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# INFLAMMATORY MEDIATORS IN DIABETIC RETINOPATHY

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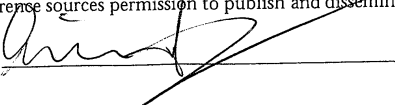
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“Trassla inte till saken genom att komma dragande med fakta”

*Groucho Marx*



# ABSTRACT

Diabetic retinopathy (DR) is the most feared complication of diabetes with an overall prevalence of 21.9-36.8% and may, if untreated, lead to severe visual disability or blindness. DR is histologically characterized by pericyte and endothelial cell loss, formation of acellular vessel strands, micro-occlusions, progressive ischemia, and finally the appearance of neovascularization and fibrosis. Hyperglycemia in the retina activates pro-inflammatory pathogenetic pathways i.e., the polyol, PKC, hexosamine, and RAS pathways, as well as AGE formation. Molecular changes result in blood flow alterations, formation of reactive oxygen species and oxidative stress, induction of inflammatory signaling systems and inflow of leukocytes, and ultimately altered gene transcription, in turn promoting the biomolecular DR characteristics. This thesis enlightens how established pathways may contribute to inflammation in DR and summarizes the results of five studies. Up-regulation of inflammatory mediators and leukocyte adhesion molecules was demonstrated in serum and eyes of diabetic subjects with both proliferative DR and no or non-proliferative DR in humans. The roles of oxidative stress as well as of inflammation in retinal ischemia-reperfusion were assessed in rats with and without diabetes, while retinal endothelial expression of VCAM-1 and leukocyte accumulation were studied in early diabetes in mice. Dyslipidemia and the anti-inflammatory effects of lipid-modulating compounds as well as an immunoregulating role of TNF- $\alpha$  were also analyzed. These studies support that inflammation, which might be aggravated by dyslipidemia, has a role in early as well as late stages of DR.

diabetic retinopathy, oxidative stress, inflammation, dyslipidemia, VCAM-1, TNF- $\alpha$



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- I. **Gustavsson C**, Agardh E, Bengtsson B, Agardh CD. TNF-alpha is an independent serum marker for proliferative diabetic retinopathy in type 1 diabetic patients. *J Diab Complic.* 2008; 22(5): 309-316.
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- V. **Gustavsson C**, Agardh E, Nilsson J, Gomez MF, Agardh CD. Vascular cellular adhesion molecule-1 (VCAM-1) in retina from diabetic and genetically modified (ApoE<sup>-/-</sup>, TNF- $\alpha$ <sup>-/-</sup>, ApoE<sup>-/-</sup>/TNF- $\alpha$ <sup>-/-</sup>) mice. *Submitted* 2009.

# ABBREVIATIONS

ACCORD-EYE	Action to Control Cardiovascular Risk in Diabetes-EYE
ACE	Angiotensin Converting Enzyme
AGE	Advanced Glycation Endproducts
ALE	Advanced Lipoxidation Endproducts
AMD	Age-related Macular Degeneration
APC	Antigen-Presenting Cell
ApoE	Apolipoprotein E
AR	Aldose Reductase
ARI	Aldose Reductase Inhibitor
ATP	Adenosine Triphosphate
AU	Arbitrary Units
BCA	Bicinchonic Acid
BMI	Body Mass Index
BRB	Blood-Retina-Barrier
CCD	Charge Coupled Device
cDNA	complementary DNA
CD	Cluster of Differentiation
CI	Confidence Interval
CRP	C-Reactive Protein
Ct	Cycle threshold
CuZnSOD	Copper Zinc superoxide dismutase

DAG	Diacyl Glycerol
DAMAD	Dipyridamole Aspirin Microangiopathy of Diabetes
DBP	Diastolic Blood Pressure
DCCT	Diabetes Control and Complications Trial
DHA	Docosahexaenoic Acid
DIRECT	Diabetic Retinopathy Candesartan Trial
DME	Diabetic Macular Edema
DNA	Deoxyribonucleic Acid
DR	Diabetic Retinopathy
DRS	Diabetic Retinopathy Study Research Group
ECM	Extracellular Matrix
eNOS	endothelial Nitric Oxide Synthetase
ET-1	Endothelin-1
ETDRS	Early Treatment Diabetic Retinopathy Study Group
FGF- $\beta$	Fibroblast Growth Factor- $\beta$
GAD	Glutamic Acid Decarboxylase
GCLc	catalytic subunit of Glutamylcysteine Ligase
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GPx1	Glutathione Peroxidase 1
GSH	Glutathione
Hb	Hemoglobin
HbA1c	Glycated Hemoglobin
HGF	Hepatocyte Growth Factor
HIF-1	Hypoxia-Inducible Factor-1

HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
hsCRP	high-sensitive CRP
iBRB	inner BRB
ICAM-1	Intercellular Adhesion Molecule-1
I-κB	Inhibitor of NF-κB
IL	Interleukin
K <sup>+</sup>	Potassium ion
KO	Knockout
LDL	Low Density Lipoprotein
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MHC	Major Histocompatibility Complex
MIF	Migration Inhibitory Factor
MnSOD	Manganese Superoxide Dismutase
NAD	Nicotine Amide Dinucleotide
NADH	Nicotine Amide Dinucleotide Hydrogen
NADPH	Nicotine Amide Dinucleotide Phosphate
NF-κB	Nuclear Factor-κB
NO	Nitric Oxide
NOS	Nitric Oxide Synthetase
NPDR	Non-Proliferative Diabetic Retinopathy
NSAID	Non-Steroidal Anti-Inflammatory Drugs
PARP	Poly-Adenosine DiPhosphate Ribose Polymerase
PDR	Proliferative Diabetic Retinopathy

PEDF	Pigment Epithelium Derived Factor
PKC	Protein Kinase C
Ppib	Cyclophilin b
$\omega$ 3-PUFA	omega3-Poly- Unsaturated Fatty Acid
RAGE	Receptor for AGE
RANTES	Regulated upon Activation Normal T cell Expressed and Secreted
RAS	Renin Angiotensin System
RBX	Ruboxistaurin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
SBP	Systolic Blood Pressure
SDH	Sorbitol Dehydrogenase
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
STZ	Streptozocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGF- $\beta$	Transforming Growth Factor- $\beta$
TLR	Toll-Like Receptor
TNF- $\alpha$	Tumor Necrosis Factor- $\alpha$
TNFR	TNF Receptor
UKPDS	United Kingdom Prospective Diabetes Study
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLA-4	Very Late Antigen-4

VLDL

Very Low Density Lipoprotein

WESDR

Wisconsin Epidemiological Study of  
Diabetic Retinopathy

wt

wildtype

# INTRODUCTION

Diabetes is a metabolic disorder characterized by chronic hyperglycemia. The global prevalence of diabetes is 2.8% for all age groups and is expected to double before the year 2030<sup>1,2</sup>. There are approximately 400000 known diabetic subjects in Sweden, representing a prevalence of 4%<sup>3</sup>. The prevalence of diabetic retinopathy (DR) in the entire diabetic population varies between 21.9 and 36.8% in different study populations<sup>4</sup>. It increases with diabetes duration and was found to be very high (67-98%) after 20 years in the large Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR) from 1984<sup>5,6</sup>, but has since then decreased most likely due to improved metabolic control. However, there are still big variations between different populations, ages, ethnicities, retinopathy screening modalities and available treatment for diabetes<sup>7,8</sup>. If left untreated, DR leads to serious visual impairment or blindness in 37-50% of cases<sup>9,10</sup>, but treatment of focally leaking microaneurysms and diffuse leakage from dilated capillaries (focal and grid laser photocoagulation) in diabetic macular edema (DME) or the peripheral retina (panretinal photocoagulation) in proliferative diabetic retinopathy (PDR) has reduced the risk of severe visual loss by at least 50-60 %, as demonstrated by the large multicenter studies Diabetic Retinopathy Study Research Group (DRS) and Early Treatment Diabetic Retinopathy Study Group (ETDRS)<sup>11-14</sup>. Despite available treatment, blindness is still a common long-term complication of DR in Scandinavia<sup>15</sup>. Since laser treatment is not generally available in many countries, DR accounts for 4.8% of the global blindness burden<sup>16,17</sup>. In Western countries, where the impact of other ophthalmological diseases is lower, the corresponding figure for diabetes-induced blindness is 17%, ranked as the third most important reason for severe visual disability or blindness after age-related macular degeneration (AMD) and glaucoma<sup>17</sup>.

The pathogenesis of DR is not entirely known. The large population-based studies of the 1990's demonstrated the importance of good blood glucose control<sup>18,19</sup> and blood pressure control<sup>20</sup> for reducing the risk of development and progression of DR, but hyperglycemia and hypertension per se do not explain the pathogenetic mechanisms. Although several pathways of importance for DR, such as increased formation of advanced glycation endproducts (AGE), polyol pathway shunting, and protein kinase C (PKC) activation have been demonstrated, blocking

those pathways in human studies have not been successful. Recently, inflammation has been suggested to be involved in the development of retinopathy in diabetes<sup>21,22</sup>. In this thesis, I will enlighten by which mechanisms inflammation may provoke the development and progression of DR, examine how established pathways may contribute, analyze the association between inflammation and DR in diabetic subjects with different degrees of retinopathy, and finally evaluate up-regulation of retinal inflammatory mediators in two different diabetic animal models.

## Histopathology of diabetic retinopathy

The retina is a complex structure composed of several tissue layers from the retinal pigment epithelium to the vitreous, and of several cell types, i.e., photoreceptors, second order neurons and ganglion cells (neurons), microglia (resident macrophages), microglia (Müller cells and astrocytes) and vascular cells (endothelial cells and pericytes)<sup>23</sup>. There is a continuously on-going intimate crosstalk between these entities. The arterial microcirculation is an end artery system anastomosing with the venous microcirculation in a fine-meshed capillary network. In retinal capillaries, endothelial cells and pericytes, which seem to have endothelial cell regulating properties, share a common basement membrane<sup>24</sup>. Endothelial cell membranes are likewise interfused by tight junctions responsible for the inner blood-retina-barrier (iBRB)<sup>23</sup>. Surrounding microglia monitor the local environment, react to stress and may, if required, release inflammatory cytokines and phagocytose damaged or necrotic cells<sup>23</sup>.

In diabetes, retinal changes most probably take place from early on a molecular level. One early measurable sign is a dysfunctional autoregulation resulting in decreased blood flow, that may at least partly be due to decreased availability of nitric oxide (NO) and increased levels of endothelin (ET) in the retina after a short period of diabetes<sup>25</sup>. Early histological findings are pericyte loss, capillary microaneurysm formation and endothelial cell death leading to the appearance of non-perfused acellular vessels, designated ghost vessels<sup>21,24</sup>. Pericyte loss leads both to a dysregulated vascular tone and to phenotype changes of endothelial cells<sup>24</sup>. Endothelial cell tight junctions disrupt, with an increased permeability and exudation of plasma proteins reacting with components of the extracellular matrix (ECM)<sup>23,26</sup>. Altered properties of the BRB in diabetes also include an increased membrane transport by pinocytosis through the endothelium, as demonstrated in diabetic rats, dogs, and humans<sup>25</sup>. Intraretinal hemorrhages and exudation of lipids, proteins and fluid from dysfunctional vessels<sup>27</sup> may result in sight-threatening DME, which may occur when reabsorption of fluid is insufficient. The common basement membrane of endothelial cells and pericytes becomes thicker, leading to increased vessel wall rigidity<sup>25,27</sup>. Basement membrane thickening is thought to depend on both increased production as well as decreased degradation of ECM proteins, above all collagen IV, laminin and fibronectin<sup>24</sup>, and may in addition to the resulting increased rigidity also have implications for the interaction between the vessel wall and components of the ECM, as shown by increased expression of integrin- $\beta$ 1 responsible for such cross-talk<sup>25</sup>. Basement membrane thickening, loss of pericytes and endothelial cells as well as transformation of remaining endothelial cells to a more prothrombotic phenotype

lead to local tissue ischemia<sup>25</sup>. This local ischemia is further aggravated by the formation of micro-occlusions, believed to be caused by leukostasis or platelet aggregation, biochemical vessel wall disturbances (endothelial dysfunction), and endothelium toxic compounds in the surrounding environment, e.g. from glial cells or activated inflammatory cells, causing endothelial cell death<sup>21,22,24</sup>. In addition to retinal vascular alterations, diabetes also damages non-vascular retinal cells, such as ganglion cells and glial cells, and it is possible that neuronal degeneration occurs before or in parallel with vascular degeneration in DR<sup>25</sup>. Extensive neuronal apoptosis was demonstrated in the retina of rats after only one month of diabetes, and was sustained for at least 12 months<sup>28</sup>. Several studies have also demonstrated electroretinogram disturbances early in diabetes<sup>25</sup>. Since the communication and interdependence between the retinal vascular system and the neuronal network is very intense, the neuronal degeneration of the retina may well initiate or aggravate diabetic vascular disturbances<sup>24</sup>. Müller cells, one type of the glial cells in the retina, are of importance for the uptake of glucose and retinal neuronal nourishment supply, regulate the retinal blood flow secondary to metabolic demands and may, due to their relative abundance of aldose reductase (AR), contribute to the activation of the polyol pathway in diabetes<sup>25</sup>. Müller cells are also of importance for the clearance of glutamate generated in the retina in photoreceptor signaling, and accumulation of glutamate is potentially harmful for retinal neurons<sup>29</sup>. Diabetes also seems to induce changes in glial cells that make them more “reactive”, leading to up-regulation of cell adhesion molecules, tissue repair and scar formation molecules, and inflammatory mediators<sup>25</sup>.

Capillary occlusion and ghost vessels lead to an increasingly severe retinal ischemia, with deficient oxygenation, nourishment starvation and accumulation of waste products<sup>29</sup>. Non-proliferative diabetic retinopathy (NPDR) proceeds to proliferative diabetic retinopathy (PDR) once the hypoxia induces the expression of angiogenic factors, e.g. hepatocyte growth factor (HGF), hypoxia inducible factor-1 (HIF-1), vascular endothelial growth factor (VEGF), fibroblast growth factor- $\beta$  (FGF- $\beta$ ) and several others<sup>30</sup>, leading to neovascularization, fibrosis, and gliosis. During neovascularization, new vessels develop from existing ones by sprouting and migration of endothelial cells, endothelial cell proliferation, and tube formation<sup>27,30,31</sup>. ECM proteolysis and cell adhesion via matrix binding integrins are necessary to enable the newly formed vessels to penetrate the tissue. Neovascularization may be initiated not only by ischemia or the local production of angiogenic factors, but also by inflammation, all present in DR<sup>31</sup>.

## Definition of inflammation

The concept "inflammation" comes from Latin *inflammatio*, in turn evolved from *inflammo*, meaning "setting on fire". Inflammation is in wide senses the body defense against harmful factors, e.g. toxins, trauma, or infection. Four cardinal signs of inflammation are known since antiquity, i.e., redness (*rubor*), swelling (*tumor*), pain (*dolor*) and local heating (*calor*)<sup>32</sup>. Yet another aspect that is often added is inadequate function (*functio laesa*). Depending on triggering agents, the characteristics of inflammation may differ. Inflammation may be exudative with outflow of fluid, purulent with an increase in leukocytes, fibrinous with increased formation of fibrin, granulomatous with limited cores of infection surrounded by inflammatory cells, and/or necrotic with tissue necrosis<sup>26,33</sup>. The course of inflammation may be defined as acute or chronic. In the acute reaction, there is initial capillary dilatation, exudation of plasma through the capillary wall, activation of the complement system, and formation of chemotactic factors in the tissue. Tissue macrophages and mast cells that localize damage or a pathogen, produce a multitude of inflammatory mediators including chemokines, cytokines, vasoactive amines, and proteolytic enzymes. Inflammatory cytokines and their receptors initiate signal transduction events generally promoting phosphorylation and degradation of the inhibitor of Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) (I- $\kappa$ B) followed by the translocation and activation of NF- $\kappa$ B in the nucleus, inducing inflammation<sup>34</sup>. Adhesion molecules, expressed at increased levels in the endothelium during inflammatory conditions, and chemokines expressed along a gradient in the damaged tissue are needed for the adhesion, homing, and subsequent transmigration of blood-born leukocytes to the site of damage. The coagulation system is activated, leading to fibrinogen accumulation in the tissue. Micro-occlusions may appear in the capillary circulation. Later, various kinds of leukocytes, primarily neutrophils, migrate from the widened capillaries to the damaged tissue. The activated vascular endothelium may selectively permit leukocytes to pass while the erythrocytes are kept in the vessel, at least as long as the endothelium is intact. The type of leukocytes accumulating at the inflammatory site is largely dependent on the type of adhesion molecules expressed on endothelial and surrounding tissue cells. Granulocytes and macrophages gather to phagocytose bacteria and dead tissue, while lymphocytes are responsible for the formation of antibodies. Activated neutrophils have the capability to disarm or kill pathogens by the release of toxins, radical oxygen species (ROS), radical nitrogen species and different enzymes. Unfortunately, this affects not only pathogens but also the surrounding tissue<sup>33</sup>.

After the initial phase, necrotic tissue and dead microbes, leukocytes, and other waste material are removed from the organ and, if needed, new vessels start to

form. New cells are created and scar tissue may form. A successful repair phase is mainly mediated by tissue macrophages, which regulate the shift from pro-inflammatory to anti-inflammatory environment by the release of resolvines, protectines, and various growth factors<sup>26</sup>. A controlled inflammatory reaction in the case of damage or disease is beneficial, but the balance is disturbed when the inflammation for some reason becomes dysregulated, like e.g. in septic chock. There is no clear-cut delimitation between beneficial and harmful outcome. Chronic inflammation evolves when resolution of the initial inflammatory phase fails, e.g. because the inflammatory agents cannot be removed, or because the inflammatory reaction is too intense with degeneration of tissue as a result<sup>26</sup>. The molecular mechanisms behind chronic systemic inflammation are not completely understood, but a dysregulated homeostasis in the diseased tissue seems to be involved.

## Inflammation in diabetes

Chronic inflammation is characteristic for several chronic diseases like diabetes. Type 1 diabetes (T1DM) is an autoimmune disease distinguished by autoantibodies associated with local inflammation, insulinitis, and the destruction of pancreatic insulin-producing beta-cells, but lately accumulated evidence indicate that there might also be systemic inflammation in T1DM<sup>35,36</sup>. In type 2 diabetes (T2DM), which is characterized by a relative insulin resistance later followed by decreased insulin production, the scenario is confounded by the metabolic syndrome, in which several factors may contribute to inflammation. Hyperglycemia, obesity, dyslipidemia, deficient fiber intake, and decreased muscle mass may all contribute to an increased systemic inflammation<sup>37</sup>. Common for all types of diabetes is the presence of pro-inflammatory factors that may also be relevant for the pathogenesis of DR. A broken iBRB due to a damaged endothelium leads to exudation of several inflammatory mediators, K<sup>+</sup> ions, and adenosine triphosphate (ATP), that may all activate the AGE receptor (RAGE) and/or toll-like receptors (TLRs), thereby eliciting a powerful inflammatory response<sup>26</sup>. Hyperglycemia *per se* also leads to increased formation of AGE and activation of the AGE/RAGE complex<sup>38</sup>. Damage of the vessel endothelium also leads to the leakage of plasma proteins and platelets<sup>26</sup>. Factor XII from plasma may in contact with collagen and ECM products become activated and elicit the kallikrein-kinin and the coagulation cascades, and activated platelets release inflammatory mediators such as serotonin and thromboxan<sup>26</sup>. Hyperglycemia further activates several alternative glucose metabolic pathways, which contribute both to increased oxidative stress and to inflammation<sup>21,22,24,27,31,38-43</sup>.

## The eye- an immunoprivileged zone

The eye has an immunoregulatory system, which has been described as immune privilege<sup>44</sup>. This phenomenon is best characterized for the anterior part of the eye. Implants and allografts survive better within an immunoprivileged zone, such as the cornea or anterior chamber of the eye, whereas a similar implant or graft would be rejected within a non-privileged zone, e.g. the skin<sup>44-46</sup>. Immunoprivileged sites have been demonstrated in the eye, the brain, and the testes<sup>44</sup>. Immune privilege is complex, involving both physical barriers and immunological regulations, which make cells of the immune system tolerant to a specific antigen. It also involves the production of immunomodulatory cytokines and neuropeptides, functionally unique antigen-presenting cells (APC) in the iris and the retina, complement inhibitors, and probably several yet unknown factors<sup>44-46</sup>. The immune privilege of the retina is largely undefined<sup>44</sup>, but is dependent on both physical properties like the inner and outer BRBs<sup>47</sup>, and biochemical compounds like the naturally anti-inflammatory pigment epithelial derived factor (PEDF) released from the retinal pigment epithelial layer (RPE). Further, transforming growth factor- $\beta$  (TGF- $\beta$ ), as well as the cell death receptor/ligand pair Fas/Fas ligand, may be of special interest in the regulation of ocular neovascularization<sup>31</sup>, and probably also in vessel permeability regulation and leukocyte adhesion<sup>22</sup>. The outer BRB, i.e., the RPE, seems to be particularly important for the maintenance of the retinal immune privilege due to its ability to express class II major histocompatibility complex (MHC) molecules, suggesting that the RPE is able to interact with T-cells to induce antigen tolerance<sup>44,48</sup>. The retina is devoid of well-defined lymphatics but abundant in APC, and resident microglia can undergo changes and migrate in response to various stimuli<sup>44</sup>. It is possible that diabetes-induced disturbances in the immune privilege, e.g. down-regulation of the anti-inflammatory cytokines PEDF or TGF- $\beta$  in addition to a physical break-down of the iBRB, might be of relevance for DR pathology<sup>31</sup>.

## Major pathogenetic pathways and inflammation in diabetic retinopathy

Low-grade, chronic inflammation is proposed to be a key factor in the pathogenesis of DR<sup>21,22,49-51</sup>. The polyol pathway, increased PKC activation, increased formation of AGE and increased oxidative stress may all evoke pro-inflammatory and pro-apoptotic intracellular signaling pathways<sup>38</sup>. Endothelial activation, resulting in up-regulation of inflammatory mediators and adhesion molecules<sup>52</sup> as well as accumulation of sticky leukocytes in retinal vessels (leukostasis), may lead to areas of non-perfusion and ischemia, ultimately causing apoptosis or proliferation of various retinal cell types<sup>24,51,53,54</sup>, e.g. loss of pericytes and endothelial cells, followed by uncontrolled growth of endothelial cells. Inflammation and endothelial dysfunction also lead to increased permeability, with loss of the iBRB integrity and extravasation of fluid, lipids and proteins into the extracellular space. The contact between plasma proteins and components of ECM may evoke intense inflammatory signaling as previously described<sup>26</sup> and hyperglycemia-induced local up-regulation of the renin angiotensin system (RAS) may start a vicious cycle<sup>43</sup>.

Leukostasis in diabetes, i.e., accumulation and adhesion of leukocytes to the vessel wall, and an increased expression of adhesion molecules, primarily intercellular adhesion molecule-1 (ICAM-1), has been demonstrated in several studies<sup>53,55-59</sup> and may be associated with insufficient perfusion<sup>60</sup>. Diabetes-induced leukostasis was found to be associated with increased permeability, which could be attenuated by the administration of anti-ICAM-1-antibodies<sup>53</sup>, further stressing the involvement of adhesion molecules in inflammation in DR. Leukocytes from diabetic subjects may have altered properties. One study on diabetic rats demonstrated increased expression of neutrophil surface integrins, responsible for the anchoring of leukocytes to adhesion molecules on endothelial and tissue cells<sup>61</sup>. Increased leukostasis in diabetes might have several implications for the pathogenesis of DR. Micro-obliterations, which occur in the small retinal vessels and cause decreased perfusion and increased ischemia, has been suggested as one mechanism. Leukocyte-mediated breakdown of the endothelial tight-junctions or direct endothelial cell damage is another explanation. Leukocytes may also be involved in later neovascularization, since monocytes have been found in the tips of sprouting new vessels in experimental neovascularization<sup>22</sup>. In one study, delayed clearance of leukocytes from the circulation was demonstrated in diabetes, although leukocyte accumulation in tissues was actually decreased<sup>62</sup>. Leukocyte migration, chemotaxis, and phagocytosis, as well as bacterial killing are also dysfunctional in diabetic subjects<sup>62</sup>. Increased glycosylation may be one reason for increased leukocyte stickiness or leukocyte dysfunction in diabetes<sup>63</sup>. The exact mechanisms

by which leukocytes or leukostasis would be of relevance for the development or progression of retinopathy in diabetes are not known.

### **The polyol pathway**

When there is an excess of glucose in the cellular cytosol, glucose metabolism may be shifted into the polyol, or sorbitol, pathway. Glucose is first reduced to sorbitol in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction by the enzyme AR, which is the rate-limiting enzyme of the polyol pathway. During normal circumstances, AR is responsible for the inactivation of toxic aldehydes formed during cellular metabolism<sup>64,65</sup>. Virtually all human retinal cell types affected in diabetes contain AR<sup>65</sup>. Polymorphism in the AR gene promoter region is associated with increased vulnerability to and faster progression of DR<sup>66</sup>. The polyol pathway leads to an accumulation of sorbitol, which in large quantities may cause osmotic damage to the cell<sup>24,38,65,67</sup>. However, osmosis seems to be of minor importance in DR. In the next step, sorbitol is transformed into fructose and hydrogen by sorbitol dehydrogenase (SDH) in a NAD<sup>+</sup>-dependent reaction<sup>65,67</sup>. The final product of this chain, fructose, functions as a substrate for AGE<sup>38,65,67</sup> and the increased production of NADPH may lead to increased formation of glyceraldehyde 3-phosphate involved in production of AGE<sup>24</sup> and PKC<sup>67</sup>. The polyol pathway also depletes the sources of NAD<sup>+</sup>, thereby decreasing the cellular formation of the anti-oxidative enzyme glutathione (GSH) and possibly affecting other enzymes necessary for the cellular anti-oxidative defense<sup>38,64,65,67</sup>. Thus, the polyol pathway increases the production of AGE and exposes the cell to oxidative stress. Lately, it has also been proposed that products of the polyol pathway may act directly on mitogen-activated protein kinases (MAPKs) and poly-adenosine diphosphate ribose polymerase (PARP) in the hyperglycemic cell, thereby directly increasing cellular oxidative stress as well as activating inflammatory signaling systems<sup>24,67</sup>. Also, administration of aldose reductase inhibitors (ARI) has been demonstrated to reduce the nuclear translocation of the inflammatory transcription factor NF- $\kappa$ B, PKC activation, adhesion molecule expression and neutrophil activation<sup>68</sup>, further supporting the role of AR in hyperglycemia-induced inflammation.

