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Obstetrics and Gynaecology
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**DIABETIC RETINOPATHY
AND PREGNANCY**

**by
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Academic Dissertation

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the Medical Faculty of the University of Helsinki,
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ABBREVIATIONS

AGEs	Advanced glycation end products
AM	Adrenomedullin
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
Ang II	Angiotensin II
ANOVA	Repeated measures analysis of variance
ANP	Atrial natriuretic peptide
AU	Arbitrary unit
BDR	Background diabetic retinopathy
BFS	Blue Field Entoptic Simulation test
BMI	Body mass index
BNP	Brain natriuretic peptide
BRB	Blood-retinal barrier
CDI	Colour Doppler imaging
CNP	C-type natriuretic peptide
CPD	Cycles per degree
CRA	Central retinal artery
CS	Contrast sensitivity
CWS	Cotton wool spot
DC	Photodetector sensitivity value
DCCT	Diabetes Control and Complications Trial Study
DM	Diabetes mellitus
DR	Diabetic retinopathy
EC	Endothelial cell
ELAM-1	Endothelial leukocyte-adhesion molecule (E-selectin)
ELISA	Enzyme-linked immunosorbent assay
ETDRS	Early Treatment of Diabetic Retinopathy Study
ET-1	Endothelin-1
FAG	Fluorescein angiography
GDM	Gestational diabetes mellitus
GF	Growth factor
HbA1c	Haemoglobin A1c
HP	Haptoglobin
HRF	Heidelberg Retinal Flowmetry (Heidelberg Engineering, GmbH, Heidelberg, Germany)
HRT	Heidelberg Retinal Tomography (Heidelberg Engineering, GmbH, Heidelberg, Germany)
ICAM-1	Intercellular adhesion molecule-1
IDDM	Insulin dependent diabetes mellitus
IGF-1	Insulin like growth factor-1

IL-6 Interleukin-6
IOP Intraocular pressure
IRMA Intraretinal microvascular abnormalities
MA Microaneurysm
NO Nitric oxide
NPDR Non-proliferative diabetic retinopathy
OCT Optical coherence tomography
ONH Optic nerve head
PDR Proliferative diabetic retinopathy
PEDF Pigment epithelium-derived factor
PGH Placental growth hormone
PGI2 Prostacyclin
PIH Pregnancy induced hypertension
PKC- β Protein kinase C beta
PRA Plasma renin activity
RAS Renin-angiotensin-system
RPF Retinal blood flow
RP Retinopathy
SD Standard deviation
SLO Scanning laser ophthalmoscopy
TGF- α Transforming growth factor α
TGF- β Transforming growth factor β
VARP Volume above the reference plane
VCAM-1 Vascular cell adhesion molecule-1
hVEGF-A Human vascular endothelial growth factor A
VEGF Vascular endothelial growth factor
sVEGFR-1 Soluble receptor of vascular endothelial growth factor type 1

ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, which will be referred to in the text by their Roman numerals **I** to **V**.

I Loukovaara S, Kaaja R, Immonen I. Macular capillary blood flow velocity by blue-field entoptoscopy in diabetic and healthy women during pregnancy and the postpartum period. *Graefes Arch Clin Exp Ophthalmol* 2002;240:977-982.

II Loukovaara S, Harju M, Kaaja R, Immonen I. Retinal capillary blood flow in diabetic and nondiabetic women during pregnancy and postpartum period. *Invest Ophthalmol Vis Sci* 2003;44:1486-1491.

III Loukovaara S, Harju M, Kaaja R, Immonen I. Topographic change in the central macula coupled with contrast sensitivity loss in diabetic pregnancy. *Graefes Arch Clin Exp Ophthalmol* 2003;241:607-614.

IV Loukovaara S, Immonen I, Yandle T, Nicholls G, Hiilesmaa V, Kaaja R. Vasoactive mediators and retinopathy during Type I diabetic pregnancy. (Submitted)

V Loukovaara S, Immonen I, Koistinen R, Rudge J, Teramo K, Laatikainen L, Hiilesmaa V, Kaaja R. Angiopoietic factors and retinopathy in pregnancies complicated with Type I diabetes. *Diabetes Medicine* 2003, in press.

1. ABSTRACT

The purpose of the present prospective study was to gain better understanding about the characteristics and pathogenetic mechanisms of diabetic retinopathy (DR) during pregnancy in women with type I diabetes. The aim was to compare retinal capillary blood flow in women with type I diabetes with nondiabetic control women during pregnancy and postpartum and with nonpregnant diabetic women using two different methods. The hypothesis was that progression of DR during pregnancy is associated with increased retinal capillary blood flow in diabetic women (I, II).

The third study was carried out to reveal whether macular topographical changes occur in diabetic compared to nondiabetic women during pregnancy and postpartum. In addition, the loss of contrast sensitivity (CS) was suspected of being related to macular thickening during diabetic pregnancy. The fourth study was carried out to evaluate the role of various systemic vasoactive mediators in the development or progression of DR during pregnancy and postpartum. The fifth study aimed to clarify the role of various systemic angiopoietic factors in the development or progression of DR during pregnancy and postpartum.

Firstly, in a prospective sub-study of 46 pregnant women with diabetes and 11 nondiabetic pregnant women macular capillary blood flow velocity was measured by psychophysical blue-field entoptic simulation test. In diabetic women, the macular capillary blood flow velocity was higher than in nondiabetic women throughout pregnancy and postpartum. Further, capillary blood flow velocity seemed to depend on the grade of DR. Diabetic women with no or very mild retinopathy had lower macular capillary blood flow velocities than those with more severe retinopathy, but higher velocities than nondiabetic women. A temporal increase from the first trimester to the postpartum period was observed in diabetic but not in nondiabetic women. These data supported the concept that capillary hyperperfusion may play a role in the development of DR during pregnancy.

Secondly, perimacular capillary blood flow was measured in 32 pregnant women with type I diabetes and 11 nondiabetic pregnant women by confocal laser Doppler flowmetry throughout pregnancy and postpartum in a prospective sub-study (II). The flow values were higher in diabetic women during pregnancy, compared to nondiabetic pregnant women or nonpregnant diabetic women. In diabetic women with mostly minimal to moderate retinopathy, no clear correlation between flow values and progression of DR could be observed. These results indicated that retinal capillary blood flow responds to pregnancy in a different manner in diabetic women compared to nondiabetic women, which may be related to impaired autoregulation of capillary blood flow in diabetes.

Thirdly, in a prospective sub-study of 46 diabetic women and 11 nondiabetic controls macular surface topography was measured by confocal scanning laser tomography throughout pregnancy and postpartum (III). In diabetic women, especially in those with clear progression of DR, the macula was slightly more elevated than in nondiabetic controls. Furthermore, CS was lower in diabetic than in nondiabetic women at mid-spatial frequencies, and loss of CS was correlated with macular elevation during the third trimester in diabetic women even in the absence of retinopathy.

Fourthly, in a prospective sub-study of 53 pregnant diabetic and 9 nondiabetic women DR was graded from fundus photographs (IV). Plasma markers of renin-angiotensin-system (RAS) (plasma renin activity, angiotensin II, aldosterone), natriuretic peptides (ANP, BNP, CNP), and adrenomedullin were measured during the first and third trimesters, and at 3 months postpartum. Diabetic pregnancy was associated with lower levels of PRA and ANP compared to nondiabetic pregnancy. But no clear associations between the vasoactive hormones and progression of retinopathy could be detected.

Fifthly, in a prospective sub-study of 26 pregnant women with type I diabetes and 8 nondiabetic controls plasma levels of angiopoietin-1 and -2, hVEGF-A and total soluble VEGF receptor-1 were measured during the first and third trimesters, and at 3 months postpartum (V). Levels of Ang-2 were lower in the diabetic than in nondiabetic women during pregnancy. At baseline, levels of angiopoietic factors showed no correlation with severity of DR. At 3 months postpartum, hVEGF-A levels were lowest in diabetic women with progression of retinopathy. The circulating levels of angiopoietic factors appeared not to be connected with the progression of retinopathy during pregnancy.

2. INTRODUCTION

Despite advances in medical and surgical management, diabetic retinopathy (DR) is still a major global health problem. It is one of the leading causes of visual disability in the industrialized countries (Aiello 1998), and it is becoming an epidemic to modernising and urbanising population also elsewhere (Gupta & Gupta 2000). Estimates of the prevalence of DR vary, but the Wisconsin epidemiological study of DR (WESDR) has documented a higher rate in those with earlier age of onset of type I diabetes, approaching 98% after 15 years of disease (Klein 1984b). In Finland, by 25 years of type I diabetes, 94% of patients are affected by NPDR, and 38% by PDR (Tiina Virtamo, personal communication). The prevalence of DR is 12.1% in Finland among diabetic people aged from 18 to 64, being the most frequent cause of new cases of blindness (28.3%) in the same age group (National Research and Development Centre for Welfare and Health in Finland, The Finnish Register of Visual Impairment, 2001). The first half of this time period mentioned above also corresponds to years of peak fertility and childbearing in diabetic women (Elman *et al.* 1990).

DR is a microvascular complication of diabetes. It develops as a result of several processes (Garner 1993). The spectrum of lesions extend from mild nonproliferative abnormalities, characterized by increased vascular permeability (local oedema and lipoprotein deposits), to moderate and severe nonproliferative diabetic retinopathy (NPDR), characterized by vascular closure (acellular capillaries and areas of ischemia), and further to proliferative diabetic retinopathy (PDR), characterized by the growth of abnormal blood vessels (neovascularization) on the retina and posterior surface of the vitreous, leading finally to the contraction of the fibrovascular proliferations and the vitreous (Larson 1960, Dobree 1964, Davis 1965, Tolentino *et al.* 1966, Ferris *et al.* 1999, American Diabetes Association 2000), and potentially to loss of function of the eye.

Etiologic factors of DR have been extensively investigated. Firstly, hyperglycaemia has been found to be the primary factor for the development of DR (Pirart 1978, Bresnick & Palta 1987, Klein *et al.* 1987). Exposure to prolonged hyperglycaemia causes first reversible, then irreversible changes in retinal structure. Secondly, various genetic, hormonal, immunologic and environmental influences have been suggested as important contributing factors to the development or progression of DR. In the Diabetes Control and Complications Trial (DCCT), a clear relationship was demonstrated in type I diabetes between hyperglycaemia and diabetic microvascular complications, including retinopathy, nephropathy, and neuropathy (DCCT 1993). Accordingly, it revealed

significantly reduced risk of progression of retinopathy by 63%, of macular oedema by 26%, and the need for laser treatment by 51% in patients with type I diabetes treated with intensive insulin therapy compared to conventional therapy (DCCT 1993). Intensive glycemic control and normoglycaemia are also the cornerstones of management for pregnant women with type I diabetes, being the most important factors associated with improved maternal and neonatal outcome (Rosenn & Miodovnik 2000).

DR affects 20 to 30% of diabetic women in the reproductive age group (Reece *et al.* 1996). The effects of pregnancy on DR are not completely clear. Several studies suggest that pregnancy in type I diabetic women may aggravate DR (Laatikainen *et al.* 1980, Dibble *et al.* 1982, Moloney & Drury 1982, Soubrane 1985, Serup 1986, Klein 1990, Reece 1994, Hellstedt *et al.* 1997b). However, pregnancy seems to have no long-term detrimental effects on the progression of DR (Kaaja *et al.* 1996). During pregnancy, it is unclear to what extent the irreversible component of DR progression is caused by pregnancy per se or whether it merely reflects the natural history of a progressive disease (Rosenn & Miodovnik 2000).

Hormonal milieu alters during pregnancy. It has been suggested that circulating and local factors such as growth hormone (GH), insulin like growth factor-1 (IGF-1), and other angiogenic factors may contribute to the progression of DR during pregnancy (Lauszus *et al.* 2003). It has been shown that angiogenic factors produced by placenta result in vessel proliferation *in vivo* and in endothelial cell cultures *in vitro* (Rosenn & Miodovnik 2000). Despite hormonal changes occurring during pregnancy, it is unclear whether other changes related to progression of DR are caused by the metabolic changes, such as rapid improvement of glycemic control, by the cardiovascular changes, such as pre-eclampsia or pregnancy induced hypertension (PIH), or by hemodynamic stresses of pregnancy, labour and delivery.

3. REVIEW OF THE LITERATURE

3.1. TYPE I DIABETES MELLITUS

3.1.1 Definition, pathogenesis, genetic aspects and incidence of type I diabetes mellitus

The process leading to type I diabetes mellitus may start in early infancy or already in utero (Hämäläinen & Knip, 2002). Type I DM develops predominantly in childhood or young adulthood. It results from a disorder of immunoregulation. In type I diabetes there is a selective destruction of insulin-secreting β -cells within the pancreas. It is believed that autoreactive T cells belonging to a T helper 1 subset and their characteristic cytokine products, interferon gamma and interleukin-2 cause islet inflammation (insulitis) and destruction of the β -cells of the pancreas (Gepts 1965, Suarez-Pinzon & Rabinovitch 2001). Islet cell antibodies are present in the serum of more than 85% of the diabetic patients.

Type I DM is caused by the synergistic effects of genetic susceptibility, environmental (exogenous) factors, and immunological factors. Human leukocyte antigen (HLA) locus on chromosome 6 harbours at least one susceptibility gene for type I DM. Haptoglobin genotype HP 1-1 has been shown to provide protection against DR when compared to HP 2-1 and HP 2-2 genotypes (Nakhoul *et al.* 2000). However, genetic effects explain only 70-75% of the susceptibility to type I diabetes, and environmental effects such as diet and viral infections (coxsackie-B, rubella, enteroviruses, cytomegalovirus, varicella zoster) may explain the rest (Kaprio *et al.* 1992).

