"Diabetic retinopathy;

from glycation to clinical aspects"

by

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List of errata

- **Page 5 within 4.3:** The title of Paper III; ”Serum levels of the Advanced Glycation End product hydroimidazolone is associated with retinopathy in type 1 diabetes patients” *should read:* ”Serum levels of the Advanced Glycation End product hydroimidazolone are associated with retinopathy in type 1 diabetes patients”.
- **Pages 2 and 15:** “**5.5**” *should read* “**5.4**”
- **Page 7 within 5.2:** "Organs mainly affected are the eyes, kidneys and nerves (15); diabetic retinopathy." *should read:* "Organs mainly affected are the eyes, kidneys and nerves (15) with diabetic retinopathy, nephropathy and neuropathy, respectively."
- **Page 28:** "(Blood tests were collected after overnight fasting." *Omit* initial parenthesis.

To my patients

Contents

1. Preface *"There is only one thing about which I am certain, and that is that there is very little about which one can be certain." W. Somerset Maugham*

2. Acknowledgements
This piece of work was carried out during intervals between 2000 and 2007 at the Diabetes Research Centre of Aker and Ullevål University Hospitals, Oslo, with the facilities of Aker University Hospital and the Hormone Laboratory, Oslo. Financial support has been granted by the Norwegian Foundation for Health and Rehabilitation after recommendations from the Norwegian Diabetes Association; the Research Forum of Ullevål University Hospital (FUS) and the Research Forum of Aker University Hospital (FAS); the Diabetes Research Centre of Aker and Ullevål University Hospitals, the Regional Health Board (Helse Øst), my own pocket and the Remembrance Foundation of Inger Holm.

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To the ones not mentioned; thank you for the open doors.

3. Abbreviations

α2β1	α2β1 trans-membrane cell-linker
ADRB3	β3 adrenergic receptor gene
AGE(s)	advanced glycation end product(s)
ALR	aldose reductase
ALT-711	alagebrium chloride
AMD	age-related macular degeneration
BRB	blood-retinal barrier
BSA	bovine serum albumin
CEC	carboxyethylcysteine
CEL	carboxyethyllysine
CI	confidence interval
CMC	carboxymethylcysteine
CML	carboxymethyllysine
CSME	clinically significant macular edema
DAG	diacylglycerol
DCCT	diabetes control and complications study
DELFIA	dissociation enhanced lanthanide fluoro-immunoassay
DNA	deoxyribonucleic acid
DNR	diabetes and no retinopathy
DOLD	3-Deoxyglucosone-derived lysine dimer
DR	diabetic retinopathy
ELISA	enzyme-linked immunosorbent assay
ETDRS	early treatment of diabetic retinopathy study
GOLD	glyoxal imidazolium crosslink
HbA1c	hemoglobin A1c
HFE	HLA-H antigen
HPLC	high performance liquid chromatography
iBRB	inner blood-retinal barrier
ICAM-1	intercellular adhesion molecule
KHL	keyhole limpet hemocyanin
LC-MS	liquid chromatography mass spectrometry
LMW	low–molecular weight
ME	macular edema
MG	methylglyoxal
MG-BSA	methylglyoxal-modified bovine serum albumin
MG-H1	methylglyoxal hydroimidazolone isomer 1
MOLD	methylglyoxal imidazolium crosslink
MS	mass spectrometry
MSMS	tandem mass spectrometry
NADPH	reduced form of Nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NPDR	non-proliferative diabetic retinopathy

NVD	neovascularization of the optic disc
NVE	neovascularization elsewhere (than of the optic disc)
oBRB	outer blood-retinal barrier
OR	odds ratio
PAI-1	plasminogen activator inhibitor-1
PDR	proliferative diabetic retinopathy
PKC	protein kinase C
PTB	N-phenacylthiazolium bromide
RAGE	receptor for AGE
ROS	reactive oxygen species
RPE	retinal pigment epithelium
RR	relative risk
SD	standard deviation
sRAGE	endogenous secretory RAGE
TGF-β1	transforming growth factor- β1
UDP-GlcNAc	N-acetyl glucosamine
UKPDS	United Kingdom Prospective Diabetes Study
VCAM-1	endothelial vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

4. List of papers

1. Increased serum levels of the specific advanced glycation end product methylglyoxal-derived hydroimidazolone are associated with retinopathy in patients with type 2 diabetes mellitus.
(Metabolism. 2006; 55 (2):232-6.)

2. Increased vitreous levels of hydroimidazolone in type 2 diabetes patients are associated with retinopathy.
(Acta Ophthalmol Scand. 2007; 85(6):618-22.)

3. Serum levels of the Advanced Glycation End product hydroimidazolone is associated with retinopathy in type 1 diabetes patients.
(Submitted)

4. Low cumulative incidence of proliferative retinopathy in childhood-onset type 1 diabetes in Norway.
(Diabetologia. 2006; 49(10):2281-90.)

5. Introduction:

5.1 Diabetes

Diabetes mellitus is a heterogeneous group of diseases characterized by high glucose levels in the blood and urine. The earliest known mentioning of the word "diabetes" dates back to a roll of papyrus from the 3rd Egyptian dynasty and the physician Hesy-Ra. "Diabetes" means "going through", referring to the polyuria which is one of the symptoms in diabetes. The Latin word "mellitus" means "honey-sweet", referring to the sweet taste of the urine from a diabetic subject (1).

The two main forms are type 1- and type 2-diabetes mellitus.

The signs and symptoms of diabetes mellitus are caused by lack of insulin or inability to respond to insulin, or both. Type 1-diabetes is characterized by autoimmune destruction of the pancreatic beta cells, causing insulin deficiency. Type 2-diabetes is due to a *relative* insulin deficit, its pathogenic mechanisms combining resistance to insulin with a relative defect of insulin secretion. There are huge variations in incidence rates of diabetes mellitus type 1- between different geographical areas and ethnical groups, the Scandinavian countries ranging among the highest (2). The prevalence of both type 1- and type 2-diabetes seems to increase worldwide, Norway included (3). Studies linking WHO's global database with demographic estimates of the UN calculate a drastic increase in the prevalence of diabetes mellitus on a world basis within the next 20 years, estimating the number of adults with diabetes in the world rising (from 135 million in 1995) to 300 million in the year 2025 (3). In Norway today, 90,000 – 120,000 people have been diagnosed with diabetes, but it is estimated that there are almost as many undiagnosed cases (4). How to face this epidemic challenge of diabetes is an up-to-date political issue (5). Additional challenges as diabetes increases not only include how to deal with the disease itself, but how to deal with its vascular complications; especially since preventive measures produce better individual health and far less health expenses (6) (7). As of yet, there are no Norwegian figures available, but the cost of treatment of diabetes and its complications in Sweden amounts to 8% of its direct healthcare expenses (8). Type 2-diabetes among persons at risk of developing the disease can be primarily prevented or postponed through life-style intervening measures (9).

5.2 Hyperglycemia and vascular complications of diabetes

Diabetes leads to disease in both large and small vessels (macro- and microvascular complications).

Macrovascular complications are due to atherosclerosis and can occur in large blood vessels in any part of the body, affecting the brain, heart, and peripheral arteries (e.g of the lower extremities) giving rise to stroke, cardiac heart disease and peripheral arterial disease. The risk for - and burden of - macrovascular mortality and morbidity are increasing (10) (11), in particular when microvascular disease already exists (12) (13) (14).

Microvascular complications are due to progressive pathology of the small arterioles. Organs mainly affected are the eyes, kidneys and nerves (15); diabetic retinopathy. Diabetic retinopathy is the most important cause of permanent visual impairment and/or secondary blindness in the working-age population in the Western world (16) (17). The findings from the small, but early Oslo and Stockholm studies were confirmed in the DCCT, stating that decreasing blood glucose levels by intensive insulin treatment could prevent or delay the development of diabetic microvascular complications (18) (19) (20) (21). This was later also confirmed in type 2-diabetes in the UKPDS (22). However, with time the vast majority of all patients with diabetes develops background retinopathy, as many as 40–50% observed progressing to proliferative retinopathy within 25 years of diabetes onset (23). Over the last decades, proliferative retinopathy is declining in many centres (24) (25) (26).

Susceptibility to diabetic microvascular complications reveals a great interindividual variation (27). Not all patients with diabetes develop complications, but *multiple* vascular complications occur in almost 20 % of patients (28). It has also been speculated whether there may be a chronological order in which microvascular complications develop (29) (30). In longitudinal studies, diabetic patients with retinopathy at baseline are at increased risk of developing diabetes-associated peripheral neuropathy (31) and coronary heart disease (32).

In type 1-diabetes, almost all patients develop signs of retinopathy in the first 20 years.
In type 2-diabetes, up to a third of patients have retinopathy at the time of diagnosis (33), increasing to two-thirds within 20 years. However; as the incidence and prevalence of both type 1- and type 2-diabetes are increasing, on a population level complications are expected to increase. It is thus important for the prevention of diabetic retinopathy and its progression that organized measures are implemented. However, in order to identify treatable retinopathy and apply further prophylactic measures, joint medical efforts are warranted. Inevitable and silent progression of diabetic retinopathy may otherwise be overlooked until the patients experience vision loss. In general, current recommendations apply for retinopathy screening to be undertaken annually.