Experimental studies on ARI in diabetic animals have shown promising results, especially on the rate of retinal pericyte drop out. ARI administration diminishes pericyte apoptosis, endothelial cell death, complement deposition, Müller cell reactivity, and neuronal cell death in diabetic rats<sup>65</sup>. AR knockout (KO) mice were found to have less ischemia and diabetes-induced retinal damage<sup>69,70</sup>. It has also been demonstrated that polymorphisms in the AR gene are linked to increased susceptibility of microvascular complications<sup>71-73</sup>. There has been only one large study on the effects of ARI on humans with DR, the Sorbinil Retinopathy Study<sup>74</sup>. Due to toxic effects demonstrated in earlier, smaller clinical studies<sup>65</sup>,

much lower doses than those given in experimental studies were administered. At those low doses, there was no reduction in retinopathy progression after three years of follow-up<sup>74</sup>. Recently, a new selective ARI, ARI-809, was developed and has shown positive effects on diabetes-induced retinal vascular changes in rats<sup>65,75</sup>. It has been proposed that the actual benefits of ARI on DR in animals were due to anti-inflammatory effects of the drug rather than the AR inhibition *per se*, because anti-inflammatory effects of ARI have been demonstrated also during normoglycemic conditions<sup>21</sup> and in retinal ischemia-reperfusion<sup>76</sup>.

### **Protein kinase C**

PKC is a family of signal transducers for a variety of growth factors, hormones, neurotransmitters, and cytokines<sup>77</sup>. Increased levels of glucose lead to increased *de novo* synthesis of diacylglycerol (DAG), a potent activator of PKC<sup>24</sup>. PKC may also be activated by an increased NADH/NAD<sup>+</sup> ratio<sup>68</sup>, and thus PKC activation signaling systems increase in diabetic cells<sup>38,77</sup>. Out of at least twelve isoforms of PKC, PKC- $\beta$  has been demonstrated as the most important isoform in DR, e.g. by causing increased vascular permeability, reduced vascular regular tone, increased leukocyte adhesion, increased induction of the angiogenic growth factor VEGF<sup>24,77</sup>, and increased formation of ECM components<sup>38</sup>. Activation of PKC may also result in increased smooth muscle contractility<sup>77</sup> by decreasing vasodilator endothelial nitric oxide synthetase (eNOS) and increasing vasoconstrictor ET-1<sup>64</sup>. PKC is involved in pro-inflammatory NF- $\kappa$ B activation and subsequent cytokine up-regulation, promotes increased MAPK and PARP activity, and stimulates neutrophil superoxide release<sup>68</sup>. The PKC-inhibitor Ruboxistaurin (LY333531, RBX) is a highly specific inhibitor of the PKC- $\beta$  isoform, and has shown good effects on diabetes-induced retinal edema in diabetic animal models<sup>77</sup>. Phase III-studies on humans have shown beneficial effects on visual acuity over time<sup>78,79</sup>, especially in subjects with DME, who also needed less focal laser treatment. Retinopathy progression was however not halted by RBX.

### **Advanced glycation endproducts – the AGE/RAGE axis**

Glycation, a process in which glucose residues are coupled to proteins, lipids, and deoxyribonucleic acid (DNA), is a normal consequence of aging. Reducing sugars react with free amino groups to first create reversible Amadori products, then irreversible products with the formation of crosslinks<sup>80</sup>. The rate of glycation is grossly accelerated during chronic hyperglycemia, and especially under the conditions of oxidative stress<sup>23,81</sup>. AGE clearance via the kidneys may also be perturbed in case of diabetic kidney dysfunction<sup>81</sup>. Dicarbonyls formed at an accelerated rhythm during chronic hyperglycemia are more prone to glycation compared to glucose, which leads to intracellular formation of AGE with

subsequent intracellular signaling activation and modification of intracellular proteins<sup>81</sup>. Intracellular production of AGE has been proposed to damage cells by gene transcription alterations, cell-matrix interaction disruption, and finally AGE-receptor activation<sup>64,81</sup>.

Several AGE-binding molecules have been described and many of the adverse effects caused by advanced glycation are thought to be mediated via AGE-receptors, RAGE being by far the best characterized<sup>80</sup>. AGE/RAGE binding elicits powerful inflammatory signaling cascade systems like PKC-activation, tyrosine phosphorylation, NF- $\kappa$ B transcription, up-regulation of ICAM-1, and leukostasis<sup>21,80,81</sup>. AGE also have the capacity to stimulate monocyte migration<sup>68</sup>. The precise pathogenetic role of AGE in DR is not completely understood. AGE promotes pericyte apoptosis, probably due to a propensity for pericytes to accumulate AGE resulting in impaired phospholipid metabolism and protein production, while endothelial cells exposed to AGE become primarily pro-angiogenic, at least partially explained by AGE-induced VEGF up-regulation<sup>24,80,81</sup>. ECM proteins become glycated and AGE accumulation may contribute to basement membrane thickening<sup>81</sup>. Neuroglia are also sensitive to high levels of AGE<sup>80</sup>. An association between serum levels of AGE and retinopathy occurrence has been demonstrated in both T1DM and T2DM subjects<sup>82,83</sup>. RAGE is physiologically present in a soluble form, which captures AGE and thereby efficiently decreases the action of the AGE/RAGE complex. Administration of the soluble receptor has shown promising effects on vessel damage in diabetic animals<sup>81,84</sup>, but so far effects in humans have not been published. An important pharmacological strategy for inhibition of the chemical reaction that causes AGE formation came up with aminoguanidine, which is a potent inhibitor of AGE-mediated crosslinking. Aminoguanidine has been shown to prevent the formation of retinal microaneurysms, acellular capillaries, and pericyte loss in diabetic animals<sup>85-88</sup>. Placebo-controlled clinical trials have been conducted in T1DM and T2DM subjects, demonstrating reduced retinopathy progression<sup>89</sup>, but further clinical evaluation has been limited as a result of concerns over long-term toxicity<sup>81</sup>. A different approach would be to break up crosslinks that have already formed<sup>90,91</sup>. The AGE-inhibitor ALT-711 has shown promising effects in subjects with diabetic cardiomyopathy, but its effect in DR has not yet been evaluated<sup>80,92</sup>. Attempts have also been made to scavenge the pre-AGE substrates i.e., the reversible Amadori products, but scavengers have not yet been tested regarding retinopathy, either in animal or human studies<sup>80</sup>.

## **Hexosamine**

Increased flux of glucose through glycolysis leads to increased formation of fructose-6-phosphate, which may cause up-regulation of mediators interfering with

gene transcription factors, ultimately resulting in altered gene transcription<sup>64,93</sup>. Increased activation of the hexosamine pathway has been demonstrated in diabetic glomerulopathy and cardiomyopathy, but its role in retinopathy is not clear. Hexosamine activation has been suggested to contribute to RAS up-regulation in diabetic retina<sup>43</sup>, yet another way to elicit retinal inflammation.

### **The renin angiotensin system (RAS)**

Control of hypertension has been recognized as an important measure to delay the progression of DR as shown in the United Kingdom Prospective Diabetes Study (UKPDS)<sup>19</sup>. However, the preferable type of antihypertensive treatment for DR remains debatable. In the UKPDS, the progression of microvascular endpoints did not differ regarding retinopathy endpoints in the group treated with angiotensin converting enzyme (ACE) inhibitors compared to the group treated with  $\beta$ -blockers<sup>20</sup>. The Diabetic Retinopathy Candesartan Trial (DIRECT) study, which compared the angiotensin II-receptor blocker candesartan with placebo, declared a barely significant effect on the incidence of DR, but failed to show any effect on the progression rate when adjusting for the blood pressure effect<sup>94,95</sup>. A recent study declared significantly beneficial effects of enalapril and losartan, both interfering with RAS, on progression of DR. However, no other antihypertensive compound was used in the study, and data were not adjusted for blood pressure effects<sup>96</sup>. Treatment with losartan or captopril has been demonstrated to decrease diabetes-induced leukostasis as well as the formation of acellular capillaries and pericyte ghosts in diabetic rats and mice<sup>97,98</sup>. Local angiotensin II synthesis is increased in diabetic end organ injury, demonstrated in both the kidney and the retina, and may be coupled to increased hexosamine shunting or increased formation of succinate through the citric acid cycle in hyperglycemia, either stimulating pre-formed renin release or directly affecting angiotensinogen gene expression<sup>43</sup>. Vitreous accumulation of plasma pro-renin from the systemic circulation, and increased intraocular levels of angiotensin and ACE have been documented in association with DR<sup>43</sup>. In addition, RAS may be involved in induction of oxidative stress through increased ROS formation, advanced glycation, PKC activation and polyol shunting<sup>43</sup>. RAS is thus possibly involved in the development and/or progression of DR.

### **Evidence of inflammation in diabetic retinopathy**

In humans, biochemical study opportunities of DR are limited. The presence and/or association of inflammatory mediators can be analyzed in serum/plasma, aqueous humor and vitreous of diabetic subjects with various degrees of DR, but access to the retina is limited to post-mortem material. Several studies have demonstrated an association between inflammatory mediators in serum or plasma and DR, although

the impact of various inflammatory mediators may vary<sup>99-105</sup>. Reports on serum levels of adhesion molecules have been very inconsistent. Some studies have demonstrated increased serum levels of adhesion molecules in diabetic subjects compared to healthy control subjects as well as correlation to DR severity<sup>106,107</sup>, while others have reported conflicting results<sup>108,109,110,102,111-114</sup>.

Analyses on aqueous humor are more attractive, allowing relatively easy access to intraocular study material, as well as access to a reasonably healthy control group. However, aqueous humor studies are relatively few. Funatsu et al. (2001) found increased aqueous humor levels of interleukin-6 (IL-6) in diabetic versus non-diabetic subjects and they were correlated to VEGF levels<sup>115</sup> and to the degree of DME<sup>116</sup>. Tashimo et al. found increased aqueous humor levels of leukocyte attractants migration inhibition factor (MIF) and monocyte chemoattractant protein-1 (MCP-1) in diabetic compared to non-diabetic subjects and they were correlated to the severity of retinopathy<sup>117</sup>. Lately, two studies demonstrating increased levels of various inflammatory mediators in aqueous humor from diabetic subjects have been published, supporting a pro-inflammatory intra-ocular status in subjects with DR<sup>118,119</sup>. The relevance of serum inflammatory mediators for progression of DR has been questioned<sup>103</sup>.

Vitreous sampling provides a better source for analysis because of its proximity to the retina. However, since vitrectomy is not regularly performed on healthy subjects, the control tissue often originates from people with other vitreoretinal diseases, or from cadavers, making comparisons complicated. Franks et al. demonstrated already in 1992 increased levels of interleukin-1 (IL-1), IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in pooled samples of vitreous and aqueous humor from subjects with PDR or uveitis compared to healthy cadaver controls<sup>120</sup>. Similar results have been reported in several later studies<sup>105,121-128,129,130</sup>. Some studies have reported increased vitreous levels of soluble adhesion molecules and leukocyte attractants in diabetic subjects compared to control subjects, but there are often also increased vitreous levels in subjects with other vitreoretinal diseases compared to cadavers<sup>121,130-134</sup>. Demircan et al.(2006) found increased levels of TNF- $\alpha$  and IL-1 in both vitreous and serum of diabetic subjects with PDR compared to vitreous cadaver control subjects and serum samples of healthy individuals<sup>135</sup>. Recently, increased vitreous levels of IL-6, TNF- $\alpha$ , sICAM-1 and sVCAM-1 from diabetic subjects with PDR compared to non-diabetic subjects were reported<sup>136</sup>. There are however other studies that report unaltered or even decreased levels of inflammatory mediators in the vitreous of diabetic compared to non-diabetic subjects<sup>137-139</sup>. Reasons for study discrepancies may be the different selection of inflammatory mediators analyzed, the severity of DR, and differing analytical techniques.

Hughes et al. have performed an immunohistochemical staining for ICAM-1 on post-mortem retina but found no difference between diabetic and non-diabetic subjects<sup>140</sup>. A few studies performed on diabetic epiretinal membranes have demonstrated up-regulation of adhesion molecules and leukocyte markers as well as increased NF- $\kappa$ B activity in these tissues<sup>137,141-146</sup>, but the number of study subjects and the availability of control groups are limited in those studies, making interpretation difficult.

Also, effects of anti-inflammatory treatments for DR using corticosteroids or non-steroidal anti-inflammatory drugs (NSAID) have been studied in humans. Repeated intravitreal injections of steroids was shown to decrease diabetes-induced vascular permeability and to reduce visual deterioration secondary to DME<sup>147</sup>. However, after a couple of years, intravitreal steroids were not superior to traditional laser treatment<sup>147,148</sup>. Moreover, steroid treatment was associated with severe side-effects i.e., cataract, glaucoma, and endophthalmitis<sup>148</sup>. NSAID have been demonstrated to reduce diabetes-induced capillary degeneration in diabetic rats<sup>149</sup> and dogs<sup>85</sup>, but treatment outcome in humans has been conflicting i.e., NSAID did not affect the course of DR in the ETDRS study<sup>150</sup> but postponed the development of retinal microaneurysms in the Dipyridamole Aspirin Microangiopathy of Diabetes (DAMAD) Study<sup>151</sup>.

In the present thesis, I have analyzed associations between inflammatory mediators and different stages of DR. In study I, the association between serum inflammatory mediators and PDR, independent of other known risk factors, was analyzed in a cohort of T1DM subjects. In study II, the levels of inflammatory mediators were analyzed in the vitreous of diabetic subjects with active PDR compared to non-diabetic subjects with other vitreoretinal diseases, as well as in the aqueous humor of diabetic subjects with no or NPDR compared to healthy subjects, thus enabling insight in the ocular domain of people with both late and early stages of DR. The clinical findings have been explored in experimental animal models. In studies III and IV, oxidative stress and inflammation in response to ischemia-reperfusion was studied in rats. In study V, endothelial activation and expression of VCAM-1 in the retinal endothelial vessel wall in early diabetes was studied in mice with differing genetic characteristics.

## Dyslipidemia and inflammation in diabetic retinopathy

The retina is a lipid dense tissue, which might be affected by lipid disturbances, but the role of dyslipidemia in DR has been little studied<sup>152</sup>. Lipid peroxidation may be an important source of ROS formation and can create a form of advanced lipoxidation products (ALE)<sup>80</sup>. Adipose tissue is considered an active hormonal organ, which may increase insulin resistance and it is the source of inflammatory cytokines such as TNF- $\alpha$  and IL-6<sup>37</sup>. Adipose tissue contains activated macrophages and contributes substantially to inflammation in vascular dysfunction by the release of inflammatory mediators. Levels of adiponectin, which has known anti-inflammatory and anti-apoptotic properties, decrease with increased obesity<sup>52</sup>. Normoglycemic individuals with obesity also demonstrate increased levels of ROS and inflammatory mediators, as well as decreased levels of adiponectin. Oxidized low-density lipoproteins (LDL) are also known to trigger inflammation, including up-regulation of adhesion molecules, chemokines, and angiogenic factors<sup>153</sup>. Subjects with dyslipidemia have been shown to have increased prevalence of lipoprotein deposits in the retina and particularly triglycerides, LDL cholesterol, and apolipoprotein B levels, seem to be positively associated with the severity of DR<sup>154</sup>. One large study on fibrates, a type of lipid-modulating agent, reported decreased need for laser treatment in diabetic subjects and slower retinopathy progression rate, although the incidence of new DR cases was not affected<sup>155</sup>.

### **Apolipoprotein E**

Apolipoprotein E (ApoE) is a glycoprotein composed of chylomicrons, very low density lipoprotein (VLDL), and LDL cholesterol. Its main task is to maintain plasma lipid homeostasis<sup>156</sup> and it is the principal lipid carrier in the central nervous system<sup>157</sup>. ApoE is involved in the development of obesity and insulin resistance, and its depletion in the ApoE deficient KO mice leads to hypercholesterolemia and atherosclerotic plaque formation<sup>158</sup>. ApoE may also be involved in the retinal regulation of docosahexaenoic acid (DHA)<sup>159</sup> and in the pathogenesis of dry AMD<sup>160</sup>, but its role in DR is unknown. In the present study we assessed a possible involvement of ApoE in retinal vascular activation by the use of ApoE<sup>-/-</sup> mice with or without diabetes (study V).

### **Docosahexaenoic acid (DHA)**

DHA is a major dietary omega-3-polyunsaturated fatty acid ( $\omega$ 3-PUFA), a structural lipid of retinal photoreceptors<sup>161,162</sup>, also present in substantial amounts in retinal endothelial cells<sup>163</sup>. DHA has been demonstrated to protect retinal photoreceptors from ischemia and oxidative stress as well as from inflammation<sup>164</sup>,

an effect that may be due to decreased nucleus translocation or phosphorylation of NF- $\kappa$ B<sup>31</sup>. It has been suggested that the administration of  $\omega$ 3-PUFAs may attenuate and delay DR in animals, but the role of DHA in the pathogenesis of DR has not been further studied. In study IV, we examined a possible anti-inflammatory effect of DHA administered before retinal ischemia-reperfusion in diabetic and non-diabetic rats.

### **Statins**

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) lipid-lowering drugs (statins) are widely used in macrovascular disease and have beneficial effects on cardiovascular risk in diabetic subjects, an effect that is at least partially anti-inflammatory<sup>165</sup>. The administration of statins has been demonstrated to reduce the histological effects of ischemia-reperfusion injury as well as increased expression of P-selectin and ICAM-1 in rat retinas<sup>166</sup>. In addition, statins seem to protect from iBRB breakdown in diabetic rats<sup>167</sup> and mice<sup>168</sup>, to decrease inflammation, and to increase the production of eNOS in brain tissue<sup>169</sup>. In diabetic subjects, statins have neither proven to decrease retinopathy progression nor to maintain visual acuity, although reduction of hard exudates has been reported<sup>170,171</sup>. There is one large trial currently enrolling subjects for the study of ophthalmological outcomes in diabetic subjects on statin therapy, Action to Control Cardiovascular Risk in Diabetes-EYE (ACCORD-EYE)<sup>172</sup>. In study IV, we examined a possible anti-inflammatory effect of pravastatin administered before retinal ischemia-reperfusion in diabetic and non-diabetic rats.

## Ischemia, oxidative stress and inflammation

A free radical is defined as any species that has one or more unpaired electrons<sup>173</sup>. It is a reaction prone molecule because of its desire to gain an electron from neighboring molecules. Under normal physiological conditions, approximately 0.5-5% of the oxygen consumed in the body is transformed into ROS. ROS are primarily formed through mitochondrial respiration, which generates energy and fuels ATP production, ion transport, and metabolism in a series of oxidative reactions<sup>174</sup>. Excess in ROS activity creates oxidative stress. Increased levels of ROS may lead to protein and lipid oxidation, DNA strand breakage, damage to membrane ion transporters and other membrane components, and depletion of NAD<sup>+</sup> and ATP<sup>29,175</sup>. The retina has a defense system of anti-oxidative enzymes for ROS detoxification. Superoxide dismutases (SOD) convert the superoxide radical to hydrogen peroxide. Next, hydrogen peroxide is converted to water and oxygen by catalase and glutathione peroxidase (GPx1) in the presence of GSH. The catalytic subunit of glutamylcysteine ligase (GCLc) is needed for the *de novo* synthesis of GSH<sup>41,42,176</sup>. Other ROS are the hydroxyl radicals, and in addition to ROS, reactive nitrogen species may also contribute to oxidative stress.

Ischemia is a feature of several retinal conditions, such as arterial occlusion, venous occlusive disorders, retinopathy of prematurity and DR<sup>29</sup>, but despite substantial work, the contribution of ischemia to those diseases is not fully understood<sup>177</sup>. In DR, ischemia is likely to be due to micro-occlusions and the formation of acellular vessel strands, which no longer can supply blood to the tissue<sup>177</sup>. In addition to deprivation of oxygen and nourishment, ischemia leads to waste accumulation, disruption of cellular energy metabolism, and increased oxidative stress as well as induction of inflammation<sup>29,178-180</sup>. Increased lipid oxygenation is another event in retinal ischemia possibly aggravating both oxidative stress and inflammation<sup>25,175</sup>. Reperfusion after initial ischemia may paradoxically increase tissue damage, possibly due to increased neurotransmitter toxicity or ROS formation<sup>181-183</sup>. Increased ROS formation may occur in diabetes by several mechanisms. Due to hyperglycemia-derived electron-donors from the citric acid cycle, hyperglycemia can disrupt mitochondrial function and substantially increase superoxide production through the mitochondrial electron transport chain<sup>40</sup>. ROS may also increase in diabetes through increased polyol pathway shunting, increased PKC activation, and AGE protein alterations<sup>175</sup>. Superoxide leads to direct damage on the mitochondrial and cellular lipids, further aggravating the vicious cycle of oxidative stress.

Animal studies have demonstrated that oxidative stress may contribute to DR<sup>41</sup> and over-expression of SOD seems to inhibit retinopathy development in

diabetic rodents<sup>184,185</sup>. In humans however, studies regarding oxidative stress in diabetes have given conflicting results<sup>23</sup> and intervention with antioxidants have been disappointing, suggesting that oxidative stress is not the major driving force in the pathogenesis of DR<sup>25</sup>, which was also supported by another study of ours on retinal pericytes<sup>186</sup>. In studies III and IV, I have explored the retinal expressions of anti-oxidative enzymes and inflammatory mediators in ischemia-reperfusion injury and/or diabetes.

## Inflammatory mediators in the present thesis

In the present thesis, I have studied the various cytokines IL-1 $\beta$ <sup>21,187</sup>, IL-6<sup>188</sup>, interleukin-8 (IL-8)<sup>189,190</sup>, TNF- $\alpha$ <sup>191,192</sup>, TNF-receptor 1 (TNFR1)<sup>191</sup>, TNF-receptor 2 (TNFR2)<sup>191</sup>, FGF- $\beta$ <sup>30</sup>, VEGF<sup>193</sup>, and Caspase-1<sup>194</sup>, adhesion molecules vascular cell adhesion molecule-1 (VCAM-1)<sup>195,196</sup>, ICAM-1<sup>195,196</sup> and P-selectin<sup>197</sup>, as well as the leukocyte chemoattractants granulocyte monocyte colony stimulating factor (GM-CSF)<sup>190</sup>, MCP-1<sup>190</sup>, Regulated on Activation, Normal T Expressed and Secreted (RANTES)<sup>198</sup>, in humans in studies I and II and/or in animals in studies IV and V. A panel of inflammatory mediators and their basic functions is available in Table 1. Anti-oxidative enzymes analyzed in study III were manganese superoxide dismutase (MnSOD)<sup>199,200</sup>, copper zinc superoxide dismutase (CuZnSOD)<sup>199-201</sup>, responsible for the conversion of the superoxide ion to hydrogen peroxide, catalase<sup>199</sup>, which together with glutathione peroxidase (GPx1)<sup>202,203</sup> converts hydrogen peroxide to oxygen and water in the presence of glutathione (GSH)<sup>176,199</sup>, and the catalytic subunit of glutamylcystein ligase (GCLc)<sup>176</sup> is needed for synthesis of GSH.

Table 1. Panel of inflammatory cytokines, chemokines, growth factors and receptors analyzed in studies I, II, IV and V.

<b>Abbreviation</b>	<b>Full name</b>	<b>Basic functions</b>
IL-1 $\beta$	Interleukin 1 beta	Acute-phase protein induction Apoptosis Bacterial defense Bone marrow activation Chemokine induction Fever IL-6 induction (Inflammation suppression) T- and B-cell activation
IL-6	Interleukin 6	Acute-phase protein induction B-cell maturation Chemokine-directed leukocyte recruitment Modulation of adhesion molecule expression Regulation of leukocyte apoptosis T-cell homing and adhesion control
IL-8/CCL8	Interleukin 8	Angiogenesis Chemokine-directed leukocyte recruitment Hypoxia defense Neutrophil chemotaxis
TNF- $\alpha$	Tumor necrosis factor alpha	Acute-phase protein induction Apoptosis Cell proliferation Cell survival

		Immune defense Pro-inflammatory trigger Regulation of NOS expression and/or activation
FGF- $\beta$ / FGF-2/ bFGF	Fibroblast growth factor beta	Angiogenesis Endothelial cell migration and proliferation Tissue repair
GM-CSF	Granulocyte monocyte colony stimulating factor	Granulocyte and monocyte stimulation and maturation
MCP-1	Monocyte chemotactic protein 1	Monocyte, T cell and dendritic cell recruitment
VEGF	Vascular endothelial growth factor	Development, maintenance and remodeling of the vasculature Enhanced microvascular permeability Monocyte migration
RANTES/ CCL5	Regulated on Activation, Normal T Expressed and Secreted	Chemoattractant for lymphocytes, granulocytes, eosinophils and dendritic cells Macrophage activation Microbial defense
TNFR1	TNF receptor 1	Activation of kinase systems and NF- $\kappa$ B DNA fragmentation and apoptosis Primarily proinflammatory and proliferative signaling
TNFR2	TNF receptor 2	Apoptosis Counteractions of TNFR1 Endothelial cell activation
P-selectin	P -selectin	First contact between endothelial cells and leukocytes and platelets

VCAM-1	Vascular cell adhesion molecule 1	Firm adhesion of leukocytes to vascular endothelium Early marker of endothelial dysfunction
ICAM-1	Intercellular adhesion molecule 1	Firm adhesion of leukocytes, vascular endothelial cells, other cell types
Caspase-1	Caspase 1	Pro-inflammatory cytokine up-regulated very early , responsible for cleavage of other cytokines

# AIMS

- I. To study the association between inflammatory mediators in serum and PDR in T1DM subjects.
- II. To study associations between inflammatory mediators in the vitreous and advanced stages of retinopathy as well as between inflammatory mediators in the aqueous humor and early stages of retinopathy in diabetic subjects.
- III. To study the mRNA expressions of anti-oxidative enzymes in ischemic retinas of rats.
- IV. To study the mRNA expressions of inflammatory mediators in ischemic and non-ischemic retinas of diabetic and non-diabetic rats pretreated with lipid modulating compounds.
- V. To study early endothelial activation of VCAM-1 in retinas of diabetic and non-diabetic genetically modified (ApoE<sup>-/-</sup>, TNF- $\alpha$ <sup>-/-</sup>, ApoE<sup>-/-</sup>/TNF- $\alpha$ <sup>-/-</sup>) mice.

# SUBJECTS

## Human subjects (studies I and II)

Study I included 128 T1DM subjects, 69 males and 59 females with different degrees of retinopathy (66 with PDR and 62 without retinopathy or with NPDR) recruited from the local register Diabetes 2000 in Malmö, Sweden. Classification of T1DM in this register is based on the presence of glutamic acid decarboxylase (GAD) antibodies. Study I subject characteristics are presented in Table 2. Study II included 26 diabetic subjects (14 with T1DM and 12 with T2DM) with PDR and 27 non-diabetic control subjects undergoing vitrectomy for vitreoretinal diseases other than PDR, as well as 22 diabetic subjects (all with T2DM) without retinopathy or with NPDR and 18 non-diabetic control subjects undergoing cataract surgery. Study II subjects were considered to have T1DM if age at onset of disease was  $\leq 30$  years and insulin treatment was necessary within one year of diagnosis, otherwise to have T2DM. Study II subject characteristics are presented in Table 3. For both studies, initial clinical assessment included a medical history of concomitant diseases, medication, smoking habits, body height and weight, waist circumference, and blood pressure measurements. Subjects were considered hypertensive if the systolic blood pressure was  $>130$  mm Hg or diastolic blood pressure was  $>80$  mm Hg and/or if they had antihypertensive medication. The first morning urine specimen was collected for analysis of urinary albumin concentration from all subjects. Subjects were considered to have albuminuria if urinary albumin was  $\geq 0.020$ g/L and/or if they were treated with ACE-inhibitors or angiotensin II receptor blockers. None had nephropathy requiring dialysis or transplantation. Those who had had myocardial infarction and/or cerebrovascular insult were considered to have macrovascular disease.