Type I DM shows a wide variation in incidence and prevalence in different populations. Its prevalence has increased dramatically worldwide during the past few decades, and it is expected to increase even more in the future (Green *et al.* 1996, Sanchez-Thorin 1998). The incidence of type I diabetes is increasing in children (Bruno *et al.* 2001, Schoenle *et al.* 20001), and it is record-high of 45 per 100 000 person years in 1996 in Finland (Tuomilehto *et al.* 1999). There is a 0.7% absolute risk of type I DM before age 15 in Finland.

Familial aggregation of type I DM is a well-known phenomenon demonstrated in many epidemiological studies. However, it is remarkable that 85 to 90% of new type I DM

cases occur in families with no previous history of the disease among the first-degree relatives. It has been estimated that the chance of a type I diabetic woman having a diabetic child is 1.6 to 2.6%, and the chance of a diabetic father 6.0% (Jovanovic-Peterson 1989, Veijola *et al.* 1996).

3.2. DIABETIC RETINOPATHY

3.2.1 Historical review

The existence of retinal disease in diabetic patients was postulated in the first part of the 19th century. Eduard Jaeger published the first report on diabetic maculopathy in 1856. But not until the second half of the 20th century, was it proved that DR really represented a unique vasculopathy. The role of growth factors in the progression of DR became obvious in 1953, when Poulsen suggested the influence of the pituitary system on the course of DR in a classic report documenting regression of DR in a patient after pituitary infarction (Poulsen 1953).

3.2.2 Pathogenesis

The pathogenetic mechanisms of DR are not fully-known. Basement membrane thickening and pericyte loss have been established as the histological hallmarks of early DR (Kohner 1989, Archer 1999). Primarily, DR is a disease of the retinal capillary endothelial cells (ECs) (Kohner 1989, Kohner 1993, Archer 1999). Since retina is one of the few tissues, which does not require insulin to transport glucose into the cell, hyperglycaemia leads to high intracellular glucose levels. In hyperglycaemia, cells are adapted to abnormally high blood glucose values, and with rapid normalization of blood glucose values ECs undergo apoptosis (Li *et al.* 1996, Mizuntani *et al.* 1996, Jousen *et al.* 2001, Lorenzi & Gerhardinger 2001), with eventual blood-retinal barrier breakdown (Poulaki *et al.* 2002). The consequences of retinal microvascular cell apoptosis can account for various features of DR (Lorenzi & Gerhardinger 2001). It has been shown that prolonged exposure to hyperglycaemia leads to progressive dysfunction of the endothelium through a number of potential pathways (Barnett 1993, Giugliano *et al.* 1996, Stehouwer *et al.* 1997, Funatsu & Yamashita 2002). These biochemical mechanisms include sorbitol (polyol) pathway, nonenzymatic protein glycation (advanced glycation end products, AGEs), oxidative stress (generation of reactive oxygen species, free radicals, and impaired antioxidant mechanisms), protein kinase C beta (PKC- β) and

the renin-angiotensin system (RAS). These abnormal pathways may influence various vasoactive factors and cytokines to mediate functional and structural changes of DR (Candido & Allen 2002). For example AGEs cause increased oxidative stress and subsequent cell death. Furthermore, retinal ECs are much more susceptible to oxidative stress and increased vascular permeability compared to brain-derived ECs (Grammas & Rideen 2002).

To date, it is understood that the pathogenesis of DR does not only deal with the retinal vessels. In addition, multiple other cell types in the retina are affected early by diabetes (Lorenzi & Gerhardinger 2001).

Fig. 1. Schematic diagram of the pathogenesis of diabetic retinopathy. NO, nitric oxide; PGI2, prostacyclin; VEGF, vascular endothelial growth factor; TGFβ, transforming growth factor beta; AGEs, advanced glycation endproducts; PIGF, placenta growth factor; PEDF, pigment epithelium-derived factor (Cai & Boulton 2002).

3.2.3 Hemodynamic changes

Retina is metabolically an extremely active sheet of neural tissue with the highest oxygen consumption per weight when compared to any other human tissue (Frank 1995). It is remarkable that human retinal circulation is a closed vascular system. The blood supply of the inner layers of retina is derived from the central retinal artery (CRA), except

in 15% of individuals who also have a cilioretinal artery. The CRA branches on the surface of the optic nerve head (ONH) to supply four major quadrants of the retina. There are two main levels of capillary network in the retina (dual vascular supply). A delicate network of retinal vessels feeds the inner plexus at the level of the ganglion cell layer. The avascular outer retina depends on the extensively fenestrated capillary plexus of the choriocapillaries at the level of inner nuclear layer.

Retinal capillaries are most dense in the macula, except in the fovea where there is a 500 μm -diameter capillary free zone. The lumen diameter of retinal capillaries is 3.5-6 μm . The retinal capillary bed is nonfenestrated, i.e. there are tight junctions between endothelial cells (ECs) that form the inner blood-retinal barrier (BRB).

Pericyte pseudopodia encircle the retinal capillary and contain the main contractile machinery (Shepro & Morel 1993, Chakravarthy & Gardiner 1999). Pericytes provide vascular stability and control proliferation of ECs (Hammes *et al.* 2002). Both ECs and pericytes have the capacity to autoregulate permeability and perfusion and to fine-tune homeostasis at the microvascular level (Hirschi & D'Amore 1996). In retina, the pericyte-EC ratio is 1:1.

It is crucial to understand the retinal microcirculation since many late complications of DM stem from its damage. In experimental animals, high glucose concentration has been shown to result in a considerable increase in blood flow (Sullivan *et al.* 1990). Hemodynamic explanations for the development of microvascular complications have also been proposed in humans (Zatz & Brenner 1986). According to some studies, diabetes is associated with an early reduction in retinal blood flow (RBF) (Patel *et al.* 1992, Bursell *et al.* 1996, Clermont *et al.* 1997, Konno *et al.* 1996) followed by gradual increase in RBF as DR progresses to nonproliferative and more advanced levels (Patel *et al.* 1992, Clermont *et al.* 1997, Yoshida *et al.* 1983, Grunwald *et al.* 1992). To date, the mechanisms underlying this biphasic change in RBF are still not completely understood.

Increased RBF is known to cause increased shear stress (the frictional force generated by blood flow) in ECs (Kohner *et al.* 1993). Although retinal microcirculation is not under neurogenic control, retina possesses intrinsic autoregulatory capacity, i.e., the ability to maintain RBF reasonably constant in the face of changing perfusion pressure (Kohner *et al.* 1995). In addition, it is known that retinal resistance elements respond to local hypoxia and hypercapnia, and that in diabetes this mechanism is impaired (Fallon *et al.* 1987).

3.2.4 Blood rheologic, haematologic, immunologic and inflammatory abnormalities

Endothelium is a complex tissue possessing multiple synthetic functions, and anticoagulant activity (Risau 1995, Porta 1996, Calles-Escandon & Cipolla 2001). ECs function in the control of cell and nutrient trafficking, in the regulation of vasomotor tone, in the maintenance of blood fluidity (hemostasis), and in the angiogenesis (Aird 2003). The structure and function of ECs are differentially regulated in space and time (heterogeneity) (Aird 2003). Endothelium releases agents that mediate vasodilatation (endothelium-derived relaxing factors) such as prostacyclin (PGI₂), and nitric oxide (NO), and agents that mediate vasoconstriction (endothelium-derived contracting factors) such as angiotensin II and endothelin-1 (ET-1). In diabetes, vascular endothelium shows an impaired synthesis or action of vasodilators in humans (Johnstone *et al.* 1993), and increased vasoconstrictor release such as ET-1 (Yamauchi *et al.* 1990) resulting in an imbalance of vascular homeostasis.

Abnormal blood rheology (decreased red cell deformability, increased red cell and platelet adhesiveness and aggregation and increased plasma viscosity) (Bertram *et al.* 1992, Matsubara *et al.* 2000), and increased levels of prostacyclin, fibrinogen, von Willebrand factor, and plasmin activator have been reported and measured in diabetic patients (Papadaki *et al.* 1999).

The renin-angiotensin system (RAS) is activated in the setting of chronic hyperglycaemia (Anderson *et al.* 1993). Furthermore, local RAS is known to be involved in the regulation of blood flow and development of neovascularization in diabetic retina (Wilkinson-Berka *et al.* 2001, Kida *et al.* 2003). Serum total renin concentration may be increased in diabetic patients with active PDR (Mäkimattila *et al.* 1998), but it may also be elevated before the development of retinopathy (Kordonouri *et al.* 2000).

Furthermore, it has been suggested that immunologic mechanisms may play a role in the pathogenesis of DR via immune complex deposition (Andreani 1980). DR may partially be a low-grade inflammatory disease (Adamis 2002, Gardner *et al.* 2002). Emigration of circulating leukocytes into tissues (extravasation) is controlled by the expression of cell surface adhesion molecules: Endothelial leukocyte-adhesion molecule (ELAM-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) (Gearing *et al.* 1992). Adhesion molecules and cell surface glycoconjugates are the main mediators of interactions between circulating blood cells and retinal capillary ECs

(Ruggiero *et al.* 1997). Normally, the intact endothelium expresses undetectable or low levels of ELAM-1 and VCAM-1 (Aird 2003). Increased levels of ELAM-1, VCAM-1 and ICAM-1 have been reported in the diabetic patients (Calles-Escandon & Cipolla 2001).

3.2.5 Structural changes

Histologic studies of human eyes with DR (Engerman 1989) and experimental studies in dogs and rats with DR have revealed that the initial lesion is the loss of intramural capillary pericytes (Kador *et al.* 1988, Robinson *et al.* 1989). The loss of pericytes leads to microaneurysm (MA) formation. Retinal capillary closure is the result of occlusion by blood cells, such as (activated) leukocytes (Schröder *et al.* 1991, Stitt *et al.* 1995, Miyamoto *et al.* 1997 & 1998), induced by alterations in thrombogenicity of the endothelial surface (Forrester 1987, Merimee 1990). This process leads to retinal ischemia, which promotes angiogenesis, the formation of blood vessels by sprouting from pre-existing ones. Retinal neovascularization induced by the production or release of various angiogenic factors, such as vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin like growth factor-1 (IGF-1), placenta growth factor (PlGF), connective tissue growth factor (CTGF), hepatocyte growth factor (HGF), transforming growth factor alpha and beta (TGF- α , TGF- β), and angiopoietin-2 (Ang-2) can cause vitreous haemorrhage, and potentially permanent loss of vision (Boulton *et al.* 1997 & 1998, Cai & Boulton 2002, Witmer *et al.* 2003).

3.2.6 Development of diabetic macular oedema

Breakdown of the BRB is the most important pathophysiological factor involved in the pathogenesis of diabetic macular oedema (Klein *et al.* 1984a, Antcliff & Marshall 1999). Macular oedema is defined as retinal thickening resulting from accumulation of extracellular fluid in Henle's layer and in the inner nuclear layer of the retina from leaking capillaries and MAs (Tso 1980). Due to increased vascular permeability, lipoproteins accumulate in the outer plexiform layer. These clinically observed hard exudates within the outer retina are often associated with retinal damage which may be potentially sight threatening, especially if these lesions are situated beneath the centre of macula.

3.2.7 Other aspects

DR is not merely a vascular disease, since retinal function may change prior to the onset of clinically manifest vascular lesions (Ewing *et al.* 1998). Neurodegeneration of the retina is also a critical component of DR (Vadala *et al.* 2002, Barber 2003). Previous studies have revealed that contrast sensitivity (CS) may be significantly altered in diabetic patients with normal visual acuity in the early phases of DR (Khosla *et al.* 1991, di Leo *et al.* 1992, Hellstedt *et al.* 1997a) or even before occurrence of DR (Ghafour *et al.* 1982, di Leo *et al.* 1992, Banford *et al.* 1994). Scanning laser polarimetry has also revealed significant nerve fibre layer defect in the superior segment of retina in patients with type I diabetes without RP (Lopes de Faria *et al.* 2002).

3.2.8 Treatment

Prior to the era of laser treatment, hypophysectomy (Kingsley *et al.* 1983) and abortion (Beetham 1950, White 1974) were used as choices of treatment in diabetic patients with PDR. Photocoagulation, introduced by Professor G. Meyer-Schwickerath in the 1950s, revolutionized the treatment and prognosis of DR. Early instrumentation, based on solar beams, was replaced by xenon arc and argon laser photocoagulation in the 1970s. Starting from 1976, panretinal laser photocoagulation (PRP) was shown to be the effective treatment of PDR and the sight-threatening lesions of diabetic macular oedema (Diabetic Retinopathy Study Research Group 1976 & 1982, ETDRS report 1987, Ferris 1993). Laser photocoagulation destroys ischemic outer retinal tissue, leading to reduction of release of angiogenic factors, and improvement in oxygenation (normoxia) of the inner retina (Stefansson 1992). PRP may also decrease oxygen consumption of the outer retina due to loss of photoreceptors (Lahdenranta *et al.* 2001). Vitrectomy per se may be a sufficient treatment for PDR, offering also sight-restoring possibilities for complications of neovascularization, i.e., haemorrhage, tractional retinal detachment, and fibrovascular membrane formation (Diabetic Retinopathy Vitrectomy Study Research Group 1985, Smiddy *et al.* 1995). In some diabetic cases, peeling of the internal limiting membrane needs to be combined with vitrectomy to restore vision.

In the nearby future, pharmacologic treatment of DR will most likely advance (Fong 2002). It is possible that treatment with antioxidants such as vitamin E supplementation (Bursell *et al.* 1999), alpha tocopherol therapy (Jialal *et al.* 2002), protein kinase C (PKC) β inhibitors (LY333531) (Frank 2002), angiotensin converting enzyme (ACE) inhibitors (Cordonnier *et al.* 2001), integrin antagonists (Witmer *et al.* 2003) and somatostatin

analogues (Grant & Caballero 2002) may prevent the development and/or progression of DR. In addition, subconjunctival or intravitreal steroids may reduce diabetic macular oedema (Estafanous *et al.* 2000, Jonas & Sofker 2001). Anti-inflammatory drugs may also be beneficial in prevention of DR (Adamis 2002). Furthermore, gene therapy is promising for delivery of anti-angiogenic proteins; however, problems do remain in developing safe viral or non-viral vector (Witmer *et al.* 2003).