5.3 Diabetic retinopathy; background.

Diabetic retinopathy was first recognized after the invention of the direct ophthalmoscope in the middle of the 19th century (34). In 1856, Eduard Jäger observed and described diabetic macular changes (35), his findings and interpretations meeting skepticism from the establishment of ophthalmology. Histopathological proof came in 1872 with Nettleship's publication (36). The proliferative changes of diabetic retinopathy, including vitreous hemorrhages and retinal detachment due to traction, were described in 1876 by Wilhelm Mantz (37). With the discovery of insulin in 1921 by Banting and Best (38) the treatment of diabetes was revolutionized. The expected "final cure" for diabetes appeared too optimistic, though, before the growing awareness of the association between diabetes and its complications. In insulin treated survivors, the prevalence of diabetic complications increased dramatically, too, further challenging the patients, clinicians and researchers. Dr. Elliot P. Joslin (already in 1931, only 10 years after the discovery of insulin) concisely described his clinical observations: "With the advent of insulin, we moved from the era of diabetic coma to the era of diabetic complications" (39). Ballantyne described in 1943 diabetic retinopathy as a unique vasculopathy rather than caused by hypertension and atherosclerosis (40). Finally, in 1954, the work of Knud Lundbæk created acceptance for the concept of diabetic microvasculopathy as a diabetic entity (41).

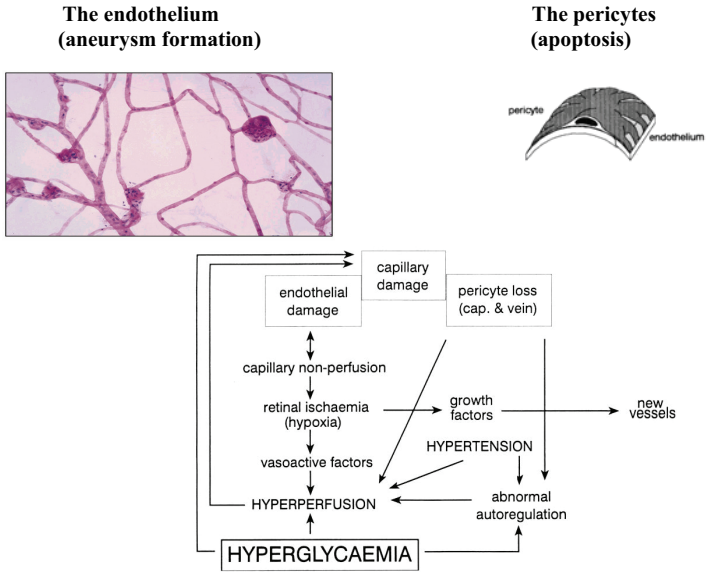
In the following second half of the 20th century, experimental treatment modalities evolved with the changing needs of the diabetic patient. Meyer-Schwickerath pioneered photocoagulation. His principles of using light on retinal conditions (initially reflected sunlight, and later light from a xenon arc) were applied and improved by several others (Morón-Salas, Wetzig, Amalric, Okun, Wessing et al) (42). Laser treatment eventually turned out the most successful means of photocoagulation (43) (44), still used world-wide. Several *surgical* approaches for treating diabetic retinopathy were also developed, even experimental procedures such as pituitary ablation (45). In 1968, The Airlie House Symposium gathered devoted ophthalmologists and diabetologists to define the future course for research and development in the field of diabetic retinopathy. Systematic approaches to the growing challenges of diabetic retinopathy continue to produce valuable insight. Basic epidemiological studies on both type 1 and type 2 diabetes have shown beneficial effects of glycemic control on onset and progression of diabetes retinopathy (21) (22).

Pathogenesis

Diabetic retinopathy is caused by deranged systemic and local (retinal) metabolism, potentially capable of destroying the architecture and specialized function of this vascularized neural tissue of the eye. Alterations in and between the functional and supportive cells of the retina occur before ophthalmoscopical *signs* of retinopathy become clinically evident. As vascular and neural cell defects seem to depend on one another, one might question at what point diabetic retinopathy actually starts. Electroretinographically observed alterations of retinal nerve cell function may occur quite early in the course of retinopathy (46), and neuroretinal dysfunction in general (measured by ERG, dark adaptation, contrast sensitivity, colour vision) has in fact been shown even before vascular lesions are seen on the fluorescein angiogram (47). In addition, *symptoms* such as vision changes can be transitory or occur late, hence an insufficient parameter for both severity grading and visual prognosis.

Apoptosis of the contractile pericytes that embrace the microvessels denotes a primal *anatomical* change, hereby facilitating later microaneurysm development (48) **(See Fig. 1)**.

Fig. 1 Early capillary targets (effects) in the development of diabetic retinopathy



Schematic diagram of pathogenesis of diabetic retinopathy
(From Kohner, EM et al 1995, *Diabetes*)

In the earliest, preclinical stages of diabetic retinopathy, the retinal blood flow increases. Leukocytes and monocytes later adhere to the vessel walls, and localized hypoxia results from capillary closure (49) (50) (51). Further autoregulatory and hemodynamic dysfunction cause the retinal veins to dilate, eventually appearing bead-like. Cellular and intercellular barrier derangement may cause intraretinal hemorrhages and leakage of plasma and precipitation of its lipid and protein constituents (48). Of all tissues, retina has the highest rate of oxygen consumption (52). When poor perfusion (revealed by fluorescein angiography) and microinfarctions of the retinal neurons occur (denoted by soft exudates), ischemia may include larger retinal areas. Further progression may trigger neovascularization, initiated by sprouting of new (but leaky!) retinal vessels due to stimulation by growth factors. These factors may leak from the systemic circulation or be of intraocular origin. VEGF is considered the most important, whereas examples of others are insulinlike growth factor 1, hepatocyte growth factor and basic fibroblast growth factor (53) (54) (55). At this stage, the clinical condition of PDR is reached, and panretinal laser treatment will decrease the stimuli for further worsening.

Macular edema is caused by increased vascular permeability in the macular area. Generalized macular edema is probably due to a widespread blood-retinal barrier (BRB) breakdown, secondary to glucose-induced microvascular damage. Anatomically, there are actually two blood-retinal barriers that may break due to ocular disease: The outer BRB consists of the retinal pigment epithelium, regulating trans-epithelial diffusion by the intercellular tight junctions, further separating the choroidal circulation from the retina. The inner BRB consists of tight junctions of the endothelial cells of retinal vessels and provides a selective mechanism for the retina to regulate its environment during varying metabolic demands. It is most fragile in diabetic retinopathy; “retinal disease of diabetes mellitus”.

Diabetic retinitis, the old descriptive term of proposed inflammatory changes of the retinae in diabetic patients, may in part be justified etymologically: Compared with patients with less severe retinopathy, patients with severe NPDR or worse have elevated serum levels of chemokines, supporting the hypothesis that inflammation is involved in the pathogenesis of retinopathy (56).

Risk

The longer the *duration* of diabetes, the greater the risk of diabetic retinopathy (57) (58). The *chronic hyperglycemia* or total glycemic load associated with diabetes over time is thought to be the primary cause of diabetic retinopathy (DR) (**See Fig 2.**) (21).

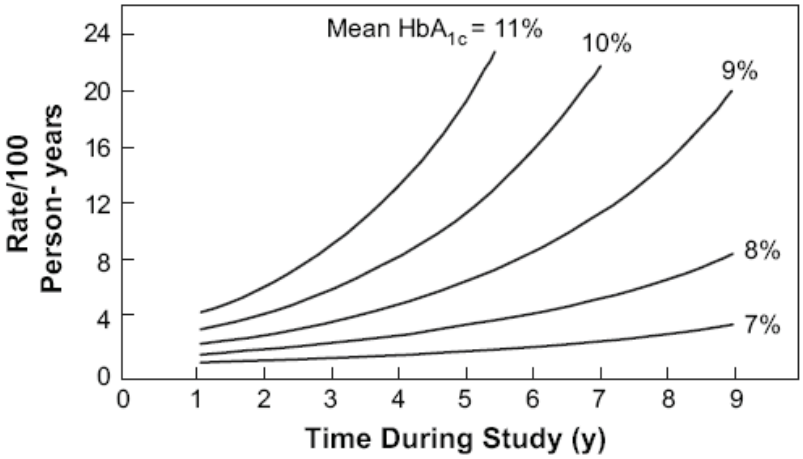


Fig. 2. Absolute risk of sustained retinopathy progression by HbA1c and years of follow-up in DCCT participants. (*Data from* Diabetes Control and Complications Trial Research Group. The relationship of glycemic exposure (HbA1c) to the risk of development and progression of retinopathy in the diabetes control and complications trial. Diabetes 1995;44:968-83.)

One of the most important treatable risk factor besides hyperglycemia is *hypertension*; both in patients with diabetes type 1 (59) (60) and type 2 (61).

Indirectly, underlying *genetic* predispositions for the background disease (diabetes mellitus) may be of etiological importance for its complications, too. Especially in diabetic *nephro*pathy, direct genetic associations have been revealed (62), already suggested by its much lower incidence over time compared to retinopathy. Among the most intriguing candidate genes in diabetic *reti*nopathy involve the expression of VEGF (vascular endothelial growth factor), ALR (rate limiting enzyme of the polyol pathway), RAGE (receptor for AGE), ICAM-1 (intercellular adhesion molecule), ADRB3 (β3 adrenergic receptor gene), HFE (or HLA-H antigen), and α2β1 integrin (specific trans-membrane cell-linker)(63). Evidence suggests that *familial factors* strongly influence the susceptibility to complications, especially regarding nephropathy (64) (65) (66). Recent studies report of female preponderance for diabetic complications (67) and genome-wide linkage analysis linking chromosome 1p to diabetic retinopathy (68).