Blood was collected for measurements of glycated hemoglobin ( $HbA_{1c}$ ), plasma creatinine, and serum levels of inflammatory mediators, and urine samples were collected for analysis of urinary albumin concentration. In study II, vitreous was collected for the analysis of vitreous inflammatory mediators, total protein and hemoglobin (Hb), and aqueous humor was collected for the analysis of inflammatory mediators. The inflammatory mediators analyzed in study I were high-sensitive C-reactive protein (hsCRP), IL-1 $\beta$ , IL-6, TNF- $\alpha$ , sICAM-1, sVCAM-1 and P-selectin and in study II IL-1 $\beta$ , IL-6 and TNF- $\alpha$  for subjects

undergoing vitrectomy and IL-6, IL-8, TNF- $\alpha$ , TNF-R1, TNF-R2, MCP-1, GM-CSF, RANTES, FGF- $\beta$  and VEGF for subjects undergoing cataract surgery.

Retinopathy grading was performed on study I subjects and on study II subjects undergoing cataract surgery based on seven standard field fundus photographs according to the ETDRS<sup>204</sup>.

Table 2. Baseline characteristics of subjects in study I.

	<b>PDR (n=66)</b>	<b>No/NPDR (n=62)</b>	<b>p value</b>
D1TM/D2TM (n)	66	62	-
Sex (m/f, n)	32/34	37/25	0.206
Age (years)	49.5 (23.0-77.0)	40.0 (22.0-74.0)	0.035
Onset (years)	16.0 (4.0-38.0)	21.5 (1.0-59.0)	0.019
Duration (years)	31.5 (6.0-60.0)	21.0 (3.0-61.0)	<0.001
SBP (mm Hg)	134 (91-201)	136 (107-192)	0.473
DBP (mm Hg)	73 (54-98)	76 (31-101)	0.138
Hypertension (n)	59	54	0.688
BMI (kg/m <sup>2</sup> )	24.1 (18.8-48.4)	24.1 (17.5-32.8)	0.985
Waist (cm)	96.0 (73.0-129.0)	93.0 (79.0-122.0)	0.378
HbA1c (%)	8.0 (4.5-14.8)	7.4 (5.1-11.1)	0.070
Smoking (n)	13	9	0.439
DME (n)	16	12	0.392
Albuminuria (n)	43	21	<0.001
Macrovascular disease (n)	13	7	0.192

Values are expressed as median (range) unless otherwise specified. Mann-Whitney U-test.

Table 3. Baseline characteristics of subjects in study II.

	<b>Vitreous PDR (n=26)</b>	<b>Vitreous control (n=27)</b>	<b>p value</b>	<b>Aqueous No/NPDR (n=22)</b>	<b>Aqueous Control (n=18)</b>	<b>p value</b>
<b>D1TM/ D2TM</b>	14/12	-	-	0/22	-	-
<b>Retinopathy</b>	PDR (26)	-	-	No (18) Mild (2) Moderate (2) Severe (0) PDR (0)	-	-
<b>Op. Diagnosis</b>	VH (22) EM (3) Cataract Debris (1)	VH (1) EM (2) Cataract Debris (1) Macular hole (7) RD (16)	-	Cataract (22)	Cataract (18)	-
<b>Sex (m/f)</b>	15/11	13/14	0.491	10/12	11/7	0.320
<b>Age (years)</b>	55 (24-81)	65 (40-82)	0.010	71.5 (62-84)	72.5 (45-86)	0.882
<b>Onset (years)</b>	29 (5-65)	-	-	62 (38-64)	-	-
<b>Duration (years)</b>	20 (4-60)	-	-	10 (0-34)	-	-

	<b>Vitreous PDR (n=26)</b>	<b>Vitreous control (n=27)</b>	<b><i>p</i> value</b>	<b>Aqueous No/NPDR (n=22)</b>	<b>Aqueous Control (n=18)</b>	<b><i>p</i> value</b>
<b>SBP (mmHg)</b>	137 (90-200)	143 (99-193)	0.631	166 (136-187)	136 (108-155)	<0.001
<b>DBP (mmHg)</b>	76 (35-98)	79 (65-102)	0.098	82.5 (67-106)	77.5 (64-95)	0.157
<b>Hyper- tension</b>	17/9	10/17	0.041	14/8	8/10	0.046
<b>BMI (kg/m<sup>2</sup>)</b>	26.1 (19.8-42.1)	26.1 (18.6-35.5)	0.364	29.9 (23.3-39.7)	26.4 (17.8-30.1)	0.003
<b>Waist (cm)</b>	99 (79-176)	103 (80-161)	0.866	101 (83-133)	97 (69-111)	0.037
<b>HbA1c (%)</b>	7.6 (4.8-14.8)	4.5 (4.0-5.1)	<0.001	5.8 (4.9-5.3)	4.8 (4.4-5.6)	<0.001
<b>Smoking</b>	9/17	5/22	0.188	2/20	3/15	0.506
<b>Albumi- nuria</b>	24	9	<0.001	12	5	0.046
<b>Macro- vascular disease</b>	2/24	4/23	0.418	4/18	3/15	0.901

Values are expressed as median (range) or numbers of persons unless otherwise specified. Mann-Whitney U-test. Op.=Operation, VH= Vitreous Hemorrhage, EM = Epiretinal Membrane, RD = Retinal detachment

## Rats (studies III and IV)

Female Wistar rats (body weight 200-250 g) from Taconic (Lille Skensved, Denmark) were used in studies III (n=64) and IV (n=50). In study IV diabetes was induced in 50% of the animals by an intraperitoneal injection of streptozocin (STZ) (freeze-dried, Zanosar, Pharmacia-Upjohn, Kalamazoo, MI) (60 mg/kg body weight in 0.9% sodium chloride, yielding 100 mg of STZ and 22 mg citric acid/ml; pH 3.5-4.5). Only rats with a blood glucose value >15 mmol/L were included in study IV and kept for one month. In diabetic rats, mean glucose levels increased from  $5.9\pm 0.5$  (mean $\pm$ SD) mmol/L at baseline to  $28.5\pm 3.0$  mmol/L at the time of surgery. Mean blood glucose levels of non-diabetic control rats in study IV was  $6.0\pm 0.5$  mmol/L.

In study III whole retina was collected for analysis of the mRNA and protein expression levels of the endogenous antioxidant enzymes MnSOD, CuZnSOD, catalase, GPx1 and GCLc, and in study IV for analysis of the mRNA expression levels of the inflammatory mediators TNF- $\alpha$ , IL-6, Caspase-1, IL-1 $\beta$ , P-selectin, VCAM-1 and ICAM-1.

## Mice (study V)

In study V C57BL/6 wildtype (wt) (n=28) and ApoE<sup>-/-</sup> (n=29) mice purchased from Taconic (Lille Skensved, Denmark) and TNF- $\alpha$ <sup>-/-</sup> (B6, 129-Tnf<sup>emlGkl</sup>) mice from the Jackson Laboratory (Charles River, Sulzfeld, Germany) were used. TNF $\alpha$ <sup>-/-</sup> and ApoE<sup>-/-</sup> mice were intercrossed and F7 TNF $\alpha$ <sup>-/-</sup> (n=24) and F10 ApoE<sup>-/-</sup>/TNF $\alpha$ <sup>-/-</sup> (n=30) progeny were used in the experiments. At an age of 22 weeks, 50% of the mice received intraperitoneal injections of STZ (Sigma-Aldrich, Stockholm, Sweden; 60 mg in citrate buffer per kg body weight, pH 4.5) or vehicle (citrate buffer) once a day for 5 days. The numbers of mice included in the studies were 28 C57BL/6 wt (16 STZ and 12 vehicle), 29 ApoE<sup>-/-</sup> (17 STZ and 12 vehicle), 24 TNF $\alpha$ <sup>-/-</sup> (14 STZ and 10 vehicle) and 30 ApoE<sup>-/-</sup>/TNF $\alpha$ <sup>-/-</sup> mice (15 STZ and 15 vehicle). Retina was collected for immunohistochemical analysis of VCAM-1 in retinal vessels in wholemount retina and of cluster of differentiation 45 (CD45) positive leukocytes in retinal tissue sections, and for total real time reverse transcription-polymerase chain reaction (RT-PCR) analysis of messenger ribonucleic acid (mRNA) expression levels of TNF- $\alpha$  and VCAM-1.

# METHODS

## Analysis of inflammatory mediators in serum and vitreous (studies I and II)

The serum inflammatory mediator levels in study I and the vitreous and corresponding serum inflammatory mediator levels in study II were analyzed after centrifugation by Immulite 1000 (Diagnostic Products Corporation Scandinavia AB, Mölndal, Sweden), a highly sensitive, semi-automatic chemiluminescent technique using a single bead covered with antibodies against the desired protein. With this method the detection limits for the cytokines are for IL-1 $\beta$  5 pg/ml, IL-6 2.8 pg/ml, and TNF- $\alpha$  4 pg/ml.

Increased levels of a particular protein in the vitreous may emanate from an increased intraocular production or accumulation of plasma protein through leakage across the damaged iBRB. In order to estimate the intraocular protein production, cytokine levels were adjusted to the total protein in vitreous and serum according to the method of Hernández et al. (2004)<sup>205</sup> with the following algebra formula:

$$[X]_{\text{corrected}} = [X]_{\text{measured}}_{\text{vitreous}} * [\text{total protein}]_{\text{serum}} - [X]_{\text{measured}}_{\text{serum}} * [\text{total protein}]_{\text{vitreous}} / ([\text{total protein}]_{\text{serum}} - [\text{total protein}]_{\text{vitreous}})$$

The ratio between corrected intravitreal and serum levels was then calculated.

Measurement of vitreous Hb was performed by photometry (HemoCue® Plasma/low Hb Photometer; HemoCue AB, Ängelholm, Sweden). The detection range was 30-3000 mg/L. Vitreous Hb was detected in three specimens. Quantification of total protein levels of centrifuged vitreous (1:2) and serum (1:10) was performed by bicinchonic acid (BCA) colorimetric quantification (BCA Protein Assay Reagent Kit, Pierce; Rockford, IL, USA) according to the manufacturer's instructions.

## Analysis of inflammatory mediators in serum and aqueous humor (study II)

The aqueous humor and corresponding serum inflammatory mediator levels in study II were analyzed by Milliplex 26 multiplex array assay

[#MPXHCYTO60KPMX26 (premixed)] on a Luminex reader (Luminex 100™ IS, 200™, HTS; Invitrogen, Paisley, UK) according to the manufacturer's instructions. For RANTES, aqueous humor was diluted 1:100 in Assay Buffer as suggested by the manufacturer. The plate was run on the Luminex reader and data was analyzed by xMAP software on the Luminex device. This highly sensitive, semi-automatic chemiluminescent technique uses multiple beads for the detection of several antigens at the same time, allowing assessment of several different proteins in the sparse aqueous humor sample possible to extract from human subjects and has recently been assessed for aqueous humor measurements<sup>206,207</sup>.

## Ischemia-reperfusion (studies III and IV)

In studies III and IV, the left eye of each rat was submitted to ischemia-reperfusion to mimic retinal ischemia in humans. Retinal ischemia was induced under stereomicroscopic retinal blood flow observation (Wild M650, Heerbrugg, Switzerland) in anesthetized animals by ligation of the vessels and the accompanying optic nerve behind the left eye bulb<sup>208</sup> using a 5-0 silk suture (Ethicon, Sollentuna, Sweden). The ligature was gently tightened until complete cessation of the retinal blood flow was observed, and then maintained for 45 minutes. Reperfusion was established by removing the ligature resulting in a visibly restored blood flow, anesthesia was disconnected and no analgesics were administered. The animals were euthanized with CO<sub>2</sub> after 0, 3, 6 or 24 hours of reperfusion. After euthanization, each eye was immediately enucleated, the lens was removed and the retina was gently peeled off from the pigment epithelium, snap frozen on dry ice, and stored at -80°C for real time RT-PCR analysis in both studies and Western blot in study III. The right eye served as control.

## Simultaneous extraction of RNA and protein (study III)

The protein and RNA extraction protocol was performed according to our protocol for simultaneous extraction of mRNA and protein from limited amounts of tissue such as the rat retina<sup>209</sup>. A preexisting method<sup>210</sup> was modified by a step of ultrafiltration in an Amicon-15 ultrafiltration tube (Millipore, Molsheim, France) to separate DNA and protein.

## Real time RT-PCR (studies III, IV and V)

cDNA was synthesized from 2µg of RNA using the SuperScript II RNase H<sup>-</sup> RT (Invitrogen Life Technologies, Paisley, UK) protocol as described<sup>209</sup> in study III or 200 U RevertAid RNase H<sup>-</sup> RT (Fermentas, Helsingborg, Sweden) and 250 ng random hexamer (Amersham Biosciences, Uppsala, Sweden) primer in studies IV and V. mRNA levels were analyzed with the real time RT-PCR 7900HT system (Applied Biosystems, Stockholm, Sweden) using 5-10 ng of cDNA (depending on assay) as described by Applied Biosystems. In study III PrimerExpress 2.0 software (Applied Biosystems, Stockholm, Sweden) was used for design of primers and TaqMan probes (FAM-TAMRA). Each primer and probe set were selected to span over an intron-exon boundary, and blasted for specificity for the rat genome against the total genome data base<sup>211</sup> and the primer sequences are presented in study III. In studies IV and V TaqMan primers and probes were purchased from Applied Biosystems (assays on demand) and probes were dually labeled with 6-FAM (reporter) on the 5' end and TAMRA (quencher) on the 3' end. Cyclophilin b (Ppib) was used as endogenous control, as suggested by Stürzenbaum & Kille in 2001<sup>212</sup> in all three studies. Relative mRNA expression levels were calculated using the comparative cycle threshold (Ct) method as described below.

## Western blot (study III)

Separation of proteins was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories AB, Sundbyberg, Sweden). The gel was transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (0.2µm, Immune-Blot PVDF membrane, Bio-Rad Laboratories AB, Sundbyberg, Sweden) and incubated with commercially available antibodies and chemiluminescent detection solution (SuperSignal West Dura Extended Duration Substrate, Pierce, Rockford, IL, USA). Detection was performed using a Fuji LAS3000 Charge Coupled Device (CCD) camera (Fujifilm, Stockholm, Sweden). Since detection of GPx1 in one single retina was not possible it was necessary to pool extracts from four individual rats and hence analyses on all enzymes were performed on pooled retinas. A volume corresponding to 50µg total protein for each of 4 single-extracted samples was mixed to give a total volume of approximately 200-250µl. The chemiluminescent detection of the membranes was measured using MultiGauge v 2.2 software (Fujifilm, Stockholm, Sweden). Signal strength was given as arbitrary units (AU) for each target as well as the endogenous control of a pooled sample. Each pooled sample was normalized against the endogenous control by calculating the ratio between the AU value of the target and the AU value of the

endogenous control. Thereafter, the relative quantity for each target was obtained by calculating the ratio between the normalized value of the ischemic eye and the control eye. For quantification, we used the same comparative Ct-method as for mRNA, using ratios instead of delta ( $\Delta$ ) values.

## Immunofluorescence (study V)

Immunofluorescence was used in study V for detection of retinal vessel VCAM-1 expression in retinal wholemounts and of leukocytes in retinal tissue sections. We used a Zeiss LSM 5 Pascal scanning laser confocal microscope. By staining retinal tissue with anti-nucleic antibodies with one fluorophore, SYTOX Green (Invitrogen, Paisley, UK), with an emission wave length of 523 nm (green), and VCAM-1 (CD106; #M/K-2; Chemicon International, Inc., Millipore, Solna, Sweden) or leukocytes (CD45 directly coupled to allophycocyanin; I3/2.3, ab25519; Abcam plc, Cambridge, UK) with a different fluorophore, Cy5 (Jackson ImmunoResearch Laboratories, Charles River, Sulzfeld, Germany) with an emission wave length of  $>650$ nm (red), we were able to detect the tissue localization of VCAM-1 as well as tissue accumulation of leukocytes. In the retinal wholemounts used for VCAM-1 staining, VCAM-1 expression was detected mainly in vessels. In retinal tissue sections it is difficult to differentiate vessels from other cell types. Therefore, detection of leukocytes was assessed as the number of CD45 positive cells per retinal tissue area. Specificity of immune staining was confirmed by the absence of fluorescence in arteries incubated with either primary or secondary antibodies. For leukocytes, the spleen was used as positive control. At least three images were taken from each vessel branch, i.e. 46-129 pictures per wholemount retina for the detection of VCAM-1, and at least eight images per retinal tissue section, i.e.,  $\geq 24$  images per retina for the detection of CD45 positive cells. Images were subsequently analyzed for mean pixel intensity (range 0 to 255 grayscale values – after background subtraction) and vessel size, using the same settings for all images, thereby giving quantifiable relative expression levels.

# STATISTICAL METHODS

Non-parametric statistical analysis was performed using Wilcoxon Rank Sum test, Mann-Whitney U-test and Spearman correlation analysis when appropriate in all studies, except for PCR results of study III, in which parametric testing was performed. In study I, multiple logistic regression analysis was performed to separate individual determinants for PDR. For multiple comparisons, post hoc adjustment was performed. A *p*-value of <0.05 was considered statistically significant. All statistical calculations were executed on SPSS versions 12.0-15.0 for Windows (studies I, III, IV, V) or version 17.0 for Macintosh (study II). Illustrations in study V were performed using Graph Pad Prism 4.

## Multiple logistic regression analysis (study I)

In study I, multiple logistic regression analysis was performed on all factors which differed between subjects with PDR and subjects with no/NPDR to assess their individual strength for PDR. Logistic analysis was performed since the dependent variable (retinopathy) only could assume two values (PDR or no/NPDR). Independent variables, e.g. cytokine levels, blood pressure or tobacco use were also dichotomized because data were skewed. Since there was no *à priori* information on which level that was considered to be normal or abnormal for cytokines, the median value was used as cut off. In case of a known reference value, the upper reference value was chosen as cut off. Non-continuous variables were likewise dichotomized and subsequently analyzed in the same way. In a first step, the association of each factor with PDR was assessed in a univariate regression analysis. All significantly associated factors were then assessed in a multiple regression model, and only those factors that were still significantly associated with PDR were considered independent.

## Comparative Ct method (studies III, IV and V)

In real time RT-PCR relative quantification is calculated as gene expression changes in a given sample relative to a reference, in this case the endogenous control Ppib<sup>212</sup>. The relative mRNA expression levels of anti-oxidative enzymes in

study III and of inflammatory mediators in studies IV and V were calculated with the comparative cycle threshold (Ct) method as stated by Applied Biosystems (Stockholm, Sweden)<sup>213</sup>. This method compares the Ct value, extracted from the exponential PCR curve, of a given gene target with that of Ppib, which is expected not to change in the experimental setting. First the Ct value of the Ppib was subtracted from the target and control Ct values, yielding  $\Delta\text{Ct}$  values. Next, the  $\Delta\text{Ct}$  value of the control was subtracted from the  $\Delta\text{Ct}$  value of the target, hence yielding a  $\Delta\Delta\text{Ct}$  value. The time point for reaching the exponential phase for the PCR reaction, i.e., the Ct value, is different for each target depending on how much cDNA template that is available from start. This means that the higher the gene expression level is, the higher the cDNA template level will be and the sooner the Ct value will be reached. Because each cycle of PCR replication doubles the amount of DNA, the relative expression level is obtained by raising the  $\Delta\Delta\text{Ct}$  value to the negative power of 2 ( $2^{-\Delta\Delta\text{Ct}}$ ).

# RESULTS

## Human subjects (studies I and II)

PDR subjects in study I were older ( $p=0.035$ ), younger at diabetes onset ( $p=0.019$ ), had longer diabetes duration ( $p<0.001$ ), exhibited more frequent albuminuria ( $p<0.001$ ) and a higher intake of possible interfering medication (immunomodulating compounds) ( $p=0.027$ ) than no/NPDR subjects. In study II diabetic subjects in the vitrectomy group was younger ( $p=0.010$ ) than non-diabetic control subjects, whereas there was no age difference between diabetic and non-diabetic control subjects in the cataract group. Hypertension was more common in diabetic vitrectomy subjects ( $p=0.041$ ) and systolic blood pressure was higher in diabetic cataract subjects ( $p<0.001$ ) than in control subjects. BMI and waist circumference were higher in diabetic vitrectomy than in non-diabetic control subjects ( $p=0.003$  and  $p=0.037$ , respectively). The frequency of albuminuria was higher in diabetic subjects in both the vitrectomy and cataract groups compared to non-diabetic control subjects ( $p<0.001$  and  $p=0.046$ , respectively). The intake of possible interfering medication was lower in diabetic cataract than in non-diabetic control subjects ( $p=0.046$ ). Serum levels of inflammatory mediators in study I and II are presented in Table 4 a and b. Vitreous and aqueous humor levels of inflammatory mediators in study II are presented in Tables 5 and 6. Significant results are presented more in detail in the text below.

## Serum inflammatory mediator levels (studies I and II)

### *Serum IL-6*

Serum IL-6 was detectable in 19 PDR (28.8%) and in 10 no/NPDR subjects (16.1%) in study I, in 16 diabetic vitrectomy (61.5%), 14 control vitrectomy (51.9%), 21 diabetic cataract (95.5%) and in 18 control cataract (90%) subjects in study II. Serum IL-6 levels tended to be higher in PDR than in no/NPDR subjects ( $p=0.058$ ). Serum IL-6 levels did not differ between diabetic and non-diabetic subjects in any of the groups.

### *Serum TNF- $\alpha$*

Serum TNF- $\alpha$  was detectable in 52 PDR subjects (79%) and in 55 no/NPDR subjects (89.4%) in study I, in all 26 (100%) diabetic vitrectomy subjects, in 24 non-diabetic control vitrectomy subjects (88.8%) and in all 40 cataract subjects(100%) in study II. Serum TNF- $\alpha$  levels were higher in PDR compared to no/NPDR subjects ( $p=0.009$ ) in study I and remained an independent determinant marker for PDR in the multiple logistic regression analysis [Odds Ratio (OR)=2.385; 95% Confidence Interval (CI) 1.035-5.495;  $p=0.041$ ]. Serum TNF- $\alpha$  levels were higher in diabetic than in non-diabetic control subjects in both the vitrectomy and in the cataract group ( $p<0.001$  and  $p=0.021$ , respectively) and correlated in both cases with body mass index (BMI) ( $R=0.386-0.442$ ;  $p\leq 0.014$ ) in study II.

### *Serum TNFR2*

Serum TNFR2 levels were analyzed in cataract subjects in study II only and were higher in diabetic than in non-diabetic subjects, ( $p=0.047$ ). There was a positive correlation between serum TNFR2 levels and BMI ( $p=0.037$ ,  $R=0.303$ ).

### *Serum sVCAM-1*

Serum sVCAM-1 levels were analyzed in study I subjects only and were higher in PDR than in no/NPDR subjects ( $p<0.001$ ), but did not reach independent significance for PDR in multiple logistic regression analysis.

### *Serum P-selectin*

Serum P-selectin levels were analyzed in study I subjects only and were higher in PDR than in no/NPDR subjects ( $p=0.017$ ), but did not reach independent significance for PDR in multiple logistic regression analysis.

### *Serum FGF- $\beta$*

Serum FGF- $\beta$  levels were analyzed in cataract subjects in study II only and were higher in diabetic than in non-diabetic subjects ( $p=0.037$ ) and did not correlate with any systemic condition or medication.

## **Vitreous inflammatory mediator levels (study II)**

### *Vitreous IL-6*

IL-6 was detected in all vitreous samples and was higher in PDR compared to non-diabetic control subjects ( $p=0.021$ ). The ratio between intravitreal and serum levels of IL-6 was 55.5:1 in PDR subjects and 16.0:1 in non-diabetic control subjects. Vitreous IL-6 levels did not correlate with any systemic condition or any medication.

### *Vitreous TNF- $\alpha$*

TNF- $\alpha$  was detected in all vitreous samples and was lower in PDR than in non-diabetic control subjects ( $p=0.034$ ). The ratio between intravitreal and serum TNF- $\alpha$  levels was 2:1 in PDR and 3.4:1 in non-diabetic control subjects. Vitreous TNF- $\alpha$  levels did not correlate with any systemic condition or medication.

## **Aqueous humor inflammatory mediator levels (study II)**

### *Aqueous humor IL-6*

Aqueous humor IL-6 was detected in 21 no/NPDR (95.5%) and in 15 non-diabetic control (83.3%) subjects. Aqueous humor IL-6 levels were higher in no/NPDR than in non-diabetic control subjects ( $p=0.008$ ). Aqueous humor IL-6 levels did not correlate with any systemic condition or medication.

## **Possible interfering diseases**

To exclude any influence on inflammatory mediators of diseases or health conditions other than diabetes, information on concomitant diseases or health conditions (hypothyreosis, current infections, unspecified heart conditions, current pregnancy, malignancy, recent surgery, rheumatoid arthritis, or celiac disease) were included in the statistical analysis. There were no differences between PDR and no/NPDR subjects in study I or between diabetic and non-diabetic subjects in study II.

## **Possible interfering medication**

To exclude any interference of medication, the use of possible immunomodulating medication was included in the statistical model; aspirin or non-steroid anti-

inflammatory drugs, statins, methotrexate, azathioprine, tetralysal, cyclosporine or steroids during the last month. The frequency of possible interfering medication was higher in PDR than in no/NPDR subjects in study I ( $p=0.027$ ), but did not influence the final outcome in the multiple logistic regression analysis. A separate analysis on the influence of statin use on serum inflammatory mediator levels in PDR subjects of study I was performed, but there were no significant differences between statin and non-statin users. The frequency of possible interfering medication did not differ between PDR and non-diabetic control vitrectomy subjects, but was lower in no/NPDR than in non-diabetic control cataract subjects ( $p=0.046$ ). However, in study II none of the inflammatory mediators studied had any correlation with any medication.

### **Multiple logistic regression analysis**

In the stepwise multiple logistic regression analysis of study I, three factors remained independent determinants for PDR, i.e., diabetes duration (OR=4.919; 95% CI 2.191-11.042;  $p<0.001$ ), presence of albuminuria (OR=2.286; 95% CI 1.016-5.145;  $p=0.046$ ), and serum TNF- $\alpha$  levels (OR=2.385; 95% CI 1.035-5.495;  $p=0.041$ ).

Table 4a. Serum inflammatory mediator levels in study I subjects.

