by clinical assessment of fundal height, performed as part of the standard antenatal check. This is usually supplemented by serial ultrasound biometry in diabetic pregnancy due to the increased incidence of growth disorders. It is routine in many units to perform these fortnightly in uncomplicated pregnancies. Clinical examination and ultrasound biometry are however both relatively poor predictors of eventual birth weight in diabetic pregnancy. In a prospective study of 181 pregnancies clinical examination performed at 34 weeks gestation had a sensitivity of 42% for detecting fetal birthweight > 95th centile, ultrasound had a 46% sensitivity (Johnstone et al 1996). More complex derivations of fetal weight obtained by utilising an increased number of ultrasound derived measurements add little to the detection rate of macrosomia (McLaren et al 1995). It is estimated that only when fetal weight is 4700g can the clinician be 90% suspicious that fetal weight is at least 4000g on the basis of ultrasound assessment. The detection of increased fetal growth velocity may be valuable in promoting improved maternal glycaemic control and allows assessment of the safety of vaginal delivery.

Detection of reduced growth velocity is an indication to increase fetal monitoring. This may be performed by more frequent ultrasound assessments of fetal wellbeing: liquor volume, umbilical artery doppler assessments or biophysical scoring. Acute assessment of fetal wellbeing can be performed be cardiotocography. All these assessments have limited predictive value in non-diabetic pregnancy. In diabetic pregnancy when fetal decompensation may occur quickly their limitations should be recognised (Johnstone 1997).

Routine use of umbilical artery doppler measurements in diabetic pregnancies has not been shown to be of value in screening for adverse fetal outcome in the absence of intrauterine growth restriction (Johnstone et al 1992, Salvesen et al 1993).

1.5.4 Timing of delivery

Iatrogenic preterm delivery may be indicated by the development of fetal or maternal compromise during pregnancy. In the uncomplicated pregnancy there is controversy as to the optimal timing of delivery. (Drury 1986, Molsted-Pretersen and Kuhl 1986). Concern about the risks of late unexplained fetal stillbirth and macrosomia favours delivery before 38 weeks gestation, however this places the infant at risk from disorders of prematurity to which the diabetic infant is more prone.

1.5.5 Method of delivery

Women with diabetes have a high rate of caesarean section even after controlling for compounding factors (Remsberg et al 1999). Estimated fetal weight > 4.5kg may

indicate delivery by elective caesarean section (Landon et al 1990). Conway and Langer (1988) reported a 50% decrease in shoulder dystocia when they used ultrasound to detect an estimated fetal weight greater then 4.25kg at term and preformed elective caesarean section at this cut off. This increased their caesarean section rate in diabetic pregnancies by 15%.

1.5.6 Management of delivery

Due to the higher risks of intrauterine asphyxia continuous fetal monitoring should be performed during labour by cardiotocography. This should be supplemented with fetal scalp pH measurements as indicated, and there should be a lower threshold to resort to caesarean delivery if there is suspected fetal distress. An experienced obstetrician should be available for delivery due to the increased risk of shoulder dystocia. This is particularly indicated after slow progress in labour or for an assisted delivery.

Maternal glycaemic control is achieved by an intravenous infusion of glucose and insulin. This should be adjusted on the basis of at least hourly blood glucose estimations (Beard and Maresh 1989).

1.5.7 Post-partum management

With delivery of the fetus and placenta maternal insulin sensitivity rapidly reverts to the non-pregnant state. The insulin/glucose infusion can be discontinued after vaginal delivery and the women re-established on her pre-pregnancy regime with her next meal. The infusion is usually continued after caesarean delivery. Insulin may not be required for many hours, its need and dose should be assessed by regular capillary glucose measurements.

A paediatrician skilled in resuscitation should be present at the delivery particularly if maternal glycaemic control has been poor. The risk of congenital abnormalities should be recognised and respiratory rate monitored as an indicator of cardiac diseases or respiratory compromise due to reduced surfactant production. The neonate requires early feeding and should be closely observed for hypoglycaemia. Formal capillary heal testing is usually performed within the first 1-2 hours of life.

Chapter 2

Vascular endothelial dysfunction in diabetes

2.1.1 Introduction: glycaemic control and diabetic vascular complications

If there is one single systemic factor implicated in aetiology of the microvascular and macrovascular pathologies of diabetes it is hyperglycaemia. The relationship between the degree of glycaemic control achieved with insulin and the development of diabetic vascular complications has been extensively studied. Initial clinical studies in humans were flawed by the inability to accurately and objectively quantify the degree of glycaemic control and the severity of the vascular complications. These studies were retrospective or non-controlled non-randomised studies. With time it has become apparent that the degree of glycation of various plasma and cellular proteins, haemoglobin in particular, reflects an integrated measurement of antecedent blood glucose. This development coupled with the introduction of widespread reliable techniques for self-monitoring of blood glucose have enhanced the ability to assess glycaemic control reliably. New techniques of insulin delivery and more complex regimes have enabled motivated diabetic populations to approach euglycaemia. In addition quantitative assessments of diabetic microvascular complications have been developed. These advancements have aided the study of glycaemic control and diabetic complications.

The mechanism through which diabetes is causally related to vascular pathology is yet to be fully elucidated. Vascular endothelial dysfunction is implicated and is likely to play a central role. Endothelial dependent vasodilatation of blood vessels obtained from diabetic subjects and of blood vessels from non-diabetic subjects exposed to hyperglycaemia is abnormal. Intensive study of endothelial cell function both in-vitro and indirectly in-vivo has suggested many biochemical mechanisms that could be involved.

Utilising knowledge of these mechanisms studies of endothelial function are valuable in assessing the response of diabetic subjects to differing treatments such as intensified insulin regimes and different physiological conditions including pregnancy.

2.1.2 Clinical studies of glycaemic control

Whole animal studies provided the first convincing evidence that the clinically detectable vascular complications of diabetes are a direct consequence of metabolic derangement in general and suggest the role of hyperglycaemia in particular. A number of animal models of diabetes exist. The chosen species is made diabetic by administration of specific islet toxins: Steptozotocin (STZ) in rats and dogs, or

Alloxan in rabbits. In these whole animal studies microvascular pathology is more amenable to clinical study than macrovascular disease. This reflects the short time course of many studies and accessibility of the pathology to visualisation and quantification. In addition animal models may not perform well as models of human macrovascular disease. The rat model in particular appears to be relatively protected against the development of atheroslerotic macrovascular disease.

The most notable whole animal studies were performed by Engerman and colleagues using a canine model (Engerman and Kern 1986 & 1987), the retina of which are highly comparable to that of the human. After the induction of diabetes, the dogs were randomly assigned to 5 years of either poor control, a single daily injection of insulin and an unrestricted diet, or good control, using twice daily insulin injection prior to two prescribed meals. The retinae were studied in life by indirect opthalmoscopy, fundal photography and fluorescein angiography and after sacrifice by light and electron microscopy. At the end of the 5-year period there was significant increase in the frequency of all studied retinal lesions in the poorly controlled dogs compared to well-controlled dogs. The central role of hyperglycaemia in particular, rather than other metabolic disturbances in general, in the aetiology of these vascular lesions was supported by the similar retinal response in dogs fed high galactose diets. In these dogs the level of glycated haemoglobin was increased whilst lipids and branched chain amino acids were not.

For many years there was a paucity of high quality human data regarding glycaemic control and the incidence or progression of vascular diseases despite numerous studies. Retrospective and prospective uncontrolled studies noted a correlation between blood glucose or HbA1c and the development and extent of microvascular disease but by their nature could not address any causative role. More recently several small prospective studies and one large prospective randomised controlled clinical trial have examined the effect of glucose control on the progression of diabetic microvascular complications.

The smaller studies, including those from the Oslo, Steno and Kroc groups, were unable to convincingly demonstrate a beneficial effect of near normoglycaemia on the vascular complications of diabetes. The Oslo study (Dahl-Jorgensen et al 1986) followed 45 insulin dependent diabetic subjects randomised to either one of two intensive insulin regimes (continuous insulin infusion or multiple (5-6)-insulin injections/day) or to conventional twice-daily insulin injections. Mean blood glucose after 2 years was 5.3 mmol/L, 6.4 mmol/L and 7.9 mmol/L and HBA1c levels were 8.7%, 9.1% and 10.2% in the respectively treated groups. After two years fewer retinal microaneurysms and haemorrhages were seen in the two intensively treated groups compared to the conventionally treated group, however this difference was not maintained at 4 year follow up. Mean urinary albumin secretion did not change or differ significantly between groups. The Steno study (Lauritzen et at 1985) randomised 30 patients with advanced background retinopathy to continuous subcutaneous insulin infusion [CSII] or conventional therapy. Mean blood glucose and HBA1c values were lower in the intensively treated group. At two years 4 patients in the CSII group and 5 in the conventional treatment group developed proliferative retinopathy. In a similar study of non-proliferative retinopathy the Kroc

collaborative study group 1984) randomised 70 diabetic subjects to CSII or conventional therapy was unable to demonstrate a significant benefit of intensive therapy on diabetic microvascular complications at both 8 months and 2 years of follow up.

The inability of these studies to demonstrate the role of poor glycaemic control in the development of diabetic vascular dysfunction has subsequently been shown, with the publication of the Diabetes Control and Complications Trial (1993), to reflect their small size, limited follow up periods and failure to address primary prevention.

The Diabetes Control and Complications Trial (The DCCT Research Group 1993) is the most important study of diabetic control and vascular complications to date. This was a large prospective randomised clinical trial. It studied 1,441 non-obese diabetic subjects recruited from 29 clinical centres during 1983-89. The subjects aged between 13-39 years were spit into two cohorts to address the questions: Will intensive therapy prevent the development of diabetic microvascular disease? (primary prevention); and will intensive therapy affect the progression of early microvascular disease? (secondary prevention). Patients with diabetes of 1-5 year's duration without retinopathy or microalbuminaemia participated in a primary prevention trial. Patients with diabetes of 1-15 year's duration, who had already mild to moderate non-proliferative retinopathy and mild/moderate nephropathy (<2000mg/day albumin excretion), participated in a secondary prevention trial. In both trials the patients were randomly assigned to receive conventional treatment (no more than 2 insulin injections per day) or receive intensive insulin therapy (3-4 insulin injection/day or CSII; self-monitoring of blood glucose at least 4 times per day). The subjects were followed an average of 6.5 years. The intensively treated groups maintained a mean HbA1c of 7.2% over this time period compared to a HBA1c of 8.9% in the conventionally treated group.

Over the follow up period intensive treatment produced substantial benefits. The risks of de novo development (primary prevention trial) or of progression (secondary prevention trial) of retinopathy were reduced by 76% and 54% respectively. In the two trials combined the development of microalbuminuria was reduced by 35% and of macroalbuminuria by 56% in the intensively treated cohorts.

The follow up period of the DCCT, 6.5 years is inadequate to fully determine the effects of glycaemic control on the development of macrovascular pathology. The calculated risk reduction for all macrovascular events combined (myocardial infarction, angina, and peripheral vascular disease) was 41%, but this change was not statistically significant (The DCCT Research Group 1993).

2.2.1 The role of the endothelium in vascular pathology

The endothelium is a key regulator of vascular function (Furchgott and Zawadzki 1980). Vascular pathologies in general frequently reflect specific endothelial pathologies. The endothelium is not, even under resting physiological conditions, simply the inert lining of blood vessels. It forms an active interface between the blood and the underlying tissue.

In the 'resting' state the endothelium regulates vascular tone and maintains blood fluidity by inhibiting coagulation and the adhesion of blood leukocytes and platelets. It also maintains a selective permeability to proteins and fluid. In response to a vast array of circulating stimuli such as infection or tissue injury the endothelial cell becomes activated. Activation of the endothelium can alter vessel tone, promote thrombus formation and the adhesion of blood cells such as neutrophils and platelets to its surface, and increase the transmigration of fluid and cells into the underling tissue. These functions are mediated through the production of numerous regulatory substances (Wu and Thiagarajan 1996).

Molecules important in maintaining the inactivated thromboresistant 'resting' state of the endothelium include prostacyclin (PGI2) and nitric oxide (NO). These molecules are secreted from the endothelium and act in a paracrine fashion to inhibit platelet and leukocyte adhesion and activation. They also exert a vasodilatory action on smooth muscle cells, which determines resting vascular tone (Moncada and Vane 1979, Moncada et al 1991). The endothelium synthesises and expresses on its surface other specific anticoagulant factors such as thrombomodulin (Esmon 1993) and heparan sulphate (Rosenberg 1994). The complex of thrombomodulin and thrombin acts as a catalytic surface for generation of activated protein C. It also binds and inhibits thrombin and factor Xa. Heparan sulphate, a proteoglycan expressed on the surface of the endothelium functions as a cofactor for antithrombin III enhancing substantially its ability to bind thrombin. The endothelium can also promote fibrinolysis by synthesis of tissue plasminogen activator (tPA).

In the activated state the endothelium may modify the secretion and expression of these factors in addition to provoked expression or secretion of prothrombotic and vasoactive molecules. von Willebrand factor produced by the endothelium is pivotal in haemostasis and thrombosis (Handlin and Wagner 1989). It acts as the essential ligand in platelet adhesion. It also binds and stabilises the activity of factor VIII. The endothelium may choose to inhibit fibrinolysis via synthesis and release of plasminogen activator inhibitor (PAI-1). Vasoactive factors produced by the activated endothelium include endothelin, prostaglandin endoperoxide (PGH2) and thromboxane (TXA2). Lipid based activators i.e. platelet activating factor (PAF) and chemotactic cytokines such as interleukin 6 and interleukin 8 may also be produced. The endothelium has also been recently shown to express specific cell adhesion molecules. These are intimately involved in the recruitment of leukocytes to the endothelial surface (Figure 2.1), which can lead to further endothelial cell activation.

Endothelial cell activation is not an all or nothing response. The endothelium orchestrates it metabolism in response to the nature, strength and duration of multiple

stimuli. It perpetually modifies this response by interacting with other cells in its environment such as neutrophils, which may or may not have been exposed to the same or additional stimuli.

Excessive stimulation of the endothelium such as chronic exposure to a variety of stimuli, including cytokines, microbial toxins and immunologic agents is likely to occur in disease states and to underlie specific vascular pathologies. Such abnormal stimulation of the endothelium is often termed endothelium dysfunction. In this state the normal vasodilatory and thromboresistant actions of the endothelium appear to be overwhelmed or lost.

2.2.2 The neutrophil and endothelial function

The neutrophil is a circulating leukocyte the main function of which is to mediate acute inflammation by accumulating in tissues damaged physically, chemically or by toxins. Neutrophils therefore intimately interact with the endothelium. Neutrophil activation may be a consequence or cause of endothelial activation.

Once activated neutrophils can further endothelial activation or disease states, and have been implicated in endothelial dysfunction and vascular damage (Harlan 1987). Activation of neutrophils can trigger their degranulation and release of elastase and other proteases. These enzymes can destroy the integrity of the endothelial cell layer, vascular basement membrane and the subendothelial matrix. The neutrophil may also release toxic oxygen radicals which can result in lipid peroxidation and endothelial cell damage. Leukotrienes produced via arachadonic acid metabolism in the neutrophils may exert a vasoactive function via the endothelium and may promote activation of further neutrophils (Bray 1983). Endothelial damage promotes endothelial expression of further molecules including specific cell adhesion molecules which will further the inflammation response recruiting not only neutrophils but also monocytes and lymphocytes to the area.

2.2.3 Cell adhesion Molecules: structure and function

Expression of cell adhesion molecules provides an important mechanism by which a cell can interact with its environment. The environment of any cell is a complex mix of potential signals. The endothelial cell for example is exposed to circulating soluble molecules, circulating cells, neighbouring endothelial cells and insoluble molecules that form the matrices of tissues. The cell can sample its environment via many surface molecules that form part of its cell membrane. Of these molecules cell adhesion molecules are principally responsible for interaction with other cells and matrix molecules.

Adhesion molecules were so named because they were discovered to effect adhesion of one cell to another or to its neighbouring matrix. The existence of these molecules followed studies of leukocyte diapedesis from blood to tissue at sites of inflammation and in the study of cell migration in embryology. Increasing study of these molecules

has shown that they are involved in a diverse range of other biological events. It has also demonstrated that they do not simply act to effect adhesion rather they effect cell-cell or cell-matrix interaction in which cell-cell or cell-matrix attachment is often only the first step.

A vast number of different cell adhesion molecules are now recognised. Rationalisation of these molecules has enabled them to be classified into six families on the basis of chemical, structural or functional studies: the immunoglobulin-like superfamily, the cadherins, the integrins, the receptor protein phosphatases, the selectins and the hyaluronate receptors. The basic structure of the integrin, selectin and immunoglobulin cell adhesion molecules is shown in Figure 2.2.

The immunoglobulin superfamily are so classified because they all contain one or more of a common Ig–like repeat within the extracellular domain, however they exhibit a broad range of functional diversity. Members of this family play a critical role in the development of the nervous system, in immune and inflammatory responses and in embryonic development (Springer 1990, Tessier-Lavigne and Goodman 1996).

The cadherins represent a family of adhesion molecules that play a critical role in cell-cell interactions. They are involved in embryonic development, formation of the epithelial layers of skin and intestine, and axonal formation in the nervous system (Takeichi 1990).

The integrins regulate both cell-cell and cell-extracellular matrix protein interactions. They play a key role in organogenesis, tissue remodelling, thrombosis and leukocyte migration (Shattil and Ginsberg 1997, Springer 1994, Hynes 1992). Integrins are composed of two subunits, α and β, and are assigned to subfamilies according to the type of these subunits. $\beta 2$ integrins are restricted to leukocytes and are important in their interaction with the endothelium.

Hyaluronate receptors are classified functionally rather than structurally. Members of this group interact with hyaluronate, an abundant structural saccharide component of extracellular matrices that is believed to play an important role in a variety of physiological and pathological tissue processes including inflammation, cell growth, cell migration and tumour progression.

Selectins are the most recently discovered group of adhesion molecules. They play a key role in the recruitment of leukocytes from the circulation to sites of inflammation and are important in fighting infection and in wound healing (Springer 1994, Carlos 1994). The selectins are divided into subgroups E, P and L denoting their association with endothelial cells, platelets or leukocytes respectively.

Despite this diversity, all cell adhesion molecules share three important characteristics (Freemont 1998). First, they are all glycoproteins and all act as a molecular link between the outside and inside of the cell. As such they are in general membrane-spanning proteins with extracellular, intramembranous and cytoplasmic domains. Second, all adhesion molecules 'work' by an external stimulus attaching to

the extracellular domain and altering its structure. All but hyaluronate receptors are involved in cell-cell interactions. Cells of the same or differing origins can interact (homotypic or heterotypic adhesion). Integrins and hyaluronate receptors are involved in cell-matrix interactions. The molecule that binds to the cell adhesion molecule is very specific and is known as its receptor or ligand. The ligands for cell adhesion molecules are other cell adhesion molecules either of the same or different class, or specific matrix molecules. Third, cell adhesion molecules are attached to other molecules within the cell through which they are able to influence the function of the cell. The cytoplasmic domain effects either cell adhesion molecule linkage to the cytoskeleton (cadherins and beta 1 and beta 3 integrins) or linkage to and activation of second messenger systems involved in cytoplasmic or nuclear metabolism. The second messenger systems activated are not specific but the same messenger systems that are activated by many other receptor-ligand binding systems such as cytokine-cytokine receptors (Schwartz et al 1994).

As well as allowing signal passage from the outside of the cell to the inside in response to ligand binding, cell adhesion molecules are also involved in the passage of information from the inside of the cell to the outside usually by modifying their ligand binding affinities.

2.2.4 Cell Adhesion Molecules and endothelial function

Cell types differ in their function and therefore differ in their membrane repertoire of cell adhesion molecules. Endothelial cells need to interact with circulating soluble molecules and circulating blood cells principally leukocytes and platelets. Cell adhesion molecules clearly play an important role in the interaction of the endothelial cell with circulating leukocytes (Bevilacqua 1993).

Three of the main endothelial expressed cell adhesion molecules associated with leukocyte activation are: E-Selectin, a member of the selectin family of cell adhesion molecules, intercellular cell adhesion molecule-1 (ICAM-1) and vascular endothelial cell adhesion molecule-1 (VCAM-1) from the immunoglobulin superfamily.

E-Selectin and ICAM-1 interact with a number of leukocytes including neutrophils. E-Selectin is an endothelial glycoprotein that consists of a carbohydrate recognition domain, an epidermal growth factor repeat and a number of complement regulatory repeats. It is expressed in low levels on the endothelial cell surface but upon activation of the cell its synthesis is increased along with its transportation to the cell surface. It is synthesised and expressed in response to inflammatory stimuli including the cytokines interleukin 1 (IL-1) and tumour necrosis factor (TNF) in addition to bacterial endotoxin. Its expression reaches a maximal level after 4-6 hours of endothelial stimulation. Endothelial expression of E-Selectin provokes the initial loose attachment of the leukocyte to the endothelium via weak bonds with its ligand. In the presence of fluid sheer stress, these weak bonds appear to allow the subsequent rolling of the leukocyte along the endothelial surface (McEver 1991, Lasky 1992 and Bevilacqua 1993). The ligands of the selectins are still subject to intense investigation (Varki and Nelson 1997) and are believed to be cell surface glycans

that possess a specific sialyl-LewisX-type structure that is also seen in blood group antigens.

Intercellular cell adhesion molecule-1 is a member of the immunoglobulin-like superfamily. Its structure contains five immunoglobulin domains. It expression on the endothelium is unregulated in response to Il-1 TNF and endotoxin. The half-life of its expression at a few days is much longer than that of E-Selectin. Leukocyte adhesion to ICAM-1 occurs after interaction of the leukocyte to E-Selectin. ICAM-1 mediates firm leukocyte adherence through interaction with its integrin ligands expressed on the leukocyte surface. Leukocyte integrin activation appears to signal a change in leukocyte shape so that it stretches out along the endothelial surface and may aid transendothelial migration (Springer 1990).

Vascular endothelial cell adhesion molecule-1 is similar in structure to ICAM-1. It is expressed in two forms containing six or seven Ig repeats. Its expression is up-regulated by IL-1, IL-4, TNF and endotoxin over a similar time period as ICAM-1. It binds to two integrin family members, $alpha_4beta_1$ and $alpha_4beta_7$ through which it supports the adhesion and interaction with lymphocytes, monocytes and eosinophils with the endothelium (Prober 1990, Petruzzelli 1999). It does not interact with neutrophils, as they do not express its specific ligands.

2.3.1 The study of endothelial function

Studies of endothelial function can be performed in several ways. Macroscopic vessels can be biopsied and studied in-vitro in tissue baths. These studies, on the whole, assess the ability of the endothelium to regulate vascular tone. Altered endothelium function is inferred from altered response to known vasodilators that primarily exert their effect via the endothelium (acetylcholine and ADP), compared to vasodilators acting directly on smooth muscle cells (sodium nitroprusside).

Release of mediators from endothelial cells in response to specific 'pure' stimuli such as hyperglycaemia can only be assessed in vitro using these isolated vessel preparations or by studying endothelial cell cultures. These studies are required to unravel the complex biochemistry of endothelial function.

As the endothelium interacts continually with its complex environment, which contains mixed stimuli, in-vivo studies of endothelial function would be ideal. However in-vivo studies are limited primarily to the assay of circulating factors released by the endothelium or to the study of circulating cells such as platelets and neutrophils, whose functions are known to be closely related to that of the endothelium. In-vivo studies of the vasoactive function of the endothelium have recently been possible by techniques such as venous plethosmography or laser doppler, however only perfusion changes in superficial tissue beds are accessible to study.

2.3.2 Circulating concentration of cell adhesion molecules: a novel marker of endothelial function?

ELISAs capable of detecting soluble forms of cell adhesion molecules in peripheral blood or in supernatants of cytokine activated endothelial cells have been developed (Gearing et al 1992). These circulating cell adhesion molecules appear to be structurally identical to those expressed on the cell surface suggesting that they arise from proteolytic cleavage of these molecules. Circulating concentrations of endothelial expressed cell adhesion molecules such as ICAM-1, VCAM-1 and E-Selectin are detectable in healthy subjects and may reflect the cumulative effect of multiple small stresses on the endothelium occurring during a normal day. The exact mechanism of their production and the physiologic function of these shed molecules remain unclear. They may reflect an excretory process of the endothelium by which it down-regulates its adhesivness. In the circulation they may function to limit the adhesion cascade by blocking leukocyte ligands. Alternatively they may upregulate recruitment of leukocytes to the endothelium through ligand occupancy and cell activation.

Elevated circulating concentrations of cell adhesion molecules have been found in a number of inflammatory and vascular disease states including hypertension, chronic renal failure and diabetes and appear to provide useful information about disease activity (Gearing et al 1992). As a measure of endothelial activation and / or dysfunction E-Selectin expressed exclusively by the endothelial cell has the potential

to perform as a selective marker. Raised levels have been reported in variant angina, ischaemic heart disease and diabetes (Gearing et al 1992, Miwa et al 1997, Blann et al 1996). Increased levels may provide prognostic information and have been demonstrated to predict progression of peripheral atherosclerosis (Belch et al 1997). ICAM-1 and VCAM-1 are expressed by other cells in addition to the endothelium; therefore some caution may be required in utilising the circulating concentrations of these markers as an index of endothelial activation. Increased levels have been found in specific cardiovascular pathologies (Blann and Lip 2000) suggesting that their assay may nevertheless be of prognostic value.

2.3.3 Evidence of endothelial dysfunction in diabetes

2.3.3.1 Whole vessel studies of endothelial function in diabetes

Reduced endothelial dependent relaxation has been shown to occur in numerous in-vitro studies of isolated arteries from diabetic animal models. Using isolated vessel preparations most investigators have demonstrated a reduced vasodilatory response of blood vessels from diabetic animals to acetylcholine (Oyama et al 1986, Kamata et al 1989). In these vessels acetylcholine is known to exert its vasodilatory response by stimulation of endothelial nitric oxide synthase and NO production. NO then acts on the smooth muscle cells to stimulate guanylate cyclase, increase cyclic GMP and effect vasodilatation. By demonstrating a normal vasodilatory response to exogenously replaced NO donors, the above studies suggested that the abnormal vasodilatory response lay at the level of the endothelium. Normalised vasodilatory responses of aortic rings have been shown in diabetic rats models treated with insulin confirming the role of diabetic metabolism in vascular dysfunction (Takiguchi 1988).

Few studies have assessed the effect of diabetes on smaller vessels in in-vitro. Using the technique of small vessel "myography" resistance sized arteries (250μm in diameter) have also been shown to demonstrate abnormal relaxation to acetylcholine when prepared from a diabetic rat model (Taylor et al 1992, Poston and Taylor 1995). In a cohort of diabetic rats who had their glycaemic control restored by insulin implants the endothelial dysfunction of these small vessels was reduced (Taylor 1994). Studies of small vessels from human diabetic subjects appear to support this work. Blunted responses to acetylcholine have been shown to occur in corpus cavernosum obtained from diabetic men compared to non-diabetic men (De Tejada et al 1989) and in isolated small arteries from subcutaneous fat biopsies obtained from diabetic compared with non-diabetic subjects (McNally et al 1994).

The use of forearm venous plethosmography in studies of diabetic subjects have produced conflicting results. This is probably reflective of differing study designs. They do although in general support abnormal vascular function in diabetes. Two studies of the vascular response to nitric oxide synthase inhibitor have demonstrated poorer vasoconstrictor effects in diabetic subjects, suggesting a reduced basal release of NO in these subjects (Calver et al 1992, Elliot et al 1993).

2.3.3.2 Plasma markers of endothelial dysfunction in diabetes

A vast range of agents synthesised by the endothelium have been measured as circulating concentrations by assays performed on peripheral blood. Raised concentrations of agents such as angiotensin converting enzyme (Schmitz 1985), Tissue plasminogen activator (Jensen 1989), endothelin (Takahshi 1990) and von Willebrand factor (Stehouwer 1991) have been found in diabetic subjects and provide indirect evidence of endothelial activation or dysfunction in diabetes.

Circulating levels of specific cell adhesion molecules have been demonstrated to be increased in a number of studies on diabetic subjects. Gearing at al (1992) included 31 diabetic subjects on which to test their assay of circulating cell adhesion molecules. Significantly raised mean concentrations of E-Selectin (23.4 units/ml) and ICAM-1 (94.5 units/ml) were found in these diabetic subjects compared to the mean values detected in healthy non-diabetic subjects (E-Selectin = 10.8 u/ml, ICAM-1 = 56.5 units/ml). They also demonstrated a smaller but still significantly elevated circulating concentration of VCAM-1 in diabetic (69.6 u/ml) compared to non-diabetic subjects (54.7 u/ml). Lampeter at al (1992) studied circulating concentrations of ICAM-1 and L-Selectin (expressed by lympocytes). They demonstrated increased concentrations of these molecules diabetic patients and family members at risk of developing diabetes (HLA-DR-3 and DR4+).

Further indirect evidence of endothelial dysfunction in diabetic subjects is derived from studies of circulating neutrophils and platelets. Circulating concentrations of neutrophil elastase provide an indirect measure of neutrophil activation and degranulation in vivo. The concentration of neutrophil elastase is normally very low. Raised concentrations are a potential index of vascular pathology. In previous studies our group has detected significantly raised circulating concentration of neutrophil elastase in diabetic non-pregnant women compared to non-diabetic non-pregnant women (Greer et al 1989) suggesting increased in-vivo activation of neutrophils as a consequence of diabetes.

Platelets like neutrophils interact closely with the endothelium. Activation of neutrophils and platelets therefore share a number of common pathways involving endothelial activation. They can also directly interact with each other. Evidence of increased platelet reactivity in diabetes has been suggested by studies on platelet rich plasma preparations prepared from blood drawn from diabetic subjects (Halushka et al 1983).

2.4.1 The aetiology of vascular dysfunction in diabetes: hyperglycaemia

Numerous studies have aimed to determine the specific underlying aetiology of vascular pathology in diabetes. Evidence that the vascular dysfunction may be directly related to glucose concentration and not other metabolic derangement is suggested by in-vitro studies of blood vessels.

Rings of isolated normal rabbit aorta when exposed to elevated media concentrations of glucose develop an abnormal relaxation to acetylcholine and ADP (Tesfamariam et al 1990). This impaired vascular response characteristically mimics the abnormal response rings aortic tissue from rabbits made diabetic by treatment with the alloxan. (Tesfamariam and Cohen 1991). By altering the concentration of glucose to which the vessel was exposed it was demonstrated that the resultant altered vascular reactivity was concentration and time dependent. Exposure of pig coronary arteries to elevated glucose concentrations has produced similar findings (Cohen and Tesfamariam 1992).

2.4.2 Mechanisms of hyperglycaemia induced endothelial dysfunction

Glucose and its metabolites are utilised in a number of intracellular pathways therefore there are many mechanisms by which hyperglycaemia may result in cellular dysfunction (Cohen 1993). These mechanisms have been investigated primarily by utilising endothelial cell culture preparations. The most investigated of these mechanisms include: (1) increased cellular sorbitol concentrations, (2) increased cellular NADPH/NADP ratio, (3) altered protein kinase C activity and (4) non-enzymatic glycation.

In most cells including the vascular endothelium glucose can be converted to sorbitol via the enzyme aldose reductase. This is a rapid reaction, however the subsequent metabolism of sorbitol to fructose occurs at a slower rate. Therefore hyperglycaemia increases cellular sorbitol concentrations. As sorbitol does not readily diffuse across cell membranes accumulation may cause dysfunction via an osmotic effect (Gonzalez 1984). Sorbitol also competitively inhibits myoinositol uptake resulting in abnormal inositol signalling. (Green 1987). Synthesis of sorbitol through the polyol pathway utilises NADPH resulting in a reduced NADPH / NADP ratio. Depleted cellular NADPH may cause oxidative stress, as it is required for the antioxidant activity of glutathione reductase. Indeed a number of free radical scavengers have been shown to prevent impaired endothelium dependent relaxation caused by elevated glucose (Tesfamariam and Cohen 1992). NADPH is also required for the action of nitric oxide synthase and therefore sorbitol formation may reduce endothelial cell NO production.

Cell culture studies demonstrate an increased activity of protein kinase C (PKC) when exposed to elevated glucose concentrations (Lee et al 1989). Phosphorylation by PKC is a key mechanism of intracellular signalling known to regulate the activities of many cellular processes including prostanoid production (Tesfemariam et al 1991) and possible inactivation of nitric oxide synthase.

Another mechanism by which hyperglycaemia may cause cellular dysfunction is by the formation of advanced glycosylation end products (ACEs). Glucose can form covalent bonds by non-enzymatic glycation, which may over time lead to the formation of ACE products in an irreversible reaction. (Brownlee 1988). This may occur in the extracellular pool affecting plasma proteins such as low density

lipoprotein (LDL), albumin and haemoglobin. Accumulation of ACE products within basement membranes renders them resistant to degradation and may therefore lead to thickening of the basement membrane.

ACE formation may alter surface ligand conformation and therefore disrupt signalling. They may cross-link DNA nucleotides and have a deleterious effect on DNA breakage and repair. The binding of ACEs to macrophages triggers an increase in the release of cytokines such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) which increase vascular permeability and alter coagulation homeostasis (Vlassara 1989). ACEs may bind to specific receptors. Receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules can be expressed by the endothelium. Occupancy of this receptor may activate cellular second messenger systems (Schmidt et al 1999). ACEs also quench the effect of nitric oxide in its vasodilator action. Recently ACEs have been shown to mediate prolonged activation of endothelial nuclear factor κβ (Bierhaus et al 1997). Activation of nuclear factor κβ is one of the final steps via which the vast range of intracellular cytoplasmic second messenger systems exert their effect on nuclear transcription. Acting in this way ACEs could chronically drive endothelial activation. Inhibition of ACE formation in animal models is associated with reduced diabetic vasculopathy (Bacala et al 1991).

In addition to the complex interacting mechanisms discussed above, all of which would lead to an endothelial vasopressor response to hyperglycaemia, there is some evidence that hyperglycaemia may exert a vasodilatory effect on the endothelium. Acting to raise endothelial cell calcium, D-glucose may increase the synthesis of NO and prostacyclin (Graier et al 1993). Increased vasodilatation has been documented to occur in the early stages of diabetes. This vasodilatation will increase sheer stress, which may initially promote further release of vasodilatory factors from the endothelium but in the longer term increased shear is likely to result in vascular damage. This has been postulated as an alternative primary cause of vascular damage in diabetes. Prolonged exposure to elevated glucose acting in this manner would be difficult to distinguish from the other mechanisms of hyperglycaemic vascular dysfunction discussed above.

2.4.3 Alternative mechanisms of endothelial dysfunction in diabetes.

Diabetes causes a disorder of other metabolic pathways other than that of glucose. Disorders of lipid metabolism have been implicated in the aetiology of vascular disease particularly macrovascular arteriosclerosis and is likely to contribute to the vascular pathology of diabetes. Diabetic subjects have been shown to have elevated hepatic synthesis of very-low-density lipoprotein (VLDL), leading to raised triglyceride and decreased high-density lipoprotein (HDL) levels. Low density lipoproteins are particularly sensitive to oxidation and oxidised LDLs are recognised to be potent stimuli of leukocyte adhesion to endothelium through stimulation of neutrophil adhesion receptors CD11B/CD18 (Lehr et al 1995). Oxidised LDLs may also trigger endothelial dysfunction directly. There is evidence that this lipid

derangement may be the result of raised systemic insulin levels (in treated type 1 diabetics and in type 2 diabetics). Insulin stimulates hepatic production of LDLs. Experimental effects of hyperinsulinaemia include proliferation and migration of vascular smooth muscle cells, cholesterol synthesis and cellular binding of LDL, leading to arterial wall thickening and atheromatous lesions (Stout 1990). High insulin levels are also associated with inhibition of fibrinolytic processes, mainly though an effect on PAI-1 (Juhan-Vague et al 1989).

2.5.1 Pregnancy and endothelial function

Uncomplicated pregnancy occurring in healthy non-diabetic woman is associated with altered endothelial function as suggested by documented changes in vascular activity, and both the closely related coagulation and fibrinolytic systems (Greer 2000). In normal pregnancy the vasculature has to adapt to an increased circulating volume and it accommodates this in association with a fall in blood pressure. This must occur via vasodilation. Normal pregnancy is associated with enhanced NO release and prostacyclin production (Lopez-Farre et al 1995).

There is evidence of a low grade of disseminated intravascular coagulation with increased coagulation, fibrinolysis and platelet activation which involve, as a cause or consequence, altered release of factors from the endothelium. The coagulation factors VIII, X, XII and von Willebrand factor are increased in pregnancy. Von Willebrand factor is synthesised by the endothelium (Stirling et al 1984).

Fibrinolytic activity is impaired during pregnancy (Halligan et al 1994). This is largely the consequence of placental derived plasminogen activator inhibitor Type 2 (PAI-2) but there is also increased concentrations of the endothelial derived inhibitor of plasminogen activator (PAI-1). Tissue plasminogen activator (t-PA) which is derived from the endothelium also increases in normal pregnancy, as do plasminogen concentrations.