3.3. DIABETIC PREGNANCY

3.3.1 Historical review

Prior to the discovery of insulin by Frederick Banting, J.J.R. Macleod, James Collip, and Charles Best at the University of Toronto in 1921, life expectancy of the type I diabetic patient was short (Davidorf & Chambers 1993). In the literature before the year 1922, fewer than 100 successful pregnancies were reported in diabetic women, with a greater than 90% infant mortality rate and a 30% maternal mortality rate (Davidorf & Chambers 1993). Accordingly, only few young diabetic women lived to childbearing age (Gabbe 1993). To date, the reported incidence of maternal mortality of pregnant type I diabetic women has reduced to 0.5% (Cousins 1987, Leinonen *et al.* 2001). However, it is noteworthy that mortality of pregnant type I diabetic women is still 109 times greater than that of the general population and 3.4 times greater than that of the nonpregnant type I diabetic women when calculated in person-years (Leinonen *et al.* 2001).

3.3.2 Epidemiology

Type I DM affects approximately 0.2% to 0.4% of all pregnancies (Engelgau *et al.* 1995, von Kries *et al.* 1997). In Finland, in the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital about 1.4% of all pregnancies are diabetic, because diabetic pregnancies from nearby district hospitals are centralized in the university clinic. During the period from 1991 to 1995, it was shown that insulin-treated diabetes complicated 4.5/1000 births in Finland, majority of the cases being type I diabetes (Vääräsmäki 2001). Accordingly, the incidence of type I DM increased from 3.9/1000 to 5.0/1000, the prevalence being 2.9/1000 among pregnant women in Finland.

3.3.3 Classification of diabetes mellitus during pregnancy

The White classification, presented by Dr. Priscilla White (White 1949, White 1978), categorizes pregnant diabetic women according to the mode of therapy (diet/insulin), age at the onset of diabetes, duration of diabetes, and the degree of vascular disease/compromise at the onset of the current pregnancy. This classification proves still to be useful since progression of DR during pregnancy is associated with advanced class (McElvy *et al.* 2001).

A Pregnant women with gestational diabetes mellitus (GDM)

B Age at onset of diabetes over 20 years, or duration of diabetes less than 10 years, no vascular lesions

C Age at onset of diabetes 10-19 years or duration of diabetes 10-19 years, no vascular lesions

D Age at onset of diabetes less than 10 years or duration of diabetes over 20 years, and hypertension or background diabetic retinopathy is found

F Nephropathy

R Proliferative retinopathy

RF Renal disease and proliferative retinopathy

G Multiple failures in pregnancy

H Arteriosclerotic heart disease

T Pregnancy after renal transplantation

3.3.4 Insulin therapy

Experimental data suggest that insulin treatment could be an important factor in the pathogenesis of DR (De Juan *et al.* 2000). Because women with type I diabetes have an absolute deficiency of beta cells and insulin secretion, exogenous insulin is needed to maintain normal blood glucose levels. Insulins of the lowest immunogenicity (Menon *et al.* 1990) and human insulin are mostly recommended to be used in treating women with type I diabetes before and during their childbearing years (Knip 1988, Teramo *et al.* 1993). Recently, short-acting insulin analogue, insulin lispro, has been used successfully during pregnancy both in women with type I diabetes (Buchbinder *et al.* 2000, Persson *et al.* 2002, Loukovaara *et al.* 2003, Masson *et al.* 2003), and with GDM (Jovanovic *et al.* 1999).

3.3.5 Pre-conception care

The main therapeutic challenge as regards the treatment of type I diabetic patients is to achieve near-normoglycaemia with minimal risk of hypoglycaemia. During pregnancy, normoglycemia is the most important factor for successful outcome of pregnancy (DCCT 1996). Furthermore, normalisation of blood sugar values should already be achieved before conception since most foetal organogenesis is complete by the seventh week of gestation (Ylinen *et al.* 1984, Johnston 1985, Reece & Homko 2000, Suhonen *et al.* 2000).

Diabetic pregnancy is a high-risk obstetrical situation (Hawthorne *et al.* 1997, Hadden 1999); 30% of diabetic women with no observable DR, and 70% with BDR at the inception of pregnancy develop obstetric complications (Price *et al.* 1984). On the other hand, as regards the perinatal outcome, the pregnancies in 43% of the diabetic women with PDR had an unfavourable outcome compared with 13% of those with no RP or NPDR (Klein *et al.* 1988).

Pre-conception care reduces the incidence of foetal malformations and spontaneous abortions of pregnant diabetic women in specialist centres where intensive medical surveillance is paid to the metabolic, hemodynamic, and cardiovascular problems associated with pregnancy (Kitzmilller *et al.* 1996, Star & Carpenter 1998, Jovanovic 2000), the outcome approaching that of the nondiabetic population (Kitzmilller *et al.* 1996). In unselected population, however, the infants of women with type I diabetes have a 10-fold risk of congenital malformations (Casson *et al.* 1997). There seem to be no racial differences as foetal and maternal outcomes for Indo-Asian and Caucasian women with diabetes have been reported to be similar (Dunne *et al.* 2000).

3.4 DIABETIC RETINOPATHY DURING PREGNANCY

3.4.1 Natural course

The structural changes in retina are identical in both the pregnant and nonpregnant diabetic patient. Prospective studies have revealed that although up to 33% of diabetic women who have no DR immediately before or early in pregnancy develop some background RP changes during pregnancy, the DR is usually mild in degree, does not require intervention, and the course is often "waxing and waning" with regression

postpartum (Moloney & Drury 1982, Ohrt 1984, Serup 1986, Phelps *et al.* 1986, Klein *et al.* 1990, Chew *et al.* 1995, Axer-Siegel *et al.* 1996, Lapolla *et al.* 1998, Sheth 2002).

First of all, it is known that MAs without other components of DR have no apparent clinical importance except as being a marker of the development of DR (Ferris *et al.* 1999). During pregnancy, MAs and haemorrhages are the components of RP, which increase most commonly (Phelps *et al.* 1986). Furthermore, the total number of MAs present in the retina correlates with the risk of progression of DR (Kohner & Sleightholm 1986, Klein *et al.* 1989). Prospective study with fluorescein angiography (FAG) revealed that the number of MAs increase progressively during pregnancy in diabetic women with mild BDR, and regress postpartum, although not necessarily returning to preconception levels (Soubrane 1985). In another study, continuous turnover of MAs was evident during pregnancy with MA count increasing during pregnancy, being highest 3 months postpartum, the disappearance rate exceeding the formation rate 6 months postpartum (Hellstedt *et al.* 1997b).

Secondly, cotton wool spots (CWSs), infarcted areas of the nerve fibre layer of the retina, are associated with low fasting blood sugar (Moloney & Drury 1982) and rapidly achieved strict metabolic control (Brinchmann-Hansen *et al.* 1985, Laatikainen *et al.* 1987). CWSs develop in some type I diabetic women with BDR during advancing pregnancy. Postpartum, they usually regress unlike the lesions that are characteristic of the preproliferative stage of DR such as intraretinal microvascular abnormalities (IRMA) (Laatikainen *et al.* 1980). Lastly, pre-existing PDR can show progressive deterioration during pregnancy (Dibble *et al.* 1982, American Diabetes Association 2002).

3.4.2 Risk factors associated with the progression of diabetic retinopathy

Pregnancy per se increases the risk of DR progression (Klein *et al.* 1990, Jovanovic-Peterson & Peterson 1991, DCCT 2000). Duration of diabetes before conception is the prime risk factor for the presence, severity, and progression of DR during pregnancy (Horvat *et al.* 1980, Laatikainen *et al.* 1980, Moloney & Drury 1982, Sinclair *et al.* 1985, Sunness 1988, Ayed *et al.* 1992, Axer-Siegel *et al.* 1996, Lauszus *et al.* 2000, Temple *et al.* 2001). Other crucial factors for the progression of DR during pregnancy are chronic and pregnancy-induced hypertension (PIH) (Klein *et al.* 1990, Rosenn *et al.* 1992, Lövestam-Adrian *et al.* 1997), hyperglycaemia (Klein *et al.* 1990, Chew *et al.* 1995, Larinkari *et al.* 1982), rapid normalization of blood glucose levels (Phelps *et al.* 1986,

Laatikainen *et al.* 1987, Lövestam-Adrian *et al.* 1997), poor glycemic control of diabetes at conception (Chew *et al.* 1995, Lövestam-Adrian *et al.* 1997, Lauszus *et al.* 2000), and stage of DR at baseline (Sinclair *et al.* 1985, Jovanovic-Peterson & Peterson 1991, Rosenn *et al.* 1992, Davidorf & Chambers 1993, Eter & Spitznas 1997, Chew *et al.* 1995, Axer-Siegel *et al.* 1996, Lapolla *et al.* 1998, Temple *et al.* 2001).

3.4.3 Long-term consequences of pregnancy on diabetic retinopathy

Pregnant women with type I DM do not seem to have an increased risk of DR in the long-term (Hemachandra *et al.* 1995). The number of pregnancies is not considered to increase the risk of the progression of DR (Klein & Klein 1984). The prevalence of DR was lower in multiparous women (≥ 2 pregnancies) (34%) compared with women who had only one (45%) or no (48%) pregnancies (Chaturvedi *et al.* 1995). Accordingly, the rate of PDR was 8% in multiparous, 7% in uniparous, and 16% in nulliparous women. In other previous studies, progression of DR occurred less often in parous than in nulliparous women (Kaaja *et al.* 1996, Vääräsmäki *et al.* 2002). One explanation may be that the diabetic women who become pregnant and give birth belong to those who are most motivated as regards the treatment of diabetes as a whole (Kaaja *et al.* 1996).

3.4.4 Management

The indications for treatment and the results of laser photocoagulation seem to be as efficacious in pregnant as nonpregnant diabetic women (Hercules *et al.* 1980, Frank 1986, Sunness 1988). Diabetic women with completely regressed PDR, either spontaneous or laser-induced, are very unlikely to have further proliferation during pregnancy (Cassar *et al.* 1978, Moloney & Drury 1982).

Some type I diabetic women develop severe macular oedema associated with preproliferative or PDR (Agardh 2002), proteinuria and mild hypertension during pregnancy (Sinclair *et al.* 1984). These women may require laser photocoagulation to treat macular changes, but laser can also worsen oedema in those with a compromised macular capillary circulation (ischemic capillaropathy). Macular oedema may regress spontaneously after delivery in some diabetic women. However, it may persist and cause long-term or potentially permanent visual loss in others (Sinclair *et al.* 1984, Conway *et al.* 1991). Salt-restriction diets and diuretics have been used to treat macular oedema in diabetic women during pregnancy, but with limited success (Cassar *et al.* 1978).

Some type I diabetic women may present with acute optic disc oedema (pseudopapilledema) during pregnancy. This pseudopapilledema is considered a relatively benign manifestation of DM, being unrelated to the level of baseline DR, not adversely affected by pregnancy, and not requiring treatment (Pavan *et al.* 1980, Ward *et al.* 1984).

3.4.5 Recommendations

All type I diabetic women are recommended to attend ophthalmic examination during early pregnancy (Eter & Spitznas 1997, Ferris *et al.* 1999, DCCT 2000, American Diabetes Association 2000, Jovanovic 2000, Dinn *et al.* 2003). Those diabetic women who have DR diagnosed before or during early pregnancy, those who have no DR but have particularly poor glycemic control and those with nephropathy and hypertension (Soubrane & Coscas 1998) need an intensive ophthalmic surveillance (comprehensive eye examination in the first trimester with a close follow-up throughout pregnancy and the first year postpartum) (Cundy 2001). As regards White's classification, classes B and C are not recommended to attend eye examinations every trimester, but rigorous follow-up is warranted in classes D-R (Puza & Malee 1996).

Generally, women with type I DM should be encouraged to plan pregnancies early in life (Johnston 1980, Lauszus *et al.* 2000). Normalization of maternal blood glucose values is necessary during pregnancy for foetal well-being (Ylinen *et al.* 1984, Johnston 1985, Reece & Homko 2000, Suhonen *et al.* 2000), but the blood glucose values should be normalized slowly (over 6-8 months) before conception (Jovanovic-Peterson & Peterson 1991, American Diabetes Association 2002). During pregnancy, tight glycemic control is recommended to avoid progression of DR (Lauszus *et al.* 2000).

Colour fundus photography and laser treatment are safe during pregnancy (Sunness 1988). Fluorescein angiography (FAG) can usually be avoided (Elman *et al.* 1990), although it has been used also during pregnancy in diabetic women (Soubrane *et al.* 1985). Although teratogenic effects on the foetus have not been identified, FAG is nowadays not recommended to be used during pregnancy, unless absolutely necessary. Indocyanine green (ICG) is generally not used for retinal angiography during diabetic pregnancy (Fineman *et al.* 2001), despite of its use as a chromodiagnostic agent in the evaluation of hemodynamic changes such as evaluation of hepatic function and cardiac output during pregnancy (Robson *et al.* 1989 & 1990).

Laser photocoagulation should be performed when needed according to the recommendations of the Diabetic Retinopathy Study (DRS) and Early Treatment Diabetic Retinopathy Study (ETDRS), despite the possibility that DR may regress spontaneously after delivery (Moloney & Drury 1982, Ohrt 1984, Serup 1994). Diabetic women with preproliferative or PDR are recommended to deliver by elective caesarean section by obstetric indications (Sunness 1988, Rosenn & Miodovnik 2000). Valsalva manoeuvre, vascular pressure generated during the second stage of labour, is known to represent a postcapillary process, being unlikely to cause haemorrhage from retinal neovascularization (Elman *et al.* 1990). Recently, there has been, however, discussion on whether natural delivery should be avoided in all type I diabetic women to avoid the additional pressure-overload during delivery (Schannwell *et al.* 2003).