An association between nephropathy and retinopathy has clinically been shown, and a previous study (29) demonstrated that diabetic nephropathy is strongly associated with diabetic retinopathy. Other recent studies have confirmed the presence of diabetic retinopathy itself revealing patients at risk of diabetic nephropathy (69); in type 1 diabetics maybe even more so (70). Squandrito and Cucinotta (1991) reported that the severity of diabetic nephropathy in type 1- and type 2-diabetes increases the prevalence of diabetic retinopathy (71). However, both complications have hyperglycemia in common, making it difficult to dissect the relationship.

Unfavourable lipid profiles have been associated with the development and progression of diabetic retinopathy (72), and a new study suggests that lowering lipids by fenofibrate reduces the need for laser treatment (73).

Clinical aspects

Preserving vision is, however, the most important treatment goal for both patient and ophthalmologist, being far simpler and more successful a task in the *early* stages of retinopathy development, and – even better; *before* its evolvement, when preventive measures may be applied more efficiently. Additional helpful management strategies as DR progresses to macular edema (ME/CSME) and/or PDR include retinal laser therapy, albeit often at the expense of functioning retina (74). With retinal traction and/or non-resorbing vitreous hemorrhage, vitreoretinal surgery may become necessary.

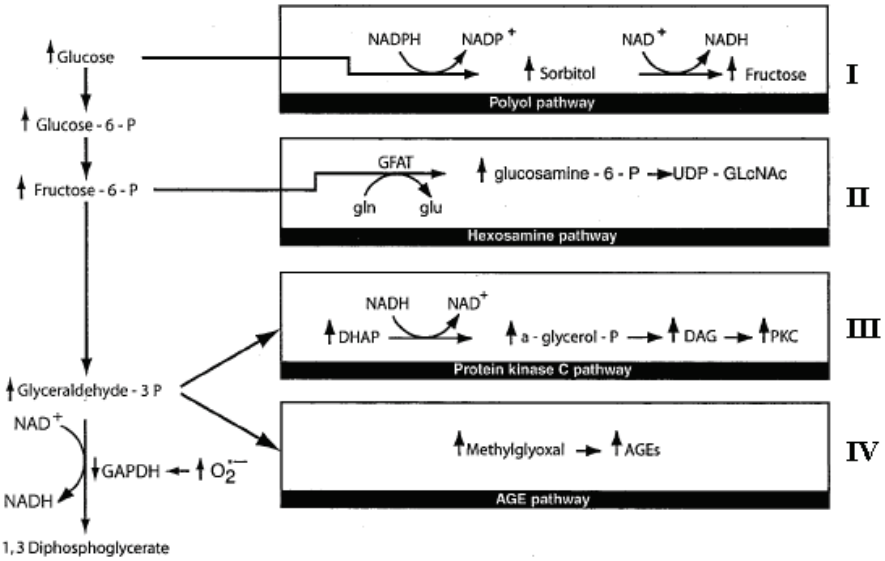
Classification

Diabetic retinopathy is *classified* according to stage and treatment indications, also revealing its potential vision threat (75). The terms no retinopathy (DNR), non-proliferative retinopathy (NPDR) and proliferative retinopathy (PDR) of diabetes reflect the chronological development of this complication left untreated/suboptimally treated; with or without macular edema (ME) or clinically significant macular edema (CSME). As mentioned, these latter entities may coexist with DR. Although uncommon, they may be solely present.

Biochemical mechanisms and ROS

A definite biochemical explanation of diabetic retinopathy has not been established, but interaction of various pathways is obvious. Four main candidate mechanisms for the deleterious effect of hyperglycemia are the following (76) (See **Fig. 3**):

Fig. 3 Hyperglycemia and cell damage



Overall categorization of signaling pathways involved in diabetic complications.
An example of ROS is the free radical $O2^{\bullet -}$ (superoxide; bottom left).
(From Michael Brownlee, *Nature* 2001)

I Polyol/Aldose reductase pathway

II Hexosamine pathway

III Protein kinase C pathway with activation of vascular endothelial growth factor

IV Advanced Glycation End products pathway (77).

I Increased glucose flux through the polyol or aldose reductase pathway will lead to sorbitol; a reaction catalyzed by the enzyme aldose reductase. The depletion of cellular cofactor NADPH may decrease NO production in endothelial cells, altering the redox balance of the cell. NADPH is also an essential cofactor for regeneration of the important intracellular antioxidant, reduced glutathione, hereby increasing the susceptibility to intracellular oxidative stress. Increased intracellular sorbitol may reduce levels of myo-inositol, which in animal studies is associated with the development of neuropathy, nephropathy and retinopathy (78).

II Increased hexosamine pathway activity: Some of the fructose-6-phosphate (Fructose-6-P) from glycolysis is diverted via N-acetyl glucosamine (UDP-GlcNAc), changing gene expression of transcription factors by modification of serine and threonine residues.

(79) (e.g. increased modification of transcription factor Sp1 increases expression of TGF-β1 and PAI-1, promoting blood vessel pathology.)

III Increased protein kinase C pathway activity: Hyperglycemia increases intracellular content of glyceraldehyde-3-phosphate, which stimulates synthesis of diacylglycerol (DAG); further activating protein kinase C (PKC). Activation of the β-isoforms of PKC in the vessels of nerves, kidneys and retinae of diabetic animals may produce vascular damage including increased permeability, altered blood flow, NO dysregulation and leukocyte adhesion.

IV Increased production of AGE and its precursors appear to cause cellular dysfunction via three routes (see also **Fig 5**):

1. By modification of *intra*cellular protein; regulatory proteins of gene transcription among the most important.
2. By modification of *inter*cellular signaling between cell and matrix after diffusion of AGE precursors out of the cell.
3. By modification of *extra*cellular proteins of the bloodstream (e.g. albumin), enabling AGE-receptors interaction and activation. This in turn causes vascular pathology from production of inflammatory cytokines and growth factors.

ROS and oxidative stress

Reactive oxygen species (ROS) are highly reactive molecules in biological systems, potentially harmful when insufficiently removed. Oxidative stress is the result of a relative imbalance of ROS and antioxidative status and may interfere with pathways **I – IV**. Generation of ROS occurs when oxygen is converted to the free radical $O_2^{\cdot -}$ (superoxide), which then is dismutated to H_2O_2 by the enzyme superoxide dismutase. H_2O_2 may be enzymatically converted to H_2O by catalase or glutathione peroxidase, or to $HO^{\cdot}$ by reaction with copper or iron (80).

ROS have various sources, including normal oxidative phosphorylation in the mitochondria; and various effects, including cell membrane dysfunction. If DNA is altered, the expression of a range of signaling or enzymatic proteins may also be altered, via modified transcription factors. Oxidative stress is central in diabetic complications, and there is most probably an interaction between ROS and AGEs (81) (76). AGEs – directly or indirectly – stimulate ROS production (82) and studies have shown a decreased formation of AGEs due to antioxidant effect (83). In diabetes, the formation of ROS/AGE may be a self-perpetuating cycle (84).

Potential sources of ROS in the Maillard reaction are many; auto-oxidation of glucose; Schiff bases; Amadori adducts and AGEs (85). **(See Fig. 3 and Fig. 4)**

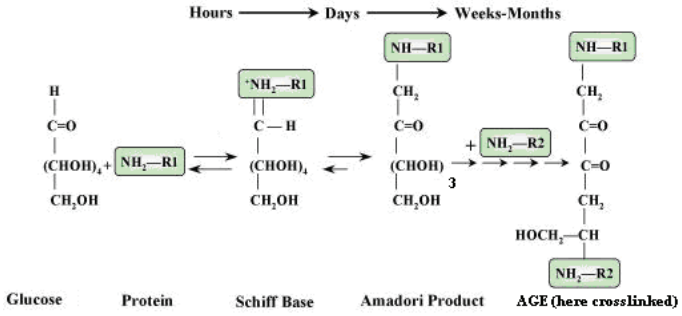
5.5 AGEs (Advanced Glycation End products): Background, pathologic relevance and detection

MAILLARD, Louis Camille (1878-1936)**.**

AGEs will now be described in more detail, as this is a central topic in the present dissertation.

The basis for formation of AGEs – the Maillard reaction – takes place when reducing sugars react non-enzymatically with amino acids on proteins forming Advanced Glycation End products; AGEs **(See Fig. 4A)**. It was first described in Louis Camille Maillard's scientific paper of 1912; "*Action des acides aminés sur les sucres: formation des mélanoidines par voie méthodique*" (86).

Fig. 4A Possible pathways for formation of Advanced Glycation End products, AGEs.



(corrected from www.google.no)

The formation of AGEs occurs in most foods during heating and plays a central role in the development of color, aroma and flavor, texture and nutritional value of cooked and processed foods.

However, these processes were later also revealed in biological systems.
HbA1c, which reflects mean level of blood glucose, is an Amadori product (**see Fig. 4A**) and the most important predictor for development and progression of vascular complications in diabetes (21) (19).