Platelet activation is suggested by in-vitro studies of platelet rich plasma prepared from pregnant subjects and is supported by evidence of increased platelet degranulation (increased circulating thromboglobulin levels) in vivo (Douglas et al 1982). Studies of platelet aggregation in whole blood (more reflective of their normal milieu than plasma preparations) have however in contrast not demonstrated increased aggregation in healthy women as a consequence of pregnancy (Greer et al 1988).

Increased concentrations of plasma neutrophil elastase have been demonstrated in uncomplicated pregnancies. Suggesting that normal pregnancy is associated with a degree of increased neutrophil activation in pregnancy (Greer et al 1989).

The cause of this altered intravascular state and endothelial function in normal pregnancy is unknown but the actions of the hormone oestrogen and altered lipid profiles have been proposed as aetiological factors.

2.5.2 Endothelial dysfunction and preeclampsia

Pre-eclampsia is the most common and most extensively studied vascular pathology occurring in pregnancy. Endothelial dysfunction is considered to be a key pathological process underlying this condition (Greer 2000). Reduced production of prostacyclin has been documented, as has a related tendency to vasoconstriction with increased vascular responsiveness to angiotensin II. Circulating endothelial-derived factors such as von Willebrand factor, tPA and PAI are increased, as are circulating concentrations of cell adhesion molecules, endothelin and fibronectin.

Associated with this endothelial dysfunction there is evidence of increased platelet and neutrophil activation in preeclampsia. Neutrophil activation is suggested by increased circulating concentrations of neutrophil elastase (Greer et al 1989). Elastase levels correlated with that of uric acid, a marker of disease severity and with von Willebrand factor concentrations, a marker of endothelial damage (Greer et al 1991). The circulating platelet count is often reduced in preeclampsia and platelet derived β-thrombomodulin is increased suggesting platelet activation and consumption in preeclampsia. Our group has studied platelet aggregation in vitro using whole blood samples. Increased platelet aggregation was observed in blood drawn from women with preeclampsia (sampled between 28-39 weeks gestation) compared to blood drawn at a similar mean gestation from matched uncomplicated pregnant woman (Greer et al 1988).

Our group has also previously studied circulating concentrations of defined endothelial cell adhesion molecules in preeclampsia (Lyall et al 1994). Raised levels of VCAM-1 (mean (SE): 842(50) ng/ml) were demonstrated in the plasma of women whose pregnancies were complicated by preeclampsia when compared to matched controls (mean (SE): 560(48) ng/ml). Circulating concentrations of E-Selectin and ICAM-1 were not significantly different in pre-eclamptic subjects compared to non-pre-eclamptic subjects.

2.5.3 Diabetic pregnancy and endothelial dysfunction

Studies of endothelial dysfunction in pregnancies complicated by maternal diabetes are presently limited. Plasma endothelin-1 concentrations have been studied in diabetic pregnancy and found to be increased compared to those measured in non-diabetic pregnancies (Wolf et al 1997). By performing platelet aggregation studies in whole blood our unit has assessed platelet function in diabetic pregnancy (Greer et al 1988). Blood from ten diabetic women during pregnancy was compared to that of 14 women with uncomplicated non-diabetic pregnancies and 12 non-diabetic pregnancies complicated by hypertension (as described previously). There was increased platelet aggregation to a known aggregatory stimulus, ADP, in the both the hypertensive group and the diabetic group. No diabetic pregnancy was complicated by hypertension in this study. The increased platelet aggregation was more marked in the hypertensive compared to the diabetic group.

Neutrophil activation in diabetic pregnancy has also been assessed by this group by measuring plasma concentrations of neutrophil elastase by specific radioimmunoassay (Greer et al 1989). Neutrophil elastase was increased in normal pregnancy [median (range): 30(15-102) ng/ml]: compared to the normal non-pregnant state [17(8-27) ng/ml]. It was increased in plasma from non-pregnant diabetic women [23(15-38) ng/ml] compared to non-pregnant controls and diabetic pregnancies [46(16-38) ng/ml] compared to non-diabetic pregnancies.

These studies suggest that pregnancy in diabetic women may be associated with increased endothelial activation or dysfunction. This may contribute to the vascular complications previously documented to occur with a higher frequency in diabetic women during pregnancy, or may at least make them more susceptible to these complications. Further study of endothelial dysfunction was therefore warranted to further assess this risk and to assess how this risk could be modified by therapeutic interventions.

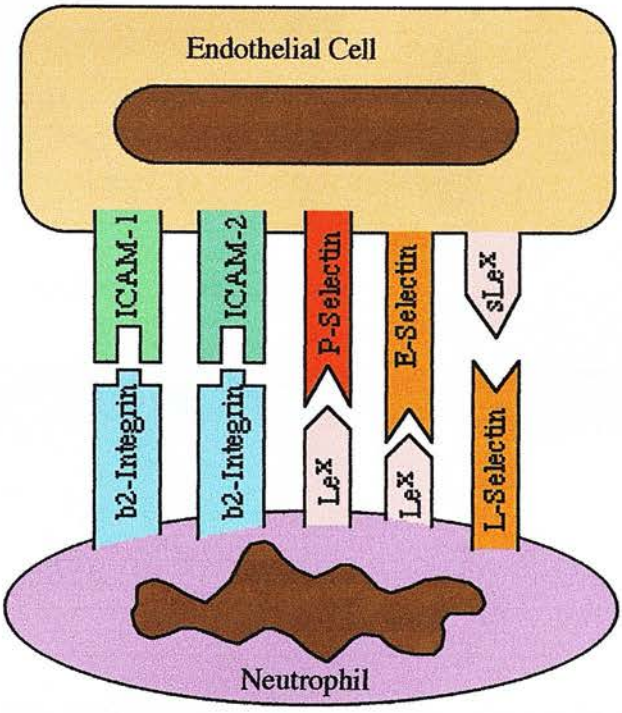


Figure 2.1 Endothelial-neutrophil interaction via the expression of cell adhesion molecules.

The interaction of circulating neutrophils with the endothelium is complex. Attachment activation, adhesion and migration though the endothelial layer occur in specific steps each effected by cell adhesion molecules. Selectins play a central role in initial attachment and activation by interaction with their ligand (sLe^X). Immunoglobulin-like adhesion molecules (ICAMs) and integrins interact with each other to mediate firm adhesion.

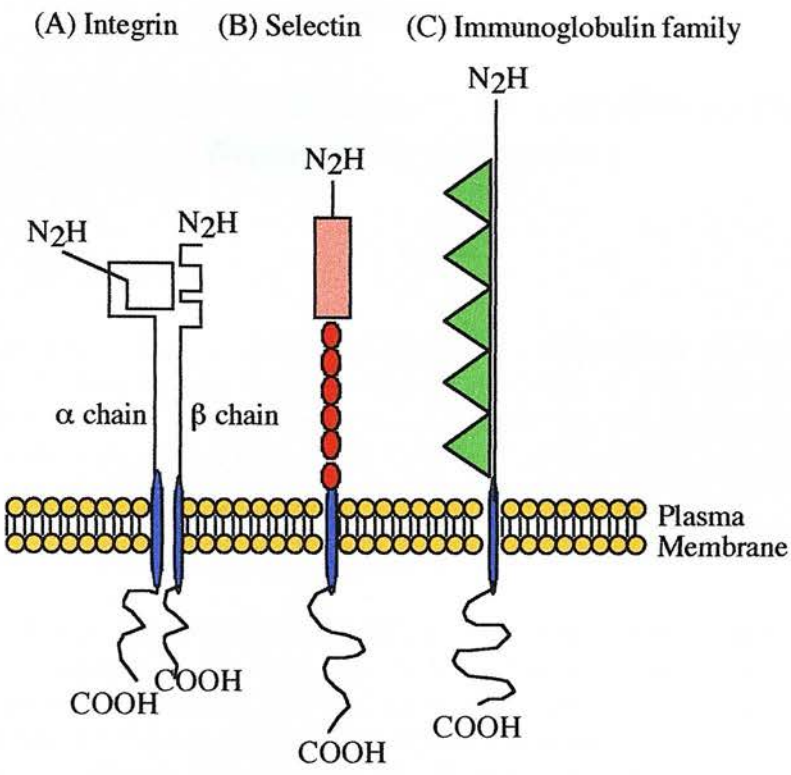


Figure 2.2 The general structure of adhesion molecules

Schematic representation of three classes of adhesion molecules, the Integrins, Selectins and Immunoglobulin (Ig) family members.
The Integrins are noncovalently linked heterodimers composed of alpha and beta subunits. The Selectins comprise of a N-terminal lectin and EGF-type domain (▫), followed by two-nine complement repeats (●). The Ig superfamily are the most structurally diverse and contain two-five Ig repeats (◁). Most cell adhesion molecules have a transmembrane domain and a cytoplasmic domain. The cytoplasmic domain activates second messenger systems which may lead to a variety of cellular effects.

Chapter 3

Circulating Cell Adhesion Molecule Concentrations in Diabetic Women during Pregnancy

3.1 Introduction

Despite recent major advances in both obstetric care and medical management of diabetes, these women remain at greater risk of morbidity during their pregnancy than non-diabetic women. Two factors that contribute to this maternal morbidity are an increased risk of pre-eclampsia (Gardner 1990) and progression of microvascular disease, in particular retinopathy (Moloney 1982, Laatikainen 1987, Klein 1990). The pregnancy–related mechanisms behind these conditions are unknown but are associated with vascular damage and dysfunction.

It is recognised that the endothelium itself can play a key role in such vascular pathology by a variety of mechanisms. One such mechanism is via the expression of an array of leukocyte specific cell adhesion molecules, each of which is induced by a variety of stimuli and will recognise specific ligands on various subsets of leukocytes promoting their attachment and activation (Bevilacqua 1993). Surface expression of these cell adhesion molecules is associated with their shedding into the peripheral circulation.

Circulating concentrations of cell adhesion molecules are increased in diabetic subjects and those at risk of developing diabetes (Gearing et al 1992, Lampeter et al 1992). Circulating concentrations of these molecules are also increased in a range of vascular pathologies (Gearing et al 1992, Miwa et al 1997, Bann et al 1996, Belch et al 1997). In pregnancy increased levels of one cell adhesion molecule VCAM-1 have been documented in pre-eclampsia (Lyall et al 1994); a vascular pathology specific to pregnancy. In these pregnant women the increased concentration of VCAM-1 occurred in addition to other potential mechanisms of vascular dysfunction; increased platelet reactivity (Greer et al 1988) and increased neutrophil activation and degranulation (Greer et al 1989). The latter two markers of vascular dysfunction have also been documented to occur in diabetic pregnancy (Greer et al 1988 & 1989).

On the basis of this evidence we hypothesised that in diabetic women pregnancy is associated with an increased endothelial expression and shedding of cell adhesion molecules. If endothelial expression of cell adhesion molecules is increased this may be associated with both the leukocyte activation and the increased incidence of vascular pathology documented to occur.

The stimulus for vascular dysfunction in diabetic pregnancy could be directly related to pregnancy per se or related to a pregnancy effect on another factor of which a key candidate would be maternal glycaemic control. Maternal glucose levels may effect changes in circulating cell adhesion molecule concentrations by a variety of

mechanisms previously discussed, i.e. increased endothelial expression of cell adhesion molecules may be provoked in response to Il-1 release from leukocytes driven by exposure to advanced glycated end products. The effect of pregnancy on vascular function in diabetic women must thus be assessed in parallel to glycaemic control in pregnancy.

In clinical studies plasma concentrations of von Willebrand factor act as a useful confirmatory marker of vascular dysfunction. von Willebrand factor is a high molecular weight glycoprotein that is synthesised by the vascular endothelium (Bloom 1973), and megakaryocytes (Nachman 1977). It is constituatively released from endothelial cells under normal conditions. In the presence of a number of stimuli, principally vasoactive and pro-coagulant it is released from activated platelets (Koutts 1978) and in increased concentrations from endothelial cells. It acts to mediate the adhesion of platelets to collagen I and III exposed beneath the vascular endothelial layer (Ruggeri 1987). Increased plasma von Willebrand factor concentrations have been demonstrated in acute infections (Pottinger 1989), vascular diseases (Belch 1987) and diabetes (Silveira 1992) and as such circulating concentrations of this factor has been used as one of the principle measures of vascular damage and dysfunction in vivo and in vitro. In diabetes increased expression has been associated with both retinopathy and nephropathy (Porta 1987, Coller 1978), and poor glycaemic control. von Willebrand factor concentrations are increased and correlate with that neutrophil activation (elastase levels) in women whose pregnancies were complicated by pre-eclampsia (Greer et al 1991).

The aim of this work was to determine the circulating concentrations of three endothelial cell adhesion molecules — E-Selectin, ICAM-1 and VCAM-1 — throughout diabetic pregnancy, to compare these to the concentrations found throughout non-diabetic pregnancy and in non-pregnant diabetic women. E-Selectin and ICAM –1 were chosen for analysis as these molecules are intimately involved in neutrophil activation and recruitment to the endothelium, and as we have evidence of neutrophil activation in diabetic pregnancy. VCAM-1 was studied as the circulating concentration of this molecule was found to be selectively elevated in pregnancies complicated by pre-eclampsia, a vascular pathology specific to pregnancy. We aimed to correlate circulating cell adhesion molecule concentrations with maternal glycaemic control, plasma von Willebrand concentrations and clinical outcome.

3.2 Materials and Methods

3.2.1 Subjects

Pregnant women (n = 26) with type 1 insulin–dependent diabetes attending the combined antenatal-diabetic clinic at Glasgow Royal Maternity Hospital were recruited into the study during an 18 month period. Approximately one third of these women had previously attended the general diabetic clinic at Glasgow Royal Infirmary, and therefore had received formal pre-pregnancy advice. Blood samples were drawn when attendance coincided with the following gestational ages, 8–12 (n = 15), 18 (n = 15), 28 (n = 16), 32 (n = 16) and 36 (n = 15) weeks' gestation. Ten

women were sampled longitudinally throughout all five designated gestations of pregnancy. At each time point studied, additional blood was drawn to assess glycaemic control by glycated haemoglobin measurement (HbA1c).

Healthy non-diabetic women (n = 58) attending a low–risk antenatal clinic were studied as controls. The initial aim of the study was to sample control women longitudinally throughout their pregnancy in a similar manner to the diabetic women, however this proved problematic for a number of reasons. First, low risk women, in contrast to diabetic women, are now seen infrequently at their base maternity unit for review. Most of their care is provided in the community. Following their booking (12 week) visit the majority will next be reviewed at the hospital around 36 or 41 weeks gestation on the discretion of their consultant. A few may be seen at 28 weeks gestation depending on the provision of their community care. Second, scheduled return appointments were not infrequently altered at patient request to accommodate social circumstances. This, and the fact that cohorts of women booking at the same clinic would frequently not have even original planned review appointments at the same clinic, made an attempt to longitudinally sample the same control woman throughout pregnancy time consuming and often unsuccessful. Third, women who have access to convenient community based care are understandably unwilling to attend hospital specifically for study blood letting. Finally, blood sampling in the community was not possible due to the lack of centrifuge facilities. For these reasons, and the time constraints of the author, control women were eventually all recruited and sampled cross-sectionally at 12 (n = 20), 28 (n = 19) and 36 (n = 19) weeks' gestation. Non-diabetic women incurring complications during pregnancy were excluded from the study.

Non-pregnant diabetic and non-pregnant non-diabetic women were also studied (n = 22 and n = 28 respectively). Glycated haemoglobin was measured at the time of sampling in the diabetic group. All subjects gave informed consent. The study was approved by the local ethics committee.

All samples drawn were analysed for cell adhesion molecule concentrations. The sample size for CAM analysis was calculated on the basis of 90% power with significance set at the 5% level. This indicated a minimal sample size of 12 women to compare pregnant and non-pregnant groups, based on data from a previous report of cell adhesion molecule concentrations in pregnant and non-pregnant women (Lyall 1994).

Samples drawn at 36 weeks gestation in the diabetic and non-diabetic pregnant groups, the non-pregnant diabetic samples and the non-pregnant non-diabetic samples were additionally analysed for von Willebrand concentrations.

3.2.2 Preparation of samples

Peripheral venous blood was drawn from the subjects using a 20-ml syringe and 21-gauge needle. Blood was drawn without the use of a tourniquet, as it is known that von Willebrand factor, and therefore probably many other markers of endothelial

function, is increased by venous stasis (Porta et al 1982). This may result from both haemoconcentration and damage to the vascular endothelium (Stehouwer et al 1991). The blood was transferred into pre-chilled tubes containing lithium heparin as anticoagulant. Plasma rather than serum (in which coagulant factors have been removed) is necessary for the determination of circulating von Willebrand concentrations. Heparin was chosen as the anticoagulant as cell adhesion molecule concentrations have been found to be higher in heparin-plasma samples than citrate-plasma samples. Plasma was prepared by centrifugation at 2000 times g at 4°C within 5 minutes of blood sample collection. Aliquots of plasma were stored at -70°C until analysis.

The cell adhesion molecules were measured by a commercially available enzyme-linked immunosorbent assay (R&D Systems Europe Ltd., Abingdon, Oxon, UK).
Von Willebrand factor concentrations was measured by an established in-house enzyme-linked immunosorbent assay. Rabbit anti-human polyclonal anti-vWF antibody was used as the capture antibody. Peroxidase-conjugated rabbit anti-human polyclonal anti-vWF antibody was used as the signal antibody (Ingerslev 1987).

3.3 Statistical analysis

3.3.1 Clinical data

Maternal age of studied groups was compared by Student's *t* test. Parity, smoking habit and White class (for diabetic groups) (White 1949) were compared by X^2 analysis.

3.3.2 Maternal glycaemic control (diabetic groups)

Haemoglobin A1c values were normally distributed. The effect of gestation on HbA1c values was assessed by multiple measures analysis of variance. Subsequent comparison of gestational to non-pregnant HbA1c values was assessed by Student's *t* test.

3.3.3 Cell adhesion molecule concentrations

Cell adhesion molecule concentrations approximated normal distribution in pregnant but not non-pregnant groups. Data from non-pregnant diabetic and non-diabetic subjects were analysed by the Mann-Whitney *U* test. During pregnancy, change relative to gestation within the diabetic subgroup (n = 10) was assessed by multiple measures analysis of variance, and within the control group by simple analysis of variance. Descriptive summary statistics for the comparison of pregnant groups is presented as mean [SD]. Comparison of non-pregnant data and non-pregnant to pregnant data is presented at median (range) due to the lack of normal distribution of the non-pregnant data.

If CAM concentrations were found to change significantly with gestation then individual gestational time points could be compared between diabetic and control groups at 12, 28 and 36 weeks gestation (Student's *t* test), and to non-pregnant values (Mann-Whitney *U* test).

If there was no change in CAM concentrations with gestation, pregnancy was then statistically assessed as a single time point in subsequent analysis of this data, and data available from women not sampled longitudinally throughout pregnancy could be included for analysis. For any diabetic women sampled more than once during her pregnancy one of the available values was selected randomly to represent pregnancy. (Available values were separately printed, folded to conceal the printed value, then placed and mixed in a vessel, from which one was drawn by a non-author). In this situation subsequent comparison between the pregnant diabetic and non-diabetic groups was assessed by Student *t* test and used data available from all diabetic subjects sampled (n = 28). The Mann-Whitney *U* test was used to compare pregnant and non-pregnant women. In a similar manner, comparison of the pregnant diabetic group and the control pregnant group allowed all control pregnancies sampled (n = 58) to be presented for analysis.

3.3.4 Von Willebrand factor concentrations

Von Willebrand factor concentrations approximated normal distribution in pregnant and non-pregnant groups. Statistical analysis between pairs of groups was performed by Student's *t* test.

3.4 Results

3.4.1 Clinical data

There was no significant difference between the groups in terms of number of previous pregnancies, smoking habit or White class (Table 3.1). Diabetic women were younger than non-diabetic controls. One diabetic woman suffered progressive retinopathy during pregnancy and required laser photocoagulation for proliferative disease and early delivery (at 36 weeks' gestation). No diabetic woman developed pre-eclampsia or gestational hypertension.

3.4.2 Maternal glycaemic control

Glycaemic control in diabetic groups, as assessed by HbA1c determination, improved significantly with gestation. By 18 weeks' gestation HbA1c measurements were reduced significantly compared to those seen in non-pregnant diabetic women and remained so for the remainder of pregnancy (Table 3.1).

3.4.3 Cell adhesion molecule data

All cell adhesion molecule concentrations were above the detection limit of the applied assay (ICAM-1: 7ng/ml in plasma prior to dilution, VCAM-1: 100ng/ml, E-selectin: 2ng/ml). Comparison of the non-pregnant groups (Figure 3.1) demonstrated significantly increased median [range] concentrations of the cell adhesion molecules E-selectin (63.0 [20.2–107.0] ng/ml) and ICAM-1 (281.5 [171.6–778.4] ng/ml) but not VCAM-1 (459.7 [301.0–909.7] ng/ml) in diabetic women compared to those found in non-diabetic women (43.5 [18.1–93.2], 243.6 [174.4–329.2] and 476.0 [253.8–929.4] ng/ml respectively).

In pregnancy there was no significant change in the measured concentrations of the cell adhesion molecules: E-selectin and ICAM-1 with respect to gestation in either the diabetic (n=10) or control group (mean values throughout gestation for these groups are shown in Table 3.2 and Table 3.3 respectively). No significant difference in E-selectin and ICAM-1 concentrations was found on comparison of diabetic pregnant (n=28) and non-diabetic pregnant data (n = 58), (E-selectin, mean [standard deviation]: 50.2 [16.0] ng/ml vrs 46.5 [17.6] ng/ml and ICAM-1: 274.5 [107.5] ng/ml vrs 287.2 [99.0] ng/ml respectively). Comparison of values in pregnant and non-pregnant women revealed that the median [range] circulating concentration of E-selectin was significantly lower ($P < .05$) in pregnant diabetic women (49.9 [21.2–75.3] ng/ml) compared to that found in non-pregnant diabetic women. There was no significant difference in the concentration of ICAM-1 between diabetic groups, or of either measured cell adhesion molecule between non-diabetic pregnant and non-pregnant groups (Table 3.4).

The circulating concentration of VCAM-1 changed significantly with respect to gestation in the diabetic pregnant subgroup but not the non-diabetic pregnant group.

Mean values at each gestation for both groups are given in Table 3.2. However there was no significant difference in VCAM-1 concentrations between diabetic and non-diabetic women at any corresponding gestation. (All available diabetic data was used in this and the following analyses). Comparison of values in pregnant and non-pregnant diabetic women revealed a significant difference ($P < .02$) between diabetic women sampled at 36 weeks gestation ($n = 15$) (median [range]: 582.8 [387.0–848.6] ng/ml) and non-pregnant diabetic women ($n = 22$) (459.7 [301.0–909.7] ng/ml), but not between any other gestational and non-pregnant data. There was no significant difference between control pregnant and non-pregnant control VCAM-1 data.

3.4.4 von Willebrand factor data

The mean [SD] concentrations of von Willebrand factor in the non-pregnant diabetic women (95.6 [27.5]) and non-pregnant non-diabetic women (84.6 [35.5] u/ml) were not statistically different. The mean [SD] von Willebrand factor levels were significantly higher in pregnant diabetic women (200.1 [78.5] u/ml, $P < 0.001$) compared to non-pregnant diabetic women. The mean [SD] von Willebrand concentration was also significantly raised in the pregnant non-diabetic women (189.5 [48.5] u/ml, $P < 0.001$) when compared to non-pregnant non-diabetic women. There was no significant difference in von Willebrand factor concentrations between pregnant diabetic and pregnant non-diabetic groups.

3.5 Discussion

Our finding that circulating concentrations of E-selectin and ICAM-1 are selectively elevated in non-pregnant diabetic women compared with those measured in non-pregnant non-diabetic women corresponds with previous studies in the general diabetic population (Gearing 1992, Lampeter 1992). Increased endothelial expression of these cell adhesion molecules, reflected by increased circulating concentrations, provides a mechanism for the neutrophil activation and the vascular damage demonstrated to occur in diabetes.

Clinical studies have demonstrated an increased incidence of vascular pathology in diabetic women as a consequence of pregnancy. Based on these studies we hypothesised that pregnancy in diabetic women would be associated with further increased circulating concentrations of cell adhesion molecules. However, we found no such increase in the circulating concentration of E-Selectin and ICAM-1 in the pregnant diabetic group compared to the non-pregnant diabetic group or with increasing gestation. Indeed, the concentration of E-Selectin, actually decreased from that seen in the non-pregnant diabetic women as a consequence of pregnancy.

Altered shedding, dilution or excretion of cell adhesion molecules may occur as a result of pregnancy so that the circulating concentrations of these molecules may no longer represent their endothelial expression. However, the constant circulating concentration of these cell adhesion molecules found throughout non diabetic pregnancy and between non-diabetic pregnant and non-diabetic non-pregnant groups suggests that this is not the case, as we would not expect any change in the endothelial expression of cell adhesion molecules during uncomplicated pregnancy in this group of women. Therefore, although the possibility of altered renal clearance in pregnant diabetic women can not be excluded, the circulating concentrations of E-selectin and ICAM-1 measured in the present population of diabetic women suggests that pregnancy per se is not associated with increased endothelial expression of these cell adhesion molecules.

The specific population of diabetic women participating in the present study may account for apparent discrepancy between these findings and the outcome of the referred clinical studies. Studies specifically determining the effect of pregnancy on retinopathy such as that by Klein et al (1990) and the large Diabetes in Early Pregnancy Study (Chew 1995) have stressed the importance of poor glycaemic control at the onset of pregnancy, rapid improvement of glycaemic control during pregnancy and advanced retinopathy before pregnancy as the major risk factors for progression of retinopathy. Such factors have clearly been proven to be pivotal to progression of retinopathy in the short-term upon the sudden introduction of tight glycaemic control in the non-pregnant state (Kroc Collaborative study Group 1984, Lauritzen 1985, Dahl-Jorgensen 1986). The women in the present study demonstrated reasonable glycaemic control at the onset of pregnancy, and although their control improved significantly during pregnancy this may not have been so abrupt as to provoke increased endothelial expression of cell adhesion molecules, vascular damage and retinal pathology.

Clinical study (Garner 1990) demonstrating an increased incidence in pre-eclampsia in pregnancies to diabetic women has shown that, as for progressive microvascular disease, the incidence of this complication is increased in women with advanced diabetic disease as classified by White class. The limited number of diabetic women participating in our study and the lack of women with advanced diabetic disease may explain why no woman in the present study developed this complication. We cannot assess from this study whether women with more advanced diabetes would demonstrate increased endothelial expression of cell adhesion molecules during pregnancy which could in turn be associated with vascular complications, and this requires further investigation. Only one woman with proliferative retinopathy during pregnancy was sampled during this study. The concentrations of cell adhesion molecules found within the plasma from this woman (E-selectin 48.1 ng/ml and ICAM-1 263.6 ng/ml at 36 weeks' gestation), were not outside the range of concentrations measured in diabetic women without complication.

It is tempting to speculate that the decreased circulating concentration of E-selectin found in our population of diabetic women during pregnancy reflected their improved glycaemic control as a consequence of this event. Hyperglycaemia has been proven to cause vascular damage in animal models (Engerman and Klein 1986) and improved glycaemic control is associated universally with delayed onset and progression of microvascular disease in both animal and clinical studies when maintained over the long term (Godine 1988). The introduction of improved glycaemic control over a short period of time, such as during pregnancy, obviously has a more complex relationship with vascular pathology. It is possible that the small but significant improvement in glycaemic control in our population of women, who demonstrated good glycaemic control before the onset of pregnancy, was of a magnitude to provoke only a positive effect on the endothelial environment. We did not directly correlate this short term improvement in glycaemic control, as assessed by HbA1c measurements, with E-selectin concentrations due to this complex relationship and because part of the reduction in HbA1c values may be explained by a physiologic increased flux of red blood cells into the circulation during pregnancy (Lind 1979)

No difference in the circulating concentration of VCAM-1 was found between non-pregnant diabetic and non-diabetic women. The ligand recognised by VCAM-1, very late antigen-4, is not expressed on neutrophils but on other leukocytes important in the later stages of inflammation. A selective increased endothelial expression of E-selectin and ICAM-1 in diabetic subjects may emphasise that the vascular endothelial damage occurring in this disease is an ongoing acute process reflecting short–term fluctuations in glycaemic control, emphasising the need for improved control, such as that demonstrated on a daily basis during pregnancy, in the general diabetic population.

The significance of the change in VCAM-1 concentration during diabetic pregnancy is unclear. A similar pattern of circulating VCAM-1 concentration was seen throughout non-diabetic pregnancy but did not attain statistical significance. This pattern, an initial decrease and then progressive increase, resembles the physiologic change in blood pressure during pregnancy and hence could be a response to, or an

altered shedding due to, this characteristic. Due to this pattern of VCAM-1 levels in diabetic pregnancy VCAM-1 concentrations were significantly increased above those measured in non-pregnant diabetic subjects at 36 weeks gestation but not at any other measured gestational time-point. Of note the circulating concentration of VCAM-1 has been demonstrated to be increased in pre-eclampsia (mean: 841.9 [SD 49.7 ng/ml] compared to control pregnant women (mean: 560.2 [SD 47.9 ng/ml]) when sampled in the third trimester of pregnancy (Lyall 1994). The mean VCAM-1 concentration can be seen to be much greater in the pre-eclamptic subjects than seen in our diabetic cohort at 36 weeks gestation, suggesting that this finding at this gestation in diabetic women is of little significance. This is further suggested by the finding that the VCAM-1 concentrations at 36 weeks gestation in diabetic pregnancy actually approximated the VCAM-1 concentrations found in the control pregnant subjects both in the study of CAM concentrations in pre-eclampsia and the present study. Furthermore if increased endothelial expression of VCAM-1 provides a mechanism for vascular dysfunction this obviously is not primarily neutrophil mediated.

We have demonstrated that pregnancy is associated with increased circulating concentrations of von Willebrand factor in both diabetic and non-diabetic women. This finding has previously been described in studies of uncomplicated pregnancies in healthy subjects (Bergmann 1991, Deng 1994) and is thought to reflect a hormonal / oestrogen (Norris et al 1997) or lipidaemic (Sattar et al 1997) effect to promote coagulation and fibrinolysis. Studies of pregnant women have however demonstrated further increased circulating concentrations of von Willebrand factor in pregnancies complicated by conditions associated with vascular damage i.e. pre-eclampsia (Bergmann 1991). The degree of elevation of circulating values correlated with the severity of pre-eclampsia (Deng 1994). Furthermore abnormally increased circulating concentrations of von Willebrand factor have been found pregnancies which later progress to become complicated by pre-eclampsia, suggesting that von Willebrand factor concentration is a sensitive indicator of vascular damage and dysfunction.

In the present study the circulating concentration of von Willebrand factor was not significantly different in diabetic women compared to non-diabetic women during pregnancy. This finding correlates with our demonstration that the circulating concentrations of the specific cell adhesion molecules: E-selectin, ICAM-1 and VCAM-1 were not increased in diabetic women during pregnancy, and supports our suggestion that the vascular damage and/or dysfunction are not increased in this population of diabetic women during pregnancy.

In contrast to previous studies we did not demonstrate that von Willebrand factor concentrations were significantly elevated in non-pregnant subjects as a consequence of diabetes. This is contrary to what we would have expected given our finding of increased circulating concentrations of the cell adhesion molecules E-Selectin and ICAM-1 in these women. However although not significant, the measured mean concentration of von Willebrand factor in the diabetic non-pregnant women did tend to be raised above that of non-pregnant non-diabetic women. We must therefore consider that the power of the present study may not have been sufficient to

statistically demonstrate such a difference between these non-pregnant groups, reflecting the small number of women studied and the variance of von Willebrand factor concentrations within these groups.

Alternatively it may be a true finding that significantly elevated CAM concentrations occurs in isolation of von Willebrand concentrations in our population of non-pregnant diabetic subjects. These women demonstrated reasonable glycaemic control and did not, on the whole, suffer any diabetic vascular complication other than mild background retinopathy. The systemic indices of their perhaps mild vascular dysfunction may not be uniform. The possibility of locally raised von Willebrand factor concentrations in specific vascular beds i.e. the retina and the kidney cannot be excluded.

Recently our laboratory has assessed endothelial function in diabetic pregnancy utilising small artery wire myography preparations (Ang et al 2002). The vessels (mean diameter 295 microns) were dissected from subcutaneous fat biopsies from woman with pre-existing type 1 diabetes during pregnancy and non-pregnant diabetic subjects. There was no difference in the response of these vessels to nitric oxide on comparison of these groups, supporting our suggestion that pregnancy does not increase vascular dysfunction in diabetic women.

One limitation of this study was the inability to sample some diabetic women and all the control women longitudinally throughout pregnancy. This created a number of statistical difficulties. Any change in the concentration of CAM throughout pregnancy had to be analysed by different methods in diabetic and control groups. In longitudinal data, each data point has a relationship/direct influence with the next. Multiple measures analysis of variance is the most appropriate and most sensitive analysis of such data. This was therefore applied to the diabetic data but could only include for analysis the 10 women with complete longitudinal data sets. The cross-sectional sampling of control pregnant women allowed assessment only by simple analysis of variance, which is less sensitive. Subsequently, once it was demonstrated that the concentrations of certain CAMs (E-Selectin and ICAM-1) did not change during pregnancy, comparison of diabetic to control pregnancy values utilised random gestational timed samples rather that simply choosing to compare samples taken at a single gestation time point i.e. 36 weeks. This appears more "noisy" but is in fact more statistically correct, as it avoids the introduction of any unintentional bias to the analysis. Previously published studies have avoided these statistical challenges by sampling women only once during pregnancy, typically at 34-36 weeks gestation. However, although our statistical analysis is more complex than in these studies it is equally valid, and has greater ability to establish the effect of the dynamics of pregnancy on circulating markers of endothelial dysfunction.

In conclusion, our findings do not support the hypothesis that increased endothelial expression and shedding of cell adhesion molecules occurs in diabetic women as a consequence of pregnancy. Rather, our findings suggest that the endothelial stimulus to neutrophil activation and, hence, vascular damage, may actually decline in these women during pregnancy possibly as a consequence of their improved glycaemic

control during this period, a feature which may be reassuring to both patient and physician alike.

3.6 Recent advances in the study of cell adhesion molecules

Since the completion of our experimental work in the study of CAMs in diabetic pregnancy, the study of CAMs out-with pregnancy has continued to advance at a rapid rate. The study of CAMs remains an exciting area of research for a number of reasons; circulating concentrations of CAMs act as useful tools to investigate the pathophysiology of cardiovascular disease, they appear stratify disease severity and prognosis (Blann and Lip 2000). In addition, the ability to 'blockade' CAM ligand binding sites has opened a number of therapeutic possibilities in the management of specific forms of cardiovascular disease (Harlan and Winn 2002).

The complex pathophysiology of diabetic vascular complications remains at the forefront of study. Recent studies in the non-pregnant type 2 diabetic populations have demonstrated that elevated circulating levels of E-Selectin and vWF are correlated more closely to LDL cholesterol rather than HbA1c levels (Steiner et al 1994). Thus, promoting greater study of the primary aetiological role of 'insulin resistance' in macrovascular disease (Chen et al 1999). Advances in this area will aid our understanding of the aetiology and complications of gestational diabetes and type 2 diabetes affecting pregnancy, and may have implications for the optimum insulin replacement profiles in type 1 diabetic subjects.

The therapeutic possibilities of CAM ligand blockade advanced from the demonstration that knockout mice deficient in E-Selectin, P-Selectin or ICAM-1 develop less atherosclerosis than other mice (Mayadas et al 1993, Nageh et al 1997). Blockade of leukocyte adhesion to the endothelium by monoclonal antibodies or other antagonists to CAMs has been demonstrated to reduce vascular injury in animal models. The disruption of selectin-carbohydrate ligands and integrin–immunoglobulin superfamily appear to be the most efficacious (Cornejo et al 1997). Trials of such therapy to reduce inflammatory based tissue injury in human diseases (multiple sclerosis, inflammatory bowel disease and psoriasis) have shown some success (Biogen: Press release 2004, Genentech: Press Release 2001). The results of similar studies in vascular ischaemia-reperfusion disorders (stroke, myocardial infarction, and haemorrhagic shock) have however been disappointing to date (Dove 2000). Other strategies to disrupt the leukocyte-CAM interaction include the disruption of second messenger systems with anti-sense oligonucleotides (Schreiber et al 2001), and the inhibition of nuclear factor-κß (Barnes et al 1997), both of which have the potential to reduce the 'inside-out' signalling of CAM activation.