Postpartum, the role of an obstetrician/ophthalmologist must switch to a preventive mode to formulate a reproductive health plan for women with type I DM. Contraception, planning of future pregnancies, and various long-term life-style changes are known to be essential in the prevention of future diabetic complications (Kjos 2000).

3.5 MECHANISMS OF PROGRESSION OF DIABETIC RETINOPATHY DURING PREGNANCY

The pathogenetic mechanisms of DR progression during pregnancy are not fully understood. Pathogenesis of DR during pregnancy is probably multifactorial. During pregnancy, physiological changes occur in the cardiovascular, hormonal, metabolic, haematologic, and immunologic systems (Thornburg *et al.* 2000). By some of these mechanisms, DR can deteriorate during pregnancy even in those diabetic women with good metabolic control and minimal DR (Soubrane *et al.* 1985, Hellstedt *et al.* 1996).

3.5.1 Cardiovascular and hemodynamic factors

Maternal physiology is associated with profound cardiovascular alterations during pregnancy (Robson *et al.* 1989, Duvekot & Peeters 1994, Thornburg *et al.* 2000). Generalized vasodilatation of the vascular system (Friedman *et al.* 1991) occurs early in pregnancy, prior to fully complete placentation (Chapman *et al.* 1998) with an increase in blood flow, cardiac output and circulating plasma volume (Gant *et al.* 1973). During pregnancy, cardiac output is gradually increased by 40% by term and plasma volume by 20%, and peripheral resistance is decreased (Sunness 1988). All of these changes cause hyperdynamic blood circulation.

Previous studies have demonstrated endothelial cell dysfunction in diabetes, suggesting that it is a main mechanism underlying the complications associated with diabetes (Johnstone *et al.* 1994, McNally *et al.* 1994). Recently, however, a report provided evidence that pregnant women with well-controlled type I diabetes might also have normal endothelial function in subcutaneous small arteries (Ang *et al.* 2002). On the other hand, once structural changes have occurred in the retinal vasculature (basement membrane thickening, loss of pericyte and smooth muscle cells), the capillary bed becomes unresponsive and autoregulative capacity gradually fails (Ciulla *et al.* 2002).

Impaired vascular reactivity during the second trimester of pregnancy has been associated with maternal type I diabetes (Savvidou *et al.* 2002). Increased ocular blood flow has been suggested to play a major role in the pathophysiology of DR (Schmetterer & Wolzt 1999), as well as increased RBF in patients with insulin-dependent diabetes mellitus, even before the onset of DR (Grunwald *et al.* 1996). Increased RBF has also been associated with increasing severity of DR during pregnancy (Chen *et al.* 1994) with the lack of change of RBF in normal pregnancy. In that study a 14% to 19% increase in RBF was found in those diabetic women with progression of DR during pregnancy. In diabetes, retinal autoregulation has been found to be impaired during pregnancy (Hellstedt 1997).

In addition, long-lasting vasoconstrictor ET-1 produced by ECs has been increased during diabetic pregnancy, which may further cause EC damage (Wolff *et al.* 1997, Best *et al.* 1999). The plasma values of aldosterone, renin, angiotensinogen, and angiotensin II are increased during pregnancy (Immonen 1983). The tissues of the eye express the components of the renin-angiotensin-system (RAS), and local RAS is activated in the eyes of diabetic patients with retinopathy (Williams 1998, Wilkinson-Berka *et al.* 2001, Strain & Chaturvedi 2002). It is a less studied subject whether RAS is altered in diabetic women with or without retinopathy during pregnancy.

The eventual return of pregnancy-induced hemodynamic changes to normal nonpregnant values is a gradual process postpartum. Changes are still detectable 6 to 8 weeks after delivery (Taylor & Lind 1979).

3.5.2 Hormonal and biochemical factors

During pregnancy, hormonal milieu alters with placenta playing an important role in synthesis of various hormones (Desoye & Shafrir 1996, Reis *et al.* 2002). Placental growth hormone (PGH) is known to replace progressively pituitary growth hormone in the maternal circulation from midgestation (Frankenne *et al.* 1988). Placental hormones have been investigated as biochemical markers of gestational diseases (Reis *et al.* 2002), but due to their abundance in the maternal circulation they could be useful markers also in the study of type I diabetes. During pregnancy, the values of progesterone and human placental lactogen or PGH have been increased in patients with PDR (Larinkari *et al.* 1982).

PGH is also known to be a major regulator of maternal IGF-1 (Caufriez *et al.* 1990 & 1993). A gross elevation of IGF-1 has been shown to occur in diabetic adults with rapidly progressing PDR (Merimee *et al.* 1983). In addition, IGF-1 has been involved in the worsening of DR during puberty and pregnancy (Bhaumick *et al.* 1986, Klein *et al.* 1990b, Chantelau 1998, Lauszus *et al.* 2003).

Accordingly, various other growth factors, hormones, and intraocular inflammatory mediators such as cytokines can alter vascular permeability in retinal capillaries. Growth factors involved in angiogenesis are among others fibroblast growth factor (FGF), TGF- α , TGF- β , tumor necrosis factor α (TNF- α) and VEGF (Forrester *et al.* 1993, Aiello *et al.* 1994, Archer 1999). Especially, VEGF that was isolated in the late 1980s (Bonn 1996), is a pluripotential angiogenic factor that might play a major role in the proliferation and migration of ECs and neovascularization (Ferrara 1995). VEGF, formerly known as vascular permeability factor (VPF), is expressed by vascular, neuronal and glial cells (Stitt *et al.* 1998). VEGF levels are shown to be elevated also in the vitreous of patients with preretinal neovascularization (Aiello 1997).

3.5.3 Metabolic and immunologic factors

During pregnancy, the total metabolism of the mother is increased due to foetal demands, as well as extra work performed by the cardiovascular, respiratory and other systems. One of the main mechanisms of DR progression during pregnancy is thought to be metabolic. Good glycemic control is the cornerstone for both maternal and foetal well-being during pregnancy. Worsening of DR has been correlated with the degree of improvement of glycemic control obtained with the institution of intensive therapy

performed before and during pregnancy (Phelps *et al.* 1986, Laatikainen *et al.* 1987, Chew *et al.* 1995, Lövestam-Adrian *et al.* 1997). Some other metabolic manifestations of diabetes, such as altered fatty-acid or protein metabolism or absolute insulin deficiency, may also cause diabetic complications (Nathan 1996).

Insulin is a key regulator of metabolism and significant changes in insulin sensitivity have been documented during pregnancy. In the early diabetic pregnancy, insulin requirements are usually decreased, whereas by the second half of pregnancy they usually increase (Atkin *et al.* 1996, Catalano *et al.* 1998). This insulin resistance may have an impact even on development or progression of DR during pregnancy.

In addition, changes in the immune system may predispose to retinal microcirculatory changes in diabetic women. Levels of circulating immune complexes have been shown to be increased in patients with PDR. However, both cell-mediated and humoral immune system responses are relatively suppressed during pregnancy, which means that the changes in the immune system are unlikely to play a major role in contributing to progression of DR during pregnancy (Davidorf & Chambers 1993).

3.6 METHODS FOR MEASUREMENT OF RETINAL BLOOD FLOW AND TOPOGRAPHY

Ophthalmoscopy and fundus photography provide a lot of information of the retina's anatomy and vasculature. But much more sophisticated methods are needed for the study of retinal blood flow and topography.

3.6.1 Measurement of retinal blood flow

The psychophysical blue field entoptic simulation technique (BFS) has been used to study the velocity of leukocytes flowing in perimacular capillaries both in nonpregnant and pregnant diabetic patients. Fallon *et al.* (1986) found increased velocities in nonpregnant patients with BDR, and Sinclair (1991) reported increased velocities in diabetic patients, concluding that capillary obstruction may focally occur within retina, being associated with vasodilatation in the adjacent microvasculature. Hellstedt *et al.* (1996) found leukocyte velocities to be generally increased in pregnant diabetic women compared to nondiabetic women, although the velocity increased towards term also in nondiabetic women.

In addition, scanning laser ophthalmoscopy (SLO) has been used for the measurement of macular capillary particle velocities. SLO studies have shown reduced perifoveal flow velocities in nonpregnant diabetic patients when compared with nondiabetic subjects (Arend *et al.* 1991, Arend *et al.* 1994, Wolf *et al.* 1991). The differences between SLO and blue field entoptic simulation technique may be related to the different vascular measuring sites and/or measurements of different phenomena. Unlike the BFS that measures perimacular capillary leukocyte velocity, the SLO quantifies the velocity of erythrocyte aggregates in the capillary lumen of the para- and perifoveal network (Arend *et al.* 1995). In addition, since velocity measurements with SLO require intravenous fluorescein, it is not recommended to be used in studies with pregnant women.

Confocal scanning laser Doppler flowmetry has not widely been used in the measurement of retinal capillary blood flow in DR (Cuyper *et al.* 2000). Its main implementation has been in the investigation of ONH in different entities of glaucoma (Chung *et al.* 1999b). Until now, previous studies on RBF in pregnant diabetic women have revealed both increased (Chen *et al.* 1994) and decreased (Schocket *et al.* 1999) volumetric blood flow.

Additionally, colour Doppler imaging (CDI) has been used to measure blood flow velocity in the ophthalmic artery and central retinal artery (CRA) in patients with DR. In a recent study, reduced blood flow velocity was found in the CRA of nonpregnant diabetic patients, with even further decrease as RP progressed (MacKinnon *et al.* 2000).

3.6.2 Measurement of retinal topography

The use of confocal laser technology offers quantitative, multispectral and 3-dimensional retinal imaging (Sharp *et al.* 1999). To date, commercial SLOs are available with a choice of wavelengths; for example the confocal scanning laser tomography (Heidelberg Retinal Tomography) uses visible diode laser of 670 nm wavelength. 3-dimensional imaging enables to build a topographic representation of the retina. The variability of tomographic measurements is, however, relatively high due to numerous factors: movement of the eye during measurement, changes in the position of the study subject's head, and cataract (Zambarakji *et al.* 1998). Despite of that the application of SLO technique in the analysis of diabetic retina/macula is important. However, to date these new imaging techniques have not yet been established as routine diagnostic means for study of DR.

Furthermore, optical coherence tomography (OCT) has been used to assess quantitatively retinal thickness in diabetic patients with and without clinically significant macular oedema (CSMO) (Yang *et al.* 2001, Sanchez-Tocino *et al.* 2002). In addition, the retinal thickness analyser (RTA) has been used in the investigation of retinal changes. Both OCT and RTA have been shown to be reliable measurements of foveal thickness quantitatively in normal subjects (Konno *et al.* 2001).

4. AIMS OF THE STUDY

The present studies were undertaken to investigate characteristics and pathogenetic mechanisms of progression of DR in women with type I diabetes mellitus during pregnancy. The specific aims were:

1. To evaluate macular/retinal blood flow in women with type I diabetes and nondiabetic controls, and to find out whether the changes in macular/retinal blood flow are associated with the progression of DR during pregnancy and postpartum **(I, II)**.
2. To evaluate whether contrast sensitivity loss is coupled with topographic change in the central macula in diabetic pregnancy **(III)**.
3. To evaluate the role of various systemic vasoactive mediators in the development and/or progression of DR during pregnancy and postpartum **(IV)**.
4. To evaluate the role of various systemic angiopoietic factors in the development and/or progression of DR during pregnancy and postpartum **(V)**.

5. SUBJECTS AND METHODS

5.1 STUDY DESIGN

This prospective study was conducted between November 1998 and January 2002 in the Departments of Ophthalmology and Obstetrics and Gynaecology, Helsinki University Central Hospital. The study protocol was accepted by the Ethics Committee of Helsinki University Hospital, Department of Obstetrics and Gynaecology. The tenets of the Helsinki Declaration were followed. Informed consent was obtained from all subjects.

5.1.1 Diabetic and nondiabetic women

Altogether 72 women with insulin-dependent diabetes could be enrolled to this prospective study at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, as soon as their pregnancy was diagnosed (usually between 5 and 10 weeks of gestation). Of these, the first 57 consecutively studied women (from November 1998 to May 2001) were included in Studies I, II and III (Fig 1). Additional 15 diabetic women (by the end of study in January 2002) could be investigated for Studies IV and V. In addition, 11 nonpregnant diabetic women were examined as prepregnancy planning women, and they were used in the final analysis in Study II. All diabetic women were referred to the Department of Ophthalmology, where they were seen for clinical evaluation and scientific studies once in each trimester, at the 12th to 14th week, at the 24th to 26th, and 34th to 36th week of gestation, and at 3 and 6 months postpartum.

Fifteen nondiabetic pregnant women attending the Department of Obstetrics and Gynaecology for monitoring of normal pregnancy could be recruited to participate as controls in the study. They were studied at the 12th to 14th week, and 34th to 36th week of gestation, and at 3 months postpartum.

Nine diabetic women were excluded either because of obstetric complications or coexisting eye disease: one because of spontaneous abortion, one for an induced abortion due to a high glycosylated haemoglobin level (12-13%), six for preterm delivery, and one for retinitis pigmentosa. Four control women were unable to attend all eye examinations because of obstetric complications: early foetal loss, preeclampsia, preterm uterine contractions, and preterm delivery.

Figure 1 shows the five sub-studies and the diabetic women included in each of them. Eleven nondiabetic women served as controls in Studies I, II, and III. In Study IV, eight nondiabetic women could be used as controls, and in Study V nine women served as controls.

5.1.2 Clinical data collection

Diabetic women were examined at the out-patient clinic of the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital according to recommendations given in Finland in 1993 (Teramo *et al.* 1993) as soon as pregnancy was verified in maternity welfare clinics (usually before the tenth week of gestation). The severity of DM was classified according to White (White 1978). A diabetologist-internist, an obstetrician, and a special nurse belonged to the care team.