AGEs; definition and examples
The term "AGEs" refers to posttranslationally glycated modifications on end-standing aminogroups on proteins, lipoproteins, lipids and nucleic acids that non-enzymatically have undergone irreversible dehydration and condensation processes via various reactive intermediates. There may be several modifications per molecule and several different proteins may be modified. The modification itself is often referred to as an "adduct". (**see Fig. 4A**).
There are several alternative routes into forming AGEs, and the predominant substrate fuelling the glycation is glucose. However, carbohydrates and sugars other than glucose, such as glyceraldehyde, fructose and ribose also glycate to form AGEs.
Early glycation products may later form *advanced* glycation end products, like glyoxal from auto-oxidative glycation. Glyoxal may, through further steps of oxidation (glyc*oxid*ation) form N^{ε}-(carboxymethyl)lysine (CML), whereas methylglyoxal (MG) may form hydroimidazolones and e.g. argpyrimidine. MG levels are increased in diabetes, and hydroimidazolone is one of the most important AGEs (87). CML and other AGEs may also form without carbohydrates from lipid peroxidation, phospholipids and the nucleotides of DNA. When oxidation accompanies glycation, examples of additional glyc*oxi*dation products to CML are CEL and pentosidine, the latter with intrinsic *fluorescent* properties (80). Tissue and plasma fluorescence may be used as indirect markers of accumulation of AGEs. In addition to CML and pentosidine, glyoxal-lysine dimer (GOLD) is considered marker of *glycoxidation* (88).
Examples of AGEs formed through *non*oxidative processes are pyrraline, MOLD and DOLD. A list of biologically important AGEs are found in **Table 1** (89).

Table 1 *Biologically important AGEs*

Non-cross-linking monolysyl adducts
N^{ε}-carboxymethyllysine (CML)
N^{ε}-carboxyethyllysine (CEL)
Pyrraline
Cross-linking bislysyl adducts
Glyoxal-derived lysine dimer (GOLD)
Methylglyoxal-derived lysine dimer (MOLD)
3-Deoxyglucosone-derived lysine dimer (DOLD)
Hydroimidazolones
Glyoxal-derived hydroimidazolone (G-H)
Methylglyoxal hydroimidazolone (MG-H)
3-Deoxyglucosone-derived lysine dimer (3DG-H)
Other cross-linking compounds
Pentosidine*

AGEs, advanced glycation end products. *Forms intermolecular links between arginine and lysine residues.

(From Bohlender, J.M. et al (2005) *Am J Renal Physiol*)

Methylglyoxal derived AGEs

In addition to sugars, many different aldehydes and ketones can form AGEs in vivo. Methylglyoxal (MG) is an α-oxoaldehyde capable of provoking oxidative stress, and is present at higher concentrations in diabetes (90). It generates from spontaneous decomposition of triose-phosphate intermediates in aerobic glycolysis and *oxidative degradation* of both carbohydrates (pentoses and ascorbate) and lipids (arachidonate) (91). It is also a substrate of the glyoxalase system (92), which detoxifies it to D-lactate.

By irreversible glycation, methylglyoxal forms AGEs both intracellularly and extracellularly. It may modify a range of different proteins in different compartments, from collagen in tissue to proteins of the circulation. The type of AGE modification formed is determined by the free aminogroup to which it binds and modifies.

There are three main aminoacids that react non-enzymatically with methylglyoxal forming physiological AGEs; cysteine, lysine and arginine.

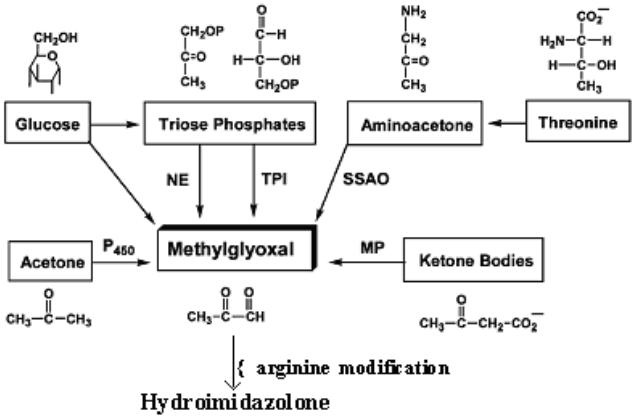
Examples of *cysteine* modifications are CMC and CEC, which are increased in plasma of diabetic nephropathy (93).

N(epsilon)-(carboxyethyl)lysine (CEL) and the imidazolium crosslink, methylglyoxal-lysine dimer (MOLD) are examples of AGEs formed from MG and *lysine*. Lysine modifications may also crosslink with eachother via lysine bridging forming imidozolysine.

When MG reacts with *arginine*, argpyrimidine may form, which is a *fluorescent* AGE. However, the main AGEs formed when MG reacts with arginine in proteins are isomers of hydroimidazolone called methylglyoxal hydroimidazolones, of which MG-H1 (N-α-acetyl-N-

δ-(5-hydro-5-methyl)-4-imidazolone is the one quantitatively dominating in vivo, often referred to as hydroimidazolone only, and of special focus of this thesis **(See Fig. 4B)**.

Fig. 4B Metabolic origins of methylglyoxal; precursor of hydroimidazolone



NE, non-enzymatic; TPI, triose phosphate isomerase; SSAO, semicarbazide-sensitive amine oxidase; MP, myeloperoxidase; P450, ethanol-inducible cytochrome P450-2E1. (From: Vander Jagt, DL and Hunsaker, LA. 2003, *Chemico-Biological interactions*)

AGEs and disease

The formation of AGEs is observed in the human body at all ages and increases as a process of *normal* aging, contributing to cross-linking of extracellular, long-lived proteins, and may lead to browning and fluorescence, and – where these reactions are accelerated – to development of diabetic *complications* and inflammatory processes linked to neurodegenerative diseases (94), hypertension, rheumatoid arthritis and atherosclerosis (95). There are several reasons for AGEs believed to be of *pathogenic importance* in diabetic vascular complications **(see Table 2)**:

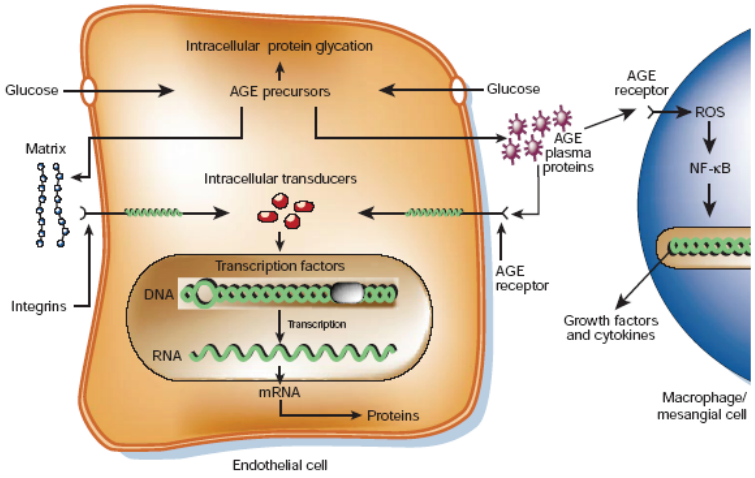
Table 2.

Background for pathogenic involvement of AGEs in vascular complications of diabetes.

Pathogenic importance	AGEs / findings	References
AGEs measured in skin collagen from human biopsies predict progression of diabetic microvascular complications better than HbA1c	CML	(96)
Injection of AGE modified proteins in non-diabetic experimental animals produces effects similar to diabetic late complications	in vitro modified rat serum albumin	(97)
Inhibition of AGEs reduces diabetic retinopathy, nephropathy and neuropathy in experimental animals	PAS-positive vasculature deposits in hypertensive rats / Radioimmunoassay-AGEs measured as AGE-RNase per μmol hydroxyproline and tissue fluorescence in end organ of diabetic rats	(98) (99) (100)
A positive correlation has been shown between content of AGEs in human skin collagen and diabetic retinopathy and nephropathy	ELISA for skin collagen AGEs; pentosidine and fluorescent AGE measurements / fructoselysine, CML, pentosidine and fluorescence	(101) (102)
Associations have been found between serum levels of AGEs and human diabetic nephropathy and cardiovascular disease	AGEs with DELFIA and polyclonal antibody / non-CML AGEs	(103) (104)
AGE receptor interactions	(see under next *italic* headline)	

Figure 5 depicts the three routes as mentioned in **6.3 IV** 1-3, through which AGEs may cause cellular dysfunction:

Fig. 5 Increased production of AGE precursors and their pathologic consequences



From Brownlee, M. (Nature, 2001)

Circulating AGEs; AGE/RAGE interaction

The liver and kidney are both involved in the catabolism and excretion of AGEs from the circulation (105) (106). Renal tissue is among the targets of AGEs that cause cellular damage and reduce kidney function. Hence, a reduced clearance of AGEs from the circulation may further increase both cause and effect of damage from glycation (107). Clinical studies have reported increased serum levels of CML in subjects with type 1- (108) and type 2-diabetes (90) and elevated levels of CML are associated with microvascular complications of diabetes such as retinopathy (101). In addition, patients with type 2-diabetes have increased circulating levels of the AGE hydroimidazolone (109) and its precursor MG (90). Positive associations between serum levels of hydroimidazolone and retinopathy have been shown in clinical studies of type 1-diabetes (110). The effects of circulating AGEs may be caused by interaction with receptors or without receptors involved (See **Fig. 5**). There are several AGE receptors, of which the multiligand receptor RAGE probably is among the most important, participating in chronic inflammatory and immune responses (111). Proposed endogenous ligands for RAGE other than certain AGEs (like CML), are S100/calgranulins (a family of closely related calcium-binding polypeptides that accumulate extracellularly at sites of chronic inflammation); amphoterin (or protein HMGB1, released by cells undergoing necrosis) and

amyloid (amyloid -β peptide accumulating in Alzheimer's disease; amyloid A accumulating in systemic amyloidosis).