Although the use of such therapeutic moieties are not yet established, the use of circulating CAM levels to stratify the risk of cardiovascular disease progression is supported by a number of studies. Raised ICAM-1 predicted future acute coronary events in a population with previously documented coronary vascular disease (Haim et al 2001), whilst raised VCAM-1 was the strongest predictor of future cardiovascular deaths in a similar population (Blankenberg et al 2001). Raised CAM levels, specifically ICAM-1 (Ridker et al 1998, Hwang et al 1997) and P-Selectin (Ridker et al 2001), have also been shown to predict future cardiovascular disease in apparently healthy individuals. Acting as such a marker of severe disease or active

disease raised circulating CAM concentrations could be used successfully to target the use of established therapeutic agents and reduce morbidity and mortality.

The value of such advances in these areas of CAM research has at present little implications for the management of diabetes in pregnancy. The therapeutic use of CAM blockade in the treatment of vascular condition arising in or affecting pregnancy is unlikely ever to be justified due to the importance of CAMs in fetal structural development, even if placental transmission of putative therapeutic agents could be made negligible. However, as the value of soluble CAM concentrations in cardiovascular risk assignment becomes established, certain diabetic women, especially those with an apparent absence of clinically evident vascular complications, may be advantaged by selection for more intensive care/insulin regimes on the basis of circulating CAM levels in early pregnancy. Future research into the roles of cell surface CAMs and of circulating 'soluble' CAMs are therefore keenly awaited.

Study Group	Category or weeks gestation	No.	Maternal Age (years)	No. primigravida	No. smokers	White Class	HbA1c (%)
Diabetic	non-pregnant	22	27.5 (6.9) [a]	10	4	2B,11C,8D,1R	8.5 (2.3)
Diabetic	12	15	24.5 (5.3) [b]	4	1	6C,6D,1B	7.6 (1.4)
Diabetic	18	15	24.7 (5.6)	5	1	6C,7D,2B	6.5 (1.2) †
Diabetic	28	16	24.0 (5.0)	7	2	9C,7D	6.3 (0.7) ‡
Diabetic	32	16	25.0 (4.7)	4	3	8C,6D,1B,1R	6.3 (0.8) ‡
Diabetic	36	15	24.7 (4.5) [c]	7	3	9C,5D,1R	6.4 (0.7) §
Diabetic	subgroup	10	24.3 (5.4)	4	1	5C,5D	
Control	non-pregnant	28	30.7 (4.2) [a]	11	4		
Control	12	20	28.8 (5.8) [b]	9	7		
Control	28	19	27.1 (5.6)	10	4		
Control	36	19	28.4 (4.7) [c]	11	5		

Table 3.1 Clinical Characteristics of Diabetic and Control Groups (for the study of circulating cell adhesion molecule concentrations).

Data are presented as mean (standard deviation). [a,b,c] Comparison of maternal age between study groups at corresponding gestations was assessed by Student *t* test ($P < .05$). *HbA1c values changed significantly with gestation in the diabetic subgroup ($P < .002$, data not shown). Comparison of gestational with non-pregnant HbA1c values was assessed by Student *t test*. HbA1c normal reference range = 3.4 – 5.2%. † $P < .02$, ‡ $P = .002$, § $P < 0.05$.

Study group	Weeks gestation	No.	E-Selectin ng/ml	ICAM-1 ng/ml	VCAM-1 ng/ml
Diabetic	12	10	55.1 (16.2)	261.9 (44.8)	422.0 (132.0) *
Diabetic	18	10	54.4 (13.9)	274.8 (61.8)	390.2 (79.3) *
Diabetic	28	10	55.1 (16.4)	263.5 (41.2)	400.8 (113.2) *
Diabetic	32	10	57.9 (20.0)	271.6 (87.1)	494.1 (144.2) *
Diabetic	36	10	55.1 (13.2)	248.7 (44.7)	560.8 (129.3) *

Table 3.2 Plasma cell adhesion molecule concentrations throughout pregnancy in women with diabetes.

Data are presented as mean (standard deviation). The values are calculated from the assay results of the subgroup of 10 diabetic subjects sampled longitudinally throughout pregnancy. Change in concentration with respect to gestation in this diabetic group was assessed by multiple measures analysis of variance. VCAM-1 concentration changed significantly with gestation in the diabetic population.

*$P < .05$.

ICAM = intracellular cell adhesion molecule. VCAM = vascular endothelial cell adhesion molecule.

Study group	Weeks gestation	No.	E-Selectin ng/ml	ICAM-1 ng/ml	VCAM-1 ng/ml
Control	12	20	44.7 (9.8)	268.6 (102.7)	446.8 (117.1)
Control	28	19	48.8 (20.9)	293.9 (89.2)	420.1 (134.9)
Control	36	19	45.8 (19.7)	293.6 (101.6)	502.6 (139.2)

Table 3.3 Plasma Cell Adhesion Molecule Concentrations during pregnancy in control subjects.

The subjects were sampled cross-sectionally at each gestational time point. Data are presented as mean (standard deviation). There was no change in the concentration of any cell adhesion molecule with respect to gestation in the control group as assessed by analysis of variance.

ICAM = intracellular cell adhesion molecule. VCAM = vascular endothelial cell adhesion molecule.

Study group	No.	E-selectin ng/ml	ICAM-1 ng/ml
Diabetic non-pregnant	22	63.0 (20.2 – 107.0)*	281.5 (171.6 – 778.4)
Diabetic pregnant	28	49.9 (21.2 – 75.3)*	252.0 (180.9 – 777.3)
Control non-pregnant	28	43.5 (18.1 – 93.2)	243.6 (174.4 – 329.2)
Control pregnant	58	45.3 (22.0 – 98.9)	260.8 (170.3 – 574.2)

Table 3.4 Plasma Cell Adhesion Molecule Concentrations: Comparison of pregnant and non-pregnant groups (E-Selectin and ICAM-1)

Data are presented as median (range). There was no significant difference in E-Selectin or ICAM-1 concentrations on the comparison of women with and women without diabetes during pregnancy. This contrasts the findings on comparing non-pregnant data (figure 3.1). E-Selectin concentrations were significantly lower in diabetic women during pregnancy than in non-pregnant diabetic women. * $P < 0.05$.
ICAM = intracellular cell adhesion molecule.

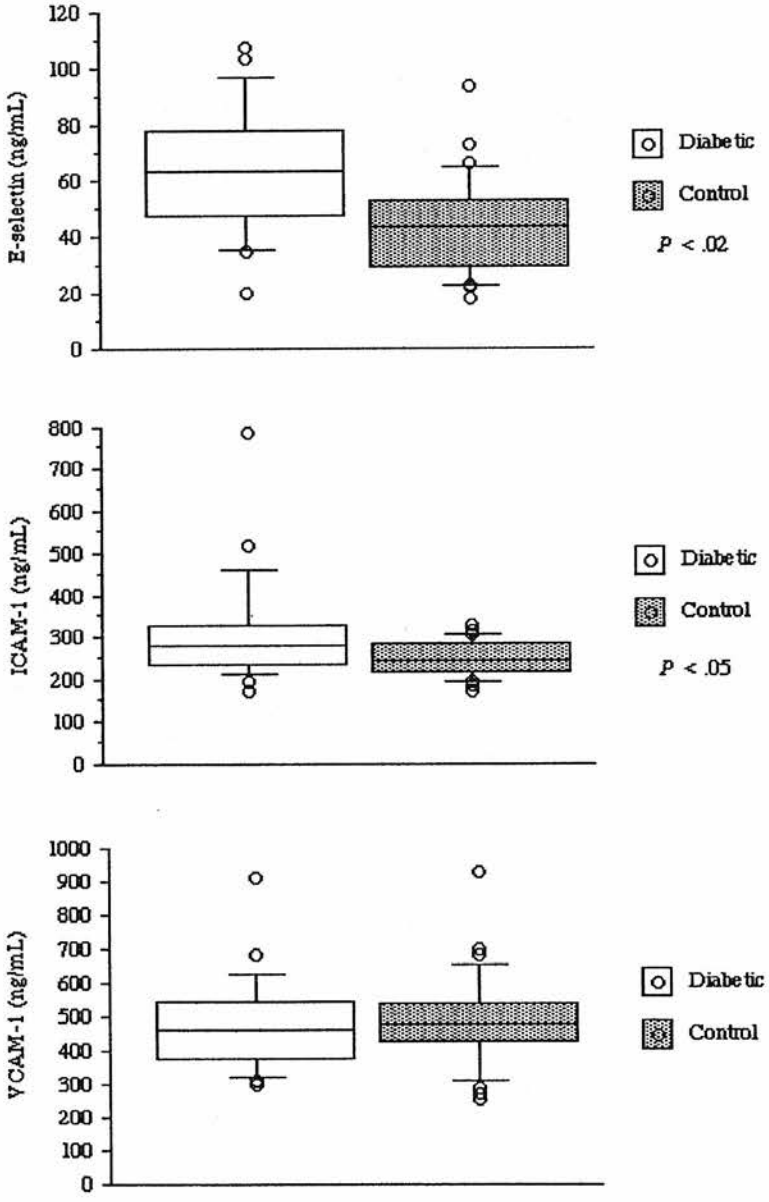


Figure 3.1 Plasma cell adhesion molecule concentrations: Comparison of non-pregnant groups.

Box plots (Median, 25th and 75th percentile, 10th and 90th percentile) of plasma cell adhesion molecule concentrations in non-pregnant diabetic and control women. The Mann -Whitney *U* test was used to compare diabetic and control data.

ICAM = intercellular cell adhesion molecule. VCAM = vascular endothelial cell adhesion molecule.

Chapter 4

Maternal diabetes mellitus and placental terminal villus structure

4.1.1 Pathophysiology of the placenta in relation to fetal complications.

The placenta forms the interface of the maternal and fetal environments. The fetus has an integral relationship with the placenta and is reliant on its multiple functions. The placenta effects the transfer of maternal nutrients and oxygen to the fetus and the reverse transfer of metabolic waste products such as carbon dioxide, urea and bilirubin to the maternal circulation. It is involved in the synthesis of various hormones. It also provides an immunological barrier, protecting the fetus from the maternal immune system in addition to certain bacteriological and viral pathogens.

Placental structure and function frequently reflect and may contribute to the aetiology of a number of fetal and maternal complications. Pathological examination of the human placenta has demonstrated characteristic placental lesions in the presence of many maternal complications, such as pre-eclampsia, anti-phospholipid syndrome (APS) and anaemia, and fetal conditions such as intrauterine growth restriction and hydrops. Characterisation of placental abnormalities has helped decipher the pathophysiology of certain fetal complications: placental infarction in maternal APS and terminal villous deficiency in IUGR (Macara et al 1996). The placenta from pregnancies complicated by maternal diabetes mellitus has been subject to numerous studies but a lack of consensus of characteristic findings remains.

4.1.2 The development of the human placenta

Fertilisation of the oocyte initiates development of two specialised structures: the fetus and the placenta. The fertilised oocyte or zygote undergoes a series of rapid mitotic divisions. When successive cleavages have resulted in a 12 to 16 cell structure, 3 days post fertilisation, the period of the morula begins. Fluid filled spaces appears inside this compact mass of cells. This blastocyst cavity separates the cells into two parts: the inner cell mass, from which forms the embryo and the outer cell layer termed the trophoblast which gives rise to the placenta.

At implantation (day 5-6) the trophoblast attaches to the endometrial epithelium (Schlafke 1975). Rapid proliferation of the trophoblast occurs within the innermost layer of the trophoblast, the cytotrophoblast. Cells from this layer migrate peripherally and lose their cell borders forming an outer multinucleated mass, the syncytial trophoblast. Finger like processes of the syncytial trophoblast invade the endometrial stroma and form lacunae. When the trophoblast invades the maternal

vasculature blood enters the lacunae and a primitive placental circulation is established (Moore 1982)

4.1.3 The development of placental villi

Proliferation of the cytotrophoblast to form finger like extensions covered with syncytial trophoblast herald the formation of the first villi (Castellucci 1982). From day 15 secondary villi are formed when mesenchyme extending from the embryo grows into the villi and differentiates into connective tissue (Luckett 1978). When the blood vessels form in this connective tissue the tertiary villi are formed (Demir 1989) and a primitive blood supply from the fetal circulation to the placenta is established. From the 6^{th} week post fertilisation these mesenchymal villi branch, giving rise to others by trophoblastic sprouting and vascularisation. At this stage in placental development there is no formal intervillous circulation and the developing placenta and fetal tissues are hypoxic relative to the mother (Rodesche et al 1992). This may serve to protect the developing embryonic tissues from exposure toxic free radical formation. It is not until the 10-12 week post fertilisation that the intervillous space is perfused with maternal blood. This follows trophoblast invasion of the maternal uterine spiral arteries (Hustin et al 1988) and establishes the haemochorial relationship between fetal and maternal blood in the human placenta.

The mesenchymal villi develop a reticular stroma and at this stage of development are termed immature intermediate villi (Kohnen 1994). The connective tissue in the stroma of these villi becomes denser as they develop into fibrosed stem villi. The development of additional immature intermediate villi ceases at the end of the second trimester. Transformation of immature intermediate villi into stem villi continues and therefore the number of stem villi becomes finite.

At the beginning of the third trimester the mesenchymal villi switch from developing into immature intermediate villi to differentiation into mature intermediate villi (Castellucci 1990). These villi do not further differentiate but instead give rise to terminal villi.

4.1.4 The terminal placental villus

Terminal villi form when the longitudinal growth of the capillaries within the mature intermediate villi exceeds the long longitudinal growth of these villi. The capillaries become coiled and form loops that bulge through the trophoblast surface layer (Kaufmann et al 1985 & 1988). The bud-like terminal villi are therefore formed passively, rather than by active differentiation. Due to the mechanism of their formation the trophoblast layer of the terminal villi is stretched and thinned. These thinned areas are where the distance dividing the maternal and fetal blood is at its minimal. Especially thinned areas overlying capillary loops are termed epithelial plates or vasculosyncytial membranes. Terminal villi thus effect the vast majority of feto-maternal and maternal-fetal transfer of substances.

4.1.5 The basic structure of the human placenta at term.

The basic structure of the placenta at term (Figure 4.1) consists of several main stem villi (rami chorii) each of which branches dichotomously to form a further 3-30 generations of stem villi (ramuli chorii). These in turn have given rise to mature intermediate villi and thereafter to terminal villi (Castellucci 1990, Leiser 1991).

Stem villi are identified histologically by their dense fibrous stoma that reaches to the trophoblast surface. The trophoblast surface of stem villi may be damaged and replaced by fibrinoid in term placentae. Stem villi contain large blood vessels that have an identifiable media and adventitia on light microscopy. At term they account for 20% of villous volume.

The mature intermediate villi account for 30 – 40 % of villous volume. They in general have a smaller diameter (60 – 150 μm) than stem villi, and demonstrate an absence of stromal fibrosis and vessels with an identifiable media. They instead have a loose connective tissue rich in cells and poor in fibres and contain slender capillaries. At term a small proportion of villous volume is composed of immature intermediate villi (<10 %) that are identifiable by net like connective tissue forming stromal channels and proportionally to their size relatively few fetal vessels.

Terminal villi account for 40% or more of villous volume at term. They average 40 – 80 μm in diameter, contain scant amounts of connective tissue fibres and cells. Capillary lumens account for approximately half of their stromal volume, and bulge against 30 – 40% of the surface trophoblast forming the 'vasculosyncytial membranes' (Kaufmann 1985).

4.1.6 Placental Transport Functions

The trans-placental passage of substances is either passive, determined by concentration gradients, or by unidirectional facilitated or active, carrier-mediated transport. Oxygen and carbon dioxide are small molecules and cross the placenta by simple diffusion down a concentration gradient. Passage across the placental barrier is usually rapid and therefore their rate of exchange is largely determined by their delivery to the area i.e. is blood flow limited. Maternal blood flow in the intervillous space and fetal flow through the villous capillaries determine the delivery to and removal of substances at the area of exchange and set the concentration gradients.

Transport of glucose as an energy supply to the fetus also occurs down a concentration gradient but is effected by placental facilitated diffusion. The glucose transporter GLUT1 has been identified in both the microvillous and basal plasma membranes of the syncytium. This transport system for D-glucose becomes saturated only at high, supra-physiological maternal concentrations of glucose (>20 mmol/l) (Hauguel et al 1986). Fetal arterial or plasma D-glucose is therefore a function of the maternal concentrations until extreme maternal hyperglycaemia occurs (Morriss et al 1975).

Other nutrients may be transported to the fetus against a concentration gradient. Amino acids concentrations are higher in fetal than maternal plasma and. An optimal fetal supply is determined by active transport systems. Transfer of these substances to the fetus is therefore potentially less sensitive to maternal concentrations and to placental structural than that of oxygen and glucose.

The active functions of the placenta require an energy source. The placenta utilises glucose as its main energy supply. Although it is estimated that the placenta can utilise one-third of transported glucose for its own metabolic process, it only appears to be capable of limited metabolism of this glucose to lactate via anaerobic glycolysis. The lactate is passed to the fetus where it is further metabolised/ oxidised through the Krebs cycle and therefore provides the fetus with energy equivalent to glucose. The placenta therefore utilises little of energy substrate passing from the mother to the fetus (Desoye and Shafrir 1996).

4.1.7 Regulation of placental blood flow

Utero-placental and feto-placental blood flows are major determinants of maternal-fetal exchange. Utero-placental blood flow is compromised in maternal pre-eclampsia as a consequence of inadequate trophoblast invasion of the uterine spiral arteries. Blood circulating around the intervillous space may be impeded by the architecture and density of the villous tree. A reduction in utero-placental blood flow by 35-45% has been documented in maternal diabetes (Nylund et al 1982) presumably reflecting an increased villous mass. Recent stereolological studies have however calculated that the intervillous volume is conserved in diabetic pregnancies when the mother achieves good glycaemic control (Mayhew et al 1994).

Feto-placenta blood flow may be influenced by the architecture of the villous vascular tree and the vascular tone of vessels with a smooth muscle component, such as the arteries and arterioles of the stem villi. The placental is not innervated (Reilly & Russell 1977); thus vascular tone of the feto-placental circulation may only be regulated by local concentration changes in vasoconstrictor or vasodilator substances.

Nitric oxide is likely to be a key vasodilatory agent. Nitric oxide synthase has been located by immunocytochemistry in both the feto-placental vascular endothelium and in the villous syncytiotrophoblast (Myatt et al 1993). Placental vascular sensitivity to NO has been demonstrated in perfused cotyledons (Myatt et al 1991). Other vasodilatory agents such as atrial natiuretic peptide (ANP) and prostacyclin may play additional roles. ANP receptors have been characterised in smooth muscle cells from stem vessels (McQueen et al 1991). However ANP has not been documented to be synthesised by the normal term placenta (Inglis et al 1993) and may therefore act in an endocrine fashion after production by the fetal heart (Wharton et al 1988). The role of prostacyclin is unclear, as inhibition of its synthesis has been documented to have no effect on the pressure required to perfuse an isolated cotyledon (Jacobson et al 1991).

Thromboxane, endothelin and angiotensin II have all been demonstrated to have a vasoconstrictive effect on the feto-placental circulation (Mak et al 1984, Myatt et al 1991, McQueen et al 1991). Thromboxane may be produced by the trophoblast, arterial smooth muscle cells (Templeton et al 1991) or by circulating platelets. Endothelial cells are known to synthesise ET-1, and synthesis of ET-1 has been demonstrated in the human placenta (Benigni et al 1991). Angiotensin II may act in an endocrine manner following production by the fetal kidney. There is also some evidence to support local production of angiotensin within the placenta (Ihara et al 1987).

The production of the vasoactive substances is likely to play a role in local regulation of blood flow in the placenta at term and possibly during development. The precise mechanisms of such a vaso-regulatory system are not yet deciphered. Studies of pregnancies complicated by IUGR have however suggested that it is the structure of the distal villous tree that has a more important influence on feto-placental blood flow. The vessels of the distal villous tree do not contain smooth muscle therefore their vascular resistance will not be influenced by vasoactive substances. It is the architecture of these small diameter vessels, particularly their length and branching patterns that appear to play a key role in feto-placental blood flow impedance (Krebs et al 1996).

4.2.1 The study of placental structure

Gross examination of placenta should include placental weight and macroscopic appearance. Placental weight correlates poorly with fetal weight and may be altered in specific pathological conditions: increased in diabetes and fetal hydrops and reduced in IUGR and preeclampsia. Macroscopic lesions such as areas of infarction and haemorrhage visible to the naked eye and are valuable findings.

Light microscopy is used to study the placenta in more detail. It allows individual villous types to be visualised, allowing assessment of numbers and basic structure. To aid assessment of structural components specific stains of the tissue sections can be used, such as the Masson trichrome technique, which accentuates stromal staining. More specific structural proteins can be targeted for staining by immunohistochemical techniques and can to advance the study of tissue morphology at this magnification.

Histomorphometric techniques have been devised to calculate an estimated quantification of villous or specific tissue volumes. These stereological studies lay a grid system of points and intersections over the viewed light microscopy image. Tissue volumes and surface areas are estimated by counting tissue overlying grid points and intersections and entering these values into established formulae (Aherne & Dunhill 1966, Boyd et al 1986, Teasdale 1981,1983,1985). Profile counting of structures such as villi can be used to derive length densities (Mayhew et al 1992). The densities are corrected for tissue shrinkage during processing. Component densities can be converted into absolute quantities using estimates of placental

volume calculated from organ weight and density (Assumed to be 1.05g/ml). (Mayhew et al 1994).

Electron microscopy allows the tissue to be studied at higher magnification. It thus allows structures indiscernible to light microscopic analysis to be visualised i.e. collagen fibres or intracellular organelles, and measured i.e. the thickness of the trophoblast basal lamina.

4.2.2 Placental vascular casts: an alternative method of ultrastructure determination.

Many studies may be limited by their two dimensional analysis of villous sections. Three-dimensional studies of placental villi may improve our understanding of pathological findings. This may be of particular importance in determining the precise structure of the intravillous vascular tree. The vascular infrastructure of placental villi has been studied in animal and human placenta by microvascular casting techniques (Thiriot and Panigel 1978, Habashi et al 1983, Lee and Yen 1983 and Leiser et al 1985) and have allowed detailed analysis of the villous vascular tree. The technique employs a low viscosity plastic to perfuse one or several adjacent cotyledons via cannulation of an extra cotyledonar artery. The low viscosity of the polymer liquid is essential, and has been perfected by Leiser (1985) for this technique to allow uniform filling of placental microvessels at low pressure. Following polymerisation and hardening of the vascular cast the overlying tissue is corroded from the cast by immersion in a strong alkaline solution. The three dimensional vascular network may then be analysed by scanning electron microscopy. Using this technique our department has successfully studied and detailed placental terminal villous vasculature abnormalities in pregnancies complicated by intrauterine growth restriction (Krebs et al 1996).

4.3.1 Placental angiogenesis and villous structure in pregnancies complicated by intrauterine growth restriction.

In certain pregnancies complicated by IUGR Doppler studies have observed that the diastolic component of the arterial flow rate in the fetal umbilical artery was reduced, absent or even reversed (Jouppila et al 1984, Laurin et al 1987, Fairley et al 1991), indicating increased vascular impedance (Adamson et al 1980). Recent studies of terminal villous structure and terminal villous vascular development in such pregnancies have recently been performed in our department (Macara et al 1996, Krebs et al 1996). These studies have greatly advanced the understanding of the pathophysiology of this condition.

Macara et al (1996) studied the anatomical composition of placenta from pregnancies complicated by severe intrinsic intrauterine growth restriction associated with absent arterial flow in the fetal umbilical artery. They demonstrated that there was significantly less villous tissue in these placentae than in matched controls, as could be expected reduced placental weight. However importantly they demonstrated that this was a selective reduction in the volume of peripheral villi in these IUGR placentae. Placental vascular casts were also prepared from these IUGR pregnancies. Analysis of these casts by scanning electron microscopy demonstrated the peripheral vascular network to be abnormal, being composed of fewer vessels which were elongated, and lacked branches, coiling and sinusoidal dilatations (Krebs et al 1996). As vessel coiling and branching underlies terminal villous formation these findings are consistent with reduced terminal villous formation and therefore numbers.

The cross-sectional ultrastructure of the terminal villi was studied in detail by transmission electron microscopy of perfusion fixed placental tissue obtained from the same pregnancies (Macara et al 1996). The terminal villi were smaller in diameter than those seen in control placentae. They demonstrated increased numbers of syncytial trophoblast nuclei, reduced cytotrophoblast nuclei, thickened trophoblast basal lamina and increased stroma. They noted that these findings were not consistent with villous hypoxia in vivo rather they suggested increased oxygenation of the villi. Further immunohistochemical studies performed on paraffin embedded tissue demonstrated increased stromal deposition of collagen and laminin and a reduced numbers of proliferating cytotrophobast in these villi, further evidence that these villi appear to have been exposed to increased oxygen tensions.

These studies demonstrated that the pattern of vessel growth and terminal villous structure in these IUGR pregnancies was not characteristic of placental ischaemia or hypoxia. Indeed the morphology of the terminal villi suggest that that these villi were exposed to greater than normal partial pressures of oxygen delivered by the maternal blood. A primary disorder of placental angiogenesis was therefore implicated in the aetiology of this 'intrinsic' type of IUGR. This is an important observation and has implications in the management of such IUGR pregnancies. Attempts to supplement maternal oxygen or to dilate the maternal circulation are unlikely to be of therapeutic benefit and may be inappropriate (Kingdom et al 1997).

4.3.2 Fetal hypoxia: Placental angiogenesis and villous structure.

By defining the pathological basis of fetal hypoxia in severe 'intrinsic' intrauterine growth restriction Kingdom and Kaufmann (1997) were able to move away from the belief that fetal hypoxia is always secondary to placental hypoxia. They proposed varying mechanisms of fetal hypoxia: (1) pre-placental hypoxia, in conditions such as maternal anaemia, heart disease or pregnancy at high altitude, (2) utero-placental hypoxia in pre-eclampsia and (3) post-placental hypoxia in 'intrinsic' IUGR.

In pre-placental hypoxia there is a reduction of the oxygen content of blood flowing from the mother though the normally dilated spiral arteries and bathing the placental villi. All these conditions are associated with the same characteristic placental villous structure. Capillary volume capacity is increased (Jackson et al 1987). There is increased capillary branching (Ali et al 1996) and the volume of the cytotrophoblast is increased in pregnancy at high altitude. Hypoxic hypoxia induced in the guinea pig model is associated with increased elaboration of villous vasculature in addition to decreased density of the stromal matrix (Scheffen et al 1990). Maternal anaemia is also associated with evidence of increased angiogenesis, with increased capillary volume (Burton et al 1996) and branching (Kadirov et al 1996). Proliferation and volume of cytotrophoblast is increased in an inverse relationship to the severity of anaemia (Kadirov et al 1996).

Pre-eclampsia is associated with utero-placental hypoxia. Aberrant invasion of the spiral arterioles by proliferating trophoblast in the second trimester results in these vessels maintaining their high resistance to maternal blood flow. A limited flow of normally oxygenated blood is available to bathe the placenta. Capillary volume fraction is increased (Salvatore 1968, Burton et al 1996) and there is an increased amount of villous cytotrophoblast (Fox 1964).

In pre-placental hypoxia and utero-placental hypoxia the placental villi are hypoxic in series with the fetus. Placental histological findings are therefore similar in both conditions with evidence of increased angiogenesis and cytotrophoblast proliferation. This is in contrast to the histological findings in placenta from 'intrinsic' IUGR pregnancies. The lack of angiogenesis and reduced cytotrophoblast volume in these placental villi demonstrates that they are well oxygenated but fetal hypoxia still occurs. The deficient placental angiogenesis is suggested to restrict the ability of the fetus to extract oxygen from the placenta, therefore giving rise to the term post-placental hypoxia.

4.3.3 The control of placental angiogenesis

Capillary growth and branching are important in the formation of the placenta and its development as low impedance circuit. Angiogenesis is stimulated by the release of growth factors from oxygen deprived villous tissues. Studies have demonstrated that placental tissue expresses a number of angiogenic growth factors such as basic Fibroblast Growth Factor (bFGF) (Ogawa 1991, Shreeniwas et al 1991), Vascular Endothelial derived Growth Factor (VEGF) (Sunderkotter et al 1994, Sharkey et al

1993, Ahmed et al 1995) and Placenta Growth Factor (PlGF) (Ahmed et al 1997) and their receptors including VEGFR-1 (Flt-1) and VEGFR-2 (fllk-1/KDR) (Ahmed et al 2000). The villous macrophage appears to be the dominant site of production of bFGF and VEGF and is implicated to play a key role in villous oxygen-sensitive angiogenesis.

Vascularisation of the placental villi starts at day 21 post conception (Demir et al 1989) and is the result of local de novo formation of capillaries from the mesenchyme of the secondary villi. The appearance of macrophages in these villi predates capillary formation consistent with a role in angiogenesis. Their expression of angiogenic growth factors suggests they perform this function via a paracrine mechanism (Desmir et al 1989, Ahmed et al 1995).

Villous vasculature increases by branching until the end of the first trimester. The vessels in the largest villi become surrounded by adventitia and the villi differentiate into stem villi. From 26 weeks of gestation until term the villus vasculature undergoes a change from branching to non-branching angiogenesis. This is the stage of formation of the mature intermediate villi and from then on angiogenesis determines the capacity of the villi for gas exchange.

VEGF appears to perform as a key angiogenic factor. VEGF expression in the human placenta has been localised by in-situ hybridisation and immunohistochemical techniques to the villous trophoblast and macrophages of both fetal and maternal origin. If exerts its effects by binding to two tyrosine kinase receptors VEGFR-1/ Flt-1 and VEGR/KDR present on endothelial cells (de Vries et al 1992).

PlGF belongs to the VEGF family and shares 53% homology with VEGF (Magilone et al 1991). Its expression in the human placenta has been localised to the villous synctiototrophoblast and the media of larger stem vessels (Shore et al 1997, Khaliq et al 1996). In in-vivo and in-vitro studies VEGF binds to both FLt-1 and KDR receptors and performs as a potent stimulus for endothelial proliferation, migration and production of the plasminogen activators required for proteolytic degradation of the extracellular matrix. PlGF appears to act as a less potent stimulus for endothelial cell chemotaxis and proliferation. PlGF binds to FLt-1 but not the KDR receptor.

In the human placenta it is proposed that the type of angiogenesis; branching or non-branching is dependent upon the relative expression of VEGF, PlGF and the VEGF receptors (Kaufmann et al 1985, Leiser et al 1985). VEGF and KDR are most intense during early gestation and decline as pregnancy advances (Jackson et al 1994). PlGF and Flt-1 expression increases towards term. VEGF appears to promote the initial capillary formation and branching angiogenesis. PlGF appears to be more involved in non-branching angiogenesis, the formation of intermediate villi and with increasing vessel length the subsequent formation of terminal villi when villous length overtaken.

Oxygen saturation may be a major regulator of the balance between VEGF and PLGF function. In placental tissues VEGF is up-regulated by hypoxia and down-regulated by hyperoxia (Shore et al 1997, Wheeler et al 1995). Hypoxia has been

demonstrated to down regulate PlGF mRNA and protein in human placenta (Khaliq et al 1999). The importance of the interaction of growth factors in establishing placental angioarchitecture is emerging in studies of IUGR pregnancies. PlGF expression is significantly increased in these pregnancies (Ahmed et al 2000), supporting the suggestion that the villi are "hyperoxic" (Kingdom and Kaufmann 1997) and provides a mechanism, a premature PlGF to VEGF dominance that may account abnormal villous vascualture in this condition.

4.4 The placenta from pregnancies complicated by maternal diabetes.

The placenta from diabetic pregnancies has been subject to many studies however there remains a lack of consensus as to the presence and nature of any characteristic findings. The earliest studies of diabetic pregnancies commented on the increased weight and blood content of the placentae and correlated this to the increased size of the fetus (Klebe 1974). Recent studies of placentae from diabetic pregnancies have suggested that placental (and fetal) weight is normalised if good maternal glycaemic control is achieved throughout pregnancy and there is an absence of any additional complications such as pre-eclampsia and intrauterine growth restriction (Mayhew 1994).

Structural studies of placenta from diabetic pregnancies have largely been based at the light microscopy level. Observed abnormalities are varied and have included increased villous oedema (Aladjem 1967, Fox 1969, Jones and Fox 1976a), immaturity (Laurin et al 1987) increased cytotrophoblast numbers, syncytial trophoblast knot formation and increased macrophage numbers (Fox 1969, Jones and Fox 1976b) in addition to increased stromal fibrosis (Jones and Fox 1976a). Increased oedema and increased cytotrophoblast numbers are often described as villous immaturity in these studies. Most studies have reported abnormalities of the villous vasculature. The majority of studies reported an increased total surface area of capillaries, however, this has been in association either with (Teasdale1981, 1983,1985, Boyd 1986, Bjork & Persson 1984, or without (Clavero et al 1963) a reported increased surface area of the villi.

The varied pathological findings between studies reflect to some extent the differing methodologies of the studies, but most importantly the differing populations of diabetic women sampled. The majority of studies fail to define their populations of diabetic women (with regard to their duration and severity of diabetes) and could include placenta obtained from women with recent onset diabetes and those with advanced diabetic vascular complications. Some studies also contain placentae from diabetic pregnancies complicated by other pathological conditions. The difficulty of interpreting the published literature of diabetic placenta may be illustrated by considering in more detail the revered studies by Boyd (1986) and Teasdale (1981-85).

Boyd et al (1986) used morphometric techniques to compare villous surface area and the volume of parenchymal tissue to that of non-parenchymal tissue in fourteen

diabetic pregnancies and twenty-two control pregnancies. Parenchymal tissue was termed to compose of villi (including fetal vessels) and the maternal intervillous space i.e. the volume of placenta potentially effecting nutrient exchange. Non-parenchyma comprised chorionic and decidual plates, fetal vessels with a diameter greater than 1mm. They demonstrated that the total volume of placenta was larger in diabetes (+12%), however the volume of parenchymal tissue was disproportionately increased (+20%), as was villous surface area (+52%). The difference was maintained despite correcting for an increased birthweight in these diabetic pregnancies. This data suggested that despite just being larger, placentae from diabetic pregnancies had altered differentiation with apparent increased nutrient exchange capacity per volume. The diabetic subjects were described as having diabetes of 3-23 years gestation and who achieved 'moderate' or 'good' diabetic control during pregnancy. None developed preeclampsia or hypertension during pregnancy.

The exhaustive studies performed by Teasdale (1981-85) also used morphometric techniques to assess relative quantities of parenchyma and non-parenchymal tissue in diabetic pregnancies. Again parenchyma was defined as the part of the placenta that contained the structures or compartments that are strictly concerned in metabolic exchange between the mother and fetus. In these studies parenchyma composed the intervillous space, the trophoblast volume and the fetal capillaries of both peripheral and stem villi. Non-parenchyma included the connective tissue of the villi. Teasdale separately studied placenta from pregnancies from women classified as white class A (1981), B (1983) and C (1985). His results suggested a tendency to an increased surface area of exchange in these placenta compared to controls but confusingly the volume of parenchyma to non-parenchyma tissue was relatively increased in class A diabetic pregnancies reduced in class B diabetic pregnancies but conserved in placenta from White class C pregnancies.

The definition of parenchyma differs between studies. Teasdale attempted clarify the previous confusing pathological findings of diabetic placenta by stratifying maternal diabetic disease. Both studies did not assess maternal glycaemic control, which may be a more important variable in placental differentiation.

In reviewing the published studies of diabetic placental pathology it is important to note that the current management of diabetes now outdates the majority. The management of diabetes in general, and diabetes in pregnancy has improved substantially over the past 20 years. The findings of the quoted studies, even if consistent, may not be representative of placental structure in current populations of diabetic women who have the potential to achieve far superior glycaemic control than their predecessors.

There are few recent studies of diabetic placentae. The most notable are from Mayhew et al (1994) and use stereographical techniques to compare the structure of placentae from pregnancies to women with diabetes who demonstrated good glycaemic control with that from non-diabetic control pregnancies. Placentae were obtained from two groups of diabetic women, 39 women classified as White's group A-C and 16 women with a longer history of diabetes or diabetes complicated by

vascular pathology (White groups D-F/R), in addition placentae from non-diabetic control pregnancies (n = 34). Good glycaemic control was advocated (aiming for a mean blood glucose ≤ 6 mmol/L, HbA1c values were < 7% in all groups at term). All pregnancies were delivered after 37 weeks gestation with no evidence of fetal malformation. Their study demonstrated that despite a tendency to increased placental weights in pregnancies to diabetic women classed in A, B and C White Classes, there was no significant difference in fetal and placental weights between control pregnancies and any class of diabetic pregnancy suggesting that improved maternal glycaemic control normalises findings.

At an ultrastructural level they calculated that the volume of peripheral villi, the intervillous space, non-parenchyma and fibrin deposits were similar in diabetic and control placentae. On assessment of villous composition they found that these volume of fetal capillaries was significantly increased in diabetic placentae. They found comparable volumes of cytotrophoblast, syncytiotrophoblast and stromal connective tissue in diabetic and control placental villi. They additionally calculated that diabetic placental villi had both an increased length and surface area of capillaries. The villi themselves were of increased length but not surface area in diabetic placentae. These findings suggest that in diabetic pregnancies the placental villi are hypervascularised. They concluded that the villi in placentae from diabetic pregnancies appear potentially better adapted to effect nutrient exchange raising the possibility that the fetuses of diabetic mothers may be trying to adapt to a state of chronic hypoxia.