	<b>PDR (n=66)</b>	<b>No/NPDR (n=62)</b>	<b><i>p</i> value</b>
<b>IL-1<math>\beta</math> (pg/ml)</b>	Nd	Nd	-
<b>TNF-<math>\alpha</math> (pg/ml)</b>	7.0 (4.0-17.0) (n=55)	6.0 (4.0-25.0) (n=52)	<0.001
<b>sVCAM-1 (ng/ml)</b>	860 (360-2120)	700 (310-1820)	<0.001
<b>sICAM-1 (ng/ml)</b>	290 (150-620)	280 (160-560)	0.154
<b>P-selectin (ng/ml)</b>	180 (39-400)	150 (42-440)	0.017
<b>hsCRP (mg/l)</b>	1.6 (0.0-46.0)	1.3 (0.2-35.4)	0.250

Values are presented as median (range). Mann-Whitney U-test. Nd = Non detectable.

Table 4b. Serum inflammatory mediator levels in study II subjects.

	IL1 β	IL-6	IL-8	TNF- α	TNFR 1	TNFR 2	GM- CSF	MCP-1	VEGF	FGF- β
V PDR (n=26)	Nd	3.2 (2.7- 11.0)	-	9.0 (5.0- 53.0)	-	-	-	-	-	-
V ctrl (n=27)	Nd	3.0 (2.7- 21.0)	-	6.7 (3.0- 11.0)	-	-	-	-	-	-
P value	-	0.509	-	<0.00 1	-	-	-	-	-	-
A No/ NPDR (n=22)	-	259.8 (3.3- 1367.8 )	61.0 (7.2- 247.0 )	27.8 (6.8- 153.7 )	1964.5 (100- 936.3)	8653 (322- 22615.3)	102.1 (3.8- 3264.2)	1736.4 (634.7- 4554.7)	606.7 (39- 1777.5)	57.5 (32.3- 355.5)
A ctrl (n=18)	-	49.2 (3.2- 1992.5)	34.6 (10.3 - )	16.5 (4.1- 42.4)	1628.4 (714.4- 3134.9)	6564 (3703.3- 12754.4)	28.8 (3.5- 651.4)	1781.2 (489.4- 5371.5)	390.6 (62- 1129.2)	42.4 (3.4- 67.9)
P value	-	0.270	0.103	0.037	0.302	0.047	0.333	1.0	0.242	0.037

Values are expressed in pg/ml and presented as median (range). Mann-Whitney U-test Serum protein levels in cataract subjects (study II) was analyzed with Luminex technique. V= vitreous, A = Aqueous, ctrl = control

Table 5. Vitreous inflammatory mediator levels (study II)

	<b>PDR (n=26)</b>	<b>Non-diabetic control (n=27)</b>	<b>p value</b>
<b>IL-1<math>\beta</math> (pg/ml)</b>	Nd	Nd	-
<b>IL-6 (pg/ml)</b>	157.5 (25.0-1401.0)	44.0 (5.0-4425.0)	0.021
<b>TNF-<math>\alpha</math> (pg/ml)</b>	18.0 (8.0-46.0)	22.0 (13.0-47.0)	0.034

Values are presented as median (range). Mann-Whitney U-test.

Table 6. Aqueous humor inflammatory mediator levels (study II)

	<b>no/NPDR (n=22)</b>	<b>Non-diabetic control (n=18)</b>	<b>p value</b>
<b>IL-6 (pg/ml)</b>	12.5 (4.4-79.5)	4.9 (3.3-66.5)	0.008
<b>IL-8 (pg/ml)</b>	6.8 (3.2-96.0)	4.4 (3.2-69.5)	0.103
<b>TNF-<math>\alpha</math> (pg/ml)</b>	61.5 (14.0-67.0)	74.5 (3.3-78.0)	0.480
<b>TNF-R1</b>	485.2 (366.9-622.0)	456.2 (303.3-956.9)	0.414
<b>TNF-R2</b>	403.6 (320.4-534.0)	356.8 (258.6-1168.1)	0.277
<b>GM-CSF</b>	102.1 (3.8-3264.2)	28.8 (3.5-651.4)	0.333
<b>MCP-1</b>	1736.4 (634.7-4554.7)	1781.2 (489.4-5371.5)	1.0
<b>RANTES</b>	19.5 (3.2-101.5)	44.8 (5.3-91.5)	0.285
<b>VEGF</b>	53.4 (24.0-136.5)	30.4 (6.8-140.4)	0.157
<b>FGF-2</b>	21.2 (3.3-200.9)	22.2 (3.4-79.5)	0.620

Values are presented as median (range). Wilcoxon rank sum test, Mann-Whitney U-test and Dunnett's post hoc correction.

# Anti-oxidative enzymes in rat retinal ischemia-reperfusion (study III)

## **mRNA expression levels of anti-oxidative enzymes**

The study III results of real time RT-PCR are shown in Table 7. All expression values given are related to the endogenous control Ppib and then compared to the control eye. All ischemic eyes were subjected to ischemia for 45 minutes and then reperfusion for 0, 3, 6 or 24 hours before enucleation. Significant results are presented more in detail in the text below.

### *GPx1*

After 24 hours of reperfusion the mRNA expression of GPx1 was increased by a factor of 1.14 ( $p=0.028$ ) in the ischemic compared to the non-ischemic eyes.

### *Catalase*

After 24 hours of reperfusion the mRNA expression of catalase declined by a factor of 0.82 ( $p=0.022$ ) in the ischemic compared to the non-ischemic eyes.

### *CuZnSOD and MnSOD*

There was a tendency for decreased mRNA expression of both CuZnSOD and MnSOD after 24 hours of reperfusion by a factor of 0.86 ( $p=0.055$ ) and 0.88 ( $p=0.053$ ), respectively, between the ischemic and the non-ischemic eyes.

### *GCLc*

After 6 hours of reperfusion the mRNA expression of GCLc was increased by a factor of 1.14 ( $p=0.034$ ), but after 24 hours of reperfusion there was instead a decline by a factor of 0.80 ( $p<0.001$ ) in the ischemic compared to the non-ischemic eyes.

## **Protein expression levels of anti-oxidative enzymes**

No significant changes in protein levels between ischemic and control eyes were observed for any of the antioxidants, regardless of group.

Table 7. mRNA expression levels of anti-oxidative enzymes after ischemia-reperfusion (study III)

	<b>Reperfusion time (h)</b>	<b>Ischemic eyes <math>2^{-\Delta\Delta CT}</math></b> <b>(SD range)</b>	<b>Non-ischemic eyes <math>2^{-\Delta\Delta CT}</math></b> <b>(SD range)</b>	<b>p value</b>
<b>GPx1</b>	0	1.05 (0.87-1.25)	1 (0.88-1.14)	0.514
	3	1.06 (0.94-1.19)	1 (0.87-1.14)	0.327
	6	1.03 (0.91-1.17)	1 (0.86-1.16)	0.643
	24	1.14(1.01-1.28)	1 (0.88-1.14)	0.028
<b>Catalase</b>	0	1.04 (0.93-1.16)	1 (0.79-1.27)	0.785
	3	0.92 (0.81-1.06)	1 (0.76-1.32)	0.285
	6	0.88 (0.7-1.1)	1 (0.89-1.12)	0.191
	4	0.82 (0.7-0.98)	1 (0.89-1.20)	0.022
<b>CuZnSOD</b>	0	0.93 (0.78-1.26)	1 (0.87-1.15)	0.350
	3	0.97 (0.84-1.15)	1 (0.93-1.08)	0.535
	6	0.99 (0.85-1.15)	1 (0.85-1.17)	0.868
	24	0.86 (0.73-1.02)	1 (0.84-1.20)	0.055
<b>MnSOD</b>	0	0.95 (0.81-1.11)	1 (0.84-1.19)	0.444
	3	0.99 (0.88-1.11)	1 (0.88-1.13)	0.789
	6	1.27 (0.82-1.17)	1 (0.79-1.27)	0.106
	24	0.88 (0.74-1.03)	1 (0.87-1.14)	0.053
<b>GCLc</b>	0	1.0 (0.85-1.17)	1 (0.86-1.14)	0.963
	3	1.11 (0.96-1.3)	1 (0.85-1.17)	0.165
	6	1.14 (1.01-1.28)	1 (0.87-1.15)	0.034
	24	0.8 (0.71-0.89)	1 (0.89-1.13)	<0.001

Relative mRNA expression levels of anti-oxidative enzymes in ischemic eyes (relative to control eyes). Values are given as  $2^{-\Delta\Delta CT}$  (SD and range) according to the comparative Ct method. Student's paired T-test and Bonferroni correction.

## Inflammatory mediators in diabetic and non-diabetic rat retinal ischemia-reperfusion (study IV)

The study IV results of real time RT-PCR are shown in Table 8. All ischemic eyes were subjected to ischemia for 45 minutes and followed by reperfusion for one or 24 hours before enucleation. 50% of the rats were studied four weeks after STZ – induced diabetes as previously described. Further, rats were treated by intravenous injection of DHA, pravastatin or vehicle five minutes before ischemia-reperfusion. Significant results are presented more in detail in the text below.

### **Ischemia-induced mRNA expression levels of inflammatory mediators in non-diabetic rat retinas**

In the non-diabetic rat group I (one hour of reperfusion) and II (24 hours of reperfusion), retinal ischemia resulted in a 13.2-fold ( $p=0.005$ ) and a 4.9-fold ( $p=0.012$ ) increased expression of TNF- $\alpha$ , respectively, a 3.9-fold ( $p=0.005$ ) and a 8.9-fold ( $p=0.017$ ) increased expression of IL-1 $\beta$ , respectively, and a 1.9-fold ( $p=0.009$ ) and a 2.6-fold ( $p=0.025$ ) increased expression of ICAM-1, respectively, as compared to non-ischemic eyes. In group II there was also a 5.6-fold ( $p=0.012$ ) increased expression of IL-6 in ischemic compared to non-ischemic eyes. Injection of DHA or pravastatin had no effect on ischemia-induced expressions in non-diabetic rats.

### **Ischemia-induced mRNA expression levels of inflammatory mediators in diabetic rat retinas**

In the diabetic rat groups I and II, retinal ischemia resulted in a 14.9-fold ( $p=0.012$ ) and a 4.0-fold ( $p=0.017$ ) increased expression of TNF- $\alpha$ , respectively, a 4.8-fold ( $p=0.012$ ) and a 26.9-fold ( $p=0.012$ ) increased expression of IL-1 $\beta$ , respectively, and a 1.8-fold ( $p=0.012$ ) and a 2.0-fold ( $p=0.017$ ) increased expression of ICAM-1, respectively, as compared to non-ischemic eyes. In group II there was also a 22.2-fold ( $p=0.012$ ) increased expression of IL-6 in ischemic compared to non-ischemic eyes. Injection of pravastatin five minutes prior to ligation reduced the ischemia-induced increased expression of IL-6 from a 22.2-fold to a 3.3-fold ( $p=0.016$ ) and of ICAM-1 from a 2.0-fold to a 1.3-fold ( $p=0.012$ ) expression after 24 hours of reperfusion (group II). DHA had no effect on ischemia-induced expressions in diabetic rats.

### **Diabetes-induced mRNA expression levels of inflammatory mediators in non-ischemic retinas (control eyes)**

In order to find out whether there was an up-regulation of inflammatory mediators triggered by diabetes *per se* in the absence of ischemia, the mRNA expression levels of inflammatory mediators were measured in diabetic compared to non-diabetic control eyes (right eyes not subjected to ischemia-reperfusion). Rats still belonged to group I (one hour reperfusion after initial ischemia in the left eye) or group II (24 hours of reperfusion after initial ischemia in the left eye). In the diabetic control groups I and II there were 3.3-fold ( $p=0.010$ ) and 2.8-fold ( $p=0.046$ ) increased expressions of caspase-1, respectively, 2.8-fold ( $p=0.021$ ) and 2.6-fold ( $p=0.027$ ) increased expressions of VCAM-1, respectively, and 2.2-fold ( $p=0.004$ ) and 2.2-fold ( $p=0.016$ ) increased expressions of ICAM-1, respectively, compared to non-diabetic control retinas. In group I, there was an additional 6.1-fold up-regulation ( $p=0.041$ ) and a 2.2-fold up-regulation of IL-1 $\beta$  ( $p=0.026$ ) in diabetic compared to non-diabetic control retinas. DHA reduced the diabetes-induced mRNA expressions of IL-6 ( $p=0.037$ ) and of VCAM-1 ( $p=0.012$ ) in group I, and pravastatin reduced the diabetes-increased mRNA expressions of IL-6 ( $p=0.012$ ) in group I.

Table 8. mRNA expression levels of inflammatory mediators after ischemia-reperfusion and in diabetic compared to non-diabetic non-ischemic retinas.

	<b>IL-1<math>\beta</math></b>	<b>IL-6</b>	<b>TNF-<math>\alpha</math></b>	<b>ICAM-1</b>
<b>Reperfusion time (h)</b>	1/24	1/24	1/24	1/24
<b>Non-diabetic left ischemia(n=10)</b>	3.9 $\pm$ 0.7 8.9 $\pm$ 2.0	1.8 $\pm$ 0.2 5.6 $\pm$ 1.2	13.2 $\pm$ 2.2 4.9 $\pm$ 1.1	1.9 $\pm$ 0.1 2.6 $\pm$ 0.5
<b>Non-diabetic right control (n=10)</b>	1.2 $\pm$ 0.2 1.8 $\pm$ 0.6	1.7 $\pm$ 0.6 1.7 $\pm$ 0.7	1.5 $\pm$ 0.4 1.3 $\pm$ 0.4	1.1 $\pm$ 0.2 1.1 $\pm$ 0.2
<b><i>p</i> value</b>	0.005/ 0.017	>0.05/ 0.012	0.005/ 0.012	0.009/ 0.025
<b>Diabetic left ischemic (n=8)</b>	4.8 $\pm$ 0.5 26.9 $\pm$ 10.1	2.8 $\pm$ 0.5 22.2 $\pm$ 10.8	14.9 $\pm$ 3.9 4.0 $\pm$ 0.9	1.8 $\pm$ 0.1 2.0 $\pm$ 0.2
<b>Diabetic right control (n=8)</b>	1.1 $\pm$ 0.2 1.2 $\pm$ 0.3	1.5 $\pm$ 0.4 1.8 $\pm$ 0.8	1.3 $\pm$ 0.3 1.2 $\pm$ 0.2	1.0 $\pm$ 0.1 1.1 $\pm$ 0.1
<b><i>p</i> value</b>	0.012/ 0.012	>0.05 0.012	0.012 0.017	0.012 / 0.017
<b>Diabetic right control (n=8)</b>	2.2 $\pm$ 0.3 1.9 $\pm$ 0.4	1.7 $\pm$ 0.6 1.7 $\pm$ 0.7	1.5 $\pm$ 0.4 1.3 $\pm$ 0.4	2.2 $\pm$ 0.2 2.2 $\pm$ 0.3
<b>Non-diabetic right control (n=8)</b>	1.2 $\pm$ 0.2 1.8 $\pm$ 0.6	6.1 $\pm$ 1.4 1.9 $\pm$ 0.8	2.1 $\pm$ 1.4 1.9 $\pm$ 1.1	1.1 $\pm$ 0.2 1.1 $\pm$ 0.2
<b><i>p</i> value</b>	>0.05/ 0.026	0.041/ >0.05	>0.05/ >0.05	0.004/ 0.016

	<b>VCAM-1</b>	<b>P-selectin</b>	<b>Caspase-1</b>
<b>Reperfusion time (h)</b>	1/24	1/ 24	1/ 24
<b>Non-diabetic left ischemia (n=10)</b>	1.0±0.1 1.1±0.3	0.9±0.1 2.2±0.8	1.0±0.0 1.6±0.2
<b>Non-diabetic right control (n=10)</b>	1.4±0.5 1.3±0.4	1.5±0.5 1.9±0.9	1.3±0.4 1.6±0.7
<b>p value</b>	>0.05/ >0.05	>0.05/ >0.05	>0.05/ >0.05
<b>Diabetic left ischemic (n=8)</b>	0.9±0.0 1.1±0.2	1.0±0.1 2.3±0.8	0.9±0.1 1.7±0.2
<b>Diabetic right control (n=8)</b>	1.1±0.1 1.1±0.2	1.4±0.5 1.6±0.5	1.4±0.4 1.3±0.4
<b>p value</b>	>0.05/ >0.05	>0.05/ >0.05	>0.05/ >0.05
<b>Diabetic right control (n=8)</b>	2.8±0.3 2.6±0.4	1.5±1.5 2.0±1.6	3.3±0.8 2.8±0.8
<b>Non-diabetic right control (n=8)</b>	1.4±0.5 1.3±0.4	1.5±0.5 2.0±1.6	1.3±0.4 1.6±0.7
<b>p value</b>	0.021/ 0.016	>0.05/ >0.05	0.010/ 0.046

Values are given as means ± SEM. Mann-Whitney U-test.

## VCAM-1 expression in retinal vessels of genetically modified mice with eight weeks of diabetes (study V)

In study V, the VCAM-1 expression in retinal vessels was studied by immunofluorescence in wt, ApoE<sup>-/-</sup>, TNF- $\alpha$ <sup>-/-</sup> and ApoE<sup>-/-</sup>/TNF- $\alpha$ <sup>-/-</sup> mice with or without eight weeks after STZ-induced diabetes. In addition, leukocyte accumulation in retinal tissue sections was studied in wt and ApoE<sup>-/-</sup> mice. Mean blood glucose was 7.5±1.1 mmol/L in non-diabetic animals and 17.6±6.2 mmol/L in diabetic animals ( $p<0.001$ ). The mean serum cholesterol level in wt and TNF- $\alpha$ <sup>-/-</sup> animals was 76.6±59.0 mg/dl and in ApoE<sup>-/-</sup> animals 515.5±283.8 mg/dl ( $p<0.001$ ). VCAM-1 expression images and results as well as CD45 accumulation images and results are presented in Figures 1 and 2. Significant results are presented more in detail in the text below.

### Immunohistochemical analysis of VCAM-1

#### *Influence of diabetes*

The VCAM-1 expression level in the ApoE<sup>-/-</sup> group was decreased in diabetic compared to non-diabetic animals ( $p<0.001$ ). [Figure 1]. There was a weak ( $R^2=0.284$ ;  $p=0.010$ ) correlation between vessel diameter and VCAM-1 expression in all groups, higher in larger arteries. The VCAM-1 expression was unaffected or decreased in diabetic animals of all genotypes, the effect being more pronounced in ApoE<sup>-/-</sup> mice, whereas increased expression in diabetic animals was observed only in the largest vessels of ApoE<sup>-/-</sup>/TNF- $\alpha$ <sup>-/-</sup> mice. The number of VCAM-1 positive vessels, i.e., VCAM-1 expression higher than mean background intensity in each retina, was calculated as percentage of total number of vessels. The percentage of VCAM-1 positive vessels in wt and ApoE<sup>-/-</sup> mice was lower in diabetic animals ( $p=0.033$  and  $p<0.001$ , respectively), whereas no differences were observed in the TNF- $\alpha$  deficient genotypes.

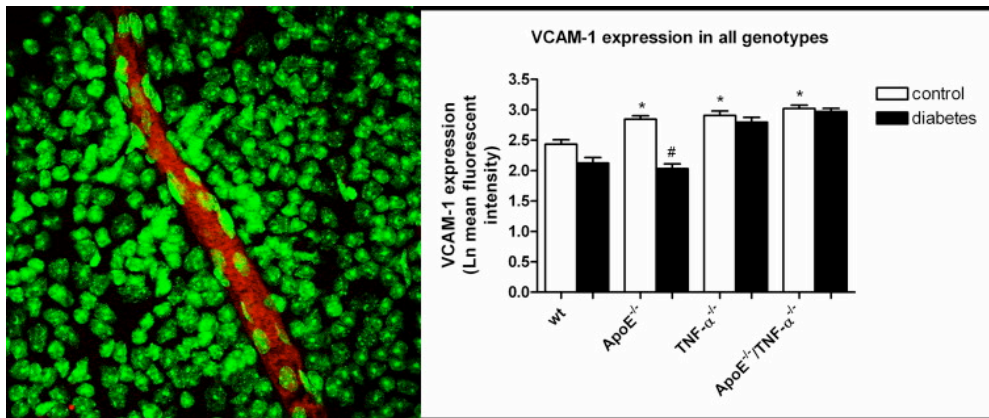
#### *Influence of dyslipidemia and TNF- $\alpha$ deficiency on basal VCAM-1 expression*

Hypercholesterolemic ApoE<sup>-/-</sup> mice expressed higher VCAM-1 levels than wt mice ( $p<0.001$ ). TNF- $\alpha$  deficient genotypes expressed higher VCAM-1 levels in both wt and ApoE<sup>-/-</sup> mice ( $p<0.001$  and  $p=0.013$ , respectively) [Figure 1].

## Immunohistochemical analysis of leukocytes

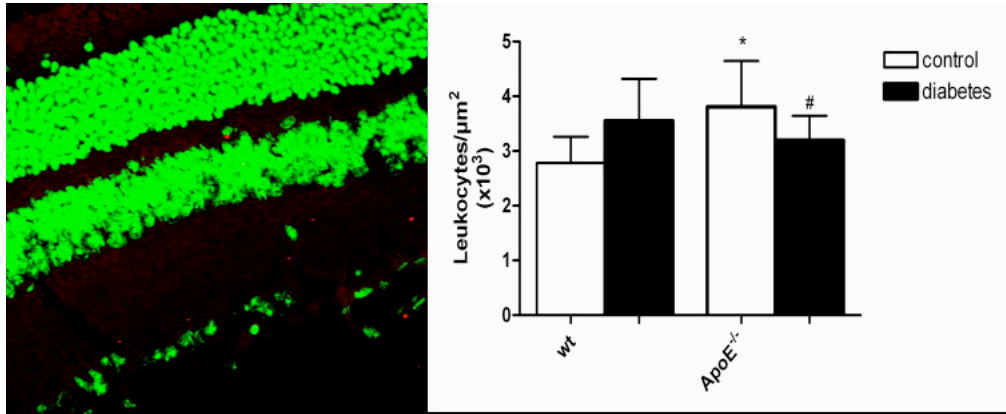
The number of leukocytes per  $\mu\text{m}^2$  retina was higher in ApoE<sup>-/-</sup> compared to wt mice ( $p=0.026$ ), but lower in diabetic compared to non-diabetic ApoE<sup>-/-</sup> mice ( $p=0.039$ ). These results were consistent with the VCAM-1 expression differences [Figure 2].

Figure 1.



Confocal image of VCAM-1 staining (red) and nuclei (green), and graph showing VCAM-1 expression results in all genotypes. VCAM-1 expression was higher in ApoE<sup>-/-</sup>, TNF- $\alpha$ <sup>-/-</sup> and ApoE<sup>-/-</sup>/TNF- $\alpha$ <sup>-/-</sup> mouse retinal vessels than in wt ( $*=p<0.001$ ). VCAM-1 was lower in diabetic than in non-diabetic ApoE<sup>-/-</sup> animals ( $\#=p<0.001$ ).

Figure 2.



Confocal image of CD45+ leukocytes (red dots) and nuclei (green), and graph showing leukocyte counts/ $\mu\text{m}^2$  in wt and ApoE<sup>-/-</sup> mouse retinal sections. Leukocyte numbers were increased in ApoE<sup>-/-</sup> compared to wt animals (\*= $p=0.026$ ), but decreased in diabetic compared to non-diabetic ApoE<sup>-/-</sup> animals (#= $p=0.039$ ).

### Real time RT-PCR

The retinal TNF- $\alpha$  mRNA expression levels were close to zero in relation to the endogenous control in TNF- $\alpha$ <sup>-/-</sup> and ApoE<sup>-/-</sup>/TNF- $\alpha$ <sup>-/-</sup> animals ( $0.0029 \pm 0.0017$  and  $0.01712 \pm 0.0308$ , respectively), confirming TNF- $\alpha$  deletion. VCAM-1 mRNA expression levels did not differ between non-diabetic and diabetic mice.

# DISCUSSION

## Inflammatory mediators are increased in late and early diabetic retinopathy in human subjects (study I and II)

In this thesis, I have summarized and analyzed the presence of inflammatory mediators in diabetic human subjects with different degrees of retinopathy, in diabetic rat retina subjected to ischemia-reperfusion, and in diabetic retina of mice of various genetic characteristics. In study I, we demonstrated increased serum levels of TNF- $\alpha$  independent of other known risk factors in T1DM subjects with PDR compared to those with no retinopathy or NPDR, suggesting that inflammation might be of significance for PDR in diabetic subjects. The results were supported by the findings in study II i.e., increased serum levels of TNF- $\alpha$ , TNFR2, and FGF- $\beta$  in diabetic compared to non-diabetic subjects. In study II, we proceeded to assess intraocular inflammatory mediator levels in subjects with and without diabetes. Diabetic subjects with active PDR had increased levels of both TNF- $\alpha$  and IL-6 in the vitreous as compared to serum. Similar results were found in the control group, which consisted of subjects with other vitreoretinal diseases. The results suggest that inflammation is evident late in the course of vitreoretinal disease, including PDR. To explore inflammatory mediators early in the course of DR, aqueous humor from diabetic subjects with no retinopathy or with NPDR was analyzed. A broad range of mediators was studied, but only IL-6 was increased in diabetic compared to non-diabetic subjects. The results indicate, that an inflammatory intraocular process starts early in diabetes and accelerates in the later ischemic stages of DR. To gain a better insight in the up-regulation of inflammatory mediators in late as well as in early stages of DR, experimental studies on two diabetic animal models were performed, one ischemic model in which ischemia-reperfusion mimics late stages of DR and another non-ischemic model which rather mimics the conditions in early DR.

## Oxidative stress is not pronounced in rat retinal ischemia (study III)

Oxidative stress has been suggested to be involved in the various possible pro-inflammatory pathways of DR<sup>41</sup>, such as the polyol pathway, PKC- $\beta$  activation, AGE formation, flux into the hexosamine pathway, and RAS up-regulation<sup>24,38,43,65,67,68,175</sup>. Hence, we first examined if there was an up-regulation of anti-oxidative enzymes as an indirect sign of oxidative stress in retinal ischemia. In study III, we therefore analyzed both the mRNA and the protein expression levels of several anti-oxidative enzymes in rat retina subjected to ischemia followed by reperfusion. Ligation of the optic nerve and accompanying vessels has earlier been successfully used<sup>208,214,215</sup> as a method allowing reversible and visually controllable blood flow strangulation. Several studies have also shown 45 minutes of ischemia to be a sufficient time for the induction of an altered gene transcription without causing necrosis<sup>29,178,216-220</sup>. The results of study III demonstrated minimally increased mRNA expression of GCLc after six hours and of GPx1 after 24 hours of reperfusion and the expression of catalase and GCLc declined after 24 hours of reperfusion, indicating that the endogenous anti-oxidative system in rat retina does not respond particularly well to ischemia/reperfusion, and perhaps that oxidative stress is not pronounced in retinal ischemia. The retina has been suggested to be comparatively resistant to ischemia as compared to brain tissue, presumably due to intravitreal glucose and intraretinal glycogen storage, or to the photoreceptors' ability of anaerobic energy exploitation<sup>29</sup>.