A truncated, *soluble* form of RAGE also exists; sRAGE (endogenous secretory RAGE), which is capable of binding extracellular ligands without cell contact. sRAGE in excess can competitively bind ligands meant for RAGE, thus preventing cellular signalling mediated via this receptor. The balance between levels and actions of RAGE and sRAGE may be central in AGE-mediated pathology (80). AGE/RAGE interaction activates a cascade of signal transductions, of which the PKC pathway is one. Generation of reactive oxygen species (ROS) may follow, triggering NF-κB. This in turn elicits release of proinflammatory cytokines, expression of adhesion molecules and growth factors (e.g. VCAM-1, TGF-B1, VEGF), all implicated in the pathogenesis of diabetic complications, like diabetic retinopathy (111) (80).

Whether hydroimidazolones interact with RAGE is uncertain. However, methylglyoxal modification of arginine residues by may be particularly damaging because arginine residues occur at a high frequency in substrate and ligand recognition sites in enzymes and receptors (112).

AGEs, VEGF and the eye

Retinal pericytes have a low regenerative capacity. Loss of these supportive cells is an early event in the course of diabetic retinopathy (48). As AGEs in vitro are toxic to bovine retinal pericytes and cells of the microvasculature (113) (114), they may play an important role in pericyte loss. Further, capillary non-perfusion and closure gradually increase hypoxia and stimulation of growth factors **(Fig. 1)**. VEGF is produced in the eye by retinal pigment epithelium (RPE) cells and is up-regulated by hypoxia. It is considered to be involved in the progression of diabetic retinopathy (115) (116) as it stimulates vascular permeability and new vessel growth. It has been shown that AGEs induce VEGF expression in retinal cell culture and animals. There are four major biologically active human isoforms. $VEGF_{165}$ predominates in the human eye, appearing to be responsible for pathological ocular neovascularization (117). If AGE modified protein is injected in non-diabetic rats, VEGF in the eye is up-regulated (118), producing dysfunction of the inner BRB (119). Utilizing "anti-VEGF" as a therapeutic concept has expanded over the last few years: VEGF antibodies are used to reduce neovascularization and edema of not only cancer but also ophthalmologic entities such as AMD and ME of DR (120) (121).

Interestingly, AGEs are associated with degenerative changes of other tissues of the eye as well:

- Accumulation of AGEs (CML) is found on the corneal basement membrane suggesting a causative role in corneal epithelial disorders in diabetes (122).
- Methylglyoxal hydroimidazolones are quantitatively major AGEs of the lens proteins in humans. These lens protein modifications may stimulate further glycation, oxidation, and protein aggregation to form cataract (123).
- Accumulation of AGEs (pyrraline) is found at the optics disc; in the cribriform plate and around vessels of the optic nerve, possibly contributing to the development of neuropathy of the optic disc and nerve in diabetes (124).
- Elevated levels of AGEs (non-CML, hydroimidazolone) have recently been described in the human vitreous of diabetic patients compared to non-diabetic controls (125) (126).

AGEs; different methods for detection

1) *Immunoassays* are widely used in the field of AGE research. The immunoassay used in the present study was developed by Kilhovd et al (109) based on work by Berg et al with minor modifications (110) in our determination of Nα-acetyl-Nδ-(5-hydro-5-methyl)-4-imidazolone (MG-H1) which is a methylglyoxal (MG) -modified arginine compound. The antibodies of the DELFIA assay (dissociation enhanced lanthanide fluoro-immunoassay) used are marked with Europium chelate for fluorimetric visualization. An advantage of the DELFIA system compared to the enzyme linked immunoassay (ELISA) is its ability to diminish interfering background fluorescence when applying a *delayed* fluorescent visualization technique. With either immunoassay the quantification is relative, depending on the quality of the standard quantification.

2) *Separation methods coupled to MS (mass spectrometry):* Due to the complexity of biological samples, *chromatographic techniques* (e.g. HPLC; high pressure liquid chromatography) have been used for initial separation of molecules into relatively homogenous groups. This may be used in combination with later, *spectrometric* methods, such as LC-MS (liquid chromatography mass spectrometry). MS may be performed in a coupled fashion called MS/MS, or tandem mass spectrometry. These are all methods for a more absolute quantification with a relatively high sensitivity.

With mass spectrometry (MS), the constituents of chemical samples are measured as the mass-to-charge ratio of ions: The sample is ionized; ions of different masses are separated, and finally; their relative abundance is measured by ion flux intensity. This is the golden standard for AGE measurements at the present time. However, initial preparation of the AGE sample is still necessary before LC-MSMS (127), usually by enzymatic digestion or acid hydrolysis. This implies that the intact modification of the protein is not measured, but rather *peptide* fragments of the modified proteins.

3) *Autofluorescence* may be measured using an autofluorescence meter applied to the skin (128). This method is not AGE specific, as compounds other than AGEs also may fluoresce. It measures both glycation and oxidation adduct fluorophores, but the phenomenon of fluorescence in tissue and plasma can be used as a marker for the presence of AGEs. Over time, tissue fluorescence increases in diabetes (83), (129), (130) as observed within the kidney, the retina, the skin and other sites of diabetic microvascular pathology. Some studies have suggested that fluorescent AGEs may be better associated with microvascular complications than with non-fluorescent compounds such as CML (131). It is thought that incomplete degradation of AGE-modified proteins from the diet or endogenous sources produce so called low–molecular weight (LMW) AGEs. A simple and indirect way of measuring tissue florescence is by measuring fluorescence in the LMW fraction of serum (108). The findings of Januszewski et al support the association between LMW AGEs and end-organ damage in diabetes (132).

6. Aims of research

- To measure and stepwise compare serum hydroimidazolone within a group of diabetic patients with different degree of retinopathy
- To measure and compare in patients with diabetes and in age matched controls:
    - hydroimidazolone in vitreous of patients with diabetes and in age matched controls
    - hydroimidazolone and VEGF both in serum and in vitreous fluid
- To compare retinopathy from fundus photographs in a follow-up study of patients with type 1 diabetes
- To apply a new, clinical method of retinopathy classification on fundus photographs

7. Subjects and methods

7.1 Subjects

Paper 1

From a Scandinavian outpatient clinic we recruited 227 patients with type 2 diabetes mellitus – 124 men and 103 women – with retinopathy ranging from none to proliferative. At the time of diagnosis, 221 patients were older than 30 years and 6 patients were younger than 30 years, but were not in need of insulin treatment. 86 had no retinopathy; 89 had NPDR and 52 had PDR. The retinopathy group (NPDR + PDR) was cross-sectionally compared to the non-retinopathy group (DNR). The two groups differed significantly on several parameters; hence logistic regression analysis was applied for comparison regarding retinopathy and serum levels of hydroimidazolone. Patients with plasma creatinine values > 200 mg/dl were excluded as this level of reduced kidney function may be associated with increased serum hydroimidazolone values.

Paper 2

Vitreous from 23 consecutive patients with type 2-diabetes and median known diabetes duration of 12 years were included and compared to 32 non-diabetic and age-matched control subjects who also underwent vitrectomy. The median age of both groups was 67 years. Vitrectomy within the last 6 months before hospitalization and vitreous of reddish colour were criteria for exclusion; as were plasma creatinine values > 200 mg/dl. Serum and vitreous parameters were cross-sectionally compared with regard to hydroimidazolone and retinopathy, in particular.

Paper 3

We randomly selected 61 patients with diabetes mellitus type 1 from a Scandinavian outpatient clinic for comparison of degree of retinopathy and serum levels of hydroimidazolone. DNR patients had a mean duration of diabetes of 14 years, which was significantly lower than the mean duration of 20 years in the DR group. The ratio PDR/NPDR was 36/11. Quartiles of serum hydroimidazolone were compared with occurrence of retinopathy.

Paper 4

In 1989, of the reachable 1868 incident type 1 diabetes cases in Norway from the period between 1973 and 1982, 600 were randomly selected for participation in the study. The baseline examination for diabetic complications of the 368 enrolled took place in 1989/-90. 355 were eligible for follow-up examination in 2002/-03.

All new-onset cases of type 1 diabetes occurring in Norwegian children below 15 years of age within the decade 1973/-82 were retrospectively registered by the Norwegian Childhood Registry between 1985 and 1986. The analyses at follow-up included 294 subjects with proper retinal photographs. The participants belonged to different hospital catchment areas, and the main investigator visited 24 different hospitals all over Norway. The examination included a medical history, blood pressure measurements, collection of overnight timed urine, random venous blood samples and fundus photography. Patient characteristics at baseline, divided into participants and non-participants at follow-up, were compared.

7.2 Methods

MG modification of BSA was prepared by incubating BSA in sodium phosphate buffer (100 mM, pH 7.4 and 37˚C) with 1 mM MG for 4 days, before dialyzed against ammonium bicarbonate buffer (30 mM, pH 7.9 and 4˚C) and lyophilized to dryness. Preparations of lyophilized MG-BSA were stored in liquid nitrogen. As determined by Thornalley's group (133) using HPLC amino acid analysis, this low-modified MG-BSA contains 23% modified arginine residues per molecule of serum albumin.