In parallel studies (Mayhew et al 1993) they aimed to assess the oxygen diffusive the diabetic placentae. The oxygen conductance of a placenta was calculated by estimating and summating the conductance of six tissue compartments; maternal erythrocytes, maternal plasma, trophoblast, stroma (including capillary endothelium), fetal plasma and fetal erythrocytes. This complex analysis aims to assess the ease with which oxygen can dissociate from maternal haemoglobin, pass through tissue in soluble form and associate with fetal haemoglobin. The physiochemical variables of conductance (chemical reaction rates of oxygen and maternal and fetal blood, solubility of oxygen in plasma and villous tissue) are fixed and were taken from previously published data therefore placental conductance was calculated to be altered by stereologically derived physical quantities such as erythrocyte surface and volume of stroma. The total oxygen diffusive conductance was calculated to be increased in diabetic placentae particularly those in diabetic white groups A-C (36 $ml.min^{-1}.kPa^{-1}$) compared to control placenta (29. $ml.min^{-1}.kPa^{-1}$). The main changes were documented to occur on the fetal side with increased stromal conductance, increased fetal plasma conductance and increased erythrocyte conductance. The villous stoma and trophoblast were calculated to be the major contributors (80%) to total resistance to total oxygen diffusion. Notably there was no difference in trophoblast conductance between groups.

Further analysis of a subgroup (n=16) of these diabetic placenta and control placentae (n=6) was performed at a later data and aimed to compare derived star volumes of villous 'domains' and intervillous pores. The derived star volumes are a measure of three dimensional spaces (and how this relates in four random directions

to an arbitrary test point) and aims to measure the ease by which maternal blood can bathe the villous tree and the efficiency of the villous tree in interacting with this bathing blood. The star volumes of peripheral villi and intervillous spaces did not alter significantly between groups confirming a lack of aberration of the maternal side of the placenta in pregnancies complicated by diabetes.

There have been few studies placentae from diabetic pregnancies based at the electron microscope level. Thickening of the trophoblast basement membrane is the most consistently noted abnormality (Okudara 1966, Fox 1969, Jones and Fox 1976). The most recently dated study (Honda 1992) also demonstrated this abnormality in addition to suggesting that the terminal villi were of smaller diameter in diabetic pregnancies, that there are abnormalities of villous branching (hyper of hypo-ramification), increased syncytial knot formations and decreased number of vascular syncytial membranes

In summary studies of diabetic placentae have produced varying results. They have often failed to define the severity of diabetic disease in the studied women making comparisons of study populations and results impossible. Additionally not until recently have studies recognised the importance of co-assessing maternal glycaemic control. Despite these limitations aberrations of villous vascular development appears to be a consistent finding in diabetic placenta and has persisted despite improvements in diabetic management and maternal glycaemic control over time. The importance of aberrant placental angiogenesis has recently been highlighted in studies of intrauterine growth restriction and hypoxia. To explain the increased risk of intrauterine stillbirth and hypoxia documented to occur in diabetic pregnancies further study of villous development in this condition is required.

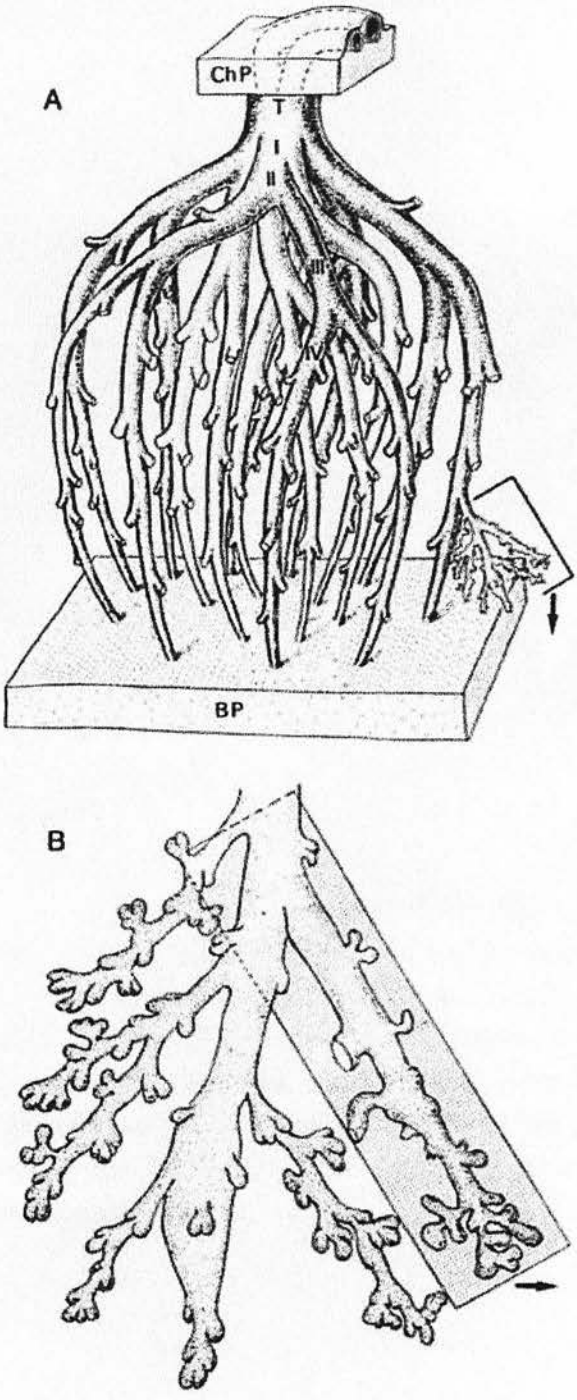


Figure 4.1(A-B) The basic structure of the human placenta at term.
(adapted from Bernirschke and Kaufmann 1995)

(A-B) A diagrammatic representation of the branching pattern of the villous tree.
Ch P = Chorionic plate; T = truncus chorrii; I-IV = generations of rami.
The vessels are shown within the distal villous tree in figure 4.1(C).

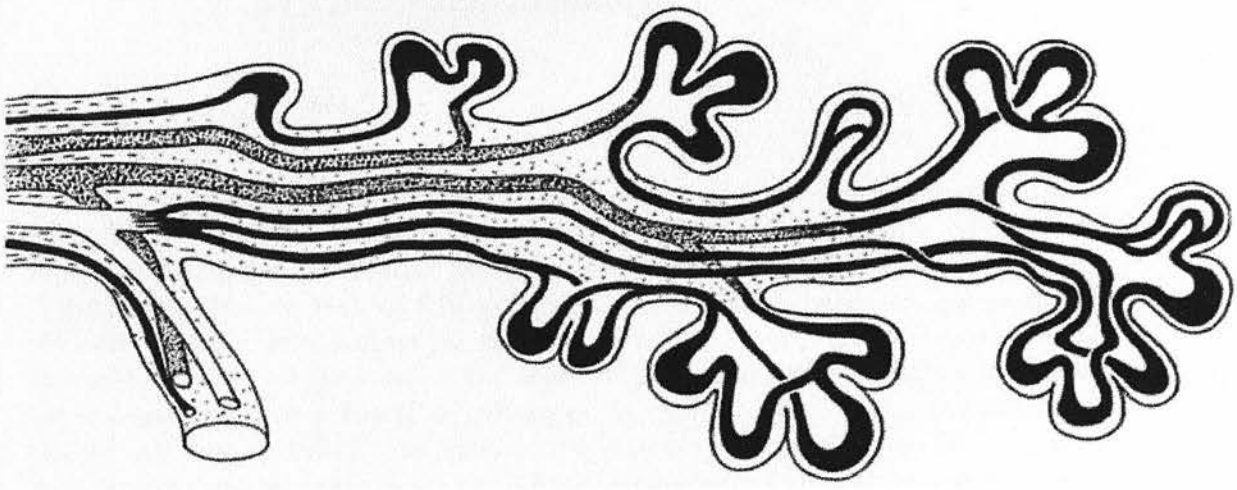


Figure 4.1(C) The basic structure of the human placenta at term.
(adapted from Bernischke and Kaufmann 1995)

(C) Fetal vessel architecture of an ending stem villous of the last ramulus (line hatched) and a terminal convolute composed of one mature intermediate villus (point shaded) together with its terminal villi (unshaded). Arterioles are point shaded, capillaries are black and venules are unshaded. Note the serial arrangement of capillary loops supplying several terminal villi.

Chapter 5

The three dimensional vascular structure of the placental terminal villus in diabetic pregnancy.

5.1 Introduction

Maternal diabetes mellitus is associated with a range of fetal complications. Perinatal mortality rate has fallen from greater than 50% to 2-3% between 1921, when insulin was discovered, and the present day. This reflects improved maternal blood glucose control, improved methods of fetal surveillance, avoidance of unnecessary premature delivery and the introduction of neonatal intensive care. The loss rate however remains 2-3 times higher than that seen in the non-diabetic population. Sudden intrauterine death contributes significantly to this raised perinatal mortality rate (Beard and Lowey 1982). The fetus of the diabetic mother also remains at risk of fetal distress and asphyxia in labour. The pathophysiology of these complications is poorly understood. It is hypothesised that the diabetic fetus is prone to hypoxia in utero (Casson et al 1980).

Development of the peripheral placental villous 'tree' and its vascular network serves to increase the placental surface area available for materno-fetal exchange and therefore fetal oxygen delivery. Analysis of placental structure often demonstrates specific abnormalities in association with fetal hypoxia, specifically that the peripheral villous vascular ultrastructure is altered. This may reflect or contribute to the fetal hypoxia. (Kingdom and Kaufmann 1997).

In a recent stereological study of placenta from diabetic pregnancies Mayhew et al (1994) demonstrated that the peripheral villi appeared hypercapillarised in the placenta obtained from diabetic pregnancies compared to those examined in matched non-pregnant control cases. The volume of the peripheral villi was not statistically different in placenta from diabetic and the control pregnancies, therefore individual placental villi appeared better developed to effect materno-fetal exchange in the placenta obtained from the diabetic subjects.

The terminal villus is where the majority of materno-fetal exchange occurs. Recently our department (Krebs et al 1996) has published studies of this specific specialised villus. Using a microvascular casting technique this group was able to demonstrate the three-dimensional vascular ultrastructure of the terminal villi in pregnancies complicated by intrauterine growth restriction and matched control cases. Using this technique they were able to clearly demonstrate aberrant (hypovascular) development of the terminal villi in placenta from growth restricted pregnancies suggesting a role in the aetiology of this condition.

The aim of the present study was to employ microvascular casting techniques to study in detail the three dimensional vascular structure of the terminal villi in

placentae of well controlled diabetic pregnancies. To correlate our findings to fetal outcome and to the recent light microscopy studies.

5.2 Materials and methods

5.2.1 Subjects

Placentae were studied from ten well-controlled insulin dependent diabetic women and ten gestationally matched healthy non-diabetic women. The women were matched for parity and smoking habit. Diabetic women were younger (mean: 24.1 years [SD 4.4] than non-diabetic women (mean: 30.6 years [SD 4.74]); $p<0.01$. The average duration of diabetes was 12.3 years [SD 4.1] in this cohort of diabetic women. The diabetic women were seen at least fortnightly in a specialised diabetic-antenatal clinic. Insulin requirements were adjusted on the basis of twice weekly 4 and 7 point blood glucose measurements. Diabetic control was also assessed by HbA1c determination at each clinic visit. The diabetic pregnancies were electively delivered at 38 to 39 weeks gestation if they were undelivered at this time.

The non-diabetic women were recruited from the low risk antenatal clinic and all had an uncomplicated antenatal course. In all pregnancies, both diabetic and non-diabetic gestation was confirmed by ultrasound within the first 16 weeks and all resulted in the delivery of a singleton neonate, which was structurally normal in development. Clinical characteristics of individual cases are given in Tables 5.1 and 5.2.

5.2.2 Preparation of the vascular casts

The placentae were obtained immediately post delivery, briefly examined for any gross pathology. Preparation of the vascular casts followed the preparation of tissue for transmission electron microscopy studies (chapter 6) but occurred within 30 minutes of delivery.

A chorionic branch of the umbilical artery with its corresponding vein was identified on the fetal surface of the area of the placenta to be assessed. The artery was cannulated with a 16-gauge cannula (Venflon), and secured with a single suture (2/0 chromic catgut) to prevent backflow of the perfusate. The venous outflow was punctured to complete the open vascular circuit. The cotyledon vascular circuit was then flushed with a minimum of 100 mls 0.9% sodium chloride until the venous effluent was clear of fetal blood. A low viscosity plastic polymer was then prepared as follows: 0.5g of catalyst was dissolved in 5 ml of methyl methacrylate. This was then added to a disposable glass beaker containing 20 ml of Mercox CL-2B resin base. The contents of the beaker were then stirred with a glass rod for 30 seconds. The resultant plastic polymer was then drawn into a 50ml syringe and infused into the chorionic artery until the venous effluent was pure polymer. The vein was then ligated and infusion continued until resistance was felt. The arterial site was then clamped. The perfused and immediately adjacent placental cotyledons were grossly dissected from the remaining placental mass. They were then allowed to sit undisturbed for 20 – 30 minutes until the polymer had set. The polymer perfused area

of the placenta was then grossly dissected from the remaining placental mass, and left overnight at room temperature to ensure complete polymerisation of the cast.

The placental tissue was subsequently corroded from the cast by alternate (6 hour) immersions in 20% potassium hydroxide (at 40 °C) and distilled water. At least six cycles of the immersion process were performed per cast to ensure that the cast was free of any tissue debris and the final immersion solutions were clear. A resultant placental vascular cast is depicted in Figure 5.1. The casts were then immersed in fresh distilled water and fractured along lines of natural cleavage into samples approximately 0.5 to 1 cm square. Random fractured sections of the cast were sputtered with gold for 3 minutes. They were then coded and examined, blinded to clinical data, in a digital scanning electron microscope. All casts were stored in a desiccator until analysis (Leiser et al 1985).

5.2.3 Analysis of the vascular casts

Each cast was initially viewed at low power (x100) to determine areas were the intermediate and terminal villous vasculature could be easily delineated. A terminal capillary loop was defined as the total capillary structure branching from the intermediate villous vasculature, which formed one spatial discrete capillary system. Analysis of the terminal capillary loops was performed at high magnification (x500). Four areas of each vascular cast were examined allowing an average of 35 consecutive terminal capillary loops to be measured (range 27-42)

Each capillary loop was assessed in the horizontal plane by rotating the cast appropriately. The following capillary parameters were assessed by the author: (1) the length of the terminal capillary loop, defined as the distance from the base of the capillary loop to its tip, (2) branching, the number of visible branching points (3) the degree of capillary coiling, (defined subjectively as 0 = no coiling, 1 = mild coiling, 2 = moderate coiling and 3 = high degree of coiling), and (4) the degree of any capillary dilatations, (again defined subjectively from 0 to 3 with increasing degree of dilatation). A typical analysis is demonstrated in Figures 5.2 and 5.3.

5.3 Statistical analysis of the vascular casts

Length data were log (x) transformed and branching data were square root transformed prior to analysis to resemble normal distribution. The data for capillary length and number of capillary branches were expressed as geographic mean (standard deviation). Diabetic and non-diabetic data were compared by Student's t test. Inter-group comparisons of coiling and dilatation data were performed by Chi-Square analysis. P values < 0.05 were considered to be significant.

5.4 Results

5.4.1 Comparison of clinical data

The mean birth weight was significantly greater in diabetic compared to control pregnancies (4.27kg [SD 0.91] vs. 3.42kg [0.46]; $p<0.02$). The mean placental weight was also significantly greater in diabetic compared to control pregnancies (937.5g [174.4] vs. 716.0g [151.1]; $p<0.05$). The placental to fetal weight ratio was not significantly different in diabetic and control cases.

5.4.2 Comparison of vascular casts

There was no gross abnormality of the placenta collected from diabetic pregnancies compared to control pregnancies. Low powered views and initial high-powered views of the plastic casts demonstrated that all casts appeared highly comparable. Typical capillary loops from a control placenta are shown at a 100-fold magnification in Figure 5.4, and at a 500-fold magnification in Figure 5.5. Corresponding images of capillary loops from a diabetic case are shown in Figures 5.6 and 5.7.

The capillary loops in the diabetic casts when compared en mass were significantly longer than those in the control cases (mean 8.55 $x10^{-5}$ m [SD 2.86] vs. 8.11 [SD 2.68]; $p<0.05$. The mean capillary lengths of the capillary loops in each cast are illustrated in Figure 5.8.

The terminal capillary loops also demonstrated increased visible branching points in the diabetic placental casts compared to control casts (2.52 [SD1.86] vs. 2.25 [1.72]; $p<0.05$). The mean numbers of capillary branching points in each cast are illustrated in Figure 5.9. There was no significant difference in either the degree of capillary coiling or dilatations between diabetic and control cases.

5.5 Discussion

Placental studies can be compromised by inadequate sampling techniques. The human placenta varies in its anatomy in different regions. Globally the areas immediately beneath the cord and at the periphery will clearly differ in volume of large stem villi they contain. The areas immediately beneath the chorionic plate are less readily bathed by maternal blood. Areas of infarction are sometimes evident. However even with the main bulk of the placenta considerable heterogeneity exists. The placental villi are arranged in placentomes (Shuhmann 1981). This represents a functional unit: a villus tree and its maternal blood supply. The maternal blood enters at the centre of the villus tree, where stem and immature intermediate villi are concentrated, and exits the intervillous space near clefts between neighbouring villous trees, where mature intermediate and their terminal villi are concentrated.

Providing a central parabasal placentome is adequately studied the findings can representative of the whole placenta (Bacon et al 1986). This is the methodology employed in this study: casting a number of central placentomes and then analysing random fractured sections. However, it should be noted that this is not a true stereologic morphometric study, which requires initial random sampling and then systematic sampling around this site (Mayhew and Burton 1988). An attempt to improve the validity of our data, analyses on the first 4 casts were repeated using measurement data from 10, 15, 20 25, 30 and 35 terminal capillaries (Maraca 1995). This demonstrated that there were no difference in the means and standard deviations obtained for 20 or more capillary loops. Measurements of > 20 capillary loops were therefore obtained for each cast. As scanning electron microscopy can only adequately assess the capillary loops exposed on the surface of the fractured cast specimen, all capillary visualised in random areas were analysed, generating numbers for analysis between 27 and 42, in an attempt to avoid introducing selection bias i.e. of particularly evident capillary loops. These limitations of our study should be acknowledged when assessing our findings.

We have demonstrated that the terminal villous capillaries are of increased length and have an increased number of visible branching points in placentae examined from diabetic pregnancies compared to control pregnancies. Although to date studies of placentae obtained from diabetic pregnancies have produced conflicting results, these findings support a recent study of gycaemically controlled diabetic pregnancies, in which increased villous vasculature has been demonstrated (Mayhew et al1994). In this previous study the placental villi were described as being better developed in diabetic pregnancies, with an increased volume, surface area, length and mean diameter of the terminal villous vasculature.

Terminal villi begin to form early in the second trimester of pregnancy. They occur where the stimulus for vascular growth in the intermediate villi is greater than the growth of these villi themselves. They therefore form as grape like outpouchings of these capillaries, carrying before them a surface layer of trophoblast (Kaufmann and Burton 1994, Benirschke and Kaufmann 1985). Further stimulation of vascular growth results in increased length and number of these terminal villous capillary loops and villi. The increased terminal capillary length demonstrated in this study

might suggest that the vascular stimulus to capillary growth is increased in diabetic pregnancies. One of the most potent stimuli to angiogenesis is hypoxia.

Oxygen deprived tissues release a range of angiogenic growth factors such as bFGF (Ogawa 1991), VEGF (Sunderkotter 1994, Wheeler, Elcock and Anthony 1995) PIGF (Ahmed, Whittle and Khaliq 1997). Increased placental angiogenesis has been demonstrated to occur in situations in which there is decreased oxygen delivery to the placenta. This may arise either by reduced oxygenation of the maternal blood; pregnancies at high altitude (Jackson, Mayhew and Hass, 1987) or maternal anaemia (Fox 1978, Burton 1996), or where there is reduced flow of normally oxygenated blood to the placenta e.g. in pre-eclampsia (Salvatore 1968, Burton et al 1996). No diabetic women studied developed pre-eclampsia during the studied pregnancy, and none of the diabetic women studied were anaemic therefore the angiogenic stimulus to terminal villous capillary growth was not as a result of such pre-placental or utero-placental mechanisms.

Maternal diabetes may however cause a unique form extra villous hypoxia. It has been suggested that the fetal metabolism is greater in diabetic pregnancies than non-diabetic pregnancies. This occurs due to the increased circulating concentrations of insulin and resultant organomegally of these fetuses (the Pedersen hypothesis). The increased fetal metabolic rate results in increased fetal consumption and demand for oxygen. This in turn requires that more oxygen be delivered to the fetus from the placenta and therefore that the placenta must extract more oxygen from the maternal blood in the intervillous space. The resultant relative hypoxia in the intervillous space may be the trigger for increased terminal villous angiogenesis.

The metabolism of placenta itself is also likely to be increased in diabetic pregnancies contributing to a local 'hypoxic' environment. Alternatively the increased fetal growth and metabolism may not play an aetiological role in increased placental angiogenesis rather both may occur as a direct result of a maternal signal i.e. insulin like growth factors.

The finding that capillary branching is increased in diabetic placentae is notable. Firstly such detailed analysis of terminal villous capillary structure can only be inferred but not visualised by any other histological techniques (i.e. stereological studies). Secondly as increased branching occurs in association with increased capillary length important information can be gained regarding the function of these terminal capillary loop systems. The feto-placental vascular tree in order to function efficiently needs to act as a high flow low impedance circulation. The terminal villi by nature of their formation arise in series. In order to maintain low resistance in such an increasing circuit capillary branching and reanastamosis occur so blood may flow through part of the capillary system in parallel. These adaptations may maintain a low total resistance of a more volumous capillary vascular system (Kaufmann 1988). Therefore the increased capillary growth demonstrated in diabetic placentae may not be pathological but a continuation of the normal physiological development of the placenta resulting, as suggested by Mayhew (1994), a better developed villus able to extract oxygen more efficiently from the immediate surrounding environment.

The mean terminal capillary lengths in the present study were stikingly shorter than those measured in pregnancies complicated by intrauterine growth restriction (217.8μm (SD71.9) and the gestationally matched control placentae (136.9μm (SD 29.8). The average gestation of the studied pregnancies was 31 weeks in both the IUGR and control groups. The increased length of these peripheral capillary systems reflects a lack of terminal villous formation. In the control group this reflects the normal limited development of the peripheral villous tree at this gestation. As terminal capillary loops form from the intermediate villus capillary network the average length of the most peripheral capillary system will reduce. In essence the ratio of terminal villi to intermediate villous capillary systems measured will increase. The disproportionately increased length of the terminal capillary loop systems measured in the IUGR group indicates a failure of the normal development of the peripheral vascular villous tree. This is supported by findings that the volume of the peripheral vascular tree (intermediate and terminal) villi is reduced in IUGR pregnancies (Jackson et al 1995).

The mean gestation of the diabetic pregnancies studied in this study [37.2 weeks (SD 1.1)] was less than the mean gestation of the control pregnancies [38.6 weeks (SD 0.70]; $p<0.005$. This difference could have a confounding effect on the capillary indices assessed for the reasons discussed above. However at these later gestations it is unlikely that de novo terminal villus formation is significant, rather it is the 'differentiation" of the numerous formed terminal villi that is important in placental adaptation to fetal demands. Studies of placentae from accurately dated pregnancies progressing to 42 and 43 weeks' gestation have found the terminal villi to be elongated, branched and notched (Kaufmann and Bernische), supporting this hypothesis. Gestational effects, outwith the first trimester, are after all a response to fetal growth and demands and not a reflection of time per se. Placental oxygen diffusive conductance (Mayhew et al 1993), and fetal oxygen uptake (Hofman 1983, Bell et al 1985) are more closely correlated to fetal weight gain than to gestation. The increased length and branching of the terminal capillary loops in diabetic cases is more likely to reflect the increased fetal demands of these pregnancies than a gestational artefact.

The significant difference between diabetic and control indices measured reflects the large number of terminal villous capillary loops assessed in each group. Given the small differences in the mean capillary length and visible branching points between the studied groups analysis of fewer capillary loops would not have had sufficient power to demonstrate these differences. It could therefore be argued that these small differences are unlikely to be functionally significant given the presumed functional reserve of the human placenta. Certainly the differences demonstrated in the present study are not of the same magnitude as the pathological differences seen on comparison of villi from IUGR and matched control pregnancies. We would argue however that the total number terminal capillary loops in an entire placenta is vastly greater than that studied here suggesting that when extrapolated to the entire organ our findings may have a valid significance.

Previous studies of diabetic pregnancies have produced conflicting results, several indicating severe pathology of the placental tree. The women in the present study were intensively reviewed throughout their pregnancies; as a result demonstrated good and continually improving glycaemic control throughout pregnancy. Such improved control is likely to have influenced placental development and resulted in the lack of any major demonstrable abnormality of the placental tree. Such 'normalisation' of placental structure in diabetic pregnancies is reassuring to the obstetrician, however our findings suggest that despite good maternal well-being and good diabetic control the terminal villus may still to undergo adaptive measures to safeguard fetal well-being.

Subject Number	TEM Studies	SEM Studies	Maternal Age (years)	Parity	Smoker	Gestation Delivery (weeks)	Birth Weight (kg)	Birth Weight Centile	Fetal Sex	Placental Weight (g)
156	√	√	24	1	No	35	2.94	50-90	Male	755
158	√	√	25	0	No	37	3.38	50-90	Female	640
159	√		27	1	No	37	4.63	>97	Female	990
160		√	16	0	No	36	3.48	90-95	Female	880
162	√	√	28	1	Yes	37	5.07	>97	Male	1010
163	√		27	2	No	38	3.98	95-97	Male	620
165		√	23	1	No	39	4.63	>97	Male	990
166		√	27	1	No	38	4.30	>97	Female	930
188	√		29	0	No	38	3.96	95-97	Male	660
218	√	√	22	2	No	38	4.67	>97	Male	1120
232	√	√	32	1	No	38	5.84	>97	Female	1030
237	√	√	20	0	No	37	3.55	50-90	Male	800
247		√	24	0	Yes	37	4.85	>97	Female	1220
268	√		25	0	Yes	38	3.94	95-97	Male	860

Table 5.1 Clinical characteristics of the pregnant diabetic cohort: placental studies.

The diabetic cases assessed by transmission electron microscopy (TEM) were also the cases studied in the light microscopic immunohistochemical studies. The vascular casts of cases 159, 163,188, and 268 contained large areas of extravasation and were not of sufficient quality to allow analysis by scanning electron microscopy. They were substituted by analysis of vascular casts made from cases 160,165,166 and 247.

Subject Number	TEM Studies	SEM Studies	Maternal Age (years)	Parity	Smoker	Gestation Delivery (weeks)	Birth Weight (kg)	Birth Weight Centile	Fetal Sex	Placental Weight (g)
183	√	√	32	1	No	40	3.43	50	Male	810
184	√		34	1	No	40	4.06	90-95	Male	700
219	√		29	3	No	38	3.56	50-90	Female	760
220	√	√	30	1	No	38	3.42	50-90	Male	610
225	√	√	27	1	No	38	4.19	95-97	Male	970
227	√	√	23	1	Yes	39	3.80	50-90	Male	720
264	√	√	34	2	No	39	3.96	90-95	Male	880
283		√	35	0	No	39	3.26	10-50	Female	840
284		√	32	0	No	38	3.26	90-95	Female	440
381	√	√	39	3	No	38	2.66	10-50	Male	660
382	√	√	27	0	Yes	39	3.26	10-50	Female	620
384	√	√	27	1	No	38	2.98	10-50	female	720

Table 5.2 Clinical characteristics of the pregnant control cohort: placental studies.

The control cases assessed by transmission electron microscopy (TEM) were also the cases studied in the light microscopic immunohistochemical studies. They were randomly selected. The vascular casts of cases 184 and 219 contained large areas of extravasation and were not of sufficient quality to allow analysis by scanning electron microscopy. They were substituted by analysis of vascular casts obtained from cases 283 and 284.

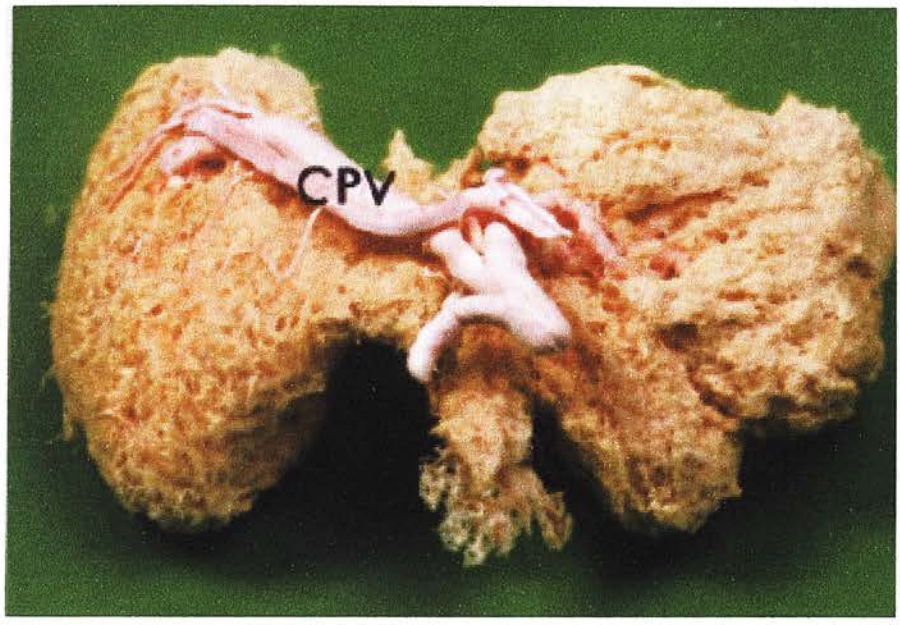


Figure 5.1 A placental vascular cast (macroscopic image)

A vascular cast of one whole placental cotyledon can be seen (left) with its associated chorionic plate vessels (CPV). The upper vessel is the artery through which the plastic polymer is infused into the cotyledon vascular circuit. The lower vessel is the vein. Once the venous effluent consists of pure plastic this vessel is ligated to allow optimal filling of the cast. The placental tissue has been corroded from the cast by immersion in a strong alkali solution.

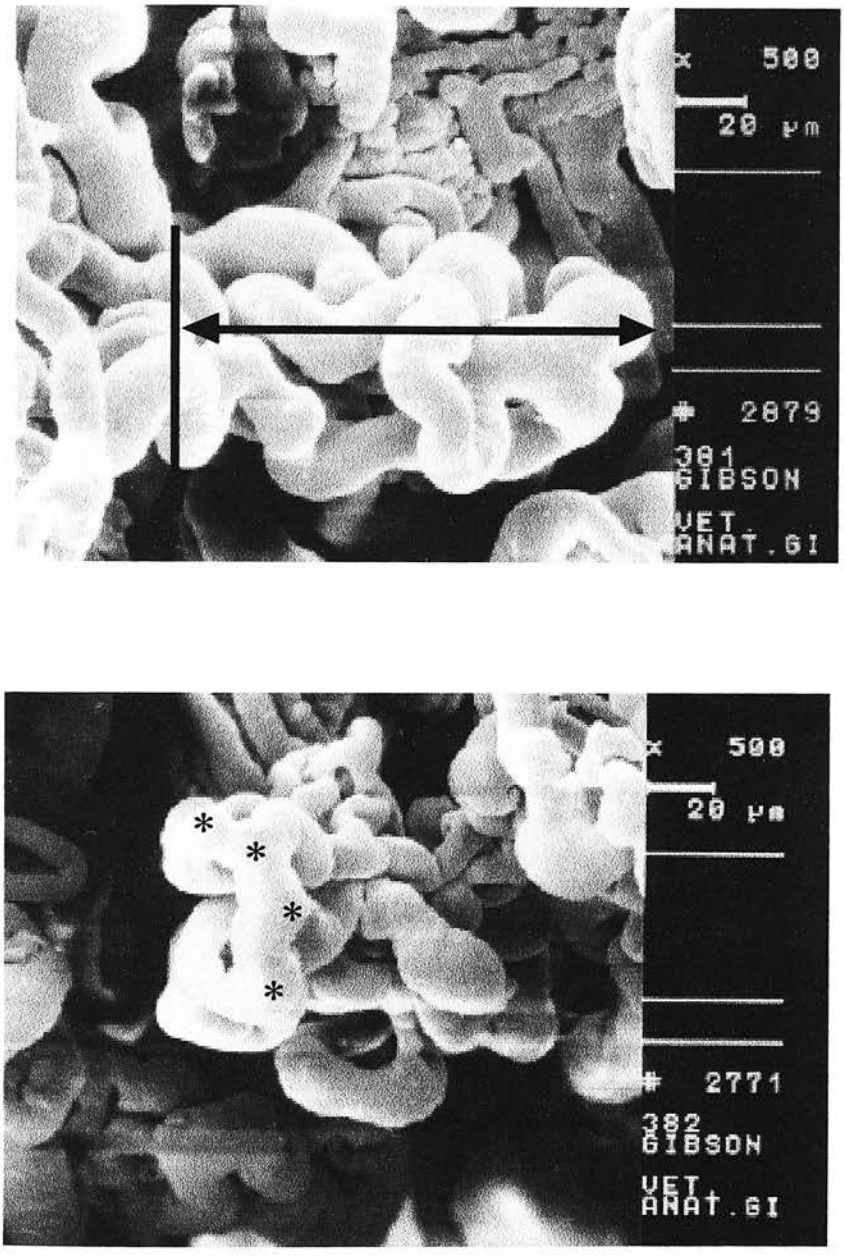


Figure 5.2 Assessment of terminal capillary loop length and branching.

The upper micrograph demonstrates the measurement of the terminal capillary loop length. The capillary loop was rotated to lie in the horizontal plane. The length was measured from the base of the capillary loop, where it arose from the intermediate villus vasculature, to its tip.
The lower micrograph shows a terminal capillary loop system demonstrating 4 visible branching points (*)
The terminal villus capillary loops were slightly longer and more branched in the diabetic cases compared to control cases; $p<0.05$.

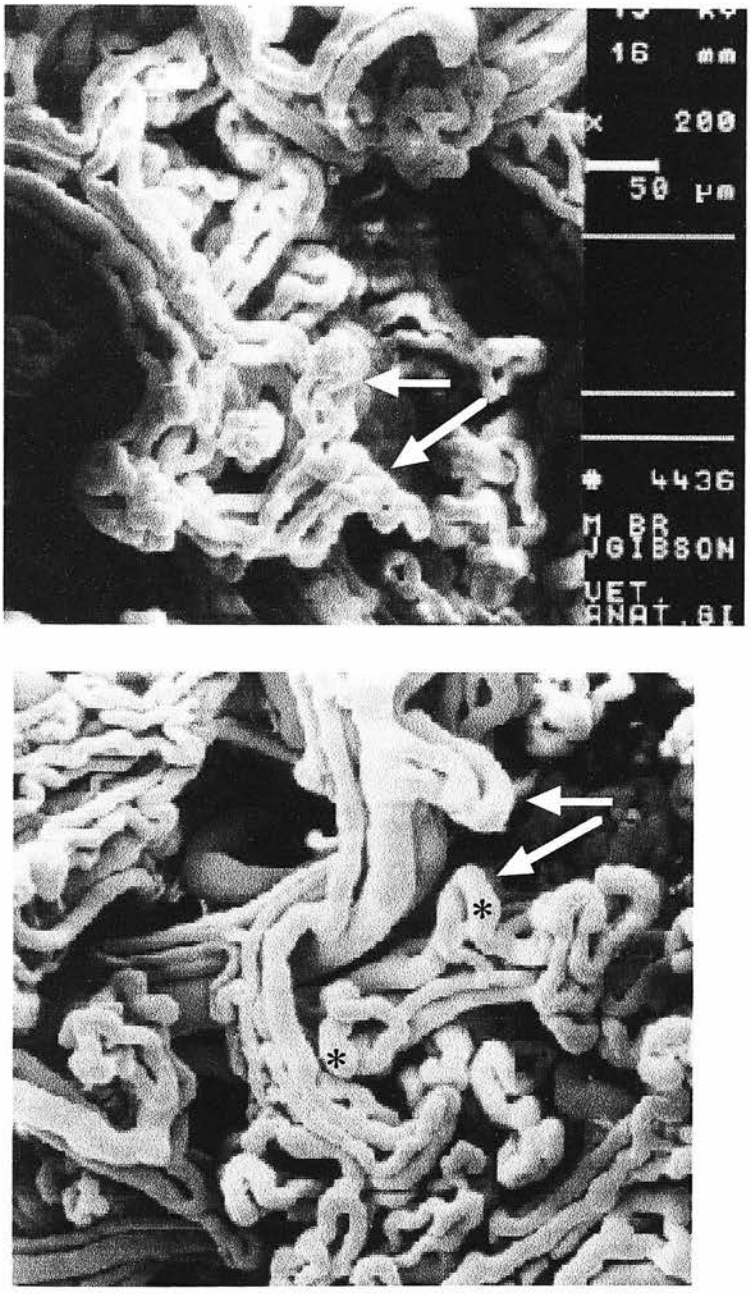


Figure 5.3 Assessment of terminal capillary loop coiling and dilatation.