The clinical data on age of the study subject, age at the onset of diabetes, duration of diabetes, pre-pregnancy planning, pre-pregnancy BMI (kg/m²), weight gain during pregnancy, other diseases, parity (nulliparous/parous), smoking, systolic and diastolic blood pressure (mmHg) in each trimester, proteinuria, mean HbA1c in each trimester, number of hypoglycemic events measured in 24-h glucose profile and reported in each trimester, dosage of daily insulin in each trimester, type of insulin used, and previous children with malformations or macrosomia were recorded on a computer data base. The information about index pregnancy and the infant was also collected into the database.

5.2 METHODS

5.2.1 Measurement of serum glycosylated haemoglobin concentration

The concentration of glycosylated haemoglobin (HbA1c) was used as an index of glycemic control. Serum glycosylated HbA1c concentrations were measured by ion-exchange high-performance liquid chromatography (HPLC) (Diamat; Bio-Rad Laboratories, Hercules, CA, USA). HbA1c concentration was measured every two weeks throughout pregnancy in the laboratory of the Department of Obstetrics and Gynaecology. Four values of HbA1c were used in the study: the mean values of all HbA1c measurements taken during the first, second and third trimester, as well as the mean value for the whole pregnancy. HbA1c concentration was also measured in nonpregnant diabetic women who were participating in prepregnancy planning, but it was not measured in the nondiabetic controls. During each visit, the actual blood glucose was also measured with blood glucose meter from fingertip in the diabetic women (Glucometer Elite, Bayer Diagnostics Manufacturing Ltd., Kyoto Dalichi Kagaku Co., Ltd, Japan).

In addition, all diabetic women used reflectance meters at home for blood glucose monitoring throughout pregnancy and postpartum.

5.2.2 Measurement of blood pressure

Blood pressure (BP) was measured after a 15-minute rest with the subject in a sitting position. The measurements were carried out as a part of the routine obstetric clinical follow-up by midwives and nurses with long clinical experience. Preeclampsia was defined as blood pressure $\geq 140/90$ mmHg with proteinuria according to a dipstick test or $\geq 0.3\text{g}/24$ h after 20 weeks of gestation (Gifford *et al.* 1990). Pregnancy induced hypertension (PIH) was defined as blood pressure $\geq 140/90$ mmHg without proteinuria, and chronic hypertension was defined as hypertension diagnosed before pregnancy or before 20 weeks of gestation (Gifford *et al.* 1990).

5.2.3 Ophthalmic examination

Complete ophthalmic examination was performed in each trimester and 3 and 6 months postpartum for diabetic women, and in the first and third trimester and 3 months postpartum for the nondiabetic controls. It was also performed in prepregnancy planning period (altogether 11 diabetic women). Diabetic women who complained of visual symptoms were examined more frequently when necessary.

The ophthalmic examination included measurement of visual acuity on a Snellen chart, measurement of intraocular pressure (IOP) with Goldmann applanation tonometer, biomicroscopic examination of the anterior segment and indirect ophthalmoscopy through dilated pupil with a 60 and/or 90 dioptre (D) Volk lens.

5.2.4 Fundus photography

Colour fundus photography of both eyes was performed through dilated pupils (by the use of 2 drops of tropicamide, 5mg/mL) by a trained operator using a retinal camera (TRC 50IA, Topcon Corp., Tokyo, Japan; Elitecrome 100 film; Eastman Kodak, Rochester, NY, USA). The severity of DR was assessed by means of two 50 degree colour slides, one centred at the macula and the other at the ONH. The photographs taken during pregnancy were compared with those taken before pregnancy, if previous photographs were available. Ocular history, including laser photocoagulation, was checked from the hospital records. Patients who showed significant deterioration in DR underwent FAG 1 to 3 months postpartum.

The retinal photographs were evaluated by a retinal specialist, who was masked to all clinical information. Retinopathy was graded by using a modification of the ETDRS grading system, with ETDRS standard pictures serving for grading of the severity of retinopathy (ETDRS Study Group, 1991). For each eye, the maximum grade of DR lesions was determined to produce an overall severity level for that eye (RP level). Retinal findings were classified into the following groups: (1) no retinopathy (RP level 10), (2) very mild retinopathy (RP level 20), (3) mild retinopathy (RP level 35), (4) moderate retinopathy (RP level 43), (5) moderate retinopathy, more extensive IRMAs, (RP level 47), (6) severe NPDR (RP level 53), and (7) PDR (RP level >53). Retinopathy levels from both eyes were combined to give the final score of DR severity for each patient (scale 1 – 11), where a score of one means no retinopathy, and a score of eleven

represents PDR (DCCT Study 1993). In addition, the number of MAs was counted in each fundus image.

5.2.5 Measurement of retinal blood flow I: Blue field entoptic simulation test (BFS-2000)

Perifoveal leukocyte velocities were measured by a blue-field entoptic simulation technique (BFS-2000, Oculix, Inc., Berwyn, PA, USA) using a method described previously (Loebl & Riva 1978, Riva & Petrig 1980). In BFS technique, the study subject matches the velocity and density of simulated leukocytes seen on a computer screen to the velocity and density of her own leukocytes seen entoptically in the perifoveal capillaries visualized by looking into a blue 430-nm background. Both eyes were examined during each follow-up time in diabetic and nondiabetic women, with the right eye examined first. Mean leukocyte velocity from both eyes was calculated for each session. Only those diabetic and nondiabetic women who were able to match the velocity of leukocytes with an accuracy greater than 80% both in a preliminary screening procedure and a proper blue-field entoptic simulation were included in the final statistical analysis.

5.2.6 Measurement of retinal blood flow II: Confocal scanning laser Doppler flowmetry (HRF)

Measurement of retinal blood flow (RBF) was performed with a confocal scanning laser Doppler flowmetry (Heidelberg Retinal Flowmetry [HRF], Heidelberg Engineering GmbH, Heidelberg Germany) that maps blood flow within the fundus and produces blood flow readings in arbitrary units (AU). The principles of this apparatus have been described in detail elsewhere (Michelson *et al.* 1996). The scanned area was a rectangle ($20^{\circ} \times 2.5^{\circ}$) composed of 64 horizontal lines of 256 points. Images centred below the fovea were obtained from each eye after bilateral pupillary dilatation with tropicamide. Three or more repeated images were obtained from each eye.

5.2.6.1. Small square analysis

Images were analysed by placing a 10 x 10-pixel square on an area of interest free of motion artifacts and major vessels. The mean flow value in the 10 x 10-pixel square was recorded, and the mean of three such recordings from three separate locations of the

perfusion map was calculated. The position of the three squares together with the outline of major blood vessels, was drawn on a transparent overlay placed on the computer screen to allow the same areas to be measured during follow-up. The squares were placed in a nonparallel line on the perfusion map to avoid the possible coinciding motion artifacts in the follow-up images. The distance from fovea was measured to make sure that the measurements were not made from the foveal avascular area.

5.2.6.2 Pointwise analysis

The same images were also analysed by pointwise analysis. This technique has been described in detail previously (Harris *et al.* 1997, Kagemann *et al.* 1998, Chung *et al.* 1999b, Jonescu-Cuypers *et al.* 2001). A single pixel-sized cursor was swept across the same areas that were analysed with the 10 x10-pixel squares. One hundred pixels per area were recorded in three different locations corresponding the areas investigated in the small square analysis. The 300 flow values were entered into a log file. The log files were sorted by flow, and different percentiles (25th, 50th, 75th, and 90th) of the set of individual pixel flow values were counted. The percentage of pixels with a flow of zero was calculated. The photodetector sensitivity (DC) from all the analysed pixels was measured. Pixels with DC values <70 or >200 were excluded.

5.2.7 Measurement of contrast sensitivity (CS)

CS was measured with Vistech 6500 Contrast Test System, Form 00984 (Vistech Consultants, Inc., Dayton, Ohio, USA), sine wave gratings (1.5, 3.0, 6.0, 12.0, and 18 cycles per degree, cpd). This measurement was performed in each eye separately with the best corrected vision. Illumination was constant (within the range of 103 to 240 cd/m² with the Vistech light meter), and the viewing distance was 3 m.

5.2.8 Measurement of macular topography (HRT)

Macular topography was analysed with the HRT (Heidelberg Retinal Tomography, Heidelberg Engineering GmbH, Heidelberg Germany, software version 2.01). The reproducibility of results and technical details of this instrument have been described in detail elsewhere (Menenez *et al.* 1995, Zambarakji *et al.* 1998). Briefly, the camera control panel is coupled to a computer system with a high-resolution monitor. The 670-nm wavelength diode laser is used to image the macula and to scan the retina point by

point. A series of 32 confocal images, parallel images taken in a plane perpendicular to the optical axis, are recorded in 1.6 s. The computer software adjusts for small eye-movements, aligns the 32 images, and then compiles the images into a three-dimensional image. All HRT scans were taken by the same operator. All eyes were dilated with tropicamide. The refractive error and keratometry values of the eyes were entered into the database before image acquisition. Keratometry values were used for correction of magnification errors. The patients were positioned in a chin-rest position and were asked to fixate on a fixation object within one meter in front of them. All scans were centred on the fovea. A minimum of three 20 x 20-degree field scans approved by the internal quality program of the software were obtained from both eyes at each examination. The scan depth was kept constant during repeated HRT image acquisition. The scan of best-quality, judged by the details seen on the monitor, of the three available scans at each time-point, was chosen. For stereometric analysis, 1.0-, 1.5-, 2.0-, and 3.0-mm diameter computer-generated circles were centred at the fovea by means of the circle-draw facility. The reference plane was adjusted to the lowest point in the height variation of the contour line at each examination. The volume above the reference plane (VARP) was calculated by computer software.

5.2.9 Laboratory methods

5.2.9.1 Sample collection

Blood samples of subjects in a sitting position were collected from the antecubital vein into chilled tubes by a trained nurse through careful venepuncture (avoiding haemolysis). The samples were centrifuged at 7000 rpm for 10 minutes at 4°C, immediately frozen and stored at -70°C until assayed.

5.2.9.2 Systemic vasoactive hormones

Radioimmunoassay (RIA) was used for measurements of plasma renin activity (PRA) (Dunn & Espiner 1976), angiotensin II (Ang II) (Nicholls & Espiner 1976), aldosterone (Lun *et al.* 1983), atrial (A-type) natriuretic peptide (ANP) (Yandle *et al.* 1986), brain (B-type) natriuretic peptide (BNP) (Yandle *et al.* 1993), C-type natriuretic peptide (CNP) (Hunt *et al.* 1994), and adrenomedullin (AM) (Lewis *et al.* 1998). Samples were transported on dry ice by World Courier Service to the Endocrine Laboratory in

Christchurch Hospital, New-Zealand. All samples from each study subject were measured in a single assay to avoid interassay variability. Intra-assay coefficients of variation were between 5 and 9%.

5.2.9.3 Systemic angiopoietic factors

Plasma angiopoietin-1 (Ang-1) and -2 (Ang-2) concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) (Regeneron Pharmaceuticals, Inc., Tarrytown, NY, USA). Samples to Regeneron Pharmaceuticals, Inc., Tarrytown, NY, USA were transported on dry ice by World Courier Service.

Plasma vascular endothelial growth factor (hVEGF-A) and its total soluble receptor (sVEGFR-1) were quantified by ELISA according to the manufacturer's instructions (Quantikine recombinant human (rh) VEGF, R&D Systems Europe Ltd, Abingdon, UK and RELIATech GmbH, Braunschweig, Germany) (Hornig *et al.* 1999, Vuorela *et al.* 2000).

Plasma hVEGF-A concentrations were measured at 3 months postpartum in diabetic and nondiabetic women. Its total soluble receptor (sVEGFR-1) concentrations were measured in both groups during the first and third trimester, and at 3 months postpartum.

5.3 STATISTICAL METHODS AND DATA ANALYSIS

Statistical analyses were performed using the following software BMDP Statistical software (version 7.0 for Windows, BMDP, Los Angeles, CA, USA) in Studies I and II, and SPSS Statistical software (version 8.0 for Windows, SPSS Inc., Chicago, IL, USA) in Study III, VI, and SPSS Statistical software (version 9.0 for Windows, SPSS Inc., Chicago, IL, USA) in Studies IV, V. Normality tests were carried out on the data, and differences were considered statistically significant when $p < 0.05$.

Repeated measures (ANOVA) was performed to study temporal changes in retinal capillary blood flow during pregnancy and postpartum and differences between pregnant diabetic and nondiabetic women (I, II), to compare temporal changes in CS and macular topography during pregnancy and postpartum within the groups of diabetic and nondiabetic women, as well as between these groups (III), and to compare temporal changes in vasoactive hormones (PRA, Ang II, aldosterone, ANP, BNP, CNP, AM)

during pregnancy and postpartum between the groups of diabetic and nondiabetic women (IV), and to compare temporal changes in angiopoietic cytokines (Ang-1 and -2, sVEGFR-1) during pregnancy and postpartum between the groups of diabetic and nondiabetic women (V). Skewness of Ang-1 and Ang-2 was corrected by logarithmic transformation (V).

For the comparison of pointwise analysis results, multivariate ANOVA was performed (I). One-way ANOVA with Bonferroni correction was used in the comparison of macular capillary blood flow values between pregnant diabetic women and non-pregnant diabetic controls (I). Frequencies of RP level were calculated with Chi-Square Test between the groups throughout pregnancy and postpartum (III). Mann-Whitney U-Test was performed to compare groups (hVEGF-A) (V).

Nonparametric Spearman's rank correlation coefficient was used for correlation analyses (III, IV, V). Multivariate logistic regression analysis was performed with the enter procedure (III, IV, V).

6. RESULTS

6.1 PERIMACULAR MICROCIRCULATORY FLOW VELOCITY MEASURED WITH BLUE-FIELD ENTOTIC SIMULATION TEST (I)

6.1.1 Baseline retinopathy level and progression of diabetic retinopathy

Data from 46 out of 57 diabetic women were analysed in this sub-study. Twenty-nine (63%) of the 46 diabetic women had no or minimal DR at the first trimester visit (DCCT score ≤ 3), while 17 (37%) had more advanced DR. Of these women, four had a DCCT score of 4, seven a score of 5, three a score of 6, two a score of 7, and one a score of 8. By the third month postpartum, 20 diabetic women (44%) had a DCCT score ≤ 3 and 25 (56%) > 3 . One was excluded due to bad quality of fundus photographs.