## Inflammatory mediators are increased in ischemic and diabetic rat retina (study IV)

In study IV, we demonstrated prominently increased mRNA expression levels of inflammatory mediators in rat retina subjected to ischemia followed by reperfusion, which is in accordance with other studies<sup>179,180,221-226</sup>. In retinal ischemia, inflammatory mediators have been suggested to be associated with up-regulation of VEGF and subsequent leukocyte infiltration<sup>227,228</sup>. Leukocyte recruitment is a well-recognized phenomenon of ischemia-reperfusion injury in other localizations of the body and may contribute to elicit inflammation and up-regulation of inflammatory mediators<sup>229</sup>. It is possible, that the leukostasis demonstrated in DR<sup>21,22,51,53</sup> is associated with ischemia-reperfusion. Even though the anti-oxidative enzyme systems of the retina were not particularly up-regulated by ischemia-reperfusion in our study III, it is probable that ischemia leads to increased ROS production, possibly also as a result of leukocyte activation<sup>230</sup>. Ischemia leads to IL-1 $\beta$ -induced expression of inducible (i)NOS as well as to activation of TLR<sup>230</sup>, resulting in blood

flow autoregulation changes and activation of pro-inflammatory NF- $\kappa$ B-induced pathways also demonstrated in diabetes<sup>26</sup>. In study IV, we could not demonstrate further increased mRNA expression levels of inflammatory mediators in retinal ischemia of rats with concomitant STZ-induced diabetes for four weeks, perhaps because the inflammatory response to ischemia-reperfusion was already maximal. By contrast, there was a rather modest up-regulation of inflammatory mediators, above all adhesion molecules, in diabetic rat retina not subjected to ischemia. The results might suggest a diabetes-activated retinal inflammation, further aggravated by ischemia as suggested<sup>231</sup> and in accordance with the results from the human studies I and II.

## Retinal endothelial activation and expression of VCAM-1 is not pronounced in early diabetes (study V)

The results of studies I, II, and IV suggest that there is an association between inflammation and DR, and that ischemia seems to elicit a strong inflammatory activation in the retina. Also in early diabetes, there may be an increased intraocular inflammation as demonstrated by increased aqueous humor cytokine levels in diabetic subjects with no or at most modest NPDR compared to the non-diabetic subjects in study II. In study V, we explored signs of retinal inflammation in mice with early diabetes. We focused on an inflammatory mediator that was associated with PDR in study I, i.e., VCAM-1, considered an early marker of endothelial activation and readily up-regulated in inflammatory conditions<sup>24,195,196</sup>. VCAM-1 may play a role in diabetic endothelial activation, as suggested by increased levels of soluble (s)VCAM-1 along with other endothelial activation markers in serum of diabetic subjects with varying retinopathy severity<sup>106,107,113,136,232-237</sup>. Increased intraocular VCAM-1 levels in diabetic compared to non-diabetic subjects, and in correlation to retinopathy severity, have likewise been demonstrated<sup>54,136,205,237-240</sup>. In our study V, however, diabetes did not change the expression of VCAM-1 in wt mouse retinal vessels. In fact, diabetes decreased the VCAM-1 expression in mice with concomitant hypercholesterolemia (ApoE<sup>-/-</sup> mice). Experimental data on VCAM-1 in diabetic retina is scarce. The only immunohistochemical study performed on rat retina, demonstrated absent expression of VCAM-1 in diabetic compared to non-diabetic rats<sup>241</sup>. Differences between experimental and clinical data in various studies might result from measuring different entities. In serum, vitreous, and aqueous humor, the soluble form of VCAM-1 is measured, the origin of which may be either shedding from membrane-bound VCAM-1 in the vessel wall endothelium, or the result of alternative splicing<sup>242</sup>. Measurements of sVCAM-1 do not necessarily reflect measurements of vessel wall VCAM-1-expression, which to a large extent probably emanate from membrane-bound VCAM-1.

It is possible, that eight weeks of diabetes is a suboptimal time point for the study of VCAM-1 up-regulation in the vessel wall, perhaps reflecting that a diabetes-induced retinal endothelial activation has not yet occurred. However, there are studies stating that VCAM-1 is of less importance for leukocyte adhesion and subsequent transmigration across the blood-brain or the blood-retina-barriers than other adhesion molecules, such as ICAM-1 or very late antigen-4 (VLA-4)<sup>243,244</sup>. One might speculate, that an explanation for the unaltered or decreased VCAM-1 expression in study V is up-regulation of other adhesion molecules instead. Increased shedding of sVCAM-1 from the endothelium in diabetes, perhaps accelerated by dyslipidemia, is another option, however not explored in our study.

## Dyslipidemia may be pro-inflammatory and lipid-modulating compounds may have anti-inflammatory effects in diabetic retinopathy (studies IV and V)

In study V, there was a higher overall VCAM-1 expression in ApoE<sup>-/-</sup> compared to wt mice, indicating that dyslipidemia may contribute to retinal endothelial activation, as previously described<sup>245</sup>. The role of dyslipidemia in DR has been little studied<sup>152</sup>. Long-term effects of dyslipidemia may be of importance for progression of DR, as shown by Barile et al.<sup>246</sup>, who demonstrated accelerated diabetes-induced retinal damage in ApoE<sup>-/-</sup> mice after six months of diabetes compared to normolipidemic mice. Acceleration of DR in these mice was associated with an increased activation of the AGE/RAGE-axis constituting a plausible link between dyslipidemia, endothelial activation/inflammation and DR. ApoE is a glycoprotein responsible for the maintenance of plasma lipid homeostasis<sup>156</sup>, the principal lipid carrier in the central nervous system<sup>157</sup> and involved in the retinal regulation of DHA<sup>159</sup>. DHA is an  $\omega$ 3-PUFA of major importance for the structure of photoreceptors<sup>161,162</sup> and may have anti-inflammatory properties. The anti-inflammatory effects of DHA were explored in study IV. DHA administered five minutes prior to ischemia-reperfusion did not affect the ischemia-induced mRNA-expression of inflammatory mediators, but reduced diabetes-induced mRNA-expression of IL-6 and VCAM-1. Likewise, pravastatin administered five minutes before ischemia-reperfusion did not affect the ischemia-induced mRNA expression of inflammatory mediators in non-diabetic rats, but reduced mRNA-expression of IL-6 and ICAM-1 in diabetic rats. Together, the results suggest that dyslipidemia might have a yet unrevealed contribution to retinal inflammation in diabetes. The influence of dyslipidemia on retinal inflammation and endothelial activation and its role in DR seems to be multifaceted and might constitute an interesting area for further research.

## TNF- $\alpha$ plays a complex regulatory role in diabetic retinopathy (studies I, II, IV and V)

Serum TNF- $\alpha$  levels were increased in diabetic subjects with PDR compared to those with no retinopathy or with NPDR in study I and in diabetic compared to non-diabetic subjects in study II. Further, intravitreal TNF- $\alpha$  levels were increased in PDR subjects, whereas aqueous humor TNF- $\alpha$  levels in subjects with no retinopathy or NPDR were not increased. In study IV, the mRNA expression levels of TNF- $\alpha$  were increased in rat retina subjected to ischemia-reperfusion regardless of diabetes, while diabetes *per se* did not influence the expression pattern. The results may indicate that an up-regulation of TNF- $\alpha$  is most important in the later stages of DR. However, they may also reflect a general diabetes-induced inflammation since serum TNF- $\alpha$  levels were increased also in diabetic subjects with no/NPDR compared to non-diabetic subjects in study II. Because of the complex and puzzling TNF- $\alpha$  scenario, we included a TNF- $\alpha$  KO mouse in study V. Contrary to expected, the basal retinal vessel wall VCAM-1 expression was increased, and neither diabetes nor dyslipidemia influenced this pattern in KO mice. TNF- $\alpha$  is a complex molecule with pro-angiogenic as well as inflammation regulating properties<sup>191,247</sup> and a clear association with DR has been demonstrated in several studies<sup>21,22,31,68,248</sup>. TNF- $\alpha$  has been suggested as a possible link between metabolic dysregulation and inflammation and/or vascular dysfunction in diabetes<sup>192,245</sup>. In addition, TNF- $\alpha$  is involved in the up-regulation of receptors for adhesion molecules on leukocytes as well as on endothelial cells and may thereby contribute to leukostasis in DR. Inhibition of TNF- $\alpha$  has been shown to suppress leukostasis and signs of retinopathy in diabetic rats<sup>59</sup>, and plasma or serum from diabetic subjects with retinopathy was demonstrated to increase TNF- $\alpha$ -associated activity in cultured human myelocytic or bovine retinal endothelial cells<sup>107,249</sup>. Intravitreal injection of a TNF- $\alpha$  inhibitor decreased pericyte and endothelial cell apoptosis and the formation of acellular vessels in the retinas of rats with eight weeks of diabetes<sup>250</sup>. However, although TNF- $\alpha$  is one of the key cytokines in inflammation, the pathways directly or indirectly activated upon TNF- $\alpha$  signaling may vary widely and lead to different outcomes depending on cell and receptor types as well as on environmental factors<sup>191</sup>. Both inflammatory and anti-inflammatory TNF- $\alpha$  actions have been described<sup>251,252</sup>. Through its complex signaling systems with positive and negative feedback loops<sup>191</sup>, TNF- $\alpha$  possibly protects against an overheated inflammation<sup>247</sup>. One explanation for the divergent actions of TNF- $\alpha$  during various circumstances may be differential expression of its three functionally different receptor types, TNFR1, TNFR2 and TNFR3<sup>191,247</sup>, also present in soluble forms. TNFR3 has no known biological activity other than acting as a “decoy” competitor to the other two receptors. TNFR1 is considered to be the main TNF-receptor, present on most cell types. TNFR1-activation mainly induces

pro-inflammatory and proliferative signaling pathways, but may also confer apoptosis. The favor of either of these dichotomic pathways is regulated in a complex and not fully known way by several interacting inter- and intracellular signaling complexes<sup>191</sup>. TNFR2 signaling occurs primarily in endothelial and hematopoietic cells, does not seem to mediate inflammation, believed to have neuroprotective properties<sup>192,251,252</sup>, but may also enhance apoptotic TNFR1 signaling<sup>191</sup>. Activation of the endothelial TNFR2 may be of importance in vascular disease, supported by the increased serum TNFR2 levels in subjects with no or NPDR compared to non-diabetic subjects in study II. A similar finding has been reported earlier by Limb et al<sup>101</sup>, who assessed intravitreal and serum levels of TNF- $\alpha$ , and soluble TNF-receptors in diabetic subjects with PDR and non-diabetic subjects with other vitreoretinal diseases compared to non-diabetic controls. Limb et al. concluded that increased levels of sTNFRs might attenuate the activities of TNF- $\alpha$  under certain conditions.

It cannot be entirely excluded that the increased expression of VCAM-1 in retinal vessels of TNF- $\alpha$ <sup>-/-</sup> mice in study V, which is in accordance with another study of ours on cerebral vessels<sup>253</sup>, may be a reflection of an immune incompetence in these mice<sup>251,252,254</sup>. Treatment with a TNF-receptor inhibitor might have yielded different results. A recent study demonstrated attenuated diabetes-induced retinal cell apoptosis by the administration of the soluble TNF-receptor etanercept in diabetic rats<sup>255</sup>. Our results regarding TNF- $\alpha$  and its receptors in studies I, II, IV and V suggest that TNF- $\alpha$  plays a complicated role and may have both deleterious and protective properties in the pathogenesis of DR.

## Retinal leukocyte attraction and accumulation are not pronounced in early diabetes, but may play a role in later stages (studies I, II, IV and V)

Leukocyte functions and behavior may be altered in diabetes with changed motility patterns, differential expression of surface markers and anchoring molecules, as well as increased ROS production and release<sup>61,62,256,257</sup>. There are several studies reporting leukostasis in diabetic retinopathy<sup>49,53,59,84,97,98,258-262</sup>, a phenomenon that may lead to micro-occlusions and secondary ischemia, but leukocytes may also contribute directly to endothelial damage through the release of ROS and inflammatory mediators<sup>22</sup>. Leukocytes might thereby contribute directly to the break-down of the iBRB, but a damaged iBRB in DR may inversely also facilitate for leukocytes to enter retinal tissue. In addition, a disruption of the ophthalmic immune privilege in DR is perhaps of importance for leukocyte transendothelial migration into the retinal tissue<sup>22,31</sup>. In the present thesis, leukostasis was not

studied, but expression of adhesion molecules, chemokines and leukocyte growth factors may be seen as indirect indicators of leukocyte mobilization in DR. The accumulation of leukocytes was studied in retinal tissue sections in study V. There were indeed increased serum levels of VCAM-1 and P-selectin in T1DM subjects with PDR compared to those with no/NPDR in study I as well as an ischemia-induced increased mRNA-expression of ICAM-1 and a diabetes-induced mRNA expression of both ICAM-1 and VCAM-1 in study IV. However, we could neither demonstrate increased vessel wall expression of VCAM-1 nor increased retinal accumulation of leukocytes in diabetic mice in study V, and protein levels of leukocyte chemokines or growth factors in aqueous humor of diabetic subjects with no/NPDR were not increased. The results might indicate that leukocytes play a more significant role in later stages of DR, but further studies comparing the influence of leukocytes and leukostasis during different stages of DR are needed.

## Final remarks

To conclude, I have demonstrated an association between inflammation and DR in diabetic human subjects with no, mild and proliferative retinopathy, as well as in diabetic animal models with and without retinal ischemia. In contrast to inflammation, oxidative stress in ischemic retina did not appear pronounced since there was no up-regulation of the anti-oxidative defense system in retinal ischemia-reperfusion. The association between inflammation and DR seemed to be strongest in late, ischemic stages, as suggested by increased serum and intraocular levels of inflammatory mediators in PDR subjects, and increased mRNA-expression levels of inflammatory mediators in diabetic ischemic rat retina. Inflammatory mediator levels in no/NPDR subjects and in non-ischemic diabetic mouse retina were modest or absent. Likewise, the involvement of leukocytes was most clearly demonstrated in late, ischemic DR, as compared to early DR. In human PDR subjects as well as in diabetic ischemic rat retinas, there was an up-regulation of leukocyte adhesion molecules, but leukocyte chemokines or growth factors were not increased in no/NPDR subjects, and the accumulation of leukocytes in retinal tissue sections of early diabetic mice was unaltered or decreased. I have also demonstrated that dyslipidemia may contribute to endothelial activation and up-regulation of VCAM-1 in the retina and that DHA and statins given prior to ischemia-reperfusion may have some anti-inflammatory effects in diabetic retina, suggesting that dyslipidemia intervention might be a useful adjuvant in DR prevention and/or treatment. The results on TNF- $\alpha$  indicate that this cytokine plays a complex role in DR and may have both harmful and protective properties that need to be addressed in further studies.

# POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Sockersjuka (diabetes) är en ämnesomsättningssjukdom som karakteriseras av ett kroniskt förhöjt blodsocker. Förekomsten av diabetes runt om i världen är 2.8% för alla åldrar. I Sverige är motsvarande siffra ca 4 %. En av de mest fruktade komplikationerna till diabetes är diabetesretinopati, som utgörs av kärlförändringar i näthinnan och som obehandlad kan leda till allvarlig synnedsättning eller blindhet. Diabetesretinopati är en viktig orsak till blindhet i världen, och kommer i de industrialiserade länderna på tredje plats efter åldersförändringar i gula fläcken och grön starr, trots att det idag finns väl etablerad behandling i form av laser, en behandling som har minskat allvarlig synnedsättning till följd av diabetesretinopati med 50-60%. Orsaken till diabetesretinopati är inte helt känd. Två stora befolkningsbaserade studier på 1990-talet visade att det är viktigt inte bara med god blodsockerkontroll utan också god blodtryckskontroll för att förhindra uppkomst och utveckling av diabetesretinopati. Trots god kontroll av både blodsocker och blodtryck fortsätter en del personer med diabetes att utveckla kärlförändringar, medan andra nästan inte får några förändringar alls. Det måste alltså finnas andra förklaringar. Flera olika biologiska system ökar aktiviteten vid diabetes, men ännu har man inte kunnat bromsa uppkomst eller utveckling av diabetesretinopati hos människa genom att blockera dessa system, trots att goda resultat i många fall har påvisats i experimentella djurmodeller. På senare tid har hypotesen framförts, att det i själva verket är inflammation i näthinnan som leder till de skador som kännetecknar diabetesretinopati. I den här avhandlingen har jag undersökt sambandet mellan inflammation och diabetesretinopati hos människor med olika grader av förändringar och hos djur i två olika diabetesmodeller. I den ena djurmodellen stryps blodförsörjningen till näthinnan för att efterlikna förhållandena hos människa vid sen diabetesretinopati, i den andra modellen påverkas inte blodflödet, utan motsvarar förhållandena vid tidig diabetes.

Näthinnan är en komplex struktur bestående av flera olika cellager och flera olika celltyper. Det pågår ständigt ett intensivt utbyte av information mellan de olika cellerna. Ett fint nätverk av små blodkärl försörjer näthinnan med syre och

näring. I dessa blodkärl delar de celler som klär insidan av kärlet, endotelceller, och stödjeceller, pericyter, ett gemensamt omgivande skikt. Endotelcellerna är så intimt sammankopplade med varandra att bara vissa ämnen tillåts att passera. Redan tidigt vid diabetes förtjockas det gemensamma omgivande skiktet, pericyterna försvinner och endotelcellernas förbindelser luckras upp, så att stora molekyler och vätska från blodbanan kan sippra ut i näthinnan. Så småningom dör också en del av endotelcellerna och efterlämnar tomma blodkärl, som inte längre kan försörja näthinnan med syre och näring och borttransporten av nedbrytningsprodukter försämras. Resultatet blir en lokal syrebrist och ansamling av giftiga produkter. Vid uttalad syrebrist bildas nya, sköra, lättblödande blodkärl som efter hand omvandlas till bindväv i näthinnan. Nybildning av kärl kan orsakas inte bara av syrebrist och lokal produktion av tillväxtfaktorer, utan också av inflammation.

Inflammation är i vid mening kroppens sätt att försvara sig mot skadliga faktorer, t ex skada, gifter eller infektion. Man brukar tala om fem kardinaltecken, rodnad (rubor), värme (calor), svullnad (tumor), smärta (dolor) och nedsatt funktion (functio laesa). Olika utlösande faktorer sätter igång olika inflammatoriska reaktioner, men gemensamt är ett inflöde av vita blodkroppar och ökad bildning av inflammatoriska faktorer. Små blodproppar och områden med blödningar uppstår. Efter en initial intensiv inflammation upphör den inflammatoriska reaktionen i de flesta fall och följs av borttransport av död vävnad, döda bakterier och vita blodkroppar. Ibland övergår inflammationen emellertid i en kronisk fas, vilket kan bero på att det kvarstår skadliga faktorer i vävnaden eller på att den initiala inflammationen av olika skäl blev så intensiv att vävnaden skadats så att den inte längre fungerar normalt. Kronisk inflammation förekommer vid flera sjukdomar, bla vid diabetes.

I näthinnan leder det höga blodsockret till att kemiska ämnen i blodet flödar ut i näthinnan och reagerar med molekyler i vävnaden så att skadliga produkter bildas. I blodkärlen bildas proppar och de vita blodkropparna fastnar. Vid diabetes har man också kunnat visa att de vita blodkropparna rör sig klumpigare, fastnar lättare, har ett ökat antal vidhäftningsställen på sin yta och släpper ut fler giftiga ämnen när de aktiveras. Ansamling av vita blodkroppar innebär ökad bildning av inflammatoriska faktorer och direkt skada på kärlväggen.

Inflammatoriska faktorer kan hos människa studeras i blodprov och i prov från ögats kammarvatten och glaskropp. Däremot kan man inte ta prov från näthinnan. Flera studier har gjorts som har visat ökade halter av inflammatoriska faktorer i blod, kammarvatten och glaskropp från personer med diabetes jämfört med dem utan, samt från personer med allvarlig diabetesretinopati jämfört med dem med mild diabetesretinopati. Det finns emellertid också studier som har visat motsatta resultat. I den här avhandlingen ingår fem studier som undersöker

sambanden mellan inflammation och den allvarliga diabetesretinopati som uppkommer sent vid diabetes samt mellan inflammation och lindrig, tidig diabetesretinopati i studie I och II, syrebrist i näthinnan i studie III, inflammation vid syrebrist i näthinnan i studie IV och blodkärlsaktivering, inflammation och inflöde av vita blodkroppar i näthinnan vid diabetes i studie V. Om rubbningar i blodfetterna kan påverka blodkärlsaktivering har undersökts i studie V. Vidare har effekten av den specifika inflammatoriska faktorn tumörnekrosfaktor-alfa (TNF- $\alpha$ ) för blodkärlsaktivering vid diabetes studerats specifikt i studie V.

I studie I analyserades halten av inflammatoriska faktorer i blodet från personer med diabetes med allvarlig syrebrist i näthinnan och nybildade kärl (PDR), och jämförde med halten hos personer utan eller med lindrigare diabetesretinopati. Det visade sig att tre faktorer var förhöjda i serum från personer med PDR, nämligen den inflammatoriska cytokinen TNF- $\alpha$  och de två adhesionsmolekylerna P-selektin och vaskulär celladhesionsmolekyl-1 (VCAM-1), vilka ansvarar för vidhäftning av vita blodkroppar till blodkärlsväggen. Efter att ha uteslutit andra kända riskfaktorer för PDR, såsom ålder, diabetesduration, blodsockerkontroll, blodtryck, midjemått, kroppsmassa och njurfunktion, kvarstod TNF- $\alpha$  som en oberoende markör för PDR. Resultatet talar för att TNF- $\alpha$  och inflammation är av betydelse i de senare stadierna av diabetesretinopati.

För att få en bättre uppfattning om förekomst av inflammatoriska faktorer i själva ögat utfördes mätningar på glaskropp och kammarvatten från personer med diabetes. TNF- $\alpha$  och den inflammatoriska faktorn interleukin-6 (IL-6) var båda förhöjda i glaskropp från dem med allvarlig retinopati och IL-6 dessutom i kammarvatten från dem utan eller med lindrig retinopati. Resultaten tyder alltså på att diabetesretinopati är kopplat till inflammation både i sena och tidiga stadier, men att kopplingen är mest uttalad vid uttalad syrebrist i sena stadier av sjukdomen.

I studie III och IV utsattes näthinna från råttor för syrebrist genom att blodflödet tillfälligt ströps. Vid strypning av blodflödet kan blodkärlen inte förse näthinnan med syre och näring och kan inte heller transportera bort slaggprodukter. Flera studier har föreslagit att sk oxidativ stress med bildande av skadliga fria syreradikaler förekommer vid syrebrist och diabetes i näthinnan. Ögat har väl utvecklade försvarssystem mot oxidativ stress. I studie III analyserades därför om dessa försvarssystem aktiveras vid strypning av blodflödet. Det visade sig emellertid att så inte var fallet, och syrebrist i näthinnan orsakar alltså inte någon nämnvärd oxidativ stress. I studie IV analyserades i stället förekomsten av inflammatoriska faktorer under samma förhållanden. De inflammatoriska faktorerna interleukin-1beta (IL-1 $\beta$ ), TNF- $\alpha$ , intercellulär adhesionsmolekyl-1 (ICAM-1) och interleukin-6 (IL-6) var kraftigt ökade hos både icke-diabetiska och diabetiska djur. Således verkar syrebrist i näthinnan framkalla en ordentlig inflammatorisk reaktion.

I studie V studerades uttrycket av VCAM-1 i näthinnans blodkärl hos möss med diabetes av kort duration. VCAM-1 är en inflammatorisk faktor och dessutom en markör för hur blodkärlens endotelceller aktiveras vid inflammation. Det fanns ingen skillnad i VCAM-1 mellan möss med och utan diabetes. Däremot hade möss där en gen av betydelse för fettomsättningen tagits bort och som därför har förhöjda halter av kolesterol i blodet högre VCAM-1 i blodkärlen. Det kan tala för att rubbningar i blodfetterna kan vara av betydelse för inflammation i näthinnan.

I studie V studerades hur den enskilda inflammatoriska faktorn TNF- $\alpha$  påverkar VCAM-1 i blodkärlen genom användandet av möss där genen för TNF- $\alpha$  tagits bort, men i stället för en förväntad sänkning av VCAM-1 hos dessa möss var VCAM-1 förhöjt. Detta påverkades inte av diabetes. Resultatet kan tolkas som att TNF- $\alpha$  skyddar i stället för att ha skadliga effekter förknippade med inflammation. Sammantaget talar resultaten från studierna på människa, råttor och mus för att TNF- $\alpha$  är en komplicerad molekyl som kan ha både skadliga och skyddande effekter i näthinnan vid diabetes.

I denna avhandling har jag visat att diabetesretinopati är förknippad med inflammation redan i tidiga stadier och än mer uttalat i senare stadier av retinopatin. Det förefaller också som om nivåerna av inflammatoriska faktorer kan vara kopplade till graden av diabetesretinopati hos människa. Hos råttor med diabetes kunde jag i en modell som stryper blodförsörjningen till näthinnan och därmed liknar situationen i sena stadier av diabetesretinopati hos människa visa att inflammatoriska faktorer är kraftigt ökade, medan oxidativ stress inte tycks vara särskilt uttalad. Rubbningar i blodfettbalansen leder till ett ökat uttryck av VCAM-1 i näthinnans blodkärl, vilket talar för att blodfetter kan vara av betydelse för utvecklingen av inflammation i näthinnan. Det finns också tecken på att inflammatoriska faktorer av särskild betydelse för vita blodkroppar ökar vid sen diabetesretinopati med syrebrist, medan kopplingen till tidig diabetesretinopati inte är lika tydlig. Den inflammatoriska faktorn TNF- $\alpha$  tycks vara starkt kopplad till diabetesretinopati, men dess roll i sammanhanget är komplicerad, och kan vara av både skadlig och skyddande natur. .

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# Paper I





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## TNF- $\alpha$ is an independent serum marker for proliferative retinopathy in type 1 diabetic patients<sup>☆</sup>

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### Abstract

**Purpose:** This study aimed to determine if there are any associations between serum levels of inflammatory markers and proliferative retinopathy (PDR) in type 1 diabetic patients. **Design:** A cross-sectional design was utilized for this study. **Methods:** One hundred twenty-eight type 1 diabetic patients underwent stereo fundus photography according to the Early Treatment Diabetic Retinopathy Study and were divided into two retinopathy groups: no or nonproliferative retinopathy (NDR/NPDR;  $n=62$ ) and PDR ( $n=66$ ). Serum levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, soluble vascular cellular adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), P-selectin, and high-sensitivity C-reactive protein (hsCRP) were analyzed. Statistical analysis was performed using nonparametric Mann–Whitney U test and multivariate logistic regression analysis. **Results:** Patients with PDR had higher levels of TNF- $\alpha$  [7.0 pg/ml (<4–17) vs. 6.0 pg/ml (<4–25);  $P=.009$ ], sVCAM-1 [860 ng/ml (360–2120) vs. 700 ng/ml (310–1820);  $P<.001$ ], and P-selectin [180 ng/ml (39–400) vs. 150 ng/ml (42–440);  $P=.017$ ; figures are expressed as median (range)]. There were no differences in serum levels of sICAM-1 or hsCRP. IL-1 $\beta$  was not detectable in any patient, and IL-6 was detectable in only 22.7% of the patients. In multivariate logistic regression analysis, TNF- $\alpha$  was the single, persistent, independent determinant inflammatory marker for PDR. **Conclusion:** The association between TNF- $\alpha$  and PDR in type 1 diabetic patients suggests that inflammation might play a role in the pathogenesis of proliferative diabetic retinopathy.