In the upper micrograph two terminal villi capillary loop systems demonstrate a moderate (++) degree of coiling (arrowed). In the lower micrograph two capillary systems demonstrate no degree of coiling (arrowed).
There is evidence of capillary dilatations in the lower micrograph (*).
There was no difference between diabetic cases and control cases on comparison of these parameters.

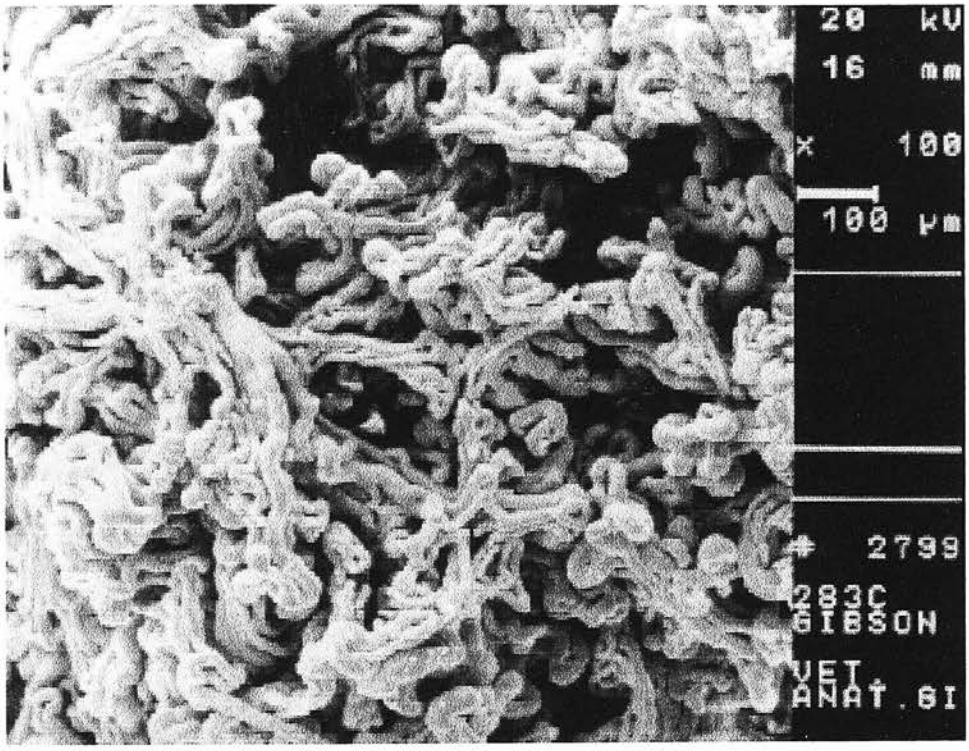


Figure 5.4 Placental vascular cast from a control case (x100)

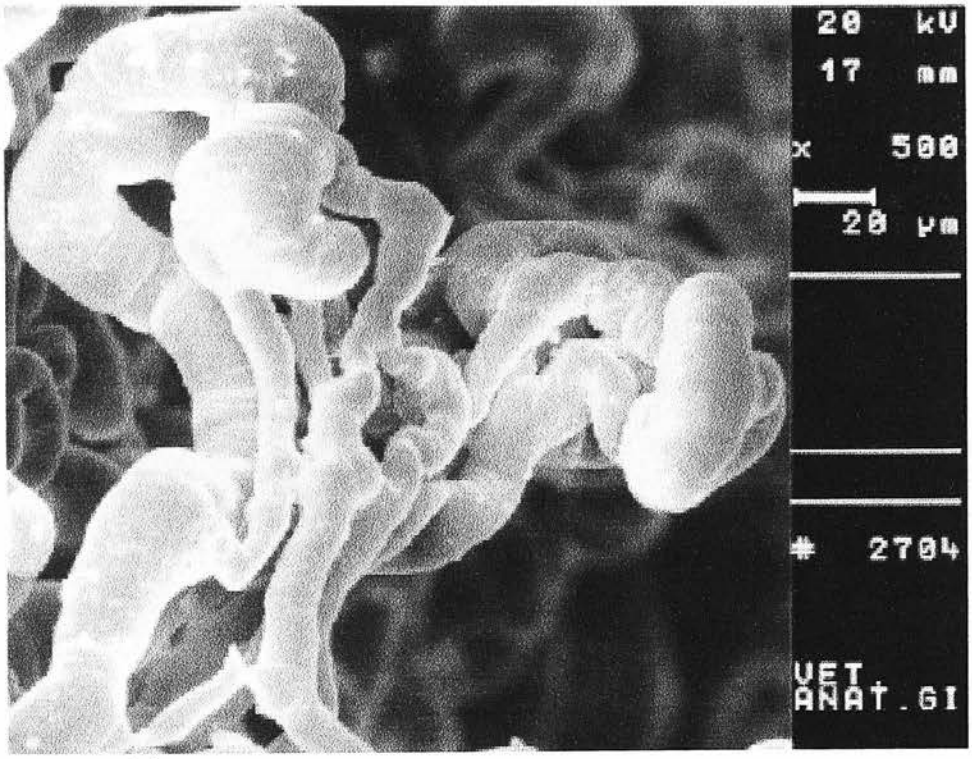


Figure 5.5 Terminal villous capillary loops from a control case (x500)

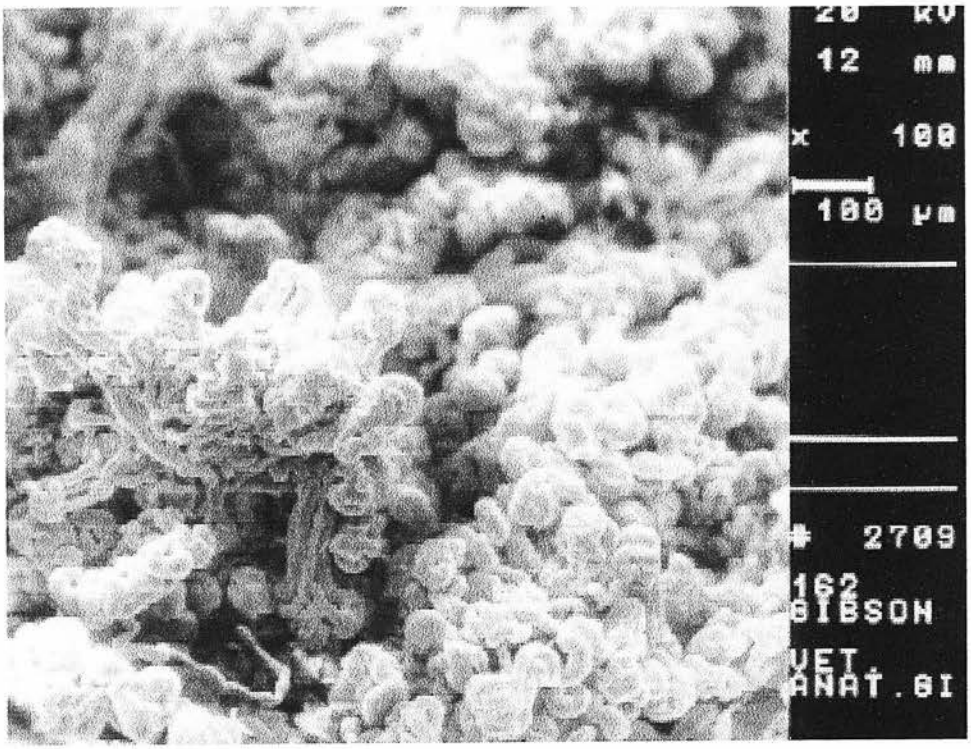


Figure 5.6 Placental vascular cast from a diabetic case (x100).

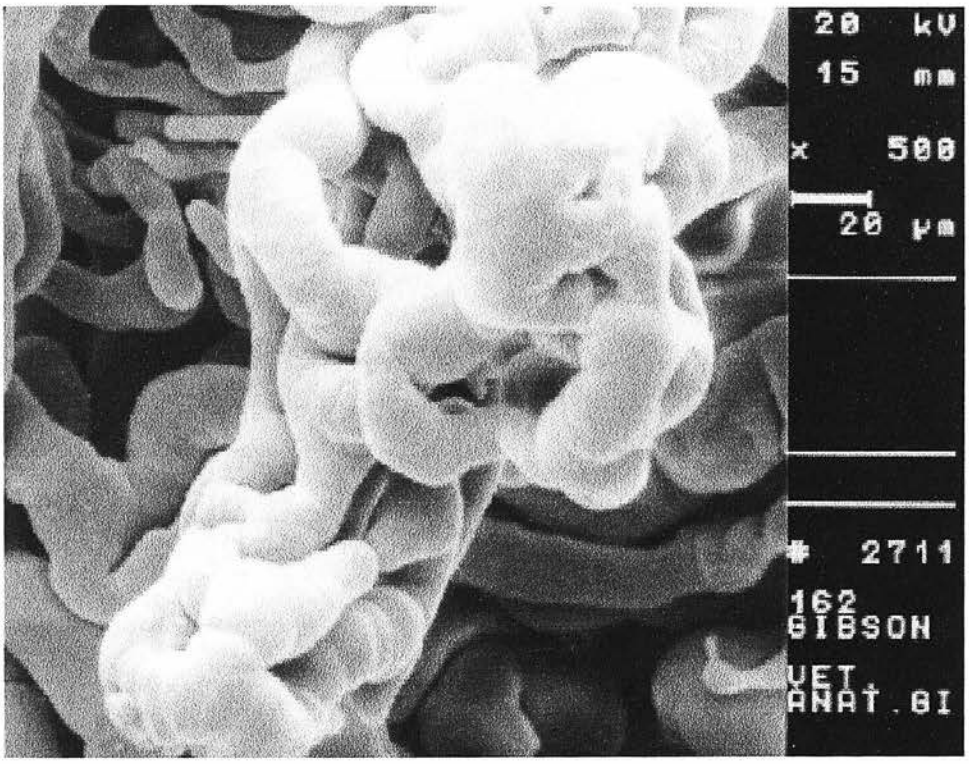


Figure 5.7 Terminal villous capillary loops from a diabetic case (x500)

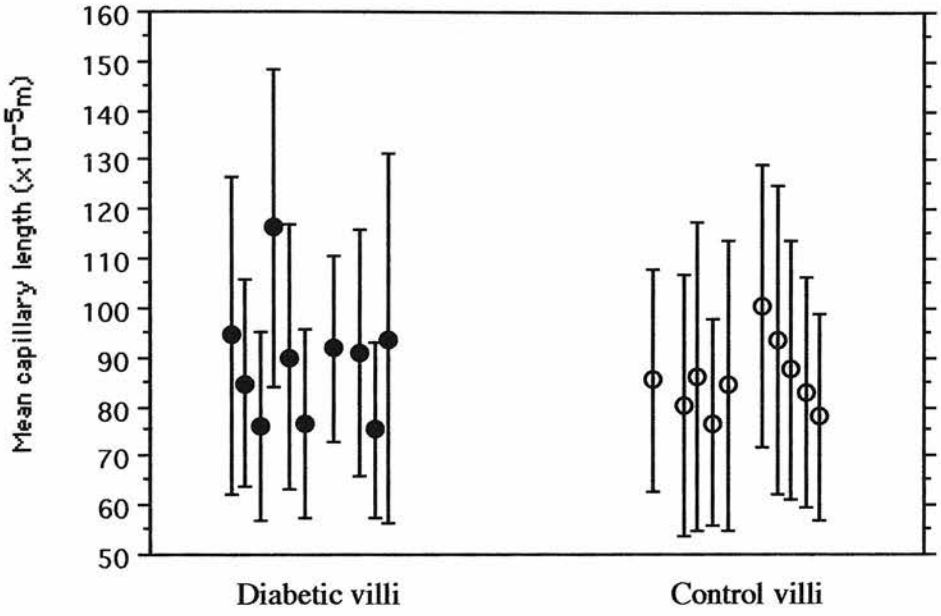


Figure 5.8 Mean terminal villous capillary length: diabetic vrs. control cases.

The mean length (± one standard deviation) of the capillaries in each of the 10 diabetic and the 10 gestationally matched control placental vascular casts.
The capillaries in the diabetic cases were slightly but significantly longer than those measured in the control cases; $p < 0.05$.

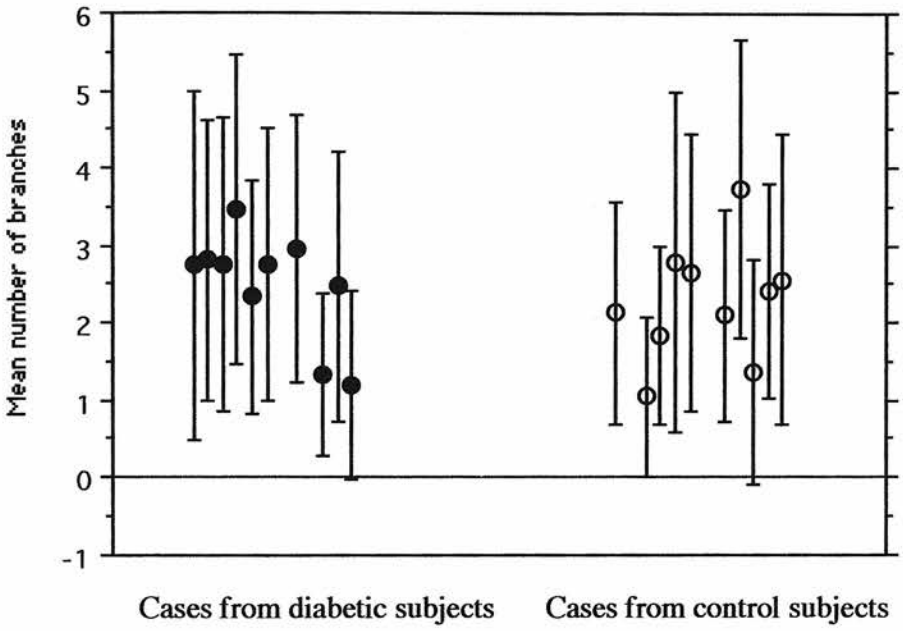


Figure 5.9 Mean number of visible capillary branches: diabetic vrs. control cases.

The mean number (± one standard deviation) of visible capillary branching points of each of the 10 diabetic and the10 control placental vascular casts.
The number of terminal villus capillary branches was slightly but significantly greater in diabetic compared to control cases; $p < 0.05$.

Chapter 6

The cross-sectional ultrastructure of the terminal villus in diabetic pregnancy

6.1 Introduction

Maternal insulin dependent diabetes is associated with a range of fetal complications. The incidence of many of these complications has reduced with improved maternal glycaemic control. However the risk of fetal compromise remains increased in diabetic pregnancies compared to non-diabetic pregnancies. Of particular importance the fetus of the diabetic mother appears to be of increased risk of intrauterine hypoxia manifest by an increased risk of unexplained stillbirth and fetal distress in labour.

By analysis of vascular casts prepared from placentae from pregnancies complicated by maternal diabetes we have demonstrated that the terminal villous capillary ultrastructure, although grossly comparable to control cases, demonstrates increased length and branching in these diabetic cases, despite apparent good maternal glycaemic control. This finding supports a previous study that suggested that the peripheral villous vascular tree is of greater volume in pregnancies complicated by maternal diabetes, possibly as an adaptation to fetal hypoxia (Mayhew et al 1994).

Fetal hypoxia occurring in association with placental hypoxia can be associated with other villous ultrastructural characteristics in addition to increased capillary volume (Kingdom & Kaufmann 1997, Bernishcke and Kaufmann 1985). To further investigate the possibility of placental hypoxia in diabetic pregnancies the cross-sectional ultrastructure of terminal placenta villi from such pregnancies was assessed by transmission electron microscopy and complemented by light microscopic immunohistochemical studies.

6.2 Materials and methods

6.2.1 Subjects

Placentae were obtained from ten insulin dependent diabetic women and ten gestationally matched healthy non-diabetic women. The women were matched for parity and smoking habit. Diabetic women were younger (mean age = 25.9 [SD 3.5] years) than non-diabetic women (30.2 [SD 4.6] years; $p < 0.05$)
Clinical characteristics of individual cases are given in Figures 5.1 and 5.2.

Diabetic control as assessed by HbA1c determination was found to improve significantly with gestation in the diabetic pregnant group, (mean [SD] HbA1c = 7.13 [0.84]% at 12 weeks gestation, 6.01 [0.48]% at 36 weeks gestation, $P < 0.01$, non-diabetic reference range = 3.4 – 5.2%)

6.2.2 Preparation of tissue for transmission electron microscopy.

Within 5 minutes of delivery a random placental cotyledon was perfusion fixed to secure tissue of sufficient quality for transmission electron microscopic assessment. A chorionic plate artery and its corresponding artery were identified. The artery and vein were prepared as described in section 5.2.2. Venous puncture of the vein ensured optimal fixation of the entire specimen and was necessary to limit any artefact that might arise from feto-maternal fluid shift. (Burton et al 1988). The artery was initially flushed with 10 mls of normal (0.9%) saline to irrigate the vascular bed. Immediately thereafter the artery was perfused at a constant pressure with 20 mls of 2.5% neutral (pH 7.2): buffered gluteraldehyde over a 5 minute until the perfused tissue became pale and firm. Thereafter approximately 10 – 15 2 mm blocks of fixed tissue were randomly excised from a central area above the basal plate. The excised blocks were stored in 15-20 mls of the of 2.5% gluteraldehyde until they were processed for examination by transmission electron microscopy.

6.2.3 Tissue processing for transmission electron microscopy.

Five random tissue blocks were rinsed in phosphate buffer, then post-fixed for 45 minutes in 1% osmium tetroxide. The blocks were then rinsed again in phosphate buffer and then dehydrated by immersion in ascending concentrations of ethyl alcohol. They were then embedded in araldite resin and polymerised at 60°C for 24 hours.

6.2.4 Analysis of tissue by transmission electron microscopy.

Three blocks of tissue were examined per case. The tissue blocks were coded to allow analysis blinded to clinical details. From each tissue block semithin (0.5 – 4.0μm) sections were prepared with an LKB ultramicrotome. These were stained with toludine blue to allow visualisation of the cut section by light microscopy. When the section visualised contained terminal villus cross-sections the area corresponding to this was isolated from the tissue block using a standard paring technique. Ultrafine sections of this area were then cut with the LKB ultramicrotome using a fresh class knife. The cut sections collected into a water filled trough mounted on the knife surface. The floating sections were than captured onto a copper plated grid. Each copper grid retrieved an average of 2 sections. The sections were then stained by immersion in uranyl acetate for 10 minutes and then lead citrate for a subsequent 2 minutes. The sections were then stored in a grid box until analysis.

The grids were viewed in a Philips CM 10 transmission electron microscope. By viewing the three prepared sections per case analysis of a minimum of seven (usually 10-14) terminal villous cross-sections was possible per case. Villous sections that appeared longitudinal, oblique or branched were excluded from analysis. Each terminal villus was viewed and photographed at three standard final magnifications. The total villous composition and stromal content was assessed at x 575 and x 2700 magnification. Areas comprising vasculosyncytial membranes were viewed at x 14850.

6.2.5 TEM assessment of villus parameters

Terminal villi were identified for analysis using the morphometric characteristics described by Kaufmann et al (1985) (see section 4.1.5). The following parameters were assessed in detail: villous diameter, capillary diameter, number of capillary cross-sections per villus, cytotrophoblast (nuclear) number, syncytiotrophoblast (nuclear) number, stromal composition, trophoblast basal lamina (densa) thickness, and the thickness (diffusion distance) of the vasculosyncytial membranes.

Villus diameter derived by measuring the villus in a random set of two perpendicular planes and then calculating the average. Capillary diameter was defined as the smallest measurable diameter of the capillary section (Figure 6.1). This was to avoid skewing the data by the inclusion of oblique capillary sections. All capillary sections per villous were assessed. The thickness of the lamina densa was measured in the areas were this layer was clearly defined and was within the region of a defined vasculosyncytial membrane (Figure 6.2). The minimal thickness of this layer was measured for each photographed 'membrane'. The minimal thickness of the entire vasculosyncytial membrane (outer trophoblast to inner capillary margins) was measured in each instance. The density of the villus stroma was assessed qualitatively in each case, assessing in particular the presence of collagen fibers and of background fibrillar material (Figure 6.1).

6.2.6 Preparation of tissue for light microscopic studies.

Following delivery, and subsequent to obtainment of tissue for TEM studies and initial cast preparation for SEM studies, full thickness blocks of placentae were cut from the remaining specimens. Several blocks from the diabetic and control pregnancies were taken in a random fashion, avoiding the area immediately beneath the umbilical cord insertion and at the extreme periphery of the placentae. The placental blocks were immersed in neutral buffered 4% formalin solution for 24 hours to effect fixation. The tissue blocks were then processed in the standard manner for paraffin embedding.

6.2.7 Tissue processing for immunohistochemisty.

Serial sections of tissue (3-5um) of tissue were cut from one random parraffin block obtained from 10 diabetic and 10 control cases. Individual sections were mounted onto glass slides. The glass slides had been pre-treated to aid adhesion of the tissue sections to the slides; the slides had been immersed in ethyl alcohol, incubated with 2% APES for 20 seconds, then washed in acetone and aqua dest (1 minute each). The cut sections mounted on the glass slides were incubated at 37°C overnight. Then stored in 'air-tight' slide boxes until analysis.

From each case one cut section was prepared for each primary antibody. Prior to exposure to the antibody the sections were deparaffinised in xylene (2 x 10 minutes) and graded ethanol (100%, 96% and 90% ethyl alcohol for 5 minutes each). Endogenous peroxidase activity within the specimen was then blocked by incubation with 1% hydrogen peroxidase in methanol. The sections were then washed in PBS [sol]. The sections to be stained for Ki-67 antibody binding were immersed in 10 mM citric acid and pre-treated by microwave (4 x 5 minutes at 600 Watts) to expose the antigen.

Immunohistochemistry
The following primary antibodies were employed: Collagen I (1 in 50 dilution), Collagen IV (1 in 90 dilution) both from Europath UK, Collagen III (1 in 50 dilution, Biogenesis, UK), Laminin (1 in 2000 dilution) and Fibronectin (1 in 500 dilution) both from Dako UK, Ki-67 (1 in 20 dilution, Dianova Germany). The sections for each antibody were all processed simultaneously in a single run. A commercial steptavidin-biotin technique was utilised (Histostain SP, Zymed ,CA).

Ki-67:
The slides were incubated with 10% non-immune goat serum to eliminate background staining. The sections were then incubated with the primary antibody, Ki-67 (diluted 1:20 in PBS [sol] with 1.5% BSA; Key et al 1993) for 60 minutes. The slides were washed three times in PBS [sol] then incubated with the secondary biotinylated goat anti-rabbit antibody for 30 minutes. The slides were then washed again in PBS[sol] and the sections were then incubated for 5 minutes with the steptavidin-peroxidase conjugate, followed by a further wash in PBS[sol]. The bound

antibody was visualised by incubating the sections with an AEC chromagen / hydrogen peroxide mixture for 15 minutes. The slides were then washed in distilled water (2 x 5 minutes) and then counter-stained with haematoxylin (3 minutes).

A negative control was generated by replacing the primary antibody with PBS[sol] /1.5%BSA. The control remained negative. A positive control slide was generated by including a section of a tonsil gland. The rapidly dividing lymphocyte population within in this structure stained positive with Ki-67.

Stromal staining:
The polyclonal antibodies; Fibronectin, Collagen I, Collagen III, Collagen IV.
The sections were covered with a serum blocker [(20%) normal swine serum in PBS] for 20 minutes to reduce non-specific background staining. After tipping the blocker from the slides. The primary antibody was added to the sections. After an incubation period of 18 hours (at 4°C) the sections were washed in PBS [sol] (3 x 10 minutes). A secondary antibody (swine anti-rabbit 1:100 in 4% NSS/PBS) was then applied to the sections. After a 45-minute period the sections were then washed three times in PBS. Peroxidase conjugated steptavidin (1:800 in 4% NSS/PBS) was then applied to the sections. After a further 45-minute incubation period the sections were washed again PBS (x3). AEC was then applied to the sections for 15 minutes. The sections were then washed twice in distilled water and then counterstained with haematoxylin for 3 minutes before mounting with coverslips.

The monoclonal antibody: Laminin.
A similar protocol to above was followed with the following modifications: 2% BSA in PBS was used as the initial blocking solution, the secondary antibody applied was peroxidase conjugated rabbit anti-mouse (1:50 in 2% BSA/PBS), peroxidase conjugated avidin (1:100 in 4% NSS/PBS) was used as the third layer.

Negative controls for each stromal antibody were generated by omitting the primary antiserum. The specificity of the procedure was verified using pre-absorbed specific antiserum. Positive controls were generated by applying each antibody to sections of hepatic and vascular tissue. The staining patterns of which were known from previous laboratory work.

6.2.7 Analysis of tissue by light microscopy.

Stained slides were blinded and coded prior to analysis. Ki-67 antibody positively stained trophoblast nuclei were counted in 50 terminal villi per case. In addition stained nuclei in the stromal compartment were also counted.

Analysis of specimens stained for collagens I, III and IV, laminin and fibronectin was performed in a visually qualitative method by the author. Staining was scored (0, +, ++, +++, ++++) in the following locations: the area immediately underlying the trophoblast and the capillaries, representing their basement membranes, and in the stromal core of the terminal villus.

6.3.1 Statistical analysis of TEM assessed villous parameters

All parameters approximated normal distribution. Mean values were compared by student's t test. A P value of < 0.05 was taken to indicate a significant finding.

6.3.2 Statistical analysis of immunohistochemical studies

The number of cell nuclei positively stained with Ki-67 in each compartment in diabetic and control placentae were compared by student's t test. The numbers (of stained cells per compartment in 50 villi) were log (x) transformed prior to analysis.

The density of stromal staining for Fibronectin, Laminin, and Collagens I, III and IV in the diabetic and control groups were compared by Chi- square analysis.

6.4 Results

6.4.1 Clinical data

The mean birth weight was significantly greater in diabetic compared to control pregnancies (4.20kg [0.86] vs. 3.52kg [0.49]; $p<0.05$). The mean placental weight was not significantly different in diabetic compared to control pregnancies (848.5g [181.1] vs. 734.0g [112.0]). The placental to fetal weight ratio was also not significantly different in diabetic and control cases.

6.4.2 Transmission electron microscopy data

The terminal villi of diabetic placentae were visually grossly comparable to those of control placentae. Specifically, we demonstrated no significant difference in villous diameter, villous capillary diameter, cytotrophoblast or syncytiotrophoblast nuclei number between study groups. However, the measured thickness of the trophoblast basal lamina (Figure 6.3) and the diffusion distance across the vasculosyncytial membranes (Figure 6.4) were increased in the terminal villi from diabetic compared to control placentae. There were also an increased number of capillary cross-sections per villus in diabetic pregnancies (Figure 6.5). Absolute values are given in Table 6.1. Typical images of trophoblast basal lamina thickness and vasculosyncytial membrane thickness in diabetic and control cases are shown in Figures 6.6 and 6.7. Terminal villus cross-sections from a control case are shown in Figure 6.8 and from a diabetic case in Figure 6.9.

6.4.3 Immunohistochemical data

There was no significant difference in the numbers of Ki-67 stained cytotrophoblast cells in diabetic (mean 28 per 50 villi, SE 0.52) and control pregnancies (mean 23 per 50 villi, SE 1.63). There was also no significant difference on comparison of Ki-67 stained stromal cells in diabetic (mean 10.5 per 50 villi, SE 2.4) and control placentae (6.5 per 50 villi, SE 0.5). Typical staining patterns of nuclei are shown in Figure 6.10. Positive and negative control slides are shown in Figure 6.11.

Typical staining patterns of collagens, laminin and fibronectin were not significant different on comparison of diabetic and cases. A typical assessment (of villi studied from either of the two groups) of the areas comprising the trophoblast basement membrane, the capillary basement membrane and the stromal compartment is given in Table 6.2 and typical staining patterns depicted in Figure 6.12-6.16.

6.5 Discussion

In the previous chapter we have demonstrated that there is evidence of increased vascularity of the terminal placental villi in diabetic compared to control pregnancies. We have confirmed this finding in the present cross-sectional studies of villous ultrastructure; transmission electron microscopic studies have demonstrated an increased mean number of capillary cross-sections per villus diabetic cases compared to control cases whilst the mean capillary diameter was conserved. These complimentary findings support published morphometric data that the volume of the peripheral villus vasculature is increased in diabetic pregnancies (Mayhew et al 1994). One of the most potent stimulators of angiogenesis is hypoxia. We have therefore assessed in more detail the overall ultrastructure of terminal villi in diabetic pregnancies, in particular looking for other ultrastructural adaptations that could be associated with villous hypoxia in these pregnancies.

The external surface of the terminal villi is composed of trophoblast and as such the trophoblast is directly exposed to the maternal environment. Abnormalities of trophoblast structure and function have been shown to occur in response to a number of maternal disease states and conditions. The trophoblast is a metabolically active structure. Damaged or aged trophoblast nuclei are shed from the villous surface after they have been sequestered or pushed into knots. The syncytiotrophoblast is a specialised structure that has lost the capacity to replicate. It is the mitotic function of the underlying cytotrophoblast to ensue an adequate syncytiotrophoblast compartment. The mitotic index of the cytotrophoblast may therefore provide a measure of the metabolic strain of the syncytial trophoblast. An increase in the mitosis of cytotrophoblast, and an increased thickness of the cytotrophoblast layer (Fox 1964, Piotrowicz et al 1969, Bernishke and Kaufmann 1985) is seen in hypoxic conditions. Indeed the proliferation rate of the cytotrophoblast has been shown to be inversely related to villous oxygen content (Arnholdt et al 1991).

In our study similar numbers of cytotrophoblast and syncytiotrophoblast nuclei were seen in diabetic and control terminal villi. This finding is consistent with recent studies of diabetic placenta specifically that the volume and nuclear content of the trophoblast is similar in diabetic and control pregnancies (Mayhew1994). These findings could be consistent with either similar rates of production and loss of trophoblast or an increased production and increased loss rate with no overall change in total mass of trophoblast.

By utilising immunohistochemistry to demonstrate actively dividing cells (Ki-67 staining nuclei) we have provided evidence that the mitotic index of the cytotrophoblast is comparable in diabetic and control placentae. Our finding that the cytotrophoblast layer is not increased nor demonstrates increased turnover argues against significant whole villous hypoxic in diabetic pregnancies.

Increased thickness of the trophoblast basement membrane has been demonstrated to occur in a number of previous electron microscopy studies of placental ultrastructure in pregnancies complicated by maternal diabetes mellitus (Okudaira et al 1966, Fox 1969, Jones and Fox 1976). Increased thickness of basement membranes is also a

common pathological feature demonstrated to occur in a range diabetic complications outwith pregnancy, such as retinopathy and in nephropathy (in association with both glomerular and tubular cells) and neuropathy (Williamson and Kilo 1973, Sternberg et al 1985, Ziyadeh et al 1989, Ziyadeh 1993).

Increased thickness of the trophoblast basement lamina may reflect an increased production or a reduced breakdown and remodelling of this layer. Increased trophoblast turnover stimulates synthesis of basement membrane contents. This has been proposed as the probable mechanism as to the increased thickness of this layer previously demonstrated to occur in ischaemic villi (Fox 1978). Assessment of trophoblast turnover in our study does not support such a mechanism and indeed other studies have suggested that increased thickness of the trophoblast basement membrane is not a characteristic feature of villous hypoxia per se. Studies of rhesus monkey placenta (a similar hemochorial placenta to human) in which villous oxygen content is increased not decreased, either by ligation of the umbilical cord or removal of the fetus, is associated with increased thickness of the trophoblast basal lamina. (Panigel and Myers 1972). This occurs in addition to increased villous stoma density. Assessment of stromal density in our study of villi from diabetic pregnancies, both by transmission electron microscopy and by immunohistochemistry has demonstrated that the stromal content is not altered in diabetic compared to control pregnancies, suggesting that these villi are not significantly hypoxic or 'hyperoxic'. Increased trophoblast basal lamina thickness in diabetic placentae must therefore be secondary to a different mechanism than that occurring in the rhesus monkey placenta or in IUGR.

Hyperglycaemia may exert a direct effect on basement membrane production. Glucose has been demonstrated to increase the production of collagen IV, the predominant constituent of the basement membrane, in in-vitro studies of murine proximal tubule cells (Zlyadeh et al 1990). Reduced breakdown of the basement membrane may also result from hyperglycaemia. Non-enzymatic glycation of the structural composition of the basement membrane and subsequent formation of advanced glycosylation end products (ACEs) may make the basement membrane aberrantly resistant to degradation (Lubec et al 1980). Multiple factors thus can mediate the regulation of basal lamina production and therefore the thickness of this layer is not a sensitive indicator of villous oxygen content.

The average thickness of the vasculosyncytial membrane was found to be increased in diabetic pregnancies. As this structure represents a key diffusional barrier to oxygen transfer it may be expected that this layer would thin when the fetal placental unit had a tendency to hypoxia. This has been documented to occur by both active remodelling of plasma distribution by the trophoblast and by the passive peripheralisation and indentation of capillaries as their increasing length outstrips that of the villus. In theory hypoxia could adversely affect active remodelling of the vasculosyncytial membrane regions by causing increased turnover of the syncytial trophoblast. Our data of trophoblast turnover do not support such a mechanism underlying this finding in diabetic pregnancies. It is possible that increased metabolic activity of the trophoblast in the areas of nutrient exchange, secondary to increased

maternal plasma glucose concentrations could be responsible for the increased thickness observed in this layer in this area.

Reduction in the diffusional barrier of vasculosyncytial membranes to comparable distances seen in control villi may not be required in diabetic pregnancy. Transfer of oxygen from the mother to the fetus is dependent on a range of other factors such as the uterine artery and umbilical partial pressures of oxygen, flow rates, maternal and fetal oxyhaemoglobin saturation curve characteristics. The oxygen gradient between the maternal and fetal blood may be sufficiently high as to diminish the importance of this dividing 'membrane' at least within the small range of thickness differences demonstrated in our study. The increased capillarisation of the terminal villi in diabetic pregnancies may also, by providing increased a total surface area for exchange, reduce the necessity of very short individual VSM widths.

In the discussion section of chapter 5 we highlighted the limitations of placental studies with regard to sampling techniques. A sampling error can be magnified during analysis; selected placental blocks, giving rise to selective sections given rise to selected fields of view and then to selected measurements (Mayhew and Burton 1988). This is particularly of concern in TEM studies due to the small fields of view. We attempted to validate our light microscopy data by taking large full thickness placental blocks and sections. We attempted to validate our TEM data by sampling from central region of the parabasal plate (Bacon et al 1986) and to sample a number a number of blocks, from a number of placentae in each experimental group (Gundersen and Østerby 1981).

In combination our placental studies have demonstrated there are some, but not consistent, changes in the terminal villus ultrastructure that could represent a tendency to villus hypoxia in diabetic pregnancies. Of the changes demonstrated increased vascularity is the only factor that could reflect villous hypoxia. Increased thickness of the vasculosyncytial membranes and trophoblast basal lamina are not indicative of trophoblast hypoxia. They could however if occurring in response to an alternative stimuli predispose to intravillous hypoxia by increasing the diffusional barrier to oxygen, however the composition of intravillous stroma is not altered in diabetic cases arguing against such a putative chain of events.

Angiogenesis itself may occur in response to an alternative stimulus other than hypoxia. Vascular growth can occur in response to increased vascular sheer stress. Increased growth of the fetus or metabolic requirements of the fetus could result in a hyperdynamic circulation. Long-term sheer stress initiates remodelling and proliferation of the vascular endothelial cells (Hudlicka et al 1992, Karimu and Burton 1994, Resnick and Grimbrone 1995).

In conclusion we have demonstrated small but significant differences in components intimately involved in nutrient exchange between groups. Although some of these features may be associated with villus hypoxia, the overall morphology of the terminal villus in diabetic pregnancies is not suggestive of severe feto-placental hypoxia.

Parameter	Units	Diabetic	Control
Villous diameter	(x 10^{-5} m)	5.76 [1.20]	5.65 [1.16]
Capillary diameter	(x 10^{-5} m)	1.35 [0.47]	1.37 [.049]
Capillary cross-sections	(no.)	3.80 [0.53]	3.21 [0.36]†
VSM thickness	(x 10^{-6} m)	2.98 [1.02]	2.61 [0.98]*
Basal lamina thickness	(x 10^{-8} m)	6.83 [2.00]	6.26 [1.42]†
Syncytiotrophoblast nuclei	(no.)	8.6 [5.4]	7.5 [4.1]
Cytotrophoblast nuclei	(no.)	0.98 [1.1]	0.84 [1.0]

Table 6.1. Terminal villus ultrastructural characteristics: diabetic vs. control cases.