Progression of DR by one score unit occurred in five women, and progression of more than one unit occurred in ten women. Two diabetic women were treated by undergoing panphotocoagulation before the third trimester examination. Focal laser treatment for small areas of neovascularization was given to four diabetic women (three months postpartum in two cases and six months postpartum in two cases). Macular capillary blood flow velocity did not differ between laser-treated and non-laser-treated diabetic women ($p=0.69$).

6.1.2 Macular blood flow and the level of diabetic retinopathy

Macular capillary blood flow velocity was higher in diabetic than in nondiabetic women during pregnancy and postpartum. In diabetic women, the macular capillary blood flow velocity was 0.94 ± 0.27 mm/s (mean $\pm$ SD) during the first trimester, 1.00 ± 0.28 mm/s during the third trimester and 1.03 ± 0.24 mm/s three months postpartum, compared with values of 0.71 ± 0.20 , 0.77 ± 0.20 and 0.82 ± 0.19 mm/s, respectively, in nondiabetic women (repeated measures ANOVA, $p=0.0026$ between groups).

Macular capillary blood flow velocity was higher in diabetic women with a DCCT score > 3 at baseline compared with those with a DCCT score ≤ 3 at baseline. A similar trend was present when laser-treated patients were excluded from the analyses, though the difference was then not statistically significant (Table 1).

Table 1. Macular capillary blood flow velocities (mm/s) (mean $\pm$ SD) in diabetic and control women during pregnancy and the postpartum period.

	1 st trimester	3 rd trimester	3 months postpartum	P between subgroups	P within group	P vs. controls
All diabetic women (n = 46)	0.94 $\pm$ 0.27	1.00 $\pm$ 0.28	1.03 $\pm$ 0.24		0.0294	0.0026
DCCT score $\leq$ 3 at baseline (n = 27)	0.88 $\pm$ 0.25	0.93 $\pm$ 0.25	1.03 $\pm$ 0.26	0.0164		0.0167
DCCT score >3 at baseline (n = 16)	1.08 $\pm$ 0.23	1.18 $\pm$ 0.23	1.07 $\pm$ 0.22			<0.00005
Non-laser-treated diabetic women (n = 40)	0.93 $\pm$ 0.27	0.98 $\pm$ 0.27	1.03 $\pm$ 0.24		0.0301	0.0048
DCCT score $\leq$ 3 at baseline (n = 27)	0.88 $\pm$ 0.25	0.93 $\pm$ 0.25	1.03 $\pm$ 0.26	0.0571		0.0167
DCCT score >3 at baseline (n = 11)	1.09 $\pm$ 0.22	1.14 $\pm$ 0.21	1.05 $\pm$ 0.22			0.0002
Laser-treated diabetic women (n = 6)	1.00 $\pm$ 0.28	1.19 $\pm$ 0.29	1.08 $\pm$ 0.23		0.0763	0.0036
Control women (n = 11)	0.71 $\pm$ 0.20	0.77 $\pm$ 0.20	0.82 $\pm$ 0.19		0.2449	

6.1.3 Subgroup analysis

The diabetic women were also divided into subgroups to study the effects of duration of diabetes, severity of DR, progression of DR, and mean serum HbA1c concentration on retinal capillary blood flow velocity. These factors were tested: 1) duration of diabetes ≤ 17 or > 17 years, 2) change in mean MA count from baseline to the third trimester, 3) change in mean MA count or DCCT score from baseline to the third trimester, and 4) HbA1c $\leq$ or $>$ the mean value in each trimester. No significant differences in flow values categorized according to these criteria were found.

6.2 RETINAL CAPILLARY BLOOD FLOW MEASURED WITH CONFOCAL SCANNING LASER DOPPLER FLOWMETRY DURING PREGNANCY IN DIABETIC WOMEN, NONDIABETIC CONTROLS AND NONPREGNANT DIABETIC WOMEN (II)

6.2.1 Baseline retinopathy level and progression of diabetic retinopathy

Data from 32 out of 57 diabetic women were analysed in this sub-study. Of the 32 diabetic women, 17 (53.1%) had an RP level of 10 in the study eye at baseline. Six (18.8%) had RP level 20, seven (21.9%) RP level 35, and two (6.3%) RP level 43. Two of the diabetic women had undergone panphotocoagulation at baseline. By the third trimester, 13 (40.6%) had RP level 10, four (12.5%) RP level 20, ten (31.3%) RP level 35, 3 (9.3%) RP level 43, and two (6.3%) RP level > 53 . Significant progression of DR was thus observed in only those two patients who developed bilateral PDR, one during her second and the other during her third trimester. These two patients underwent panphotocoagulation in the study eye during pregnancy. After panphotocoagulation, both patients showed a moderate decrease in the flow value.

During pregnancy, 19 (59.4%) of the diabetic women had no progression in the level of RP, 4 (12.5%) progressed one level, and 9 (28.1%) progressed more than one level. Because of “the waxing and waning” course of RP, i.e., increase in BRP lesions (MAs, CWSs, haemorrhages) during pregnancy and decrease in these lesions postpartum, by 6 months postpartum 22 (68.8%) diabetic women had the same level of RP as at baseline, and 10 (31.3%) had progressed. In addition, three of these patients had received local laser treatment after the 3-month follow-up. All patients who were found to have active

neovascularization were given laser therapy. All the fundus photographs of nondiabetic control subjects were graded as RP level 10 in the masked grading.

6.2.2 Blood flow between diabetic and nondiabetic women

Retinal capillary blood flow values were higher in the diabetic than in the nondiabetic women throughout pregnancy and postpartum (multivariate ANOVA, $p=0.009$). In women with diabetes, blood flow tended to increase during pregnancy until the third trimester, and to be lower 3 and 6 months postpartum. However, this trend was not statistically significant. The results were essentially the same with small square mean value and the mean values of the 50th percentile, 75th percentile, and 90th percentile of pointwise analyses (repeated measures ANOVA, Table 2). Among the nondiabetic women, there were non-significant decreases from the first trimester values to the third trimester values and the 3-month postpartum values.

6.2.3 Subgroup analysis

The pregnant diabetic women were divided into subgroups to study the effects of duration of diabetes, severity of RP, progression of RP, and mean serum HbA1c concentration on retinal capillary blood flow. The mean ($\pm$ SD) HbA1c value in pregnant diabetic women was $7.17\pm0.96\%$ in the first, $6.44\pm0.89\%$ in the second, and $6.74\pm1.18\%$ in the third trimester. These subgroups were compared using the small square mean blood flow value and the mean value of the 25th, 50th, 75th, and 90th percentile pointwise-analysis blood flow values. No significant differences in flow values categorized according to these criteria appeared during pregnancy or postpartum.

6.2.4 Blood flow related to laser treatment

To study blood flow in the diabetic women with the most severe progression of DR during pregnancy, those with diabetes were grouped according to whether they needed laser treatment or not during the follow-up (Table 3). No difference appeared in the retinal capillary blood flow between laser-treated and non-laser-treated diabetic women, but flow values were higher in diabetic than in the nondiabetic women.

Table 3. Individual pixel flow in the 75th percentile by point-wise analysis in diabetic women laser-treated (n=5) during follow-up, in not laser-treated diabetic women (n=27), and in nondiabetic control women (n=11). Data are expressed as the mean $\pm$ SD in AU. Of the laser-treated diabetic women, 2 received panphotocoagulation treatment, and 3 received local laser treatment between the second and third trimester. P values based on repeated measures ANOVA.

Patient group	first trimester	third trimester	3 months postpartum	P within group	P vs. controls
Laser-treated	341 $\pm$ 63	391 $\pm$ 59	395 $\pm$ 57	0.486	0.004
Not laser-treated	381 $\pm$ 124	393 $\pm$ 86	365 $\pm$ 80	0.340	0.008
Controls	324 $\pm$ 57	301 $\pm$ 36	307 $\pm$ 57	0.423	

6.2.5 Blood flow values in pregnant versus nonpregnant diabetic women

Blood flow values measured in the pregnant diabetic women were also compared with those measured in the nonpregnant diabetic women during the third trimester. Values in those nonpregnant were the following: small square mean value 201 $\pm$ 36, mean value of the 50th percentile of the pixel flow values of the pointwise analysis 150 $\pm$ 43, mean value of the 75th percentile of the pixel flow values of the pointwise analysis 316 $\pm$ 49, and mean value of the 90th percentile of the pixel flow values of the pointwise analysis 470 $\pm$ 70.

The small square mean value did not differ between nonpregnant and pregnant diabetic women during the first trimester (p=0.58), but the value was lower in the nonpregnant diabetic women during the third trimester (p=0.023), and 3 months postpartum (p=0.05). For the mean value of the 75th percentile of the pixel flow values of the pointwise analysis, the results between these two groups were essentially the same (p=0.49, p=0.012, p=0.084).

6.3 CONTRAST SENSITIVITY AND MACULAR TOPOGRAPHY (III)

6.3.1 Contrast sensitivity

Contrast sensitivity measurements were available from 47 out of 57 diabetic women. CS measured at 3 and 6 cycles per degree (cpd) was lower in diabetic than in nondiabetic women throughout pregnancy and postpartum ($p = 0.012$ and $p = 0.043$). At 1.5 cpd, 12 cpd and 18 cpd, no difference appeared between the groups. A temporal improvement occurred in CS values in both groups at 3 and 6 cpd ($p < 0.001$ and $p = 0.039$) from the first trimester to the third trimester and postpartum.

6.3.2 Topographic measurements with HRT

HRT analyses could be successfully completed in 46 out of 57 diabetic women. Macular topographic measurements showed that the volume above the reference-plane parameter (VARP) was greater in diabetic than in nondiabetic women throughout pregnancy and postpartum, when measured with the 1.5-mm diameter circle ($p = 0.036$). Furthermore, measured with the circles of 1.0, 2.0 or 3.0 mm diameter, the values were higher in diabetic women but did not reach statistical significance. No statistically significant temporal changes appeared in macular topography during pregnancy in either group, but nondiabetic women showed a trend toward a postpartum decrease in 1.0 and 1.5 mm diameter VARP measurements.

6.3.3 Correlation between VARP and CS

Both VARP and CS measurements were available from 45 diabetic women. During the third trimester, the mean VARP measurements with 1.5- and 2.0-diameter circles were correlated with CS at 3 and 6 cpd in diabetic pregnant women, with a statistically significant negative correlation between 1.5 mm VARP and CS at 6 cpd ($r = -0.471$, $p = 0.001$, Fig. 1), and between 2.0 mm VARP and CS at 6 cpd ($r = -0.446$, $p = 0.002$). The correlations between VARP and CS were also tested during other timepoints in diabetic and nondiabetic women, with no other statistically significant differences.

Fig 1. Correlation between contrast sensitivity (CS) at 6 cpd and 1.5 mm volume above reference plane (VARP) during the third trimester in diabetic women ($n=45$).

6.4 VASOACTIVE HORMONES (IV)

6.4.1 Retinopathy status

Data from 63 out of 72 diabetic women could be analysed in sub-study IV. Thirty-three (52.3%) diabetic women had mild retinopathy (DCCT score ≤ 3) and 30 (47.6%) had more severe retinopathy (DCCT score >3) in the first trimester. By the third trimester 23 (36.5%) of the women had a DCCT score ≤ 3 , and 40 (63.5%) a DCCT score >3 .

Ten (15.9%) diabetic women had received laser treatment prior to the current pregnancy; seven (11.1%) had received panphotocoagulation, and three (4.8%) local laser treatment. Seven (11.1%) diabetic women developed proliferative changes during the study requiring subsequent laser treatment. In addition, one diabetic women received laser treatment because of leaking microaneurysms, and one because of local vitreous traction and haemorrhage from an avulsed temporal vein.

6.4.2 Vasoactive hormones in diabetic and nondiabetic women

Plasma RA, Ang II and aldosterone levels were higher during pregnancy than at 3 months postpartum in both diabetic and nondiabetic women (Table 4). Levels of PRA, Ang II and aldosterone were lower in diabetic than nondiabetic women throughout pregnancy and postpartum, although this difference was statistically significant for PRA only ($p < 0.0001$). The statistically significant difference in PRA between diabetic and nondiabetic women existed also when grouped either by progression of RP or baseline RP. However, there were no differences in Ang II, aldosterone or AM between diabetic and nondiabetic women when grouped by progression of RP or baseline RP.

Table 4. Plasma concentrations of markers of renin-angiotensin-system and adrenomedullin in diabetic and nondiabetic women throughout pregnancy and postpartum.

	1 st trimester	3 rd trimester	3mo pp	p-value
Ang-II (pmol/l)				
With diabetes (n=53)	11.4 ± 8.1	19.7 ± 16.6	5.1 ± 3.4	0.187
Controls (n=9)	12.1 ± 5.2	29.2 ± 18.6	5.1 ± 2.5	
PRA (pmol/l/h)				
With diabetes (n=48)	2.7 ± 0.9	2.5 ± 1.0	0.9 ± 0.5	<0.0001
Controls (n=9)	3.0 ± 0.9	4.7 ± 2.5	1.2 ± 0.7	
Aldosterone (pmol/l)				
With diabetes (n=53)	727 ± 344	1916 ± 930	230 ± 194	0.108
Controls (n=9)	970 ± 287	2260 ± 1262	307 ± 157	
AM (pmol/l)				
With diabetes (n=52)	4.8 ± 2.6	6.7 ± 4.1	4.8 ± 1.3	0.509
Controls (n=9)	4.8 ± 0.9	8.3 ± 3.8	4.7 ± 1.2	

Ang II, Angiotensin II; PRA, plasma renin activity; AM, adrenomedullin.

Values are mean ± SD. P-values calculated with repeated measures ANOVA.