Hydroimidazolone immunoassay

We have previously developed specific solid-phase, time-resolved competitive immunoassays (DELFIA Wallac, Turku, Finland) for determining AGEs in serum (103); further developed by Kilhovd et al (109). The primary antiserum of the assay is a monoclonal anti-hydroimidazolone (IG7) antiserum. (It was obtained by injecting mice with keyhole limpet haemocyanin (KHL) and modified by incubating with 70 mmol/l of MG for 6 hours at 37˚C. Epitope specificity of the anti-hydroimidazolone antibody was evaluated using both dot blots and an indirect competitive ELISA. The IG7 antibody reacted specifically with Nα-acetyl-Nδ-(5-hydro-5-methyl)-4-imidazolone, showed 1% cross-reaction against its oxidized form methylimidazolone, and to some extent recognized the analogue glyoxal derived arginine hydroimidazolone compound. It did not react with Nα-acetyl-argpyrimidine, bis(Nα-

acetyl)lys-4-methyl-imidazolium chloride or carboxyethyllysine.) The IG7 antibody relatively specifically recognized MG-induced modifications of arginine residues, and did not cross-react with the imidazolones produced by the reaction of 3-deoxyglucosone with arginine (134), (135).

Essentially as described by Kilhovd et al (109), levels of hydroimidazolone immunoreactivity were determined with this monoclonal anti-MG-modified arginine antibody, except that we coated the microtiter wells in 0.05 mol/L Tris buffer (pH 7.8) and set up our assay in duplicates: Microtiter strips of 12 wells each were coated with 0.1 ml of MG-modified BSA (25 µg/ml) diluted in 0.05 mol/L Tris buffer (pH 7.8). They were then covered, and incubated overnight while shaking at room temperature. The strips were further stored at 4˚C before washed. The wells were washed 6 times in DELFIA washing buffer before use. Duplicates of 100 µl MG-modified BSA standard or serum (diluted 1:4) were added to each well along with 50 µl of anti-hydroimidazolone (IG7) antiserum diluted 1:5000 in DELFIA assay buffer*****. 7 standard solutions of 0, 2.5, 5, 10, 20, 40 and 100 µg/ml MG modified BSA were used per assay. The strips were incubated while shaking in room temperature for two hours, and then washed six times in washing buffer. 100µl/well of Europium-labeled anti-mouse-IgG-antibodies were then added in a final concentration of 0.1 µg/ml in DELFIA assay buffer. (The indicator antibody for the DELFIA was marked with Europium chelate for fluorimetric visualization). All strips were incubated while shaking for one hour in room temperature, before subsequently washed 6 times and incubated for 5 minutes while shaking with DELFIA Enhancement solution prior to measurement of the Europium-ion chelate specific fluorescence in a 1232 DELFIA Fluorometer.

This assay was applied in paper 1, 2 and 3. The arbitrary hydroimidazolone unit (U) was defined as the competitive activity of 1 µg of MG-modified BSA standard, expressed as U/mg protein.

*****When determining *vitreous* levels of hydroimidazolone, the same assay was used. Vitreous samples were set up in non-diluted triplicates.

VEGF assay

We determined serum and vitreous levels of the angiogenic isoforms of natural human $VEGF_{165}$. The Quantikine ® VEGF immunoassay (R & D Systems, Inc., Minneapolis, MN, USA) was used. Optical density was determined at 450 nm using a Rosys Anthos HT2 micro

plate absorption spectrophotometer (Anthos Labtec Instruments, Salzburg, Austria). A correction wavelength of 620 nm was used.

Vitreous hemoglobin

Harboe's method together with updated validations for measuring haemoglobin (Hb) in micromolar concentrations were used (136). We measured absorbance with an Ultrospec® 3300 pro spectrophotometer (Amersham Biosciences, Cambridge, UK; acquired by General Electric Company, Fair- field, CT, USA) at wavelengths of 380 nm, 415 nm and 450 nm. The following equation then expresses in g/l the concentration of hemoglobin (137): $C_{Hb} = 1.67 \times A415 - 0.83 \times A380 - 0.83 \times A450$.

Vitreous albumin

Albumin was measured in duplicates of 50 µl vitreous samples using the Tina-quant immunoturbidimetric albumin assay (F. Hoffmann-La- Roche Ltd, Basel, Switzerland).

Asessment of Retinopathy

Paper I & Paper III: After pupillary dilation, stereo photographs from 7 standard fields were taken of each eye, using a 30˚ fundus camera (Topcon TRC-50, Tokyo, Japan). Grading was performed in a masked fashion. The patients were characterized according to the ETDRS level of retinopathy in the worse affected eye by a centrally located, highly skilled team. If former treatment with panretinal photocoagulation had been given, retinopathy was automatically classified as PDR; that is, ETDRS level 61.

We then grouped these results in three categories: DNR, NPDR and PDR for comparison of retinopathy with other parameters.

Paper II: The patients were clinically diagnosed during their period of hospitalization: Diagnoses were pre- and peroperatively confirmed by the eye surgeon through mydriatic pupils, using indirect ophthalmoscope and operating microscope, respectively.

Paper IV: In paper 4, the photographic procedure of taking colour retinal photographs of each eye using Kodachrome 64 ISO 35-mm colour slide film (Kodak, Rochester, NY, USA) with a non-mydriatic 45˚ retinal camera (45NM-CR; Canon, Tokyo, Japan) was applied by the same investigator (T. Skrivarhaug) after instilling one drop of cyclopentolate 1% and epinephrine 10% in the cul-de-sac of each eye to obtain dilated pupils. The standard procedure of centering the photographs midway between the fovea and the temporal edge of

the optic disc was executed in the exact same way at follow-up and baseline. For back-up safety in case of reduced quality affecting the readability of one or more pictures, double sets of pictures were taken of each eye. Without knowledge of the subjects' identity, the pictures were graded centrally for retinopathy (by D. Fosmark), status of the worse eye deciding level of retinopathy. Between the extremes PDR and DNR; NPDR was further sub-classified with a new, simplified *clinical* method **(Table 1)** based on evidence from the ETDRS and the WESDR (Diabetic Retinopathy Disease Severity Scale (75)). Macular edema was not detectable using our method. Patients with fibrous proliferations, vitreous hemorrhage or scars from panretinal photocoagulation were assigned to the PDR category.

HbA1c was analyzed with high-performance liquid chromatography (VARIAN II Hemoglobin A1c program, BioRad, Hercules, CA) (reference range, 4.0%-5.3%).

(Blood tests were collected after overnight fasting.

Three overnight timed urine samples were collected at home.

Creatinine in urine was analyzed using a kinetic method (Beckman Synchron LX20, Brea, CA).

Urinary albumin was measured using nephelometry (IMMAGE, Beckman Coulter) or turbidimetry (Beckman Synchron LX20). Normal values for urine albumin urine creatinine ratio were < 2.0 mg/mmol for men and < 2.8 mg/mmol for women.

Plasma creatinine was analyzed with a kinetic method (Beckman Synchron LX20). The Reference ranges are 51- 88 μmol/L (women) and 63-105 μmol/L (men).

Lipids (triglycerides and total cholesterol) were measured with standard enzymatic methods at a centralized lab.

Statistical analyses

In all papers, statistical analyses were performed using the SPSS software, version 12.0.0 (SPSS, Chicago, IL). Two-sided Mann-Whitney U test was used when comparing medians of

continuous data not normally distributed. The Spearman correlation test was used when studying the association between two continuous variables. For conservative estimates, we used logistic regression analysis according to Katz (138). A significance level of 5% was used for each test.

Paper I: Unadjusted comparisons between patients with and without retinopathy were performed using Student's t-tests (2-sided) for continuous variables and exact Fisher tests (2-sided) for dichotomous variables. Logistic regression analysis, with retinopathy as dependent variable, was used to study the impact of selected variables; hydroimidazolone, plasma creatinine, urinary albumin-creatinine ratio, HbA1c, diabetes duration, age, and blood pressure. (These variables were enabled for multiple regression analyses as initial bivariate unadjusted analyses showed significant associations with retinopathy).

Paper II: Spearman's test for correlations was used for correlation testing. Logistic regression was used for correction of vitreous albumin when comparing vitreous VEGF between controls and diabetes patients.

Paper III: A linear-by-linear association chi-square test was used when studying the association between quartiles of hydroimidazolone and retinopathy. We used a two-sided exact Fisher test when comparing the prevalence of retinopathy between two groups. When comparing HbA1c and age between two groups, an independent two-sample t-test was used; and for the comparison of duration of diabetes, a two-sided Mann-Whitney test was used. The Spearman correlation test was used when studying the association between two continuous variables.

Paper IV: We estimated the cumulative incidence of PDR from diabetes onset until follow-up, using a Kaplan-Meier plot. To assess declining incidence of PDR with year of diagnosis, the patients were divided into two groups: 1973–1977 (n=133) and 1978–1982 (n=161). Cox regression analysis was used to estimate the hazard ratio for association between baseline factors and PDR. The following variables were analyzed: sex, age, age at diabetes onset, diabetes duration, smoking status, arterial blood pressure, AER, HbA1c, triglycerides and total cholesterol. Variables with a p-value <0.20 were then included simultaneously in a multiple regression model.