Data are presented as mean [SD]. Comparable parameters were analysed by Student's t-test. * $P < 0.005$, † $P < 0.05$.

	Trophoblast BM	Capillary BM	Stroma
Collagen IV	+++	+++	++
Laminin	++++	+++	+
Fibronectin	+	++/+++	+
Collagen I	0	++	++
Collagen III	0	+	++/+++

Table 6.2. Qualitative assessment of stroma composition in diabetic and control cases.

There was no difference on comparison of stromal characteristics between diabetic and control cases. This table represents typical findings in any assessed case.

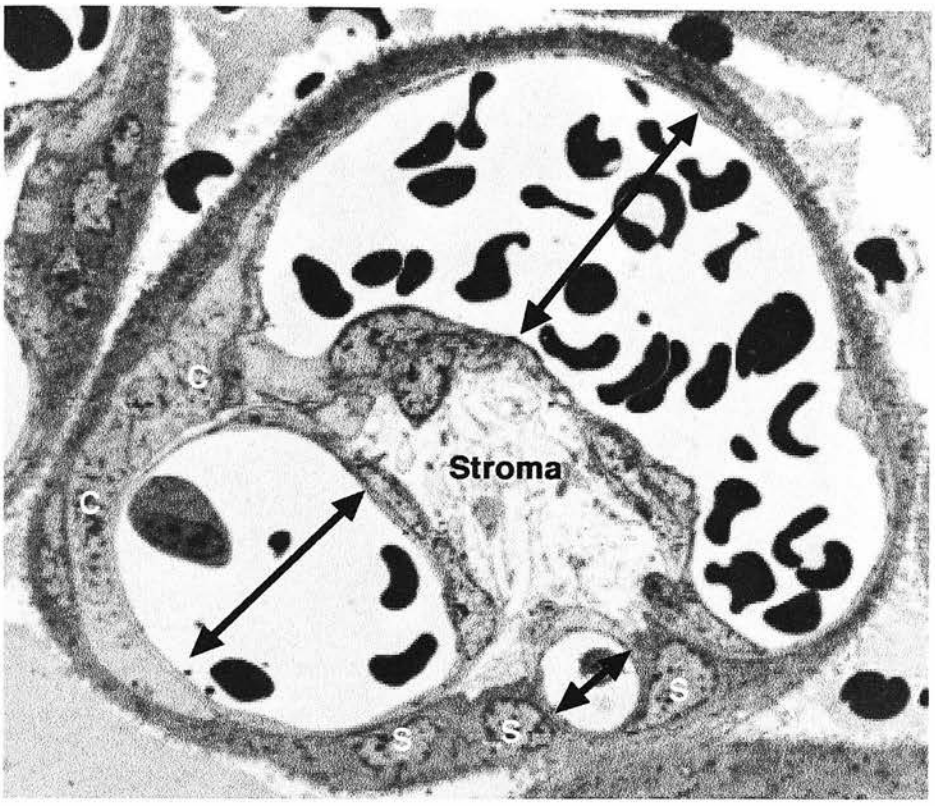


Figure 6.1 Cross-sectional ultrastructure of a terminal villus

This terminal villus contains 3 capillaries. Measurement of the shortest diameter of the capillary sections is shown (double arrows). This was necessary to avoid skewing the data by the inclusion of tangential sections of capillaries. Cytotrophoblast nuclei (C) surrounded by a pale cytoplasm and a defined cell border can be counted. Syncytiotrophoblast nuclei (S) surrounded by a darker cytoplasmic mass can also be counted. The density of the stroma was assessed in a qualitative manner. Magnification x 700.

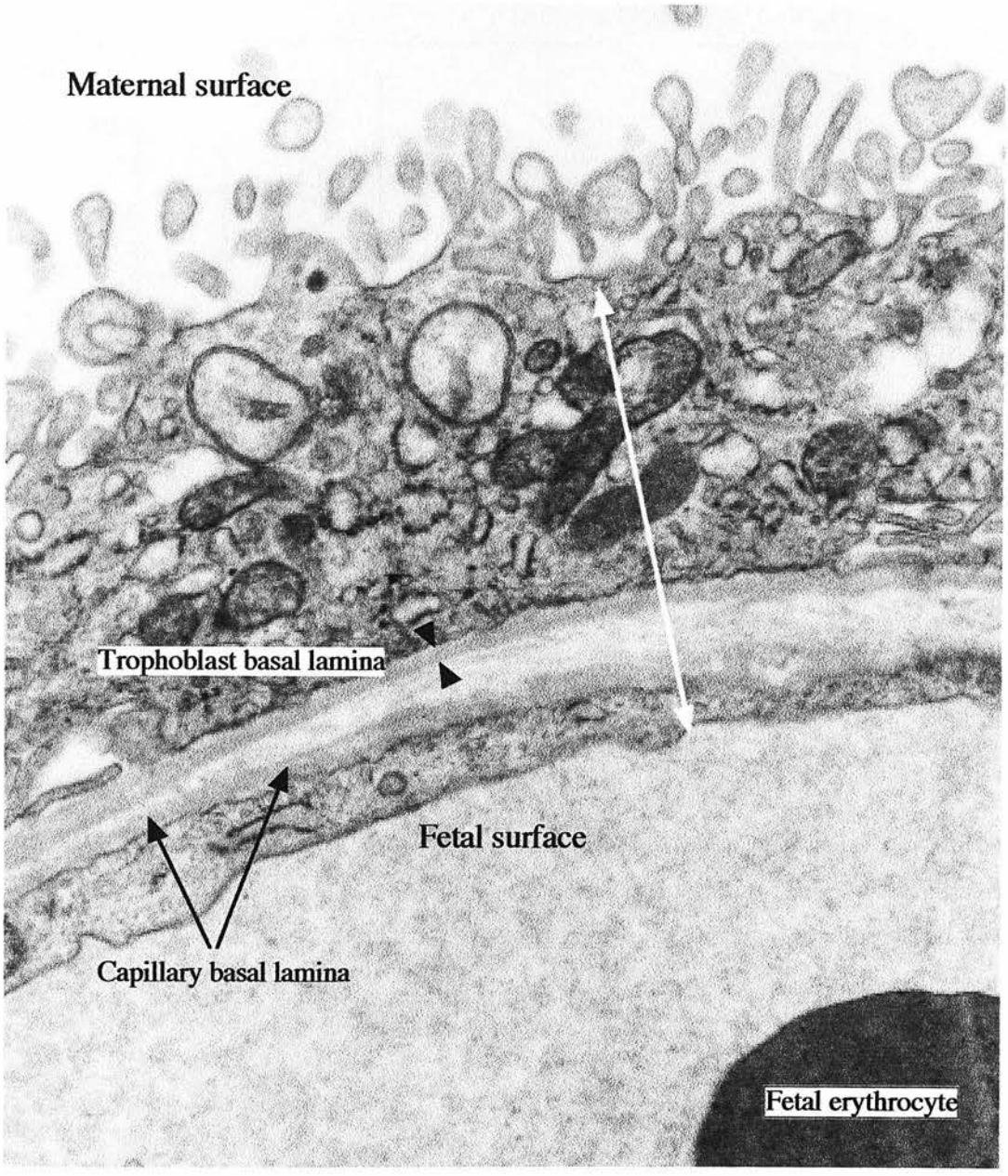


Figure 6.2 The structure of the vasculosyncytial membrane

The vasculosyncytial membrane comprises the thinnest area between the maternal and fetal surfaces of the placenta. Measurement of this distance is shown by the white double arrow. The thickness of the trophoblast basal lamina was measured in this area of the villus. The measured thickness of the basal lamina densa is depicted between the two arrowheads.
Magnification x 11500.

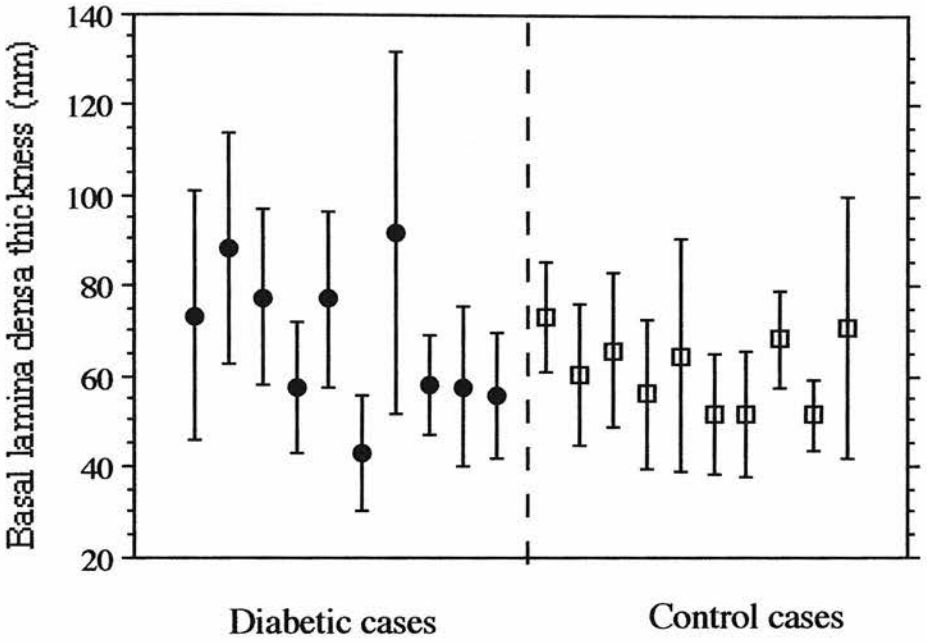


Figure 6.3 Mean trophoblast basal lamina densa thickness: diabetic vs. control cases.

The mean thickness (± one standard deviation) of the trophoblast basal lamina densa in the 10 diabetic and the 10 control cases.
The trophoblast basal lamina densa measured slightly but significantly thicker in the villous cross-sections viewed in diabetic compared to control cases; $p<0.05$.

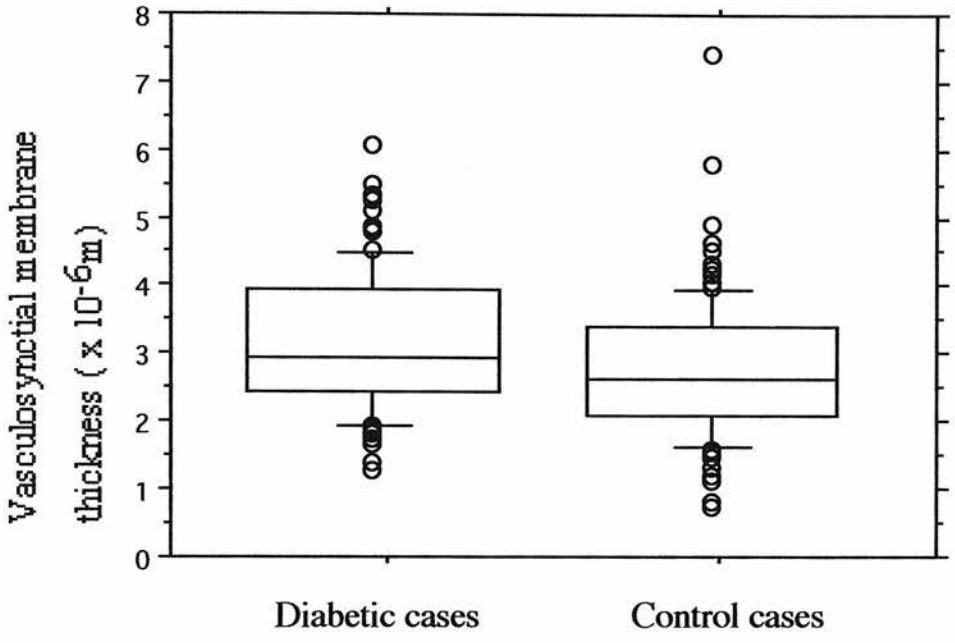


Figure 6.4 Median (range) vasculosyncytial membrane thickness: diabetic vs. control cases.

Box plots (Median, 25th and 75th percentiles, 10th and 90th percentiles) of vasculosyncytial membrane thickness in diabetic and control cases.
This plot demonstrates that outlying measurements occur in both diabetic and control cases. The vasculosyncytial membranes measured slightly but significantly thicker in the villous cross-sections viewed in diabetic compared to control cases; $p<0.05$.

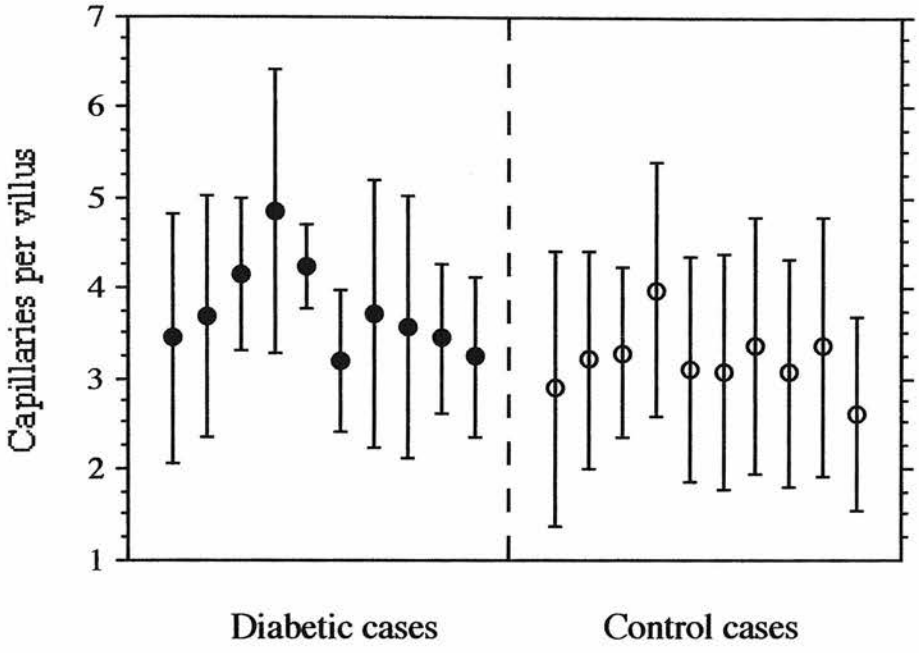


Figure 6.5 Capillary cross-sections per villus: diabetic vs. control cases.

The mean number (± one standard deviation) of capillary cross-sections viewed in villi from the 10 diabetic and the 10 control cases.
Statistical analysis revealed significantly more capillaries per villus in diabetic compared to control cases; $p<0.05$.

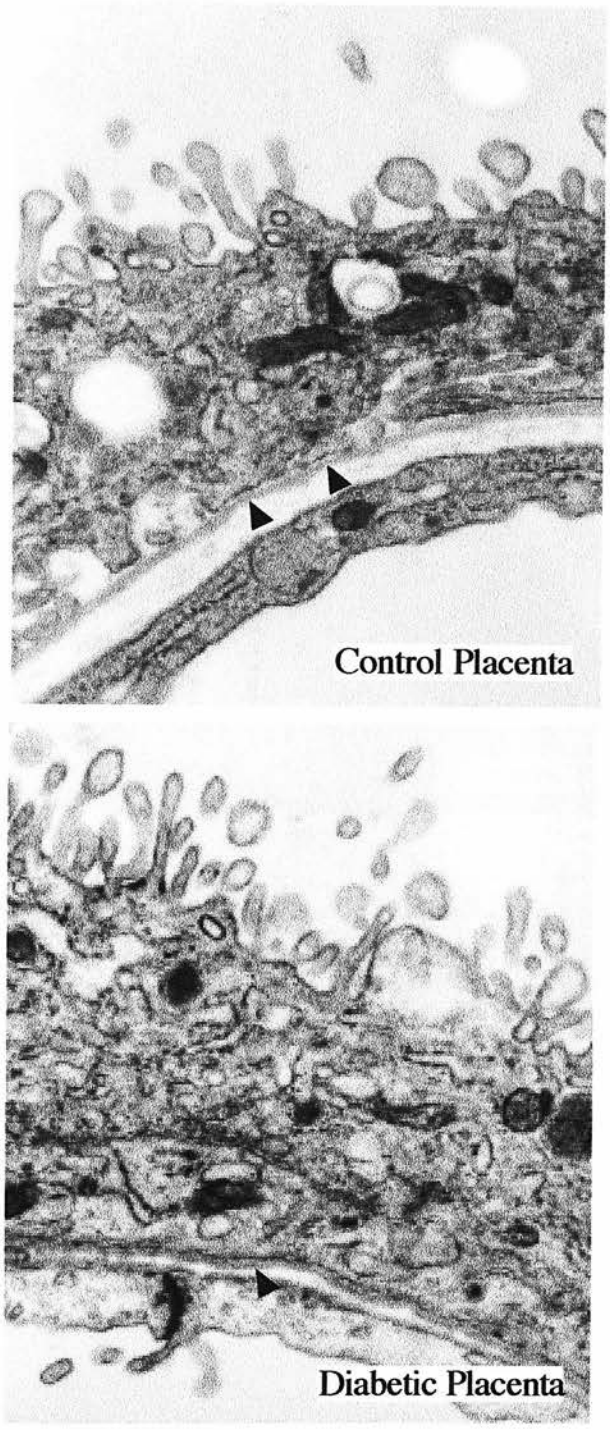


Figure 6.6 Trophoblast basal lamina densa thickness: comparison of a diabetic and a control case.

The thickness of the trophoblast basal lamina (densa) was measured where discrete cross sections could be easily identified (arrowheads). The trophoblast basal lamina cross-sections, in the regions defined as vascular syncytial membranes, are shown in a control case (upper) and a diabetic case (lower). Magnification x 11500.

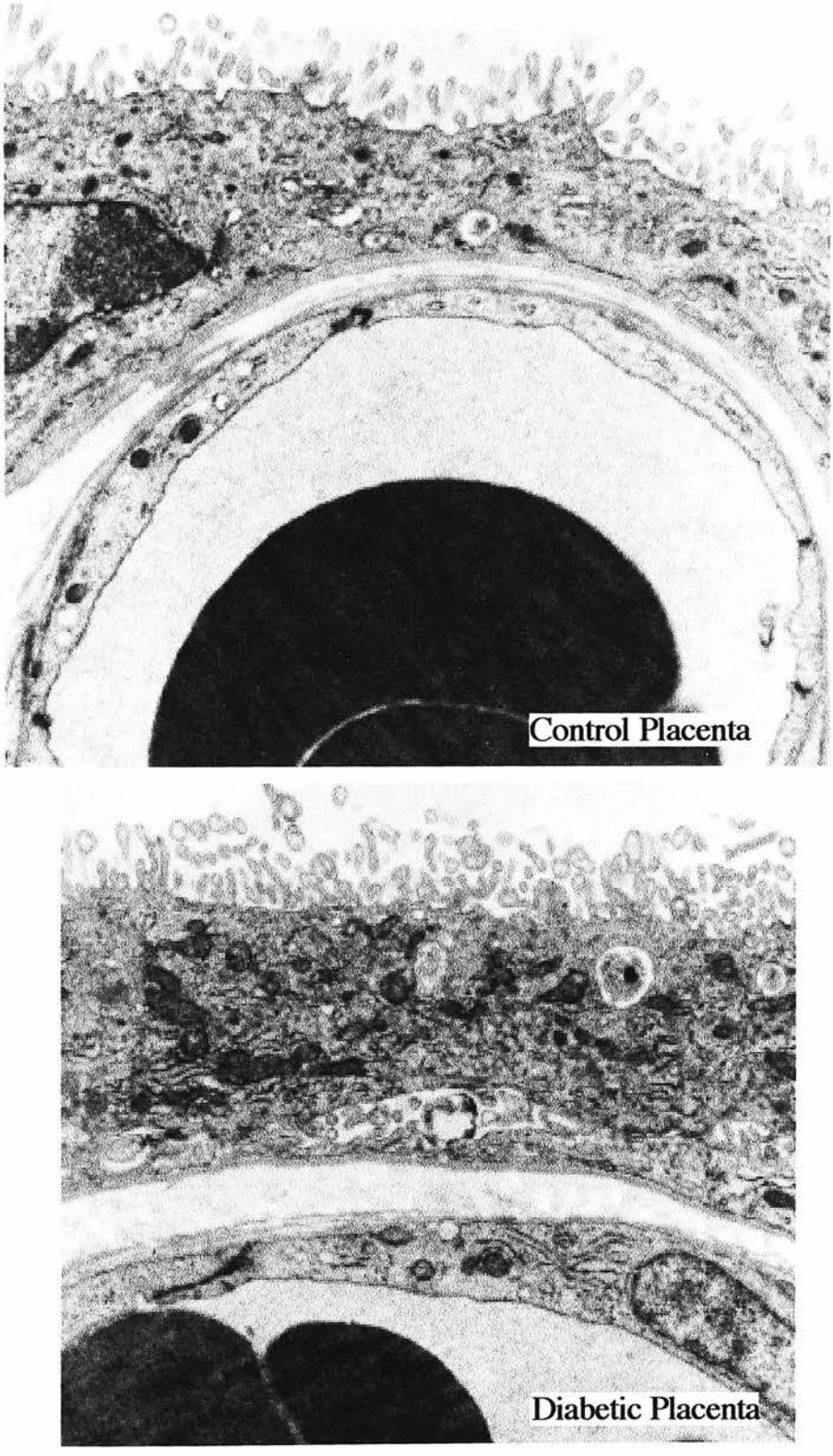


Figure 6.7 Vasculosyncytial membrane thickness: comparison of a diabetic and a control case.

A typical vasculosyncytial membrane from a control case (upper) and a diabetic case (lower). Magnification x 6600.

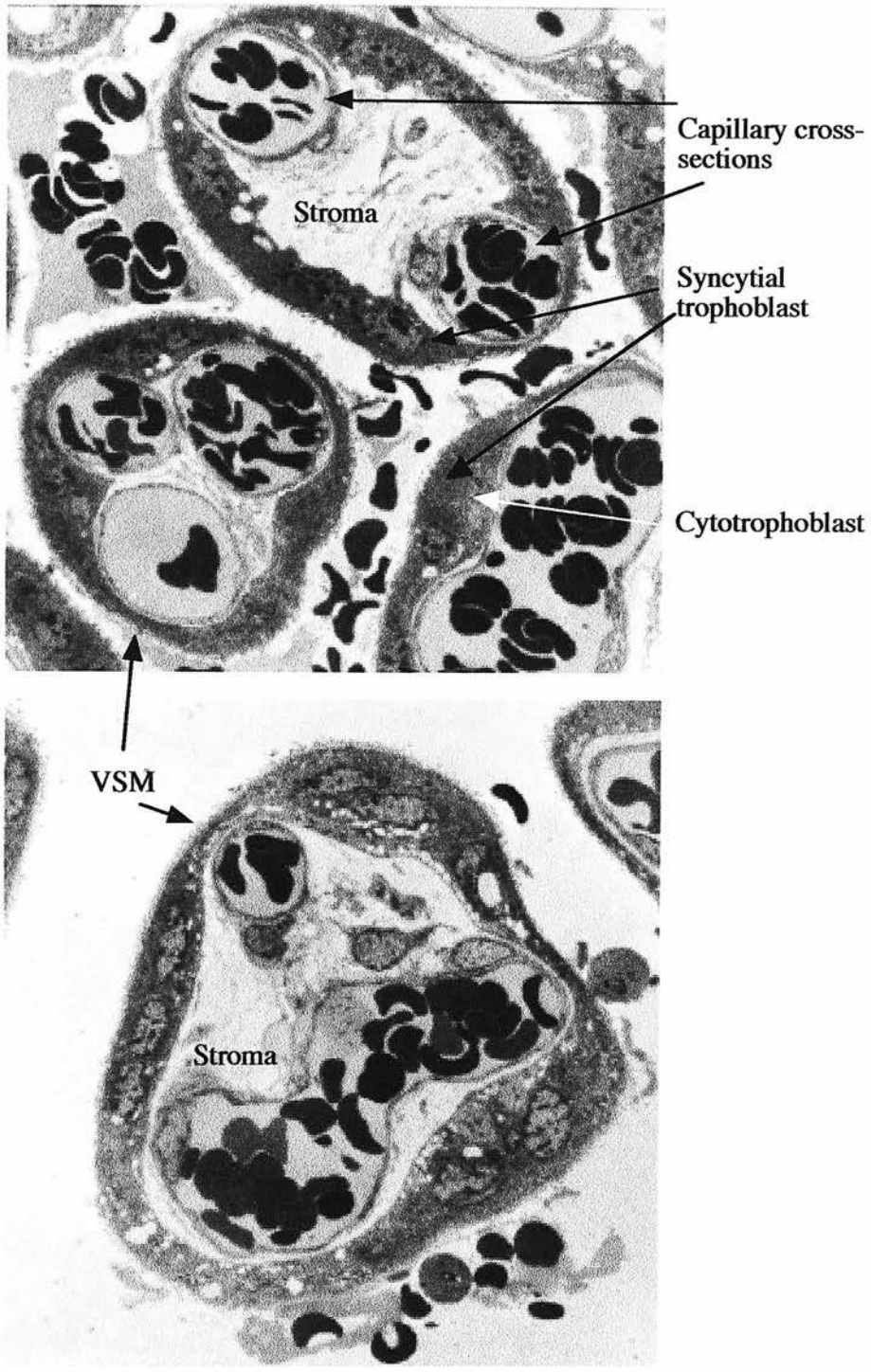


Figure 6.8 The cross-sectional structure of terminal villi from a control case.

The upper villi contain 2-3 capillary cross-sections and the lower villus contains 2. The syncytial trophoblast cytoplasm stains denser that the cytotrophoblast. The central villous core of stroma can be easily visualised. VSM = vasculosyncytial membrane. Magnification x 700.

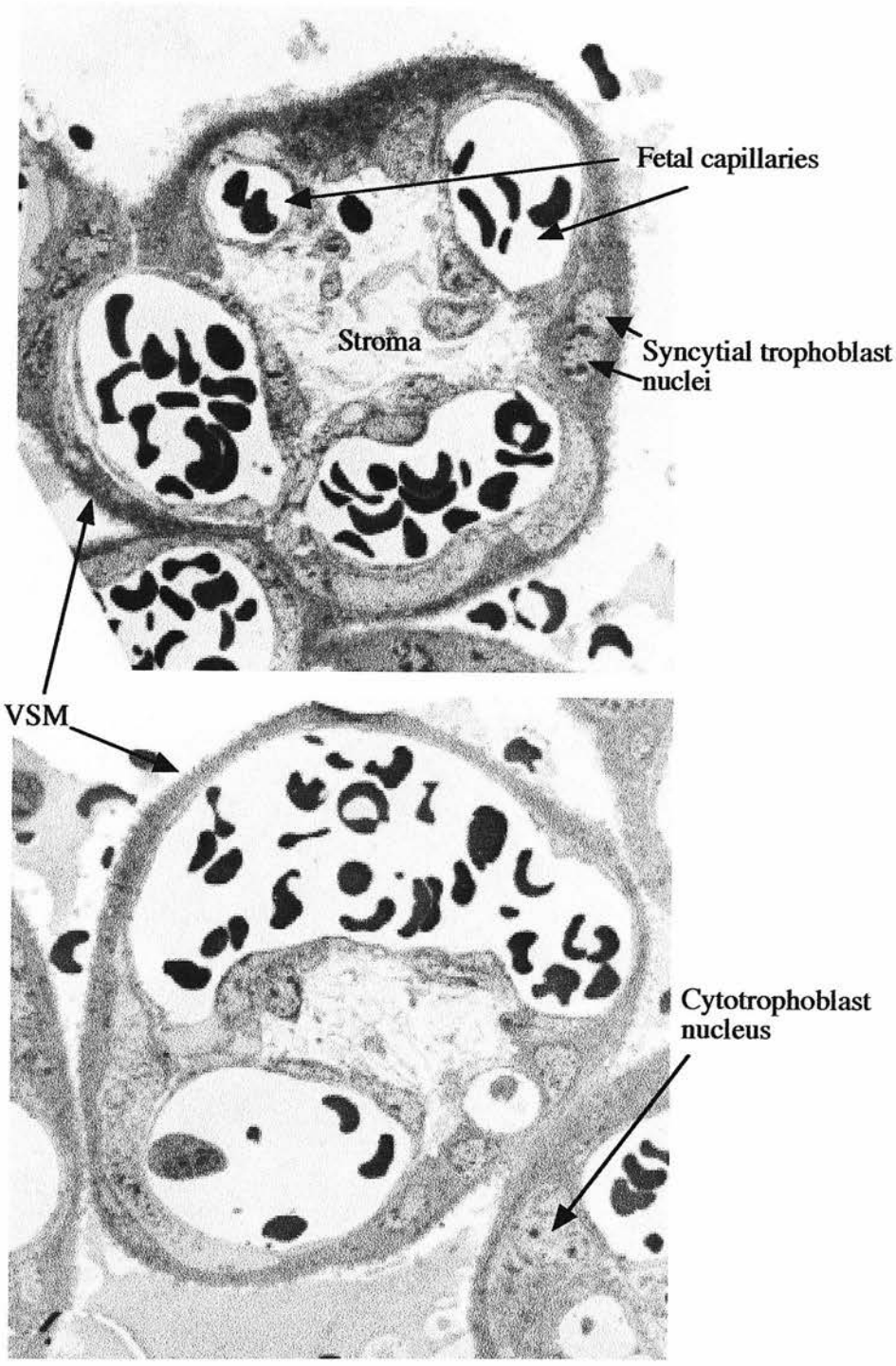


Figure 6.9 The cross-sectional structure of terminal villi from a diabetic case.

The upper villus contains four capillary cross-sections and the lower villus contains three. The cytoplasm of the syncytial trophoblast stains darker than that the cytotrophoblast, reflecting its higher rate of metabolic activity.
The central villous core of stroma can be easily visualised.
VSM= vasculosyncytial membrane. magnification x 700.

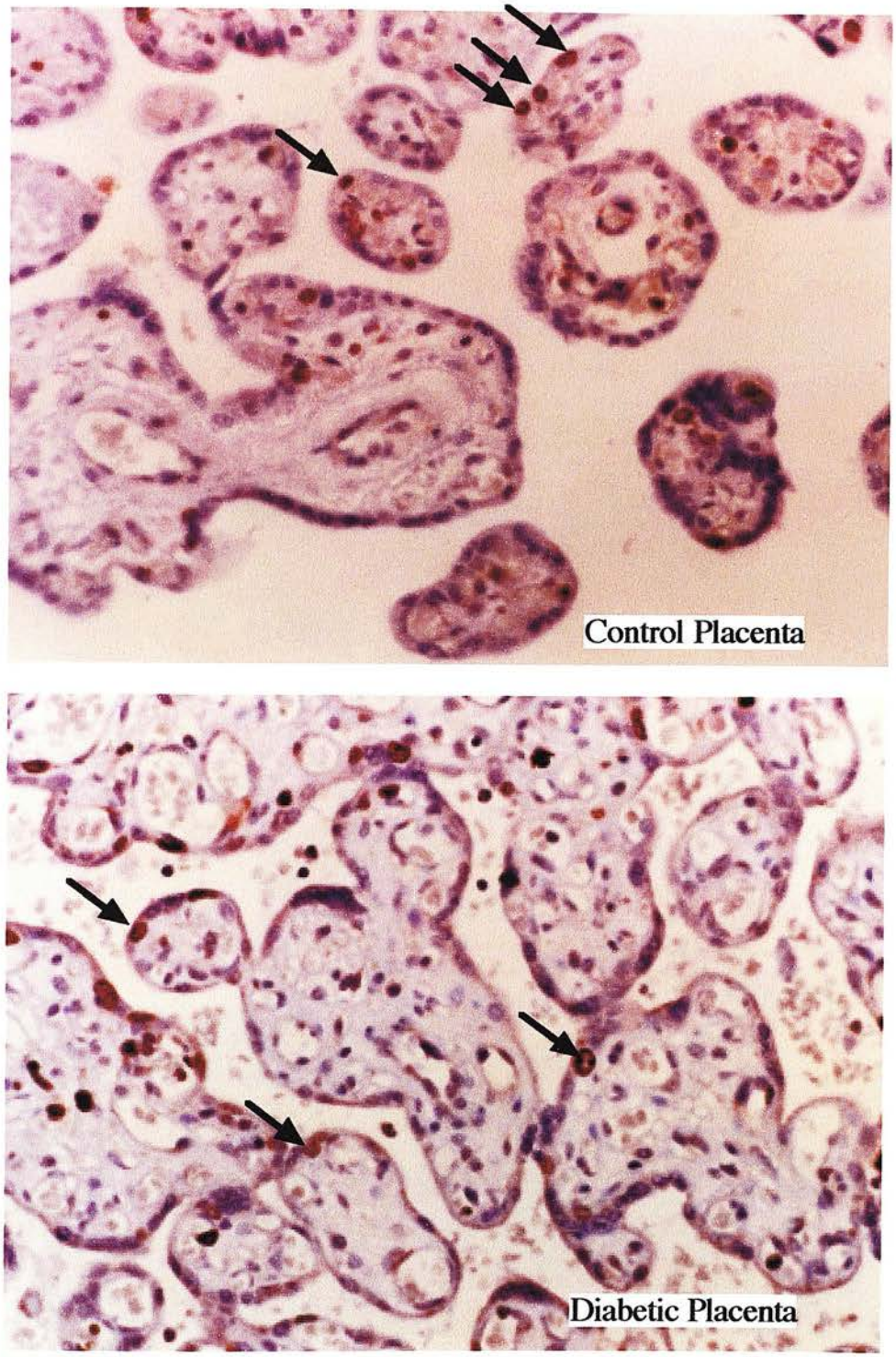


Figure 6.10 Cytotrophoblast mitotic activity: Ki-67 staining.

Actively dividing cells are stained positive by the Ki-67 antibody (arrows). There was no significant difference in the number of positively stained cytotrophoblast cells per villus on comparative analysis of control cases (top) and diabetic cases (bottom). Magnification x 320.

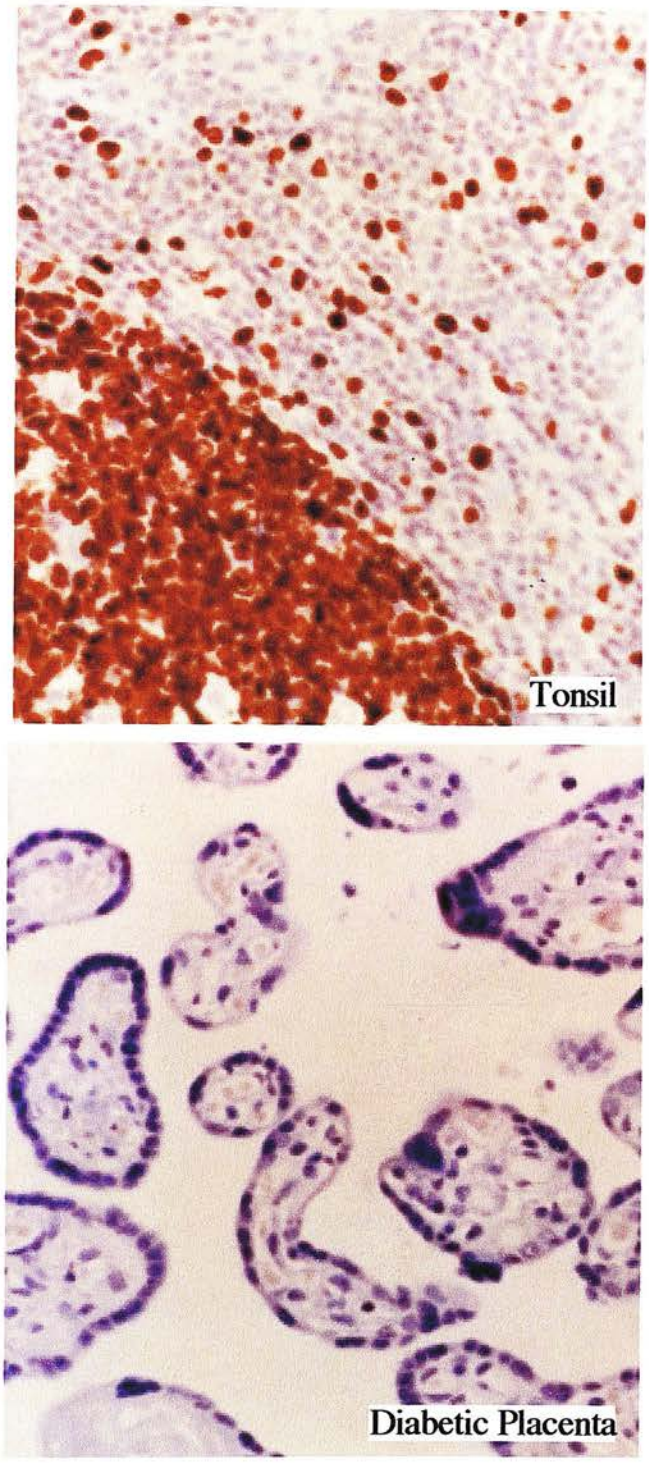


Figure 6.11 Positive and negative control slides for Ki-67 immunohistochemistry.