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**Keywords:** Cytokines; Adhesion molecules; Diabetic retinopathy

### 1. Introduction

Diabetic retinopathy is presently estimated to account for 4.8% of global blindness (World Health Organization, 2006), a figure that is expected to increase due to a growing diabetic population. In the Western countries, diabetic retinopathy accounts for 4–15% of serious visual impairment and blindness (World Health Organization, 2006).

The DCCT (DCCT Research Group, 1993) and the UKPDS (UKPDS Group, 1998) studies documented the importance of blood glucose control to prevent the development and progression of diabetic microvascular complica-

tions. However, the increased blood glucose concentrations did not account for all the risk for development of and progression to sight-threatening retinopathy. There is evidence that chronic inflammation may be involved (Bhavasar, 2006; Wellen & Hotamisligil, 2005). Schram, Chaturvedi, Schalkwijk, Fuller, and Stehouwer (2005) performed a cross-sectional study on patients involved in the EURODIAB trial (EURODIAB, 1994) and demonstrated an elevated score of serum inflammatory markers in patients with type 1 diabetes, defined as age at onset before 36 years, with a continuous need for insulin treatment within 1 year of diagnosis and with micro- as well as macrovascular complications. Furthermore, Meleth et al. (2005) showed elevated levels of inflammatory markers in a mixed group of type 1 and type 2 diabetic patients having at least severe nonproliferative diabetic retinopathy.

There are several inflammatory markers that might be involved in the pathogenic processes of diabetic retinopathy, as mediators between leukocytes or as regulators of

<sup>☆</sup> The authors state that there is no duality of interest.

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leukocyte adhesion and activation in retinal tissue. In the present study, we aimed at gaining further information on a possible role for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, soluble vascular cellular adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), and P-selectin in the pathogenesis of proliferative retinopathy (PDR) in patients with type 1 diabetes, verified with the presence of GAD antibodies early in the course of disease.

## 2. Methods

### 2.1. Subjects

A cross-sectional study assessing serum levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, sVCAM-1, sICAM-1, P-selectin, and high-sensitivity C-reactive protein (hsCRP) in patients with and without proliferative diabetic retinopathy according to fundus photographic grading in agreement with the Early Treatment Diabetic Retinopathy Study (ETDRS) scale (ETDRS Group, 1991) was approved by the Ethics Committee of Malmö/Lund and performed in accordance with the Declaration of Helsinki. Two hundred one type 1 diabetic patients were recruited from the local register, Diabetes 2000, in Malmö, Sweden, of whom 131 accepted to participate in the study. Diabetes typing in this register is based on the assessment of GAD antibodies, regardless of age at diagnosis. Three patients were excluded due to ungradable fundus photographs. The remaining 128 patients comprise the sample for the present study.

Initial clinical assessment included a medical history of concomitant diseases, medication, smoking habits, body height and weight, waist measurement, and blood pressure. Blood was collected for measurements of HbA<sub>1c</sub>, plasma creatinine, hsCRP, and serum levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, sICAM-1, sVCAM-1, and P-selectin, and urine samples were collected for analysis of urinary albumin excretion. All patients were given a serial number, and all data were subsequently analyzed in a masked fashion.

Seventy patients (34.8%) chose not to participate. These patients had a somewhat shorter diabetes duration [23.0 years (2.0–71.0) vs. 25.0 years (3.0–61.0);  $P=0.48$ ] and a lower HbA<sub>1c</sub> [7.2% (3.9–12.7) vs. 7.8% (4.5–14.8);  $P=0.005$ ; figures are expressed as median (range)] than those in the study group, as well as less frequent PDR (32.9% vs. 51.6%;  $P=0.12$ ).

### 2.2. Observation procedures

After dilatation of the pupils, stereo photographs were taken from seven standard fields in each eye, using a 30° fundus camera (Topcon Inc., TRC-50, Topcon Scandinavia, Mölndal, Sweden). Grading was performed by an experienced ophthalmologist (E.A.) in a masked fashion according to the ETDRS grading scale (ETDRS Group, 1991), and patients were subsequently divided into two retinopathy groups: no or

nonproliferative retinopathy (NDR/NPDR;  $n=62$ ) and PDR ( $n=66$ ). Diabetic macular edema was assessed according to the global retinopathy scale (Wilkinson et al., 2003) and defined as retinal thickening or hard exudates within the vessel arcades. Macular edema was present in 28 (21.9%) patients, of whom 16 (57.1%) also had PDR.

Blood pressure was measured in the supine position after a 5-min rest using a sphygmomanometer. Patients were considered hypertensive if they had a blood pressure  $>130/80$  mmHg and/or were taking antihypertensive medication ( $n=113$ ). Patients were considered to have albuminuria if urinary albumin was  $\geq 0.020$  g/l and/or if they were using ACE inhibitors or angiotensin II receptor blockers ( $n=64$ ). None of the patients had renal failure; that is, they had undergone or were required to undergo kidney transplantation. Patients with a medical history of myocardial infarction and/or cerebrovascular insult were considered to have macrovascular complications ( $n=20$ ; 15.6%).

All laboratory procedures were performed on external laboratories according to the manufacturer's protocol. HbA<sub>1c</sub> was analyzed with high-performance liquid chromatography (VARIAN II Hemoglobin A1c program, BioRad, Hercules, CA, USA; reference range, 4.0–5.3%). Plasma creatinine was analyzed according to the kinetic Jaffé reaction (reference range, 51–88  $\mu\text{mol/l}$ ). Urinary albumin was analyzed via nephelometry (Image, Beckman Coulter, Brea, CA, USA) or turbidimetry (Synchron LX20, Beckman Coulter; normal value,  $<0.020$  g/l). hsCRP was analyzed through rate turbidimetry (Synchron LX20, Beckman Coulter; normal value,  $<3$  mg/l). Cytokines and adhesion molecules were analyzed via Immulite 1000 (Diagnostic Products Corporation Scandinavia AB, Mölndal, Sweden) in serum obtained by centrifugation of whole blood immediately after sampling. The samples were stored at  $-80^\circ\text{C}$  until analyses. The detection limits for the cytokines are as follows: IL-1 $\beta$ , 5 pg/ml; IL-6, 2.8 pg/ml; and TNF- $\alpha$ , 4 pg/ml. Those for the adhesion molecules are as follows: sICAM-1, 54.6 ng/ml; sVCAM-1, 210 ng/ml; and P-selectin, 16 ng/ml. Inter- and intraindividual variability ranged from 4.2% to 5.6% and from 4.6% to 7.9%, respectively.

Statistical analysis was performed using nonparametric Mann–Whitney U test and logistic regression analysis. Patients with and without PDR were tested for differences in sex, age, age at onset of diabetes, diabetes duration, hypertension, body mass index (BMI), waist measurement, smoking habits, presence of macular edema and macrovascular disease, HbA<sub>1c</sub>, albuminuria, hsCRP, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , sVCAM-1, sICAM-1, and P-selectin. For logistic regression analysis, continuous data were dichotomized using upper reference value or, if there was no reference, the median value (i.e., inflammatory markers) as split points. Univariate logistic regression was performed to identify factors to be included in the stepwise multivariate logistic regression analysis, which was performed for analysis of individual strength and the exclusion of interfering factors. A  $P$  value  $<0.05$  was considered

Table 1  
Patient characteristics in all diabetic subjects as well as in patients with and without PDR

	Total (n=128)	PDR (n=66)	NDR/NPDR (n=62)	P value
Sex (n)				
Male	69	32	37	.206
Female	59	34	25	
Age (years) <sup>a</sup>	45.5 (22.0–77.0)	49.5 (23.0–77.0)	40.0 (22.0–74.0)	.035
Age at onset (years) <sup>a</sup>	18.0 (1.0–59.0)	16.0 (4.0–38.0)	21.5 (1.0–59.0)	.019
Diabetes duration (years) <sup>a</sup>		31.5 (6.0–60.0)	21.0 (3.0–61.0)	<.001
Systolic blood pressure (mmHg)	135 (91–201)	134 (91–201)	136 (107–192)	.473
Diastolic blood pressure (mmHg)	74 (31–101)	73 (54–98)	76 (31–101)	.138
Hypertension (n)				
Yes	113	59	54	.688
No	15	7	8	
BMI (kg/m <sup>2</sup> )	24.1 (17.5–48.4)	24.1 (18.8–48.4)	24.1 (17.5–32.8)	.985
Waist (cm)	94.0 (73.0–129.0)	96.0 (73.0–129.0)	93.0 (79.0–122.0)	.378
Smoker (n)				
Yes	22	13	9	.439
No	106	53	53	
Macular edema (n)				
Yes	28	16	12	.392
No	96	46	50	
Macrovascular complication (n)				
Yes	20	13	7	.192
No	108	53	55	
HbA <sub>1c</sub> (%)	7.8 (4.5–14.8)	8.0 (4.5–14.8)	7.4 (5.1–11.1)	.070
Albuminuria <sup>a</sup> (n)				
Yes	64	43	21	<.001
No	64	23	41	
Interfering medication <sup>a</sup> (n)				
Yes	41	27	14	.027
No	87	39	48	
Interfering disease (n)				
Yes	53	32	21	.095
No	75	34	41	

Values are expressed as median (range) unless otherwise specified. The Mann–Whitney U test was employed.

<sup>a</sup> Analyzed further by logistic regression.

significant. All statistical calculations were executed on SPSS version 12.0 for Windows.

### 3. Results

Patient characteristics are described in Table 1, and inflammatory markers are described in Table 2. In the nonparametric tests, patients with PDR were older ( $P=.035$ ), were younger at diabetes onset ( $P=.019$ ), and had a longer diabetes duration ( $P<.001$ ) than patients without PDR. Levels of TNF- $\alpha$ , sVCAM-1, and P-selectin

were also higher in the PDR group ( $P=.009$ ,  $P<.001$ , and  $P=.017$ , respectively). PDR patients exhibited more frequent microalbuminuria ( $P<.001$ ) and possible interfering medication ( $P=.027$ ). No other differences between groups were seen.

#### 3.1. Inflammatory markers

##### 3.1.1. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6

TNF- $\alpha$  was detectable ( $>4.0$  pg/ml) in 108 patients (84.4%). Nondetectable levels were designated as 0 in the

Table 2  
Inflammatory markers in all diabetic subjects as well as in patients with and without PDR

	Total (n=128)	PDR (n=66)	NDR/NPDR (n=62)	P value
hsCRP (mg/l)	1.6 (0.0–46.0)	1.9 (0.0–46.0)	1.3 (0.2–35.4)	.250
TNF- $\alpha$ (pg/ml) <sup>a</sup> (n=108)	6.0 (<4.0–25.0)	7.0 (<4.0–17.0)	6.0 (<4.0–25.0)	.009
IL-6 (pg/ml) (n=29)	4.0 (<3.0–14.0)	4.0 (<3.0–14.0)	3.5 (<3.0–5.0)	.058
IL-1 $\beta$ (pg/ml)	–	–	–	–
sVCAM-1 (ng/ml) <sup>a</sup>	760 (310–2120)	860 (360–2120)	700 (310–1820)	<.001
sICAM-1 (ng/ml) <sup>a</sup>	290 (150–620)	315 (150–620)	280 (160–560)	.154
P-selectin (ng/ml) <sup>a</sup>	160 (39–440)	180 (39–400)	150 (42–440)	.017

Values are expressed as median (range). The Mann–Whitney U test was employed.

<sup>a</sup> Analyzed further by logistic regression.

Table 3  
Multivariate logistic regression analysis (forward stepwise)

Variable	Reference	P value	Exp (B)	95% CI for Exp (B)	
				Lower	Upper
<b>Variables in the equation</b>					
Step 1					
Duration	<25.0	.000	5.953	2.764	12.823
Step 2					
Duration	<25.0	.000	5.829	2.651	12.816
TNF- $\alpha$	<6.0	.017	2.707	1.196	6.130
Step 3					
Duration	<25.0	.000	4.919	2.191	11.042
Albuminuria	No	.046	2.286	1.016	5.145
TNF- $\alpha$	<6.0	.041	2.385	1.035	5.495
<b>Variable</b>			<b>Significance of the change</b>		
<b>Model if term is removed</b>					
Step 1					
Duration			.000		
Step 2					
Duration			.000		
TNF- $\alpha$			.015		
Step 3					
Duration			.000		
Albuminuria			.045		
TNF- $\alpha$			.038		
<b>Variables</b>			<b>Significance</b>		
<b>Variables not in the equation</b>					
Step 1					
Age			.779		
Age at onset			.069		
Albuminuria			.016		
Interfering medication			.362		
TNF- $\alpha$			.015		
VCAM-1			.021		
P-selectin			.046		
Step 2					
Age			.824		
Age at onset			.177		
Albuminuria			.043		
Interfering medication			.513		
VCAM-1			.064		
P-selectin			.104		
Step 3					
Age			.640		
Age at onset			.139		
Interfering medication			.159		
VCAM-1			.090		
P-selectin			.060		

Continuous data were dichotomized using the median as split point. Albuminuria was dichotomized as no or yes. Exp (B) signifies the extra risk for PDR with each variable in this model.

statistical analysis performed using the Mann–Whitney U test. Nondetectable values were more frequently seen in patients without PDR (21.0% vs. 10.6%). The serum levels were higher in patients with PDR compared to those without ( $P=.009$ , Table 2). TNF- $\alpha$ , together with diabetes duration, remained an independent determinant factor for PDR in multivariate logistic regression analysis ( $P=.017$ , Table 3).

IL-6 was detectable in 29 patients (22.7%) only. The serum levels in patients with PDR ( $n=19$ ) showed a

tendency to be elevated compared to those without ( $n=10$ ;  $P=.058$ ; Table 2). Logistic regression analysis was not performed because of the few samples above the detection limit. IL-1 $\beta$  was not detectable in any serum sample.

### 3.1.2. sVCAM-1, sICAM-1, and P-selectin

sVCAM-1 was detectable in all serum samples. The concentration was higher in patients with PDR than in those without ( $P<.001$ ; Table 2), but sVCAM-1 did not remain an

independent determinant factor for PDR in multivariate logistic regression analysis (Table 3).

sICAM-1 was detectable in all serum samples, but the levels did not differ between patients with and those without PDR.

P-selectin was also detectable in all serum samples. The concentration was higher in patients with PDR compared to those without ( $P=.017$ ; Table 2), but P-selectin did not remain an independent determinant factor for PDR in the multivariate logistic regression analysis (Table 2).

### 3.1.3. hsCRP

hsCRP was detectable in all serum samples. Levels did not differ between patients with and those without PDR.

### 3.2. Possible interfering disease

To exclude any influence of diseases or health conditions other than diabetes, we included information on concomitant diseases or health conditions [hypothyroidism ( $n=25$ ); current infections, i.e., foot ulcers, cold ( $n=21$ ); unspecified heart conditions ( $n=15$ ); current pregnancy ( $n=3$ ); malignancy ( $n=3$ ); recent surgery ( $n=2$ ); rheumatoid arthritis ( $n=2$ ); or celiac disease ( $n=1$ )] in the statistical model. There were no differences between groups (Table 1).

### 3.3. Possible interfering medication

To exclude any interference of medication, we included the use of the following in the statistical model: aspirin or nonsteroid anti-inflammatory drugs during the last month ( $n=36$ ); statins ( $n=32$ ); immunomodulating medication such as methotrexate, azathioprine, Tetralsal, and cyclosporine ( $n=6$ ); or steroids during the last month ( $n=4$ ). The frequency of possible interfering medication was higher in the PDR group (Table 1), but this did not influence the final outcome in the logistic regression analysis (Table 3).

### 3.4. Multivariate regression analysis

In the stepwise multivariate logistic regression analysis, three factors remained as independent determinant factors for PDR: diabetes duration ( $P<.001$ ), presence of albuminuria ( $P=.046$ ), and TNF- $\alpha$  ( $P=.041$ ; Table 3).

## 4. Discussion

The present study reveals elevated levels of the inflammatory marker TNF- $\alpha$  in serum samples from type 1 diabetic patients with PDR compared to patients with no diabetic retinopathy or with NPDR, suggesting that an inflammatory process might be involved in the pathogenesis of PDR. In previous *in vitro* studies, plasma from diabetic

patients with retinopathy was shown to increase TNF- $\alpha$ -associated activity of the glycosylating enzyme core 2 GlcNAc-T on cultured human myelocytic cells (Ben-Mahmud et al., 2004), an enzyme involved in leukocyte-endothelial cell adhesion and capillary occlusion in diabetic retinopathy (Chibber, Ben-Mahmud, Mann, Zhang, & Kohner, 2003). In addition, serum from diabetic patients increased endothelial cell migration-induced activity on cultured bovine retinal endothelial cells (Olson, Whitelaw, McHardy, Pearson, & Forrester, 1997). Further, inflammatory mediators were detected in the sera of diabetic patients with different vascular complications (Araszkievicz, Zozulinska, Trepinska, & Wierusz-Wysocka, 2006; Fasching et al., 1996; Schram et al., 2005) or different degrees of diabetic retinopathy (Doganay et al., 2002; Meleth et al., 2005; Olson et al., 1997), and several studies also showed elevated inflammatory activity in the vitreous of patients with proliferative diabetic retinopathy (Abu el Asrar, Maimone, Morse, Gregory, & Reder, 1992; Hernandez et al., 2001, 2005; Limb et al., 1999; Yuuki et al., 2001). The results from the present study contribute to verify the hypothesis that diabetic retinopathy is, at least in part, an inflammatory disease (Adamis, 2002; Jousseen et al., 2004).

Our primary study focus was to identify possible inflammatory markers for PDR in type 1 diabetic patients, avoiding as much comorbidity with other diseases or diabetic complications as possible. Thus, patients were recruited on the basis of earlier retinopathy grading scores from the local Diabetes 2000 register in Malmö, Sweden. Diabetes typing in this register is based on the assessment of GAD antibodies, regardless of age at diagnosis. We chose to involve only type 1 diabetic subjects in order to avoid as many confounding factors as possible. Type 2 diabetic patients have a broader disease spectrum than type 1 diabetic subjects, and obesity in itself, as well as atherosclerosis, has been suggested to be associated with the inflammatory process (Blankenberg, Barbaux, & Tiret, 2003; Wellen & Hotamisligil, 2005). However, several different aspects of complications must also be considered in type 1 diabetes. Hence, the presence of microalbuminuria and macrovascular disease was also included and analyzed in the statistical model, as was information on concomitant disease and medication. Taking all these factors into consideration, TNF- $\alpha$  still remained an independent determinant factor for proliferative diabetic retinopathy.

Several cytokines and adhesion molecules might be involved in retinal inflammatory processes, as mediators between leukocytes or as regulators of the leukocyte adhesion and activation in retinal tissue. TNF- $\alpha$  is an angiogenic cytokine and is involved in the up-regulation of receptors for adhesion molecules on leukocytes (Abu el Asrar et al., 1992; Barouch et al., 2000; Jousseen et al., 2004; Schram et al., 2005; Yuuki et al., 2001). Inhibition of TNF- $\alpha$  was shown to suppress signs of retinopathy in diabetic rats (Jousseen, Poulaki, & Mitsiades, 2002), and plasma or serum from diabetic patients with retinopathy was demonstrated to

increase TNF- $\alpha$ -associated activity in cultured human myelocytic cells (Ben-Mahmud et al., 2004) or endothelial cell migration-induced activity on cultured bovine retinal endothelial cells (Olson et al., 1997). Recently, Ben-Mahmud et al. (2006) showed that circulating TNF- $\alpha$  levels were significantly higher in plasma from type 1 and type 2 diabetic patients with PDR and that this elevation correlated with an increased activity of the glycosylating enzyme core 2 GlcNAc-T in polymorphonuclear cells. In the present study, the level of TNF- $\alpha$  was higher in patients with PDR, and taking into account other risk factors such as age, diabetes duration, and blood pressure, TNF- $\alpha$  remained an independent determinant factor for PDR, implicating a strong link to this diabetic complication.

IL-1 $\beta$  is proinflammatory with bone-marrow-stimulating properties, and it exacerbates the expression of other cytokines, chemokines, and adhesion molecules (Dinarello, 2005). It was also shown to play a role in the development of diabetic retinopathy (Abu el Asrar et al., 1992; Franks et al., 1992; Kowluru & Odenbach, 2004). In the present study, we were not able to detect IL-1 $\beta$  in any of the sera of the study patients, which might be due to some aspect of the biomedical properties of IL-1 $\beta$ . Dinarello (2005) states, for example, that IL-1 $\beta$  secretion is tightly regulated, making it hard to measure in peripheral blood in patients with active inflammation despite reversal of disease severity with the use of IL-1 receptor antagonists. Measuring techniques based on the biochemical response to blocking its receptor IL-1Ra as suggested by Dinarello might, perhaps, detect low levels of IL-1 $\beta$  in our samples, but it cannot be excluded that our findings may represent a true low level of IL-1 $\beta$  in type 1 diabetic subjects.

IL-6 is involved in the regulation of the immune system, in the acute phase reaction and inflammation, and in the growth and development of hematopoietic cells and embryonic stem cells (Song & Kellum, 2005). IL-6 acts in synergy with IL-1 $\beta$  and TNF- $\alpha$  (Song & Kellum, 2005). Several studies demonstrated a correlation between IL-6 and diabetic retinopathy (Abu el Asrar et al., 1992; Funatsu et al., 2002; Kauffmann et al., 1994; Kojima, Yamada, & Tamai, 2001; Shimizu, Funatsu, Yamashita, Yamashita, & Hori, 2002). In the present study, we did find trends toward a positive correlation between IL-6 and PDR, but the number of samples exceeding the detection limit for IL-6 was too few to allow any further statistical analyses. IL-6 was higher also in the groups of patients with signs of nephropathy or macrovascular disease, and further investigation of this molecule in the involvement of vascular complications in diabetes might be warranted.

Both sVCAM-1 and iVCAM-1 are involved in the leukocyte adhesion mechanisms (Blankenberg, Barbaux, & Tiret, 2003), and several studies pointed out their significance in the diabetic retinopathy process (Barouch et al., 2000; Fasching et al., 1996; Jousen et al., 2004; Jousen, Poulaki, & Qin, 2002; Limb, Hickman-Casey, Hollifield, & Chignell, 1999; Matsumoto et al., 2002;

McLeod, Lefler, Merges, & Luty, 1995; Olson et al., 1997). Inhibition of sVCAM-1 diminishes leukostasis and venous leakage in rats, according to Miyamoto et al. (1999), who also claim that VEGF-induced retinal vascular permeability is mediated by this molecule (Miyamoto et al., 2000). In the present study, the concentration of sVCAM-1 was higher in sera from patients with PDR. In multivariate logistic regression, however, sVCAM-1 did not persist as an independent determinant factor for PDR, which may suggest an association between sVCAM-1 and some other factor, presumably TNF- $\alpha$ .

P-selectin is one of several molecules involved in leukocyte rolling and platelet adhesion (Blankenberg et al., 2003), which might contribute to diabetic retinopathy. Disturbed patterns of leukocytes, platelets, and neutrophils have been observed in diabetic eyes and in eyes subjected to ischemia–reperfusion injury (Barouch et al., 2000; Hatchell, Wilson, & Saloupis, 1994; Nishijima et al., 2004; Tsujikawa et al., 2000). P-selectin is up-regulated in diabetic retinal and choroidal vessels (McLeod et al., 1995; Panès et al., 1996). In the present study, we found elevated levels of P-selectin in the sera of patients with PDR, but the difference did not remain after the logistic regression analysis. P-selectin is a molecule that is up-regulated quickly in an inflammatory setting but is down-regulated just as quickly by internalization and lysosomal targeting (Blankenberg et al., 2003), making it difficult to detect in peripheral blood. Thus, the serum concentrations of P-selectin in PDR in our study might be underestimated.

In summary, the present study showed elevated inflammatory markers in the sera of type 1 diabetic patients with PDR, suggesting an overall increased inflammatory state in these patients. Alongside with diabetes duration, a well-known risk factor for development of proliferative diabetic retinopathy, and the presence of albuminuria, which may have several different reasons in this patient cohort, TNF- $\alpha$  was an independent determinant factor for PDR, not influenced by any other diabetic complication, concomitant disease, or medication.

Although serum levels do not necessarily reflect local changes within ocular tissues, we suggest that inflammation may contribute to the pathogenesis of PDR. Assessments of local inflammatory markers in the human diabetic eye, as well as studies on their retinal localization and function in diabetes using animal models, will increase our understanding of their putative role in the pathogenesis of this sight-threatening eye disease.

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## Paper III





## Expression of antioxidant enzymes in rat retinal ischemia followed by reperfusion

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### Abstract

To evaluate the expression and protein levels of antioxidant enzymes in the rat retina exposed to oxidative stress induced by ischemia-reperfusion injury, Retinal ischemia was induced in female Wistar rats by ligation of the optic nerve and vessels behind the left eye bulb, and was followed by reperfusion for 0, 3, 6, or 24 hours. The right eye served as control. RNA and protein were extracted simultaneously from each retina. Expressions of the endogenous antioxidant enzymes glutathione peroxidase (GPx1), catalase (CAT), copper/zinc superoxide dismutase, manganese superoxide dismutase, and the catalytic subunit of glutamylcysteine ligase (GCLc) were analyzed with real-time reverse transcription polymerase chain reaction and related to the endogenous control cyclophilin B. Protein levels were measured with Western blot analysis. During the early phase (0 or 3 hours) of reperfusion, no changes were seen in enzyme expression. After 6 hours, GCLc expression increased by a factor of 1.14 ( $P = .034$ ), followed by a decline of 0.80 after 24 hours ( $P = .00004$ ), according to the comparative Ct method. After 24 hours of reperfusion, GPx1 expression increased by a factor of 1.14 ( $P = .028$ ), and CAT had decreased by 0.82 ( $P = .022$ ). Expressions of copper/zinc superoxide dismutase and manganese superoxide dismutase showed a tendency toward a decrease by factors of 0.86 ( $P = .055$ ) and 0.88 ( $P = .053$ ), respectively, after 24 hours. Protein levels did not differ for any of the antioxidants, regardless of reperfusion time. The slightly increased messenger RNA expression of GPx1 after 24 hours of reperfusion with a concomitant very modest decrease in CAT and GCLc expression and no change in protein levels indicate a very modest, if any, response to oxidative stress generated by ischemia followed by reperfusion in rat retina.