In the analysis of predictive risk factors for PDR, the three subjects with PDR at baseline were not included. As the exact time of onset of NPDR was unknown, the assessment of potential predictors of retinopathy among patients without DR at baseline was analyzed similarly using logistic regression.

Sensitivity analysis of non-participation was exclusively based on HbA1c values. By using assumptions based on external data, we performed a sensitivity analysis in order to assess the potential influence of selection bias due to non-participation and other losses to follow-up (deaths and emigration) on our estimated risk of complications. The total risk in the full cohort of participants and non-participants is a weighted average of the observed risk of complications among participants and the corresponding risk among non-participants. This total risk can then be estimated under *conservative* assumptions regarding the risk among non-participants.
(Data from the DCCT indicated a 75% increase in risk of PDR per 1%-point increase in HbA1c during a mean follow-up of 6.5 years. The HbA1c difference among non-participants and participants in our material in 1990 was only 0.6%-points. A conservative assumption of 75% higher risk of PDR as compared with the participants was made, and that 50% of them had no DR at baseline while the rest had NPDR.)

8. Summary of main results (papers)

Paper 1:

- Increased serum levels of the specific advanced glycation end product methylglyoxal-derived hydroimidazolone are associated with retinopathy in patients with type 2 diabetes mellitus.

According to the ETDRS protocol, level of retinopathy was determined from retinal photographs of 227 patients with type 2 diabetes mellitus and median known diabetes duration of 14 years. 86 patients had no retinopathy (DNR), whereas non-proliferative retinopathy (NPDR) was diagnosed in 86 patients and proliferative retinopathy (PDR) in 52 patients. Median age was 66 years.
Serum levels of hydroimidazolone were increased in the group of patients with retinopathy, with a further increment as retinopathy worsened to PDR. This was found when including all patients irrespective of time elapsed from diabetes having been diagnosed, but also in the smaller group of patients with *shorter* duration of diabetes, i.e. below the median of 14 years. We found a strong association between HbA1c and diabetic retinopathy ($p<0.0001$), and the association between retinopathy and hydroimidazolone was independent of HbA1c.

Paper 2:

- Increased vitreous levels of hydroimidazolone in type 2 diabetes patients are associated with retinopathy.

Using a cross-sectional case-control study design, we compared vitreous and serum contents in 23 patients with diabetes type 2 – sixteen of which had PDR – to 32 age-matched controls also undergoing vitrectomy. Level of retinopathy was based on clinical examination. We found a positive correlation in *all* patients between vitreous and serum content of hydroimidazolone ($r=0.48$, $p<0.001$). This was also true within cases and non-cases separately.

Paper 3:

- Serum levels of the Advanced Glycation End product hydroimidazolone is associated with retinopathy occurrence in type 1 diabetes patients.

In this cross-sectional study of 61 type 1 diabetic patients, 14 had no retinopathy (DNR), 11 had NPDR and 36 had PDR. Grading of retinopathy was based on retinal 7-field stereo photographs according to the ETDRS protocol. Comparisons of serum levels of hydroimidazolone were made between patients with and without retinopathy. Hydroimidazolone quartiles were found significantly associated with retinopathy ($p=0.013$). After adjusting for duration of diabetes by logistic regression analysis, a significant difference in retinopathy present was found when comparing the lowest quartile with the rest ($p=0.022$).

Paper 4:

- Low cumulative incidence of proliferative retinopathy in childhood-onset type 1 diabetes in Norway.

294 childhood-onset type1 diabetic patients had readable fundus photographs taken and examined for retinopathy between 2002 and 2003. This was a follow-up of originally 368 patients having undergone identical examinations between 1989 and 1990; all belonging to the 10 year cohort of 1906 persons diagnosed with diabetes mellitus type 1 between 1972 and 1983. 262 of 294 (89.1%) developed diabetic retinopathy, of which 32 developed PDR. The cumulative incidence of PDR began increasing after 10 years of diabetes duration, reaching 10.9 % (95 % CI: 7.3-14.5 %) at twenty-five years. Mean diabetes duration was 19 years (range 12-29 years); mean age for diagnosis of PDR was 27 years (range 17-41 years). Significant predictors at baseline for developing retinopathy of any degree were HbA1c

(OR=3.25, 95 % CI: 1.76-6.02, p<0.001) and male gender (OR=2.51, 95 % CI: 1.06-6.00, p=0.037), whereas significant predictors for developing PDR were NPDR at baseline (RR=3.71, 95 % CI: 1.59-8.68, p=0.03), HbA1c (RR=2.05, 1.44-2.93, p<0.001) and triglycerides (RR=1.55, 1.06-1.95, p=0.019).

9. Discussion

9.1 Methods

Clinical and Photographic methods of retinopathy diagnosis

a) The "Gold standard" for epidemiological surveys of DR is the seven-field, (analogue) retinal photographic method using film and a 30˚ camera. Members of a highly skilled team, centrally located with resources at hand, applied this method on the subjects in paper 1 and 3, including later grading of the pictures according to the ETDRS standard. We grouped these results in three categories: DNR, NPDR and PDR for comparison of retinopathy with other parameters.

b) In paper 4, to obtain dilated pupils one drop of cyclopentolate 1% and epinephrine 10% was instilled in the cul-de-sac of each eye. The non-mydriatic 45˚ retinal camera (45NM-CR; Canon, Tokyo, Japan) produced colour retinal photographs of each eye using Kodachrome 64 ISO 35-mm colour slide film (Kodak, Rochester, NY, USA). The photographic procedure was applied by the same investigator (T. Skrivarhaug). The standard procedure of centering the photographs midway between the fovea and the temporal edge of the optic disc was performed in the exact same way at baseline and follow-up. For back-up safety in case of reduced quality affecting the readability of one or more pictures, double sets of pictures were taken of each eye. Without knowledge of the subjects' identity, the pictures were graded centrally for retinopathy (by D. Fosmark), status of the worse eye deciding level of retinopathy. Between the extremes PDR and DNR, NPDR was further sub-classified with a new, simplified *clinical* method (Table 1) (75). Macular edema was not detectable using our method. Patients with fibrous proliferations, vitreous hemorrhage or scars from panretinal photocoagulation were assigned to the PDR category.

c) In paper 2, all ophthalmologic diagnoses including retinopathy were given during the patients' period of hospitalization. The diagnoses were pre- and peroperatively confirmed by the eye surgeon using indirect ophthalmoscopy and operating microscopy, respectively.

When we chose methods for comparison of retinal photographs in paper 4, digital cameras were about to substitute the use of analogue equipment – including film. Yet, the extensively marketed digital techniques with resolution constantly improving, a standardized comparison with analogue photographs was missing. Hence, at follow-up, the described *one-field method* for film was chosen due not only to its simplicity, but because of identicality to the method originally used at baseline. This facilitates the comparison between the two investigations.

A more detailed yet standardized method for further *classification* of late changes in the course of retinopathy development was needed. An improved *clinical* method, newly developed in order to simplify the existing 7-field ETDRS Gold standard and to facilitate data comparisons between different countries and trials, was found applicable for our purpose. Albeit subjective, the *clinical* standard for identification of patients with retinopathy is by direct ophthalmoscopy. However, images from cameras producing single 30˚ and 45˚ fields have both been considered useful for retinopathy screening, epidemiology studies and routine care purposes. An exact agreement of 82.5 % was found when comparing ophthalmoscopy to the seven field stereo photographic method (139).

Our method using a *single* 45˚ photograph can be criticized for the possibility of under-detecting retinopathy. One relative advantage with the 45˚ field is that only one photo is needed to view the posterior pole of the retina. However, it is of lower magnification than (the one) produced with a 30˚ camera (minified 0.64x at zero diopters). The 45˚ images include areas above and below the temporal arcades, and temporal to the macula and just nasal to the disc (**see Fig. 6**).

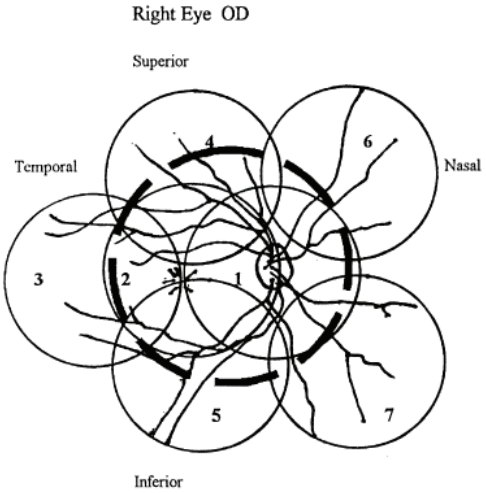


Fig. 6 Approximate field obtained with a 45˚ camera superimposed (dotted lines) on photographic field obtained with the standard seven-field mydriatic stereoscopic protocol from the Early Treatment Diabetic Retinopathy Study and a 30˚fundus camera (field 1 = disc; field 2 = macula; field 3 = temporal to macula; field 4 = superior temporal; field 5 = inferotemporal; field 6 = superior nasal; field 7 = inferior nasal).

With the use of a *digital, monochrome* camera, a study on retinopathy in 197 type 1 and type 2 diabetic patients with a *single* 45˚ image versus ophthalmoscopy revealed 100% sensitivity and 71 % specificity, the field identical to the field in our study (paper 4). Compared to the 7 field stereo photographic method, the sensitivity and specificity of the 45˚ image were 78 % and 86 %, respectively, with an exact agreement of 83 % (140).