The positive control slide was a section of tonsil gland. The rapidly dividing lymphocyte population is correctly stained with the Ki-67 antibody. The dividing cells stain brown. The negative control was generated by replacing the primary antibody with PBS[sol.]/1.5%BSA. Magnification x 320.

Figure 6.12 Distribution of collagen type IV in the terminal villi of control and diabetic cases.

Similar staining patterns for collagen IV were seen in villi from control cases (top) and diabetic cases (bottom). Magnification x 320.

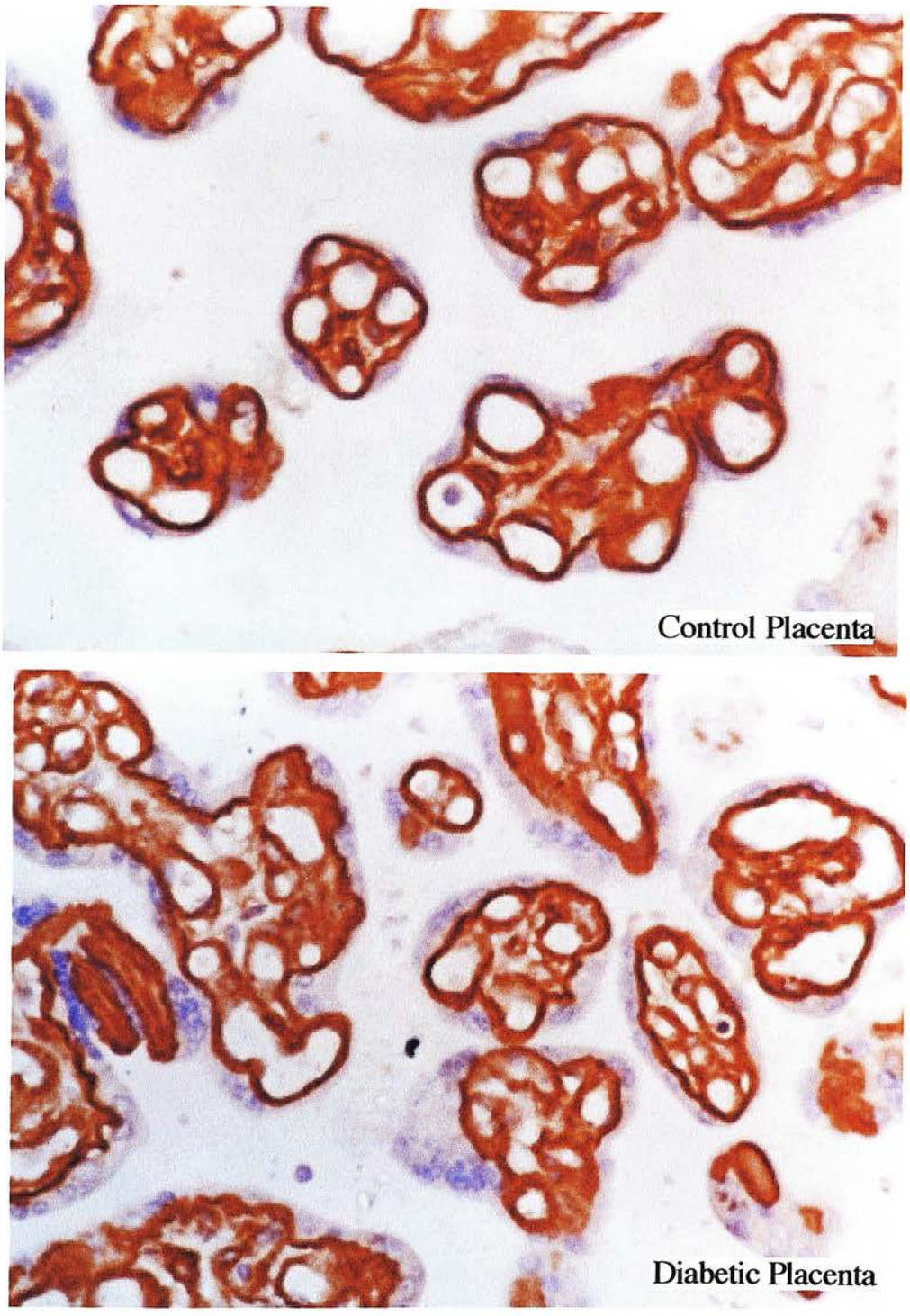


Figure 6.13 Distribution of laminin in the terminal villi from control and diabetic cases.

Similar staining patterns were seen for laminin in control cases (top) and diabetic cases (bottom). Magnification x 320.

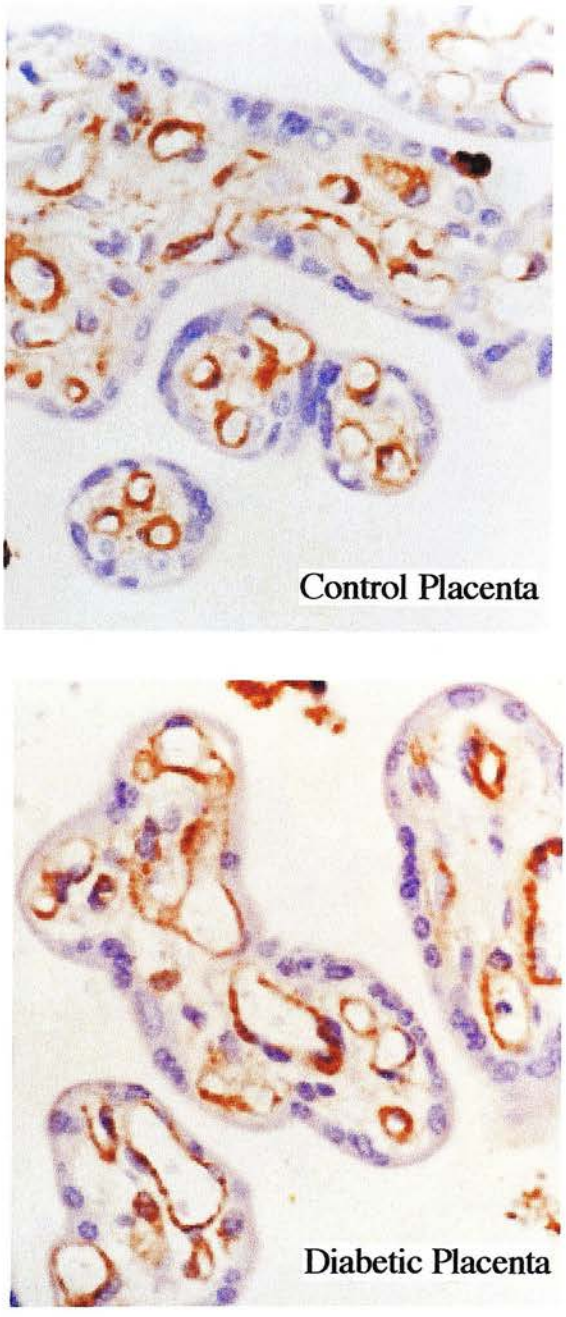


Figure 6.14 Distribution of fibronectin in the terminal villi from control and diabetic cases.

Similar staining patterns for fibronectin were seen in the viilli from control cases (top) and diabetic cases (bottom). Magnification x 320.

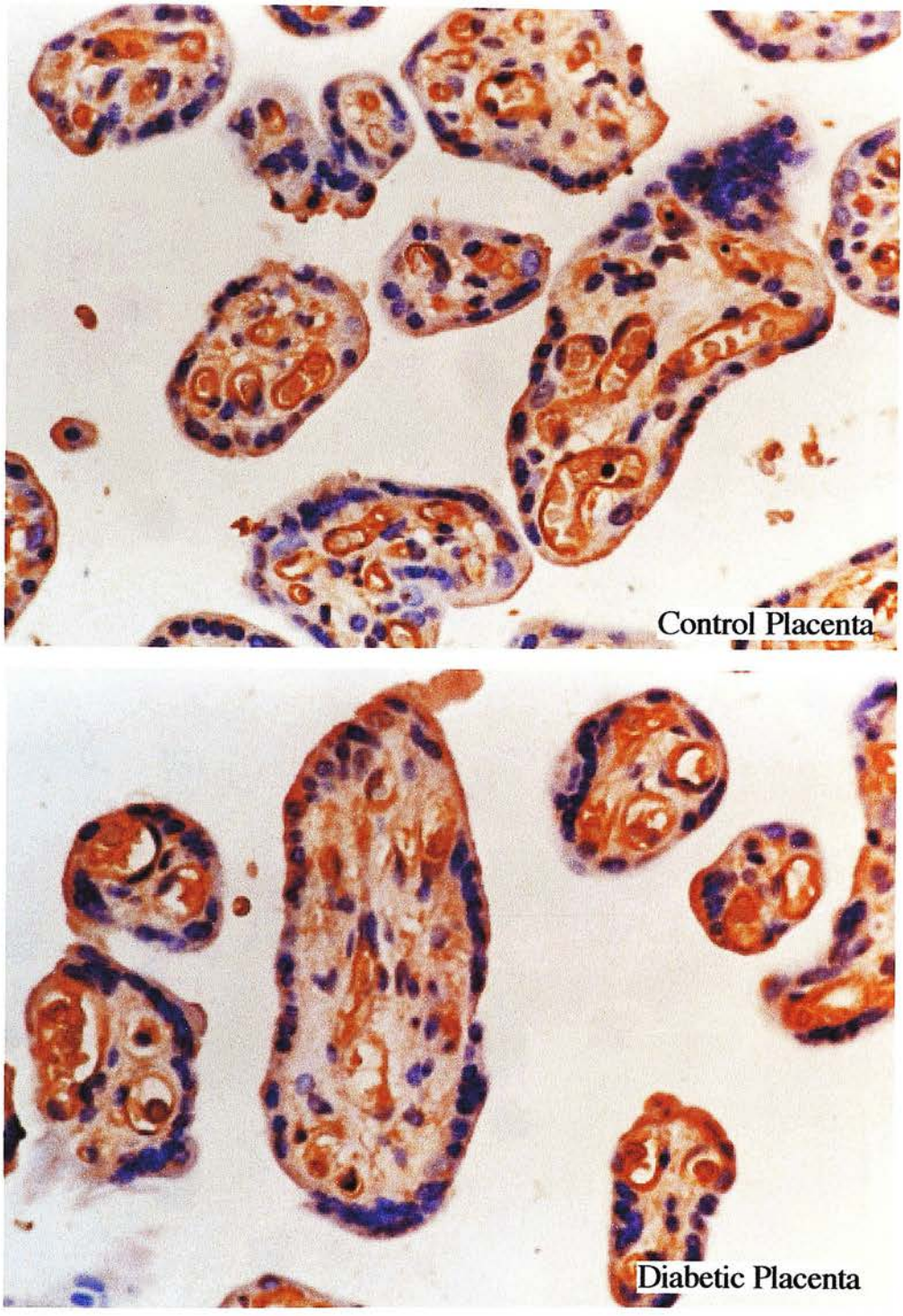


Figure 6.15 Distribution of collagen type I in the terminal villi from control and diabetic cases.

Similar staining patterns for collagen type I were seen in villi from control cases (top) and diabetic cases (bottom). Magnification x 320.

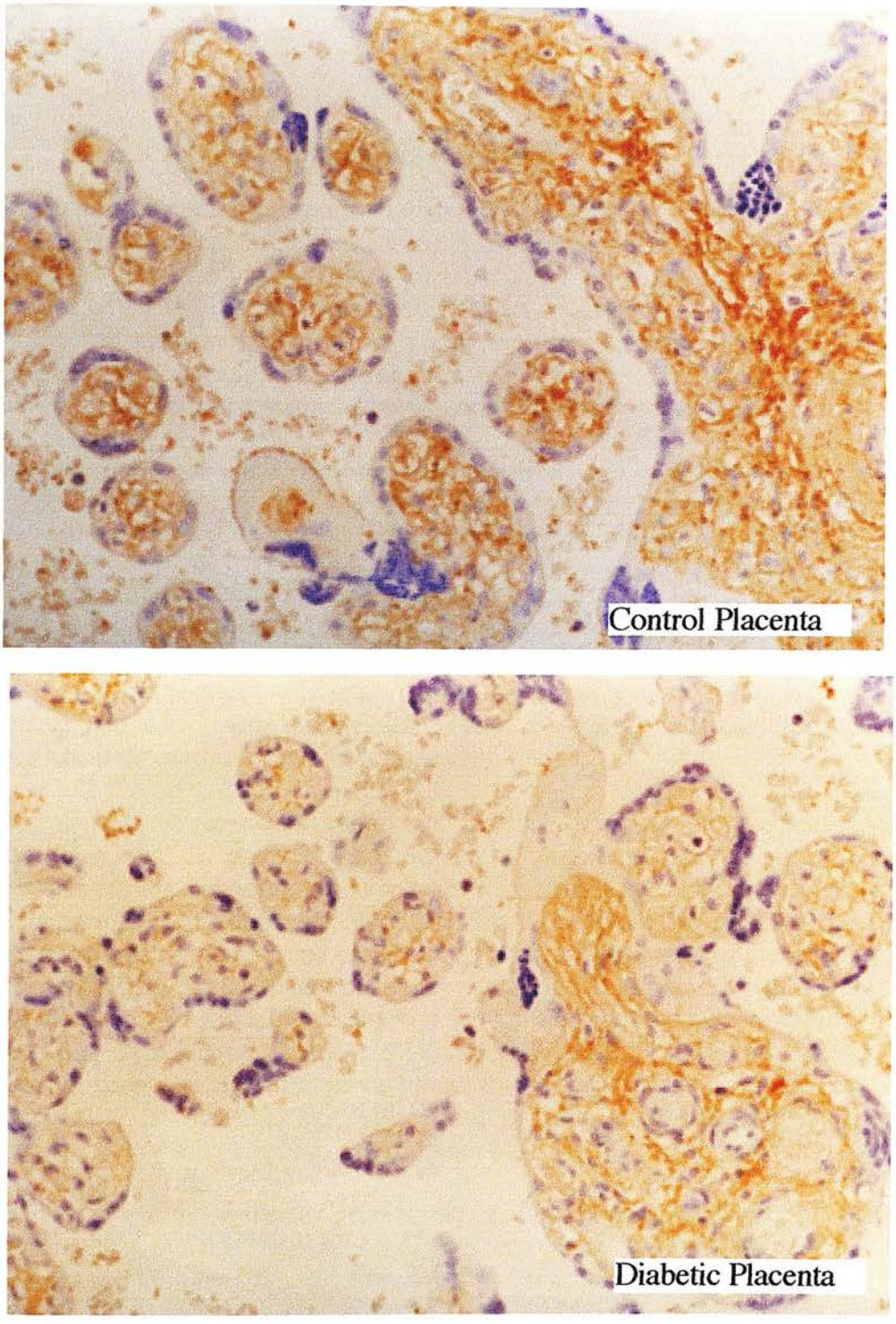


Figure 6.16 Distribution of collagen type III in the terminal villi from control and diabetic cases.

Similar staining patterns for collagen type III were seen in villi from control cases (top) and diabetic cases (bottom). Magnification x 320.

Chapter 7

General discussion

Women with insulin dependent diabetes comprise a large proportion of women with a prior medical condition considering pregnancy. Prior to the 1960s these women were actively discouraged against pregnancy due to the high incidence of maternal, fetal and perinatal complications. In the present day diabetic women now expect a successful pregnancy outcome with little or no risk to themselves. This change of expected outcome has been possible primarily due to improvements in replacement insulin regimes, and to a much lesser extent improved fetal monitoring.

Despite more optimistic outcomes for the mother and fetus population studies still indicate a significantly raised incidence of complications; especially fetal loss rates, fetal abnormalities, and raised mean birth weight in pregnancies to diabetic mothers (Casson et al 1997, Hawthorn et al 1997). The effect of pregnancy on maternal vascular pathology has not been subjected to such comparable study. The favourable findings with regard to (a lack of) progression of vascular complications reported in clinical trials of intensive maternal insulin regimes may not be mirrored in local populations. The 5-year aim of the St Vincent declaration (World Heath Organisation 1989), that women with diabetes should have a pregnancy outcome similar to that of non-diabetic women has not been achieved.

The majority of pregnant women enter pregnancy without major recognised vascular complications. In these women the risk of complications arising in pregnancy appears to be associated with glycaemic control. Of the fetal complications, spontaneous miscarriage and congenital abnormalities are the most clearly linked to maternal glucose levels, and it appears that a relatively modest degree of glycaemic control is required to reduce the incidence of these complications (Greene et al 1989, Mills et al 1988, Kitzmiller et al 1991). This degree of glycaemic control must, to be effective in this respect, be achieved prior to the first 7 weeks of pregnancy, when fetal organogenesis is occurring. To this aim pre-pregnancy clinics have been established and have proved beneficial when uptake of this service has been good (Steel 1996).

In our population of diabetic women only one-third had been seen prior to pregnancy within the regional tertiary referral diabetic centre, and therefore had access to formal pre-pregnancy management. The lack of fetal structural abnormalities in our population of diabetic pregnancies must therefore reflect the improved diabetic care and glycaemic aims of the whole (non-pregnant) diabetic population. As such this is an important acknowledgement. Unlike fetal structural abnormalities, which are usually diagnosed after 18 weeks gestation, we cannot assess the incidence of spontaneous miscarriage in our studied population of diabetic women. This complication often predates the 12-week booking appointment and therefore affected pregnancies would not present for recruitment to the study. However given the similar range of glycaemic control required to reduce the incidence of both structural

abnormalities and spontaneous miscarriage the pre-pregnancy target of glycaemic control in our population of diabetic women is likely to have also been beneficial in the later effect.

To reduce the incidence of fetal complications occurring in later pregnancy, fetal macrosomia and intrauterine death, there appears to be a stricter threshold for maternal glycaemic control. The Diabetes in Early Pregnancy Study (Jovanovic-Peterson et al 1991) has demonstrated that the risk of fetal macrosomia, due to sub-optimal maternal glycaemic control is a continuum. The risk of macrosomia is more closely related to the one-hour post-prandial maternal blood glucose concentrations than any other index of glycaemic control i.e. HbA1c values. This demonstrates that small fluctuations in maternal glycaemic control have an important influence on fetal growth.

Higher post-prandial glucose concentrations are seen in diabetic women compared to non-diabetic women even in the face of stringent glycaemic control. This occurs because currently utilised insulin preparations cannot mimic the sharp post-prandial action of endogenous insulin. Soluble insulin has a peak action around 2-4 hours after administration, which is not physiological. To reduce the risk of macrosomia improved insulin preparations are required (Johnstone 1997). New insulin preparations have been developed with quicker durations of action. Insulin Lispro is an analogue of human insulin. Its rapid absorption from the subcutaneous injection site allows for the faster peak of action, less than one hour after injection. This mimics more closely the physiological insulin release seen in non-diabetic subjects in response to a carbohydrate load. Studies demonstrate that it significantly reduces post-prandial glucose concentrations in non-pregnant diabetic subjects (Anderson et al 1997). Studies of these preparations in pregnancy are at present very limited, but currently support this finding. Jovanovic et al (1999) demonstrated a beneficial effect of this insulin in women with gestational diabetes; compared to women treated with conventional soluble insulin. The group treated with Lispro achieved lower post prandial glucose concentrations without an increased risk of hypoglycaemic events. On going clinical trials of new insulin preparations are keenly awaited.

The acknowledgement that currently achievable maternal glycaemic control is suboptimal with regard to fetal wellbeing is supported by our placental studies. The placenta and fetuses of diabetic mothers tended to be of increased weight when compared with those from control pregnancies despite apparent good glycaemic control during pregnancy. In addition subtle abnormalities of the fetal terminal villus architecture were determined in the placentae collected from the diabetic group. These findings although not, as a whole, consistent with significant feto-placental hypoxia, do represent some form of altered maternal-fetal dynamics in these key exchange units. As such these alterations may provide a marker for the increased risk of fetal compromise in late gestation.

In an attempt to reduce the fetal risks of compromise and stillbirth in diabetic pregnancies a number of studies have assessed the benefit of Doppler ultrasound of the umbilical arterial blood flow. One suggestion to explain the increased incidence of acidaemia in fetuses of diabetic mothers is that of impaired fetal placental

perfusion. If blood flow to the placenta is restricted this should be made evident by increased impedance within the umbilical artery. One isolated study (Bracero et al 1986) has demonstrated such an association between increased umbilical artery impedance and poor maternal glycaemic control. This was correlated with an increased risk of fetal complications such as stillbirth and neonatal morbidity. Other reported studies of Doppler have not supported this finding (Landon et al 1989, Dickler et al 1990, Zimmermann et al 1992 and Kofinas et al 1991). Landon et al (1989) demonstrated no significant association between impedance to flow in the umbilical arteries and maternal blood glucose or HBA1c levels. Johnstone et al (1992) studied 128 pregnancies in a Scottish population and confirmed that there appeared to be no association between impedance to flow in the umbilical arteries with either short or long term glycaemic control. In this study an antenatal diagnosis of fetal compromise (non-reassuring CTG or poor biophysical profile) was made in 7 pregnancies which were promptly delivered by caesarean section. Of these 7 pregnancies only three had abnormal Doppler characteristics. The baby in the poorest condition at birth, requiring ventilation for asphyxia had normal Doppler indices measured earlier the same day as fetal delivery. The majority of evidence therefore suggests that in maternal diabetes (without significant complicating vascular pathology), impedance to flow in the umbilical arteries is not related to either short or long term maternal glycaemic control, or fetal compromise.

The reason why Doppler assessment of umbilical artery blood flow is not an effective screening tool for fetal compromise/hypoxia may be explained by our study findings. We have demonstrated in support of work by Mayhew et al (1984), that the placenta has a more voluminous vascular capillary network in diabetic pregnancies. The terminal capillaries comprise the largest volume of placental vessels and are of the smallest diameter. Blood flow through these capillaries therefore exerts the most significant effect on the impedance of umbilical artery flow. This has clearly been demonstrated in studies of pregnancies complicated by intrauterine growth restriction (Macara et al 1994, Krebs et al 1996). In this condition the reduced numbers of terminal villi capillary loops appears to be the likely underlying mechanism by which umbilical artery blood flow is hampered by down stream resistance. In intrauterine growth restriction the paucity of terminal capillary vasculature is likely to be a causative mechanism of the fetal condition. In diabetes it is more likely that the capillary volume of the placenta reflects a primarily fetal drive. Thus in diabetes even if an increased capillary volume was reflective of fetal acidaemia or relative fetal hypoxia, the increased capillary volume by its very nature would have a protective effect on umbilical artery blood flow resistance, negating the value of assessing this resistance as an index of hypoxia.

Severe maternal hyperglycaemia may have a time dependent effect on feto-placental blood flow resistance. Ishimatsu et al (1991) demonstrated increased umbilical artery impedance is association with high maternal blood glucose levels. The umbilical artery impedance subsequently returned to normal once the maternal glucose concentration fell to within the euglycaemic range. To act in this away glucose must be having an effect on the muscularised vessels of the placenta such as those in the intermediate or stem villi. Any mechanism by which hyperglycaemia reduces prostacyclin production, or nitric oxide production could underlie this mechanism.

Doppler assessment of umbilical artery flow in this acute condition, although attractive as a test in theory, would not be useful. Hyperglycaemia itself is more readily and less expensively determined. The known adverse fetal effects of such severe maternal hyperglycaemia are established and should not require additional indicators.

Evidence of altered placental structure in diabetic pregnancies despite apparent good maternal glycaemic control coupled with the realisation that these placental adaptations are not readily assessed by current fetal monitoring techniques leaves us with difficulties in the management of these pregnancies especially near to term. Faced with the remaining increased risk of stillbirth in these pregnancies most clinicians will remain justified in elective delivery of these pregnancies once they reach expected fetal maturity i.e. around 38 weeks gestation, especially if the fetus is macrosomic.

Our study of maternal vascular dysfunction has more encouraging findings. The lack of raised levels of specific cell adhesion molecules in diabetic women during pregnancy suggests that pregnancy per se, at least in women without major pre-pregnancy vascular pathology, does not have a detrimental effect on diabetic vascular complications. It is therefore no longer justified to dissuade the majority of diabetic women on the basis of their health. They should be encouraged that improved glycaemic control is associated with a reduced measure of vascular dysfunction. Sharing such information can only improve the patient–clinician relationships, may promote attendance at pre-pregnancy clinics and improve long term diabetic care and outcomes.

The aim to correlate our maternal vascular findings with our placental findings is problematic due to the apparent vascular wellbeing in our cohort of diabetic mothers during pregnancy. However it is likely that this maternal vascular health contributed to the lack of significant placental villous structural anomalies. The benefits of glycaemic control summating at the maternal-fetal interface.

Where do we go from here?

Maternal vascular endothelial function

In our small study of endothelial function in diabetic pregnancy we have demonstrated a general "normality" of circulating cell adhesion molecules in women achieving good glycaemic control. Repeating this study in a future cohort of diabetic women, advantaged by improved insulin preparations, regimes and glycaemic control is therefore unlikely to demonstrate any additional benefit. A much larger study however would allow diabetic women to be subdivided on the basis of circulating CAM concentration centiles. This would facilitate the clinical and scientific comparison of those with the highest and lowest values, helping to decipher and weight the significance of improved glycaemic control, and other compounding factors; such as blood pressure, pre-existing clinical cardiovascular disease, and other circulating marker concentrations, on the risk of adverse cardiovascular outcome.

As indicated previously CAM concentrations measured in early pregnancy or ideally pre-pregnancy could be used to assign "risk" to the pregnancy, Increased surveillance and support of identified 'high risk' pregnancies, which may have a higher degree of subclinical cardiovascular compromise, may be of therapeutic value. If CAM assay is not readily available, as indeed it may not be outwith a research centre, circulating C Reactive Protein (CRP) values may be a useful alternative prognostic marker. This protein produced by the liver in response to IL-6, acts as a general down stream marker of inflammation. The circulating concentration of this protein is readily measured by mainstream hospital laboratories and has been investigated extensively as a marker of vascular disease progression outwith pregnancy. A comparison of CRP values to CAM, vWF values and clinical cardiovascular disease in pregnancy would be of value.

Placental studies in diabetic pregnancy

The use of CAM, or indeed CRP, concentrations to stratify diabetic pregnant women into groups of differing cardiovascular 'risk' could also aid the study of fetal outcome, and placental studies. Vascular casting, transmission and scanning electron microscopy are labour intensive, however targeted comparison of placenta from pregnancies displaying the highest and the lowest markers of maternal vascular disease could yield clear specific findings. Such a 'severity classification' of diabetes could be a superior alternative to White class in future studies of placental structure and function, aiding comparison of study populations and therefore findings.

Our work focused on the structure of the terminal villi rather than the function. Given that the structure is grossly conserved, analysis of the function of these villi, including assessment of fetal endothelial function, has the potential to be more insightful as to the basis of the continued increased fetal loss rate in diabetic pregnancies and further studies in this area are required. In-vitro perfusion studies of villous vascular circuits (placental explants) have been developed (Siman et al 2001, Sibley et al 2002). This study model may help future studies to determine what we

need to establish... which are the most critical fetal stresses: hypoxia?, acidosis?, hyperglycaemia?, hyperinsulinaemia?, or rapid fluxes in each?

Chapter 8

General materials and methods

8.1 General methods

8.1.1 Immunohistochemistry

Immunohistochemistry is a widely used technique and essentially is a specialised stain of tissue sections in which the pivotal reagent is an antibody to a specific tissue antigen (Mesa-Tejada et al. 1997). The antibody is linked directly or indirectly to an enzyme i.e. peroxidase or alkaline phosphatase, which when binds to an added substrate catalyses a reaction to convert the colourless substrate (chromogen) into coloured end product. Alternatively the antibody can be linked to a fluorescent marker (Fluroscein, texas red or rhodamine)

8.1.1.1 Antibodies

Antibodies or immunoglobulins are glycoproteins present in the serum and tissue fluids of all animals. Their production is stimulated when the lymphoid system comes in contact with immunogenic foreign molecules (antigens) and they bind specifically to the antigen that induced their formation. They are therefore an element of the adaptive immune system. A typical antibody molecule has 12 domains arranged in 2 heavy and 2 light chains linked though cystine residues by disulphide bonds so that their domains lie in pairs. The N terminus domain is the most variable and contains the antigen-binding site; the other domains are relatively constant. The constant domains of the heavy chain mediate binding of the antibody to host tissues including cells of the immune system, phagocytic cells and the first component (C1q) of the complement cascade, and determine antibody class.

There are five major classes of antibodies: Immunoglobulin (Ig)G, IgA, IgM, IgD, and IgE. Immunoglobulin G and IgM are the most frequently utilised antibodies in immunohistochemistry. Immunoglobulin M is the first antibody formed in a humoral reaction. It is largely confined to the intravascular pool and has a short half-life of 4-6 days. Immunoglobulin G is the major immunoglobulin in the hyperimmunised host, accounting for 70-75% of the total immunoglobulin pool. It is evenly distributed between the intra- and extravascular pools and has a longer survival, approximately 3 weeks.

For their use in immunohistochemical techniques antibodies may be further classified as either polyclonal or monoclonal. Polyclonal antibodies are produced by different cells and are therefore immunochemically dissimilar and react with various epitopes on the antigen to which they are raised. A number of animals are used to make polyclonal antibodies, of which the rabbit is the most commonly utilised. Monoclonal antibodies are produced by cones of plasma cells; therefore antibodies for a given clone are immunochemically identical. Monoclonal antibodies react with a specific epitope on the antigen to which they are raised. The main advantage of the use of monoclonal antibodies, compared to the use of polyclonal antibodies in immunohistochemistry is their high antigen specificity. Further advantages include their high homogeneity and absence of inter-batch variability. However their antigen recognition can be more readily disrupted by tissue fixation techniques. The single

antigen epitope to which they are raised may not survive fixation, where as other antigen epitopes may survive allowing antigen recognition by polyclonal antibodies.

8.1.1.2 Enzymes and substrates

Enzymes are proteinaceous catalysts peculiar to living matter. One of the most commonly used enzymes in immunohistochemistry is horseradish peroxidase. Horseradish peroxidase is a 40 kd molecular weight protein that contains a heme group at its active site. The heme group complexes with hydrogen peroxide in the presence of an electron donor, then subsequently dissociates to produce water and atomic oxygen. The oxygenation of the electron donor (the chromogen) results in a coloured insoluble end product.

One of the most commonly used chromogens is three, 3'- Diaminobenzidine tetrahydrochloride (DAB). Oxygenation of this compound produces a brown insoluble end product.

8.1.1.3 Staining methods

The simplest form of immunohistochemistry is the direct method. In this method an enzyme labeled antibody reacts directly with an antigen in the tissue. The subsequent addition of a substrate/chromogen concludes the reaction sequence.

More involved methods include the 2 step and 3 step indirect methods, where an unconjugated primary antibody binds to the tissue antigen. An enzyme labeled secondary antibody is then directed against the primary antibody (2-step method). Subsequently an enzyme labeled tertiary antibody may be directed against the secondary antibody (3-step method). More enzyme can therefore located to the antigen site, thereby amplifying the staining and improving the sensitivity of the staining procedure. Further improvement in sensitivity can be made by using the avidin biotin method (Marty et al 1982) in which a biotinlyated secondary antibody binds both the primary antibody and to added enzyme labeled avidin or a preformed avidin biotin enzyme complex. The strong affinity of avidin for biotin at its multiple biotin binding sites concentrates enzyme at the antigen site.

8.1.2 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assays (ELISAs) employ similar staining principles to that described for immunohistochemistry. The aim of the assay is to detect and quantify the presence of a soluble antigen in biological fluid, rather than to detect and locate an antigen in a solid tissue specimen.

An antibody is raised to the circulating antigen and is then coated onto the walls of a microtitre well plate. A further antibody to the antigen is conjugated to an enzyme. Any antigen in the biological specimen will then form a bridge between the two antibodies thereby binding the enzyme to the microtitre plate in proportional quantities to the amount of antigen present. Any unbound enzyme conjugated antibody is removed by aspiration and washing steps. The amount of conjugate in the well is detected by a reaction with an added substrate specific for the enzyme, which during a given limited time period yields a coloured product proportionate to the amount of the conjugate. The coloured sample is then quantified photometrically. By analysis of "standards" of known antigen concentration and plotting a curve of signal

versus concentration, the concentration of the antigen in the biological specimen can be determined.

8.1.3 Transmission electron microscopy

The best resolution obtainable using light microscopy is 2 um. By the end of the 19th century scientists recognised that this 2um barrier was inherent to the wavelength (4um) of visible light. It was subsequently recognised that a beam of electrons travelling in a vacuum was associated with wave properties. Using this principle along with the ability to focus a beam of electrons in a magnetic field, Ruska - Knoll developed the first transmission electron microscope in 1931. Refinements to this design allow present day electron microscopes to achieve a resolution of 0.2 nm (2 x 10-4 um) with non-biological specimens and 1-2 nm with biological specimens.

Transmission electron microscopy works along the same principles as light microscopy (Agar et al 1974, Robinson et al). In electron microscopy the "light" equivalent source is a beam of electrons. Electrons are formed from a heated tungsten filament enclosed in a cathode plate and are accelerated away from the filament towards an anode plate, producing the electron beam. The condenser "lens" assembly is a magnetic coil that focuses the beam of electrons onto the plane of the specimen. This tissue specimen is cut sufficiently thin to allow the majority of the electrons to pass through the specimen. This emergent beam is focused by an objector magnetic coil and then passes through several projector magnetic coils which form a magnified two dimensional image of the specimen. The viewing screen of the transmission electron microscope is coated with a fluorescent material which when bombarded by the projected electrons fluoresces at the visible range. Due to the grain size of the fluorescent material resolution on the viewing screen is limited however improved detail may be recorded on fine grain photographic plates when exposed from beneath the fluorescent plate.

Due to the high magnification only small areas of tissue specimen (< 0.5 mm^2) can be examined at any one time. Multiple samples from tissues that display significant heterogeneity, such as placental cotyledons should be analysed to ensure adequate structural representation (Habashi, Burton & Steven 1983).

8.1.4 Scanning electron microscopy

The scanning electron microscope produces topographical images of specimens. It is similar to the transmission electron microscope in the use of electrons as a source of short-wave radiation and electromagnetic coils as lenses, however differs in design and operating mode (Black 1974). In transmission electron microscopy the specimen image is obtained from transmitted electrons, in scanning electron microscopy when the electron beam strikes the specimen it interacts with atoms in the surface layer and a variety of signals are produced. Electrons from the electron beam strike the specimen and resulting in both elastic collisions (electron-nucleus interactions) and inelastic collisions (electron-electron interactions) with the atoms of the specimen. Secondary electrons (resulting from inelastic collision) have low energy and are detected by a scintillator, which moves closely over the specimen in parallel with the electron beam, producing a light emission. Less secondary electrons are detected from depressions than peaks in the specimen and therefore appear as dark spots on the monitor as opposed to the light spots made by the peaks. Presently scanning electron microscopes can produce magnification of specimens in the order of 100,000 x and are capable of resolving topographical details of 5 to 10nm.

8.1.5 Preparation of tissue for electron microscopy
8.1.5.1 Tissue fixation

Due to the high resolution achievable with electron microscopy it is essential that tissue specimens are fixed in a manner to minimise artifactual changes. Material for transmission electron microscopy is fixed in gluteraldehyde or paraformaldehyde, which form strong covalent bond between tissue proteins, preserving structure. Perfusion fixation of biological specimens speeds fixation of the tissue thereby reducing hypoxic degradation of the tissue and allows preservation of vessel dimensions (Kaufmann 1985). Scanning electron microscopy requires that tissue is dried in a specialised manner as soft tissues readily undergo artifactual changes due to the properties of liquids as they expend at the tissue surface when evaporating into gas.

8.1.5.2 Sputter coating

Fixed and dried tissues for analysis by scanning electron microscopy require coating with a thin layer of conductive material to stop electrons accumulating on their surface during analysis and thereby reducing obtainable resolution. Sputter coating uses ionised gas plasma (usually argon) which impinges on a suitable metal target (e.g. gold) and atoms of that target are subsequently deposited on the tissue specimen. The coating material is laid down in an even layer and penetrates well into tissue crevices.