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### 1. Introduction

Ischemia is common in several retinal conditions, such as central and branch retinal artery occlusion, anterior ischemic optic neuropathy, venous occlusive disorders, retinopathy of prematurity, glaucoma, and diabetic retinopathy [1]. Ischemia deprives the retina of oxygen and nourishment, and compromises an efficient removal of waste products. This ultimately disrupts cellular energy metabolism and leads to several harmful events [1], for example, the formation of reactive oxygen species (ROS), degradation of the antioxidant system, induction of cytokine production via transcriptional factors, leukocyte activation triggering an

inflammatory response [2–9], and extracellular accumulation of glutamate likely to be excitotoxic to neuronal elements [10–12]. Reperfusion after initial ischemia paradoxically maintains the destruction process, perhaps due to increased levels of extracellular neurotransmitters, ROS, and waste products damaging previously unharmed cells when being reoxidized [1,13]. Any imbalance between ROS and the antioxidant defense system can create a state of oxidative stress, ultimately resulting in DNA strand breakage, damage to membrane ion transporters and other membrane components, depletion of nicotinic amide adenine dinucleotide and adenosine triphosphate, and peroxidation of lipids [1]. In response to the exposure to ischemic damage, the organism strives to modulate its gene expression of several antioxidant enzymes [1]. Superoxide dismutases (SODs) convert the superoxide radical to hydrogen peroxide, which in turn is converted to water and oxygen by catalase (CAT) and glutathione peroxidase (GPx1) in the presence of glutathione, and the catalytic subunit of glutamylcysteine ligase (GCLc)

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is needed for the de novo synthesis of glutathione, necessary for the complete antioxidant to take place [14–19].

Animal models make it possible to induce ischemia in the retina and to study the tissue response in a controlled setting. The messenger RNA (mRNA) expression and protein levels of antioxidant enzymes in the rat retina after ischemia-reperfusion injury can be considered as an indirect measure of the response to oxidative stress.

We recently showed that simultaneous extraction of RNA and protein from rat retina allows parsimonious tissue handling as well as direct coupling between an altered mRNA expression and protein levels in the retina by subsequent real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis [20]. The aim of the present study was to apply those methods to rat retina subjected to ischemia-reperfusion injury to explore the expression and protein levels of retinal endogenous antioxidant enzymes (ie, GPx1, CAT, copper/zinc superoxide dismutase [CuZnSOD], manganese superoxide dismutase [MnSOD], and GCLc) in response to oxidative stress and to relate the results to an internal control, cyclophilin B (Cyp B), for quantification.

## 2. Materials and methods

### 2.1. Animals

Female Wistar rats (body weight, 200–250 g) were from Taconic (Lille Skensved, Denmark). The animals were housed in a temperature-controlled environment with free access to food and water and a 12-hour light-dark cycle. All animals were treated according to the Principles for the Care and Use of Animals in Ophthalmic and Vision Research approved by the Association for Research in Vision and Ophthalmology. The Animal Ethics Committee of Malmö/Lund approved the study.

### 2.2. Induction of transient retinal ischemia

The animals were anesthetized intraperitoneally (0.33 mL per 100 g body weight) with a mixture of pentobarbital (9.72 mg/mL, Apoteket, Malmö, Sweden), chloral hydrate (42.5 mg/mL, Merck, Sollentuna, Sweden), magnesium sulfate (86.25 mmol/L), ethanol (10% v/v), and propylene glycol (40% v/v). The pupils were dilated with 1% Cyclogyl (cyclopentolate, Alcon, Stockholm, Sweden) and a local anesthetic; 1% Tetrakain Chauvin (Novartis Ophthalmics, Copenhagen, Denmark) was applied. Retinal blood flow was observed using a corneal contact lens and a stereomicroscope (Wild M650, Wild Heerbrugg, Heerbrugg, Switzerland). Retinal ischemia was induced by ligation of the vessels and the accompanying optic nerve behind the left eye bulb [21] using a 5-0 silk suture (Ethicon, Sollentuna, Sweden). The ligature was gently tightened until complete cessation of the retinal blood flow was observed and maintained for 45 minutes. Reperfusion was established by removing the ligature, resulting in a visibly restored blood flow; anesthesia

was disconnected; and no analgesics were administered. The animals were euthanized with CO<sub>2</sub> after 0, 3, 6, or 24 hours of reperfusion. After euthanization, each eye was immediately enucleated, the lens was removed, and the retina was gently peeled off from the pigment epithelium, snap frozen on dry ice, and stored at –80°C until use. All groups comprised 16 animals with an even spread in body weight and time of surgery during the day. The right eye served as control.

### 2.3. Simultaneous extraction of RNA and protein

The protein and RNA extraction protocol has previously been described in detail [20]. In short, the retinas were homogenized in TRI Reagent (Molecular Research Center, Cincinnati, OH), phases separated, the RNA extracted as described by Chomczynski [22], the DNA discarded, and the protein was kept in solution and washed with a wash buffer and concentrated with an ultrafiltration device. RNA and protein samples were stored at –80°C.

### 2.4. Real-time RT-PCR

cDNA was synthesized from 2 µg of RNA using the SuperScript II RNase H<sup>-</sup> RT (Invitrogen Life Technologies, Paisley, UK) protocol as described [20]. mRNA levels were analyzed with the real-time RT-PCR 7900HT system (Applied Biosystems, Stockholm, Sweden) using 5 ng of cDNA as described by Applied Biosystems. PrimerExpress 2.0 software (Applied Biosystems) was used for the design of primers and TaqMan probes (FAM-TAMRA). Each primer and probe set were selected to span over an intron-exon boundary and blasted for specificity for the rat genome against the total genome data base [23]. Relative expression levels were calculated using the comparative Ct method [24,25] with Cyp B as endogenous control [26]. If the standard deviation of the duplicate Ct value differed by more than 0.16, the sample was rerun. All sequences are 5' → 3' :

- GPx1 exons 1 and 2  
Forward: CTC GGT TTC CCG TGC AAT  
Reverse: CAT ACT TGA GGG AAT TCA GAA TCT CTT  
Probe: ATT CTT GCC ATT CTC CTG ATG TCC GAA CT
- CAT exons 12 and 13  
Forward: CCC GAG TCC AGG CTC TTC T  
Reverse: CGG CCT GTA CGT AGG TGT GA  
Probe: ACC AGT ACA ACT CCC AGA AGC CTA AGA ATG CA
- CuZnSOD exons 3 and 4  
Forward: GCG GTC CAG CGG ATG A  
Reverse: GTC CTT TCC AGC AGC CAC AT  
Probe: AGG CAT GTT GGA GAC CTG GGC
- MnSOD exons 4 and 5  
Forward: TCA GGA CCC ACT GCA AGG A  
Reverse: GCG TGC TCC CAC ACA TCA  
Probe: CCA CAG GCC TTA TTC CAC TGA TGG G

5. GCLc exons 3 and 4  
 Forward: AGG AGA ACA TCA GGC TCT TTG C  
 Reverse: GTG CTC TGG CAG TGT GAA TCC  
 Probe: CGA TAA CTT CAT TTC CCA GGC TAG  
 GCT GC
6. Cyp B exons 3 and 4  
 Forward: GGA GAT GGC ACA GGA GGA AA  
 Reverse: CCATAG TGC TTC AGC TTG AAG TTC T  
 Probe: AGC ATC TAT GGT GAG CGC TTC CCA  
 GA

### 2.5. Western blot

Gel electrophoresis and Western blot procedures were performed as previously described [20]. For immunoprob- ing, Tris-Buffered Saline (pH 7.6) with 0.1% Tween 20 (TBS-T) with 0.5% gelatine was used as blocking buffer. All primary and horseradish peroxidase-conjugated secondary antibodies were commercially available (Table 1).

### 2.6. Pooling and concentration of sample extracts

It was not possible to detect GPx1 in one single retina, as the limit for detection turned out to be 12.5 to 25  $\mu\text{g}$  [20]. This necessitated pooling of extracts from 4 individuals, and to get comparable results, analyses on all enzymes were performed on pooled retinas. A volume corresponding to 50  $\mu\text{g}$  total protein for each of 4 single-extracted samples was mixed to give a total volume of approximately 200 to 250  $\mu\text{L}$ . The pooled sample volume was transferred to a Microcon YM-3, 3000 MWCO (Millipore, Molsheim, France) centrifugal filter device and inserted into a Microcon vial (Millipore), then centrifuged at  $14000 \times g$  for 45 minutes at room temperature to receive approximately 80 to 120  $\mu\text{L}$  of retentate. The sample reservoir containing the retentate was placed upside down in a new vial, centrifuged at  $1000 \times g$  for 3 minutes at room temperature to collect the retentate, and stored at  $-20^\circ\text{C}$  for later use.

Table 1  
 Antibodies and dilutions for the Western blot multiplex assays

	Primary or secondary antibody	Dilution
Assay 1	Rabbit antihuman MnSOD (S8060-10A) <sup>a</sup>	1/1000
	Sheep antihuman CuZnSOD (S8060-15) <sup>a</sup>	1/1500
	Rabbit antihuman CAT (LF-PA0060) <sup>b</sup>	1/2000
	Rabbit antihuman Cyp B (PAI-027) <sup>c</sup>	1/100000
Assay 2	Sheep antirabbit IgG (H&L) HRP (I1964-41P) <sup>a</sup>	1/100000
	Rabbit antishsheep IgG (H&L) HRP (I1904-59C) <sup>a</sup>	1/100000
	Rabbit antirat GCLc (RB-1697-PI) <sup>d</sup>	1/1000
	Rabbit antihuman GPx1 (LF-PA0019) <sup>b</sup>	1/2000
Assay 3	Rabbit antihuman Cyp B (PAI-027) <sup>c</sup>	1/750000
	Sheep antirabbit IgG (H&L) HRP (I1904-41P) <sup>a</sup>	1/50000
	Rabbit antirat GCLc (RB-1697-PI) <sup>d</sup>	1/1000
	Rabbit antihuman Cyp B (PAI-027) <sup>c</sup>	1/750000
	Sheep antirabbit IgG (H&L) HRP (I1904-41P) <sup>a</sup>	1/50000

H&L indicates heavy and light chain.

<sup>a</sup> US Biological (Swampscott, Mass).

<sup>b</sup> Lab Frontiers (Seoul, Korea).

<sup>c</sup> Affinity Bio Reagents (Sydney, Australia).

<sup>d</sup> NeoMarkers, Lab Vision (Newmarket, Suffolk, UK).

### 2.7. Quantification of proteins

Quantification of proteins was based on a fixed concentration (yielding 50  $\mu\text{g}$ ) from each extract regardless of volume. After pooling and concentrating 4 extracts from each experimental group, the gel was loaded with a fixed volume (20  $\mu\text{L}$ ) from each pool. Extracts from 16 retinas in each group resulted in 4 samples ( $n = 4$ ) for further statistical analysis. Three multiplexed assays were run to identify the antioxidants (Table 1). The results of the chemiluminescent detection of the membranes were measured using MultiGauge v 2.2 software (Fujifilm, Stockholm, Sweden). Signal strength was given as arbitrary units (AU) for each target as well as for the endogenous control of a pooled sample. The coefficient of variation was calculated for each duplicate, and if the coefficient of variation was 15% or higher, the sample was rerun. Each pooled sample was normalized against the endogenous control by calculating the ratio between the AU value of the target and the AU value of the endogenous control. Thereafter, the relative quantity for each target was obtained by calculating the ratio between the normalized value of the ischemic eye and the control eye. For quantification, we used the same comparative Ct method as for mRNA, but because Western blot results cannot be expressed exponentially, we used ratios instead of delta ( $\Delta$ ) values. The relative quantity for Cyp B was calculated to evaluate its reliability as an endogenous control.

### 2.8. Statistics

Statistical differences were evaluated using 2-tailed paired Student *t* test for real-time RT-PCR and Wilcoxon's signed rank test for 2 related samples for Western blot. For PCR, the comparative Ct method was used to calculate the relative quantity ( $2^{-\Delta\Delta\text{Ct}}$ ) as well as the standard deviation ( $\Delta\Delta\text{Ct SD}$ ) of the mRNA expression. Expression and protein levels of each enzyme at each time point were considered exploratory, and, hence, *P* values were not adjusted for multiple comparisons in this study.

## 3. Results

### 3.1. Expression of antioxidant enzymes

The results of real-time RT-PCR are shown in Table 2. All expression values given are related to the endogenous control Cyp B and then compared to the control eye.

#### 3.1.1. Glutathione peroxidase

No change in GPx1 mRNA expression levels could be observed after 0, 3, or 6 hours of reperfusion. After 24 hours of reperfusion, there was an increase by a factor of 1.14 ( $P = .028$ ) compared to the control eye.

#### 3.1.2. Catalase

No change in CAT expression levels could be observed after 0, 3, or 6 hours of reperfusion. After 24 hours of reperfusion, a decline by a factor of 0.82 ( $P = .022$ ) was seen.

Table 2  
RNA expression of antioxidant enzymes

	Reperfusion time (h)	Control eyes		Ischemic eyes		P
		$2^{-\Delta\Delta CtL}$	$\Delta\Delta CtL$	$2^{-\Delta\Delta CtR}$	$\Delta\Delta CtR$	
			SD range		SD range	
GPx1	0	1	1.14/0.88	1.05	1.25/0.87	.514
	3	1	1.14/0.87	1.06	1.19/0.94	.327
	6	1	1.16/0.86	1.03	1.17/0.91	.643
	24	1	1.14/0.88	1.14	1.28/1.01	.028
CAT	0	1	1.27/0.79	1.04	1.16/0.93	.785
	3	1	1.32/0.76	0.92	1.06/0.81	.285
	6	1	1.12/0.89	0.88	1.10/0.70	.191
	24	1	1.20/0.89	0.82	0.98/0.70	.022
CuZnSOD	0	1	1.15/0.87	0.93	1.26/0.78	.350
	3	1	1.08/0.93	0.97	1.11/0.84	.535
	6	1	1.17/0.85	0.99	1.15/0.85	.868
	24	1	1.20/0.84	0.86	1.02/0.73	.055
MnSOD	0	1	1.19/0.84	0.95	1.11/0.81	.444
	3	1	1.13/0.88	0.99	1.11/0.88	.789
	6	1	1.27/0.79	1.27	1.17/0.82	.106
	24	1	1.14/0.87	0.88	1.03/0.74	.053
GCLc	0	1	1.16/0.86	1.00	1.17/0.85	.963
	3	1	1.17/0.85	1.11	1.30/0.96	.165
	6	1	1.15/0.87	1.14	1.28/1.01	.034
	24	1	1.13/0.89	0.80	0.89/0.71	.00004

Relative quantity for each value is given as  $2^{-\Delta\Delta Ct}$  value and range of  $\Delta\Delta Ct$  SD according to the comparative Ct method. Ischemic eyes were compared to control eyes. CtL indicates left (control) eye; CtR, right (ischemic) eye. P value according to Student paired t test.

### 3.1.3. Copper/zinc superoxide dismutase

For CuZnSOD, there were no significant changes in mRNA expression for any of the groups.

### 3.1.4. Manganese superoxide dismutase

For MnSOD, there were no significant changes in mRNA expression for any of the groups. The analysis for

the 6-hour group (factor, 1.27;  $P = .11$ ) was run twice on different occasions with identical results.

### 3.1.5. Glutamylcysteine ligase

For GCLc, there was an increase in mRNA expression levels after 6 hours of reperfusion with a factor of 1.14 ( $P = .034$ ), which turned into a decrease by a factor of 0.80 ( $P = .00004$ ) after 24 hours of reperfusion. No other changes were observed.

### 3.1.6. Cyclophilin B

The mRNA expression levels for Cyp B were monitored during all assays in parallel to the gene of interest. No significant changes could be observed for Cyp B in any of the reperfusion groups (data not shown).

### 3.2. Protein levels of antioxidant enzymes

We used a new protocol for measuring total protein concentration as it was not possible to measure the pooled and concentrated sample extracts according to the BCA standard method, as the colorimetric response was too high. Dilution of the sample retentate did not affect the results, nor was there any evidence of protein in the filtrate. We assume that concentrating the samples resulted in too high concentrations of sodium dodecyl sulfate (SDS), exceeding the 5% limit of the bicinchoninic acid method (The BCA Protein Assay, Pierce Biotechnology, Rockford, IL), or that interference was caused by SDS micelles formed during the concentration process. The critical SDS level for formation of 18-kd micelles is 0.23% [27].

Antibodies and dilutions used for the multiplex detection of the targets on Western blot are shown in Table 1. The membrane detection of multiplexed assays 1 and 3 is illustrated in Figs. 1 and 2. No significant changes in protein levels between ischemic and control eyes were observed for

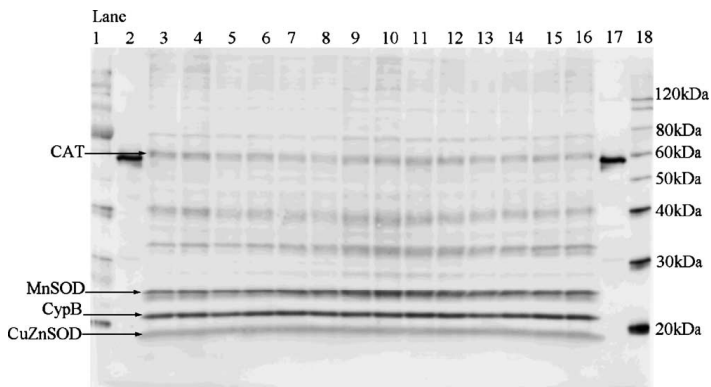


Fig. 1. Multiplexed assay 1: membrane detection of CAT, MnSOD, CuZnSOD, and Cyp B (endogenous control). Lanes 1 and 18 contained protein standards; lanes 2 and 17 contained a positive bovine CAT control; lanes 3 and 4 (0 hour), 7 and 8 (3 hours), 11 and 12 (6 hours), and 15 and 16 (24 hours) were pooled ischemic eyes; and lanes 5 and 6 (0 hour), 9 and 10 (3 hours), and 13 and 14 (6 hours) were pooled control eyes.

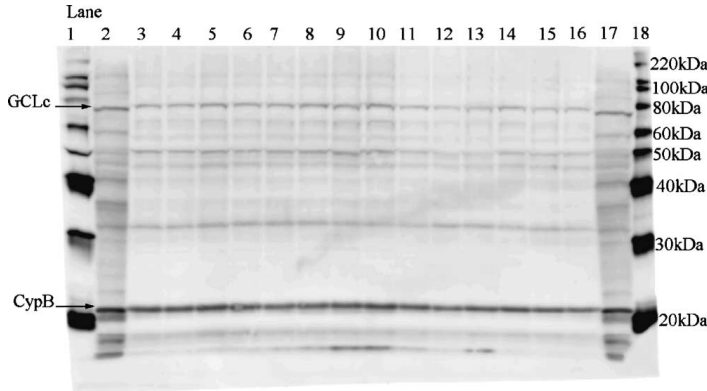


Fig. 2. Multiplexed assay 3: membrane detection of GCLc and Cyp B (endogenous control). Lanes 1 and 18 contained protein standards; lanes 2 and 17 contained a positive human GCLc cell lysate control; lanes 3 and 4, 7 and 8, and 11 and 12 were pooled (24 hours) control eyes and lanes 5 and 6, 9 and 10, 13 and 14, and 15 and 16 were pooled (24 hours) control eyes.

any of the antioxidants, regardless of group (Table 3). Omitting one CuZnSOD outlier in the ischemia followed by the 3-hour reperfusion group did not change the results. The endogenous control was stable except for one outlier in the ischemia without a reperfusion group. Omitting this outlier did not change the results.

#### 4. Discussion

Previous studies have demonstrated an elevated presence of free radicals in ischemic/reperfused rat retina, either directly by electron paramagnetic resonance [28] or indirectly by showing diminished damage after administering antioxidant drugs such as SOD, EGB 761 extracted from *Ginkgo biloba*, vitamin E, mannitol, CAT, and several other compounds [2,29,30]. Several studies have demonstrated the ischemic effect on the retina as shown by electroretinogram (ERG), either alone [31] or by modulation by administration of antioxidant drugs [31–35]. However, only a few studies have dealt with the endogenous antioxidant system in the retina, for example, in Muller cells subjected to oxidative stress [36] and in retina subjected to ischemia-reperfusion injury [37–39]. In the present study, we demonstrate that retinal ischemia followed by reperfusion induces only a slight change in endogenous antioxidant enzyme expressions. Real-time RT-PCR revealed the mRNA expression of GCLc after 6 hours of reperfusion to be modestly but significantly increased, as was the expression of GPx1 after 24 hours of reperfusion with a concomitant decline of CAT and GCLc. In this study, Cyp B was used as an endogenous control, and no changes were observed in any of the reperfusion groups. This is in line with previous studies [40–42].

Our results indicate that the endogenous antioxidant system in the rat retina does not respond particularly well to

ischemia/reperfusion induced by temporary ligation of the vessels along the optic nerve. We applied 45 minutes of ischemia, which should be sufficient to induce substantial ischemic damage. Osborne et al [1] claimed that 20 minutes of ischemia was required to cause irreversible functional ischemic injury in Wistar rat retinas as demonstrated with ERG, and 45 minutes was needed to produce histopathological changes. Block and Schwarz [31] demonstrated reversible ERG changes within 30 minutes regardless of

Table 3  
Protein levels of antioxidant enzymes

	Reperfusion time (h)	Control eyes		Ischemic eyes		P
		Median	Range	Median	Range	
GPx1	0	1.08	0.92	1.28	1.68	.465
	3	0.92	0.89	0.64	0.87	.715
	6	0.81	1.29	0.68	1.68	1.000
	24	0.85	0.79	1.86	1.12	.144
CAT	0	0.79	1.18	0.88	1.32	1.000
	3	1.05	0.45	1.00	3.00	.715
	6	0.99	0.69	1.25	1.40	.144
	24	0.58	2.01	0.84	0.71	1.000
CuZnSOD	0	0.36	2.77	1.06	1.25	.715
	3	0.98	0.32	1.14	6.46	.144
	6	0.34	2.80	1.12	0.53	.715
	24	0.41	2.78	0.90	0.99	.715
MnSOD	0	1.00	0.23	1.01	0.31	.715
	3	1.04	0.27	1.11	1.30	.465
	6	0.28	2.92	1.02	1.11	.715
	24	0.25	3.07	1.07	0.50	.715
Cyp B	0	1.10	1.30	0.95	16.07	.401
	3	0.99	0.73	0.97	1.76	.889
	6	1.08	1.45	1.00	1.21	.575
	24	1.00	1.69	1.01	0.97	.937
GCLc	24	0.93	0.43	0.98	0.20	1.000

Relative quantity for each target given as median value and range. Ischemic eyes were compared to control eyes. P value according to Wilcoxon signed ranks test.

occlusion method, but 60 minutes or more was necessary to induce irreversible changes using central retinal artery occlusion, and Hayreh et al [35] found irreversible ERG changes to occur sometime between 97 and 105 minutes. After more than 90 to 100 minutes of ischemia, cell death may occur [2,43-45], precluding further analyses of processes in viable cells. Because the goal of the present study was to measure cellular expression changes in response to ischemia-reperfusion damage, it was desirable not to exceed the time limit for viable retinal cells.

Various experimental designs have been set up for the induction of ischemia-reperfusion damage in the retina, that is, 2-vessel occlusion by bilateral clamping of the common carotid arteries [31], 4-vessel occlusion by clamping of the vertebral arteries as well as the common carotid arteries [31], central retinal artery occlusion by ligation of the optic nerve [46,47], raised intraocular pressure above the arterial opening pressure [45,46], microembolization [46,47], and laser coagulation of peripheral retinal arteries [31,48,49]. In the present study, we used the method of optic nerve ligation [21], including the central artery and vein. This method enables easy reversible occlusion, a visually controlled blood flow, and prevents additional injury caused by raised intraocular pressure as well as a global cerebral damage by occlusion of major cerebral vessels.

The various expression scenarios for the antioxidant targets involved in the present study may be the result of different turnover rates for the various enzymes and different feedback mechanisms in the biosynthetic pathways, as autoregulation by negative feedback is a key mechanism for the cellular enzyme cascades. It may well be that the enzymes are in the process of normalization after 24 hours of reperfusion, and it cannot be excluded that an altered mRNA expression actually culminates either before 3 hours or sometime between 6 and 24 hours of reperfusion, which would explain the modest expression alterations. There is also a possibility that very short or longer reperfusion periods might have triggered further gene expression. However, there are no consensual data as to which reperfusion time is the most appropriate. It seems that longer reperfusion periods are necessary to induce macroscopic and histological damage [21,30,31,38,41,45], but this does not exclude that alterations in gene expression may occur much earlier; the peak intensity of free radical production in the study of Szabo et al [2] was observed after only 3 minutes of reperfusion.

We have previously shown that all of the antioxidant enzymes measured except for GPx1 were detectable by Western blot [20]. Our intention was to analyze each retina separately. However, as we observed a slightly increased expression of GPx1 after 24 hours of reperfusion, we found it important to include also this enzyme in our Western blot analyses, and hence we decided to pool retinas. Despite this procedure, we found no significant changes in protein levels between ischemic and control eyes in any of the groups. Whereas real-time RT-PCR is a highly sensitive method

enabling the detection of very small changes in mRNA expression, Western blot analysis is less sensitive and requires larger quantities of enzymes. Thus, there might have been undetected small alterations in enzyme production, unrevealed by Western blot.

In the present exploratory study, we were not able to demonstrate a clearly altered mRNA expression of endogenous antioxidant enzymes in response to ischemia and subsequent reperfusion in rat retina and there were no correlated protein level changes. After a Bonferroni correction, only one of the observations, the decreased GCLc expression after 24 hours of reperfusion, remained significant. For the other enzymes, it remains an open question whether the small differences in expression could be real. It has been argued that the retina is comparatively insensitive to ischemic injury compared to the closely related brain tissue, which could depend on intravitreal glucose and intraretinal glycogen storage, the ability of photoreceptors to exploit energy sources anaerobically, and the expandable nature of the retinal tissue in an edematous state [1]. Moreover, because the retina is a tissue with a mixture of different cell types, the mRNA expression is necessarily a summation response. There is a possibility for one cell type being more vulnerable to oxidative stress than others, and narrowing the process down to one particular cell type may perhaps reveal a more specific response of oxidative stress effects in the retina.

#### Acknowledgment

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## Paper IV



# INFLAMMATORY MARKERS IN NONDIABETIC AND DIABETIC RAT RETINAS EXPOSED TO ISCHEMIA FOLLOWED BY REPERFUSION

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**Purpose:** To examine the retinal inflammatory response to ischemia–reperfusion in nondiabetic and diabetic rats injected with either an  $\omega$ -3-polyunsaturated fatty acid (docosahexaenoic acid [DHA]) or a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (pravastatin).

**Methods:** Diabetes was induced by an intraperitoneal injection of streptozocin, and retinal ischemia was induced by ligation of the optic nerve and vessels, followed by reperfusion for 1 hour or 24 hours. Five minutes before surgery, an intravenous injection of DHA, pravastatin, or vehicle (ethanol) was administered. The mRNA expressions of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, caspase-1, IL-1 $\beta$ , P-selectin, vascular cellular adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 were compared between ischemic and nonischemic retinas as well as diabetic and nondiabetic nonischemic retinas.