New vessels in the periphery *only* are very rare (<1 %), and new vessels at the posterior pole (in patients younger than 60 years) are significantly *more* frequent than beyond the posterior pole (141). Thus, a one-field photo of each eye covers most of the area of interest for inclusion of PDR-diagnoses. Predilection sites for retinal proliferations are the temporal arcades and second, the area nasal to the optic disc (142) (141).

In order to cover a *larger* retinal area, a *two field* photographic method with an identical 45˚ NM-Canon camera has been validated over a 5 year period; centered on the macula and the optic disc, respectively (141). Missed diagnoses of PDR amounted to 0.9 %. False positive findings occurred in 6 of 1341 readings, or 0.4 %. False NVD were recorded mostly in younger patients (≤36 years), whereas false NVE were recorded mostly in older patients (≥50 years). True NVE/NVD were found located on the temporal arcades in 48 % of the eyes and nasally to the optic disc in 42 % of the eyes, and were rarely found beyond the posterior pole (13 %; $p<0.001$) (141).

In retrospect, the two-field method might also have been applied in our setting. A larger area, particularly nasally to the optic disc, would then have been included. Still, macular edema would not have been classifiable. However, the one-field method was found sufficient for our purpose; to classify a larger group epidemiologically, and was chosen due to comparisons to be made with identical field pictures. The better of two photographs from *both* eyes was graded before the diagnosis was given by the state of the worse eye, but a small fraction of false negative PDR with our method due to a lower sensitivity is possible. However, a possibility of having undergone laser treatment *without* PDR also exists, overestimating this group. Importantly, in our material 78.6% had NPDR; of these most had *mild* NPDR (54.6%). Further, *moderate* NPDR accounted for 32.4% and *severe* NPDR amounted to only 13.0%. This clustering of relatively benign retinopathy is in keeping with our conclusion of a relatively low cumulative incidence of PDR after 25 years of diabetes. The pictures were thoroughly graded by one experienced ophthalmologist (D. Fosmark) in one session only.

Table 1. Severity of diabetic retinopathy and corresponding findings

Disease severity level	Findings on 45° retinal photographs in mydriasis
No apparent retinopathy	No abnormalities
Mild NPDR	Microaneurysms only
Moderate NPDR	More than just microaneurysms but less than severe NPDR
Severe NPDR	Any of the following: more than 20 intraretinal haemorrhages in each of four quadrants definitive venous beading in two or more quadrants prominent intraretinal microvascular abnormalities in one or more quadrant and no signs of PDR
PDR	One or more of the following: neovascularisation vitreous haemorrhage photocoagulation scars

Immunoassay method

Different methods have been developed and used for the detection of AGEs within the field of research. For the detection of the hydroimidazolone MG-H1, the DELFIA-system was chosen due to experience regarding development and use in our laboratory. The quantification of AGEs when using immunoassays gives relative results, partly due to a missing quantitative standard for the different AGEs. Arbitrary units are therefore employed. Our assay for detection had an *intra*-assay variation in the range of 12 – 15%. The *inter*-assay variation was of up to 21%. The cross-reactivity against AGE-BSA was 8% when calculated as AGE-BSA protein against MG-modified BSA protein. No cross-reactivity was found against CML-BSA or glycated albumin in the hydroimidazolone immunoassay. The IG7 antibody relatively specifically recognizes *MG*-induced modifications of arginine residues.

In use, the assay is practical as it is inexpensive, and many samples can be run within a relatively short period of time. It is also sufficient for our purpose, which is in larger samples to search for significant differences between cases and controls. Immunoassays have been criticized for their heavily modified antigen, use of arbitrary units and possible serum effects (87). However, the present assay has an acceptable dilution curve and satisfactory recovery (135): Both serum samples from diabetic patients and controls produced parallel inhibition to hydroimidazolone standard. Recovery studies have been performed by adding MG modified BSA to serum from patients and controls. The mean recovery of amount added was 115% ± 26 %-points. A linear dilution curve reflected minor serum effect.

One advantage of the DELFIA system compared to other immunoassay methods (ELISA) is its ability to diminish the background fluorescence through *delayed* fluorescence from Europium attached to the secondary antibody. Hydroimidazolone (MG-H1) is our AGE modification of focal interest due to its relative abundance in biological systems and its likelihood of playing a pathogenic role in diabetic complications.

9.2 Interpretation of results

For each paper, thorough interpretations are found in their respective sections of discussion (See Papers 1, 2, 3 and 4). In Paper 1 and 3, there exists a positive association between retinopathy and serum levels of hydroimidazolone in both type 2 and type 1 diabetes. However, as the studies are cross-sectional, no *causal* conclusions can be made. The lack of association between HbA1c and hydroimidazolone is in keeping with earlier findings; and logical, as the two are formed via partly different pathways. However, it is noteworthy that both hydroimidazolone and HbA1c were found strongly and *independently* associated with

retinopathy. Patients with clearly increased serum creatinine (>200 μmol/l) had not surprisingly clearly elevated serum hydroimidazolone, and these patients were excluded from further analysis. If hydroimidazolone is causally involved in early nephropathy is not known. In the present study, no association was shown between hydroimidazolone and urinary albumin-creatinine ratio.

We measured hemoglobin, albumin and VEGF, adapting the methods for vitreous. This enabled a more extensive discussion and thorough interpretation of the true vitreous content of hydroimidazolone in Paper 2. Intravitreal measurements of albumin used as an indirect sign of iBRB disruption are seldseen. In our study, the correlation of vitreous albumin and hydroimidazolone is explained by the increase of vitreous albumin in the PDR group, due to a breakdown of the iBRB. Hydroimidazolone most likely originated from serum, whereas VEGF was produced intraocularly. No correlation between vitreous hydroimidazolone and VEGF was found in our study. However, the number of subjects studied was small.

In Paper 4, we found a low cumulative incidence of PDR after 24 years of type1 diabetes. The study had a population based design, but there was a marked proportion of non-participants and losses for follow-up. Thus, a sensitivity analysis was done to assess any influence of selection bias on risk for retinopathy. As regards PDR, a higher cumulative incidence of 14.0% was found with this method (versus 10.9%). Still, this is relatively low. However, the proportion of subjects with NPDR was substantial (78.6%). There remains a potential for still improved glycemic control and optimization of other risk factors that can be modified, like blood pressure and triglycerides. A uniform screening system is still missing in Norway, as are registers for incident blindness. This study is a contribution to increased knowledge on microvascular complications nationwide, and as such a stimulus for further research and improvement of prophylactic measures.

10. Concluding remarks (Relevance of papers, future research)

The exact pathogenic mechanisms of diabetic retinopathy are only partly revealed. Interplay of ROS and AGEs are plausible. Most probably, the explanation consists of multiple factors. The range of functionally and structurally different AGEs also makes it difficult to point out which AGE is the most pathogenic.

Exogenous AGEs (from coffee, smoking, foods etc) and their disputed pathogenic role are not discussed in this paper. In general, a limited consumption is recommended due to their abundance in “unhealthy food”, but in particular if reduced kidney function exists (143) (144).

Elegant approaches for potential interventions in restricting the burden of *endogenous* AGEs have emerged, attacking at the following sites:

a) *AGE formation* may be inhibited by trapping of reactive (di)carbonyls in the glycation process and chelation of transition metal ions (e.g. by aminoguanidine, pyridoxamine). Unfortunately, aminoguanidine has toxic side effects which has lead to discontinuation of clinical studies (145) (146). A drug category already in use – inhibiting the angiotensin-converting enzyme – has anti-hypertensive effect but is also potent as AGE inhibitor (147).
b) *AGE cross-links* may be broken down. PTB (N-phenacylthiazolium bromide) and ALT-711 (alagebrium chloride), cleave AGE-mediated cross-links (89).
c) *AGE binding* may be inhibited. The binding of ligand to AGE receptor include soluble RAGE (sRAGE) and RAGE specific antibodies, neutralizing the effect of receptor interaction (148) (149).
d) *ROS/AGE* interaction may be targeted via antioxidants, exemplified by improved retinal blood flow in type 1 diabetics after high-dose vitamin E supplementation (150).

Productive "anti-AGEs" research makes future important *clinical* implications of this knowledge probable, including retinopathy.

Our findings strongly support the following statements:

- Serum levels of the AGE-modification hydroimidazolone (MG-H1) are increased in both type 1 and type 2 diabetic patients with retinopathy compared to those without retinopathy.
- Serum levels of hydroimidazolone (MG-H1) are increased in PDR compared to NPDR (diabetes mellitus type 2).
- Vitreous levels of hydroimidazolone (MG-H1) are increased in type 2-diabetic patients compared to age-matched controls due to leakage from serum.
- Vitreous levels of hydroimidazolone (MG-H1) are increased in PDR compared to NPDR in type 2-diabetic patients, and may originate from serum due to iBRB breakdown.
- VEGF in vitreous fluid is increased in diabetes type 2. Even higher levels are found in PDR. The increase in VEGF is not due to a spill-over effect from the circulation, even though the iBRB is disrupted, supporting that it is produced intraocularly.

  Patients with type 1 diabetes of 24 years duration and retinopathy staged from fundus photographs had 89.1% cumulative incidence for DR. 10.5% of these have PDR.

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