Levels of plasma ANP were significantly lower in diabetic than in nondiabetic women (Table 5), and this difference compared to controls was evident in diabetic women with no progression of RP. There was, however, no significant difference in ANP levels between diabetic subgroups grouped by baseline RP score. Other natriuretic peptides did not differ between the two groups.

Table 5. Plasma concentrations of natriuretic peptides in diabetic and nondiabetic women throughout pregnancy and postpartum.

	1 st trimester	3 rd trimester	3mo pp	p-value
ANP (pmol/l)				
With diabetes (n=51)	6.5 ± 2.5	9.0 ± 6.0	8.0 ± 3.3	0.04
Controls (n=9)	9.3 ± 3.6	9.1 ± 3.5	11.6 ± 5.8	
BNP (pmol/l)				
With diabetes (n=51)	3.0 ± 1.2	3.1 ± 1.8	3.9 ± 1.8	0.281
Controls (n=8)	3.6 ± 1.5	2.9 ± 1.0	5.0 ± 2.4	
CNP (pmol/l)				
With diabetes (n=51)	1.1 ± 0.5	1.4 ± 1.3	1.1 ± 0.5	0.640
Controls (n=9)	0.9 ± 0.2	1.2 ± 0.5	1.0 ± 0.3	

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide.

Values are mean ± SD. P-values calculated with repeated measures ANOVA.

6.4.3 Multivariate logistic regression analysis

A multivariate logistic regression analysis was performed to assess vasoactive hormones associated with retinopathy progression. With the retinopathy progression by the third trimester (stable DCCT score or worsening DCCT score during pregnancy) as the dependent variable, none of the measured vasoactive hormones qualified in the model.

6.5 ANGIOPOIETIC FACTORS (V)

6.5.1 Retinopathy status

Of the 26 diabetic women with sVEGFR-1 and hVEGF-A measurements available, 11 (42%) had no or mild retinopathy (DCCT score ≤ 3) and 15 (58%) more severe retinopathy (DCCT score >3) in the first trimester. By the third trimester, 9 (35%) of the women had a DCCT score ≤ 3 , and 17 (65%) a DCCT score >3 .

Seven (27%) diabetic women with VEGF measurements had received laser treatment prior to the current pregnancy. Out of these seven women, 1 (4%) had received bilateral panphotocoagulation, and 6 (23%) local laser treatment in the other eye. Seven (27%) diabetic women developed proliferative changes during the study period requiring subsequent laser treatment.

6.5.2 Angiopoietic factors in diabetic and nondiabetic women

Concentrations of angiopoietic factors in the groups are summarized in Table 6. Ang-1 levels did not change significantly between diabetic and nondiabetic women during pregnancy and postpartum. Ang-2 levels were significantly lower in diabetic women compared to nondiabetic controls during pregnancy and postpartum ($p=0.002$). In both groups, the levels of Ang-2 were 8-9 times higher during the first than the third trimester.

sVEGFR-1 was significantly higher in diabetic than in nondiabetic women during the third trimester ($p=0.014$ Mann-Whitney U-test), although there was no temporal difference between these two groups. Furthermore, hVEGF-A concentration tended to be lower in diabetic than in nondiabetic women at 3 months postpartum. Additionally, hVEGF-A concentrations were lowest in those diabetic women with progression of retinopathy ($p=0.037$).

Table 6. Plasma concentrations of angiopoietic cytokines in Type I diabetic women and nondiabetic control subjects throughout pregnancy and postpartum.

	1 st trimester	3 rd trimester	3mo pp	p-value (women with diabetes vs controls)
ANG-1 (ng/ml)				
With diabetes (15)	3.0 [1.3 - 8.1]	2.9 [1.0 - 4.7]	2.4 [1.2 - 4.6]	0.441
Controls (8)	4.5 [2.4 - 8.2]	2.6 [1.3 - 5.7]	4.8 [0.8 - 13.6]	
ANG-2 (ng/ml)				
With diabetes (15)	26.5 [12.1 - 47.7]	2.9 [0.6 - 3.5]	0.5 [0.3 - 0.7]	0.002
Controls (8)	44.3 [38.3 - 61.9]	5.7 [3.1 - 8.4]	0.9 [0.6 - 4.9]	
hVEGF-A (pg/ml)				
With diabetes (26)	ND	ND	44.2 ± 45.5	0.076*
Controls (8)	ND	ND	86.8 ± 24.7	
sVEGFR-1 (ng/ml)				
With diabetes (26)	3.1 ± 1.8	6.2 ± 3.5	1.7 ± 1.0	0.131
Controls (8)	2.6 ± 0.7	3.4 ± 1.0	2.0 ± 1.8	

Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; hVEGF-A, human vascular endothelial growth factor A; sVEGFR-1, soluble receptor of vascular endothelial growth factor (type 1). Values are median [interquartile range] or mean ± SD. P-values were calculated with repeated measures ANOVA; Ang-1 and Ang-2 were transformed to logarithms to correct skewness. *Mann-Whitney U-Test. ND, not done

6.5.3 Correlation and logistic regression analysis

Correlation analysis revealed a positive correlation between Ang-1 and -2 during the third trimester in diabetic women ($r=0.526$, $p=0.036$). A stepwise logistic regression analysis with retinopathy progression during pregnancy in diabetic women as the dependent variable revealed no association with age, duration of diabetes, or the biochemical markers of interest. Entry criterion for this model was $P=0.2$. All the studied factors were dropped from the model.

7. DISCUSSION

7.1 IMPORTANCE OF RETINAL CAPILLARY BLOOD FLOW IN THE PATHOGENESIS OF DIABETIC RETINOPATHY DURING PREGNANCY (I, II)

Progression of DR has been associated with pregnancy (Ohrt 1984, Moloney & Drury 1982, Serup 1986, Phelps *et al.* 1986, Klein *et al.* 1990, Chew *et al.* 1995, Axer-Siegel *et al.* 1996, Lapolla *et al.* 1998). Retinal blood flow measurements in early stages of DR are needed to understand the alterations in retinal hemodynamics in the development and/or progression of DR. Since the blood circulation is generally increased during pregnancy (Thornburg *et al.* 2000), pregnancy is thought to provide a good model for the study of retinal microcirculation. In diabetes, the link between chronic hyperglycaemia and the retinal microcirculatory changes is not completely understood. It has been postulated that hyperglycaemia affects the autoregulatory mechanisms of the retinal microcirculation, and that the consequent hyperdynamic circulation causes increased shear stress on capillary endothelium, starting the process that leads to the development of DR (Patel *et al.* 1992, Kohner *et al.* 1995). Diabetic subjects are known to suffer from retinal autoregulatory incapacity in proportion to disease severity (Ciulla *et al.* 2002).

The ability to determine precisely blood flow of retina is crucial in understanding the relationship of RBF to the development and/or progression of DR during pregnancy. However, the methods currently available for investigation of retinal blood flow are scarce. As regards diabetic pregnancy, it was suggested that various biochemical changes during pregnancy increase retinal capillary leukocyte flow velocity (Hellstedt *et al.* 1996) and total retinal blood flow in diabetic women (Chen *et al.* 1994). In the present study, retinal capillary blood flow was measured with more conventional technique, the BFS method, and newer, more sophisticated method, the confocal scanning laser Doppler flowmetry. These techniques are known to measure different phenomena of the retinal hemodynamics. However, with both techniques (I, II), RBF was found to be higher in diabetic than in nondiabetic women throughout pregnancy and postpartum. In addition, when measured with BFS, we found a correlation between capillary blood flow velocity and severity of DR suggesting that the velocity is higher in diabetic women with more severe DR in the first trimester than in those with no or mild DR (I). Furthermore, no difference existed in blood flow between nonpregnant diabetic and nondiabetic pregnant women, whereas pregnant diabetic women had higher flow values than nonpregnant diabetic women (II).

Taken together, BFS is an experimental technique that provides a quantitative measure of flow in the perifoveal capillary network (Loebl & Riva 1978). The pattern of moving particles (leukocytes) flying around in an area of 10 to 15 degrees of arc radius centered at the fovea in the inner nuclear and outer plexiform layers (Marshall 1935, Riva & Petrig 1980), illuminated with blue light with wavelength of 430 nm, resembles the anatomic distribution of perifoveal capillaries. If performed correctly, BFS gives reproducible data on macular leukocyte flow velocities (Yap & Brown 1994). As a non-invasive technique, it is also well suited for studying macular capillary blood flow velocity during pregnancy. However, the psychophysical and subjective nature of the method limits its usefulness. To be reliable, the technique requires good concentration and co-operation from the study subject.

Even under physiological conditions, shear stress on the endothelium stimulates the secretion of local mediators that modify vessel tone and other circulatory parameters (Shyy 2001). In diabetes, leukocyte rolling and adhesion to the capillary endothelium have been suggested as a potential mechanism causing vessel damage (Miyamoto *et al.* 1997, Schröder *et al.* 1991, Stitt *et al.* 1995). In the phenomenon called "rolling", leukocytes, when observed *in vivo*, are pushed along by the bloodstream. They are known to slow down in the capillaries, thus suggesting that they may plug retinal capillaries (Miyamoto *et al.* 1998). In pregnant diabetic women with impaired autoregulatory mechanisms (hyperdynamic circulation), this kind of additional shear stress could further damage EC structure and functions in the retinal capillary level (Kohner *et al.* 1995, Patel *et al.* 1992, Tooke 1995). In our study, however, the high leukocyte velocities in the retinal capillaries of pregnant diabetic women did not speak for generalized stagnation of leukocytes.

Scanning laser ophthalmoscopy (SLO) studies have shown reduced perifoveal flow velocities in nonpregnant diabetic patients when compared with nondiabetic subjects (Arend *et al.* 1991 & 1994, Wolf *et al.* 1991) in contrary to our results. The differences between SLO and BFS may be related to the different vascular measuring sites and/or measurements of different phenomena. The SLO quantifies the velocity of erythrocyte aggregates in the capillary lumen of the para- and perifoveal network (Arend *et al.* 1995). In addition, velocity measurements with SLO require intravenous fluorescein that is not recommended in studies with pregnant subjects. Invasive methods of measuring retinal capillary blood flow, such as those based on digital FAG, cannot either be used during pregnancy (Hossain 1999).

Previous studies on volumetric RBF in pregnant diabetic women have given conflicting results with both increased (Chen *et al.* 1994) and decreased (Schocket *et al.* 1999) blood flow values reported. Retinal capillary blood flow velocity does not necessarily correlate with volumetric flow changes in the retina. According to Bernouille's law, the velocity at a given perfusion pressure and volume depends on the diameter of the vessel. Thus, even reduced volumetric blood flow can result in an increased capillary blood flow velocity if the vessel is constricted.

Additionally, a noninvasive ultrasound method, colour Doppler imaging of the CRA circulation has revealed reduced blood flow velocities in nonpregnant diabetic patients (MacKinnon *et al.* 2000). In that study, the blood flow velocities were found to be even further reduced as RP progressed. However, in CDI technique blood flow in the CRA cannot be quantitatively calculated since the diameter of the blood vessels cannot be accurately calculated.

As regards our study with confocal scanning laser Doppler flowmetry, to our knowledge, it was the first study to use direct objective measurement of retinal capillary blood flow during pregnancy in diabetic and nondiabetic women. Our results showed that increase in RBF was at least connected with pregnancy, thus indicating an altered response of the retinal circulation to pregnancy in diabetes.

The penetration depth of HRF is 300um (Michelson *et al.* 1996) enabling non-invasive measurement of actual blood flow in superficial layers of retinal capillary bed. Despite being objective and noninvasive, HRF technique has some restrictions as small artifacts such as movements of the eye within the area of the scanned sector may cause changes in actual blood flow. In addition, brightness of the image, i.e. the photodetector sensitivity (DC), is an important factor as regards the reliability of the RBF values (Kagemann *et al.* 1999 & 2001), because overexposed areas give decreased and underexposed areas increased blood flow values (Tsang *et al.* 1999, Kagemann *et al.* 2001). Furthermore, location of the scanned area plays a role, since for example inferior temporal quadrant has been shown to have greater capillary perfusion compared to superior temporal quadrant (Chung *et al.* 1999a), and valid retinal flowmetry measurements are obtained more likely from papillomacular region than the foveal area (Cuyppers *et al.* 2000).

Retinal capillaries are arranged as flat networks in laminate fashion, and only part of them may be perfused at a particular time. This variation in capillary perfusion (shunting or capillary recruitment) is an established phenomenon in human tissues (Toussaint *et al.*

1961). Both intravascular and extravascular factors modulate the flow of red blood cells through retinal capillaries. HRF image is a 2-D projection of a 3-D retinal capillary bed (Kagemann *et al.* 1998). Since pointwise analysis was suggested of giving more accurate results of retinal capillary blood flow values (Michelson *et al.* 1998) compared to more conventional small box analysis, we decided to use both techniques. However, both techniques gave similar results with increased retinal blood flow in pregnant diabetic women in our study.

In conclusion, our data indicated that capillary circulation in the diabetic retina shows a different response to pregnancy compared with a normal retina. Our data support the concept that capillary hyperperfusion may play a contributing role in the development of DR during pregnancy. It is noticeable that most of our diabetic women had minimal to moderate DR in the beginning of pregnancy, and that more than 50% had taken part in the prepregnancy planning and reached optimal glycemic control before conception, and that glycemic control continued to be good in most of them throughout pregnancy and postpartum. The results would most likely have been different in diabetic women with more severe forms of DR and worse glycemic control. Furthermore, the direct correlation of leukocyte velocity measured with BFS and the retinal blood flow measured with HRF was not performed in our study, but it could reveal additional information of capillary microcirculation.

7.2 CONTRAST SENSITIVITY LOSS AND TOPOGRAPHIC CHANGE IN THE CENTRAL MACULA IN DIABETIC PREGNANCY (III)

Pregnancy is known to have many systemic implications due to the physiological volume-overload (Schannwell *et al.* 2003). The changes in the human eye may be either physiological or pathological, having the potential to affect all ocular tissues. Hormonal changes during pregnancy are known to lead to retention of water in the cornea, with increase in corneal thickness (Weinreb *et al.* 1988) and curvature (Park *et al.* 1992). Hormonal changes together with fluid overload might also affect retinal thickness during pregnancy.