**Results:** Ischemia induced increased expressions of TNF- $\alpha$  ( $P \leq 0.012$ ), IL-1 $\beta$  ( $P \leq 0.017$ ), ICAM-1 ( $P \leq 0.025$ ), and IL-6 ( $P = 0.012$ ), and diabetes induced increased expression of caspase-1 ( $P \leq 0.046$ ), VCAM-1 ( $P \leq 0.027$ ), ICAM-1 ( $P \leq 0.016$ ), IL-1 $\beta$  ( $P = 0.016$ ), and IL-6 ( $P = 0.041$ ). Ischemia plus diabetes did not increase these findings significantly. DHA and pravastatin had some inhibitory effects in diabetic rats ( $P \leq 0.037$ ).

**Conclusions:** Inflammation triggered by ischemia–reperfusion may play a role in diabetic retinopathy. Intervention on lipid-based structures by  $\omega$ -3-polyunsaturated fatty acids or statins seemed to have beneficial effects on inflammation in diabetes.

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Several studies have shown higher expression levels of various cytokines in retinas from rats subjected to ischemia–reperfusion injury<sup>1,2</sup> as well as in retinas from rats with diabetes.<sup>3</sup> A link between ischemia–

reperfusion and inflammation in the process of diabetic retinopathy and the development of proliferative diabetic retinopathy has been suggested. Ischemia in diabetic retinopathy leads to several harmful events, including formation of reactive oxygen species,<sup>4,5</sup> induction of cytokine production, and leukocyte activation.<sup>2,6,7</sup> Further, experimentally induced ischemia–

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reperfusion injury has been shown to elevate the retinal expressions of several inflammatory markers.<sup>8</sup>

The hypothesis that chronic low-grade inflammation may be involved in the pathogenesis of diabetic retinopathy<sup>9</sup> has lately been confirmed by several studies demonstrating elevated score levels of inflammatory markers in serum or plasma from diabetic patients<sup>10,11</sup> as well as in the vitreous from patients with proliferative diabetic retinopathy.<sup>12–14</sup> We have recently shown that tumor necrosis factor alpha (TNF)- $\alpha$  is elevated in serum from type 1 diabetic patients with proliferative diabetic retinopathy, independently of other well-known risk factors.<sup>15</sup> Lipid-based molecules may be involved in the induction of inflammation and angiogenesis. It has been suggested that the administration of  $\omega$ -3-polyunsaturated fatty acids ( $\omega$ 3-PUFAs) can protect against diabetic retinopathy<sup>16</sup> and ischemia–reperfusion injury in rat retina as shown on electroretinograms.<sup>17</sup> Docosahexaenoic acid (DHA) is a major dietary  $\omega$ 3-PUFA, and a structural lipid of the retinal photoreceptors, and may protect from several harmful events, such as ischemia and oxidative stress or inflammation.<sup>16</sup> The administration of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitory compounds (i.e., statins) has been shown to reduce the histologic effects of ischemia–reperfusion injury as well as the increased expression of P-selectin and intercellular adhesion molecule (ICAM)-1 in rat retinas.<sup>18</sup> Further, it has been shown to protect from blood–retinal barrier breakdown in diabetic rats,<sup>19</sup> to decrease inflammation, and to increase the production of endothelial nitric oxide synthase, which may contribute to endothelial relaxation after transient ischemia in brain tissue.<sup>20</sup>

Experimentally induced ischemia followed by reperfusion in diabetic rat retina can provide new knowledge on possible harmful events preceding progression to proliferative retinopathy in human diabetic eyes. The aim of the present study was to reveal a possible link between ischemia and inflammation in diabetic rat retina by exploring the mRNA expression of some inflammatory markers previously suggested to be involved in diabetic retinopathy (i.e., TNF- $\alpha$ ,<sup>21</sup> interleukin [IL]-6,<sup>22</sup> caspase-1,<sup>2</sup> IL-1 $\beta$ ,<sup>3</sup> P-selectin,<sup>23</sup> vascular cellular adhesion molecule [VCAM]-1, and ICAM-1<sup>24</sup>) as well as to reveal whether any response could be influenced by the administration of DHA (22:6n-3  $\omega$ 3-PUFA) or pravastatin.

## Materials and Methods

### Animals

Female Wistar rats (body weight, 200–250 g) were purchased from Taconic (Lille Skensved, Denmark).

The animals were housed in a temperature-controlled environment with free access to food and water during a 12-hour light–dark cycle. All animals were treated according to the Principles for the Care and Use of Animals in Ophthalmic and Vision Research approved by the Association for Research in Vision and Ophthalmology. The Animal Ethics Committee of Malmö/Lund (Sweden) approved the study.

### Induction of Diabetes

Diabetes was induced by an intraperitoneal injection of streptozocin (freeze-dried Zanosar; Pharmacia-Upjohn, Kalamazoo, MI) (60 mg/kg body weight in 0.9% sodium chloride, yielding 100 mg of streptozocin and 22 mg of citric acid/mL; pH 3.5–4.5). If the blood glucose level was  $<15$  mmol/L after 4 days, the injection was repeated once, and thereafter, only rats with blood glucose levels of  $\geq 15$  mmol/L were included and kept for 1 month. In diabetic rats, mean blood glucose levels  $\pm$  SD increased from  $5.9 \pm 0.5$  mmol/L at baseline to  $28.5 \pm 3.0$  mmol/L at the time of surgery. In nondiabetic control rats, mean blood glucose levels  $\pm$  SD were  $6.0 \pm 0.5$  mmol/L.

### Induction of Transient Retinal Ischemia

The animals were anesthetized (0.33 mL/100 g body weight) with a mixture of pentobarbital (9.72 mg/mL; Apoteket, Malmö, Sweden), chloral hydrate (42.5 mg/mL; Merck, Sollentuna, Sweden), magnesium sulfate (86.25 mmol/L), ethanol (10% vol/vol), and propylene glycol (40% vol/vol) given intraperitoneally. Diabetic rats were divided into three groups matched for blood glucose levels. Five minutes before retinal surgery, one group of diabetic rats ( $n = 8$ ) received an intravenous injection of 90% ethanol (vehicle), a second group ( $n = 8$ ) received 55  $\mu$ L/kg of DHA (Larodan Fine Chemicals, Malmö, Sweden), and a third group ( $n = 8$ ) received 50 mg/kg of pravastatin (Calbiochem/Merck, Darmstadt, Germany). The same procedure was performed for the same subgroups and numbers ( $n = 10$ ,  $n = 8$ , and  $n = 8$ , respectively) of nondiabetic rats.

The pupils were dilated with 1% Cyclogyl (cyclopentolate; Alcon, Stockholm, Sweden) and a local anesthetic, 1% Tetrakain Chauvin (Novartis Ophthalmics, Copenhagen, Denmark), was applied. Retinal blood flow was observed using a corneal contact lens and a stereomicroscope (Wild M650, Heerbrugg, Switzerland). Retinal ischemia was induced by a ligation around the optic nerve and accompanying vessels behind the left eye bulb<sup>25</sup> using a 5-0 silk suture (Ethicon, Sollentuna, Sweden). The ligation was tightened until complete cessation of the retinal blood flow

was observed and thereafter was maintained for 45 minutes. Reperfusion was established by removing the ligature, resulting in visibly restored blood flow, anesthesia was stopped, and no analgesics were administered. The right eye served as control. The animals were killed with CO<sub>2</sub> after 1 hour (group I) or 24 hours (group II) of reperfusion; each eye was immediately enucleated, the lens was removed, and the retina was peeled off from the pigment epithelium, snap frozen on dry ice, and stored at -80°C until use.

#### *Extraction of RNA*

The extraction protocol was according to Chomczynski<sup>26</sup> with a modified protein isolation step.<sup>27</sup> Each retina was homogenized in 1 mL of TRI reagent (Sigma-Aldrich, Stockholm, Sweden) supplemented with 5  $\mu$ L of Polyacryl carrier (Molecular Research Center, Cincinnati, OH) on a rotor-stator Polytron (PT1200; Kinematica AG, Littau-Lucerne, Switzerland). One hundred microliters of 1-bromo-3-chloropropane (Sigma-Aldrich) was then added, and the samples were vortexed and left for 15 minutes before phases were separated by centrifugation at 12,000g for 15 minutes at 4°C. The aqueous phase (RNA) and the red organic phase (protein) were then transferred to new tubes, while the interphase (DNA) was discarded. The RNA was precipitated with 500  $\mu$ L of isopropanol at 12,000g for 10 minutes at 4°C. The pellet was left to air dry before it was dissolved in 50  $\mu$ L of DEPC-H<sub>2</sub>O supplemented with 60 U of RNasin Plus RNase Inhibitor (Promega, Madison, WI). Total RNA quantification was performed on a spectrophotometer (Biophotometer; Eppendorf, Hamburg, Germany). The samples were stored at -80°C until analysis.

#### *Real-Time Reverse Transcriptase Polymerase Chain Reaction Analysis*

cDNA was synthesized from 2  $\mu$ g of RNA using 200 U of RevertAid RNase H RT (Fermentas, Helsingborg, Sweden) and 250 ng of random hexamer (Amersham Biosciences, Uppsala, Sweden) primer for 2 hours at 42°C. Expression of TNF- $\alpha$ , IL-6, caspase-1, IL-1 $\beta$ , P-selectin, VCAM-1, ICAM-1, and the internal control cyclophilin B mRNA levels was analyzed using real-time reverse transcriptase polymerase chain reaction analysis on a 7900HT system (Applied Biosystems, Stockholm, Sweden). TaqMan assays were from Applied Biosystems (assays on demand): TNF- $\alpha$  (Rn00562055\_m1), IL-6 (Rn00561420\_m1), caspase1 (Rn00563627\_m1), IL-1 $\beta$  (Rn005800432\_m1), ICAM-1 (Rn00564227\_m1), VCAM-1 (Rn00563627\_m1), and cyclophilin B (Rn00574762\_m1). For each reaction, 10 ng to 100 ng

of cDNA (depending on the assay), 1 $\times$  TaqMan assay mix, and 1 $\times$  TaqMan Universal PCR Master Mix was loaded in duplicate. If the SD of the duplicate threshold cycle (Ct) value differed by >0.16, the sample was later rerun. Probes were dually labeled with 6-FAM (reporter) on the 5'-end and tetramethylrhodamine (quencher) on the 3'-end.

The relative expression of enzymes was calculated using the comparative Ct method (as stated by Applied Biosystems) with cyclophilin B as endogenous control.<sup>28</sup> In short, the calculation was done by subtracting the control values from the target values in two steps yielding a  $\Delta\Delta$ Ct value that was then raised to the negative power of 2. In the first step, the template input for each retina was normalized against an endogenous control by subtracting the endogenous control Ct value from the target Ct value, yielding a  $\Delta$ Ct value. In the second step, the relative expression of the target gene was obtained by subtracting the  $\Delta$ Ct value of the control retina from the  $\Delta$ Ct value of the ischemic retina, yielding a  $\Delta\Delta$ Ct value. Each cycle of polymerase chain reaction replication will double the amount of DNA (i.e., the fluorescent signal), and the higher the amount of cDNA template from the start, the sooner the polymerase chain reaction will reach its exponential phase (i.e., the Ct value) and, hence, the reason for raising the  $\Delta\Delta$ Ct value to the negative power of 2 ( $2^{-\Delta\Delta$ Ct}) to obtain the relative expression value.

#### *Statistical Analysis*

Statistical differences were evaluated by a two-tailed Wilcoxon signed ranks test for pairs (i.e., ischemic versus contralateral control retinas) or the Mann-Whitney rank sum test for nonpairs (i.e., nonischemic diabetic control versus nondiabetic control retinas) using SPSS version 12.0.1 (SPSS, Inc., Chicago, IL). There were too many factors for adequate correction for multiple comparisons to be performed. However, hazard significances (i.e., statistical significances by chance due to the many comparisons) seem unlikely because the results were the same between groups repeatedly.  $P \leq 0.05$  was considered significant.

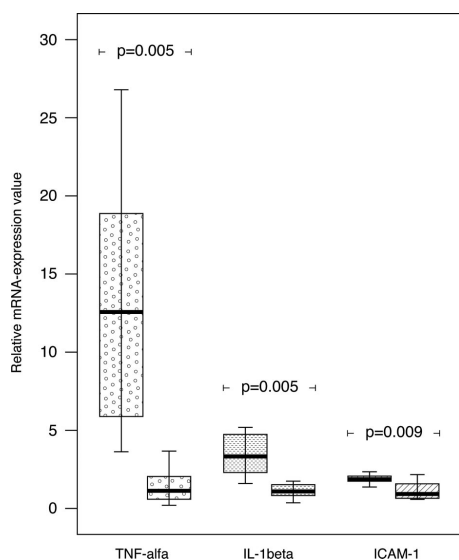
## **Results**

#### *Expression of Inflammatory Mediators After Ischemia Followed by Reperfusion*

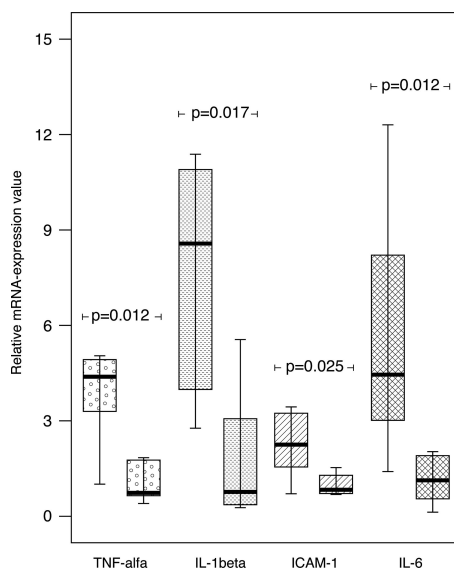
*Nondiabetic Rat Retinas.*—In nondiabetic rat groups I (1 hour of recirculation) and II (24 hours of recirculation), retinal ischemia followed by reperfusion resulted in 13.2-fold ( $P = 0.005$ ) and 4.9-fold ( $P = 0.012$ ) increased expression of TNF- $\alpha$ , respectively, 3.9-fold ( $P = 0.005$ ) and 8.9-fold ( $P = 0.017$ ) in-

creased expression of IL-1 $\beta$ , respectively, and 1.9-fold ( $P = 0.009$ ) and 2.6-fold ( $P = 0.025$ ) increased expression of ICAM-1, respectively, as compared with retinas of nonischemic control eyes. There was also 5.6-fold ( $P = 0.012$ ) increased expression of IL-6 in group II. The expressions of caspase-1, P-selectin, and VCAM-1 did not change. The magnitudes of expression between group I and group II did not differ significantly for any of the markers. Positive results are presented in Figures 1 and 2.

**Diabetic Rat Retinas.**—In diabetic rat groups I and II, retinal ischemia followed by reperfusion resulted in 11.8-fold ( $P = 0.012$ ) and 2.6-fold ( $P = 0.017$ ) increased expression of TNF- $\alpha$ , respectively, 4.0-fold ( $P = 0.012$ ) and 12.4-fold ( $P = 0.012$ ) increased expression of IL-1 $\beta$ , respectively, and 1.7-fold ( $P = 0.012$ ) and 1.8-fold ( $P = 0.017$ ) increased expression of ICAM-1, respectively. There was also 8.7-fold ( $P = 0.012$ ) increased expression of IL-6 in group II. The expressions of caspase-1, P-selectin, and VCAM-1 did not change. The magnitudes of expression between group I and group II and between nondiabetic and diabetic rat retinas did not differ significantly for any of the markers. Positive results are presented in Figure 3 and 4.



**Fig. 1.** Box plot. Relative mRNA expressions of inflammatory markers in ischemia-reperfusion (left bars) versus nonischemic control (right bars) retinas from nondiabetic rats after ischemia for 45 minutes followed by 1 hour of reperfusion. Median (minimum to maximum). TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule.



**Fig. 2.** Box plot. Relative mRNA expressions of inflammatory markers in ischemia-reperfusion (left bars) versus nonischemic control (right bars) retinas from nondiabetic rats after ischemia for 45 minutes followed by 24 hours of reperfusion. Median (minimum to maximum). TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule.

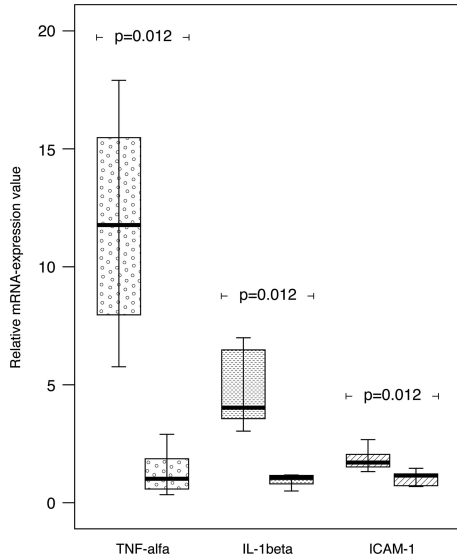
#### *Influence of DHA and Pravastatin on Expression of Inflammatory Mediators After Ischemia Followed by Reperfusion*

**Nondiabetic Rat Retinas.**—Injection of DHA or pravastatin had no effect on the ischemia-induced expressions in nondiabetic ischemic rat retinas.

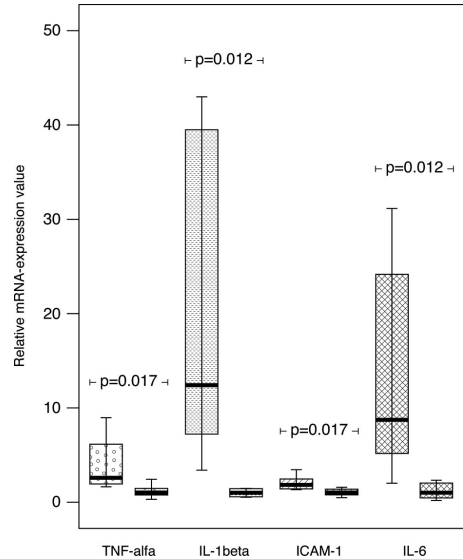
**Diabetic Rat Retinas.**—Injection of DHA had no effect on the ischemia-induced expressions in diabetic ischemic rat retinas. Injection of pravastatin reduced the increased expression of IL-6 from 8.7-fold to 3.1-fold ( $P = 0.016$ ) and ICAM-1 from 1.8-fold to 1.3-fold ( $P = 0.012$ ) in diabetic ischemic rat retinas in group II (data not shown).

#### *Expression of Inflammatory Mediators in Diabetic Versus Nondiabetic Rat Retinas Unexposed to Ischemia-Reperfusion (Control Eyes)*

Diabetic rat retinas unexposed to ischemia-reperfusion (control retinas) were compared with likewise unexposed control retinas from nondiabetic rats to find out if diabetes per se could trigger any inflammatory response in the absence of ischemia-reperfusion. In diabetic rat retina groups I and II, there was 3.3-fold



**Fig. 3.** Box plot. Relative mRNA expressions of inflammatory markers in ischemia–reperfusion (left bars) versus nonischemic control (right bars) retinas from diabetic rats after ischemia for 45 minutes followed by 1 hour of reperfusion. Median (minimum to maximum). TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule.



**Fig. 4.** Box plot. Relative mRNA expressions of inflammatory markers in ischemia–reperfusion (left bars) versus nonischemic control (right bars) retinas from diabetic rats after ischemia for 45 minutes followed by 24 hours of reperfusion. Median (minimum to maximum). TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule.

( $P = 0.010$ ) and 2.8-fold ( $P = 0.046$ ) increased expression of caspase-1, respectively, 2.8-fold ( $P = 0.021$ ) and 2.6-fold ( $P = 0.027$ ) increased expression of VCAM-1, respectively, and 2.2-fold ( $P = 0.004$ ) and 2.2-fold ( $P = 0.016$ ) increased expression of ICAM-1, respectively, as compared with nondiabetic rat control retinas. There was additional 6.1-fold upregulation of IL-6 ( $P = 0.041$ ) and 2.2-fold upregulation of IL-1 $\beta$  ( $P = 0.026$ ) in group I. The expressions of TNF- $\alpha$  and P-selectin did not differ between diabetic and nondiabetic rat retinas in either group. The magnitudes of expression between group I and group II did not differ significantly for any of the markers (data not shown).

#### *Influence of DHA and Pravastatin on Expression of Inflammatory Mediators in Diabetic Versus Nondiabetic Retinas Unexposed to Ischemia–Reperfusion (Control Eyes)*

Injection of DHA reduced the 6.1-fold upregulation of IL-6 in diabetic rat retinas by 50% ( $P = 0.037$ ) and normalized the 2.8-fold upregulation of VCAM-1 ( $P = 0.012$ ) in group I. Pravastatin normalized the upregula-

tion of IL-6 in diabetic rat retinas ( $P = 0.012$ ) in group I. No other effects were observed (data not shown).

#### Discussion

The present study revealed increased expressions of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and ICAM-1 in retinas from both nondiabetic and diabetic rats subjected to ischemia–reperfusion, which is in accordance with the results of other studies<sup>1–3,29</sup> and supports the idea that retinal ischemia results in an inflammatory tissue response. Such a response might be provoked by vascular endothelial growth factor–stimulated recruitment of inflammatory leukocytes in the ischemic retina at sites of pathologic neovascularization, as shown by Ishida et al.<sup>30,31</sup> Likewise, expression of glutaredoxin, a regulating enzyme protective against irreversible protein oxidation in oxidative stress, was recently found to be increased in hyperglycemic retinal Müller cells, with nuclear factor  $\kappa$ B–driven ICAM-1 induction correlating at sites of ischemia,<sup>32</sup> suggesting that a disturbed antioxidative defense system in hyperglycemic retina might be proinflammatory. One could speculate upon a possible correlation between the vascular endothelial growth factor–stimulated recruitment of leukocytes

and an increased adhesion of these cells to the retinal vessels in ischemic conditions with perturbation of the antioxidative system in hyperglycemia. In the present study, on cytokines and adhesion molecules, the median values of the IL-6 and IL-1 $\beta$  expressions were higher in ischemic retinas from diabetic rats, although the magnitudes of expression between nondiabetic and diabetic rat retinas did not differ significantly for any of the markers. The inflammatory response to ischemia–reperfusion could have been too extensive to reveal differences between nondiabetic and diabetic animals, but it is also possible that ischemia and hyperglycemia trigger different pathways in the inflammation cascade. In control eyes not subjected to ischemia–reperfusion, retinas from diabetic rats were shown to have elevated expression of several inflammatory markers compared with nondiabetic rat retinas. This may suggest that hyperglycemia triggers an inflammatory response in the retina, perhaps making it vulnerable to superimposed ischemic damage, as stipulated by Panès et al.<sup>33</sup> We and other researchers have recently demonstrated elevated levels of inflammatory markers in sera from patients with proliferative diabetic retinopathy.<sup>10,11,15</sup> We found a TNF- $\alpha$  elevation independent of other well-known risk factors. TNF- $\alpha$  is an extremely potent key cytokine in several inflammatory conditions and is involved indirectly in the angiogenesis process by stimulation of vascular endothelial growth factor and other proangiogenic factors<sup>34</sup>; in addition, TNF- $\alpha$  and IL-6, IL-1 $\beta$ , caspase-1, ICAM-1, VCAM-1, and P-selectin have all been implicated in diabetic retinopathy, involved in the promotion of inflammation (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ )<sup>3,21,22,35</sup> or capillary destruction and obstruction (caspase-1)<sup>36,37</sup> or as controllers of leukocyte and platelet rolling, activation, and adhesion (ICAM-1, VCAM-1, and P-selectin).<sup>38–40</sup> Studies on how inflammation may contribute to diabetic retinopathy and whether damage may be counteracted by inflammation modulating therapies are thus important.

Bioactive molecules from lipid-based structures may be involved in the development of diabetic retinopathy. In the present study, the administration of DHA or pravastatin 5 minutes before surgery did not have any effect on the inflammatory upregulation triggered by ischemia, but it depressed the diabetes-induced expression of inflammatory markers in nonischemic retinas in group I. DHA is a major dietary  $\omega$ 3-PUFA abundant in the photoreceptors of the retina, and it was found to protect from ischemia–reperfusion injury and inflammation in human retinal vascular endothelial cells<sup>41</sup> and in rabbit retina.<sup>17</sup> It was recently demonstrated that mice fed a  $\omega$ 3-PUFA-rich diet as compared with  $\omega$ 6-PUFAs had less retinopathy development in a model of retinopathy of prematurity.<sup>42</sup>

The effect of DHA on cytokine expressions in diabetic animals in the present study may support certain antiinflammatory qualities by this molecule in diabetes. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (i.e., statins) widely used in dyslipidemic and atherosclerotic conditions have been shown to exert antiinflammatory effects beside their lipid-lowering actions<sup>43</sup> and to decrease inflammation and increase the production of endothelial nitric oxide synthase, which may contribute to endothelial relaxation after transient ischemia in brain tissue.<sup>20</sup> Statins were further shown to inhibit cytokine-induced leukocyte–endothelial interaction in transient ischemia–reperfusion injury in rat retina<sup>18</sup> as well as to protect from blood–retinal barrier breakdown in diabetic rats.<sup>19</sup> It has been argued that statins may interfere with the signaling between advanced glycation end products and their receptors formed at an accelerated rate in diabetes, thereby suppressing advanced glycation end products/receptors of advanced glycation end products–elicited angiogenesis.<sup>44</sup> Miyahara et al<sup>45</sup> showed that diabetes-induced elevated expression of ICAM-1 and subsequent elevated leukocyte adhesion in rat retina were suppressed by the administration of simvastatin orally for 2 weeks after diabetes onset. Our findings seem to support that statins may have antiinflammatory effects in diabetic animals, but there is also a possibility that administration for a longer time before surgery in our study would have yielded more profound effects on the expressions of cytokines induced by ischemia–reperfusion in both nondiabetic and diabetic rats.

In conclusion, the present study revealed increased expressions of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and ICAM-1 in rat retina subjected to ischemia–reperfusion, supporting the hypothesis that retina exposed to ischemia–reperfusion elicits a profound inflammatory response. Although the magnitudes of expression between nondiabetic and diabetic rat retinas did not differ for any of the markers, the fact that there was elevated expression of several inflammatory markers in diabetic retinas unexposed to ischemia may suggest a hyperglycemia-triggered inflammatory response in diabetic retinas, which could make them more vulnerable to ischemia–reperfusion injury. A combined effect of hyperglycemia and ischemia–reperfusion, eliciting inflammation alone or together, may contribute to the pathogenesis of proliferative diabetic retinopathy. Although our study failed to demonstrate any protective effect of DHA in both nondiabetic and diabetic eyes as well as of pravastatin in nondiabetic eyes for ischemia–reperfusion–elicited inflammatory upregulation, both drugs reduced the expressions of two inflammatory markers in nonischemic diabetic control eyes. It could

be argued that the treatment period was too short or that the ischemic impact was too profound. On the other hand, the drugs had some effect in diabetic animals, but further studies are needed before it can be revealed whether  $\omega$ 3-PUFAs or statins can prevent the harmful effects of ischemia and perhaps delay progression to proliferative diabetic retinopathy.

**Key words:** cytokines, diabetic retinopathy, docosahexaenoic acid, inflammatory mediators, ischemia-reperfusion, pravastatin.

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