8.2 Clinical subjects
8.2.1.1 Diabetic pregnant subjects

The diabetic women were recruited from the specialised combined diabetic-antenatal clinic at Glasgow Royal Maternity Hospital over an eighteen-month period from August 1994. It was a requisite of recruitment that diabetic subjects were diagnosed as type 1 insulin dependent diabetic prior to pregnancy. Insulin dependent diabetes was defined as a random blood glucose greater than 11.1 mmol/L or a fasting blood glucose greater than 7.8 mmol/l. Informed consent was obtained for maternal blood sampling and post delivery collection of the placentae. Blood was sampled when clinic attendance corresponded with 12, 18, 28, 32 and 36 weeks' gestation. To improve sample numbers women advanced further than 12 weeks gestation were included in the recruitment and sampled at the relevant advancing gestations, providing relevant retrospective data could be collected from their hospital records.

8.2.1.2 Management of diabetic pregnant women.

Diabetic pregnant women were referred to the diabetic clinic as soon as pregnancy was diagnosed. They were then seen fortnightly at this clinic until 32-34 weeks gestation, thereafter they were reviewed weekly. At each visit they were reviewed by a diabetologist, an experienced midwife and a dietician. A named obstetric consultant with a special interest in diabetes was available to review the diabetic women as needed. At each visit a routine antenatal check was performed, including the measurement of maternal blood pressure and urinalysis. Fundoscopy was performed at least once each trimester of pregnancy to determine any appearance or progression of retinopathy.

8.2.1.3 Assessment of glycaemic control

Insulin requirements were adjusted on the basis of twice weekly 4 and 7 point blood glucose measurements. Diabetic control was also assessed by HbA1c determination at each clinic visit. Episodes of poor glycaemic control required patient admission to hospital for stabilisation. During labour glycaemic control was maintained using a glucose potassium intravenous infusion along with an intravenous insulin infusion. The rate of the latter was adjusted on the basis of two hourly blood glucose readings.

8.2.1.4 Assessment of fetal complications

Fetal well being was assessed by AFP screening (primarily for neural tube defects) at 15 weeks gestation. A detailed ultrasound scan for fetal anomalies was routinely performed at 18 weeks gestation. In the third trimester fetal biometry scans were performed at least fortnightly.

Elective delivery of diabetic women was planned for 38 to 39 weeks' gestation. Induction of labour was the preferred protocol for this. During labour fetal monitoring was assessed by a continuous cardiotocograph tracing. Following delivery the infants were routinely admitted to the special care unit for several hours to monitor for hypoglycaemia and then reviewed daily by the hospital paediatric staff. Fetal weight at delivery was related to birth weight centiles obtained from a local database (Small and Forbes 1983).

8.2.2 Non-diabetic pregnant subjects

Non-diabetic pregnant women to serve as a control population were recruited randomly from the low risk antenatal clinics held at the same hospital over a similar time scale from August 1994. Recruited non-diabetic pregnant women had no current or past medical illness. Parous women had previously uncomplicated pregnancies.

Recruitment of diabetic and non-diabetic women required that their pregnancies had been adequately dated with an estimated date of delivery determined by ultrasound scan before 16 weeks gestation.

8.2.3 Non-pregnant subjects

Type 1 insulin dependent non-pregnant women were recruited from the diabetic clinic at Glasgow Royal Infirmary. A hospital located within a mile of the maternity hospital in an attempt to recruit control subjects from the same geographical population. It is known however that only a third of pregnant diabetic women attending Glasgow Royal Maternity hospital attend this hospital's diabetic clinic prior to pregnancy.

Non-pregnant non-diabetic women were recruited from the staff of Glasgow Royal Infirmary and Glasgow Royal Maternity Hospital, providing they had no relevant current or past medical history.

8.3 Non- clinical materials

8.3.1 General biochemicals

The following chemical were purchased from BDH (Poole, England); acetic acid, buffer tablets (pH 4, 7.4 and 9.2), decon 75, formaldehyde, hydrochloric acid, sodium chloride, sodium citrate, calcium chloride, potassium hydroxide pellets, sodium hydroxide pellets and cupric sulphate (Anala R)(copper sulphate pentahydrate).

The following reagents were purchased from the Sigma Chemical Company (Dorset, England); 30% w/v hydrogen peroxide, Harris haematoxylin, 2% silane and tris chloride.

Ethanol, methanol and acetone (Anala R quality) were purchased from Hayman Limited (Essex, England).

Phosphate buffered saline tablets (PBS) were purchased from ICN Flow (Irvine, Scotland).

8.3.2 General apparatus

8.3.2.1 Centrifuges

Glasgow Royal Infirmary: MSE Mistral 4L was purchased from Fisons (Sussex, England) and the Damon CRU 5000 from Life Sciences (Hampshire, England).
Glasgow Royal Maternity Hospital: The Damon Clini-cool centrifuge was purchased from Life Sciences (Hampshire, England).

8.3.2.2 Refrigeration and Freezers

The Scandinova (SLF 111) fridge and the Blomberg (-20) freezer were purchased from Comet (Glasgow, Scotland). The -70 freezer was purchased from Scotlab (Coatbridge, Scotland).

8.3.2.3 Ovens

The proline power wave 800 microwave was obtained from Comet (Glasgow, Scotland). Gallenkamp Prelude incubator (Scotlab, Lanarkshire, Scotland)

8.3.2.4 Pipettes

Volumes of solutions in the range of 1-25ul were transferred accurately using digital pipettes from Scotlab (Coatbridge, Glasgow). Volumes of solutions in the range of 10-1000ul were transferred using Finn-pipettes purchased from Labsystems (Hampshire, England). Larger volumes were dispensed using standard laboratory glassware.

8.3.2.5 Balances

Measurement of weighted reagents was performed on the Stanton fine balance, Fisons (Sussex, England) and on the Sartorius balance BDH (Poole, England).

8.3.2.6 pH measurement

Measurements of pH were carried out using a Kent 7020 pH meter purchased from BDH (Poole, England). This apparatus was regularly standardised using a solution of pH 4, pH 7 or pH 9.2 buffer tablets.

8.3.2.7 Miscellaneous

Glassware was purchased from BDH (Poole, England). Plastic syringes and disposable 21G needles were obtained from Becton-Dickinson (Madrid, Spain). Glass microscope slides, coverslips and DPX mountant were purchased from BDH (Poole, England). The Corning magnetic stirrer and the whirlimixer were obtained from BDH (Poole, England)

8.3.3 General solutions and buffers

Distilled Deionised Water (ddH2O)
All solutions and buffers were prepared using ddh2O, obtained from a Milli Q Plus water purification system purchased from Millipore S.A. (Molsheim, France). Volumes approximating 500ml were autoclaved and stored at 4oC.

0.9% Sodium Chloride
9g of NaCl was dissolved into 1L ddH2O. Volumes approximating 500ml were autoclaved and stored at 4oC.

Tris buffered saline (TBS) buffer
10M Hydrochloric acid was added to 25ml 0.2M tris until a pH of 7.4 was obtained. This was made up to a final volume of 100ml with ddH2O. The Tris HCl was added to 900ml 0.9% sodium chloride to give the final solution.

Citrate Buffer
2.1g of anhydrous citric acid was dissolved in ddH2O. The pH was titrated to pH 6.0 with 2M NaOH.

0.1% Trypsin solution
0.3g of trypsin (type III from bovine pancreas), obtained from Sigma Chemicals Co. (USA) and 0.3g of CaCl were added to 300ml heated tris buffered saline. This solution was prepared immediately prior to use.

0.5% Copper sulphate solution
2g CuSO4 was dissolved in 400 ml tris buffered saline.

Phosphate buffer(PBS)
0.2M solution of disodium hydrogen orthophosphate (DHO) was prepared by dissolving 2.83g of DHO in 100mls ddH2O [Sol A]. 2.81g of citric acid was dissolved in 100ml ddH2O to make 0.1M solution of citric acid [SolB]. Neutral

phosphate buffer was prepared by adding 86ml of solution A to 17.7 ml of solution B. The buffer was stored at 4oC until use.

4% neutral buffered formalin
80 mls of 10 % formalin (containing 4% formaldehyde) was added to 120 mls phosphate buffer.

8.3.4.1 ELISA reagents and disposable apparatus

The VCAM-1, ICAM-1 and E-Selectin ELISA Kits were purchased from R+D Systems (Oxon, England). Microcentrifuge tubes, sterile pastettes and pipette tips were purchases from Alpha Laboratories (Hamphire, England). Rabbit anti-human vWf polyclonal antibody, HPR-conjugated rabbit anti-human vWF polyclonal antibody and ortho-phenylenedione (OPD) chomogen were all obtained from Dako Ltd, (High Wycombe). vWF (5th British standard for blood coagulation factors) was obtained from NIBSC (London).

8.3.4.2 ELISA non-disposable equipment

Microplate reader: Dynatech MR5000, Dynatech medical products (Guernsey, Channel Isles)

8.3.5.1 Immunohistochemistry reagents and disposable apparatus

Rabbit anti-human ki-67 (MIB-1) antibody, mouse anti- human macrophage (CD68) antibody, rabbit immunoglobulin fraction, mouse IgG negative control, biotinylated goat anti-mouse immunoglobulins, 3,3'-diaminodenzidinetetrachloride (DAB), peroxidase conjugated steptavidin and the wax pen were purchased from DAKO Ltd (High Wycombe, England) Biotinlyated goat anti-rabbit antibody was obtained from the Vector Elite ABC rabbit kit, Vector laboratories (Peterborough, England) Highly purified anti-goat collagen type I and collagen type IV from Europath Ltd (Cornwall, UK), purified anti-rabbit collagen type III from biogenesis(Bournemouth UK). Anti-laminin and anti-fibronectin from DAKO Ltd. (Buckinghamshire, UK). Goat Serum was obtained from the Scottish Antibody Production unit (SAPU): Carluke, Scotland). Normal human serum was obtained from Sigma (St Louis USA). 3-aminopropyl-triethoxy-silane was obtained from Sigma (St Louis, USA). Xylene (Anala R) was purchased from BDH

8.3.5.2 Immunohistochemistry non-disposable equipment

The Grant waterbath was obtained from Scotlab (Coatbridge, Scotland). Slide trays and racks were purchased from Philip Harris LTD (Aberdeen, Scotland)
The pressure cooker was purchased from Lakeland Plastics (UK)

Histokinette 2000 was purchased from Medical Laboratory Equipment services. The tissue Tek 3 and green uni-cassettes (to be used with this machine) were purchased from Bayer Diagnostics (Basingstoke, England). The microtome was purchased from

The light Olympus BX50 microscope was purchased from Olympus Optical Co. Ltd. (Japan).

8.3.6.1 Scanning Electron microscopy reagents and disposable apparatus

Methyl methacrlylate and hydrogen peroxide pellets were purchased from BDH Suppliers Ltd. (Poole, England). Baxter (Norfolk, England) supplied 250ml bags of 0.9% saline. Mercox® and catalyst were purchased from Japan Vilene Hospital (Hamburg, Germany). Stubs and storage boxes were purchased from Agar Scientific Ltd. (Essex, UK). Mounting glue was obtained from Neubauer Chemikalien (Munster, Germany). Venflons (21G) were purchased from Viggo Spectramed (Helsingburg, Sweden). Sutures (2/0 Chromic catgut were obtained from Ethicon (Edinburgh Scotland). Photographic film (Ilford Pan F50) was obtained from HA west (Clydebank, Scotland)

8.3.6.2 Scanning electron microscopy general apparatus

Critical point drier (CPD 750) was purchased from Biorad Microscience Division (Hertforshire, England). The Edwards coating unit was obtained from VG Scientific (Crowley, England). The Gallenkamp Prelude incubator was obtained from Scotlab (Lanarkshire, Scotland). The digital scanning electron microscope (DSM-940) and the stereomicroscope were purchased from Zeiss (Switzerland).The light microscope was obtained from Leitz-Labovert (Wetzler, Germany)

8.3.7.1 Transmission electron microscopy reagents and disposable apparatus

The following were purchased from Agar Scientific Ltd; Araldite, 25% EM grade gluteraldehyde, Kodak D19 solution, Rapid fix solutions A&B, dodecenyl succinic anhydride (DDSA), Kodak 4489 EM grade film, tri-dimethylaminomethyl phenol (DMP30) and Kodak 4489 thick base EM film. Lead nitrate and uranyl acetate were purchased from Sigma. Osmium tetroxide, glass strips, disposable vials and trufs were purchased from Leica (UK) Ltd. (Milton Keynes, England). Disposable blades were purchased from Bayer Diagnostics (Hampshire, England).

8.3.7.2 Transmission electron microscopy general apparatus

The LKB ultramicrotome was obtained from Leica (UK) Ltd. (Milton Keynes, UK). The plate degasser was obtained from VG Scientific (Crawly, England. The transmission electron microscope was obtained from Philips (UK).

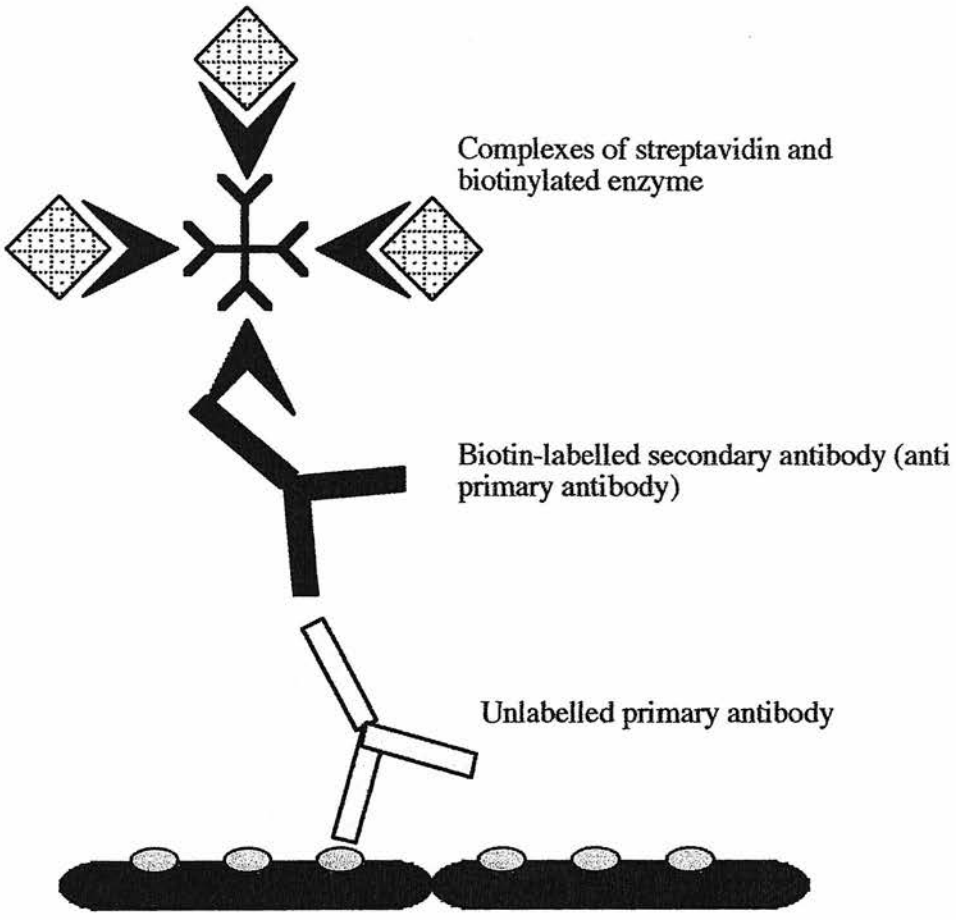
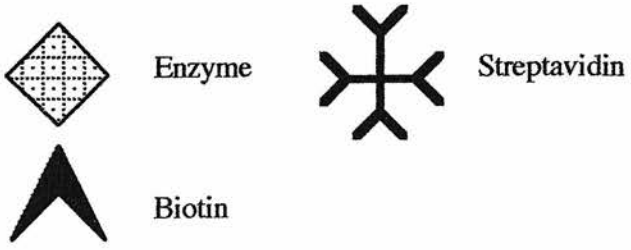


Figure 8.1 The streptavidin; biotin technique for indirect immunohistochemistry



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Appendix 1

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Circulating Cell Adhesion Molecule Concentrations in Diabetic Women During Pregnancy

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Objective: **To determine whether circulating concentrations of defined cell adhesion molecules, which are thought to reflect endothelial expression, are increased in insulin-dependent diabetic women during pregnancy.**

Methods: **Pregnant diabetic women demonstrating good glycemic control and without major complications before pregnancy were studied at 8–12 ($n = 15$), 18 ($n = 15$), 28 ($n = 16$), 32 ($n = 16$), and 36 ($n = 16$) weeks' gestation. A subgroup of ten diabetic women was sampled longitudinally through all five gestational ages. The diabetic women were compared with healthy nondiabetic women sampled cross sectionally at 12 ($n = 20$), 28 ($n = 19$), and 36 ($n = 19$) weeks' gestation. Nonpregnant diabetic ($n = 22$) and nonpregnant nondiabetic women ($n = 28$) also were studied. Plasma concentrations of the cell adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular endothelial cell adhesion molecule-1 (VCAM-1) were measured by enzyme-linked immunosorbent assay.**

Results: **Significantly higher median (range) concentrations of E-selectin (63.0 [20.2–107.0] ng/mL) and ICAM-1 (281.5 [171.6–778.4] ng/mL) but not VCAM-1 (459.7 [301.0–909.7] ng/mL) were found in nonpregnant diabetic women compared with nonpregnant nondiabetic women (43.5 [18.1–93.2], 243.6 [174.4–329.2], and 476.0 [253.8–929.4] ng/mL, respectively). During pregnancy these significant differences between diabetic and control groups were lost. The median (range) concentration of E-selectin (50.0 [21.2–96.3] ng/mL) was significantly lower in pregnant compared with nonpregnant diabetic women. The plasma concentrations of E-selectin and ICAM-1 did not change significantly with gestation in either diabetic or nondiabetic pregnant groups. Vascular endothelial cell adhesion molecule-1 concentration changed significantly with gestation in the diabetic pregnant group only.**

Conclusion: **Circulating concentrations of defined vascular cell adhesion molecules are not increased abnormally in diabetic women with good glycemic control during otherwise uncomplicated pregnancy. (Obstet Gynecol 1997;90: 874–9. © 1997 by The American College of Obstetricians and Gynecologists.)**

Despite recent major advances in both obstetric care and medical management of diabetes, women with this disease remain at greater risk for morbidity during their pregnancy than nondiabetic women. Two factors contributing to this maternal morbidity are preeclampsia[1] and progression of microvascular disease, in particular, retinopathy.[2–4] The pregnancy-related mechanisms behind these conditions are unknown but are associated with vascular damage and dysfunction.

It is well recognized that the endothelium itself can play a key role in such vascular pathology by a variety of mechanisms. One such mechanism is through the expression of an array of leukocyte-specific cell adhesion molecules, each of which is induced by a variety of stimuli and will recognize specific ligands on various subsets of leukocytes, promoting leukocyte attachment and activation.[5] Once activated, leukocytes can be powerful mediators of vascular damage and inflammation. Activation of neutrophils has been demonstrated to occur in diabetic and preeclamptic pregnancy and in nonpregnant diabetic women.[6,7] Neutrophils are acute mediators of vascular damage that release a variety of toxic agents, including elastase and other proteases, toxic oxygen radicals, and leukotrienes, on activation.[8,9]

Three of the main endothelial-expressed cell adhesion molecules associated with leukocyte activation are E-selectin, a member of the selectin family of cell adhesion molecules; and intercellular cell adhesion molecule-1 (ICAM-1) and vascular endothelial cell adhesion molecule-1 (VCAM-1) from the immunoglobulin superfamily. E-selectin and ICAM-1 interact with a number of

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leukocytes, including neutrophils. E-selectin provokes the initial loose attachment of the leukocyte to the endothelium and, in the presence of fluid sheer stress, the subsequent rolling of the leukocyte along the endothelial surface. Intercellular cell adhesion molecule-1 mediates firm adherence and leukocyte activation. Vascular endothelial cell adhesion molecule-1 supports the adhesion of lymphocytes, monocytes, and eosinophils to the endothelium.

Surface expression of these cell adhesion molecules is associated with their shedding into the peripheral circulation. The exact mechanism of this process and the physiologic function of these shed molecules remain unclear; however, circulating adhesion molecule concentrations have been shown to be increased in a number of disease states, including diabetes[10,11] and preeclampsia.[12]

We hypothesized that in diabetic women, pregnancy is associated with an increased endothelial expression and shedding of cell adhesion molecules. If endothelial expression of cell adhesion molecules is increased, this may be associated with both the leukocyte activation and the increased incidence of vascular pathology previously documented to occur in diabetic women during pregnancy.[1–4] The aim of this work was to determine the circulating concentrations of three endothelial cell adhesion molecules—E-selectin, ICAM-1, and VCAM-1—throughout diabetic pregnancy, to compare these with the concentrations found throughout nondiabetic pregnancy and in nonpregnant diabetic women, and to correlate our findings with clinical outcome.

Materials and Methods

Sample size was calculated on the basis of 90% power with significance set at the 5% level. This indicated a minimal sample size of 12 women in each group, based on data from a previous report[12] of cell adhesion molecule concentrations in pregnant and nonpregnant women. Pregnant women with type 1 insulin-dependent diabetes attending the combined antenatal-diabetic clinic at Glasgow Royal Maternity Hospital were recruited into the study during an 18-month period. Blood samples were drawn when attendance coincided with the following gestational ages: 8–12 (n = 15), 18 (n = 15), 28 (n = 16), 32 (n = 16), and 36 (n = 15) weeks' gestation. Ten women were sampled longitudinally throughout all five designated gestations of pregnancy. At each time point studied, additional blood was drawn to assess glycemic control by glycated hemoglobin measurement.

Healthy nondiabetic women attending a low-risk antenatal clinic were studied as controls and were sampled cross sectionally at 12 (n = 20), 28 (n = 19), and 36 (n = 19) weeks' gestation. Nondiabetic women incurring complications during pregnancy were excluded from the study. Nonpregnant diabetic and nonpregnant nondiabetic women also were studied (n = 22 and n = 28, respectively). Glycated hemoglobin was measured at the time of sampling in the diabetic group. All subjects gave informed consent. The study was approved by the local ethics committee.

Peripheral venous blood was collected into prechilled tubes containing lithium heparin as anticoagulant. Plasma was prepared by centrifugation at 2000 × g at 4 C within 5 minutes of blood sample collection. Aliquots of plasma were stored at −70 C until use. The cell adhesion molecules were measured by enzyme-linked immunosorbent assay (R&D Systems Europe Ltd., Abingdon, Oxon, UK).

Cell adhesion molecule concentrations approximated normal distribution in pregnant but not nonpregnant groups. Data from nonpregnant diabetic and nondiabetic subjects were analyzed by the Mann-Whitney U test. During pregnancy, change relative to gestation within the diabetic subgroup (n = 10) was assessed by multiple-measures analysis of variance and within the control group by simple analysis of variance. Subsequent comparison between the pregnant diabetic and nondiabetic groups was assessed by Student t test and used all data available from diabetic subjects. The Mann-Whitney U test was used to compare pregnant and nonpregnant women.

The effect of gestation on glycated hemoglobin values was assessed by multiple-measures analysis of variance. Subsequent comparison of pregnant and nonpregnant glycated hemoglobin values was assessed by Student t test. Maternal age of studied groups was compared by Student t test. Parity, smoking habit, and White class (for diabetic groups)[13] were compared by χ^2 analysis.

Results

There was no significant difference between the groups in terms of parity, smoking habit, or White class (Table 1). Diabetic women were younger than nondiabetic controls. One diabetic woman had progressive retinopathy during pregnancy and required laser photocoagulation for proliferative disease and early delivery (at 36 weeks' gestation). No diabetic woman developed preeclampsia or gestational hypertension. Diabetic control as assessed by glycated hemoglobin determination improved significantly with gestation. By 18 weeks' gestation, glycated hemoglobin measurements were reduced significantly compared with those seen in nonpregnant diabetic women and remained so for the remainder of pregnancy (Table 1).

Table 1. Clinical Characteristics

Study group	Category of weeks' gestation	No.	Maternal age (y)	No. of primigravidas	No. of smokers	White class	Glycated hemoglobin (%)*
Diabetic	Nonpregnant	22	27.5 (6.9)†	10	4	2B, 11C, 8D, 1R	8.5 (2.3)
Diabetic	12	15	24.5 (5.3)†	4	1	6C, 6D, 1B	7.6 (1.4)
Diabetic	18	15	24.7 (5.6)	5	1	6C, 7D, 2B	6.5 (1.2)‡
Diabetic	28	16	24.0 (5.0)	7	2	9C, 7D	6.3 (0.7)§
Diabetic	32	16	25.0 (4.7)	4	3	8C, 6D, 1B, 1R	6.3 (0.8)§
Diabetic	36	15	24.7 (4.5)†	7	3	9C, 5D, 1R	6.4 (0.7)‖
Diabetic	Subgroup	10	24.3 (5.4)	4	1	5C, 5D	
Control	Nonpregnant	28	30.7 (4.2)†	11	4		
Control	12	20	28.8 (5.8)†	9	7		
Control	28	19	27.1 (5.6)	10	4		
Control	36	19	28.4 (4.7)†	11	5		

Data are presented as mean (standard deviation).

*Glycated hemoglobin values changed significantly with gestation in the diabetic subgroup ($P < .002$, data not shown). Comparison of gestational with nonpregnant glycated hemoglobin values was assessed by Student t test. Normal reference range 3.4–5.2%.

†Comparison of maternal age between study groups at corresponding gestations was assessed by Student t test ($P < .05$).

‡$P < .02$.

§$P = .002$.

‖$P < .05$.

All cell adhesion molecule concentrations were above the detection limit of the applied assay. Comparison of the nonpregnant groups (Figure 1) demonstrated significantly increased median (range) concentrations of the cell adhesion molecules E-selectin (63.0 [20.2–107.0] ng/mL) and ICAM-1 (281.5 [171.6–778.4] ng/mL) but not VCAM-1 (459.7 [301.0–909.7] ng/mL) in diabetic women compared with those found in nondiabetic women (43.5 [18.1–93.2], 243.6 [174.4–329.2], and 476.0 [253.8–929.4] ng/mL, respectively).

In pregnancy, there was no significant change in the measured concentrations of the cell adhesion molecules E-selectin and ICAM-1 with respect to gestation in either the diabetic or control group (mean values throughout gestation for both groups are shown in Table 2). The latter observation allowed pregnancy to be assessed as a single time point in subsequent analysis of this data. For any woman sampled more than once during her pregnancy, one of the available values was selected randomly to represent pregnancy. (Available values were printed separately, folded to conceal the printed value, and then placed and mixed in a vessel, from which one was drawn by a nonauthor.) Subsequent comparisons also included the data available from the diabetic women not sampled longitudinally throughout pregnancy. Therefore, a total of 26 diabetic and 58 control "pregnancy" values were available for analysis. No significant difference was found between mean (standard deviation) diabetic pregnant E-selectin and ICAM-1 concentrations (50.2 [16.0] and 274.5 [107.5] ng/mL, respectively) and nondiabetic pregnancy cell adhesion molecule concentrations (46.5 [17.6] and 287.2 [99.0] ng/mL, respectively). Comparison of

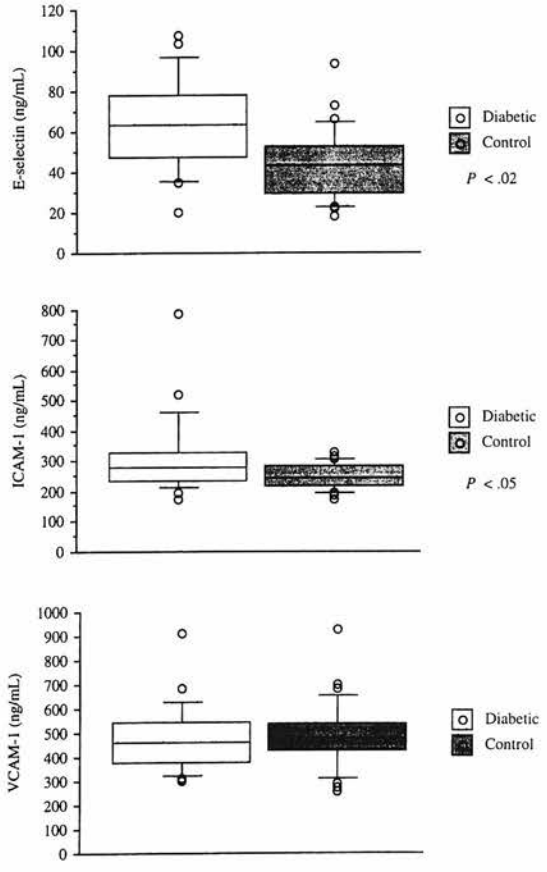

Figure 1. Box plots (median, 25th and 75th percentile, tenth and 90th percentile) of plasma cell adhesion molecule concentrations in nonpregnant diabetic women and controls. The Mann-Whitney U test was used to compare diabetic and control data. ICAM = intercellular cell adhesion molecule; VCAM = vascular endothelial cell adhesion molecule.

Table 2. Gestational Plasma Cell Adhesion Molecule Concentrations

Study group	Weeks' gestation	No.	E-selectin (ng/mL)	ICAM-1 (ng/mL)	VCAM-1 (ng/mL)
Diabetic	12	10	55.1 (16.2)	261.9 (44.8)	422.0 (132.0)*
Diabetic	18	10	54.4 (13.9)	274.8 (61.8)	390.2 (79.3)*
Diabetic	28	10	55.1 (16.4)	263.5 (41.2)	400.8 (113.2)*
Diabetic	32	10	57.9 (20.0)	271.6 (87.1)	494.1 (144.2)*
Diabetic	36	10	55.1 (13.2)	248.7 (44.7)	560.8 (129.3)*
Control	12	20	44.7 (9.8)	268.6 (102.7)	446.8 (117.1)
Control	28	19	48.8 (20.9)	293.9 (89.2)	420.1 (134.9)
Control	36	19	45.8 (19.7)	293.6 (101.6)	502.6 (139.2)

ICAM = intracellular cell adhesion molecule; VCAM = vascular endothelial cell adhesion molecule.

Data are presented as mean (standard deviation). Change in concentration with respect to gestation in the diabetic group was assessed by multiple-measures analysis of variance. Change relative to gestation in the control group was assessed by simple analysis of variance. Vascular endothelial cell adhesion molecule-1 concentration changed significantly with gestation in the diabetic population.

* $P < .05$.

values in pregnant and nonpregnant women revealed that the median (range) circulating concentration of E-selectin was significantly lower ($P < .05$) in pregnant diabetic women (50.0 [21.2–96.3] ng/mL) than in nonpregnant diabetic women (63.0 [20.2–107.0] ng/mL). There was no significant difference in the concentration of ICAM-1 between diabetic groups or of both measured cell adhesion molecules between nondiabetic pregnant and nonpregnant groups.

The circulating concentration of VCAM-1 changed significantly with respect to gestation in the diabetic pregnant subgroup but not the nondiabetic pregnant group. Mean values throughout gestation for both groups are given in Table 2. However, there was no significant difference in VCAM-1 concentrations between diabetic and nondiabetic women at any corresponding gestation. (All available diabetic data were used in this analysis and the following analyses.) Comparison of values in pregnant and nonpregnant diabetic women revealed a significant difference ($P < .02$) between diabetic women sampled at 36 weeks' gestation ($n = 15$) (median [range] 582.8 [387.0–848.6] ng/mL) and nonpregnant diabetic women ($n = 22$) (459.7 [301.0–909.7] ng/mL) but not between any other gestational or nonpregnant data. There was no significant difference between control pregnant and nonpregnant VCAM-1 data.

Discussion

Our finding that circulating concentrations of E-selectin and ICAM-1 are elevated selectively in nonpregnant diabetic women compared with those measured in nonpregnant nondiabetic women corresponds with findings of previous studies[10,11] in the general diabetic population. Increased endothelial expression of these cell adhesion molecules, reflected by increased circulating concentrations, provides a mechanism for the neutrophil activation and the vascular damage demonstrated to occur in diabetes.

Clinical studies[1–4] have demonstrated an increased incidence of vascular pathology in diabetic women as a consequence of pregnancy. Based on these studies, we hypothesized that pregnancy in diabetic women would be associated with further increased circulating concentrations of cell adhesion molecules. However, we found no such increase in the circulating concentrations of E-selectin and ICAM-1 in the pregnant diabetic group compared with the nonpregnant diabetic group or with increasing gestation. The concentration of E-selectin actually decreased from that seen in the nonpregnant diabetic women as a consequence of pregnancy.

Altered shedding, dilution, or excretion of cell adhesion molecules may occur as a result of pregnancy and therefore the circulating concentrations of these molecules may no longer represent their endothelial expression. However, the finding of constant circulating concentrations of these cell adhesion molecules throughout nondiabetic pregnancy and in nondiabetic pregnant and nondiabetic nonpregnant groups suggests that this is not the case, because we would not expect any change in the endothelial expression of cell adhesion molecules during uncomplicated pregnancy in this group of women. Therefore, although the possibility of altered renal clearance in pregnant diabetic women cannot be excluded, the circulating concentrations of E-selectin and ICAM-1 measured in the present population of diabetic women suggest that pregnancy per se is not associated with increased endothelial expression of these cell adhesion molecules.

The specific population of diabetic women participating in the present study may account for apparent discrepancy between these findings and the outcome of the referred clinical studies. Studies specifically determining the effect of pregnancy on retinopathy, such as that by Klein et al[4] and the large diabetes in early

pregnancy study,[14] have stressed the importance of poor glycemic control at the onset of pregnancy, rapid improvement of glycemic control during pregnancy, and advanced retinopathy before pregnancy as the major risk factors for progression of retinopathy. Clearly, such factors have been proven to be pivotal to progression of retinopathy on the short-term introduction of tight glycemic control in the nonpregnant state.[15–18] The women in the present study demonstrated reasonable glycemic control at the onset of pregnancy, and although their control improved significantly during pregnancy, this may not have been so abrupt as to provoke increased endothelial expression of cell adhesion molecules, vascular damage, and retinal pathology.

A clinical study[1] demonstrating an increased incidence in preeclampsia in pregnancies in diabetic women has shown that, as for progressive microvascular disease, the incidence of this complication is increased in women with advanced diabetic disease as classified by White class. The limited number of diabetic women participating in our study and the lack of women with advanced diabetic disease may explain why no woman in the present study developed this complication. We cannot assess from this study whether women with more advanced diabetes would demonstrate increased endothelial expression of cell adhesion molecules during pregnancy that could in turn be associated with vascular complications, and this requires further investigation. Only one woman with proliferative retinopathy during pregnancy was sampled during this study. The concentrations of cell adhesion molecules found within the plasma from this woman (E-selectin 48.1 ng/mL and ICAM-1 263.6 ng/mL at 36 weeks' gestation) were not outside the range of concentrations measured in diabetic women without complication.

It is tempting to speculate that the decreased circulating concentration of E-selectin found in our population of diabetic women during pregnancy reflected their improved glycemic control as a consequence of this event. Hyperglycemia has been proved to cause vascular damage in animal models,[19] and improved glycemic control is associated universally with delayed onset and progression of microvascular disease in both animal and clinical studies when maintained over the long term.[20] The introduction of improved glycemic control over a short period, such as during pregnancy, has a more complex relationship with vascular pathology. It is possible that the small but significant improvement in glycemic control in our population of women, who demonstrated good glycemic control before the onset of pregnancy, was of a magnitude to provoke only a positive effect on the endothelial environment. We did not correlate this short-term improvement in glycemic control, as assessed by glycated hemoglobin measurements, with E-selectin concentrations, because of this complex relationship and because part of the reduction in glycated hemoglobin values may be explained by a physiologic increased flux of red blood cells into the circulation during pregnancy.[21]

No difference in the circulating concentration of VCAM-1 was found between nonpregnant diabetic and nondiabetic women. The ligand recognized by VCAM-1, very late antigen-4, is expressed not on neutrophils but on other leukocytes important in the later stages of inflammation. A selective increased endothelial expression of E-selectin and ICAM-1 in diabetic subjects may emphasize that the vascular endothelial damage occurring in this disease is an ongoing acute process reflecting short-term fluctuations in glycemic control, emphasizing the need for improved control, such as that demonstrated on a daily basis during pregnancy, in the general diabetic population.

The significance of the change in VCAM-1 concentration during diabetic pregnancy is unclear. A similar pattern of circulating VCAM-1 concentration was seen throughout nondiabetic pregnancy but did not attain statistical significance. This pattern, an initial decrease and then progressive increase, resembles the physiologic change in blood pressure during pregnancy and hence could be a response to, or an altered shedding due to, this characteristic. The circulating concentration of VCAM-1 has been demonstrated to be increased in preeclampsia.[12] However, if increased endothelial expression of VCAM-1 provides a mechanism for vascular dysfunction, this dysfunction obviously is not primarily neutrophil mediated.

In conclusion, our findings do not support the hypothesis that increased endothelial expression and shedding of cell adhesion molecules occur in diabetic women as a consequence of pregnancy. Rather, our findings suggest that the endothelial stimulus to neutrophil activation and, hence, vascular damage may decline in these women during pregnancy, possibly as a consequence of their improved glycemic control during this period, a feature that may be reassuring to patient and physician alike.

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