Fluid retention is known to exacerbate retinal capillary leakage (Bresnick 1986). Histopathological study of diabetic macular oedema has revealed that fluid leaks out of the damaged retinal vessels, enters Muller cells and causes intracellular swelling, especially in the outer plexiform layer (Yanoff *et al.* 1984, Yanoff & Fine 1996). Current

slitlamp biomicroscopy and stereoscopic fundus photography are insensitive methods to detect such subtle changes in retinal thickness. Consequently, new methods are necessary for the investigation of macular structure in diabetes.

DR is not merely a vascular, but also a neurosensory disorder (Ewing *et al.* 1998). These neurosensory aspects of visual function include electroretinography, visual evoked potential (VEP: P100 and P300), CS and color vision/hue discrimination. In diabetic patients, CS has been reported to be decreased (di Leo *et al.* 1992, Banford *et al.* 1994) or unchanged (Sokol *et al.* 1985) compared to nondiabetic patients. Previously, the loss of CS in pregnant diabetic women with minimal DR was suggested to reflect pregnancy related subclinical accumulation of fluid in the retina (Hellstedt *et al.* 1997a). Recently, loss of colour CS in nonpregnant patients with type I diabetes was even related to increased retinal blood flow (Findl *et al.* 2000). Also, hyperglycaemia-related changes in the lens and in the retina have been suggested as possible mechanisms of CS loss in diabetic patients.

We suspected that retinal capillary leakage could increase and lead to fluid retention in macula in diabetic women during pregnancy, with possible adverse impact on the progression of DR. We also suspected that loss of CS and macular topography might be associated with each other. Therefore, we used a confocal scanning laser tomography to assess quantitatively the thickness of retina during pregnancy and postpartum in a group of diabetic and nondiabetic women, and correlated the results with loss of CS. We chose the use of VARP because it had been documented in previous studies (Zambarakji *et al.* 1998 and 1999).

Our study showed that CS is decreased in diabetic compared to nondiabetic women at 3 and 6 cpd throughout pregnancy and postpartum. This was in agreement with a previous study showing a selective decrease in CS at mid-spatial frequencies during diabetic pregnancy (Hellstedt *et al.* 1997a). However, our study failed to demonstrate any correlation between glycemic control or the presence of retinopathy and CS during pregnancy. Previously, a positive correlation was found between poor glycemic control and deteriorating CS in diabetic patients (di Leo *et al.* 1992, Banford *et al.* 1994). Also, changes in CS had been related to the degree of DR in nonpregnant type I diabetic patients (Hyvärinen *et al.* 1983).

We found that the surface of the central macula is elevated in diabetic women compared to nondiabetic women throughout pregnancy and postpartum. Elevation of the central macula was also most remarkable in those diabetic women with worst progression of DR during pregnancy and postpartum. In addition, our results suggested that the loss of CS in diabetic women is connected with a change in macular topography during the third trimester, even in the diabetic women with a normal macula and visual acuity. With the OCT, we would certainly have obtained more accurate information about the intraretinal changes (*in vivo* histology) in our diabetic women (Schaudig *et al.* 2000, Tadrous 2000, Otani & Kishi 2000, Yang *et al.* 2001). Confocal scanning laser instrument that we used in our study was known to assess objectively macular oedema in DR (Zambarakji *et al.* 1998), but without showing intraretinal structures.

According to our study, fluid retention in retina can be associated with loss of CS during diabetic pregnancy even in the absence of retinopathy. Metabolic abnormalities and severity of DR are most likely to contribute to loss of CS in diabetic patients, although we could not relate glycemic control or severity of DR and loss of CS. One explanation could be that more than half of our diabetic women had taken part in prepregnancy planning and reached optimal blood glucose values before current pregnancy. Most of our patients also continued with good glycemic control during pregnancy. In addition, most of them experienced only minor worsening in the RP level during pregnancy and after delivery.

7.3 VASOACTIVE HORMONES AND RETINOPATHY IN DIABETIC PREGNANCY (IV)

We had found out that retinal capillary blood circulation is hyperdynamic in diabetic compared to nondiabetic pregnant women (II). Because hyperdynamic retinal capillary blood flow and consequent endothelial cell damage have been implied as pathogenetic mechanisms in early DR, we suspected that perturbations in one or more vasoactive system might contribute and play a role in this process. Therefore, to evaluate the temporal connection of vasoconstrictive mediators of RAS (PRA, Ang II, Aldo), and on the other hand, the vasodilatory mediators of natriuretic peptides (ANP, BNP, CNP), and AM in the hemodynamics of retinal blood flow and in development or progression of DR, this additional work was performed.

Previously, activation of the RAS has been reported in nondiabetic human pregnancy (Wilson *et al.* 1980). Peripheral vasodilatation has been known to occur early in human pregnancy, and activation of RAS has been suggested (Chapman *et al.* 1998). Of

particular interest in this context is our finding that the RAS was suppressed in the diabetic women compared to the controls. Under most other circumstances the RAS is known to be suppressed in diabetes but we were unaware of comparable data in pregnant type I diabetic women. Whatever the reasons for suppression of the RAS, it is pertinent to enquire whether it might be linked in any way with the level of retinal blood flow or the stage of retinopathy in our diabetic women. For example, it might be argued that reduced activity of the RAS leads to (or at least contributes to) the increased retinal blood flow that we have observed.

In addition, we found out that plasma ANP levels were significantly lower in the diabetic women than in the controls. We might have anticipated elevated, rather than suppressed ANP levels since earlier studies have shown that diabetic patients under other circumstances have a propensity to an increased left ventricular mass and left atrial size compared with nondiabetic subjects (Grossman *et al.* 1992, Rutter *et al.* 2003). Furthermore, diabetic patients are generally reported to have elevated or normal plasma ANP levels, and variously exhibit impaired or exaggerated responsiveness to stimuli such as fluid-loading or postural change (Kahn *et al.* 1986, Dussaule *et al.* 1988, Opocher *et al.* 1989, Trevisan *et al.* 1990). Furthermore, nonpregnant diabetic patients with retinopathy have been reported to exhibit higher circulating concentrations of ANP than those without retinopathy (Yano *et al.* 1998). So far, we are not aware of studies comparable to ours, i.e., comparing pregnant type I diabetic women with control pregnant women. Whatever the explanation for the suppressed levels of ANP in our study, it is difficult to see how this could contribute to the increase in retinal blood flow or the retinopathy during pregnancy.

Our study has some limitations which need to be mentioned. The pathophysiological importance of hormones may not always be reflected accurately by their levels in plasma. This is especially true for AM which is seen primarily as a paracrine hormone acting within blood vessels rather than a classic circulating hormone. Likewise, tissue levels of some hormones including Ang II and ANP, especially in the eye and within blood vessels, might exert potent actions which may not be reflected by their levels within the circulation. Another obvious limitation is that we have measured only a small number of vasoactive substances and ignored, for example NO, the endothelins, the prostaglandins and the catecholamines. We must also point out that lack of evidence of the role of systemic vasoactive hormones on DR progression during pregnancy can be related to good glycemic control with only slight progression of retinopathy during pregnancy.

In summary, we have previously shown that retinal capillary blood flow is elevated in pregnant diabetic women compared with nondiabetic women (II). In this sub-study, we also confirm that diabetic women seem to have lower levels of PRA and ANP than nondiabetic controls. But unlike we expected, no systemic vasoactive hormones could be associated with the progression of DR. In the future, it is possible that measurements of arteriolar and venous calibre in retinal vessels might reveal more accurate information about systemic vasoactive mediators and their possible effect on retinal capillary hemodynamics during pregnancy and postpartum.

7.4 PREGNANCY INDUCED SYSTEMIC HORMONAL CHANGES, ANGIOPOIETIC FACTORS AND THEIR RELATION TO THE DEVELOPMENT AND/OR PROGRESSION OF DIABETIC RETINOPATHY (V)

In a normal adult, most vasculature is quiescent with only 0.01% of ECs undergoing division (Carmeliet & Jain 2000), probably due to the presence of endogenous angiostatic factors (Nakagawa *et al.* 2000) with the exception of angiogenesis in response to tissue injuries (wound healing and inflammation) (Paavonen *et al.* 2000) or the female reproductive organs (Ferrara *et al.* 1998). Pathologic angiogenesis is, thus, a hallmark of cancer and various ischemic and inflammatory diseases such as DR and rheumatoid arthritis.

Retina is a source of many growth factors (Forrester *et al.* 1993 & 1997). Under normal circumstances a balance exists between angiogenesis and angiostasis. After the phenomenon called "angiogenic switch", when positive regulators are predominating, the endothelium is activated (angiogenic), and when negative regulators are predominating, the endothelium remains quiescent (angiostatic) (Pepper 2001). In DR, this normal balance is disturbed, and hypoxia is known to be a strong stimulus for angiogenesis. Retinal ischemia (capillary closure) stimulates the release of chemical mediators, which have receptors in the retinal vasculature initiating neovascularization. Moreover, it is known that hypoxia-driven angiogenesis can cause blindness in premature newborns (retinopathy of prematurity) and in diabetic patients (Alon *et al.* 1995). It has also been thought that the hypoxia-driven angiogenesis plays a role in the development of choroidal neovascularization in age-related macular degeneration as well (Witmer *et al.* 2003).

To date, several angiogenic and angiostatic factors have been discovered and their functions have been investigated. *In vivo*, VEGF is one of the most important potent positive regulators of angiogenesis. It seems to play a major role in many physiologic and

pathologic conditions (Witmer *et al.* 2003). It may contribute to the development of blood-retinal barrier breakdown in human DR (Mathews *et al.* 1997). Other important growth factors involved in angiogenesis are among others IGF-1 (Forrester *et al.* 1993) and its binding proteins (IGFBPs) (Rajaram *et al.* 1997) and angiopoietins (Jones & Clemmons 1995, Otani *et al.* 2001). Angiopoietins are important for vessel wall assembly and the maintenance of vascular integrity. As regards the angiostasis, the pigment epithelium derived factor (PEDF) is one of the most potent endogenous angiogenic inhibitors.

As regards DR, GFs are implied in retinal pathology at all stages of RP, and intravitreal or systemic injections of angiogenetic GFs have been experimentally shown to cause retinopathy-like changes in animal retinas (Kern & Engerman 1996, Aiello *et al.* 1995). During pregnancy, profound angiogenesis and vascular remodelling occur in the placenta, with an active local synthesis of several angiopoietic factors (Dunk *et al.* 2000, Vuorela *et al.* 2000). It is possible that any increase in plasma levels of these various angiopoietic factors might create a milieu of systemic growth favouring even progression of DR during pregnancy.

Therefore, in the present study plasma levels of the main angiopoietic factors during pregnancy were analysed and related to progression of DR. Previously, the role of angiopoietins in DR had been unclear, although Ang-1 seemed to be protective (Joussen *et al.* 2002, Thurston *et al.* 2000), and Ang-2 capable of potentiating VEGF-induced angiogenesis and causing neovascularization (Otani *et al.* 2001). However, our study showed that progression of DR was not associated with changes in the levels of either Ang-1 or Ang-2.

Previously, VEGF had been identified as a primary initiator of proliferation of DR and as a mediator of NPDR (Smith *et al.* 1999, Aiello & Wong 2000, Witmer *et al.* 2003). Being tightly bound during pregnancy by the sVEGFR-1 which is a short-length soluble VEGF binding protein belonging to VEGF family (Vuorela *et al.* 2000), VEGF was measured only 3 months postpartum in our study, being then significantly lower in diabetic women with progression of retinopathy than in nondiabetic controls. Generally, VEGFRs are known to be expressed at low levels in many adult tissues, being upregulated in ECs during development, and in certain angiogenesis-associated pathologic situations. The association of sVEGFR-1 and DR during pregnancy is not established yet. Thus, in our study, sVEGFR-1 was measured during pregnancy and postpartum.

Unlike we originally expected, our results indicated that levels of angiopoietic factors were similar or lower in diabetic women during pregnancy and postpartum compared to nondiabetic pregnant women. This suggests that systemic angiopoietic stimuli are unlikely to be the inducing factors for the progression of DR during pregnancy. However, the very high levels of Ang-2 (hypoxia driven) during the first trimester may augment neovascularization in diabetic women, if the local balance between angiogenesis and angiostasis has already been disturbed by other factors. On the other hand, Ang-1 and the measured components of the VEGF family were not much affected by pregnancy.

8. SUMMARY AND CONCLUSIONS

This study was planned and carried out to enlighten the development of DR and possible mechanisms responsible for DR progression in women with type I DM during pregnancy and postpartum. Based on it the following conclusions can be drawn.

1. Hyperdynamic retinal blood circulation may play a role in the progression of DR during pregnancy and postpartum.
2. Loss of CS, an early functional marker of retinal affection in DR is probably mediated by thickening of the macula, suggesting an early subclinical BRB breakdown in DR.
3. Systemic vasoactive mediators (PRA, Ang II, Aldo, ANP, BNP, CNP, AM) do not seem to play a significant role in the development or progression of DR during pregnancy. RAS seems to be suppressed in diabetic women during pregnancy and postpartum.
4. Systemic circulating levels of angiopoietic factors (Ang-1, Ang-2, hVEGF-A, total soluble VEGF receptor-1) do not seem to play a significant role in the presence or progression of DR during pregnancy.

Accordingly, future research is needed to identify other potential factors related to the progression of DR during pregnancy. For example, it is possible that inflammation might play a role in the development and progression of DR during pregnancy. Systemic or vitreous studies of proinflammatory markers such as interleukin-6, soluble VCAM-1 or ICAM-1 might reveal additional information of the multifactorial development of DR. If any of these factors could be identified, the pathogenesis of DR during pregnancy would be understood more clearly in the future.

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